Characterization of the glycation of human insulin by reversed-phase HPLC.

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The non-enzymatic glycosylation (glycation) of proteins is a common post-translational modification found in diabetes under conditions of hyperglycaemia [1]. The rates and extent of glycation are dependent on both the ambient glucose concentration and the half life of the particular protein [2]. We have investigated the process of glycation with human insulin in vitro quantitating the extent of glycation by reversed-phase HPLC.

We investigated the relationships between the extent of insulin glycation and: i) the concentration of glucose, and ii) the length of exposure to glucose. Human insulin (Sigma, E. coli recombinant) was dissolved (1 mg/ml) in 0.002 N HCl. An aliquot (100 µl) of insulin was added to 900 µl phosphate buffered saline (PBS, Difco) pH 7.4, containing D-glucose (final concentration 220 mM). Samples were incubated in triplicate at 37°C with gentle agitation and the reaction stopped by freezing at -20°C at various times including 0, 2, 4, 8, 12 and 24 hr. Controls contained insulin incubated with PBS only. A separate dose dependent glycation experiment was performed with human insulin using various concentrations of D-glucose including 0, 27.5, 55, 110 and 220 mM. Samples (500 µl) were introduced via a Rheodyne model 7125 injector onto a Supelcosil LC-8 column (150 x 4.6 mm) equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 1.0 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 35% over 10 min and to 56% over 20 min, using linear gradients. The absorbance was monitored at 206 nm and peak areas were measured using a model 2221 LKB integrator.

A typical HPLC profile is shown in Fig. 2. Glycated insulin eluted at 20.2 min (Peak 1) and was followed by non-glycated insulin at 21.4 min (Peak 2). Both peaks were shown to have insulin immunoreactivity when checked by radioimmunoassay. Results showed a dose dependent increase in % glycated insulin over the range from 0 to 220 mM glucose (Fig. 1a). There was a highly significant time dependent increase in % glycated insulin over the period from 0 to 24 hr (Fig. 1b).

Insulin glycation may contribute to insulin resistance and B-cell dysfunction recognised in diabetes [3]. Glycated insulin has been shown to exhibit reduced in vivo biological potency compared to non-glycated insulin when examining glucose oxidation in mouse diaphragm muscle [4]. Further studies are underway to determine the exact sites of insulin glycation within the molecule.

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Fig. 1a. Dose dependent glycation of human insulin at 37°C over 24 hr.

Fig. 1b. Time dependent glycation of human insulin with 220 mM D-glucose

Fig. 2. RP-HPLC separation of glycated and non-glycated insulin following incubation of insulin with 220 mM D-glucose for 24 hr at 37°C.