In humans at least 75% of insulin secretion arises from punctuated insulin secretory bursts

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Pørk sen, Niels, Birgit Nyholm, Johannes D. Veldhuis, Peter C. Butler, and Ole Schmitz. In humans at least 75% of insulin secretion arises from punctuated insulin secretory bursts. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E908–E914, 1997.—Detection of insulin secretory bursts in peripheral blood is hampered by hepatic insulin extraction, dilution in the systemic insulin pool, and time-delayed damping of secretory burst amplitude. Previous studies in dogs in vivo and other experiments in vitro have shown that ~70% of all insulin is released within distinct insulin secretory bursts. To establish a method for detection and quantification of pulsatile insulin release in humans on the basis of peripheral insulin concentration measurements, we used a high-sensitivity, -specificity, and -precision insulin enzyme-linked immunosorbent assay (ELISA) and optimized an established deconvolution methodology to quantify the frequency, mass, and amplitude of insulin secretory bursts as well as to estimate the relative contribution of pulsatile insulin release to overall insulin secretion. By use of minutely sampled serum insulin concentrations measured by a highly sensitive insulin ELISA, and insulin kinetics of 2.8 min (first half-life), 5.0 min (second half-life), and a fractional slow component of 0.28, the deconvolved insulin secretion rates in 20 healthy subjects during glucose infusion (4.5 mg·kg−1·min−1) could be resolved into a series (4.7 ± 0.1 pmol·pulse−1) of approximately symmetric insulin secretory bursts with a mean mass of 87 ± 12 pmol·pulse−1 and a mean amplitude (maximal release rate) of 35 ± 4.7 pmol·pulse−1·min−1. The relative contribution of pulsatile insulin secretion was 75 ± 1.6% (range 59–85%). We conclude that insulin secretion in humans during nominal glucose stimulation consists of a series of punctuated insulin secretory bursts accounting for ≥75% of total insulin secretion.

METHODS

Subjects and Design

The protocol was approved by the Ethical Committee of the County of Aarhus and was in accordance with the Declaration of Helsinki. A total of 32 (14 female, 18 male) healthy volunteers were studied. Insulin kinetics were examined in six persons (4 male, 2 female) (protocol 1), and the appropriate stringencies for detection of pulsatile insulin secretory events were based on analysis of an insulin concentration time series obtained from three male subjects during constant suppression of endogenous insulin secretion (by somatostatin) and a known constant concomitant insulin infusion (protocol 2). Having established optimal stringencies and insulin kinetic parameters, we examined optimal sampling frequencies in three (1 female, 2 male) subjects by sampling every 30 s and using every sample vs. every second sample for pulse detection purposes (protocol 3). The above stringencies, insulin kinetic parameters, and optimal sampling frequency were applied to insulin concentration time series obtained from 20 volunteers (11 female, 9 male; age 35 ± 1.6 yr; body mass index 25 ± 0.6 kg/m2; waist-to-hip ratio 0.88 ± 0.02) to examine pulsatile and nonpulsatile insulin secretion (protocol 4). None of the volunteers was on medication or had concurrent diseases or any family history of diabetes. In each protocol the subjects were studied after an overnight fast (10 h). After catheter placement in both antecubital veins for sampling and infusion purposes, the study protocol was commenced as described in Protocols.

Protocols

Protocol 1: Insulin kinetics. The disappearance of insulin infused into the systemic circulation was examined in six
subjects. At time 0 a bolus injection of crystalline human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark; 0.04 U/kg) was injected intravenously. At 60 min a constant insulin infusion (1 mU·kg⁻¹·min⁻¹) was started and continued for 120 min, at which time the infusion was discontinued. The decay of insulin was examined for an additional 60 min. To avoid symptomatic hypoglycemia and to prevent hyperglycemia, a variable glucose infusion was given to achieve plasma glucose concentrations between 3.5 and 5.0 mM. At these glucose concentrations, endogenous insulin secretion was assumed to be trivial, and counterregulatory mechanisms would not influence insulin disappearance. Blood was collected at frequent intervals (initially every 15 s, and at later decay every 5 min) for the purpose of analyzing insulin kinetics. The decay of the serum insulin concentration after constant insulin infusion was used to estimate the second (slow) component insulin half-life, because complete distribution of insulin in the sampling pool was assumed and endogenous insulin secretion was likely to be minimal.

Protocol 2: Pulse detection stringencies. To define the optimal stringencies for pulse detection, one must avoid detection of fluctuations in insulin concentrations arising from assay noise and biological noise (fluctuations arising from sampling technique, changes in blood flow, and the like). Deconvolution stringencies (statistical criteria for allowing changes in the insulin concentration to be defined as pulses) were adjusted to ensure minimal false-positive pulse detection (type I statistical error) when applied to insulin concentration changes due to assay (SDassay) and biological (SDbiol) noise. To determine the optimal stringencies to avoid false positives due to assay variability (SDassay), we assayed 75 plasma samples obtained from pooled plasma collected from three normal volunteers. The pooled plasma was collected at the fasting state and at various times postprandially to achieve a range of representative plasma insulin concentrations. The 75 samples were assayed in triplicate and analyzed by deconvolution as a mock plasma insulin concentration time series to define signal-free false-positive pulse detection.

Similarly, to account for SDbiol, we sampled plasma from three volunteers during suppression of endogenous insulin secretion (plasma C-peptide concentrations fully suppressed) by high-dose somatostatin infusion (300 µg/h), during a constant glucose infusion (2.5 mg·kg⁻¹·min⁻¹), and during a variable glucose infusion to achieve plasma glucose concentrations of 5 mM (euglycemic clamp). The samples were analyzed for plasma insulin concentrations in triplicate, and the plasma insulin concentration time series was subjected to deconvolution analysis.

Protocol 3: Sampling frequency. To examine whether a sampling frequency of 30 s vs. 1, 2, or 3 min per sample would yield improved pulse detection during endogenous pulsatile insulin secretion, we sampled every 30 s for 75 min during a constant glucose infusion (2.5 mg·kg⁻¹·min⁻¹) in three healthy volunteers. We then employed either every data point (thirty 3 s per sample) or every second (1 min per sample), fourth (2 min per sample), or sixth (3 min per sample) data point to calculate pulsatile insulin secretion in the face of different sampling densities.

Protocol 4: In vivo measurement of human pulsatile insulin release. Insulin secretion in healthy humans was examined during a low-dose glucose infusion (4.5 mg·kg⁻¹·min⁻¹) subject to optimal pulse detection conditions (above) during a known intermediate secretory stimulus. After 60 min of glucose infusion, 1 ml of blood was collected every minute for 75 min for measurement of serum insulin concentrations. At 15-min intervals, an additional 2-ml amount of blood was collected to allow measurement of serum C-peptide.

Assays

Insulin. Serum insulin concentrations were measured in triplicate by a two-site immunospecific insulin enzyme-linked immunosorbent assay (ELISA), as previously described (1). Briefly, the assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmark) specific for insulin. The detection range of this ELISA insulin assay is 5–600 pM. At medium (150 pM), medium-high (200 pM), and high (350 pM) plasma insulin concentrations, the interassay (among triplicate) variation coefficients are 3.7, 4.0, and 4.5%. Corresponding intra-assay variations are 2.3, 2.1, and 2.0%. There is no cross-reactivity with proinsulin, split (32, 33)-insulin, and des(31, 32)-proinsulin. The antibodies cross-react 30 and 63% with split (65–66)-proinsulin and des(64,65)-proinsulin, respectively. C-peptide, insulin-like growth factors I and II, and glucagon do not cross-react (1).

C-peptide. C-peptide measurements were performed using a commercially available kit (K6218, DAKO Diagnostics, Cambridgeshire, UK). The assay is a two-site ELISA, based on two monoclonal antibodies and employing the same principles referred to in insulin. Each sample was assayed in duplicate, and the intra- and interassay (among triplicate) variation coefficients were 2.2 and 3.3%.

Calculations

Insulin kinetics. For examination of the disappearance of the insulin bolus injection, a two-compartment model with bieponential insulin decay was assumed. The estimate of the second (slower) half-life was based on insulin decay after a 60-min insulin infusion. This second half-life was then used as an initial estimate when the decay in insulin concentrations was fit after an insulin bolus injection. The effective volume of distribution under conditions of constant delivery into the sampling compartment was calculated from insulin concentration data during the constant insulin infusion. The insulin data were deconvolved using the measured insulin kinetics, resulting in insulin delivery rates per unit volume of distribution that should equal the known insulin infusion rates.

Deconvolution analysis. The plasma insulin concentration time series were analyzed by deconvolution for purposes of insulin secretory pulse detection and quantification. Deconvolution of venous insulin concentration data was performed with a multiparameter technique (20) that requires the following assumptions. The venous plasma insulin concentrations in any one subject measured in samples collected at frequent intervals were assumed to result from five determinable and correlated parameters: 1) a finite number of discrete insulin secretory bursts occurring at specific times and having 2) individual amplitudes (maximal rate of secretion attained within a burst); 3) a common half-duration (duration of an algebraically Gaussian secretory pulse at half-maximal amplitude), superimposed on a 4) basal time-invariant insulin secretory rate; and 5) a bieponential insulin disappearance model in the systemic circulation consisting of estimated half-lives of 2.8 and 5.0 min, and a fractional slow compartment of 0.28, measured as we have described. Assuming the foregoing nominal insulin disappearance values, we estimated the number, locations, amplitudes, and half-duration of insulin secretory bursts, as well as a nonnegative basal insulin secretory rate, for each data set by nonlinear least squares fitting of the multiparameter convolution integral for each insulin time series. A modified Gauss-Newton quadratically convergent iterative technique was employed with an inverse (sample variance) weighting function (21). Parameters were estimated until their predicted values and the total fitted variance both varied by <1 part in 100,000.
Asymmetric highly correlated joint variance spaces were calculated for each parameter by the Monte Carlo support-plane procedure (21). Secretory rates were expressed as mass units of insulin (pmol) released per unit distribution volume (liter) per unit time (min). The mass of hormone secreted per burst (time integral of the calculated secretory burst) was thus computed as picomole insulin released per liter of systemic distribution volume. Because the calculated values truly represent hepatic vein insulin appearance, total insulin secretion was calculated from C-peptide concentrations (18). The serum insulin concentration time series obtained during a constant insulin infusion and ablation of endogenous insulin secretion (protocol 2) were subjected to deconvolution, and stringency for detection of insulin secretory bursts was increased progressively from, first, statistical confidence intervals of 67 to 95% for individual pulse amplitudes; second, for all pulse amplitudes, considered jointly; and, finally, for all pulse amplitudes and the pulse half-duration considered jointly. The use of confidence limits allowing <1 false positive pulse per 100 samples (min) was considered optimal. When the deconvolution analysis was performed, basal secretion was adjusted to allow accommodation of 95% of troughs. Likewise, the secretory burst half-duration was initially estimated as −1 min, allowed to fit individual unimodal secretory bursts, and then fit iteratively with all amplitudes, positions, and baseline.

SDassay and SDbiol. The assay uncertainty, or SDassay, was determined from the intrasample variance of the triplicates in each data set. Furthermore, because of dilution from saline infusate, hemolysis, proteases, and unknown reasons, biological variation, or SDbiol, may occur. SDbiol under sampling conditions was calculated from the variation (SDtotal) in plasma insulin concentration time series obtained during constant insulin infusion and ablation of endogenous insulin secretion by high-dose somatostatin infusion, employing

$$SD_{\text{biol}} = \sqrt{(SD_{\text{total}})^2 - (SD_{\text{assay}})^2}$$

(1)

Statistics

All data in texts and figures are given as means ± SE. Statistical methods related to detection of pulses are described in Calculations. Analysis of insulin decay is described in Insulin.

RESULTS

Insulin Kinetics

The mean insulin decay after a constant insulin infusion was measured to be 5.0 ± 0.1 min. When this was used as the second half-life in fitting the decay after a bolus insulin injection, the first half-life was 2.8 ± 0.4 min, and the fractional slow component was 0.28 ± 0.04. In examining the second half-life on the basis of disappearance of a single bolus rather than after discontinuation of a constant infusion, similar measures of second half-life were obtained (5.2 ± 0.2 min). Examples of three individual decays obtained by use of these values after constant and bolus injections are shown in Fig. 1. The calculated effective volume of distribution at steady state was 206 ± 18 ml/kg body weight.

Pulse Detection Stringencies

Application of 95% joint confidence limits to all pulse amplitudes resulted in two false-positive pulses per 225 min (P < 0.01 as desired). SDassay was calculated to be 2.2 ± 0.3%, whereas SDbiol under the study conditions was calculated to be 3.3 ± 0.4%. The variation(s) in insulin concentration due to assay alone and due to SDbiol and SDassay considered jointly are compared with a representative serum insulin concentration profile during endogenous insulin secretion in vivo in Fig. 2.

Sampling Frequency

When the sampling intensity is varied from 1 sample/30 s to 1 sample/min, the detected insulin pulse frequency was unchanged (shown in Fig. 3). However, further increase of the sampling interval to 2 or 3 min significantly reduced the number of pulses detected, as shown in Fig. 3. Consequently, the optimal sampling intensity under the present conditions (glucose infusion, measurements in triplicate, present ELISA, healthy humans) was sampling by the minute.

In Vivo Measurement of Human Pulsatile Insulin Release

In all cases, inspection of peripheral insulin concentration profiles showed obvious oscillations in the serum insulin concentrations, consisting primarily of serial data points leading to peaks and to troughs (Figs. 2 and 4). Deconvolution analysis confirmed that the insulin concentration oscillations were due to pulsatile insulin release (Fig. 4). This insulin release was best described as serial insulin secretory bursts superim-
posed on a nonnegative basal insulin secretion rate. The frequency of insulin secretory events was $4.7 \pm 0.1 \text{ min/pulse}$. The pulses had a mean amplitude of $35 \pm 4.7 \text{ pmol}\cdot\text{l}^{-1}\cdot\text{min}^{-1}$ and a mean secretory burst mass of $87 \pm 12 \text{ pmol}\cdot\text{l}^{-1}\cdot\text{pulse}^{-1}$. This results in a mean pulsatile insulin secretion rate of $19 \pm 2.6 \text{ pmol}\cdot\text{l}^{-1}\cdot\text{min}^{-1}$. In contrast, the calculated nonpulsatile (basal) insulin secretion rate was estimated to be $6.5 \pm 1.0 \text{ pmol}\cdot\text{l}^{-1}\cdot\text{min}^{-1}$. The relative contribution of pulsatile to total insulin secretion was $75 \pm 1.6\%$, with an absolute range of $59$–$85\%$. In cases with clearly separated pulses, the relative contribution of pulsatile to nonpulsatile insulin secretion tended to be higher. This probably resulted in a minimum estimate of pulsatile insulin secretion when pulses overlapped, because a clear separation of overlapping pulses into pulsatile and nonpulsatile secretion becomes difficult at higher frequencies. However, by $F$ ratio testing, best-fit estimate(s) were similar when a comparison was made between the presence or absence of a nonpulsatile component insulin secretion (Fig. 5). After waveform-independent deconvolution analysis, or “PULSE,” the individual insulin secretory bursts were by inspection of raw data symmetric and suitably fit via a family of Gaussian distribution(s) (Fig. 6).

**DISCUSSION**

In the present studies, we first established optimal parameters for detection of insulin secretory bursts in humans by deconvolution analysis of intensively sampled peripheral serum insulin concentration time series. We applied a highly sensitive and precise ELISA insulin assay for triplicate insulin measurements to minimize within-assay experimental uncertainty. Low-dose glucose infusion was imposed to facilitate the detection of insulin secretory events and to examine the significance and nature of stimulated pulsatile insulin secretion. We found that, overall, insulin secretion consisted predominantly of high-frequency insulin secretory bursts that were partially overlapping. The insulin secretion that was not detected as pulsatile accounted for no more than 25% of all insulin secreted. The calculated insulin kinetic values were similar to literature values obtained after decay of an intravenously injected insulin bolus (6). To examine insulin kinetics a two-phase protocol was chosen that showed good agreement. Endogenously secreted insulin is unlikely to influence the calculations, because C-peptide concentrations were suppressed, and hyperinsulinemia in the absence of hyperglycemia (damped glucose concentration 3.5–5.0 mM) is known to suppress insulin secretion (19). The deconvolution pulse detection criteria employed were carefully defined after sequential analysis of mock insulin concentration time series derived from repetitive triplicate insulin measurements of pooled plasma and from triplicate insulin concentration measurements.
Fig. 4. Representative insulin concentration profile (bottom) together with deconvolution-defined best-fit curves, which are predicted concentration profiles that would result from deconvolution-calculated insulin secretion rates (top) and known insulin kinetics. Note almost superimposable raw data and best-fit curves.

Fig. 5. Representative insulin concentration profile analyzed by fitting basal (nonpulsatile) insulin secretion (A) and assuming a basal secretion of 0 (B). Best-fit concentration profile is very similar to the observed concentration time series in both cases, and it yields a similar fitted variance. Thus data show that more insulin is released in bursts, and, indeed, is compatible with a model assuming purely pulsatile insulin release.
time series obtained during a constant insulin infusion with concomitantly ablated endogenous insulin secretion. As shown earlier for pulse-analysis optimization (22), different assay(s), number(s) of replicates, and sampling procedure(s) may influence the preferred criteria.

Further increase of the blood sampling intensity to every 30 s vs. every minute did not improve the detection of endogenous insulin secretory bursts significantly. This may also be the result of optimized methods for pulse detection, the use of stimulated insulin secretion, a highly sensitive and precise insulin assay, and the application of deconvolution analysis vs. conventional pulse detection. The former has been shown to improve pulse detection (13), particularly when pulses are partially overlapping. In studies employing less sensitive or less reproducible insulin assays, increased sampling frequency and replications could potentially be advantageous. Also, the relatively shorter half-life of insulin vs. C-peptide (18) favors the use of insulin vs. C-peptide for purposes of deconvolution analysis, because consecutive insulin secretory bursts may be easier to separate. The variable insulin half-lives among different subjects, in contrast to the fairly constant C-peptide half-lives (18), were dealt with by application of a waveform-independent deconvolution method that incorporates the population variability in the insulin half-life as obtained by protocol 1.

Sampling from peripheral blood results in an inherent time lag between the secretory event and detection of insulin concentration changes in peripheral blood. This time delay also is likely to cause damping of the insulin secretory burst (13) and thereby to underrepresent the pulsatile component of overall insulin secretion. However, the present data are similar to calculated relative contributions of pulsatile to total insulin secretion in dogs, in which blood was obtained directly from the portal vein, and independent mathematical analyses were applied (15).

We report a mean interpulse interval (time from pulse peak to pulse peak) of 5 min for glucose-stimulated pulsatile insulin secretion, which is less than previously inferred in humans during euglycemia (10). However, studies employing portal vein sampling report a similar frequency at similar glucose concentrations in dogs (14) and in humans with cirrhosis (16). Our combined use of high-sensitivity assay, high-intensity blood sampling (0.5–1.0 min/sample), glucose infusion, and validated deconvolution analysis likely improves pulse detection in vivo (13). Indeed, the in vivo frequency reported here is similar to that reported in the isolated perfused pancreas (7) and in the isolated perfused islet (11). Studies have shown oscillations in the intracellular calcium concentrations that have similar periodicity (9). In aggregate, therefore, the very similar oscillatory patterns observed for membrane potentials (5), intracellular calcium (9, 11), and insulin secretion in vitro (3, 11) in individual islets conform with the oscillatory nature of peripheral insulin concentrations in vivo inferred here. The in vivo frequency tends to be slightly higher, perhaps reflecting either pulse detection differences or a decrease in pacemaker activity in the in vivo milieu.

Overall insulin secretion was resolved into a series of partially overlapping symmetric and discrete secretory events with little basal or nonpulsatile insulin secretion. To the extent that these bursts overlap, resolution of interpulse insulin secretion into basal secretion vs. ultra-high-frequency overlapping pulses is less achievable. The analysis applied here favors detection of some nonpulsatile insulin secretion if events partially overlap, and, of note, apparent basal secretion was inferred in all 20 studies. Thus our estimate of 75% pulsatile insulin secretion is likely to be a minimum value. In addition, analysis of the fitted variance ratios assuming zero vs. finite (positive) nonpulsatile insulin secretion did not consistently show a significantly better fit in the absence of assumed and/or calculated basal insulin secretion.

The quantification of insulin secretion as predominantly pulsatile also suggests that significant intrapancrpic coordination is necessary to govern the relative timing of insulin release from discrete islets. In theory, oscillating insulin release may be due to periodic stimulation, periodic inhibition, or both. For example, in the case of growth hormone (GH) release, balanced inhibition and stimulation by, respectively, GH-releasing hormone and somatostatin with concomitantly decreased inhibition and agonist-driven secretion serve to organize secretion into intermitten secretory bursts (17).

Impaired pulsatile insulin release has been reported in type II diabetics and first-degree relatives of patients
with type II diabetes (12), underlining the possible importance of disrupted secretory patterns in these states. Furthermore, a blunted insulin secretory response to an oral or intravenous glucose challenge in type II diabetes is a hallmark of this disease (4). The central role of pulsatile secretion in overall insulin release suggests a link between impaired pulsatile insulin secretion and inappropriate glucose-stimulated insulin secretion in type II diabetes.

The insulin secretory pattern reported in this study implies that most β-cells secrete simultaneously over a 2- to 4-min period, with a frequency of one burst every 5 min. The duration of nonsecretory “basal” intervals is inevitably dependent on the frequency of insulin secretory bursts. The latter may be increased with hyperglycemia (14). If the nonsecretory phase is important for restitution of β-cells and for their nonsecretory functions, then the duration of this nonsecretory phase may be important for long-term β-cell performance.

For detailed analysis of pulsatile insulin secretion, mathematical methods based on merely measuring amplitudes of peripheral insulin oscillations may be hampered by changes in the frequency of insulin secretory bursts, because the latter will cause changes in the nadir levels between pulses and thus influence the incremental and maximal (absolute) amplitude of these oscillations. For example, studies comparing pulsatile insulin secretion during different glucose concentrations, in which circumstances of different frequencies may be assumed (14), will tend to favor detection of pulsatile insulin secretion at lower glucose concentrations. Deconvolution analysis to accommodate succession of peaks and troughs should improve the appreciation of insulin secretory bursts at higher glucose concentrations (22).

Whereas it thus appears that, in healthy humans, glucose-stimulated insulin secretion mainly consists of series of punctuated insulin secretory bursts rather than basal or nonpulsatile secretion, the mechanisms by which overall secretion is regulated through changes in frequency, mass, and/or shape of pulsatile insulin secretion require further study. Moreover, the mechanisms underlying the impaired pulsatile insulin secretion observed in type II diabetes are likely to be important in understanding the characteristically blunted insulin secretory responses to relevant challenges in this disease.

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