Meal-Induced 24-Hour Profile of Circulating Glycated Insulin in Type 2 Diabetic Subjects Measured by a Novel Radioimmunoassay

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Increasing evidence supports a role for glycated insulin in the insulin-resistant state of type 2 diabetes. We measured 24-hour profiles of plasma glycated insulin, using a novel radioimmunoassay (RIA), to evaluate the effects of meal stimulation and intermittent fasting on circulating concentrations of plasma glycated insulin in type 2 diabetes. Patients (n = 6; hemoglobin A_{1c} [HbA_{1c}], 7.2% ± 0.6%; fasting plasma glucose, 7.4 ± 0.7 mmol/L; body mass index [BMI], 35.7 ± 3.5 kg/m^2; age, 56.3 ± 4.4 years) were admitted for 24 hours and received a standardized meal regimen. Half-hourly venous samples were taken for plasma glycated insulin, glucose, insulin, and C-peptide concentrations between 8 AM and midnight and 2-hourly overnight. The mean plasma glycated insulin concentration over 24 hours was 27.8 ± 1.2 pmol/L with a mean ratio of insulin:glycated insulin of 11:1. Circulating glucose, insulin, C-peptide, and glycated insulin followed a basal and meal-related pattern with most prominent increments following breakfast, lunch, and evening meal, respectively. The mean concentrations of glycated insulin during the morning, afternoon, evening, and night-time periods were 24.4 ± 2.5, 28.7 ± 2.3, 31.1 ± 2.1, and 26.2 ± 1.5 pmol/L, respectively, giving significantly higher molar ratios of insulin:glycated insulin of 18.0:1, 14.2:1, and 12.7:1 compared with 7.0:1 at night ($P < .01$ to $P < .001$). These data demonstrate that glycated insulin circulates at relatively high concentrations in type 2 diabetes with a diurnal pattern of basal and meal-stimulated release. A higher proportion of glycated insulin circulates at night suggestive of differences in metabolic clearance compared with native insulin.

FORMATION OF advanced glycation end-products (AGEs) as a consequence of long-term hyperglycemia contributes to many end-organ complications of diabetes,[1,2] including renal,[3] ophthalmic,[4] and vascular disease.[5] Increasing evidence supports the concept that secretion of glycated insulin from the pancreatic β cells under conditions of hyperglycemia contributes to insulin resistance by impairing insulin action in type 2 diabetes. Glycation of insulin has been demonstrated in both in vitro and in vivo models of type 2 diabetes.[6,7] Reduced biological activity of glycated insulin has been observed in studies using isolated adipose or muscle tissue preparations, normal mice, and in healthy human volunteers using the hyperinsulinemic-euglycemic glucose clamp technique.[8-12]

Glycation of insulin occurs rapidly in pancreatic β cells in a concentration-dependent fashion, with evidence of glycation within 2 hours exposure to hyperglycemic conditions in culture.[7] Glycated insulin can remain stable within beta cells for up to 24 hours, representing up to 27% of the total cellular insulin content.[7] Secretion of glycated insulin from β cells in vitro is readily stimulated by glucose, amino acids, and other insulin secretogogues.[7] In addition, the extent of cellular insulin glycation can be reduced by 66% to 80% by culture of isolated β cells under hyperglycemic conditions with inhibitors of protein glycation such as aminoguanidine, vitamin C, and acetylsalicylic acid.[7]

Identification of the N-terminal Phe[1] of the insulin B-chain as the site of glycation[13] has enabled production of specific antibodies and recent development of a novel sensitive radioimmunoassay (RIA) for the measurement of glycated insulin in plasma.[14] These antibodies have also been used to identify intracellular glycated insulin stores in pancreatic β cells in the islets of Langerhans of diabetic animal models using immunocytochemistry.[14,15] Previous techniques for the measurement of glycated insulin by affinity chromatography[6] were not sensitive enough to measure plasma glycated insulin in normal or diabetic states.

Using the recently established RIA,[14] we confirmed elevated circulating concentrations of plasma glycated insulin in patients with type 2 diabetes compared to age- and sex-matched healthy control subjects.[16] To further evaluate the role of plasma glycated insulin in glucose homeostasis, the present study has measured the 24-hour profiles of plasma glycated insulin and associated parameters in a group of type 2 diabetic subjects. We have demonstrated diurnal variation of glycated insulin with a pattern of basal and meal stimulated release. It is apparent that glycated insulin, like insulin, is readily released following a meal, and that a particularly high proportion of plasma glycated insulin circulates during the night.

MATERIALS AND METHODS

Subjects

Twenty-four–hour profiles of plasma glycated insulin and associated parameters were studied in 6 subjects recently diagnosed with type 2 diabetes. Characteristics of the subjects with a mean duration of diabetes of 5.2 ± 0.7 months are listed in Table 1. All were outpatients, being treated by diet therapy alone, who agreed to be hospitalized for 24 hours to take part in the study. The patients were admitted at 7:30 AM to the Endocrinology and Diabetes Centre, Royal Victoria Hospital. An intravenous cannula was inserted into the antecubital vein, which was kept patent by means of heparinized saline. Hemoglobin A_{1c} (HbA_{1c}) was 7.2% ± 0.6% with a mean fasting plasma glucose and serum insulin of 7.4 ± 0.7 mmol/L and 110.4 ± 25.8 pmol/L, respec-

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Table 1. Characteristics of Subjects With Type 2 Diabetes

Characteristics	
Sex	3M/3F
Age (yr)	56.3 ± 4.4
BMI (kg/m^2)	35.7 ± 3.5
HbA_{1c} (%)	7.2 ± 0.6
Duration of diabetes (mo)	5.2 ± 0.7
Fasting plasma glucose (mmol/L)	7.4 ± 0.7
Serum creatinine (μmol/L)	78 ± 4.9
Mean 24-h insulin (pmol/L)	289.4 ± 22.8
Mean 24-h glycated insulin (pmol/L)	27.8 ± 1.2
Mean 24-h ratio insulin:glycated insulin	11:1
Mean plasma glycated insulin (pmol/L) (ratio insulin:glycated insulin)	
Morning (8 AM to 12:30 PM)	24.4 ± 2.5 (18.0:1*)
Afternoon (1 PM to 5:30 PM)	28.7 ± 2.3 (14.2:1†)
Evening (6 PM to 10:30 PM)	31.1 ± 2.1 (12.7:1*)
Night (11 PM to 6 AM)	26.2 ± 1.5 (7.0:1)

NOTE. Values are mean ± SEM.

*$P < .001$ and †$P < 0.01$ compared to ratio of insulin: glycated insulin recorded at night (11 PM to 6 AM).

tively. The subjects were served a standardised meal regimen at 8:30 and 10:30 AM, and 12:30, 3:30, 5:30, and 9:30 PM (Table 2). Venous blood samples were taken throughout the 24-hour period, at 2, 4, 6, and 8 AM, and half-hourly thereafter until midnight. Plasma glucose, insulin, C-peptide, and glycated insulin were determined at each time-point as outlined below. Blood samples were also taken at 9:30 AM from normal healthy subjects (n = 10; age, 62.9 ± 6.7 years; body mass index [BMI], 24.1 ± 1.3 kg/m^2) and analyzed for plasma glucose, HbA_{1c}, glycated insulin, and insulin. This study was approved by the Ethics Committee of The Queen's University of Belfast and carried out following informed consent from all subjects.

Biochemical Analyses

Development of a specific RIA for glycated insulin has been described in detail elsewhere.[14] In brief, an N-terminally glycated synthetic insulin peptide, closely related to the amino-terminal sequence of the insulin B-chain (Phe-Val-Asn-Gln-His-Leu-Tyr-Lys) was linked to ovalbumin using glutaraldehyde and used to raise specific antibodies in guinea pigs. This peptide comprised the naturally occurring 1-6 sequence of insulin B-chain with a Tyr and Lys substituted at positions 7 and 8, respectively. For radiometric determination of circulating glycated insulin, the insulin peptide was glycated under hyperglycemic reducing conditions and iodinated using the solid-phase iodogen method[17] generating a high–specific activity mono-iodinated I^{125}-tyrosylated glycated peptide tracer. Antiserum G3/B/vi was used to establish a dextran-coated charcoal RIA with a glycated insulin standard curve in the presence of insulin-free serum. Assay sensitivity was 9 pmol/L with an intra-assay coefficient of variation of 1.8%. The glycated insulin antibody cross-reacted 52% with glycated proinsulin; however, cross-reaction with nonglycated insulin, proinsulin, and other pancreatic hormones was negligible.[14]

Serum insulin was determined using the Abbott IMX insulin microparticulate enzyme immunoassay (MEIA; Abbott Laboratories, Berkshire, UK), which has a sensitivity of 6 pmol/L and an intra-assay coefficient of variation of 4%. Cross-reactivity with proinsulin was less than 0.005% with no detectable reaction with C-peptide. Cross-reactivity with glycated proinsulin was concentration-dependent, representing about 50%. C-peptide was measured using a commercial kit (Dako Diagnostics, Ely, Cambridgeshire, UK). Glucose concentrations were measured in plasma using the glucose oxidase method.[18] Percent HbA_{1c} was measured in whole blood by ion-exchange high-performance liquid chromatography (HPLC) using the Menari HA-8140 kit (BIOMEN, Berkshire, UK). Serum creatinine was determined using the Johnston and Johnston Vitros 950 analyzer (Orthoclinical Diagnostics, Buckinghamshire, UK) using a multilayered dry slide aminohydrolase technique.[19]

Statistical Analysis

All experimental data are expressed as mean ± SEM. Significant differences between groups of data were assessed using analysis of variance (ANOVA) or unpaired Student's *t* test as appropriate; statistical significance was assumed if $P < .05$.

RESULTS

Study participants comprised 6 diabetic subjects (3 men and 3 women), with a mean age of 56.3 ± 4.4 years and BMI of 35.7 ± 3.5 kg/m^2 (Table 1). Circulating glucose, insulin, C-peptide, and glycated insulin concentrations followed a basal and meal-related pattern of change over 24 hours (Fig 1). Distinct increments were observed following breakfast (8:30 AM), lunch (12:30 PM), and evening meal (5:30 PM). A reasonably close temporal association existed between changes in each of the 4 parameters. Thus, glycated insulin concentrations, like those of insulin and C-peptide, showed a distinct diurnal pattern, although the duration of the stimulatory peaks appeared relatively short.

The mean circulating concentrations of glycated and nonglycated insulin over 24 hours were 27.8 ± 1.2 pmol/L and 289.4 ± 22.8 pmol/L, respectively. As a rough guide, this gives a ratio of insulin: glycated insulin of 11:1 over 24 hours (Table 1). The mean concentrations of glycated insulin during the morning, afternoon, evening, and night-time periods were 24.4 ± 2.5, 28.7 ± 2.3, 31.1 ± 2.1, and 26.2 ± 1.5 pmol/L, respectively. Corresponding ratios insulin:glycated insulin were 18.0:1 ($P < .001$), 14.2:1 ($P < .01$), and 12.7:1 ($P <$

Table 2. Details of Macronutrient Content of Meals and Snacks Eaten by Subjects During the 24-Hour Study Period

Time Served	Composition of Meal/Snack	Carbohydrate (g)	Protein (g)	Fat (g)	Fiber (g)
8:30 AM	Cereal; milk; bread; tea/coffee	67	11	15	10
10:30 AM	Digestive biscuit; tea/coffee	12	2	4	1
12:30 PM	Cooked ham; mashed potato; mixed vegetables; yoghurt; tea/coffee	40	16	8	7
3:30 PM	Digestive biscuit; tea/coffee	12	2	4	1
5:30 PM	Chicken salad; wheaten bread; yoghurt; tea/coffee	42	19	21	11
9:30 PM	Digestive biscuit; tea/coffee	24	4	8	2

NOTE. Total approximate energy content of food eaten over the 24-hour period was 1,500 kcal.

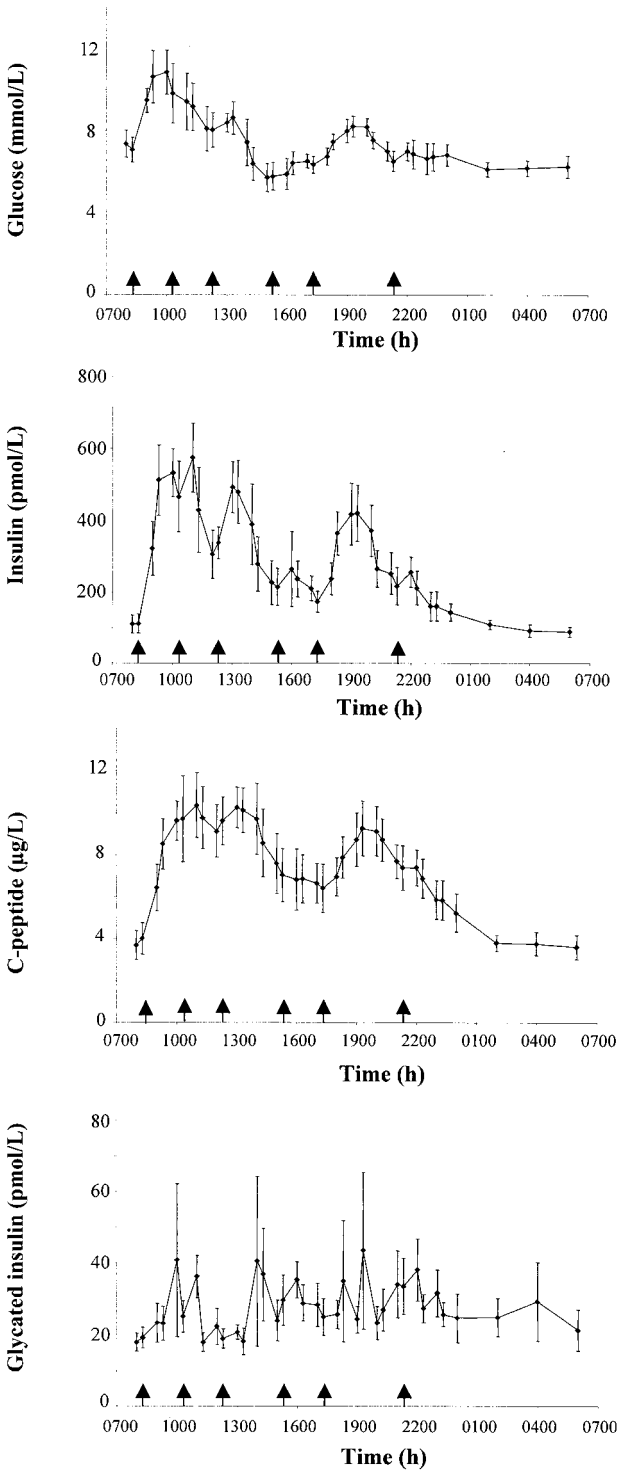

Fig 1. Twenty-four–hour profiles of circulating concentrations of glucose, insulin, C-peptide, and glycated insulin in subjects with type 2 diabetes. Values are mean ± SEM for 6 subjects. Meals (breakfast 0830 h; lunch 1230 h; evening meal 1730 h; and snacks 1030, 1530, and 2130 h) were eaten at the times denoted by the arrows.

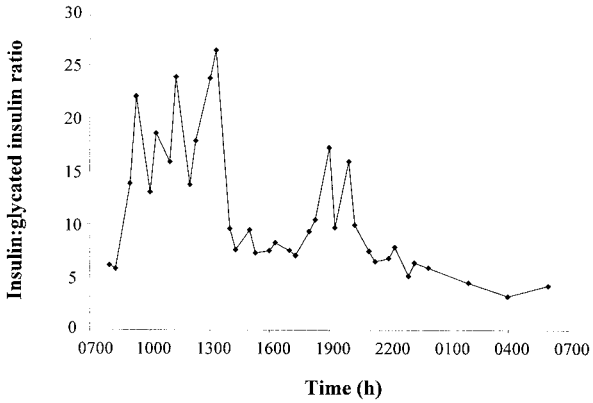

Fig 2. Twenty-four–hour profile of circulating insulin relative to glycated insulin in subjects with type 2 diabetes. Values are mean insulin:glycated insulin ratio for 6 subjects.

.001) compared with the higher value of 7.0:1 at night (Table 1 and Fig 2). Glycated insulin concentrations in normal healthy controls (n = 10; glucose, 5.1 ± 0.4 mmol/L; HbA_{1c}, 5.9% ± 0.3%; insulin, 185.8 ± 30.0 pmol/L) was 8.8 ± 0.3 pmol/L.

DISCUSSION

One of the major pathophysiological consequences of long-term hyperglycemia is an increase in the non-enzymatic glycation of proteins.[20-24] Glycation results from the non-enzymatic reaction between a reducing sugar aldehyde group and an available amino group of amino acids, peptides, or proteins. This process involves a reversible condensation reaction that produces an aldimine or Schiff base, which readily dissociates or then undergoes rearrangement to form the more stable Amadori product; this eventually leads to the formation of irreversible AGEs.[23]

Increasing evidence now exists to support the concept that insulin is glycated in the pancreatic β cell under conditions of hyperglycemia and that such structural modification contributes to insulin resistance by impairing insulin action.[22,25] Glycation has been shown to occur readily in the pancreas of normal and diabetic animal models of diabetes.[6] The glycation process is rapid and glycated insulin is secreted from isolated pancreatic islets and clonal insulin-secreting cells maintained under hyperglycemic conditions in tissue culture.[6,7,15] Previous studies have shown that glycated insulin exhibits a reduced ability to regulate plasma glucose homeostasis in vivo and to stimulate glucose uptake and oxidation by isolated diaphragm and abdominal muscle in vitro.[10,11]

With the advent of a sensitive and specific RIA for glycated insulin it has now been possible to begin to evaluate the possible pathological significance of circulating glycated insulin in human subjects with type 2 diabetes.[16] Initial studies have focused on measurement of plasma glycated insulin in subjects under variable degrees of glycemic control. Preliminary studies employing antiserum raised against the synthetic glycated insulin peptide in rabbit, indicate significant elevations in plasma glycated insulin in a large series of type 2 diabetic patients compared to nondiabetic control subjects in a mid-morning

nonfasted sample.[16] Interestingly, higher plasma levels of glycated insulin were detected in moderately and well-controlled diabetic subjects compared to subjects with poor glycemic control, which may reflect the combined effects of glucose toxicity and β-cell failure on the insulin secretory apparatus.

To characterize further the role of glycated insulin in the pathogenesis of type 2 diabetes, we have studied 24-hour profiles to determine the effects of daily meals, preprandial and postprandial periods, and overnight fasting on circulating concentrations of glycated insulin and associated parameters. Consistent with early observations,[26,27] distinct and temporally associated changes were observed in circulating glucose, insulin, and C-peptide concentrations corresponding with breakfast, lunch, and evening meals. Our results indicate a pattern of basal and meal stimulated secretion of plasma glycated insulin, which approximately mirrors changes of insulin and C-peptide. Mean morning, afternoon, and evening plasma glycated insulin levels were comparable and peak plasma glycated insulin followed a meal stimulated pattern with maximal rises of 113%, 115%, and 75% following breakfast, lunch, and evening meal, respectively. Although differences in cross-reactivity of antisera for glycated proinsulin and glycated insulin need to be borne in mind, the ratio of immunoreactive insulin:glycated insulin was approximately 11:1, with relatively higher concentrations of the glycated peptide being found at night. This may partly reflect possible differences in clearance of the 2 peptides, although accumulation of slightly delayed and relatively less sustained meal-induced increases of glycated insulin may clearly contribute. Circulating concentrations of glycated insulin were detected in normal healthy subjects confirming earlier studies that glycation of insulin occurs under normoglycaemic conditions.[6] However, as concentrations of glycated insulin were considerably lower in controls than type 2 diabetic subjects, a meal-stimulated pattern of glycated insulin release from the pancreatic β cell was not explored.

Previous in vitro and in vivo studies support the concept that insulin is glycated under conditions of hyperglycemia within the pancreatic β cells where it is stored in secretory granules prior to cosecretion with insulin.[6,7,14,15] The present demonstration of diurnal variation of plasma glycated insulin with a pattern of basal and meal stimulated release supports this view. Relatively small differences in the timing, magnitude and duration of changes in circulating glycated insulin suggest small differences in the kinetics of secretion and metabolic clearance compared with native insulin. Since glycated insulin exhibits impaired biological activity, the concentrations measured in type 2 diabetic patients may contribute to insulin resistance and to the well-known diurnal variations of glucose intolerance in these patients.

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