

# Deficiency of Phosphofructo-1-Kinase/Muscle Subtype in Humans Is Associated With Impairment of Insulin Secretory Oscillations

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In healthy humans, insulin is secreted in an oscillatory manner. While the underlying mechanisms generating these oscillations are not fully established, increasing evidence suggests a central role for phosphofructo-1-kinase/muscle subtype (PFK1-M), which also serves as the predominantly active PFK1 subtype in the pancreatic  $\beta$ -cell. The fact that normal oscillatory secretion is impaired in subjects with impaired glucose tolerance and healthy relatives of patients with type 2 diabetes suggests that this defect may be involved in the secretory dysfunction. To evaluate a possible link between inherited PFK1-M deficiency in humans (Tarui's disease or glycogenosis type VII) and altered insulin oscillations, *in vivo* studies were performed. We determined basal insulin oscillations during 2 h of frequent plasma sampling in two related teen-aged individuals with homozygous and heterozygous PFK1-M deficiency compared with nondeficient, unrelated control subjects. As predicted by the underlying hypothesis, normal oscillations in insulin secretion were completely abolished in the individual with homozygous deficiency of PFK1-M and significantly impaired in the heterozygous individual, as shown by spectral density and autocorrelation analyses. Thus, deficiency of PFK1-M subtype in humans appears to be associated with an impaired oscillatory insulin secretion pattern and may contribute to the commonly observed secretion defects occurring in type 2 diabetes. *Diabetes* 48:1557–1561, 1999

**W**hile the observation of oscillatory insulin secretion was first described decades ago (1–4), its relevance to the pathogenesis of type 2 diabetes was repeatedly shown in later years, as impaired oscillations were proposed to be an early step in the development of type 2 diabetes (5,6). Thus, the origin of

reduced pulsatility may be one important factor contributing to the impaired  $\beta$ -cell function observed in type 2 diabetes (5–8). Recent studies by Tornheim (8) and others have demonstrated that pulsatile insulin secretion may be linked to glycolytic oscillations in the pancreatic  $\beta$ -cell, defining the central stimulus-secretion coupling process. In addition to the  $\beta$ -cell-specific function of glucokinase, which has been shown to be associated with a maturity-onset diabetes of the young subtype (9), the rate-limiting step in glycolysis is the phosphorylating enzyme phosphofructo-1-kinase (PFK1) (EC 2.7.1.11) (10). PFK1 occurs in humans as three distinct subtypes encoded on different chromosomes. According to their predominant expression, these subtypes were named muscle (PFK1-M), liver, and platelet subtypes (11). PFK1-M has been shown to be regulated autocatalytically in an AMP-dependent oscillatory manner in muscle (12) as well as in pancreatic  $\beta$ -cells (13). Furthermore, PFK1-M has been demonstrated to be the predominantly active subtype in both of these tissues (11,13). Thus, oscillations in glycolysis have been proposed to generate changes in the ATP/ADP ratio of the  $\beta$ -cell, leading to regulation of  $K_{ATP}$  channels triggering oscillatory insulin secretion (8,13). While this coupling of PFK1-M-dependent ATP oscillations to pulsatile insulin secretion appears to be a convincing hypothesis supported by experimental evidence, direct proof—obtained by a PFK1-M knockout model, for instance—is lacking (8).

We therefore decided to study humans with an inherited deficiency of PFK1-M, a rarely diagnosed (frequency 1:100,000 [14]) disorder called glycogenosis type VII or Tarui's disease (15). The clinical features of this disease include excess glycogen storage, myopathy, hemolysis, and lack of exercise-induced lactate increase in peripheral venous blood (16,17). While only homozygously affected individuals exhibit these features, heterozygous deficiency is asymptomatic and detectable only by measuring reduced PFK1 activity in red blood cells (RBCs). Recently, we demonstrated that homozygous PFK1-M deficiency predisposes to type 2 diabetes (18). Individuals with homozygous PFK1-M deficiency exhibited a loss of intravenous glucose-induced first-phase insulin secretion, while both homo- and heterozygous PFK1-M deficiency led to peripheral insulin resistance.

To gain a better understanding of a possible link between PFK1-M activity and the regulation of  $K_{ATP}$  channels in the pancreatic  $\beta$ -cell, we evaluated alterations in basal oscillatory insulin secretion in a naturally occurring human knockout model for PFK1-M. In the present study, we found a significant impairment in the pulsatility of insulin secretion in these patients, suggesting a link between PFK1-M activity and insulin oscillations.

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IVGTT, intravenous glucose tolerance test; OGTT, oral glucose tolerance test; PFK1, phosphofructo-1-kinase; PFK1-M, phosphofructo-1-kinase/muscle subtype; RBC, red blood cell; RIA, radioimmunoassay.

RESEARCH DESIGN AND METHODS

**Patients.** Two brothers were evaluated in this study. Both parents were also available, but the mother had overt type 2 diabetes and the father had elevated fasting blood glucose; they were therefore excluded (19). The subjects evaluated were born in the Ukraine and were of Ashkenazi-Jewish origin. The clinical characteristics of these individuals are published elsewhere (17,18) and are summarized in Table 1. The older brother (brother I, age 19 years) showed exercise-induced myopathy and hemolysis as well as excess glycogen storage, typically observed in Tarui's disease, while the younger brother (brother II, age 13 years) had no clinically apparent symptoms. Brother I had a history of insulin-treated diabetes during a hepatitis A infection. At that time, he was treated with steroids of unknown dosage.

Biopsies from the quadriceps muscle and subsequent histochemical staining showed absence of PFK1-M in brother I. Determination of total PFK1 activity in protein extracts of RBCs (performed by Dr. E. van Schaftingen, Bruxelles) showed a ~50% reduction in brother I and a ~25% reduction in brother II. Since PFK1-M contributes 50% of total PFK1 activity in RBCs (20), these results are compatible with a complete deficiency of PFK1-M in brother I and a ~50% reduction in brother II (Table 1). Subsequent genetic analysis revealed that both brothers had a G-to-A point mutation at the first nucleotide of intron 5, changing the invariant GT of the splice donor site to AT and leading to exon 5 deletion by missplicing (Table 1). Brother I additionally had a single base deletion of the nucleotide 2003 within exon 22, resulting in a frameshift leading to a stop codon 47 nucleotides downstream. This predicted the generation of a truncated PFK1-M protein with 16 altered amino acids at the COOH-terminus. Therefore, brother I was shown to be homozygously PFK1-M deficient, while brother II was heterozygously deficient for PFK1-M protein (Table 1). Subsequent phenotyping for disturbances of glucose metabolism revealed both insulin resistance and impaired  $\beta$ -cell function. While a hyperinsulinemic-euglycemic clamp did not reveal significant insulin resistance, an octreotide-based quantification of insulin resistance (21) showed marked insulin resistance in the homozygous brother I. The heterozygous brother II was also shown to be significantly more resistant than matched control subjects, although his resistance was less marked than that of his older brother (Table 1). Oral glucose tolerance tests (OGTTs) of both brothers were shown to be normal using a 75-g glucose load based on World Health Organization criteria (Table 1), while a 100-g (irregular) load revealed impaired glucose tolerance only in the homozygously affected brother I (18). Determination of  $\beta$ -cell function by intravenous glucose tolerance test (IVGTT; 0.33 g glucose per kilogram body weight) showed at least a 50% loss of first-phase insulin secretion (3–5 min after stimulus) in the homozygously affected brother I, whereas the first-phase secretion did not appear to be significantly altered in the heterozygously affected brother II (Table 1).

Since no unaffected individuals (carrying two nonmutant/wild-type PFK1-M alleles) could be found in the family—a typical feature of this compound heterozygous, therefore pseudodominant, disease (22)—control subjects for these parameters were unrelated and matched by age, BMI, and sex (Table 1). For comparison of insulin oscillatory patterns, we selected data from a single subject representative of seven unrelated healthy male subjects. This subject was somewhat older (28 years) than the two brothers and had a BMI of 22 kg/m<sup>2</sup>.

**Methods.** All experiments were performed according to German ethics committee regulations after obtaining written informed consent by the subjects and (for brother II) by both parents.

**Sampling.** After 3 days of carbohydrate-rich meals and an overnight fast, at 8:00 A.M. both patients had a 14-gauge cannula placed in the left forearm in the distal radial vein. After 30 min, the right forearm was placed into a sterilized water bath heated to 44°C to obtain arterialized blood samples as described earlier (23). From 9:00 to 11:00 A.M., blood was drawn every 2 min into tubes containing EDTA (Sarstedt, Hamburg, Germany) in a volume of ~2 ml and immediately placed on wet ice. The samples were processed in a refrigerated centrifuge, and the plasma was frozen at -70°C for <4 weeks.

**Analysis of insulin.** Insulin was measured in each plasma sample, in quadruplicate, by radioimmunoassay (RIA) using our own anti-porcine insulin guinea pig antiserum, [<sup>125</sup>I]-labeled insulin as tracer, human insulin standard (Novo A/S, Bagsvaerd, Denmark), and Dextran-coated charcoal to separate bound from free insulin. The sensitivity of the assay was 3.9 mU/l, the interassay coefficient of variation was <3.8%, and the intra-assay coefficient of variation was <3.1%.

**Pulse analysis.** Each plasma insulin profile was analyzed using the pulse-detection computer program Ultra (24). This program eliminates all insulin peaks for which the incremental or decremental magnitude will not enter a measurement error-based threshold. It will also by interpolation estimate missing values. The threshold used was set to two times the intra-assay coefficient of variation, which has been shown to minimize both the false-negative and false-positive errors (24).

**Spectral and autocorrelation analysis.** Before the time series analysis, the data were linearly stationarized to overcome problems with long-term trends. We used the statistical software STATISTICA (StatSoft, Tulsa, OK) to perform both spectral analysis and autocorrelations. When assessing spectral density function, Fast Fourier Transforms (FFT) and a Blackman and Tukey smoothed window were applied on all insulin profiles and normalized regarding their standard deviation to make the spectral density comparable between subjects (25,26). The power spectra were then transformed, being dependent on lag time. Autocorrelation analysis was performed by calculating the coefficient of correlation between a time series and a copy of itself lagged progressively 1 to 50 samples horizontally with respect to the original data. If any regular oscillatory pattern existed with period time *T*, there would be a nadir (below zero) at lag time *t* = *T*/2 (that is, the series would coincide) and a positive peak at lag time *t* = *T*. The significance of the autocorrelations was checked with Box-Ljung statistics (27). A correlation coefficient >0.23 was regarded by the software as statistically significant (*P* < 0.05).

RESULTS

To obtain information concerning the oscillatory insulin secretion in the PFK1-M-deficient individuals (brothers I and II), a frequent sampling technique was used. Putative alterations of secretory oscillations during basal insulin secretion were quantified by multiple sampling of arterialized venous blood over a period of 2 h. Potential assay-dependent pseudo-oscillations (so-called noise) in the study design was evaluated in healthy subjects (*n* = 3) infused during 6 h with octreotide (0.2 mg · kg<sup>-1</sup> · h<sup>-1</sup>), glucagon (0.5 ng · kg<sup>-1</sup> · min<sup>-1</sup>), and insulin (0.1 mU · kg<sup>-1</sup> · min<sup>-1</sup>). Plasma C-peptide levels were suppressed to values below detection levels. Frequent sampling of plasma insulin levels during the last 2 h did not reveal any sign of significant oscilla-

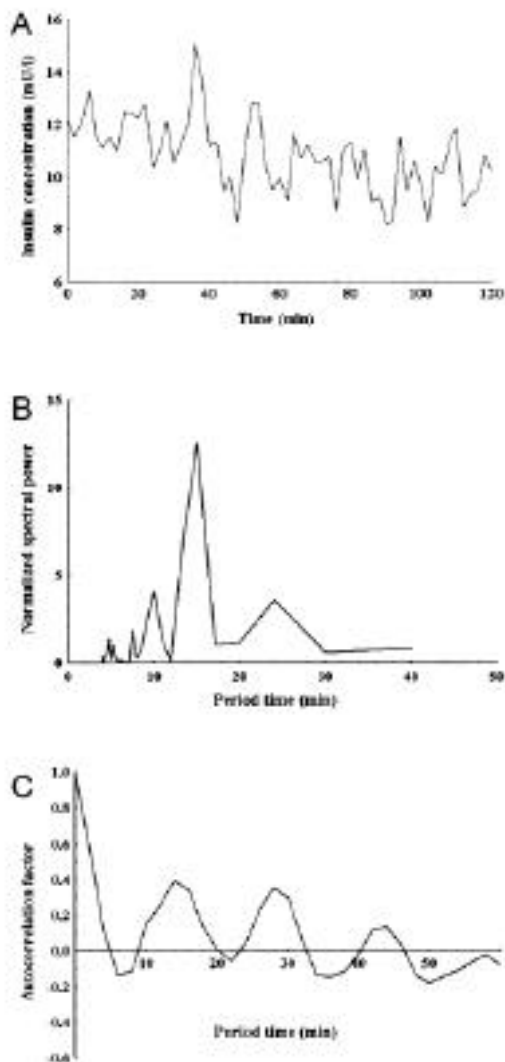
TABLE 1  
Characteristics of PFK1-M-deficient study subjects and matched control subjects

	Brother I	Brother II	Control subjects
Age (years)	19	13	21.1 ± 2.78
BMI (kg/m <sup>2</sup> )	17.8	16.5	19.8 ± 3.1
PFK1 activity in RBCs (μmol · min <sup>-1</sup> · g <sup>-1</sup> )	2.9	4.2	6.0 ± 0.1
Missense mutation PFK1-M intron 5	G A	G A	No
Deletion PFK1-M exon 22	C	No	No
PFK1-M deficiency status	Homozygous	Heterozygous	No
Fasting blood glucose (mg %)	97 ± 3	83 ± 2	86 ± 5
OGTT (75 g) maximum blood glucose	139	128	133.2 ± 25.2
Fasting plasma insulin (mU/l)	17	8.5	7 ± 3.5
IVGTT/first-phase insulin peak (mU/l)	52	164	162 ± 21
Insulin sensitivity test/steady-state blood glucose (mg %)	218	148	104 ± 27
Fructosamine reflecting average blood glucose (μmol/l)	256	247	242 ± 9.5

Data are *n* or means ± SE. Adapted from Ristow et al. (18).

tions (data not shown). Hence, nonspecific noise for methodological reasons is not likely to contribute to the oscillations observed in patients and control subjects. While a recent study by Pørksen et al. (28) recommends a sampling frequency of one per minute, we obtained samples every 2 min because the experimental portion of the study was performed before Pørksen et al. published their data. Insulin levels were determined by RIA in quadruplicate for each sample. The SD of each measurement was <5% of the mean (data not shown).

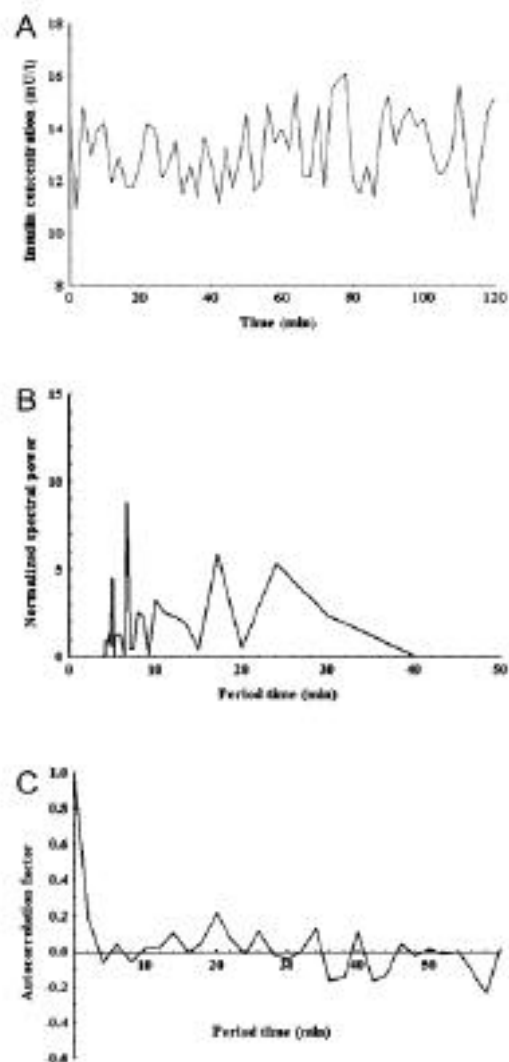
The results of insulin determinations derived from fasting individuals were plotted against time on the *x*-axis (Figs. 1A, 2A, and 3A). Whereas Fig. 1A shows the results of a typical control individual, demonstrating regular oscillations of insulin secretion, Fig. 2A depicts the insulin determinations of brother I, homozygously deficient for PFK1-M, and Fig. 3A, the heterozygously affected brother II. Since these raw data are difficult to interpret for oscillatory patterns, spectral and autocorrelation analyses were applied.



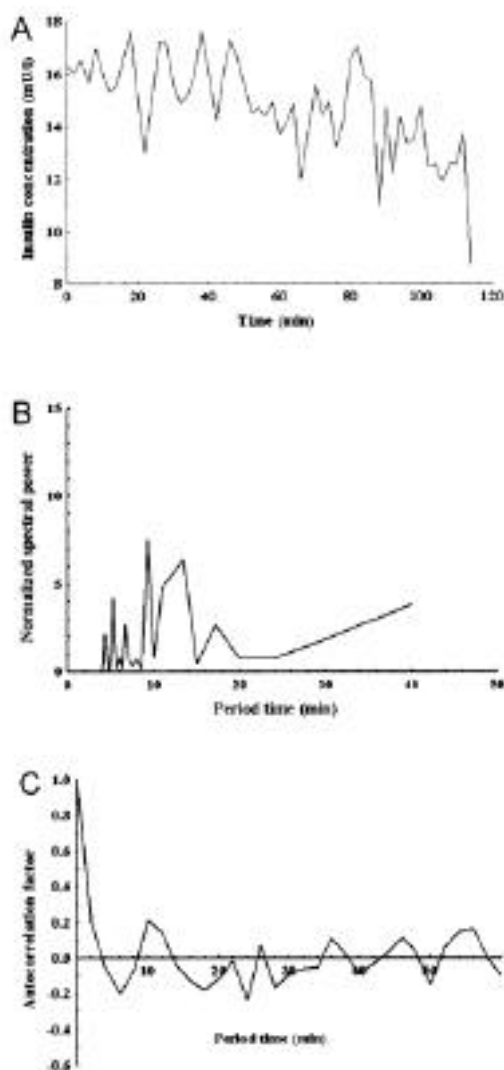
**FIG. 1.** Basal insulin secretion (A), spectral analysis (B), and autocorrelation analysis (C) of plasma insulin oscillations in a healthy control subject. Blood samples were drawn every 2 min over a 2-h period. Insulin was determined in quadruplicate as described in METHODS. Spectral analysis demonstrated a prominent peak of oscillations with a period of about 14 min, which is highly significant ( $P = 0.00001$ ) according to autocorrelation analysis (correlation coefficient 0.36).

First, to determine the time period of these oscillations, spectral analyses (as described in METHODS) were performed. The analysis for the control subject shows a period of about 14 min, consistent with previously published data (29), as shown in Fig. 1B. The spectral analysis for the homozygously deficient brother I (Fig. 2B), however, does not reveal any regular oscillation patterns, while the analysis for the heterozygously affected brother II suggests oscillations with a period between 10 and 12 min (Fig. 3B).

Second, to evaluate these oscillation period times generated by spectral analysis for significance, autocorrelation analyses (as described in METHODS) were used. As expected from the literature and our previous data, the 14-min time period was highly significant ( $P = 0.00001$ ) according to autocorrelation analysis (Fig. 1C) for the healthy control subject. Brother I, homozygously deficient for PFK1-M, appeared to lack regular oscillations according to spectral analysis (Fig. 2B); this was verified by the lack of a significant autocorrelation



**FIG. 2.** Basal insulin secretion (A), spectral analysis (B), and autocorrelation analysis (C) of plasma insulin oscillations in a homozygously PFK1-M-deficient subject (brother I). Blood samples were drawn every 2 min over a 2-h period. Insulin was determined in quadruplicate as described in METHODS. Spectral analysis revealed a scatter of peaks with different period times; none were significant according to the autocorrelation analysis, thus indicating a lack of pulsatility.



**FIG. 3.** Basal insulin secretion (A), spectral analysis (B), and autocorrelation analysis (C) of plasma insulin oscillations in a heterozygously PFK1-M-deficient subject (brother II). Blood samples were drawn every 2 min over a 2-h period. Insulin was determined in quadruplicate as described in METHODS. Spectral analysis suggested a peak of oscillating activity with a period of 10–12 min, which was also evident in the autocorrelation analysis. The latter did not reach significance, however, and was therefore consistent with a decrease of pulsatility.

factor (Fig. 2C). In the heterozygously affected brother II, although spectral analysis suggested a peak of insulin oscillations with a period of 10–12 min (Fig. 3B), it did not achieve statistical significance in the autocorrelation analysis (Fig. 3C) applying the threshold correlation coefficient of 0.23 as generated by the STATISTICA software.

Concerning a summary of oscillation patterns of all seven control subjects, the following results were obtained. The autocorrelation coefficients were significant ( $P < 0.05$ ) in five of the seven control subjects, with a mean  $\pm$  SE of  $0.25 \pm 0.04$  (range 0.10–0.39). The spectral density in the control subjects was  $10.2 \pm 1.7$  (range 3.1–17.8) for an oscillation period of  $13.8 \pm 1.1$  min (range 11–20).

In summary, homozygous as well as heterozygous PFK1-M deficiency may be associated with an impairment of regular oscillatory insulin secretion in humans. Together with our previously published data (18), this impairment of pulsatility is

likely to contribute to the development of type 2 diabetes subsequent to a reduced activity of PFK1-M in these individuals.

## DISCUSSION

Normal oscillatory secretion of insulin is known to be impaired in patients with type 2 diabetes (6,30) as well as their immediate relatives (5) and subjects with impaired glucose tolerance (30). Therefore, this early defect may be involved in the pathogenesis of type 2 diabetes (5,6). Furthermore, it has been demonstrated that pulsatile insulin administration has a greater hypoglycemic effect than continuous delivery (31). The physiologic basis of these oscillations has been extensively studied in both isolated rat islets and insulin-producing rodent cell lines (8). Recent studies from Tornheim and colleagues (8,13) have shown that PFK1-M might be a key metabolic regulator of insulin oscillations. In this context, it has been suggested that PFK1-M governs oscillations in the glycolytic flux in the  $\beta$ -cell, which in turn induces oscillations in the cytosolic ATP/ADP ratio as well as  $\text{Ca}^{2+}$  levels leading to insulin exocytosis. While a putative effect of a PFK1-M knockout situation on oscillatory insulin secretion has not been evaluated so far, we describe a significant impairment of insulin pulsatility in humans with deficiency of PFK1-M. While the homozygous deficiency of PFK1-M (brother I) leads to completely irregular oscillations according to the data of this study, the individual with the heterozygous deficiency (brother II) exhibited a borderline, yet still significant, impairment of pulsatility, one less pronounced than in the homozygous relative. It therefore appears likely that the observed impairment of oscillations is a quantitative effect directly correlated to the level of decrease in PFK1-M activity levels, especially when compared with control subjects. Furthermore, although not included in the results of this study for technical reasons (19), the prediabetic/diabetic phenotype (18) observed in older individuals carrying the same genotype (parents of brothers I and II) may also be associated with impaired insulin oscillatory patterns (6,30). Indeed, determination of insulin oscillations in the homozygously affected father showed severe impairment of pulsatility, identical to what was found in his older son (brother I). The family we evaluated did not contain any unaffected (homozygously healthy) members. In this context, an unknown inherited co-effect (such as linkage disequilibrium with the PFK1-M locus), although unlikely, cannot be fully excluded. On the other hand, a differently inherited trait leading to a diabetic phenotype in this family appears unlikely, since no family history of diabetes was reported. Furthermore, it should be emphasized that the change in oscillation patterns observed in our study subjects is different from the alterations in relatives of people with type 2 diabetes, since the latter group does not exhibit significant changes in first-phase insulin secretion during IVGTT (5), whereas homozygous PFK1-M deficiency leads to pronounced reduction of this acute-phase response (Table 1). We therefore propose an association between PFK1-M deficiency and impaired insulin pulsatility in vivo in humans. Thus, the decreased activity of PFK1-M found in these individuals is the most probable reason, especially if our previous data—loss of first-phase insulin secretion and peripheral insulin resistance in these subjects—are taken into account (18). Still, additional studies including different pedigrees or controls with a genetically identical background, although difficult to obtain, should be considered necessary.

Because oscillatory insulin secretion appears to be of major importance in the pathogenesis of type 2 diabetes (5,6,30), and because at least 70% of total secreted insulin is released in bursts (28,29), alterations in PFK1-M activity may contribute to the biochemical basis of this disease (8,18), especially if the importance of changes in mitochondrial ATP production, located downstream and thus dependent on glycolytic flux, is taken into consideration (32–34).

In summary, an inherited deficiency of PFK1-M is clearly associated with impaired insulin secretory oscillations in humans and might be an important factor in the pathogenesis of certain subtypes of type 2 diabetes.

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