Proinsulin Secretion Is a Persistent Feature of Type 1 Diabetes

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OBJECTIVE
Abnormally elevated proinsulin secretion has been reported in type 2 and early type 1 diabetes when significant C-peptide is present. We questioned whether individuals with long-standing type 1 diabetes and low or absent C-peptide secretory capacity retained the ability to make proinsulin.

RESEARCH DESIGN AND METHODS
C-peptide and proinsulin were measured in fasting and stimulated sera from 319 subjects with long-standing type 1 diabetes (≥3 years) and 12 control subjects without diabetes. We considered three categories of stimulated C-peptide: 1) C-peptide positive, with high stimulated values ≥0.2 nmol/L; 2) C-peptide positive, with low stimulated values ≥0.017 but <0.2 nmol/L; and 3) C-peptide <0.017 nmol/L. Longitudinal samples were analyzed from C-peptide–positive subjects with diabetes after 1, 2, and 4 years.

RESULTS
Of individuals with long-standing type 1 diabetes, 95.9% had detectable serum proinsulin (>3.1 pmol/L), while 89.9% of participants with stimulated C-peptide values below the limit of detection (<0.017 nmol/L; n = 99) had measurable proinsulin. Proinsulin levels remained stable over 4 years of follow-up, while C-peptide decreased slowly during longitudinal analysis. Correlations between proinsulin with C-peptide and mixed-meal stimulation of proinsulin were found only in subjects with high stimulated C-peptide values (≥0.2 nmol/L). Specifically, increases in proinsulin with mixed-meal stimulation were present only in the group with high stimulated C-peptide values, with no increases observed among subjects with low or undetectable (<0.017 nmol/L) residual C-peptide.

CONCLUSIONS
In individuals with long-duration type 1 diabetes, the ability to secrete proinsulin persists, even in those with undetectable serum C-peptide.

Type 1 diabetes results from autoimmune-mediated destruction of the pancreatic β-cell, resulting in the need for exogenous insulin treatment (1). The classic paradigm that type 1 diabetes leads to a complete loss of β-cell mass and absolute insulin deficiency has been challenged by recent data (1). Analysis of pancreatic sections from organ donors with diabetes indicates the presence of residual insulin-containing islets many years after disease onset (2). In addition, multiple groups have reported detectable levels of serum C-peptide in cohorts of individuals with long-duration T1D (3–9). These studies have included highly selected populations, such as the Joslin
Medications, who were identified on the basis of long-term survival, as well as groups more reflective of general diabetes populations, with estimates that up to 80% of individuals with type 1 diabetes retain the ability to secrete small amounts of stimulated C-peptide (3–7).

For detection of residual β-cell mass and function, these studies have relied nearly exclusively on the measurement of C-peptide, which is generated from the processing of immature preproinsulin molecules into insulin and C-peptide. Preproinsulin processing begins with cleavage of the N-terminal signal peptide to form proinsulin within the lumen of the β-cell endoplasmic reticulum (ER) (10). Disulfide bond formation and terminal protein folding occurs in the ER and Golgi, and proinsulin is eventually cleaved into mature insulin and C-peptide by the enzymes prohormone convertase 1/3, prohormone convertase 2, and carboxypeptidase E within secretory granules (10). β-Cell dysfunction, such as that caused by inflammatory or ER stress, results in the accumulation of incompletely processed proinsulin (11–13). Thus, measurement of C-peptide secretion alone could underestimate the ability of the β-cell to initiate hormone production, while increased proinsulin secretion may provide insight into specific disease pathology.

Studies in human cohorts have suggested that abnormalities in β-cell proinsulin processing have clinical relevance to type 1 diabetes. Levels of circulating proinsulin relative to C-peptide (PI:C ratios) are elevated at the time of proinsulin relative to C-peptide (PI:C to type 1 diabetes. Levels of circulating insulin processing have clinical relevance to initiate hormone production, while increased proinsulin (11) or ER stress, results in the accumulation of C-peptide secretion alone could understate the accumulation of C-peptide secretion alone could understate the ability of the β-cell to initiate hormone production, while increased proinsulin secretion may provide insight into specific disease pathology.

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Persistent Serum Proinsulin in Type 1 Diabetes

Diabetes Care

MMTT 90-min values were used to define stimulated C-peptide or proinsulin secretion. For C-peptide–positive subjects, P i:C ratios were calculated as molar ratios × 100.

Table 1—Characteristics of the study population

| Variable                                      | C-peptide <0.017 nmol/L (n = 99) | C-peptide 0.017–0.2 nmol/L (n = 117) | C-peptide ≥0.2 nmol/L (n = 103) | Control subjects (n = 12) |
|-----------------------------------------------|-----------------------------------|--------------------------------------|---------------------------------|--------------------------|
| Age at initial MMTT (years)***               | 34.0 (17.0, 56.0)                 | 29.0 (17.0, 45.0)                    | 41.0 (28.0, 48.8)               | 20.5 (18.0, 25.8)        |
| Sex (% male)                                  | 46.5                              | 42.7                                 | 44.7                            | 50                       |
| BMI (kg/m²)                                   | 25.5 (22.0, 29.0)                 | 25.4 (22.1, 28.4)                    | 26.1 (22.7, 29.2)               | 24.7 (23.1, 28.4)        |
| Race/ethnicity (% non-Hispanic white)*        | 93.9                              | 87.2                                 | 86.7                            | 66.7                     |
| T1D duration at consent (years)***            | 16.0 (7.0, 33.0)                  | 6.0 (4.0, 11.5)                      | 7.0 (4.0, 13.0)                 | n/a                      |
| Age at diagnosis (years)***                   | 15.0 (7.0, 25.0)                  | 19.0 (11.0, 45.0)                    | 29.0 (21.5, 38.0)               | n/a                      |
| HbA1c (%)*                                    | 7.8 (5.3, 8.7)                    | 7.7 (6.8, 9.3)                       | 7.3 (6.6, 8.1)                  | n/a                      |
| GAD positive*                                 | 41.4                              | 62.4                                 | 60                              | n/a                      |
| IA2 positive**                                | 35.4                              | 44.4                                 | 22.9                            | n/a                      |
| ZnT8 positive***                              | 14.1                              | 40.17                                | 2.6                             | n/a                      |
| ≥1 high risk HLA DRB1 alleles present         | 83.2                              | 76.2                                 | 75                              | n/a                      |

Data represent values obtained at initial visit and are median (interquartile range) or percent unless otherwise indicated. AUC, area under the curve; $ZnT8 positive*** 14.1 40.17 25.7 n/a

Table 2—Fasting and stimulated C-peptide and proinsulin values

| Variable                                      | C-peptide <0.017 nmol/L (n = 99) | C-peptide 0.017–0.2 nmol/L (n = 117) | C-peptide ≥0.2 nmol/L (n = 103) | Control subjects (n = 12) |
|-----------------------------------------------|-----------------------------------|--------------------------------------|---------------------------------|--------------------------|
| Fasting C-peptide (nmol/L)                    | n/a                               | 0.023 (<0.017, 0.036)***#            | 0.156 (0.095, 0.30)*            | 0.601 (0.525, 0.800)      |
| Stimulated (90 min) C-peptide (nmol/L)        | n/a                               | 0.079 (0.040, 0.111)***#             | 0.513 (0.344, 0.847)*           | 1.84 (1.08, 2.47)         |
| Fasting proinsulin (pmol/L)                   | 13.5 (6.2, 23.8)                  | 10.9 (7.3, 21.5)                     | 15.2 (9.8, 23.8)                | 15.19 (11.8, 17.4)        |
| Stimulated (90 min) proinsulin (pmol/L)       | 11.2 (4.6, 19.7)***#              | 11.3 (7.3, 34.8)***#                | 22.5 (17.1, 34.8)               | 46.7 (34.3, 74.2)         |
| Fasting P i:C ratio                           | n/a                               | 37.7 (22.0, 58.5)***#                | 9.6 (5.9, 18.1)***              | 2.3 (2.0, 3.1)            |
| Stimulated P i:C ratio                        | n/a                               | 15.4 (8.4, 27.9)***#                 | 4.9 (2.4, 6.9)                  | 2.6 (2.4, 3.2)            |

Data represent values obtained at initial visit and are expressed as median (interquartile range). n/a, not applicable. *P < 0.05; **P < 0.01; ***P < 0.001 compared with control subjects and #P < 0.001 compared with C-peptide ≥0.2 nmol/L group using Kruskal-Wallis test with Dunn multiple comparisons test.
and 90-min time points. Table 2 displays median baseline fasting and stimulated proinsulin values grouped by C-peptide positivity. As anticipated, nearly all subjects who were C-peptide positive at baseline had detectable fasting or stimulated proinsulin, including 100% in the highest C-peptide group and 98.3% in the group with C-peptide >0.017 but ≤0.2 nmol/L. Unexpectedly, proinsulin was also detectable in 89.9% (89 of 99) of subjects with serum C-peptide levels >0.017 nmol/L. Of note, median fasting proinsulin values among each of the groups with type 1 diabetes (13.5, 10.9, and 15.2 pmol/L) were well above the recommended level of detection for the assay (3.1 pmol/L) and fell in the midpoint of the standard curve for the assay. Fasting proinsulin concentrations were not significantly different between any of the groups, and there was no reduction in presence or level of fasting proinsulin with increasing diabetes duration (Fig. 1B). For validation of these findings, targeted mass spectrometry analysis was performed on 10 samples from individuals with undetectable fasting or stimulated proinsulin, including 100% in the highest C-peptide group and 98.3% in the group with C-peptide ≥0.017 but <0.2 nmol/L. Unexpectedly, proinsulin was also detectable in 89.9% (89 of 99) of subjects with serum C-peptide levels <0.017 nmol/L. Of note, median fasting proinsulin values among each of the groups with type 1 diabetes (13.5, 10.9, and 15.2 pmol/L) were well above the recommended level of detection for the assay (3.1 pmol/L) and fell in the midpoint of the standard curve for the assay. Fasting proinsulin concentrations were not significantly different between any of the groups, and there was no reduction in presence or level of fasting proinsulin with increasing diabetes duration (Fig. 1B). 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Over the course of 4 years of follow-up, stimulated serum proinsulin levels tended to stay relatively stable. Figure 1D displays individual stimulated proinsulin values for the groups with C-peptide $\geq 0.017$ but $<0.2$ nmol/L and C-peptide $\geq 0.02$ nmol/L at each study visit. Among all participants, the intra-class correlation coefficient for repeated proinsulin values was 0.795, confirming a strong clustering of repeated measurements over the duration of the study.

We have previously shown that circulating PI:C ratios are elevated in pediatric subjects with new-onset type 1 diabetes compared with matched healthy control subjects (15). Moreover, elevations in the circulating PI:C ratio were associated with clinical progression to type 1 diabetes in autoantibody-positive individuals, suggesting utility of the PI:C ratio as an indicator of $\beta$-cell stress (14). To define whether circulating PI:C ratios differed among the groups with long-standing type 1 diabetes and control subjects, we calculated fasting and stimulated PI:C ratios for subjects with detectable C-peptide and control subjects without diabetes. Similar to findings observed at diabetes onset, fasting PI:C ratios were increased in both groups with type 1 diabetes and detectable C-peptide compared with control subjects without diabetes ($P < 0.001$ for each group compared with control subjects) (Table 2). Consistent with this measure as a reflection of $\beta$-cell dysfunction, PI:C ratios were the highest in the group with the lowest residual stimulated C-peptide secretion ($P < 0.001$ compared with group with C-peptide values $\geq 0.02$ nmol/L) (Table 2).

CONCLUSIONS

Recently, several independent groups have reported that a substantial percentage of individuals retain the ability to secrete C-peptide many years after the diagnosis of type 1 diabetes (3–8). However, less is known about the relationship between proinsulin and C-peptide secretion in long-standing type 1 diabetes. Older analyses of subjects with type 1 diabetes detected circulating proinsulin in subjects with undetectable fasting C-peptide (21–23). In addition, a recent report from a subset of adult subjects from the T1D Exchange registry described detectable circulating proinsulin in 16% of samples from C-peptide–negative subjects with long-standing type 1 diabetes. However, this analysis was performed on randomly collected samples without regard for meal stimulation, and all previous studies were cross-sectional in nature (24). Here, we analyzed longitudinal fasting and stimulated serum samples from subjects with established type 1 diabetes, using a proinsulin radioimmunoassay with negligible cross-reactivity to insulin or C-peptide. In a cohort with a wide distribution of age at diagnosis and duration of disease, we found that almost all individuals tested had detectable serum proinsulin under fasting or meal-stimulated conditions, including 89.9% of subjects with undetectable serum C-peptide (<0.017 nmol/L). Median values for the cohorts were well within the limits of detection for the proinsulin radioimmunoassay used and fell in the midpoint of the standard curve for the assay. In fact, fasting proinsulin values were similar among all groups, irrespective of stimulated C-peptide status. Taken together, these data indicate that the vast majority of subjects with long-standing type 1 diabetes retain the ability to initiate proproinsulin production and secrete proinsulin.

These data provide an important clinical measure that substantiates recently published findings quantifying elevations in proinsulin at the level of the islet. This includes increases in islet Pi:C insulin in euglycemic individuals with positive islet autoantibodies as well as in subjects with recent-onset type 1 diabetes (17). In pancreatic sections from donors with long-standing type 1 diabetes, persistence of islet insulin mRNA and proinsulin protein, despite reduced islet insulin and C-peptide content, was also recently reported (18). Our study is the first to examine longitudinal relationships of circulating C-peptide and proinsulin values in established type 1 diabetes. Our data show that although C-peptide levels decreased gradually, proinsulin levels remained stable over 4 years of follow-up.

In light of these findings, we suggest that detectable proinsulin in subjects with low or undetectable C-peptide levels provides additional information regarding $\beta$-cell hormone production over that afforded by measurement of C-peptide alone. Interestingly, our analysis indicates that in long-standing type 1 diabetes, the relationship between meal stimulation and proinsulin secretion is influenced by the level of ambient and residual $\beta$-cell function. Only subjects with highly functional $\beta$-cells (stimulated C-peptide levels $\geq 0.2$ nmol/L) exhibited increased proinsulin levels with meal stimulation. No significant increase in proinsulin values was observed with stimulation among the groups with undetectable (<0.017 nmol) or low stimulated C-peptide values. Along these lines, stimulated proinsulin and C-peptide values were correlated only in the group with high residual stimulated C-peptide secretion. The etiology of this observation is unclear but will require further testing in human samples to explore underlying mechanisms.

Many individuals in this cohort were diagnosed with type 1 diabetes as adults. Still, 130 of 133 (97.7%) of subjects diagnosed as children had detectable proinsulin, suggesting that applicability of these findings is not limited to individuals with a later age of diagnosis. Because only a small number of individuals exhibited undetectable proinsulin and C-peptide values, we were unable to adequately examine differences in clinical characteristics of this group, but future analyses are warranted to explore factors related to absolute proinsulin and C-peptide deficiency. Additionally, some subjects in the T1D Exchange cohort were diagnosed with type 1 diabetes using clinical parameters, and we cannot exclude the possibility that some individuals may have been misdiagnosed. We calculated PI:C ratios as a potential proxy for $\beta$-cell stress, which is the standard method of analysis in the field (14). While our data identified an increase in PI:C ratios in subjects with long-standing type 1 diabetes compared with a small cohort of healthy control subjects without diabetes, we acknowledge that differences between the groups were driven by lower levels of C-peptide among subjects with diabetes.

Finally, our proinsulin and C-peptide assays exhibit different sensitivities. Although more sensitive C-peptide assays are available, the Tosoh assay used here has been most widely used in large clinical networks including the T1D Exchange, TrialNet, nPOD (Network
for Pancreatic Organ Donors With Diabetes), and the Immune Tolerance Network (3,19,25,26). Additionally, standardization of C-peptide assays is not performed at lower levels of C-peptide, such as those observed in many of our subjects. The National Institutes of Health has recently highlighted issues with re-agents. To address this, we performed multiple assay validation experiments, including analysis of sensitivity, intra- and interassay variability, proinsulin recovery, reproducibility with mass spectrometry testing, and testing for specificity, with testing for human insulin, human C-peptide, proinsulin in the context of insulin autoantibodies, and proinsulin in individuals after pancreatectomy requiring exogenous insulin analogs. These results were all reassuring. However, we cannot guarantee with absolute certainty that our proinsulin assay is binding only intact proinsulin or proinsulin split products in sera from our subjects with type 1 diabetes. Analysis of additional cohorts using different proinsulin assays, including those measuring both intact and total proinsulin, should be performed.

Notwithstanding these limitations, our findings demonstrate that persistent circulating proinsulin can be detected in almost all subjects with long-standing type 1 diabetes, including 89.9% of those with low or absent C-peptide. The ability to increase proinsulin secretion under conditions of meal stimulation occurred only in those patients with significant C-peptide levels. Together, these observations suggest a potential hierarchy of β-cell dysfunction, which begins with a healthy β-cell that secretes mostly C-peptide. Early defects are characterized by increased proinsulin secretion with relatively intact C-peptide secretion. Increased progression of β-cell dysfunction is characterized by lower C-peptide secretion but retained ability to increase proinsulin in response to stimulation. Ultimately, as C-peptide levels fall further, proinsulin secretion in response to meal stimulation is blunted. Whether this hierarchy represents distinct stages of disease that are common among all individuals or whether aspects of this framework can be applied to dissect pathophysiological heterogeneity in type 1 diabetes is uncertain. However, the observation of persistent proinsulin production in late-stage disease raises the tantalizing proposition that therapies aimed at β-cell health could have utility in improving insulin secretion in type 1 diabetes.

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