Characterization of insulin glycation in insulin-secreting cells maintained in tissue culture

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Abstract

Characteristics of cellular insulin glycation were examined in the pancreatic B-cell line, BRIN-BD11. The extent of insulin glycation increased stepwise during 72 h of culture at 5-6-33-3 mmol/l glucose, attaining levels up to 27%. Glycation of insulin at 33-3 mmol/l glucose was rapid, reaching maximal values within 2 h, and not readily reversible during 2 to 24 h of subsequent exposure to 5·6 mmol/l glucose. Glycated insulin was readily secreted by BRIN-BD11 cells upon active stimulation with glucose and other secretagogues. Cellular insulin glycation was decreased by 66-80% by inhibitors of protein glycation, vitamin C, aminoguanidine or acetylsalicylic acid. Modulation of insulin-secretory activity of BRIN-BD11 cells by co-culture at high glucose with diazoxide, L-alanine or glibenclamide indicated that long-term stimulation of secretion was associated with a decrease in the extent of insulin glycation. Glycation of insulin in vitro was substantially less extensive than in BRIN-BD11 cells, although glucose-6-phosphate and glyceraldehyde-3-phosphate were 1·4-to 2·0-fold more reactive than glucose per se. These observations indicate that insulin is readily glycated and secreted from insulin-secreting cells under hyperglycaemic conditions in culture.


Introduction

One of the many consequences of hyperglycaemia associated with diabetes is the non-enzymatic glycosylation (glycation) of proteins. Glycation can lead to alterations in conformation and/or function of both structural and functional proteins (Williams et al. 1982, Shaklai et al. 1984). Chronic hyperglycaemia resulting in glycation is associated with the pathognomonic microvascular and neurological damage which is recognized in diabetes (Brownlee 1994, Yki Jarvinen 1990). The extent of protein glycation is a function of both ambient glucose concentration and the half-life of the protein (Vlassara et al. 1986). Glycation can affect blood proteins such as haemoglobin (Fluckinger & Winterhalter 1976, Kennedy et al. 1982), structural proteins including collagen (Fu et al. 1992), functional proteins such as Cu-Zn superoxide dismutase (Adachi et al. 1991) as well as peptide hormones (Dolhofer & Weiland 1979, Tarelli et al. 1994).

Recent studies in our laboratory have demonstrated significant increases in percentage glycated insulin and proinsulin in pancreatic extracts of hyperglycaemic animal models of diabetes compared with controls (Abdel-Wahab et al. 1996). Significant elevations in % glycated insulin were also observed when lean mouse islets were cultured in hyperglycaemic media (33·3 mmol/l glucose) for 24 h (Abdel-Wahab et al. 1996). The observed glycation of proinsulin in diabetes may be of relevance to the increased levels of proinsulin and split products reported in non-insulin-dependent diabetes mellitus (NIDDM). Rhodes & Alarcón (1994) outline a number of possible mechanisms which might affect proinsulin processing, leading to hyperproinsulinaemia. Thus glycation of both proinsulin and insulin in the B-cell during the stages of synthesis and storage may contribute to insulin resistance and other features associated with glucose toxicity (Simonson et al. 1992).

Previous reports have shown that glycation of insulin impairs glucose oxidation and lipogenesis in isolated rat adipocytes as well as reducing glucose disposal in vivo in humans (Dolhofer & Weiland 1979, Lapolla et al. 1988). Additionally, we have demonstrated that glycation of insulin impairs peripheral insulin action in man using the hyperinsulinaemic euglycaemic clamp technique (Boyd et al. 1995). The occurrence of glycated insulin in vivo, together with a reduction in its biological activity demand that further studies are carried out to assess the role of glycation in the pathophysiology of NIDDM. Other investigations have shown that human insulin is glycated preferentially at the amino terminal Phe1 site of the B-chain (O’Harte et al. 1994a).

The present study was initiated to examine the extent and characteristics of insulin glycation in pancreatic B-cells. To achieve this, we used the glucose-sensitive
insulin-secreting BRIN-BD11 cell line, which is derived from electrofusion of RINm5F cells with New England Deaconess Hospital rat pancreatic B-cells (McClanaghan et al. 1996). In addition, the effects of inhibitors of glycation (aminoguanidine, acetylsalicylic acid and vitamin C) and the secretion of glycated insulin were investigated.

**Materials and Methods**

**Insulin-secreting cells**

The production and characterization of insulin-secreting BRIN-BD11 cells are presented elsewhere (McClanaghan et al. 1996, Rasschaert et al. 1996). BRIN-BD11 cells used were taken from stocks cryopreserved at −140 °C in liquid nitrogen, using medium containing 80% (v/v) fetal calf serum (FCS), 10% (v/v) RPMI-1640 tissue culture medium (Gibco Life Technologies Ltd, Paisley, Strathclyde, UK) and 10% (v/v) dimethylsulphoxide. Cells used for experimentation were from passages 20–42.

**Cell culture**

BRIN-BD11 cells were cultured in RPMI-1640 tissue culture medium containing 10% (v/v) FCS, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mmol/l glucose. Cells were maintained in sterile tissue culture flasks (Corning Glass Works) at 37 °C in an atmosphere of 5% CO2 and 95% air using an LEEC incubator (Laboratory Technical Engineering, Nottingham, Notts, UK). Cells were gently washed with Hank’s balanced salt solution prior to detachment with trypsin/EDTA (Gibco Life Technologies Ltd). Detached cells were resuspended in tissue culture medium and collected after centrifugation (800 r.p.m.). Aliquots of cell suspension were stained with 1% (w/v) trypan blue and counted by means of haemocytometer for determination of total cell number. The cells were then used for experimentation immediately, or allowed to attach overnight as monolayers.

**Extraction and separation of glycated and non-glycated insulin**

Insulin was extracted from BRIN-BD11 cells by the addition of acid–ethanol (750 ml absolute ethanol: 220 ml water: 30 ml concentrated HCl) followed by sonication (3 × 10 s, on ice) using a Soniprep 150 Ultrasonic disintegrator (MSE, Loughborough, Leics, UK). After overnight incubation at 4 °C, the samples were dialysed (molecular weight cut off 2000) at 4 °C for 18 h against saline (0.9% (w/v) NaCl) to remove the acid–ethanol. Glycated and non-glycated proteins in dialysed extracts were separated using Glyco-Gel B affinity chromatography columns (Pierce and Warriner, Chester, Cheshire, UK) comprising of 1 ml m-amino-phenyl boronic acid. Samples (120 µl) were applied to the top of the columns under gravity, and the non-bound (non-glycated) material collected after washing with 8 ml wash buffer (250 mmol/l ammonium acetate, 50 mmol/l magnesium chloride, 3 mmol/l sodium azide, pH 8.5). The bound (glycated) material was eluted using 3 ml elution buffer (200 mmol/l sorbitol, 50 mmol/l sodium EDTA, 100 mmol/l Tris–HCl buffer, 3 mmol/l sodium azide, pH 8.5). Glycated and non-glycated insulin in fractions were determined by RIA (Flatt & Bailey 1981), using an antibody which exhibits full cross-reactivity with glycated insulin (Fig. 1). Rat insulin standard was obtained from Novo Industria, Copenhagen, Denmark and 125I-labelled bovine insulin (specific activity 3.0 µCi/100 ng insulin) from Amersham International plc, Amersham, Bucks, UK.

To investigate the recovery from Glyco-Gel B, a mixture of glycated (10 µg) and non-glycated (10 µg) insulin was applied to the columns, eluting samples as indicated above. Samples volumes were reduced under vacuum, using an AES 1000 Speed-Vac (Savant, USA).
Southampton, Hants, UK) and reconstituted to 1·0 ml in 0·12% (v/v) trifluoroacetic acid/water, prior to reverse-phase (RP)–HPLC separation on a C-8 Supelcosil column (4·6 × 150 mm) as described elsewhere (O’Harte et al. 1994b). Peak areas were compared with those of glycated and non-glycated insulin prior to the Glyco-Gel B step. The recovery of peptides when RP-HPLC analysis was performed was 80·7 ± 0·4% (mean ± s.e.m., n = 3).

Intracellular insulin glycation and secretory activity of BRIN-BD11 cells

To evaluate the characteristics of intracellular glycation of insulin, BRIN-BD11 cells were cultured for 2 to 72 h at a density of 1 × 10⁶ cells per flask (25 cm² sterile polypropylene) in RPMI-1640 tissue culture medium containing a range of glucose concentrations from 1·4 mmol/l to 33·3 mmol/l, inhibitors of glycation (aminoguanidine, acetaldehyde and vitamin C) or other additions as indicated in Figs 2–4 and Table 1. The media were changed every 24 h. In some experiments, aliquots were withdrawn from tissue culture for measurement of insulin release by RIA (Flatt & Bailey 1981). In these studies, results are expressed as cumulative insulin released over the culture period specified. At the end of the culture periods, BRIN-BD11 cells in each flask were trypsinized and processed for the separation and measurement of glycated and non-glycated insulin as described above. In one experimental series, secretion of glycated insulin was assessed in acute incubations using BRIN-BD11 cells precultured for 48 h with 33·3 mmol/l glucose. Following exposure to 33·3 mmol/l glucose, BRIN-BD11 cells were incubated for 20 min with glucose, L-alanine or a depolarizing concentration of K⁺ in Krebs–Ringer bicarbonate buffer (115 mmol/l NaCl, 4·7 mmol/l KCl, 1·28 mmol/l CaCl₂, 1·2 mmol/l KH₂PO₄, 1·2 mmol/l MgSO₄, 10 mmol/l NaHCO₃; pH 7·4) supplemented with 5 g/l BSA.

Glycation of insulin in vitro

For comparative purposes, the characteristics of insulin glycation were examined by incubation in vitro in PBS, pH 7·4. Insulin aliquots (1 mg/ml, 100 µl) dissolved in 2 mmol/l HCl (400 µg/ml) were incubated for 24 h at 37°C in 0·9 ml PBS containing glucose, glucose metabolites, inhibitors of glycation or modulators of insulin secretion as indicated in Fig. 6 and Table 2. Control incubations consisted of insulin and PBS only. Glycated and non-glycated insulin in dialysed samples were determined as described above.

Statistical analysis

Results are presented as mean ± s.e.m. Groups of data are compared using the unpaired Student’s t-test. Differences are considered significant when P<0·05.

Results

Effects of glucose concentration on glycation and release of cellular insulin

As indicated in Fig. 2 there was a significant increase in the percentage of insulin glycated in BRIN-BD11 cells cultured for 72 h with glucose concentrations of 1·1 mmol/l (P<0·01) and 33·3 mmol/l (P<0·001) when compared with levels cultured at 1·4 mmol/l or 5·6 mmol/l glucose. Additionally, there was a significant increase (P<0·05) in the percentage of insulin glycated at 33·3 mmol/l compared with 11·1 mmol/l glucose. Insulin release was significantly enhanced at 11·1 mmol/l and 33·3 mmol/l compared with culture at the two lower glucose concentrations (P<0·001 and P<0·01 respectively), but cellular insulin content was not significantly changed by culture at different glucose concentrations.
Figure 3 (a) Glycated cellular insulin and (b) cumulative insulin release by BRIN-BD11 cells cultured in RPMI-1640 for 2, 6, 24, 48 and 72 h at 5-6 and 33-3 mmol/l glucose. Tissue culture media were changed at 24-h intervals as appropriate. Insulin release is expressed as total insulin output over the 2-, 6-, 24-, 48- or 72-h culture periods. Values are means ± S.E.M. (n=4). *P<0.02, **P<0.01 compared with 5-6 mmol/l glucose at the same time; ΔP<0.001 compared with the same glucose concentration at 2 h.

(P>0.05; 85–120 ng/10^6 cells). Transfer of BRIN-BD11 cells from 48 h of culture at 33.3 mmol/l glucose to 5.6 mmol/l glucose was not associated with a significant decrease in the extent of glycation over 2 to 24 h (P>0.05; 16.5–20.3%).

Effects of time on glycation and release of cellular insulin

Evaluation of the time-dependency of cellular insulin glycation revealed a significant increase in the percentage of insulin glycated in BRIN-BD11 cells incubated at 33.3 mmol/l glucose (P<0.01) by 2, 6, 24, 48 h (P<0.001) compared with cells cultured for the same time at 5.6 mmol/l (Fig. 3). The extent of insulin glycation remained stable during 2 to 72 h of culture. Insulin release by BRIN-BD11 cells under these conditions was significantly increased at 33.3 mmol/l glucose (P<0.01 at 2, 6, 24 and 48 h, and P<0.02 at 72 h) compared with culture for the same time at 5.6 mmol/l glucose (Fig. 3). Changes
in glucose concentration in culture media did not significantly affect ($P^{>0.05}$) the cellular insulin content in these experiments ($87 \pm 20$ and $154 \pm 58$ ng/10$^6$ cells for 5.6 and 33.3 mmol/l glucose respectively, $n=4$).

**Effect of modulators of secretion on glycation and release of cellular insulin**

To investigate the possible relationship between secretory activity and the extent of cellular insulin glycation, BRIN-BD11 cells were cultured for 48 h with 5.6 mmol/l glucose, 33.3 mmol/l glucose, and 33.3 mmol/l glucose supplemented with either 800 µmol/l diazoxide or 10 μmol/l glibenclamide. As indicated in Table 1, inhibition of insulin release ($P^{<0.001}$) with diazoxide did not affect the glycation of cellular insulin induced by 33.3 mmol/l glucose. Glibenclamide did not influence glycation or insulin release during culture at 33.3 mmol/l glucose. However, culture for 48 h at the lower concentration of 20 mmol/l glucose in the presence of 10 μmol/l glibenclamide or 10 mmol/l L-alanine, revealed a clear association between stimulated insulin release ($P^{<0.001}$) and lower levels ($P^{<0.01}$) of cellular insulin glycation (Table 1). Cellular insulin content was not affected by glucose environment, L-alanine or glibenclamide in these experiments ($140–195$ ng/10$^6$ cells). Diazoxide decreased the insulin content by 35% ($P^{<0.001}$) compared with BRIN-BD11 cells cultured at 33.3 mmol/l glucose ($140 \pm 7$ ng/10$^6$ cells, $n=4$).

**Effects of inhibitors of glycation on glycation and release of cellular insulin**

As indicated in Fig. 4, culture for 48 h with 10 mmol/l vitamin C, 0.5 mmol/l acetylsalicylic acid or 22 mmol/l aminoguanidine significantly decreased insulin glycation ($P^{<0.01}$, $P^{<0.001}$ and $P^{<0.001}$ respectively) compared with cells cultured at 33.3 mmol/l glucose alone. The extent of glycation was significantly greater than at 5.6 mmol/l glucose in cells cultured in 33.3 mmol/l glucose alone and in cells cultured in 33.3 mmol/l glucose supplemented with aminoguanidine ($P^{<0.001}$). Vitamin C and aminoguanidine both inhibited insulin release ($P^{<0.02}$) compared with cells cultured with 5.6 mmol/l or 33.3 mmol/l glucose. These two agents also decreased the cellular insulin content by 56–67% ($P^{<0.01}$) compared with cells cultured with 33.3 mmol/l glucose alone ($189 \pm 14$ ng/10$^6$ cells, $n=4$).

**Stimulation of secretion of glycated insulin**

BRIN-BD11 cells cultured for 48 h at 33.3 mmol/l glucose were used to assess secretion of glycated insulin in response to acute stimulation. As shown in Fig. 5, 33.3 mmol/l glucose, 10 mmol/l L-alanine or 25 mmol/l K+ stimulated insulin release significantly ($P^{<0.001}$) above that observed under basal conditions with 5.6 mmol/l glucose. The percentage glycated insulin secreted under these conditions was 13–16%, differing very little between incubation conditions or from a glycated cellular insulin content of 16.0 ± 0.7% (mean ± s.e.m., $n=4$).

**Characteristics of insulin glycation in vitro**

As shown in Fig. 6, exposure of insulin to 0–110 mmol/l glucose resulted in a stepwise increase in the glycation of insulin to 16.5 ± 0.2% ($n=3$). The extent of glycation at 110 mmol/l glucose was substantially less than that observed following culture of BRIN-BD11 cells for 24 h.
at the lower concentration of 33.3 mmol/l glucose (24 ± 9% vs. 2%, n=4; Fig. 3). However, the level of in vitro glycation was increased by 39–43% (P<0.001) when insulin was exposed to glucose-6-phosphate or glyceraldehyde-3-phosphate alone compared with equimolar concentrations of glucose (Fig. 6). As indicated in Table 2, 24 h of incubation with 10 mmol/l vitamin C, 0.5 mmol/l acetylsalicylic acid or 22 mmol/l aminoguanidine significantly decreased the glycation of insulin induced by 220 mmol/l glucose (P<0.001, P<0.001 and P<0.01 respectively). In contrast, 0.8 mmol/l diazoxide or 0.1 mmol/l glibenclamide did not affect the in vitro glycation of insulin under these conditions.

**Discussion**

The current studies employed the novel BRIN-BD11 cell line to investigate the characteristics of insulin glycation in insulin-secreting cells during tissue culture. BRIN-BD11 cells express the GLUT-2 glucose transporter and exhibit a high glucokinase:hexokinase ratio with intact glucose metabolism (McClenaghan et al. 1996, Rasschaert et al. 1996). Consistent with glucose sensing as in normal pancreatic B-cells, glucose exerted both acute and
Table 2 Glycation of insulin during 24 h of incubation in vitro in PBS, pH 7.4 with 14–110 mmol/l glucose, inhibitors of glycation (vitamin C, acetylsalicylic acid or aminoguanidine) or drugs (diazoxide, glibenclamide). Values are means ± S.E.M., n = 3

<table>
<thead>
<tr>
<th>Addition (mmol/l)</th>
<th>Glucose (mmol/l)</th>
<th>Glycated insulin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
<td>1·0 ± 0·1</td>
</tr>
<tr>
<td>None</td>
<td>220</td>
<td>19·2 ± 1·9</td>
</tr>
<tr>
<td>Vitamin C (10)</td>
<td>220</td>
<td>4·9 ± 0·6***</td>
</tr>
<tr>
<td>Acetylsalicylic acid (0.5)</td>
<td>220</td>
<td>3·8 ± 0·7***</td>
</tr>
<tr>
<td>Aminoguanidine (22)</td>
<td>220</td>
<td>6·5 ± 1·0**</td>
</tr>
<tr>
<td>Diazoxide (0·8)</td>
<td>220</td>
<td>19·5 ± 5·1</td>
</tr>
<tr>
<td>Glibenclamide (0·1)</td>
<td>220</td>
<td>19·1 ± 0·2</td>
</tr>
</tbody>
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**P<0·01, ***P<0·001 compared with 220 mmol/l glucose alone. The % glycated insulin was consistently greater (P<0·01) at 220 mmol/l glucose compared with 0 mmol/l glucose.

long-term stimulatory effects on insulin release in the present study. In addition, exposure of BRIN-BD11 cells to glucose in culture resulted in the glycation of insulin as previously observed in animal models of diabetes and isolated islets cultured under hyperglycaemic conditions (Abdel-Wahab et al. 1996).

Consistent with studies in rats and mice (Abdel-Wahab et al. 1996), exposure of BRIN-BD11 cells to a hyperglycaemic environment in culture resulted in an extensive glycation of cellular insulin stores. The extent of glycation in BRIN-BD11 cells over a fixed 72-h period was concentration dependent over the range 5·6 to 33·3 mmol/l glucose with a significant enhancement of glycation at 11·1 mmol/l glucose compared with a normoglycaemic environment (5·6 mmol/l glucose). Temporal studies revealed that the process of glycation was rapid, attaining near maximal values following 2 h of exposure to 33·3 mmol/l glucose. Evidence also suggests that glycated insulin may be reasonably stable since transfer of BRIN-BD11 cells to 5·6 mmol/l after culture for 48 h at 33·3 mmol/l glucose was not associated with an appreciable decline in the extent of cellular insulin glycation. A small decrease was observed in the extent of insulin glycation secreted from BRIN-BD11 cells exposed to 5·6 mmol/l glucose in acute incubations. However, the extent of glycated insulin secreted in response to 33·3 mmol/l glucose or to a combination of 10 mmol/l l-alanine or 25 mmol/l K+ with 5·6 mmol/l glucose was not significantly different from the cellular glycated insulin in BRIN-BD11 cultured at 33·3 mmol/l glucose.

These observations suggest that glycated insulin can be formed and secreted by the regulated pathway from pancreatic B-cells at glucose concentrations likely to be encountered in diabetes. Indeed, comparison with the glucose concentration dependence for the in vitro glycation of insulin indicates that the environment within BRIN-BD11 cells may be especially favourable for
glycation. This presumably reflects transport and active metabolism of glucose by these cells (McClenaghan et al. 1996, Rasschaert et al. 1996), with rapid equilibrium of intracellular and extracellular glucose concentrations (Malaisse 1992). It may be relevant that glucose-6-phosphate and glyceraldehyde-3-phosphate are considerably more efficient at glycuting insulin and other proteins (Stevens et al. 1977, Dolhofer & Weiland 1978) than equimolar concentrations of glucose. Thus the sequence of events leading to insulin glycation can be envisaged to begin with glucose being taken up into the B-cell by the GLUT-2 transporter (Thorens et al. 1988) such that the intracellular glucose concentration matches the external hyperglycaemic milieu. Glucokinase then metabolizes glucose to glucose-6-phosphate (Lenzen 1992) which is transported across the endoplasmic reticulum membrane by a specific glucose-6-phosphate transport protein (Waddell & Burchell 1993). This enables glucose-6-phosphate to effect rapid glycation of insulin prohormone at a site where it is highly concentrated. Glycation may also continue during transport and storage of the peptides prior to secretion from the β-cell.

Modulation of secretory activity of BRIN-BD11 cells during exposure to a hyperglycaemic environment in culture provides evidence for a relationship with the extent of insulin glycation. Enhancement of secretory activity by L-alanine and glibenclamide led to an approximate 36% reduction of insulin glycation without affecting cellular insulin content. This might reflect decreases in the processing and storage time of insulin in the cells. In contrast, inhibition of insulin output in culture with diazoxide had no effect on insulin glycation. However, diazoxide was associated with a 35% decrease in cellular insulin content, suggesting that crinopathy of secretory granules may have counteracted a possible increased period of cellular insulin storage in these experiments (Skoglund et al. 1987). The established inhibitors of glycation, amino-
guanidine, acetylsalicylic acid and vitamin C (Brownlee et al. 1986, Ajiboye & Harding 1989, Stolba et al. 1991) all decreased the extent of insulin glycation during 48 h of culture at high glucose levels. These agents inhibit glycation by a variety of mechanisms, including decreasing the active aldehyde form of the sugars, acetylation of the amino groups of the protein and by competitive inhibition by attaching to the free amino groups of the protein respectively. As expected, these inhibitors decreased the in vitro glycation of insulin, suggesting that effects at the cellular level reflect uptake and inhibition of intracellular glycation. Effects of these agents on BRIN-BD11 cell function do not change this view. Thus the inhibition of insulin output and cellular insulin content observed with aminoguanidine and vitamin C might be predicted to increase rather than decrease the extent of insulin glycation.

In conclusion, these studies have demonstrated that the glycation of insulin in insulin-secreting cells is dependent on the prevailing glucose concentration, the duration of glucose exposure and both the biosynthetic and secretory activities of the cell. Glycation is rapid and glycated insulin is readily secreted from the cell in response to glucose and other secretagogues. Since glycated insulin exhibits decreased biological potency (Dolhofer & Wieland 1979, Lapolla et al. 1988, Boyd et al. 1995), these observations suggest that glycation of insulin in the pancreatic B-cells during synthesis and storage may contribute to B-cell dysfunction and pathophysiology of NIDDM (Flatt et al. 1994).

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References


