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Modification of the glyoxalase system in human red blood cells by glucose *in vitro*

Paul J. THORNALLEY*
MRC Mechanisms of Drug Toxicity Group, Pharmaceutical Sciences Institute, Aston University, Aston Triangle, Birmingham B4 7ET, U.K.

The human red-blood-cell glyoxalase system was modified by incubation with high concentrations of glucose *in vitro*. Red-blood-cell suspensions (50%, v/v) were incubated with 5 mM- and 25 mM-glucose to model normal and hyperglycaemic glucose metabolism. There was an increase in the flux of methylglyoxal metabolized to D-lactic acid via the glyoxalase pathway with high glucose concentration. The increase was approximately proportional to initial glucose concentration over the range studied (5–100 mM). The activities of glyoxalase I and glyoxalase II were not significantly changed, but the concentrations of the glyoxalase substrates, methylglyoxal and *S*-D-lactoylglutathione, and the percentage of glucotriose metabolized via the glyoxalase pathway, were significantly increased. The increase in the flux of intermediates metabolized via the glyoxalase pathway during periodic hyperglycaemia may be a biochemical factor involved in the development of chronic clinical complications associated with diabetes mellitus.

INTRODUCTION

Diabetes mellitus is a disease associated with abnormal carbohydrate metabolism, arising from insulin deficiency and/or malfunction of insulin receptors. The consequent elevation of the concentration of glucose in the blood plasma produces hyperglycaemia in tissues with insulin-independent uptake of glucose. Important examples of affected tissues are lens fibre cells, the vascular endothelial cells and red blood cells (Kinoshita, 1974; Davis, 1979; Betz *et al.*, 1983). Where the symptoms of diabetes mellitus are managed by insulin therapy and diet, there remains a periodic hyperglycaemia in these tissues. The cumulative effects of this managed periodic hyperglycaemia are thought to be a major causal factor in the development of the chronic clinical complications (cataract, retinopathy, nephropathy, neuropathy, generalized microangiopathy and arteriosclerosis) associated with diabetes (Keen & Jarrett, 1982).

There are characteristic features of red-blood-cell biology in diabetes mellitus which are thought to contribute to clinical abnormalities of the microcirculation (Davis, 1979). Red-blood-cell aggregation is increased (Schmid-Schonbein & Volger, 1976), deformability of the red-blood-cell membrane is decreased (Juhan *et al.*, 1982) and the mean lifespan of red blood cells is decreased during diabetes mellitus (Petersen *et al.*, 1977). Hyperglycaemia is thought to mediate these characteristic changes through mechanisms involving the non-enzymic glycation of haemoglobin (Gonen *et al.*, 1977) and other proteins (Schleicher *et al.*, 1981), and the intracellular accumulation of sorbitol (Malone *et al.*, 1984) formed by the reduction of glucose with NADPH catalysed by aldose reductase (Srivastava *et al.*, 1984). Another factor to be considered is the formation of α-oxoaldehydes during hyperglycaemia (Thornalley, 1985; Wolf & Dean, 1987) and their metabolism to aldonic acids via α-hydroxyacylglutathione intermediates by the glyoxalase system (Carrington & Douglas, 1986). Here I describe the modification of the glyoxalase system with change in glucose concentration in isolated human red blood cells *in vitro*. The results suggest that the flux of methylglyoxal metabolized to D-lactic acid via the glyoxalase pathway is increased during exposure to high glucose concentrations, similar to those found physiologically in hyperglycaemia associated with diabetes mellitus (10–50 mM). This produces an elevation of the cellular concentration of methylglyoxal and *S*-D-lactoylglutathione *in vitro*. This may be a biochemical factor involved in the development of chronic clinical complications associated with diabetes mellitus.

MATERIALS AND METHODS

Isolation of human red blood cells

Red blood cells were isolated from blood samples (10–20 ml) drawn daily (with informed consent of the subject) by venous puncture into tubes containing lithium heparide anticoagulant. Venous blood samples were centrifuged (2000 ***g***, 5 min) and the plasma and white blood cells were removed. The packed red-blood-cell pellet was washed three times with 4 vol. of phosphate-buffered saline (1 part 100 mM-sodium phosphate, pH 7.4; 9 parts 0.9% NaCl), and washed a fourth and final time with 4 vol. of Krebs–Ringer phosphate buffer (120 mM-NaCl/4.8 mM-KCl/1 mM-$CaCl_2$/1.2 mM-$MgSO_4$/16.5 mM-NaH_2PO_4/Na_2HPO_4, pH 7.4). Where higher glucose concentrations were used, the iso-osmoticity was preserved by decreasing the concentration of NaCl.

The modification of the red-blood-cell glyoxalase system during hyperglycaemia was modelled by incubation of 50% (v/v) red-blood-cell suspensions in Krebs–Ringer phosphate buffer with 5 mM-glucose (model for normal glycaemic control) and with 25 mM-

* Present address: Department of Chemistry and Biological Chemistry, University of Essex, Wivenhoe Park, Colchester, Essex, CO4 3SQ, U.K.

glucose (model for hyperglycaemia). Red-blood-cell suspensions were incubated for 1 h at 37 °C to attain the model steady-state glycolysis. The glyoxalase system was then characterized in normal glycaemic and hyperglycaemic models.

Assay of red-blood-cell glyoxalase activities

Red blood cells were collected by centrifugation of incubation samples (2000 **g**, 5 min). The red-blood-cell pellet was lysed with 4 vol. of ice-cold water and mixed well. The membrane fragments were sedimented by centrifugation (5000 **g**, 10 min) and the resultant lysate was assayed for glyoxalase I and glyoxalase II activities.

The activity of glyoxalase I was assayed by measuring the initial rate of formation of *S*-D-lactoylglutathione from hemithioacetal in the presence of diluted red-blood-cell lysate, monitored spectrophotometrically by the increase in absorbance at 240 nm; a value for $\Delta\epsilon_{240}$ of $2.86\,mM^{-1}\cdot cm^{-1}$ at pH 6.6 was assumed (Van der Jagt *et al.*, 1972). This initial concentration of hemithioacetal inhibits glyoxalase II (Uotila, 1973) and, hence, enables glyoxalase I activity to be determined as the initial rate of formation of *S*-D-lactoylglutathione. The hemithioacetal was prepared by preincubating 2 mM-methylglyoxal and 2 mM-GSH in 100 mM-sodium phosphate, pH 6.6, and 37 °C for 10 min; the nominal hemithioacetal concentration is 1.33 mM (Van der Jagt *et al.*, 1972). Activities of glyoxalase I are given in units/ml of packed red blood cells, where 1 unit is the amount of enzyme required to catalyse the formation of 1 μmol of *S*-D-lactoylglutathione/min under the assay conditions given.

The activity of glyoxalase II was assayed by measuring the initial rate of hydrolysis of *S*-D-lactoylglutathione in the presence of diluted red-blood-cell lysate, monitored spectrophotometrically by the decrease in absorbance at 240 nm; a $\Delta\epsilon_{240}$ value $3.10\,mM^{-1}\cdot cm^{-1}$ was assumed (Van der Jagt *et al.*, 1972). The initial concentration of *S*-D-lactoylglutathione was 0.3 mM in 50 mM-Tris/HCl, pH 7.4. Activities of glyoxalase II are given in units/ml of packed red blood cells, where 1 unit is the amount of enzyme required to catalyse the hydrolysis of 1 μmol of *S*-D-lactoylglutathione/min under the given assay conditions.

Assay of methylglyoxal

The concentration of methylglyoxal in red-blood-cell suspensions was assayed by an adaptation of the h.p.l.c. method of Ohmori *et al.* (1987), which determines α-oxoaldehydes as quinoxalines formed by incubating α-oxoaldehydes with σ-phenylenediamine. Red-blood-cell suspensions were treated with 2 vol. of ice-cold 100% ethanol, mixed well, and incubated on ice for 10 min. The precipitate was sedimented by centrifugation (5000 **g**, 10 min). The clear aqueous ethanol supernatant was incubated with 100 μM-σ-phenylenediamine for 2 h in the dark at room temperature and then extracted five times with 4 vol. of chloroform. The combined chloroform extracts were evaporated to dryness under vacuum at ambient temperature. The residue was dissolved in 0.3 ml of h.p.l.c. mobile phase and analysed for quinoxaline content. H.p.l.c. was performed on C_{18} columns (8 mm internal diameter × 10 cm; type 8MBC 1810; Waters Associates, Harrow, Middx., U.K.) with a mobile phase of 80% 10 mM-KH_2PO_4, pH 2.1, with 20% (v/v) acetonitrile. Quinoxalines were detected by flow spectrophotometry at 315 nm. The internal standard was 2,3-dimethylquinoxaline. The measurement was calibrated by assaying solutions of known methylglyoxal concentration. The recovery of methylglyoxal as 2-methylquinoxaline was about 90% in the normal metabolic range of methylglyoxal concentration.

Assay of GSH and *S*-D-lactoylglutathione

The concentration of GSH in red-blood-cell suspensions was determined by the method of Davies *et al.* (1984), which determines GSH as the chromophore *S*-2,4-dinitrophenylglutathione, formed from 1-chloro-2,4-dinitrobenzene in the presence of glutathione *S*-transferase. Red-blood-cell suspensions (50%, v/v; 1 ml) were treated with 0.5 ml of ice-cold 20% (w/v) trichloroacetic acid. The sample was mixed thoroughly and left on ice for 20 min. The precipitate was sedimented by centrifugation (5000 **g**, 10 min). The supernatant was removed and neutralized by extraction with 5 × 4 vol. of water-saturated diethyl ether. Residual ether was dispersed with nitrogen gas. A portion of the de-proteinized extract was incubated with 100 μM-1-chloro-2,4-dinitrobenzene, and 10 units of horse glutathione *S*-transferase (Sigma, Poole, Dorset, U.K.) in 50 mM-Tris/HCl, pH 7.4, at 37 °C for 1 h. The absorbance at 340 nm relative to the reference (without enzyme) was recorded. The measurement was calibrated and controlled by assaying solutions of known GSH concentration and Krebs–Ringer phosphate buffer respectively.

S-D-lactoylglutathione was determined by measuring the release of GSH in the deproteinized cell extract on the addition of glyoxalase II. For these measurements, most (about 90%) of the GSH was removed from the cell extract by incubating the cell suspension with 1 mM-*N*-ethylmaleimide for 10 min before treatment with trichloroacetic acid. This procedure produces a deproteinized extract depleted of GSH. A portion of deproteinized cell extract was incubated with 1-chloro-2,4-dinitrobenzene and glutathione *S*-transferase for 1 h as described above, and the absorbance at 340 nm relative to the reference was recorded. Glyoxalase II (1 unit; from yeast; Sigma) was added to the reference and assay samples; the samples were incubated for a further 1 h, and the absorbance at 340 nm, relative to the reference, was recorded. The measurement was calibrated and controlled by assaying solutions of known *S*-D-lactoylglutathione concentration and Krebs–Ringer phosphate buffer respectively. (This assay determines glyoxalase II 'reactive material', since only the glutathione moiety of the substrate is assayed. However, methylquinoxaline was the major quinoxaline found in the h.p.l.c. chromatogram, indicating that *S*-D-lactoylglutathione was the major glyoxalase II substrate determined by this procedure.)

Assay of the flux of methylglyoxal and the percentage of glucotriose metabolized by the glyoxalase pathway

The flux of methylglyoxal metabolized by the glyoxalase system in red blood cells was assayed by determining the rate of formation of D-lactic acid over a 2 h period. D-Lactic acid was assayed in 50% (v/v) red-blood-cell suspensions before and after incubation in Krebs–Ringer phosphate buffer at 37 °C for 2 h. The effect of glucose concentration was investigated by incubating red blood cells with 5, 25, 50 and 100 mM-glucose.

Table 1. Modification of the glyoxalase system during model hyperglycaemia *in vitro*

Parameter	Normal glycaemic control (initial ([glucose] = 5 mM) [mean ± S.D. (*n*)]	Hyperglycaemia (initial ([glucose] = 25 mM) [mean ± S.D. (*n*)]	Significance
Glyoxalase activity (units/ml of packed red blood cells)			
Glyoxalase I	57.7 ± 9.2 (4)	51.2 ± 6.2 (4)	$P > 0.10$
Glyoxalase II	18.2 ± 1.5 (4)	18.4 ± 1.6 (4)	$P > 0.10$
Glyoxalase metabolites (nmol/ml of packed red blood cells)			
Methylglyoxal	0.80 ± 0.14 (4)	1.66 ± 0.26 (4)	$P < 0.01$
S-D-Lactoylglutathione	14.9 ± 4.6 (4)	39.3 ± 7.4 (4)	$P < 0.01$
GSH	2088 ± 47 (4)	2006 ± 67 (4)	$P > 0.10$
Flux of methylglyoxal (nmol/ml of packed red blood cells/h)			
D-lactic acid formation	5.2 ± 0.9 (4)	16.8 ± 1.3 (4)	$P < 0.001$

The percentage of glucotriose metabolized via the glyoxalase pathway was assayed by determination of the rate of glucose consumption concomitant with D-lactic acid formation. The percentage was calculated as follows:

$$\text{Percentage of glucotriose metabolized by the glyoxalase pathway} = \frac{\text{Rate of D-lactic acid formation}}{2 \times \text{Rate of glucose consumption}} \times 100$$

The concentration of D-lactic acid in red-blood-cell suspensions was assayed by the method of Brandt *et al.* (1980). The concentration of glucose in red-blood-cell suspensions was assayed by the method of Raabo & Terkildsen (1960) by using a Sigma glucose assay kit (no. 510-A).

Statistical analysis

Data from model normal glycaemic control and hyperglycaemia were tested for significance of difference by using a two-tailed *t* test.

RESULTS

Modification of the red-blood-cell glyoxalase system during model hyperglycaemia *in vitro*

When 50% (v/v) red-blood-cell suspensions were incubated for 1 h in Krebs–Ringer phosphate buffer with 5 and 25 mM-glucose, there was no significant change in the activity of glyoxalase I nor a significant change in the activity of glyoxalase II (Table 1). There was also no significant change in the cellular concentration of GSH. However, the concentration of methylglyoxal was elevated by ~ 108%, and the concentration of *S*-D-lactoylglutathione was elevated by ~ 164% on increasing the initial glucose concentration from 5 to 25 mM.

The flux of methylglyoxal converted into D-lactic acid via the glyoxalase pathway in red blood cells was estimated by measuring the rate of formation of D-lactic acid. Human red blood cells do not metabolize D-lactic acid. The increase of the initial concentration from 5 to 25 mM increased the rate of D-lactic acid formation by ~ 223%. The effect of glucose concentration on the rate of formation of D-lactic acid formation in 50% red-blood-cell suspensions over the range of 5–100 mM glucose is illustrated in Fig. 1. The rate of D-lactic acid formation increased approximately proportionately with the increase in initial glucose concentration.

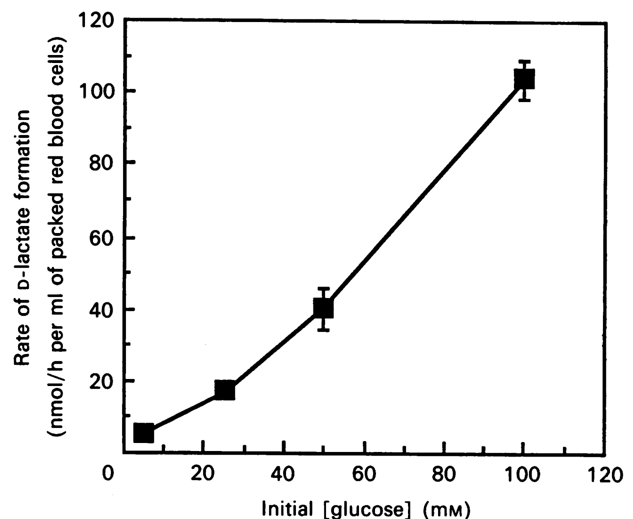


Fig. 1. Effect of glucose concentration on the rate of D-lactate formation by human red blood cells

Rates of D-lactate formation are means ± S.D. of four determinations as described in the Materials and methods section.

The percentage of glucotriose metabolized via the glyoxalase pathway in red blood cells was estimated by measuring glucose consumption concomitant with D-lactic formation (Table 2). With an initial glucose concentration of 5 mM, ~ 0.09% of glucotriose was converted into D-lactic acid, whereas with an initial glucose concentration of 50 mM, ~ 0.4% glucotriose was converted into D-lactic acid. Therefore, although the glyoxalase pathway represents only a minor fate for glucotriose in red blood cells, it increases in metabolic importance with increased concentration of glucose.

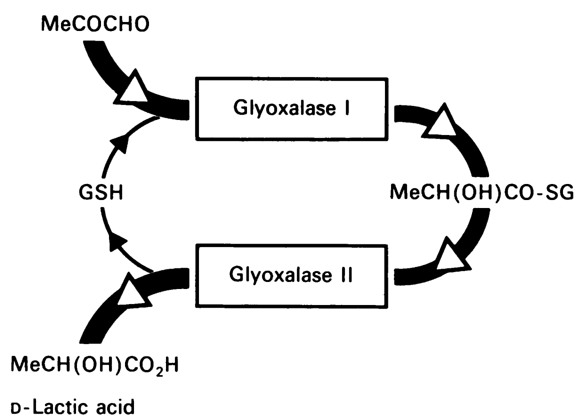
DISCUSSION

The glyoxalase system catalyses the conversion of methylglyoxal into D-lactic acid via the intermediate *S*-D-

Table 2. Rate of D-lactic acid formation and percentage of gluocotriose metabolized by the glyoxalase pathway in human red blood cells

Rates of glucose consumption and D-lactate formation were determined over 2 h in 50% red-blood-cell suspensions in Krebs–Ringer phosphate buffer, pH 7.4 and 37 °C.

Initial [glucose] (mM)	Rate of glucose consumption (nmol/h per ml of red blood cells) [mean ± S.D. (*n*)]	Rate of D-lactic acid formation (nmol/h per ml of red blood cells) [mean ± S.D. (*n*)]	Glucotriose metabolized to D-lactate (%)
5	2928 ± 130 (4)	5.2 ± 0.9 (4)	0.089 ± 0.019 (4)
25	–	16.8 ± 1.3 (4)	–
50	5208 ± 260 (4)	40.2 ± 5.6 (4)	0.386 ± 0.073 (4)
100	–	103.8 ± 5.2 (4)	–



Scheme 1. The glyoxalase pathway

lactoylglutathione. It comprises two enzymes, glyoxalase I and glyoxalase II, and a catalytic amount of GSH (Scheme 1). Glyoxalase I (EC 4.4.1.5) catalyses the formation of *S*-D-lactoylglutathione from the hemithioacetal formed non-enzymically from methylglyoxal and GSH:

$$\mathrm{MeCOCHO + GSH \rightleftharpoons MeCOCH(OH) - SG.}$$

Glyoxalase II (EC 3.1.2.6) is a thiol esterase, catalysing the hydrolysis of *S*-D-lactoylglutathione to D-lactic acid and GSH. The glyoxalase system is present in the cytosol of all cells and is the major route for the metabolism of methylglyoxal and other α-oxoaldehydes (Carrington & Douglas, 1986). Methylglyoxal is considered to be the physiological substrate and is formed from dihydroxyacetone phosphate, catalysed by methylglyoxal synthase (Hopper & Cooper, 1972; Tsai & Gracy, 1976; Ray & Ray, 1981).

The possible involvement of α-oxoaldehyde metabolism in hyperglycaemia and associated chronic clinical complications of diabetes was recognized following the observation that α-oxoaldehydes were a major product of the slow non-enzymic degradation of monosaccharides, catalysed by trace metal ions, termed 'monosaccharide autoxidation' (Wolf *et al.*, 1984; Thornalley, 1985). Later studies suggested monosaccharide autoxidation may contribute to protein modification by glucose *in vitro*, particularly with respect to the formation of chromophoric and fluorophoric pigments from glycated protein (Wolf & Dean, 1987). However, our examination of blood samples from diabetic patients (P. J. Thornalley, N. I. Hooper, A. F. Jones, J. Lunec, C. Florkowski & A. H. Barnett, unpublished work) and isolated red-blood-cell suspensions (reported above) indicated the major detectable α-oxoaldehyde was methylglyoxal, an α-oxoaldehyde formed enzymically and not by monosaccharide autoxidation.

The red-blood-cell glyoxalase system represents > 95% glyoxalase activities and *S*-D-lactoylglutathione in circulating blood. Methylglyoxal readily crosses the red-blood-cell membrane and therefore is found in both the cellular and plasma fraction of whole blood (Hooper, 1987).

In the present study, isolated human red blood cells were incubated in Krebs–Ringer phosphate buffer with 5 and 25 mM-glucose to model normal and hyperglycaemic control respectively. In the hyperglycaemic model, the activities of the glyoxalase enzymes were maintained, but the concentrations of methylglyoxal and *S*-D-lactoylglutathione were elevated. This occurred concomitant with an increase in flux of methylglyoxal metabolised via the glyoxalase pathway during hyperglycaemia, as judged by the rate of D-lactic acid formation. The increase in glyoxalase metabolic flux represented a minor, yet increased, fraction of glucotriose metabolized via the glyoxalase pathway during hyperglycaemia. The increase in metabolic flux through the glyoxalase system during hyperglycaemia is the probable cause of the increased concentrations of methylglyoxal and *S*-D-lactoylglutathione. The increase in metabolic flux during hyperglycaemia suggests that the rate of methylglyoxal formation is enhanced during hyperglycaemia in red blood cells *in vitro*. α-Oxoaldehyde metabolism via the glyoxalase system may be involved in the development of diabetic complications. From this study it is expected that periodic hyperglycaemia *in vivo* is accompanied by a periodic increase in the cellular concentrations of methylglyoxal and *S*-D-lactoylglutathione. The effect of abnormally high concentrations of these metabolites on red-cell biology is not known.

Methylglyoxal binds to, and modifies, arginine residues, and it binds to, and cross-links, lysine residues in proteins. It may thereby inactivate enzymes with arginine and lysine in the active site and may change the tertiary structure and solubility characteristics of proteins. However, most of these effects occur to a significant extent only with relatively high concentrations of

methylglyoxal (Leonici *et al.*, 1980; Schauenstein *et al.*, 1977).

S-D-Lactoylglutathione potentiates the GTP-promoted assembly of microtubules *in vitro* (Gillespie, 1975), the release of histamine from basophils (Gillespie, 1978) and may be involved in the regulation of the microtubular cytoskeleton during the functional activation of human neutrophils (Thornalley *et al.*, 1987).

Modification of the glyoxalase system during hyperglycaemia may contribute to the development of diabetic complications through methylglyoxal-mediated changes in protein solubility and aggregation characteristics (Brownlee *et al.*, 1984; Monnier *et al.*, 1986) and periodic modification of the microtubular cytoskeleton during hyperglycaemia by *S*-D-lactoylglutathione.

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