Glycation (non-enzymic glycosylation) inactivates glutathione reductase

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Non-enzymic binding of sugars to proteins (glycation) is a common biological phenomenon that is increased in diabetes. Most work has been directed towards structural proteins which may be present for many years and would continue to accumulate sugar residues. As glycation is a non-specific reaction, other proteins such as enzymes will also be susceptible to glycation and could well display altered activity. We investigated the effect of various sugars whose concentrations increase in diabetes in insulin-independent tissues on glutathione reductase, an enzyme that maintains the GSH level in cells. Glucose, glucose 6-phosphate and fructose all displayed a time-dependent inhibition of glutathione reductase activity, suggesting that these sugars glycate this enzyme. Aspirin gave some protection against the loss of activity induced by glucose.

INTRODUCTION

Non-enzymic glycosylation (glycation) was first described by Louis Maillard (1912), who observed browning of amino acids when they were heated with sugars. The 'Maillard reaction' was not shown to be important outside the food industry for many decades. The first protein demonstrated to be glycated *in vivo* was haemoglobin. Bookchin & Gallop (1968) showed that there was a hexose molecule linked to the *N*-terminal valine of the β -chain of HbA1c, a minor component of haemoglobin that increases in diabetes in humans (Rahbar *et al.*, 1969) and in animals (Koenig & Cerami, 1975). Flückiger & Winterhalter (1976) formed HbA1c *in vitro* by incubating haemoglobin with glucose. Other sugars, such as glucose 6-phosphate and fructose 1,6-bisphosphate, bind to haemoglobin *in vivo* (Bunn *et al.*, 1978). Many other plasma and structural proteins have been shown to be glycated *in vivo* with increases in diabetes (van Boekel, 1991).

The level of glycation of plasma proteins is limited by their rapid replacement. However, structural proteins that are present for many years, or even decades, such as crystallins at the centre of the lens, where there is little or no synthesis of new protein (Harding, 1991), will continue to accumulate sugar residues. Glycation impairs the function of some proteins. For example it shifts the haemoglobin-oxygen dissociation curve, possibly compromising oxygen delivery to cells (Standl & Kolb, 1973; Ditzel *et al.*, 1978); it alters the ligand-binding properties of albumin (Shaklai *et al.*, 1984) and decreases clearance of low-density lipoproteins, owing to the decrease in its ability to interact with fibroblast receptors (Sasaki *et al.*, 1982). Disruption of the surface network of charges of lens crystallins by glycation could lead to protein unfolding (Beswick & Harding, 1987), exposing thiol groups, which could become involved in both intra- and inter-molecular cross-linking, leading to high-molecular-mass aggregates which scatter light (Harding, 1973; Perry *et al.*, 1987). Glycation has also been implicated in complications associated with diabetes seen in the kidney basement membrane (Uitto *et al.*, 1982), myelin in the peripheral and central nervous system (Vlassara *et al.*, 1983), osteoporosis and osteopenia (Gundberg *et al.*, 1986).

Most interest in glycation has been directed towards structural proteins, but other proteins, such as enzymes, will be susceptible to glycation and may have their activity inhibited through

blockage of the active site itself or by structural alteration which in turn affects the active site. The enzyme superoxide dismutase (SOD) from the plasma has been shown to be glycated *in vivo*, the proportion of glycated SOD being considerably higher in diabetic patients (Adachi *et al.*, 1991). The enzyme activity was not affected, suggesting that the glycation site(s) was rather far away from the active site. However, glycation did affect affinity for heparin, which could decrease the amount of SOD bound to the cell surface, which in turn would increase the susceptibility of cells to superoxide radicals produced in the extracellular space. Glycation of $\text{Na}^+ + \text{K}^+$ -ATPase alters the kinetics of this ion pump (Garner & Spector, 1986).

Glutathione reductase maintains the level of GSH in cells. The content of this biological reducing agent decreases steadily with age in mouse erythrocytes, liver, kidney and heart (Abraham *et al.*, 1978; Hazelton & Lang, 1980), in rat liver, heart, brain, lung and spleen (Farooqui *et al.*, 1987) and in human lens and erythrocytes (Harding, 1970; Matsubara & Machado, 1991). A further decrease occurs in human cataract (Harding, 1970) and, indeed, in almost all experimental cataracts (Harding, 1991). Opacification of lens protein solutions treated with hexose was diminished by GSH (Stevens *et al.*, 1978), and GSH offered protection against cataract in diabetic rats (Ross *et al.*, 1983). The degree of decrease in GSH content of the lens correlated with the increased opacification in diabetic rats (Blakytty & Harding, 1992).

It is possible that this inability to maintain GSH could result from loss of glutathione reductase activity. Decrease in activity of this enzyme has been demonstrated in tandem with GSH loss on aging in various tissues in mouse (Abraham *et al.*, 1978), rats (Farooqui *et al.*, 1987) and human erythrocytes (Al-Turk *et al.*, 1987). Activity of glutathione reductase in erythrocytes is significantly decreased in both diabetic adults (Murakami *et al.*, 1989) and children (Ståhlberg & Hietanen, 1991). Decrease in enzyme activity has also been observed in mature, nuclear and cortical human cataracts (Friedburg & Manthey, 1973; Rogers & Augusteyn, 1978; Ohrloff *et al.*, 1984).

To determine whether the loss of GSH in diabetes is due to the inhibition of glutathione reductase through glycation, the bovine intestinal enzyme was incubated with glucose, glucose 6-phosphate and fructose, all of which are elevated in some tissues in diabetes (Gonzalez *et al.*, 1980; Jedziniak *et al.*, 1981; Mayhew

et al., 1983). Sorbitol, lacking the carbonyl group required for glycation, was used as a control, and ethanediol was used to check whether any inhibition of enzyme activity was due to changes in osmolarity of the incubation medium. Aspirin and ibuprofen decrease glycation *in vitro* (Day *et al.*, 1979; Huby & Harding, 1988; Ajiboye & Harding, 1989; Raza & Harding, 1991) and *in vivo* in rats (Rendell *et al.*, 1986; Swamy & Abraham, 1989; Blakytyn & Harding, 1992) and also partly maintain GSH levels in the lenses of streptozotocin-induced diabetic rats (Blakytyn & Harding, 1992). In the present work we also investigated whether these analgesics protected glutathione reductase against inactivation by sugars. Paracetamol was also studied; this analgesic, along with aspirin and ibuprofen, protect against cataract in diabetic rats (Swamy & Abraham, 1989; Blakytyn & Harding, 1992) and are associated with a protective effect against cataract in man (van Heyningen & Harding, 1986; Harding & van Heyningen, 1988; Harding *et al.*, 1989; Mohan *et al.*, 1989), although there is some controversy over their protective role (Harding, 1991; Isaac *et al.*, 1991; Seddon *et al.*, 1991).

MATERIALS AND METHODS

Materials

Bovine intestinal glutathione reductase, NADPH and colorimetric glucose assay kit were obtained from Sigma (Poole, Dorset, U.K.). Sil-Prep was obtained from Applied Science (State College, PA, U.S.A.). The capillary column for gas chromatography was from Scientific Glass Engineering (Milton Keynes, Bucks, U.K.). D-[¹⁴C]Sorbitol was from du Pont U.K. (Stevenage, Herts., U.K.). All other chemicals were obtained from Sigma or BDH (Lutterworth, Leics., U.K.).

Glutathione reductase

The enzymic activity of bovine intestinal glutathione reductase was determined by measuring the decrease in absorption at 339 nm on a Perkin-Elmer 55 u.v.-visible spectrophotometer as NADPH was used to reduce GSSG. The assay was based on that described by Bergmeyer (1987), with slight modifications. First 0.1 ml of 15 mM-EDTA, 0.1 ml of 63.5 mM-GSSG and enzyme solution (5 units/ml of buffer) were added to 2.73 ml of 0.12 M-potassium phosphate buffer, pH 7.2, at 37 °C (1.0 M-NaOH was added to KH_2PO_4). The solution was incubated in a water bath at 37 °C for 4.5 min, then pipetted into a quartz cuvette in the spectrophotometer and 50 μl of 9.6 mM-NADPH [in 1% (w/v) NaHCO_3] was added 5 min after the start of the incubation, stirred rapidly and the absorbance continuously monitored against a water blank (absorption range 0–1.0; response 2; slit width 2 nm; chart speed 2 cm/min). All enzyme activities were calculated from the decrease in the absorption between 0.5 and 1.5 min.

Incubation of enzyme with sugars

To determine whether various sugars affected the activity of glutathione reductase, the enzyme was incubated in the pH 7.2 buffer with glucose, glucose 6-phosphate or fructose at 0.5 M in the first set of experiments and at 5 mM in later experiments. Additionally the enzyme was incubated with sorbitol, which is a sugar alcohol not containing a carbonyl group in the open-chain form, and with 0.5 M-ethanediol, to show that any changes were due to the sugars themselves and not because of a change in the osmolarity of the medium. Stock solutions were made of the enzyme with and without sugar or ethanediol and divided into separate small sterilized glass vials with rubber tops (Wheaton) through a sterilized 0.2 μm -pore-size Millipore filter. The vials were placed in a shaking water bath at 37 °C. Individual vials

were removed at zero time and various times thereafter for assaying enzyme activity in triplicate.

Protective effect of drugs against sugar inhibition

Aspirin, paracetamol and ibuprofen were dissolved in pH 7.2 buffer and added to the enzyme solution to give a final concentration of 20 mM in the incubation solution with or without sugar (5 mM). Incubation and assay of enzyme activity was as described above.

To confirm the absence of glucose contaminant in sorbitol

Sorbitol is synthesized by reduction of glucose, hence unreduced glucose could be a contaminant. This was tested by two independent methods: colorimetrically and by g.l.c.

For the colorimetric glucose oxidase method (Sigma), three 20 μl portions were taken from 0.5 M-sorbitol in the pH 7.2 phosphate buffer and from the sorbitol incubation after 8 days and added to 0.48 ml of distilled water in Eppendorf tubes. For standards, 50 μl of glucose (1 mg/ml) in distilled water was added to 0.45 ml of distilled water. Blanks consisted of 0.5 ml distilled water alone. After shaking, 0.25 ml of 0.3 M- $\text{Ba}(\text{OH})_2$ and 0.3 M- ZnSO_4 was added. The tubes were shaken and centrifuged at 11500 g for 8 min. Supernatants (0.5 ml) from experimental and blank tubes were placed in test tubes, and 0.25 ml from the standards was added to 0.25 ml of distilled water. To this was added 2.5 ml of the colour reagent (Sigma kit: glucose oxidase, peroxidase and o-dianisidine dihydrochloride), the tubes were shaken and left at room temperature for 45 min. Absorption at 450 nm was read on a Cecil CE505 spectrophotometer set on double beam. The blank was used to zero the absorption against water so that all subsequent readings reflect absorption due to the glucose. The amount of glucose was calculated using the average reading obtained for the standards.

A second and more sensitive test was by g.l.c. The hydroxy groups of sorbitol, glucose and fructose were converted into their trimethylsilyl derivatives by dissolving approx. 10 μg of the sugar in 50 μl of Sil-Prep and leaving the solution at room temperature for 5 min. No further derivatization was needed, so 2 μl of the solution was loaded on to a Perkin-Elmer 8420 capillary gas chromatograph and passed through a wall-coated capillary column (25 m long; int. diam. 0.22 mm) with a 0.25 μm film of BP1 (methylsilicone equivalent to OV-1; bonded on to the wall of the column), using nitrogen as the carrier gas and a hydrogen flame-ionization detector.

Binding of sorbitol to soluble bovine lens proteins

Two bovine lenses (total 2.9 g) were decapsulated and homogenized in 12 times their weight of 2 mM-sodium pyrophosphate buffer, pH 7.4. After centrifugation at 12000 g for 20 min, the supernatant was dialysed three times against water (2 litres) over 3 h and then freeze-dried for storage at –20 °C.

The reaction mixture consisted of 10 mg of protein dissolved in 0.1 M-sodium pyrophosphate buffer, pH 7.4, 2.5 mg of NaN_3 (to inhibit organic growth), 5 mM-EDTA in 0.5 ml of buffer, 50 mM-sorbitol in 1 ml of buffer, 0.1 ml of radiolabelled sorbitol (sp. radioactivity 342 $\mu\text{Ci}/\text{mmol}$) and 0.9 ml of buffer, pH 7.4, to give a reaction volume of 5 ml. All solids were weighed into sterilized containers. On mixing the reaction solution it was immediately passed through a 0.2 μm -pore-size sterilized Millipore filter into an autoclaved sealed container via a sterilized needle and placed in a shaking water bath at 37 °C. A sample of 0.3 ml was immediately withdrawn by sterilized needle and syringe. From this four 10 μl samples were taken and placed in scintillation vials to which 3.5 ml of Scintran cocktail T was added. Total radioactivity in the vials was measured on a

scintillation counter to calculate the total radiolabel in the reaction mixture. Four aliquots (50 μ l) were precipitated overnight in 1 ml of 10% (w/v) trichloroacetic acid at 4 °C. The precipitates were retained on Whatman GF/C microfibre filter discs by suction and washed with 5% trichloroacetic acid. The discs were inserted into scintillation vials, dried at 70 °C for 30 min and allowed to cool at room temperature; 3.5 ml Scinttran cocktail T was added and the radioactivity was measured on a scintillation counter. At various times a 225 μ l portion was similarly removed and subdivided into 50 μ l aliquots to determine binding.

RESULTS

Experiments with 0.5 M-sugars

There was no immediate inhibition of enzyme activity by glucose, glucose 6-phosphate, fructose or sorbitol, zero time activity not being different from that of the control (Fig. 1). Therefore these sugars are not active as inhibitors in the usual sense. The three sugars and sorbitol did, however, display a time-dependent inhibition of enzyme activity (Fig. 1), suggesting non-enzymic binding to the protein molecule. Fructose had the most rapid effect. Inhibition by sorbitol was unexpected, as it should not react with proteins, since it lacks a carbonyl group and was included as a sugar control. Ethanediol did not display an inhibitory effect (results not shown), suggesting that the sugars are not inhibiting the enzyme through increased osmolarity.

Experiments with 5 mM-sugars

Even at this much lower concentration, more representative of conditions *in vivo*, at least during diabetes, all four sugars demonstrated a time-dependent inhibition of glutathione reductase activity (Fig. 2). Both fructose and glucose 6-phosphate had significantly lowered enzyme activity after 2 days [$17.2 \pm 25.4\%$ (mean $\pm$ s.d.) of initial activity; $P < 0.01$; $61.0 \pm 17.6\%$, $P < 0.025$ by Student's *t* test respectively], fructose being more effective than glucose 6-phosphate ($P < 0.05$). Glucose displayed a slower inhibitory effect, which was statistically significant after 5 days ($48.2 \pm 32.0\%$; $P < 0.05$), although not significantly different from fructose or glucose 6-phosphate in its inhibition at this stage. Sorbitol displayed a considerable delay, not being statistically significantly lower until 9 days of incubation ($74.8 \pm 13.9\%$; $P < 0.05$). With time the differences in inhibition by the sugars decrease, with that of both fructose and glucose 6-phosphate being significantly lower than that of sorbitol after 5 days ($P < 0.005$), whereas, by the end of the incubation (15 days), only that of fructose was still significantly lower than that of sorbitol ($P < 0.05$). It would seem that the three glycating sugars display non-enzymic binding to protein (glutathione reductase), but at different rates. The effect of sorbitol had to be examined more closely (see below).

Incubation with drugs

Aspirin, paracetamol and ibuprofen did not display any protection against the four compounds (results not shown) except that 20 mM-aspirin gave some protection against glucose. Until the end of the incubation with glucose, the activity was higher in the presence of aspirin. To confirm that this was a real protective effect, the incubation was repeated two further times. The combined data of the three incubations (Fig. 3) showed that aspirin provided protection against inhibition of enzyme activity by 5 mM-glucose over most of the 15 days of the experiments, being statistically significant after 5 days ($P < 0.05$ by Student's *t* test). However, aspirin did not maintain the activity at the level of the controls. Aspirin itself did not affect the activity in the absence of sugar (Fig. 3).

Purity of sorbitol and binding to proteins

Sorbitol does not contain a free carbonyl group in either the ring or open-chain forms and should therefore not be able to glycate proteins. We checked the sorbitol for unchanged glucose

Fig. 1. Inhibition of bovine intestinal glutathione reductase by 0.5 M-sugars

The sugars used were: $\circ$, glucose; $\bullet$, glucose 6-phosphate; $\triangle$, fructose; $\blacktriangle$, sorbitol. All points were calculated from the means of three sets of readings for glucose and triplicates for the other sugars.

Fig. 2. Inhibition of bovine intestinal glutathione reductase by 5 mM-sugars

Sugars and symbols were as given in Fig. 1. All points were calculated from the means of four sets of readings.

Fig. 3. Protection by aspirin (20 mM) against the inhibition of glutathione reductase by glucose (5 mM)

Key to symbols: $\circ$, glucose; $\bullet$, glucose + aspirin; $\triangle$, aspirin. All points were calculated from the means of three sets of readings.

Fig. 4. Purity of sorbitol shown by g.l.c.

The expected positions of the likely contaminants are indicated: G, glucose; F, fructose. The peak at about 5 min is the reagent peak.

Fig. 5. Binding of sorbitol to total soluble bovine lens protein

All points are the means of quadruplicate determinations.

(which is chemically reduced to produce sorbitol) or for other contaminating sugars.

The glucose oxidase method indicated less than 1% of glucose was present. As expected, with the g.l.c. method, a single peak was found with no shoulders for sorbitol (Fig. 4), which came off later than the glucose peak, the latter having shoulders for the various possible derivatives (results not shown). Fructose also gave a peak with multiple shoulders. These results indicate that the sorbitol used was free of reactive glycosylating sugars and therefore that the inhibitory effect is due to the sorbitol itself.

When radiolabelled sorbitol was incubated with lens proteins there was relatively little binding of sorbitol to the proteins, and this was all over long before sorbitol started to affect glutathione reductase activity (Fig. 5; cf. Fig. 2). After 150 h, 60 mmol of sorbitol was bound/mol of protein, whereas in a similar experiment with glucose 6-phosphate, 240 mmol of the sugar were bound/mol of protein. The rapid initial sorbitol binding which does not increase further with time (Fig. 5) would suggest that this is non-specific, whereas glucose 6-phosphate binding continues to increase with time (results not shown), being indicative of glycation.

DISCUSSION

The time-dependent inhibition of bovine intestinal glutathione reductase by glucose, glucose 6-phosphate and fructose suggests that these sugars bind non-enzymically to this enzyme. The possibility that the sugars act by exerting an osmotic effect was ruled out by the lack of inhibition by ethanediol. The rate of glycation is dependent on the percentage of sugar in the open-chain form, in which the carbonyl group is available for Schiff-base formation. The percentage for fructose is greater than that of glucose, being reflected in the former's greater reactivity with haemoglobin (Bunn & Higgins, 1991), hence fructose's more rapid inhibition of glutathione reductase activity. The more rapid inhibition by glucose 6-phosphate compared with glucose may be due to the phosphate group adding to the disruption of the enzyme, as has been suggested for the unfolding of γ -crystallin (Beswick & Harding, 1987).

The inhibitory effect of sorbitol cannot be explained in terms of it glycosylating the enzyme, owing to its lack of a carbonyl group. Although some initial binding was observed when using radiolabelled sorbitol and total bovine lens proteins, this occurred before sorbitol displayed any inhibitory effect on the enzyme, and the degree of binding did not alter with time. Sugar contaminants were not found using both colorimetric and g.l.c. methods.

Glycation of free amino groups *in vivo* has been shown to display a preference for lysyl-lysine sequences over a single lysine residue in human serum albumin (Iberg & Flückiger, 1986). Human erythrocyte glutathione reductase has such a lysyl-lysine in its catalytic centre involved in nicotinamide binding (Pai *et al.*, 1988). This is conserved in *Escherichia coli* glutathione reductase, *Trypanosoma congolense* trypanothione reductase (Shames *et al.*, 1988) and *Pseudomonas aeruginosa* glutathione reductase (Perry *et al.*, 1991). Lysine-66 in human erythrocyte glutathione reductase is involved in nicotinamide binding, whereas lysine-67 is involved in an important intersubunit hydrogen bond to *cis*-proline-468'-O (Karplus & Schulz, 1987). Binding of a sugar molecule to either of these residues could result in inhibition of enzyme activity. Additionally in the cavity next to lysine-67 of human erythrocyte glutathione reductase is a glutamic acid residue with negative charge (Karplus & Schulz, 1987). Glucose 6-phosphate could, in addition to blocking the active site, also exert electrostatic repulsion, again disrupting the dimer. Lysine-212 of human erythrocyte glutathione reductase, conserved in *E. coli*, *P. aeruginosa*, *T. congolense* and yeast dihydrolipoamide dehydrogenase, is involved in NADPH binding (Scrutton *et al.*, 1990). Glycation of this residue could also contribute to inhibition of enzyme activity. Should all these residues be conserved in

Scheme 1. A possible pathway of the decrease in reduced glutathione in diabetes via the inactivation of glutathione reductase enzymic activity by glycation

Abbreviations: Glc, glucose; G6P, glucose 6-phosphate.

bovine intestinal glutathione reductase, this could explain the inhibitory effect of the sugars on this enzyme.

The elevated levels of glucose, glucose 6-phosphate and fructose in the lens in diabetes could result in the accelerated glycation of lenticular glutathione reductase. As we have shown inhibition of enzyme activity for bovine intestinal glutathione reductase, the enzyme in the lens may similarly be affected, resulting in a decrease in GSH (Scheme 1), providing one possible explanation for early loss of GSH in diabetic cataract.

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