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

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Insulin is released in a high-frequency pulsatile secretory pattern, which is reflected as quantifiable oscillations in peripheral circulating insulin concentrations. Type 2 diabetes mellitus is characterized by a broad spectrum of abnormalities in β-cell function, including disturbed pulsatile insulin secretion as assessed by autocorrelation analysis. To achieve further insight into β-cell pathophysiology in type 2 diabetes, we examined the orderliness of the baseline serum insulin time series (blood collection every minute for 75 minutes) in 16 type 2 diabetics (fasting plasma glucose, 170 ± 10 mg/dL [mean ± SE]; serum free fatty acid [FFA], 0.794 ± 0.083 mmol/L; and known diabetes duration, 6 ± 2 years) and 15 healthy controls (serum FFA, 0.523 ± 0.055 mmol/L). We used approximate entropy (ApEn), a recently introduced scale- and model-independent measure of serial irregularity. ApEn was significantly increased in the type 2 diabetics compared with the controls (0.671 ± 0.016 *v* 0.653 ± 0.008, *P* = .04), indicating more irregular serum insulin time series in diabetics. Autocorrelation also discriminated between groups, although only when the data were pooled. Interestingly, an inverse relationship between ApEn and serum FFA was observed in the controls ($r = -.63$, $P = .01$) and diabetics ($r = -.65$, $P < .01$), whereas no relationships were found between ApEn and the age, body mass index (BMI), or plasma glucose. In conclusion, type 2 diabetes is characterized by an increased disorderliness of the fasting serum insulin time series, strongly suggesting perturbed rapid oscillatory insulin release. An inverse relationship between ApEn and fasting serum FFA among both groups might suggest a hitherto unknown stabilizing action of FFA on the high-frequency pulsatile insulin release process. This hypothesis needs to be tested in experimental designs that more specifically focus on this issue, eg, during changes in serum FFA.

TYPE 2 DIABETES MELLITUS is a heterogeneous disorder characterized by insulin resistance and absolute or relative insulin secretory deficiency. Since the classic study by Yalow and Berson,[1] an immense number of studies have highlighted β-cell dysfunction in type 2 diabetes mellitus.[2-5] Alterations include the loss or blunting of first-phase insulin secretion, decreased ability of non-glucose secretagogues to potentiate insulin secretion during hyperglycemia, delayed or attenuated secretory responses to ingestion of a mixed meal, increased circulating levels of proinsulin relative to insulin, diminished insulin secretion rate during a graded intravenous glucose infusion, alterations in ultradian oscillations of insulin secretion, and disrupted high-frequency pulsatile insulin secretion. However, some of the abnormalities have been shown to be more or less reversible when the glycemic level is nearly normalized.

The oscillatory secretion of pancreatic islet hormones is well established.[6,7] More recent studies have demonstrated that the majority of normal insulin secretion occurs in pulsatile bursts.[8,9] In healthy humans, high-frequency pulses (intervals between 6 and 13 minutes) have been reported,[7,9] together with oscillations of slower and larger amplitude and a periodicity of approximately 120 minutes.[10] The mechanisms for the synchrony of insulin secretion within and between islets, albeit putatively ascribable to an intrinsic β-cell pacemaker, are still only partly understood and enigmatic. Rapid insulin oscillations are evident in the perfused pancreas[11] and during constant glucose infusion in healthy humans.[12] However, some data show a temporal relationship between insulin oscillations and plasma glucose excursions, which may indicate that a feedback loop between β cells and the liver contributes to regulation of the insulin release process.[7]

The literature on high-frequency insulin pulses in type 2 diabetic individuals is limited. One hypothesis is that disturbed pulsatile insulin secretion in type 2 diabetes mellitus is an important link between insulin secretion and the insulin resistance of type 2 diabetes, because pulsatile insulin delivery exerts greater insulin action compared with nonpulsatile insulin delivery.[13-15] Based on autocorrelation analysis of serum insulin time series, Lang et al[16] observed irregular oscillations of the baseline insulin concentration in type 2 diabetic individuals, probably reflecting abnormalities in the series of metabolic and signaling events involved in the cyclic release of insulin. Since then, more sensitive and specific insulin assays have been developed[17] and other mathematic approaches to assess signal disruption have been presented.

We sought to add another dimension to our understanding of β-cell dysfunction in type 2 diabetes by examining time series of baseline circulating insulin (and glucose) in a group of type 2 diabetics and healthy subjects by irregularity assessment and autocorrelation analysis. To quantify irregularity, we used approximate entropy (ApEn), a recently developed scale- and model-independent statistic that quantifies the orderliness of

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serial data and is complementary to other algorithms for pulse identification.[18-21] Previously, a loss of regularity of insulin secretion has been identified in elderly people[22] and in healthy first-degree relatives of diabetics during parenteral glucose stimulation[23] using ApEn.

SUBJECTS AND METHODS

Subjects

Sixteen individuals (10 males and 6 females) with type 2 diabetes according to American Diabetes Association criteria and 15 healthy subjects (9 males and 6 females) without any family history of diabetes mellitus participated in the study. All were anti-glutamic acid decarboxylase (GAD)–negative. The mean age and body mass index (BMI) were 53 ± 2 years (mean ± SE) and 26.9 ± 0.9 kg/m^2 in the diabetic group and 40 ± 3 years and 25.5 ± 1.1 kg/m^2 in the control group. The mean known diabetes duration was 6 ± 2 years. Seven diabetics were only treated with diet, and the other 9 were treated with oral agents (sulfonylurea, n = 4; biguanides, n = 3; or both, n = 2). Antidiabetic agents were not used in the last 36 hours prior to examination. The protocol was approved by the Ethical Committee of the County of Aarhus, Denmark.

Sampling Protocol

At 8 AM after a 10-hour fast, a catheter (Venflon 17G/45 mm; BOC Ohmeda, Helsingborg, Sweden) was placed in an antecubital vein for frequent blood sampling. Sampling began 30 minutes later and continued for 75 minutes. At baseline, blood for determination of free fatty acid (FFA) and hemoglobin A_{1c} (in diabetics) was obtained. The total dead space in the cannula and the 3-way stopcock (Connecta; BOC Ohmeda) was 0.38 mL. The catheter was perfused with saline (0.9%, 1.0 mL/min). For the determination of insulin and plasma glucose, blood was collected every minute as follows. Twenty seconds before sampling, the saline infusion was stopped and 0.5 mL blood was withdrawn and discarded. From time −5 to +5 seconds, 1.5 mL blood was removed at a constant rate with a 2-mL syringe. Then, the saline infusion was repeated until 20 seconds prior to the next sampling. This sampling procedure has been previously evaluated by our group, and the so-called biologic variation was less than 4%.[24]

Assays

The plasma glucose level was measured in duplicate shortly after sampling (Beckman Instruments, Palo Alto, CA). Circulating insulin concentrations were measured in duplicate by enzyme-linked immunosorbent assay using a 2-site immunoassay[17] that does not detect proinsulin or split(32-33)- and des(31-32)-proinsulin. Split(65-66)- and des(64-65)-proinsulin cross-react 30% and 63%, respectively. The minimal detection limit was 5 pmol/L. The intraassay coefficient of variation was 2.8% and 2.4% at concentrations of 150 and 350 pmol/L, respectively. FFA was determined by a colorimetric method using a commercial kit (Wako Chemicals, Neuss, Germany).

Analytic Strategy

Although the mean circulating glucose and insulin apparently showed no trend during the 75-minute sampling period, there were considerable individual long-term trends in serum insulin presumably due to ultradian oscillations. To improve the identification of short-term oscillations, these trends were removed by first-differencing. This is a standard statistical method to stationarize time series[25] and it is applicable to a broad class of models. Furthermore, in differencing, episodic versus random distinctions present in the original data set are mostly retained. By analyzing the first-differenced process, considerable baseline information of possible significance may be discarded. However, there is a precedent for handling the nonstationarities in insulin time series via the first-differenced derivatives. To allow comparisons to earlier studies,[16,26] which, by using autocorrelation analysis, reported irregular high-frequency oscillations of circulating insulin and glucose in type 2 diabetic and prediabetic individuals, the data were also assessed using 3-point moving-average smoothing prior to autocorrelation (vide infra).

ApEn

The regularity of the serum insulin concentration time series was quantified by ApEn, a model-independent statistic described elsewhere.[18,19] ApEn is complementary to algorithms for pulse identification, which arise mechanistically from different perspectives of hormone secretion.[18,19] ApEn evaluates both dominant and subordinate patterns in data and detects changes in the underlying episodic behaviors not reflected in the peak occurrence or amplitude.[21] The algorithm has been applied to assess release processes of growth hormone, luteinizing hormone, follicle-stimulating hormone, corticotropin, parathyroid hormone, and testosterone in health and disease. ApEn calibrates an ensemble of sequential interrelationships, quantifying a continuum that ranges from totally ordered to completely random. In brief, ApEn assigns a single nonnegative number to a time series, where larger values correspond to greater apparent process randomness and smaller values correspond to more instances of recognizable patterns or consistent features in the data.

For this study, we primarily calculated ApEn values for all data sets with m equal to 1 and *r* equal to 1.0 standard deviation (SD) of the individual subject time series, applied to the first-differenced time series as already indicated. However, we also used *r* values of .7, .5, .3, and .2 SD (vide infra). Normalizing *r* to each time series SD gives ApEn a translation and scale invariance to the absolute serum concentrations.[21] Multiple previous studies that included both theoretic analysis[18,27,28] and clinical applications[20,22,23] have demonstrated that applications of ApEn with m equal to 1 produce good statistical reproducibility for ApEn for time series of the length analyzed herein. The choice of an *r* value of 1.0 SD provides a coarser-scale (macroscopic) assessment of subpattern persistence within a time series, while in other settings, group differences are more pronounced at a finer (microscopic) level, eg, with a parameter choice of *r* equal to .2 SD.[20,22,23]

Further technical discussion of the mathematic and statistical properties of ApEn, including robustness to noise and artifacts, mesh interplay, relative consistency of (m,*r*) pair choices, asymptotic normality under general assumptions, statistical bias, and error estimation for general processes, can be found elsewhere.[18,27,28] To develop a more intuitive, physiologic understanding of the ApEn definition, a multistep description of its typical algorithmic implementation, with figures, was also developed.[27] As well, Fig 1 in Schmitz et al[23] provides a schematic diagram of the core comparisons in the ApEn calculation.

Autocorrelation

Autocorrelation analysis was performed on the individual time series with and without prior smoothing to detect high-frequency pulsatility (SPSS version 8.0; SPSS, Chicago, IL). By autocorrelation analysis, the obtained data are correlated to duplicate data lagged by 1-min intervals. By definition, the autocorrelation coefficient at time lag 0 minutes is 1. By displacement of the time series, the copy gradually becomes out of phase and the correlation declines and becomes negative. If the time series shows regular oscillations, the copy will subsequently move back into phase by further displacement, resulting in a positive autocorrelation. An autocorrelation coefficient was considered significant if the first positive peak after the first trough exceeded the 95% confidence interval, or if the autocorrelogram had a sinusoidal shape and one of the subsequent peaks exceeded the confidence interval. To determine the

frequency of oscillations by autocorrelation, data from the subjects were pooled. Autocorrelation coefficients (m) were transformed to Z values by Fisher's Z transformation; the mean value was calculated and transformed to m values by the inverse procedure.

Statistical Analysis

Results are expressed as the mean ± SE. The statistical significance of differences between groups was evaluated by Student's 2-tailed unpaired *t* test or the Mann-Whitney rank sum test when data were not normally distributed. Bivariate correlations between ApEn and the BMI, age, plasma glucose, and serum FFA were tested using Spearman's rank order correlation. Differences were considered significant at a *P* value less than .05.

RESULTS

Clinical and Biochemical Characteristics of the Study Groups

The diabetic group was older (on average, 13 years) and had a slightly higher BMI (on average, 1.4 kg/m^2) than the control group (both $P < .01$). Gender distribution did not differ between the two groups. Fasting plasma glucose was 170 ± 10 mg/dL in the diabetic group, compared with 90 ± 2 md/dL in the controls ($P < .001$). Hemoglobin A_{1c} was 7.9% ± 0.5% in the diabetic (normal range, 4.8% to 6.4%). Serum insulin tended to be higher in the diabetic group, but was not statistically significant (49 ± 7 *v* 36 ± 4 pmol/L, $P = .10$). Serum FFA was elevated in the diabetic group compared with the controls (0.794 ± 0.083 *v* 0.523 ± 0.055 mmol/L, $P = .01$).

ApEn

ApEn was significantly increased in type 2 diabetic subjects (0.671 ± 0.016) compared with the healthy controls (0.653 ± 0.008, $P = .04$), indicating a more disorderly serum insulin time series in the diabetic individuals (Fig 1). However, there was a large overlap between the two groups. ApEn did not differ among diabetics treated solely with diet versus oral hypoglycemic agents ($P = .76$). Bivariate correlation analyses between ApEn and age, BMI, fasting plasma glucose, and serum FFA showed that FFA was significantly (inversely) related to ApEn in both controls ($r = -.63$, $P = .01$) and type 2 diabetics ($r = -.65$, $P < .01$) (Fig 2). There was no relationship between ApEn and the BMI, age, and fasting plasma glucose (all $P > .40$). The regularity of plasma glucose in the diabetics and controls was comparable (0.583 ± 0.004 *v* 0.604 ± 0.005, $P = .73$).

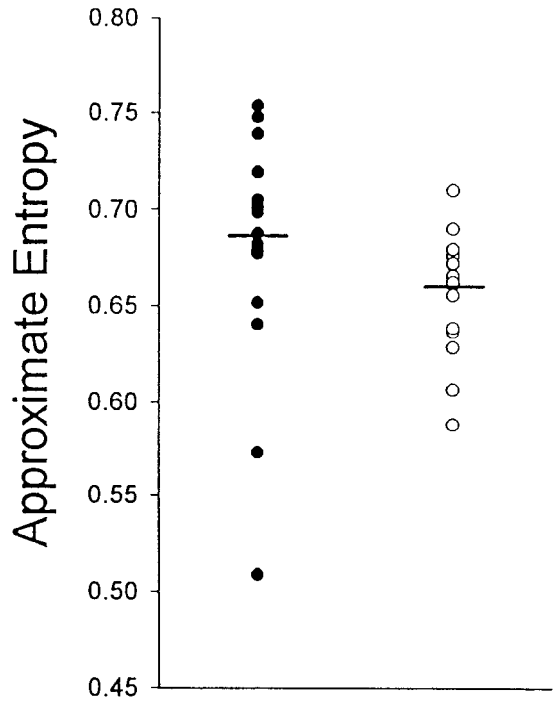

Fig 1. Approximate entropy of fasting serum insulin time series in 16 type 2 diabetics (●) and 15 controls (○).

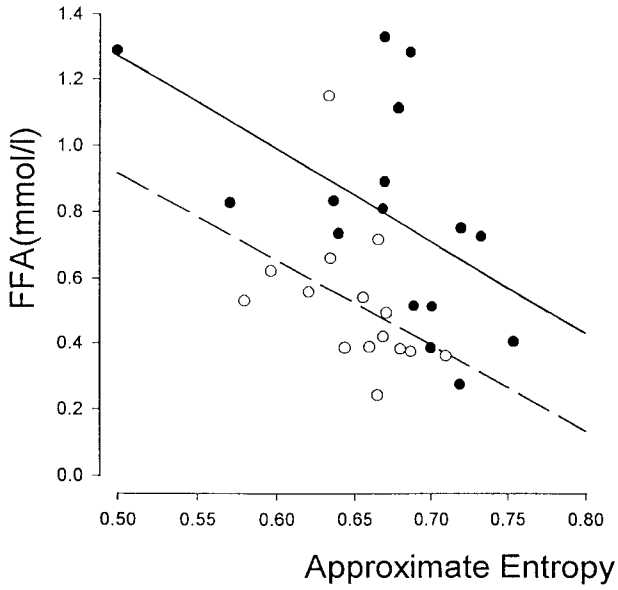

Fig 2. Relationship between approximate entropy of fasting serum insulin time series and fasting serum FFA in 16 type 2 diabetics (●, $r = -.65$, $P < .01$) and 15 controls (○, $r = -.63$, $P = .01$). The regression lines are also shown.

ApEn did not differ between the groups when applied to serum insulin time series using the finer-scale assessments (input parameter choice of $r = 50\%$ and 20% SD, data not shown). However, the inverse relationship between ApEn and FFA was robust in the controls, being statistically significant at minimum *r* values of 30% SD, whereas the relationship in type 2 diabetic individuals vanished at *r* values less than 70% SD.

Autocorrelation

Significant positive peaks of autocorrelation were observed in a similar number of type 2 diabetic individuals and controls, namely 3 of 16 and 3 of 15 participants in the two groups, respectively. Calculations were also performed on time series initially smoothed using a 3-point moving average and subsequently stationarized by differencing as described by O'Rahilly et al.[26] With this analytic strategy, the number of individuals exhibiting significant positive autocorrelation increased in both groups to 6 of 16 and 8 of 15 in the diabetic and control groups,

respectively. However, there was no statistical difference between the groups ($P = .78$).

Figure 3 shows the pooled autocorrelation data for the controls and type 2 diabetics. The controls exhibited a significant peak of regular oscillations at 11 minutes ($r = .099$, $P = .01$). Although the correlogram for diabetic individuals showed a pattern resembling a regular pulsatile pattern, the peaks did not exceed the confidence limits (at lag time = 7 minutes, $r = .043$, $P = .14$; at lag time = 15 minutes, $r = .050$, $P = .12$).

DISCUSSION

In parallel with an increased understanding of metabolic oscillations in the β cell,[29] there has been an increased interest in the high-frequency insulin release process. The current study used a very sensitive and specific insulin assay together with the ApEn algorithm to demonstrate that fasting serum insulin time series in type 2 diabetic patients are less regular than those in healthy individuals. Moreover, there was a reasonable sensitivity and specificity with ApEn in separating controls from type 2 diabetics. At a cutoff point of 0.669 for ApEn, 12 of 16 diabetics exceeded this value, in contrast to only 4 of 15 controls. It should be noted that the difference between groups was only present when a coarse-scale (macroscopic) assessment of subpattern persistence within the time series was used.

It may be argued that irregular peripherally sampled serum insulin time series do not reflect disturbed insulin secretion but instead an irregular hepatic insulin extraction. However, this argument is unlikely for two reasons. First, a study performed in dogs with portal catheters[8] demonstrated that peripherally collected insulin time series mirror the portal profiles not only in the fasting state but also in the postprandial state, with the latter characterized by changes in insulin and FFA. Second, to challenge the hypothesis, we also measured the plasma C-peptide level (which does not undergo hepatic extraction) in 4 of the time series, and found a strong relationship between ApEn on insulin time series and ApEn on C-peptide time series ($r = +.88$).

Autocorrelation analyses also discriminated between groups, but only when the data were pooled. Interestingly, pooled autocorrelation data showed more frequent oscillations in the diabetic group compared with the control group (7 *v* 11 minutes), thus confirming the early observations from Oxford.[16] However, the number of individuals who presented a significant autocorrelation coefficient did not differ between the two groups.

A novel and unexpected observation in this study is the inverse relationship between FFA and ApEn not only in the diabetic individuals but also in the controls. Although it has been recognized for years that an elevation of FFA concentrations enhances the glucose-stimulated insulin response,[30,31] a number of studies in rats[32-34] and humans[35-37] during the past few years have emphasized the pivotal role of FFA-dependent insulin secretion in glucose-stimulated insulin secretion, which may be even more important for non–glucose-stimulated insulin secretion.[38] Prentki et al[39] have hypothesized that long-chain fatty acyl-coenzyme A esters (LC-CoA) are among several signals capable of modifying β-cell release of insulin in an oscillatory manner, with a possibly permissive role for changes in intracellular calcium. Exogenous FFAs are activated to LC-CoA, which may exert significant actions on a variety of steps in β-cell metabolism, as shown mainly by Corkey's group.[38-40]

While previous in vivo studies on β-cell function have focused on the influence of FFA on quantitative insulin secretion, the current report indicates (although indirectly) for the first time in humans that FFA furthermore could be an important regulator of the high-frequency pulsatile insulin release

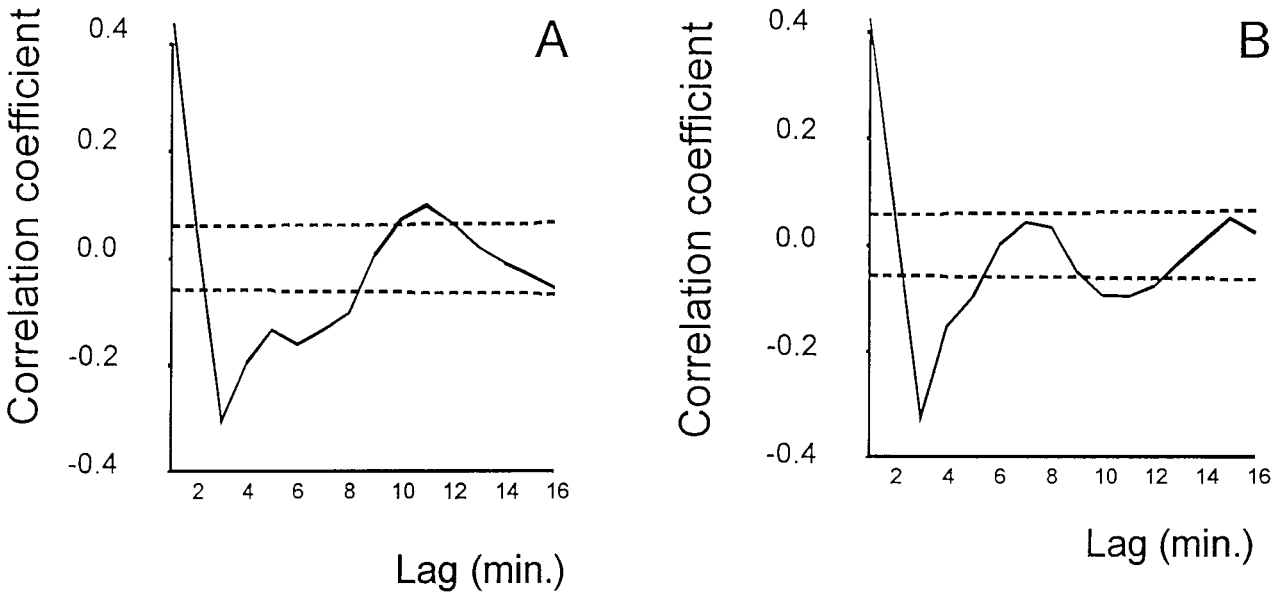

Fig 3. Pooled autocorrelograms of serum insulin time series in 15 healthy controls (A) and 16 type 2 diabetics (B) following 3-point moving-average and first-differencing. Controls exhibited a peak of regular oscillatory activity every 11 minutes, whereas the diabetics tended to maintain peaks every 7 and 15 minutes. (- - -) 95% confidence intervals for a null hypothesis of no significant autocorrelation.

process in overnight-fasted individuals in conditions with elevated FFAs and in healthy subjects. However, our observations do not allow us to exclude FFA as a surrogate measure for other yet undefined key modulators of oscillatory insulin release.

The diabetic patients in the current study had only a moderate elevation of serum FFA. It is possible that a more pronounced exposure to FFA for a longer period, which would presumably result in triglyceride accumulation in β cells,[41] would abolish or invert the reciprocal relationship between the oscillatory pattern of insulin time series and circulating FFA.

Serum FFA was inversely related to ApEn. Since serum FFA was significantly increased in the type 2 diabetics compared with the controls, the difference in ApEn between the two groups, when adjusted for FFA, would be even larger. Although the study cohorts were not matched in terms of age, BMI, and plasma glucose (per definition), there was not so much as a trend for a relationship between these variables and ApEn (in the diabetic group, $P = .86$, .79, and .47, respectively). This strongly suggests that other factors, possibly including genetic influences, may be responsible for the greater irregularity of insulin oscillations in diabetic individuals. A genetic contribution to the augmented disorderliness in the diabetic group would be in line with a previous study[23] in healthy first-degree relatives of type 2 diabetics, who exhibited an increased irregularity in glucose-stimulated insulin time series. Furthermore, it is of interest to note that Paolisso et al[37] have demonstrated that reducing plasma FFA levels in first-degree relatives of type 2 diabetics leads to an improvement of the acute insulin response, while it is apparently suppressed in other subjects.[35,36]

In contrast to the study by Meneilly et al[22] also using ApEn on insulin time series, we did not demonstrate an association between the disorderly insulin secretory pattern and age. However, the present study was not designed to detect a possible age-dependent influence on insulin secretion. Our participants had a mean age of 40 years (controls) and 53 years (type 2 diabetics), a small distinction, in contrast to the aforementioned study which examined groups with a mean age of 24 and 77 years, respectively.

In conclusion, the present study demonstrates that overnight-fasted type 2 diabetic individuals treated with diet solely or oral hypoglycemic agents display irregular high-frequency insulin oscillations, as assessed by autocorrelation analysis and for the first time by irregularity analysis via the complementary ApEn algorithm. The latter may represent an important new tool in quantifying the control of β-cell function in health and disease. No relationship was found between the disorderly serum insulin time series and the level of glycemia, but an inverse relationship was found between serum FFA and irregularity in both the controls and the diabetics. It is tempting to suggest that after an overnight fast, FFA has a regulatory and stabilizing influence on the pulsatile insulin release process. This possibility and its physiologic relevance should be further explored in studies in which serum FFA can be modulated experimentally.

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