Nephrol Dial Transplant (1996) 11 [Suppl 5]: 95–98

Nephrology
Dialysis
Transplantation

Role of polyol pathway in nonenzymatic glycation

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Abstract. In order to confirm the link between nonenzymatic glycation and the polyol pathway, we observed the effect of treatment with epalrestat (Ep), an aldose reductase inhibitor, on the concentration of advanced glycation end-products (AGEs) in erythrocytes from diabetic patients. We also examined the effect of the drug on erythrocyte fructose 3-phosphate (F3P), a novel metabolite that has been reported to relate to the polyol pathway, and ascertained the glycation capability of F3P and its possible breakdown product, 3-deoxyglucosone (3DG), by incubating the metabolites with bovine serum albumin (BSA). Incubation of BSA with F3P or 3DG resulted in a greater production of AGEs in comparison with the incubation with glucose or fructose. F3P was significantly increased in erythrocytes from diabetic patients compared with those from nondiabetic individuals and was lower in patients who had been treated with Ep than in those who were free from the compound. A treatment of patients with Ep for 1 month resulted in a significant decrease in F3P. Erythrocyte AGEs were significantly elevated in diabetic patients compared with nondiabetic individuals and tended to be lower in patients taking Ep than in those without Ep. Administration of Ep for 2 months decreased AGEs. These results show that the polyol pathway is likely to play a substantial role in the nonenzymatic glycation of proteins and the suppression of E3P as well as AGEs by an aldose reductase inhibitor may explain in part the preventive effect of the drug on diabetic complications.

Key words: polyol pathway; advanced glycation end-products; nonenzymatic glycation; erythrocyte; non-insulin-dependent diabetes mellitus

Introduction

A number of studies have demonstrated that the polyol pathway is involved in the pathogenesis of diabetic complications and that inhibitors of aldose reductase, the first enzyme of the pathway, can prevent or ameliorate the complications [1,2]. However, the mechanisms by which the pathway plays a role have not yet been fully elucidated. Some hypotheses have been advocated to explain how the polyol pathway relates to the development of diabetic complicatons. The 'osmotic or compatible osmolyte' hypothesis [3,4] is based on the assumption that the sorbitol accumulation *per se* causes osmotic alterations and further metabolic abnormalities, such as myoinositol depletion and impaired Na^+/K^+-ATPase sensitivity, that lead to tissue damage. In contrast, the 'hyperglycaemic pseudohypoxia' hypothesis [5] emphasizes that changes in cytoplasmic redox balance as a consequence of the enhanced oxidation of sorbitol to fructose rather than glucose to sorbitol plays a predominant role in the aetiology of the complications. Another explanation for the polyol pathway-mediated mechanism of diabetic complications is that a decrease in NADPH/$NADP^+$ ratio resulting from enhanced reduction of glucose to sorbitol may impair the scavenger function for oxgen free-radical or may reduce nitric oxide production [6].

In addition to these hypotheses, the link between the polyol pathway and nonenzymatic glycation has recently attracted attention. A previous study has demonstrated that the inhibition of aldose reductase is able to reduce collagen fluorescence in diabetic rats [7], suggesting an association of the pathway with nonenzymatic glycation. Fructose was considered to be responsible for the involvement of the polyol pathway in the glycation [7], yet some metabolites have recently received attention as having a more potent glycation capability. Fructose 3-phosphate (F3P) and sorbitol 3-phosphate (S3P) are novel metabolites recently identified in mammalian lenses [8] and human erythrocytes [9]. Both compounds have been reported to be increased in the diabetic state [8,9]. F3P is of particular interest since a previous study revealed that the metabolite and its breakdown product, 3-deoxyglucosone (3DG), are much more potent glycating agents than fructose [8], thus implying its possible connection to the initiation of diabetic complications such as cataract [10]. Some *in vitro* experiments have

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shown that F3P and S3P are directly produced from fructose and sorbitol by the activity of an unknown phosphokinase [9,11]. These findings indicate an association of the metabolites with the polyol pathway.

In order to assure the link between the nonenzymatic glycation and the polyol pathway, we examined the effects of an aldose reductase inhibitor on the concentration of advanced glycation end-products (AGEs) along with F3P in erythrocytes from diabetic patients, and observed the glycation capability of F3P and 3DG.

Methods

Formation of AGE-BSA by F3P and 3DG

Bovine serum albumin (BSA; 10 mg/ml) was incubated with 20 or 100 mM F3P and 3DG as well as fructose and glucose at 37°C for 16 days, and the formation of AGE-BSA was observed. The amount of AGEs was determined by competitive ELISA using monoclonal antibodies raised against AGE-BSA that was obtained by incubating BSA with glucose [12]. In brief, each well was incubated with 0.1 ml of 1.0 μg/ml of AGE-BSA in 50 mM carbonate buffer (pH 9.7) at room temperature for 2 h. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (Buffer A) and blocked with 0.25 ml of 0.5% (w/v) gelatin in 5 mM carbonate buffer (pH 9.7) for 1 h. After washing the wells three times with buffer A, a pre-mixed solution of 50 μl each of sample and the biotinylated anti-AGE antibody solution was placed in each well. The wells were incubated for 1 h at room temperature, washed again and then 0.1 ml of avidin–biotin–horseradish peroxidase complex was added to each well. After incubation for 1 h at room temperature, the wells were washed and 0.1 ml of substrate solution was added. Following incubation for 20–30 min at room temperature in the dark, absorbance at 405 nm was measured.

Determination of erythrocyte F3P

Blood samples were obtained with oral consent from 14 non-insulin-dependent diabetic patients and seven healthy volunteers in a fasting state. Seven of the patients had been treated with 150 mg of epalrestat per day for at least 1 month prior to the examination and six patients were free from the compound (controls). Fasting plasma glucose and HbA_{1c} were matched between the two groups of patients. Blood samples were also collected from three untreated patients after administration of epalrestat for 1 month.

Approximately 20 ml of blood was drawn into heparin vacutainers and centrifuged at 1000 *g* for 15 min at 4°C. After removing the buffy coat, erythrocytes were suspended in ~2 vol of phosphate-buffered saline and centrifuged again for 15 min. The obtained packed erythrocytes were stored at −70°C until use.

Erythrocyte F3P and S3P were identified by ^{31}P NMR spectroscopy by a slight modification of the methods previously described by Petersen *et al.* [9]. In brief, 6 ml of packed erythrocytes was homogenized with 2 vol of ice-cold 1.8 M perchloric acid and centrifuged at 20 000 *g* for 15 min at 4°C. The supernatant was neutralized with 20% (w/w) KOH and centrifuged under the same conditions. The extract was lyophilized and then dissolved in 40% D_2O, 50 mM CDTA and 50 mM HEPES, pH 7.5. After filtering the solution, ^{31}P NMR spectra were obtained and the quantities of the metabolites were determined by integration of NMR peaks using 1 mM phosphorylcholine as an internal standard set at 0.49 p.p.m.

Erythrocyte AGEs

Blood samples were obtained from 18 non-insulin-dependent diabetic patients and six healthy volunteers in a fasting state. Eight of the patients had been treated with 150 mg of epalrestat per day for at least 2 months prior to the examination and 10 patients were free from the compound (controls). Fasting plasma glucose and HbA_{1c} were matched between the two groups of patients. Blood samples were also collected from six untreated patients after administration of epalrestat for 2 months.

Approximately 5 ml of blood was drawn into heparin vacutainers and centrifuged at 1000 *g* for 15 min at 4°C. After washing, packed erythrocytes were stored at −70°C until use. Thawed erythrocytes were suspended in 10 vol of PBS, lysed by an ultrasound sonicator and diluted to 1 mg/ml with PBS. The amount of AGEs in the solution was determined by the competitive ELISA as described above.

Results

Formation of AGE-BSA

After incubation for 16 days, the solution containing 20 mM or 100 mM F3P or 3DG with BSA turned brownish, whereas no change was seen in the colour of the samples incubated with glucose or fructose. Figure 1 shows the time course of AGEs formation by the incubation of BSA with 20 mM concentrations of each metabolite. As can be seen, AGEs emerged on day 4 in samples containing F3P or 3DG and rapidly increased. In contrast, only a small amount of AGEs was detected in samples incubated with glucose or fructose.

One hundred micromolar glucose incubated with BSA for 16 days produced a small amount of AGEs, and fructose yielded more AGEs than glucose (Table 1). However, incubation of BSA and F3P at the same concentration resulted in the production of 100 times the amount of AGEs than fructose (Table 1).

Erythrocyte F3P levels

Figure 2 shows F3P and S3P in erythrocytes from nondiabetic volunteers and diabetic patients who were

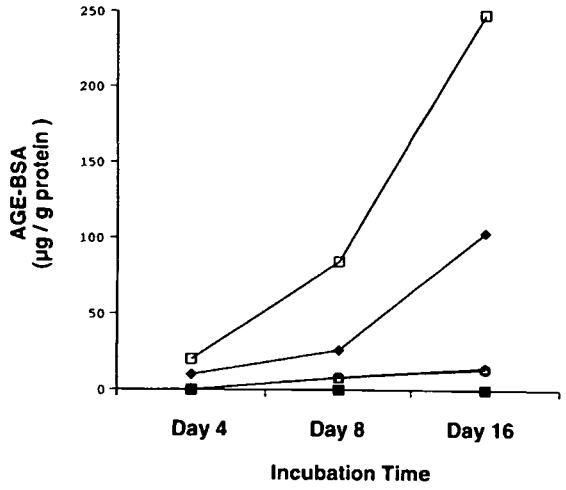

Fig. 1. Production of AGE-BSA by incubation of BSA with 20 mM glucose (O), fructose (Δ), fructose 3-phosphate (◆), 3-deoxyglucosone (□). (■), control.

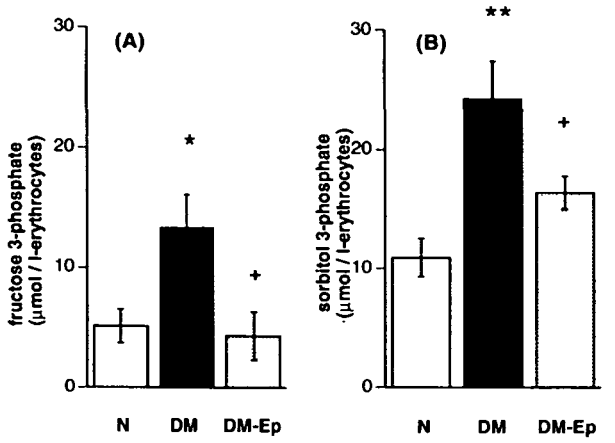

Fig. 2. Erythrocyte F3P and S3P levels in nondiabetic individuals (N) and diabetic patients (DM), and diabetic patients treated with epalrestat (DM-Ep). $^{*}P < 0.05$, $^{**}P < 0.01$ versus N, $^{+}P < 0.05$ versus DM.

Table 1. Production of AGEs by incubation of BSA with 100 mM glucose fructose and fructose 3-phosphate

Incubation (days)	AGEs-BSA (μg/g protein)		
	Glucose	Fructose	F3P
8	10.8	27.8	1550
16	13.2	30.6	4880

Table 2. Effects of treatment with epalrestat on erythrocyte fructose 3-phosphate and sorbitol 3-phosphate levels in three diabetic patients

	FPG (mM)	HbA_{1c} (%)	F3P (μmol/l erythrocytes)	S3P (μmol/l erythrocytes)
Baseline	8.1 ± 2.9	8.0 ± 0.2	8.3 ± 1.7	24.7 ± 1.9
After treatment	7.8 ± 1.8	8.0 ± 0.1	3.0 ± 2.2*	16.2 ± 0.7**

Values are expressed as means ± SE.
$^{*}P < 0.01$, $^{**}P < 0.05$ versus baseline.

free from epalrestat and those who had been treated with epalrestat for at least 1 month prior to the examination. Both F3P and S3P were significantly greater in diabetic patients than in nondiabetic individuals (5.1 ± 1.4 versus 13.3 ± 2.8 μmol/l erythrocytes, $P < 0.05$, and 10.9 ± 1.6 versus 24.2 ± 3.2 μmol/l erythrocytes, $P < 0.01$, respectively). Patients who had been treated with epalrestat showed significantly smaller amounts of both metabolites when compared with those without the medication (13.3 ± 2.8 versus 4.3 ± 1.9 μmol/l erythrocytes, $P< 0.05$, and 24.2 ± 3.2 versus 16.4 ± 1.4 mmol/l erythrocytes, $P < 0.05$, respectively). A treatment of three diabetic patients with epalrestat for 1 month resulted in a significant decrease in concentrations of both metabolites, with no changes in the plasma glucose and HbA1c (Table 2).

Erythrocyte AGEs

As shown in Figure 3, erythrocyte AGEs were significantly elevated in diabetic patients compared with nondiabetic individuals (65.8 ± 11.7 versus 25.1 ± 6.4 μg/g protein, $P < 0.05$), and showed lower in the patients who had been treated with epalrestat compared with those free from the compound [38.1 ± 12.7 versus 65.8 ± 11.7 μg/g protein, $P = 0.09$]. The treatment of patients with epalrestat for 2 months lowered, though not statistically significantly, the level of AGEs without changing plasma glucose and HbA1c (Table 3).

Discussion

It has been suggested that macromolecules such as proteins and nucleic acids are nonenzymatically glycated by glucose or some metabolites, leading to deterioration of tissue function and further to onset of diabetic complications [13,14]. This theory has been supported by the data that the inhibition of glycation by aminoguanidine is able to prevent or ameliorate all diabetic retinopathy [15], nephropathy [16] and neuropathy [17]. Although increased glucose has been considered to yield AGEs through Amadori adducts in a diabetic state [14], our data showed that the elevation of erythrocyte AGEs in diabetic patients was at least partially restored by an aldose reductase inhibitor. The results seem to indicate that the polyol pathway plays a substantial role in the formation of AGEs. Although fructose has been of interest in relating the pathway to glycation, given the superior glycation capability of F3P along with its possible breakdown product, 3DG, compared with fructose, these metabolites may account

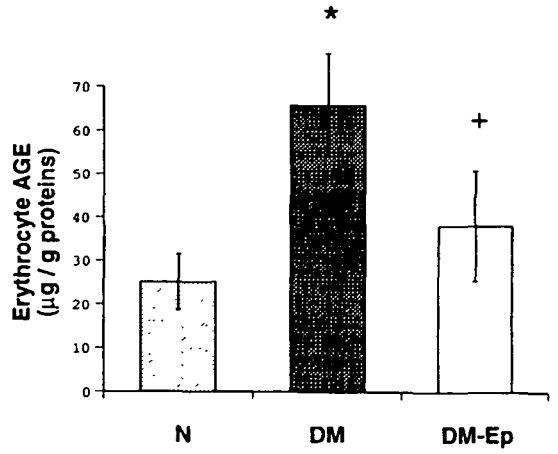

Fig. 3. Erythrocyte AGEs in non-diabetic individuals (N), diabetic patients (DM) and patients treated with epalrestat (DM-Ep). $^{*}P < 0.05$ versus N, $^{+}P = 0.09$ versus DM.

Table 3. Effects of treatment with epalrestat on erythrocyte AGEs level in six diabetic patients

	FPG (mM)	HbA_{1c} (%)	AGEs (μg/g protein)
Baseline	9.7 ± 1.2	8.4 ± 0.6	79.1 ± 24.9
After treatment	9.6 ± 1.4	9.1 ± 0.8	34.2 ± 14.4*

$^{*}P = 0.08$ versus baseline. Values are expressed as means ± SE.

for the link between the polyol pathway and glycation better than fructose. Fructose may be important as a source of these metabolites rather than as a glycating agent itself, because both F3P and 3DG have been suggested to originate from fructose [10,11,18]. The previous observations of enhanced development of diabetic complications by a fructose-rich diet in diabetic rats [19] seem concordant with this conjecture. A suppressive effect of an aldose reductase inhibitor on AGEs production could be a different explanation for beneficial effects of the drug on the development of diabetic complications.

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