Adipocyte insulin binding and insulin sensitivity in 'brittle' diabetes

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Summary. Adipocyte insulin binding and insulin sensitivity to stimulation of lipogenesis were assessed in a group of extremely 'brittle' diabetic patients who were resistant to subcutaneous insulin therapy and had required frequent and prolonged hospital admission. These patients had significantly lower maximum adipocyte insulin binding ($1.78 \pm 0.18\%$) than age-, sex- and weight-matched stable diabetic control subjects ($2.57 \pm 0.36\%$; $p < 0.05$). Scatchard analysis suggested that the decreased binding was secondary to reduced receptor affinity with no change in receptor number. Adipocytes from the brittle subjects displayed resistance to insulin stimulation of lipogenesis compared with those from diabetic or normal

control groups (half-maximal stimulation at 34 ± 4 , 15 ± 3 and 13 ± 2 pmol/l respectively; $p < 0.01$ between brittle and stable diabetic groups). In the one subject who was treated with intraperitoneal insulin, the changes in insulin binding and sensitivity were found to have reverted towards normal. The peripheral tissue abnormalities of brittle diabetes may exacerbate the clinical syndrome although the relationship of these changes to the primary cause of the syndrome is uncertain.

Key words: Brittle diabetes, adipocyte, insulin binding, insulin action, intraperitoneal insulin

The term 'brittle diabetes' has been applied in many different ways to states of unstable diabetic control. The term was originally used to describe a group of insulin-dependent diabetic patients who experienced frequent and inexplicable oscillations between hyperglycaemic ketosis and hypoglycaemia [1]. The definition was subsequently widened to include any diabetic whose life was disrupted by episodes of either hyperglycaemia or hypoglycaemia [2]. For the purpose of this study, the term 'brittle diabetes' is restricted to those subjects who were referred to this centre with extremely unstable diabetes, no cause for which could be identified, and who proved impossible to control on subcutaneous insulin despite intensive in-patient supervision. This definition, which has been used by others [3], serves to delineate those patients who present the most difficult problems of management. These patients all require high doses of insulin intramuscularly or intravenously, routes which achieve adequate plasma free insulin levels in such patients [4, 5]. Resistance to the action of insulin at the tissue level therefore could explain the poor response of brittle diabetic patients to appropriate insulin therapy.

The present studies were performed in order to test the hypothesis that the abnormal response to insulin in brittle diabetes is a consequence of pathology at the lev-

el of the insulin receptor or of the subsequent steps of insulin action. Adipose tissue was chosen for these studies, being metabolically important, capable of responding to insulin in vitro and readily accessible. The results of studies upon a group of brittle diabetic patients were compared with those of matched groups of stable insulin-dependent diabetic and non-diabetic subjects.

Subjects and methods

Subjects

Six insulin-dependent diabetic patients who had been referred to Newcastle upon Tyne from other centres throughout Great Britain with extremely unstable diabetes were studied. Prior to referral, the patients had suffered a mean of 19 episodes (range 5–35) of severe ketoacidosis and over the 6-month period immediately prior to referral had spent 80 days (range 42–120 days) in hospital. No cause for the unpredictable tendency to ketoacidosis could be identified, and no patient could be controlled on subcutaneous insulin despite intensive supervision. The control group of seven stable insulin-dependent diabetic subjects was chosen to match the brittle group with respect to age, sex, body weight and duration of diabetes (Table 1). Only subjects with sub-optimal diabetic control were selected. The brittle group were receiving more than twice the daily insulin dose of the control group, three by intramuscular injection and three by intrave-

Table 1. Clinical characteristics of the brittle diabetic, stable diabetic and normal groups

	Brittle diabetic patients (n=6)	Stable diabetic patients (n=7)	Normal subjects (n=6)
Sex	All female		
Age (years)	22 ± 2	25 ± 2	35 ± 4
Body mass index	26.9 ± 1.7	27.1 ± 1.0	23.2 ± 1.8
Duration of diabetes (years)	10.6 ± 3.0	14.3 ± 2.0	—
Insulin dose (units/day)	134 ± 17	57 ± 6	—
Fasting plasma free insulin (mU/l)	41.3 ± 18.2	11.2 ± 1.4	—
HbA _{1c} (%)	12.6 ± 1.3	11.1 ± 0.5	—
Diet – g carbohydrate/day	112 ± 6	131 ± 14	178 ± 24
– Kcal/day	1146 ± 81	1477 ± 166	2010 ± 126
– g fat/day	67 ± 5	79 ± 7	98 ± 1

Data are expressed as mean ± SEM.

There was no significant difference between the brittle and stable diabetic group apart from that in insulin dose ($p=0.001$)

nous infusion. All the control group were receiving subcutaneous insulin, one by continuous infusion and six by intermittent injection of short and intermediate acting insulins. Both diabetic groups were receiving carbohydrate-restricted diets. The brittle diabetic patients and one stable diabetic patient were studied as in-patients, and six of the stable diabetic subjects were studied as out-patients. One brittle diabetic patient, who was receiving intramuscular insulin at the time of the initial study (HbA_{1c} 12.0%; insulin dose 1.64 U · kg⁻¹ · day⁻¹; fasting plasma free insulin 10.0 mU/l), was subsequently treated with intraperitoneal insulin delivered by a Siemen's pump (Siemens, Erlangen, FRG). After 9 months, during which time diabetic control was stable (HbA_{1c} 9.1%; insulin dose 0.57 U · kg⁻¹ · day⁻¹; fasting free plasma insulin 20.4 mU/l), a repeat fat biopsy was performed. Permission for the studies was given by the Newcastle upon Tyne Health Authority Ethical Committee and each patient gave informed consent.

Sampling and preparation of adipocytes

Subcutaneous adipose tissue (2–3 g) was obtained from the lateral gluteal region by open biopsy after cutaneous infiltration of 1% lignocaine. All biopsies were taken between 0800 and 0900 h after an overnight fast. The procedure was associated with minimal discomfort.

The adipocyte isolation procedure described by Pedersen et al. was used [6]. The subcutaneous tissue sample was conveyed to the laboratory in glucose-saline (5 mmol/l glucose and 154 mmol/l NaCl). The tissue was finely chopped using scissors and incubated with collagenase (collagenase 0.5 mg/ml, human serum albumin 25 mg/ml, glucose 5 mmol/l in a Hepes buffer, pH 7.4) in a shaking water-bath (120 cpm) for 90 min at 37 °C. The isolated adipocytes were then filtered through nylon mesh and washed extensively in a 5% human albumin/Hepes buffer, pH 7.4. The mean cell diameter for each sample was determined using a micrometer eyepiece (Graticules, Tonbridge, Kent, UK) and the mean of the individual cell volumes and surface areas was calculated [6]. The adipocyte suspensions were adjusted to a lipocrit of 5% using a microhaematocrit centrifuge (Beckman Instruments, Palo Alto, USA). The cell number and total cell surface area per incubation tube were derived knowing the mean of the individual cell volumes, the lipocrit of the final suspension and

the mean of the individual cell surface areas. The coefficient of variation of the cell sizing procedure was 2.0% and that of the lipocrit determination was 2.5%.

Adipocyte insulin binding

Insulin binding to adipocytes was measured by incubating 300 µl aliquots of cell suspension with 100 µl A₁₄ labelled monoiodoinsulin [7] (final concentration 4–6 pmol/l) and 100 µl buffer or unlabelled insulin (final concentration 120–21,000 pmol/l) in duplicate at 37 °C for 60 min in a Hepes buffer (mmol/l: Na⁺, 135; K⁺, 4.8; Mg⁺⁺, 1.7; Ca⁺⁺, 2.5; H₂PO₄⁻, 0.2; HPO₄²⁻, 1.0; Hepes 10, and glucose 1.0 mmol/l, with human serum albumin 50 mg/ml, pH 7.4). The binding was stopped by adding 10 ml chilled 154 mmol/l NaCl. Silicone oil (1 ml, density 0.97 g/ml) was layered on the surface and the tubes were spun at 1,000 g for 3 min. The cell pellet was harvested using a pipe cleaner as described by Gliemann and Sonne [8]. Specific binding was calculated by subtracting the binding observed in the presence of unlabelled insulin (10 µmol/l) from the total binding for each insulin concentration. The non-specific binding averaged 1.5 ± 0.2% of cell bound insulin at a tracer concentration of 16 pmol/l. The intra-assay coefficient of variation of specific binding at a tracer concentration of 4 pmol/l was 2.3%. Binding was expressed as percentage specific binding per 10 cm² adipocyte membrane [9].

Insulin degradation

Degradation of A₁₄ labelled monoiodoinsulin under the conditions described for insulin binding was assessed by addition of trichloroacetic acid (12%) to an aliquot of the incubation medium [8]. The precipitate was separated by centrifugation at 2,000 g for 3 min and the ratio of ¹²⁵I in the supernate to ¹²⁵I in the precipitate was determined. Degradation was assessed in the presence of tracer insulin (16 pmol/l) alone and in the presence of tracer insulin together with an excess of cold insulin (10 µmol/l).

Insulin stimulation of lipogenesis

Triplicate 200 µl aliquots of adipocytes in a glucose-free Hepes buffer (composition as above apart from absence of glucose) were pre-incubated at 37 °C for 45 min with or without increasing amounts of insulin. Glucose (0.4 µCi D-(U-¹⁴C), final concentration 6 µmol/l), was added and the incubation was continued without shaking for 90 min. The reaction was stopped by adding 1 mol/l H₂SO₄ (200 µl) and the tubes were allowed to stand for 60 min before the total lipid fraction was separated using the procedure of Dole and Meinertz [10]. The amount of D-(U-¹⁴C) glucose incorporated into lipid for each triplicate set of tubes was calculated and expressed as pmol/l glucose incorporated per 90 min per 10 cm² adipocyte membrane. At the trace glucose concentration used, glucose transport is probably the rate-limiting step of lipogenesis [9].

Chemicals

Human serum albumin (>99% electrophoretically pure) was obtained from Hoechst, Hounslow, Middlesex, UK. Collagenase from *Clostridium histolyticum* (Batch no. 504–22) was obtained from P-L Biochemicals, Milwaukee, Wisconsin, USA. Crystalline porcine insulin and mono ¹²⁵I-(A₁₄Tyr) insulin were supplied by Novo Industries, Copenhagen, Denmark. U-(¹⁴C)-D-glucose (sp. act. 270 Ci/mol) was purchased from Amersham International, Bucks, UK.

Statistical analyses

Statistical analyses were carried out using the Mann-Whitney U test and Spearman rank correlation coefficient. Results are expressed as mean ± SEM.

Fig. 1. Specific insulin binding to adipocytes from brittle diabetic patients (○—○), stable diabetic patients (●—●) and normal subjects (△—△). Mean $\pm$ SEM

Fig. 2. Scatchard plots of adipocyte insulin binding for the brittle (○—○) and stable (●—●) diabetic groups. SEM bars have been omitted for clarity, but measure of vertical scatter of points may be read from Figure 1

Results

Adipocyte insulin binding

The binding displacement curves are shown in Figure 1. Insulin binding at tracer insulin concentrations was significantly reduced in the brittle group ($1.78 \pm 0.18\%$) compared with the stable diabetic group ($2.57 \pm 0.36\%$; $p < 0.05$), who themselves were not significantly different from control subjects. The difference in binding between brittle and stable diabetic patients was significant at all insulin concentrations up to 120 pmol/l . Half-maximal displacement of tracer insulin was observed at 206 ± 42 for the brittle group and $122 \pm 13 \text{ pmol/l}$ for the stable group ($p = 0.069$). This suggestion of reduced receptor affinity in the brittle group was borne out by Scatchard analysis (Fig. 2). The slope of the high affinity portion of the Scatchard plot for the brittle group was found to be reduced, but the receptor number, as assessed from the x axis intercepts, was similar in the two groups. Adipocyte insulin binding data for the normal control group are summarized in Table 2.

Table 2. Adipocyte insulin binding, insulin sensitivity and insulin degradation in the non-diabetic group, and in the stable and brittle diabetic groups

	Brittle diabetic patients ($n=6$)	Stable diabetic patients ($n=7$)	Normal control subjects ($n=6$)
Maximum specific binding to adipocytes (%)	1.78 ± 0.18	2.57 ± 0.36	2.81 ± 0.18
Insulin concentration for half maximal displacement (pmol/l)	206 ± 42	122 ± 13	113 ± 10
Basal lipogenesis (pmol/ 10 cm^2 per 90 min)	75 ± 14	61 ± 5	68 ± 13
Maximal lipogenesis (pmol/ 10 cm^2 per 90 min)	181 ± 30	187 ± 37	173 ± 29
Insulin concentration for half-maximal stimulation of lipogenesis (pmol/l)	34 ± 4	15 ± 3	13 ± 2
Total insulin degradation at 60 min (%)	1.1 ± 0.1	1.4 ± 0.1	1.2 ± 0.1

Results expressed as mean $\pm$ SEM

Adipocyte insulin degradation

Total insulin degradation was similar in the presence of cells of the brittle, stable diabetic and normal groups ($1.14 \pm 0.04\%$, $1.36 \pm 0.12\%$ and $1.21 \pm 0.11\%$, respectively at 60 min and $2.41 \pm 0.17\%$, $2.66 \pm 0.21\%$ and $2.42 \pm 0.10\%$, respectively at 120 min; Fig. 3). Tracer insulin degradation in the presence of cold insulin ($10 \text{ } \mu\text{mol/l}$) did not increase markedly between 60 and 120 min, whereas the increase in tracer insulin degradation in the absence of cold insulin increased linearly with time.

Adipocyte insulin sensitivity

Basal and maximal rates of lipogenesis were similar in the two diabetic groups (75 ± 14 , 181 ± 30 and 61 ± 5 , $187 \pm 37 \text{ pmol/l}$ per 10 cm^2 per 90 min for brittle and stable groups, respectively). The percentage stimulation of lipogenesis is shown in Figure 4. The insulin concentration required to achieve half-maximal stimulation was $34 \pm 4 \text{ pmol/l}$ for the brittle group and $14 \pm 3 \text{ pmol/l}$ for the stable group ($p < 0.01$). The data for the non-diabetic group are given in Table 2.

Relationship between adipocyte insulin binding and insulin sensitivity

There was a negative correlation between maximum specific binding to adipocytes and the insulin concentration required to achieve half-maximal stimulation of

Fig. 3. Degradation of tracer insulin in the presence of adipocytes from brittle diabetic patients (O—O) and stable diabetic patients (●—●): — in the presence of tracer insulin alone; ---- in the presence of 10 $\mu\text{mol/l}$ unlabelled insulin

Fig. 4. Percentage stimulation of lipogenesis in adipocytes from brittle (O—O) and stable (●—●) diabetic subjects. Dotted line indicates 50% stimulation

Fig. 5. Relationship between maximum specific insulin binding and insulin concentration required for half-maximal stimulation of lipogenesis in adipocytes from brittle (O) and stable (●) diabetic patients ($R_s = 0.78$; $p < 0.01$)

Fig. 6A and B. Specific insulin binding (A) and percentage stimulation of lipogenesis (B) in a brittle diabetic during intramuscular insulin therapy (●—●) and after 9 months of intraperitoneal insulin administration (O—O). Dotted lines represent half-maximal displacement of insulin (A) and 50% maximal stimulation (B)

lipogenesis (Fig. 5) for all diabetic subjects ($R_s = 0.78$, $p < 0.01$) and for the brittle diabetic patients alone ($R_s = 0.89$, $p < 0.05$).

Effects of achieving stable control

In the patient who was studied twice, maximum specific insulin binding was 1.15% during treatment with intramuscular insulin ($1.64 \text{ U} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$), and this rose to 1.90% during treatment with intraperitoneal insulin ($0.57 \text{ U} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$). The rise in binding was accompanied by an appreciable decrease in the insulin concentration required for half-maximal displacement of tracer (Fig. 6). This increase in receptor affinity was accompanied by an increase in insulin sensitivity in vitro, the insulin concentration required to achieve half-maximal stimulation after intraperitoneal insulin being almost identical to that for the stable diabetic group (Fig. 4).

Discussion

The results presented here demonstrate that this group of brittle diabetic patients have normal receptor number, but reduced adipocyte insulin receptor affinity and that this reduction in affinity is reflected in resistance to insulin stimulation of lipogenesis in vitro. The insulin

dosage in the brittle group was more than double that of the control group and the possibility of receptor down-regulation by insulin must be considered. There was, however, no relationship between insulin dose and maximum insulin binding within either group. Similarly, there was no relationship between insulin dose and half-maximal stimulation of lipogenesis. Fasting plasma free insulin levels were markedly higher in those brittle diabetic patients receiving insulin by intravenous infusion (74 ± 24 mU/l) rather than by intermittent intramuscular injection (8.7 ± 3.5 mU/l), but this bore no relationship to the extent of the reduction in insulin binding or insulin sensitivity. Furthermore, the mean fasting plasma free insulin level was higher in the control group than in the brittle subjects receiving intramuscular insulin. Dietary factors, especially fat intake, have been shown to have an important influence upon cellular insulin binding [11, 12]. The mean fat intake of the brittle diabetic group was only slightly lower than that of the stable diabetic patients and would not have been expected to affect insulin binding. The two diabetic groups were well matched with respect to age and body mass index.

Previous studies *in vivo* and *in vitro* have suggested that short-term down-regulation of insulin receptors can be mediated by reduction in cell surface receptor number [13, 14]. Studies of adipocyte insulin binding in various clinical states of insulin resistance have also suggested that reduction in adipocyte insulin binding is most often a result of reduced receptor number [15–17]. Short-term changes in insulin receptor affinity have been recorded after acute changes in food intake [18–20], after hydrocortisone [21] and after growth hormone administration [22]. Examples of chronic reduction in receptor affinity are rare, having been demonstrated during high fat diets [23], in one study of obese subjects [24] and during glucocorticoid administration in rats [25]. The reduction of receptor affinity observed in the group of brittle diabetic patients is therefore of particular interest. The cause of this change in receptor affinity is uncertain. No abnormalities in serum glucagon, growth hormone, prolactin or catecholamines have been observed in brittle diabetes and although morning cortisol levels have been noted to be somewhat elevated, mean 24-h serum cortisol levels in the same subjects were unremarkable (M. Massi-Benedetti et al., unpublished observations). Serum non-esterified fatty acid and total ketone body levels were observed to be variable, but not consistently high [28]. The syndrome of brittle diabetes is intimately related to the administration of extremely variable insulin doses over a long period of time. As insulin itself appears to be a major factor regulating the insulin receptor, the observation of an unusual adipocyte insulin receptor status is perhaps not surprising. The adipocyte insulin binding in the stable diabetic group was not significantly different from that of an age- and sex-matched group of normal subjects. This is at variance with Pedersen and Hjollund's find-

ing of reduced adipocyte insulin binding in a group of stable insulin-dependent diabetic patients [15]. In the same group of subjects, Pedersen and Hjollund observed similar rates of initial glucose transport and identical insulin sensitivity, results very similar to those of the present study. It is possible that the discrepancy in insulin binding results is a consequence of the selection criteria used in the two studies, poorly controlled stable diabetic subjects being specifically chosen as controls for the brittle diabetic subjects in the present study. Relative hypoinsulinaemia in these subjects could account for the differing results, and, indeed, the mean fasting plasma free insulin was only 50% of that reported by Pedersen and Hjollund [15]. Other possible reasons include differences in age, body weight and dietary factors, the stable diabetic group having a lower fat intake. The differences in dietary intake are small compared with those shown to be able to influence insulin binding [11, 12].

It has been suggested that increased degradation of insulin in the subcutaneous tissues may account for the poor response to subcutaneous insulin characteristic of brittle diabetic patients [26, G. V. Gill, unpublished observations]. The studies on insulin degradation presented here demonstrate that the adipocytes themselves are not responsible for any such abnormal degradation, although other subcutaneous components could be involved in insulin degradation. The similarity in insulin degradation in the presence of adipocytes from each of the groups also eliminates this as a possible cause of the observed low insulin binding to the cells of the brittle diabetic patients.

Although intramuscular insulin is sometimes effective in achieving adequate plasma free insulin levels and reasonable metabolic control in the short term [3, 4], this is often not so and long-term intravenous insulin may be required [26]. Both of these routes induce hyperinsulinaemia in the peripheral circulation, a factor which can be avoided at least in part by peritoneal delivery of insulin [27–29]. In the brittle patient restudied after a 9-month period of satisfactory metabolic control on intraperitoneal insulin therapy, both adipocyte insulin binding and insulin sensitivity reverted towards the mean of the stable diabetic group. The reversal of the abnormalities characteristic of brittle diabetes brought about by this change in route of administration and dose of insulin indicates that the reduced receptor affinity and adipocyte insulin resistance are unlikely to be primary changes. Once present, however, these changes are likely to exacerbate the clinical problems.

In conclusion, reduced adipocyte insulin receptor affinity and reduced adipocyte insulin sensitivity have been observed in brittle diabetes. These abnormalities were observed during treatment with high doses of insulin administered intramuscularly or intravenously. The reversal of the abnormalities during treatment with intraperitoneal insulin in one patient suggests that insulin receptor changes may not be the primary cause of the

syndrome, although no direct relationship of the tissue abnormalities to either insulin dose or plasma free insulin levels could be established.

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