Human erythrocyte sorbitol metabolism and the role of sorbitol dehydrogenase

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Summary. Rapid fluctuation of erythrocyte sorbitol in response to the changes in plasma glucose concentration has been reported from clinical evidence. We performed more extensive in vitro and in vivo studies focussing on how fast sorbitol was accumulated and how fast the accumulated sorbitol was oxidised in response to the changes in ambient glucose concentration. Incubation studies of intact erythrocytes from healthy subjects and diabetic patients showed that erythrocyte sorbitol increased rapidly in response to increased ambient glucose concentration and the accumulated sorbitol easily decreased according to the rapid reduction of ambient glucose concentration. In addition, the higher the glucose concentration in the medium, the more erythrocytes could accumulate sorbitol. The rapid response of sorbitol levels to ambient glucose concentration was further confirmed by the results of a 75 g oral glucose tolerance test in non-diabetic subjects and diabetic patients with gastrectomy, who showed marked early hyperglycaemia caused by rapid absorption of ingested glucose and subsequent rapid reduction of plasma glucose concentration (erythrocyte sorbitol levels changed concomitantly). These findings strongly indicate that the measurement of erythrocyte sorbitol is not useful as an index of medium or long term glycaemic control.

Key words: Human erythrocyte, aldose reductase, sorbitol, sorbitol dehydrogenase, polyol pathway.

Some earlier studies have shown good correlation between erythrocyte sorbitol concentration and HbA_1 [1–3], and the usefulness of measuring erythrocyte sorbitol as an index of medium or long term diabetic control [1, 3]. On the other hand, recent clinical studies have shown rapid fluctuation of erythrocyte sorbitol in response to rapid changes in plasma glucose concentration [4–6] and no consistent relationships between erythrocyte sorbitol levels and HbA_1 [6]. Therefore, measurement of erythrocyte sorbitol was suggested not to be a reliable indicator of diabetic control. However, only a couple of in vitro incubation studies using intact erythrocytes and high glucose concentrations have been presented, and the precise relationship between ambient glucose concentration and erythrocyte sorbitol levels has not yet been determined.

Human erythrocytes are highly permeable to glucose and this glucose transport is insulin-independent. Human erythrocyte hexokinase has a very low Km for glucose (0.05–0.08 mmol/l) and is saturated even at normal plasma glucose levels (5 mmol/l) [7], while the Km of erythrocyte aldose reductase is 9 mmol/l [8]. Therefore, it seems very reasonable that glucose utili-sation via the polyol pathway does increase during hyperglycaemia, while glucose utilisation via the glycolytic pathway increases only by a very small percentage [7]. Since the erythrocyte membrane is not permeable to sorbitol, the intracellular sorbitol level simply reflects an equilibrium between its formation by aldose reductase and its depletion by sorbitol dehydrogenase [9].

We performed in vitro and in vivo studies focussing on how fast sorbitol was accumulated and how fast the accumulated sorbitol was oxidised in response to the changes in ambient glucose concentration in order to resolve the clinical question whether measurement of erythrocyte sorbitol could serve as a reliable index of diabetic control.

Subjects and methods

Erythrocyte incubation studies

Heparinised blood was obtained from healthy subjects and diabetic patients [10], and 500 µl of washed erythrocytes were incubated with 2 ml of 0.1 mol/l HEPES buffer (pH 7.4), containing 135 mmol/l NaCl and 5 mmol/l glucose, for 1 h at 37°C in a shaking bath for equilibration. Then, glucose was added to the incubation medium to give final concentrations of 5-50 mmol/l and the incubations were continued for 5 h at 37°C. In some experiments, erythrocytes were incubated with 30 mmol/l glucose for 1 h and then with 5 mmol/l glucose. The changes of pH even after the 5 h incubations were very small (within 0.05) without any difference between 5 and 50 mmol/l glucose medium.

Oral glucose tolerance test

In two non-diabetic subjects and two diabetic patients with gastrectomy, the concentrations of plasma glucose and erythrocyte sorbitol were measured during a 75 g oral glucose tolerance test.

Biochemical procedures

Sorbitol was measured spectrophotometrically with sorbitol dehydrogenase (from sheep liver, purchased from Sigma, St.Louis, Mo, USA) by a modified method of Beutler [11]. Deproteinised and neutralised samples were added to the reaction mixture containing 2 mmol/l NAD^+ and 1 unit of sorbitol dehydrogenase. Although sorbitol dehydrogenase is not specific for sorbitol, validity of this assay has been proven [1, 12].

Glucose and fructose were assayed as reported elsewhere [13, 14].

Sorbitol dehydrogenase assay

The assay was performed by the method of Barreto et al. [15]. The effects of glycolytic intermediates, nucleotides and fructose on sorbitol dehydrogenase activity were investigated at 2- and 10-fold physiological concentrations.

Partial purification of sorbitol dehydrogenase

Erythrocytes free from leukocytes and platelets were haemolysed, and then passed through a DEAE cellulose column, pre-equilibrated with buffer A (10 mmol/l potassium phosphate, pH 7.4). The column was washed with buffer A eluting against a linear gradient of 0-0.35 mol/l KCl in buffer A. The active fractions were pooled and precipitated by ammonium sulfate of 45-60% saturation. After centrifugation, the pellet was extensively dialysed against buffer A and used for the sorbitol dehydrogenase assay. Using this approach, lactate dehydrogenase activity was separated from sorbitol dehydrogenase activity. Very low activities of glucose-6-phosphate dehydrogenase remained, but were not detected in the presence of NAD^+.

Statistical analysis

Data are given as means ± SD. The results were evaluated statistically using unpaired Student's t-test.

Results

Figure 1 shows the time-dependent formation of erythrocyte sorbitol at various concentrations of glucose, using intact erythrocytes from three healthy subjects and four diabetic patients. Although the erythrocyte sorbitol concentration increased in response to increased medium glucose concentration, this sorbitol accumulation was not proportional to the ambient glucose concentration. The higher the glucose concentration in the medium, the more erythrocytes could accumulate sorbitol.
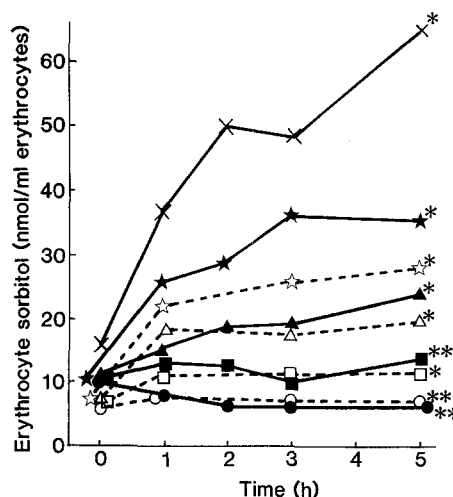

Fig. 1. The time-dependent formation of erythrocyte sorbitol. Erythrocytes from three healthy subjects were incubated with glucose (●——●, 5 mmol/l; ■——■, 10 mmol/l; ▲——▲, 20 mmol/l; ★——★, 30 mmol/l; ×——×, 50 mmol/l) for 5 h. Erythrocytes from four diabetic patients, whose HbA_1 were 11.3 ± 0.9%, were incubated with glucose (O----O, 5 mmol/l; □----□, 10 mmol/l; △-----△, 20 mmol/l; ☆-----☆, 30 mmol/l) for 5 h. The values represent means of three or four duplicate experiments. (*, $p < 0.05$, **, NS vs 0 time)
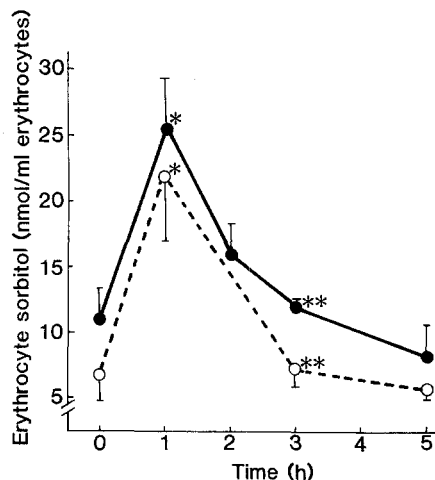

Fig. 2. The rate of oxidation of the increased sorbitol at 5 mmol/l glucose. Erythrocytes from 5 healthy (●——●) and 4 diabetic subjects (O----O) were incubated with 30 mmol/l glucose for 1 h and then for further 4 h with 5 mmol/l glucose. The values represent means ± SD. (*, $p < 0.05$; **, NS vs 0 time)
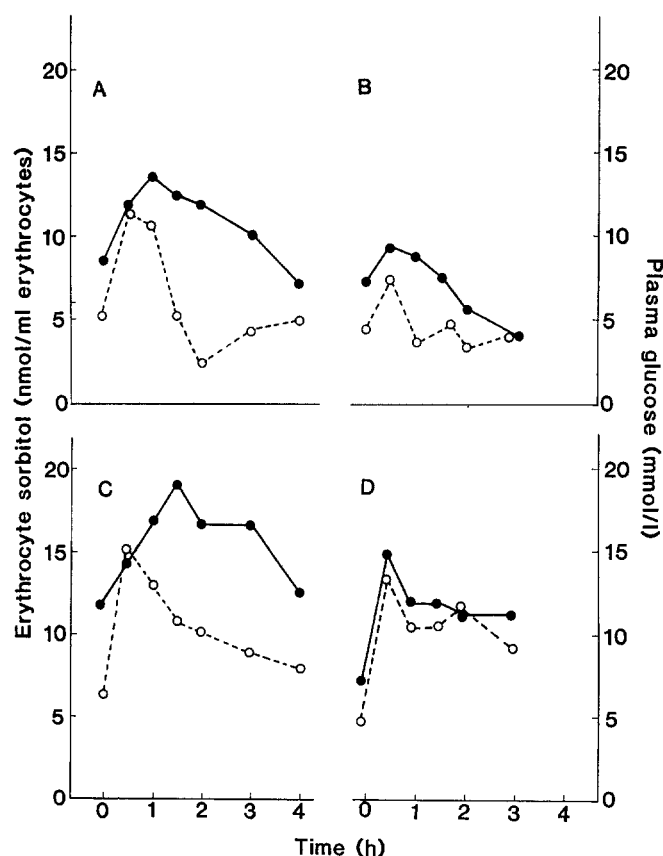
Fig. 3. Time course of plasma glucose and erythrocyte sorbitol levels in 75 g oral glucose tolerance test. Non-diabetic subjects with gastrectomy (A and B) and diabetic patients with gastrectomy (C and D). Plasma glucose (O----O), erythrocyte sorbitol (●——●)

Table 1. Sorbitol/glucose ratio in subjects with gastrectomy who were placed on a 75 g oral glucose tolerance test

Time	Sorbitol/glucose ratio (min)						
	0	30	60	90	120	180	240
Case 1	1.07	0.92	1.28	1.70	1.62	1.86	1.56
Case 2	1.50	1.11	1.13	1.13	0.98	1.24	–
Case 3	1.65	1.03	1.27	2.43	5.10	2.33	1.43
Case 4	1.58	1.27	2.38	1.65	1.74	1.00	–

Cases 1 and 2 are diabetic patients with gastrectomy. Cases 3 and 4 are non-diabetic subjects with gastrectomy. Sorbitol/glucose ratio expressed sorbitol levels (nmol/ml erythrocytes) as a function of the coincident plasma glucose concentration (mmol/l)

In Figure 2, the rate of oxidation of the increased sorbitol is indicated. In erythrocytes from 5 healthy and 4 diabetic subjects, pre-incubation levels of intracellular sorbitol were 11.1 ± 2.2 nmol/ml, 6.5 ± 1.4 nmol/ml and they reached 25.5 ± 4.3 nmol/ml, 21.8 ± 5.3 nmol/ml after 1 h incubation with 30 mmol/l glucose and returned to 12.0 ± 0.5 nmol/ml, 7.1 ± 1.2 nmol/ml within 2 h during incubation with 5 mmol/l glucose respectively. Fructose concentration (410.2 ± 169.0 μmol/l) in the medium at the end of 1 h incubation of erythrocytes from healthy subjects with 50 mmol/l glucose resulted in an approximate 8-fold

increase compared to those with 5 mmol/l glucose (51.2 ± 15.8 μmol/l). Sorbitol was not detectable in the medium. This is in agreement with a previous observation that sorbitol cannot cross the erythrocyte membrane [9]. Because the erythrocyte membrane is easily permeable to fructose [9, 16, 19], increased fructose in the medium substantially reflects increased production of fructose in erythrocytes.

Gastrectomised non-diabetic and diabetic subjects underwent a 75 g oral glucose tolerance test to investigate the relationships between plasma glucose and erythrocyte sorbitol levels (Fig. 3). Characteristically, two non-diabetic subjects showed a rapid increase of plasma glucose levels, achieving a peak of 11.6 and 7.4 mmol/l, followed by an abrupt descent to hypoglycaemic levels (Fig. 3 A and B). They, therefore, appear to be analogous to our in vitro incubation studies. In this situation, the response of erythrocyte sorbitol to the changes of plasma glucose levels was very similar to those seen in in vitro studies (Fig. 2). Two diabetic patients with gastrectomy also showed a rapid increase of plasma glucose levels, followed by a rapid increase in sorbitol levels. As plasma glucose concentration gradually decreased, erythrocyte sorbitol levels gradually returned to the fasting levels (Fig. 3 C and D). The data presented indicate that the rapid changes of sorbitol occur not only in vitro but also in vivo.

Sorbitol/glucose ratios also fluctuated widely in response to changes of plasma glucose concentration, the values in diabetic patients being similar to those in non-diabetic subjects (Table 1).

The glycolytic intermediates and nucleotides except adenosine 5'-triphosphate (ATP) and lactate were found to have no effect on sorbitol dehydrogenase activity. Three mmol/l ATP and 5 mmol/l lactate caused approximately 40% inhibition of sorbitol dehydrogenase activity. Even 1 mmol/l fructose above physiological concentration had no inhibitory effect on sorbitol dehydrogenase activity.

Discussion

Clinical application of erythrocyte sorbitol measurements as an index of medium or long term glycaemic control has been proposed [1–3], while additional clinical data has failed to demonstrate consistent relationships between erythrocyte sorbitol levels and HbA₁ [6]. As shown in Figures 1 and 2, our incubation studies indicated that erythrocyte sorbitol increased rapidly in response to the ambient glucose concentration, while accumulated sorbitol decreased easily according to the rapid reduction of glucose concentration. Similar results were also obtained during 75 g oral glucose tolerance tests. The results are consistent with the previous observation that erythrocyte sorbitol levels correlated well with coincident

plasma glucose levels [5]. These findings strongly indicate that the measurement of erythrocyte sorbitol is not useful as an index of medium or long term glycaemic control.

Another trend of using erythrocyte sorbitol measurements is the attempt to obtain representative sorbitol levels in less accessible tissues. In streptozotocin-induced diabetic rats, significant correlations of sorbitol levels between erythrocytes and the sciatic nerve or the lens were reported, proposing that erythrocyte sorbitol might be useful to monitor sorbitol levels in these tissues [3, 17], thus rendering erythrocyte sorbitol/glucose ratio helpful in assessment of the polyol pathway activities [18]. However, erythrocytes have much lower sorbitol concentrations than lens or peripheral nerve in diabetic subjects. Sorbitol levels in lens and peripheral nerve do not fluctuate as rapidly as in erythrocytes [6]. In addition, the results of the oral glucose tolerance test (Fig.3) indicate that sorbitol/glucose ratios change widely in response to changes in plasma glucose concentration. Therefore, it is concluded that erythrocyte sorbitol may not represent polyol pathway activity in other tissues.

Intracellular sorbitol levels reflect an equilibrium between production and turnover [9]; thus, the enhanced accumulation during higher glucose concentrations appears to be a result of increased synthesis and/or decreased breakdown.

Human erythrocytes are highly permeable to glucose, and the time needed to attain equilibrium between outer and inner membrane layers is 1 or 2 min [19, 20]. Therefore, entry of glucose into erythrocytes is not a rate-limiting step of intracellular glucose utilisation. Since the reduction of glucose by aldose reductase follows classical Michaelis-Menten kinetics and the reported Km of aldose reductase for glucose is 9 mmol/l [8], the calculated velocity of sorbitol synthesis at 50 mmol/l glucose should increase 2.4-fold as compared to 5 mmol/l. As shown in Figure 1, however, sorbitol synthesis increased several-fold in our present study. Therefore, the accelerated accumulation of sorbitol at the higher glucose concentration seems not to be merely due to increased availability of glucose to aldose reductase.

Aldose reductase is activated by glucose-6-phosphate and inhibited by glycerate-2,3-bisphosphate [8]. The former is consistently elevated and the latter decreased in hyperglycaemic states, both in vivo and in vitro [7, 21-23]. Therefore, sorbitol synthesis may be more activated at the higher glucose concentration.

Another possibility is a decreased rate of sorbitol breakdown. High $NADH/NAD^+$ ratios inhibit the sorbitol dehydrogenase reaction. However, increased ratios of $NADH/NAD^+$ as found in hyperglycaemic states [12] are not sufficient to reverse the reaction [24]. Among glycolytic intermediates, nucleotides and fructose, only ATP and lactate were found to have inhibitory effects on sorbitol dehydrogenase activity, but none of them were increased in diabetic patients [21] and after incubation of erythrocytes with high concentrations of glucose [12]. On the contrary, an 8-fold increase of fructose concentration after 1 h incubation with 50 mmol/l observed in our experiments may indicate the acceleration of sorbitol breakdown rather than its inhibition. This agrees with a previous report that exposure of the lens to elevated glucose levels resulted in marked increases in both sorbitol production and turnover [25]. From these findings, it is unlikely that sorbitol dehydrogenase contributes to sorbitol accumulation during hyperglycaemia.

Our studies further support recent clinical studies that erythrocyte sorbitol level is well correlated with the ambient plasma glucose concentration [5] and provide the basis to conclude that measurement of erythrocyte sorbitol means nothing more than the easy measurement of plasma glucose.

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