Immunoradiometric Assay of Human Intact Proinsulin Applied to Patients with Type 2 Diabetes, Impaired Glucose Tolerance, and Hyperandrogenism

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We describe an immunoradiometric assay for human intact proinsulin in serum. In this method, one monoclonal antibody, coated onto polyacrylamide beads, cross-reacts with proinsulins and insulin. A sandwich is formed with intact proinsulin, split (65–66) proinsulin, and des (64–65) proinsulin binding with an ¹²⁵I-labeled monoclonal antibody specific for an epitope at the intact B–C junction of proinsulin. Because split (65–66) and des (64–65) proinsulin concentrations are very low in serum, this assay essentially measures intact proinsulin. When we used 1-mL serum samples, the mean detection limit was 0.4 pmol/L. Mean proinsulin concentrations (pmol/L) were 3.4 (range 1–9.1) in healthy fasting subjects, 28.5 (9.7–101) in patients with type 2 diabetes (treated with metformin and sulfonylureas), 5.0 (1.6–9.3) in women with hyperandrogenism and normal insulinemia, 10.3 (2.6–36) in women with hyperandrogenism and hyperinsulinemia, and 8.5 (4.8–21.3) in patients with impaired glucose tolerance.

Indexing Terms: insulinemia/metformin/sulfonylureas

Insulin is produced in the β-cells of the pancreas by enzymatic cleavage of its precursor proinsulin. Proinsulin is split into several proinsulins and then into connecting (C)-peptide, insulin, and two pairs of basic amino acids (1). Insulin and C-peptide are released into the blood with small amounts of intact and split proinsulins (2). It is unclear whether circulating proinsulins have a physiological function. Plasma concentrations of human proinsulins are increased in subjects with impaired glucose tolerance (IGT) (3, 4), in type 2 diabetics (4, 5), patients with insulinoma (6), and siblings of type 1 diabetics (7) and may be associated with cardiovascular risk factors (8).

Accurate measurement of human proinsulins is difficult because polyclonal antibodies cross-react with insulin and C-peptide, and because blood proinsulin concentrations are low. The methods first used involved gel chromatography to separate proinsulin from insulin (9) or enzymatic destruction of insulin (10). However, gel chromatography is insensitive and cumbersome, and therefore unsuitable for routine analysis. Also, the enzymatic reaction yields imprecise results because degradation of insulin and proinsulin is only partial and, for insulin, is concentration-dependent (11). RIAs and IRMAs have since been developed. Indirect RIAs require a separation step (12, 13), and direct RIAs are based on the use of specific polyclonal antibodies to proinsulin (4, 7, 14–16). Proinsulin can be immunoprecipitated by using an excess of antibody to insulin or to C-peptide and quantified by measuring the insulin or C-peptide immunoreactivity of the precipitate (12, 13). Problems with both direct and indirect RIAs (with polyclonal anti-proinsulin antibodies) include poor sensitivity and a lack of specificity. Two ELISAs, which include antibodies to insulin and C-peptide, have improved sensitivity (detection limits of 1 pmol/L for polyclonal antibodies (17) and 0.1 pmol/L for monoclonal antibodies (mAbs) (18)), but poor specificity.

Until now, only two-site IRMAs or immunoenzymometric assays (IEMAs), based on the use of mAbs, have shown good sensitivity and specificity (19–21). The use of appropriate mAbs allows measurement of insulin, intact proinsulin, and split (65–66) and split (32–33) proinsulins with a detection limit between 1 and 2.5 pmol/L (19, 20) in IRMAs and 0.8 pmol/L in IEMAs (21).

Our goal was to develop a highly sensitive IRMA of human intact proinsulin, using a commercially available, specific mAb. Measurements of proinsulin in patients with various metabolic diseases involving glucose and (or) insulin disturbances (i.e., IGT and hyperinsulinemia in hyperandrogenic women) were compared with results from healthy controls and type 2 diabetics.

Materials and Methods

Antibodies

A mouse mAb, 3B7, was purified from ascitic fluid by (NH₄)₂SO₄ precipitation and DEAE chromatography. MAb 3B7 has an affinity of 5.6 × 10¹⁰ L/mol for intact proinsulin. Cross-reactivity is 100% with des (31–32) and des (64–65) proinsulins, 93% with insulin, 56% with split (32–33) proinsulin, 81% with split (65–66) proinsulin, and <0.01% with C-peptide (19). Another mouse mAb, HPI-005, was obtained from Novo-Nordisk (Bagsvaerd, Denmark). This mAb recognizes human intact proinsulin with an affinity of 3 × 10⁸ L/mol (the epitope is the intact B–C junction). It cross-reacts with split (65–66) and des (64–65) proinsulin (90%) but not with insulin, split (32–33) or des (31–32) proinsulins, C-peptide, or bovine proinsulin.
Calibrators

Split (65–66) and des (64–65) proinsulins, split (32–33) and des (31–32) proinsulins (gift of Eli Lilly, Indianapolis, IN), biosynthetic human proinsulin, insulin, and C-peptide (Sigma, Saint-Quentin Fallavier, France) calibrators were prepared by dilution in saline containing 50 g/L bovine serum albumin (BSA) and 0.1 g/L of sodium ethylmercurithiosalicylate (Sigma).

Coating Procedure

Briefly, we washed 20 mL of nonsedimenting polyacrylamide beads (Bio-Rad, Ivry sur Seine, France) with 0.03 mol/L sodium phosphate buffer, pH 6.3; suspended the beads in 6 mL of the same buffer; and mixed the suspension overnight (at 4°C) with 0.1 mg of 3B7 mAb and 2 × 10 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma). We then washed the pellet with 0.05 mol/L sodium phosphate buffer, pH 7.5, and resuspended it in the same buffer plus 9 g/L NaCl, 1 g/L BSA, 0.1 g/L sodium ethylmercurithiosalicylate, and 1 g/L Triton X-100 (diluent buffer).

Assay Procedure

The IRMA technique we developed is done in two steps. First, nonsedimenting polyacrylamide beads are coated with the first mAb (3B7) to immunoextract serum insulin and proinsulin(s); second, 125I-labeled mAb HPI-005 is used to detect and quantify intact proinsulin. We mixed 75, 150, and 200 μL of immunoabsorbent with 0.5, 1.0, and 1.5 mL of serum, respectively. After incubation for 18 h at 4°C with constant shaking, the pellet was washed with the diluent buffer and incubated again for 18 h at 4°C with 1 mL of 0.05 mol/L sodium phosphate buffer, pH 7.4, and with 9 g/L NaCl containing ~150 000 counts/min of 125I-labeled HPI-005. Finally, after washing the pellet with diluent buffer, we counted the radioactivity of the pellet in a gamma counter (Gamma Master; Pharmacia LKB, St-Quentin-en-Yvelines, France).

For iodination we used the conventional Chloramine T method with 1 mL of Na125I (CIS, Gif-sur-Yvette, France) and 0.1 mg of antibody; the iodinated mixture was separated on a Sephadex G-25 column (Pharmacia LKB).

Calculation

Comparative statistical analyses were performed on logarithmically transformed values by analysis of variance. To compare groups with respect to the relation between serum proinsulin and fasting plasma glucose (FPG) concentration in samples from patients with differing body mass index (BMI) by covariance analysis (regression technique), we used the superANOVA program (Abacus Concepts, Berkeley, CA). The limit of statistical significance was set at P < 0.05.

Subjects

The reference group consisted of 30 subjects with normal results from oral glucose tolerance tests. All the diabetic and IGT patients fulfilled World Health Organization criteria for a diagnosis of diabetes mellitus (22). Details characterizing the subjects are shown in Table 1.

Type 2 diabetic patients were treated with metformin and sulfonylureas (mean duration of treatment 10 years, range 5–29). Obesity was defined by BMI > 25.0 kg/m² in men and 27 kg/m² in women. The diagnostic criteria for hyperandrogenism were oligo- or amenorrhea, hirsutism, acne, and increased plasma concentrations of androstenedione. The hyperandrogenic women had normal oral glucose tolerance tests. Hyperinsulinaemia was defined as a fasting insulinemia > 15 mIU/L (control group range 2–15 mIU/L) when measured by an IEMA specific to insulin that does not cross-react with proinsulin (IMx Insulin; Abbott, Rungis, France). Blood was drawn from fasting subjects. After centrifugation at 4°C, serum was decanted and stored at –20°C. The study was approved by the Ethics Committee of the Centre Hospitalo-Univer-

Table 1. Details of study subjects.

<table>
<thead>
<tr>
<th>Control</th>
<th>Diabetes</th>
<th>IGT</th>
<th>HA-HI</th>
<th>HA-HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>30</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>Sex, M/F</td>
<td>10/20</td>
<td>20/10</td>
<td>15/9</td>
<td></td>
</tr>
<tr>
<td>Age, Range</td>
<td>30–60a</td>
<td>40–69</td>
<td>31–78</td>
<td>16–69b</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>Mean 22.7</td>
<td>29.1c</td>
<td>28.7c</td>
<td>23.1</td>
</tr>
<tr>
<td>FPG, mmol/L</td>
<td>Mean 4.8</td>
<td>10.1c</td>
<td>6.0a</td>
<td>4.9a</td>
</tr>
<tr>
<td>HA-HI, hyperandrogenic normoinsulinic women; HA-HI, hyperandrogenic hyperinsulinic women.</td>
<td></td>
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</tbody>
</table>

* Significantly different from diabetic patients, IGT, HA-HI, HA-HI: P < 0.01.
# Significantly different from control group, IGT, and diabetic patients: P < 0.01.
Significantly different from control group: P < 0.001; P < 0.05.
* Significantly different from diabetic patients: P < 0.001.

Results

Analytical Evaluation

A calibration curve for human proinsulin (calculated with the spline function), with concentrations from 0 to 500 pmol/L, is shown in Fig. 1. The detection limit, based on the precision profile, is 2 SD of the signal for the zero concentration calibrator. Reproducibility and the mean detection limit are shown in Table 2. Inter- and intraassay CVs calculated from serum samples measured 10 times were <10%, except for the interassay CV of 11% at a serum concentration of 3.5 pmol/L. Mean detection limits (n = 10) were 0.3, 0.4, and 0.5 pmol/L for proinsulin in 1.5-, 1.0- and 0.5-mL samples, respectively. The mean recovery of human proinsulin (2–125 pmol/L) added to two samples of human serum (containing 4.6 and 8.0 pmol/L of intact proinsulin) was 102% (range 88–109%). Serial dilution (2- to 16-fold) of human serum with saline containing 50 g/L BSA (Table 3) yielded a mean recovery of 102% (range 96–111%).

The assay cross-reacts with split (65–66) proinsulin and des (64–65) proinsulin (75% each), but not with split (32–33) and des (31–32) proinsulins or C-peptide. Insulin alone at <1000 pmol/L did not interfere with the
Table 2. Inter- and intraassay CVs and effect of sample volume on detection limits of intact proinsulin.

<table>
<thead>
<tr>
<th>Proinsulin conc, pmol/L</th>
<th>Sample vol, mL</th>
<th>1.5</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean detection limit, pmol/L*</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Intraassay CV, %</td>
<td>3.5</td>
<td>7.1</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>6.2</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>4.8</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6</td>
<td>2.3</td>
</tr>
<tr>
<td>Interassay CV, %</td>
<td>3.5</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>8</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.2</td>
<td>6.2</td>
</tr>
</tbody>
</table>

n = 10 each.

*2 SD of the signal for the zero calibrator.

assay. However, the presence of a large quantity of insulin (>640 pmol/L) diminished the recovery of proinsulin, probably by competing for the 3B7 antibody coating the polyacrylamide beads. This value must be checked after each new coating because the coating efficiency is variable. When the serum contains a high concentration of insulin, one should increase the quantity of coated beads used or dilute the sample.

Clinical Evaluation

The mean values for intact proinsulin (pmol/L) were 3.4 (range 1–9.1) in 30 healthy fasting subjects, 28.5 (9.7–101) in 30 treated type 2 diabetics, 5.0 (1.6–9.3) in 30 women with hyperandrogenism and normal insulin concentrations, 10.3 (2.6–36) in 13 women with hyperandrogenism and hyperinsulinemia, and 8.5 (4.8–21.3) in 24 IGT subjects (Fig. 2). Our statistical analyses showed no relation between log fasting proinsulin and BMI, FPG, or age.

Discussion

In this sensitive IRMA for human proinsulin in unextracted serum, we used a commercially available mAb, HPI-005, that recognizes the intact B-C junction of proinsulin and, thus, recognizes split (65–66) and des (64–65) proinsulins. Despite the weak affinity of 125I-labeled HPI-005, the low detection limit allowed us to assay serum proinsulin in fasting subjects. Serum volumes of 0.5 or 1 mL, easily obtained in most clinical situations, gave a low detection limit (<0.5 pmol/L) and a wide measurement range (up to 500 pmol/L).

The problem of interference in the presence of high insulin concentrations (>640 pmol/L) has been cited by others (18). High concentrations may occur in cases involving insulin resistance, particularly after a patient has received a glucose load. The measured proinsulin concentration then is less than the actual concentration, probably because of insulin saturation of the beads. Such interference can be easily corrected, either by increasing the quantity of beads (and thus of mAb), or by lowering the quantity of serum.

The concentrations of intact proinsulin (range 1–9.1 pmol/L) shown in Table 3 were compared between normoinsulinic (controls), hyperandrogenic normoinsulinic, hyperandrogenic hyperinsulinic, and type 2 diabetics (IGT). Differences between groups were evaluated by analysis of variance and Student's t-test. The concentrations in the IGT group were significantly higher than in the control group (P < 0.01).

Table 3. Intact proinsulin measured in two samples of human serum at several dilutions.

<table>
<thead>
<tr>
<th>Dilution, fold</th>
<th>Mean recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105</td>
</tr>
<tr>
<td>2</td>
<td>96</td>
</tr>
<tr>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>8</td>
<td>106</td>
</tr>
<tr>
<td>16</td>
<td>111</td>
</tr>
</tbody>
</table>

*Endogenous proinsulin, 220 pmol/L.

*Diluted with saline containing 50 g/L BSA.

**n = 2.
pmol/L) measured in our healthy fasting subjects (n = 30) correlated well with results from previous studies (19, 20). In our study, these subjects were easily distinguished (P < 10^{-4}) from type 2 diabetic patients treated with oral hypoglycemic drugs (n = 30), whose fasting proinsulin was >9.5 pmol/L (range 9.7–101 pmol/L). Covariance analysis showed that this increase had no relation to the increased BMI and FPG of type 2 diabetic subjects. Proinsulinemia is also increased in untreated type 2 diabetics (3). Therefore, the influence of metformin and sulfonylureas on proinsulin secretion does not seem to be a determining factor.

The 24 IGT subjects formed two subgroups: 19 with normal concentrations of proinsulin and 5 with >10 pmol/L. In the five patients with IGT and an increased proinsulinemia, three were normal for BMI and FPG. Several studies have reported increased mean concentrations of proinsulinemia in IGT (3, 4). However, IGT patients seem to be a heterogeneous group, with some being in the upper range of normal glucose tolerance, some in the lower range, and some truly with IGT (23). Follow-up studies of IGT patients may determine whether measurements of proinsulin can distinguish between these groups and whether high proinsulin is a sign of the impending development of type 2 diabetes.

As in type 2 diabetes, hyperandrogenism is often associated with insulin resistance, the mechanism of which is unknown (24). Hyperandrogenic subjects with normal insulin concentrations (n = 30) had the same fasting proinsulin concentrations as the control group. As in the IGT subjects, there were two subgroups of hyperandrogenic hyperinsulinemic subjects: nine with normal concentrations and four with >10 pmol/L. Of the four hyperandrogenic hyperinsulinemic women, two had normal BMI and FPG.

Some authors have reported a correlation between fasting proinsulin concentrations and obesity in type 2 diabetic subjects as well as in nondiabetic subjects (25). However, that relation was not found by all authors, some of whom linked the increase of proinsulin concentration to the degree of glucose intolerance (3). In our study, the statistical analysis showed no relation between log fasting proinsulin concentration and BMI or FPG in the control group or in IGT, diabetic, and hyperandrogenism subjects. These discrepancies may be due to the use of different immunoassays and to the heterogeneous populations studied.

In summary, with this specific and sensitive IRMA of human intact proinsulin we can distinguish clearly between controls and type 2 diabetics. Follow-up studies of hyperandrogenic women and glucose-intolerant patients with hyperproinsulinemia may determine whether high proinsulin concentrations are a sign of the impending development of type 2 diabetes.

References