Influence of Interindividual Variability of Aldose Reductase Protein Content on Polyol-Pathway Metabolites and Redox State in Erythrocytes in Diabetic Patients

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OBJECTIVE — To clarify the influence of interindividual difference in the level of aldose reductase on the polyol pathway–related metabolism in diabetic patients.

RESEARCH DESIGN AND METHODS — The enzyme protein content was determined by a two-site enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies to recombinant human aldose reductase in erythrocytes from 35 diabetic patients and 11 healthy volunteers. Patients were stratified into two groups by the median of aldose reductase content, and the erythrocyte sorbitol level, the fructose level, and the lactate-to-pyruvate ratio were compared between the two groups. We also examined the correlation of the enzyme content with these metabolic parameters.

RESULTS — The group of patients whose enzyme content was above the median showed a significant increase in the levels of sorbitol (34.7 ± 4.9 vs. 20.4 ± 2.0 nmol/g Hb, $P < 0.05$) and fructose (99.8 ± 17.2 vs. 45.9 ± 4.6 nmol/g Hb, $P < 0.05$), along with an elevated lactate-to-pyruvate ratio (28.6 ± 6.1 vs. 11.7 ± 1.2, $P < 0.05$), compared with patients with low enzyme levels. The aldose reductase content in erythrocytes was well correlated with its activity, and there was a significant correlation between the enzyme content and the erythrocyte sorbitol ($r = 0.58$, $P < 0.001$) or fructose ($r = 0.57$, $P < 0.001$) levels as well as between the enzyme level and the lactate-to-pyruvate ratio ($r = 0.38$, $P < 0.05$).

CONCLUSIONS — These results suggest that the interindividual variability of aldose reductase content may contribute tangibly to the polyol-pathway flux and cytoplasmic redox alteration in diabetic patients.

The metabolic abnormalities resulting from enhanced activity of the polyol pathway, a minor route of glucose catabolism of which physiological roles are still equivocal, have been implicated in the etiology of diabetic complications (1–3). Although effects of inhibition of aldose reductase (EC 1.1.1.21), the first enzyme of the polyol pathway, on the pathway-related metabolism and on the development of diabetic complications have been intensively studied by both basic experiments and clinical trials (4,5), the influence of interindividual differences in the aldose reductase level on the metabolic alterations has attracted little attention. The activity of aldose reductase has been found to vary in erythrocytes (6) and neutrophils (7) of diabetic patients, and the heterogeneity of the enzyme was also observed in postmortem human livers (8). Recently, an enzyme immunoassay for human aldose reductase was established, using monoclonal antibodies raised against the recombinant enzyme (9), and the enzyme content in erythrocytes was reported to vary by approximately threefold among diabetic patients (9). Because high levels of aldose reductase have been suggested to enhance the polyol-pathway flux in cultured cells (10), it may be postulated that together with hyperglycemia, heterogeneous levels of this enzyme contribute to the polyol-related metabolic abnormalities observed in diabetic patients.

To clarify this premise, we determined the aldose reductase content and activity in erythrocytes from diabetic and nondiabetic individuals and examined erythrocytes from the same individuals to determine the relationship of the enzyme content to the sorbitol and fructose levels, as well as to the lactate-to-pyruvate ratio, which are recognized as indicators of polyol pathway–mediated metabolic alterations (1–3,11).

RESEARCH DESIGN AND METHODS

Sample preparation

Fasting blood samples were collected from 11 healthy volunteers and 35 patients with NIDDM after informed consent was obtained according to the principles in the Declaration of Helsinki. Patients were recruited at the outpatient clinic of Nagoya University Hospital. Of the diabetic subjects, 10 were receiving insulin therapy, 21 were receiving oral hypoglycemic agents, and 4 were treated with diet therapy alone. Samples were carefully manipulated at 0–4°C to minimize metabolic alterations and enzyme activity loss. Blood was drawn into heparin vacutainers and was immediately cooled in ice. After samples were centrifuged for 5 min at 1,000g and a buffy coat was removed, the erythrocytes were washed with two volumes of ice-cold phosphate-buffered saline (PBS) and centrifuged again for 5 min. The packed erythrocytes that were obtained were stored at −70°C until use.

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Abbreviations: PBS, phosphate-buffered saline.

Immunoassay for aldose reductase

The aldose reductase protein content of erythrocytes was determined by a two-site enzyme-linked immunosorbent assay using monoclonal and polyclonal antibodies directed against recombinant human aldose reductase as described previously (9). In brief, immunoplates were coated at 4°C overnight with polyclonal antibodies (2 mg/l in PBS). After the plates were washed with 0.05% Tween 20 in PBS, they were blocked with 0.2% bovine serum albumin and 0.05% Tween 20 in PBS (blocking solution) for 1 h. After the washing procedure, 100 µl of sample solution diluted in the blocking solution was placed, and the plate was incubated for 90 min and then washed. Next, 100 µl of horseradish peroxidase-labeled Fab′ fragment solution (150 µg/l in blocking solution) was placed, and the plate was incubated for 30 min and washed. After 100 µl of substrate solution (0.5 g/l *o*-phenylenediamine and 75 mg/l hydrogen peroxide in purified water) was placed, the plate was incubated for ~30 min. Absorbance at 492 nm was measured with a microplate reader (Model 3550-UV; Bio-Rad, Richmond, CA).

Determination of aldose reductase activity

Erythrocyte aldose reductase activity was measured spectrophotometrically with DL-glyceraldehyde as substrate after separation from aldehyde reductase, an enzyme that has overlapping substrate specificity with aldose reductase, by chromatofocusing as described previously (9). Briefly, 1.5 ml of thawed erythrocytes was homogenized with an equal volume of 25 mmol/l imidazole-HCl buffer, pH 7.0, containing 5 mmol/l 2-mercaptoethanol by sonication. The homogenate was then centrifuged at 20,000*g* for 15 min at 4°C. To determine Hb concentrations, 40 µl of the supernatant was used, and the rest of the supernatant was desalted and equilibrated with the same buffer by a DG10 gel filtration column. The sample was applied to a PBE94 polybuffer exchange column (0.7 ×18 cm) after which the column was washed with 20 volumes of the buffer. The proteins were eluted with polybuffer 74 (1:8 dilution, pH 4.0) at a flow rate of 6 ml/h with a fraction volume of ~2.5 ml. An activity in each fraction was measured, and the total activity of the first peak of enzyme was calculated as aldose reductase activity. An assay mixture for measurement of the enzyme activity consisted of 50 mmol/l potassium phosphate buffer, pH 6.0, 0.4 mol/l lithium sulfate, 5 mmol/l 2-mercaptoethanol, 10 mmol/l DL-glyceraldehyde, 0.1 mmol/l NADPH, and a sample solution. The activity was measured spectrophotometrically at 340 nm, and one unit of enzyme activity was defined as the amount that oxidized 1 µmol/min of NADPH at 37°C.

Measurement of metabolites

The erythrocyte sorbitol level was determined by the enzyme fluorometric method previously reported (12), and plasma glucose was measured by the glucose oxidase method (13). The erythrocyte fructose, lactate, and pyruvate levels were determined by the enzymatic assay described previously (14–16).

Statistical analysis

Differences in the plasma glucose level, the erythrocyte sorbitol and fructose levels, the erythrocyte aldose reductase content and activity, and the erythrocyte lactate-to-pyruvate ratio between diabetic and nondiabetic groups were evaluated by the unpaired two-tailed *t* test, and those between groups of diabetic patients stratified by erythrocyte aldose reductase content were assessed by the Mann-Whitney *U* test. Simple linear regression analysis was performed to assess correlations among the erythrocyte aldose reductase content and activity, the plasma glucose level, the erythrocyte sorbitol and fructose levels, and the erythrocyte lactate-to-pyruvate ratio.

Materials

PBE94 and polybuffer 74 were purchased from Pharmacia (Uppsala, Sweden). DG10 was obtained from Bio-Rad. Maxisorp F96 immunoplates were purchased from Nunc (Kamstrup, Denmark). Horseradish peroxidase was purchased from Toyobo (Tokyo), and other chemicals were obtained from Sigma (St. Louis, MO).

RESULTS — The fasting plasma glucose and HbA_{1c} levels (means ± SEM) were significantly higher in diabetic patients than in nondiabetic individuals (9.8 ± 0.8 vs. 5.1 ± 0.2 mmol/l, $P < 0.01$, and 9.2 ± 0.4 vs. 5.2 ± 0.2%, $P < 0.01$). There was no difference in sex between the patients and the control subjects (M/F, 18/17 vs. 6/5) but the mean age was higher in the patients (54.3 ± 1.6 vs. 39.5 ± 4.4 years, $P < 0.01$). A significant increase in the levels of erythrocyte sorbitol (27.4 ± 2.8 vs.15.1 ± 2.0 nmol/g Hb, $P < 0.05$) and fructose (71.1 ± 9.6 vs. 27.0 ± 6.7 nmol/g Hb, $P < 0.05$) was observed in diabetic patients compared with nondiabetic control subjects. The mean erythrocyte lactate-to-pyruvate ratio was also elevated in diabetic patients compared with nondiabetic control subjects (19.6 ± 3.3 vs. 14.0 ± 2.6), although statistical analysis showed no significant difference because of a large interindividual variability of the values.

The patients were then stratified by the median of aldose reductase content in this group to observe the influence of the enzyme content on the metabolic alterations. Because the absolute values of the enzyme, e.g., the normal range, are not yet established, the median was used for stratification to obtain two subgroups that contained the almost equal number of patients and that were similar in the plasma glucose and HbA_{1c} levels, along with age and sex (Table 1). The group of patients whose aldose reductase content was above the median showed significantly higher levels of sorbitol (34.7 ± 4.9 vs. 20.4 ± 2.0 nmol/g Hb, $P < 0.05$) and fructose (99.8 ± 17.2 vs. 45.9 ± 4.6 nmol/g Hb, $P < 0.05$) than patients who had the enzyme content equal to or less than the median. The patients with high enzyme content also showed a significantly increased lactate-to-pyruvate ratio compared with those who had low enzyme content (28.6 ± 6.1 vs. 11.7 ± 1.2, $P < 0.05$).

Both the content and activity of erythrocyte aldose reductase varied approximately threefold among the diabetic patients and twofold in nondiabetic individuals, with the enzyme content being strongly correlated with its activity (r = 0.85, $P < 0.0001$). The distribution of the enzyme content was normal. There was a significant correlation between the enzyme content and the erythrocyte sorbitol level (r = 0.58, $P < 0.001$), the erythrocyte fructose level (r = 0.57, $P < 0.001$), or the lactate-to-pyruvate ratio (r = 0.38, $P < 0.05$) among all subjects (Fig. 1). The correlation coefficient values between the enzyme level and these metabolic parameters were better in nondiabetic individuals, whose glycemic levels were within a small range, than in diabetic patients (r = 0.60 and 0.58 for sorbitol, r = 0.82 and 0.54 for fructose, and r = 0.48 and 0.35 for lactate-to-pyruvate ratio in nondiabetic and diabetic individuals, respectively) (Fig. 1). The plasma glucose level was also correlated with the erythrocyte sorbitol level in all subjects and

Table 1—*Clinical characteristics, erythrocyte sorbitol and fructose levels, and erythrocyte lactate-to-pyruvate ratio in nondiabetic individuals and diabetic patients stratified by the median of erythrocyte aldose reductase content*

Group	*n*	Sex (M/F)	Age (years)	Fasting plasma glucose (mmol/l)	HbA_{1c} (%)	AR activity (mU/g Hb)	Sorbitol (nmol/g Hb)	Fructose (nmol/g Hb)	Lactate/ pyruvate
AR <9.4 μg/gHb	18	10/8	56.5 ± 1.8	9.5 ± 0.8	9.1 ± 0.5	12.2 ± 0.6	20.4 ± 2.0	45.9 ± 4.6	11.7 ± 1.2
AR >9.4 μg/gHb	17	8/9	51.9 ± 2.6	10.2 ± 1.3	9.2 ± 0.7	17.9 ± 1.1*	34.7 ± 4.9†	99.8 ± 17.2†	28.6 ± 6.1†

Data are means ± SEM. AR, aldose reductase. *$P < 0.001$ vs. low-AR group; †$P < 0.05$ vs. low-AR group.

in diabetic patients ($r = 0.63$, $P < 0.001$, and $r = 0.58$, $P < 0.001$, respectively), as well as with the fructose level ($r = 0.63$, $P < 0.001$, and $r = 0.57$, $P < 0.001$, respectively), although the plasma glucose level failed to show a statistically significant correlation with the erythrocyte lactate-to-pyruvate ratio ($r = 0.09$ in all samples). However, when patients were stratified into four subgroups by the glucose level of the mean + 2 SD of that in nondiabetic controls (6.4 mmol/l) along with the aldose reductase content (Fig. 2), the increase of the lactate-to-pyruvate ratio was most distinct in the high–aldose reductase group with high glucose levels (31.3 ± 7.9, $P < 0.05$ vs. the low-enzyme, high-glucose group). Similar results were seen in the sorbitol and fructose levels (38.1 ± 5.6 and 105.7 ± 21.5 nmol/g Hb in the high-enzyme, high-glucose group, $P < 0.05$ vs. the low-enzyme, high-glucose group, respectively). These results appear to indicate that hyperglycemia accentuated those metabolic changes in the patients with high enzyme content.

The erythrocyte sorbitol level was also significantly correlated with the erythrocyte fructose level ($r = 0.53$, $P < 0.001$ in all samples; $r = 0.61$, $P < 0.05$, and $r = 0.45$, $P < 0.01$, in nondiabetic and diabetic individuals, respectively). There was no significant correlation between the lactate-to-pyruvate ratio and the sorbitol or fructose levels ($r = 0.03$ and 0.27 in all subjects, respectively). Neither the enzyme content nor the enzyme activity was correlated with the plasma glucose level ($r = 0.24$ and $r = 0.24$ in all subjects, respectively) or with age ($r = 0.21$ and $r = 0.11$). No significant differences were observed in the enzyme level among groups of patients who were treated with insulin, oral hypoglycemic agents, or diet therapy, or between male and female groups.

CONCLUSIONS — Our study showed that there was a large variability in both the protein content and the activity of aldose reductase in erythrocytes from diabetic patients, and that the enzyme content was strongly correlated with the activity. There was no statistical difference in the specific activity of the enzyme among nondiabetic individuals and diabetic patients who had

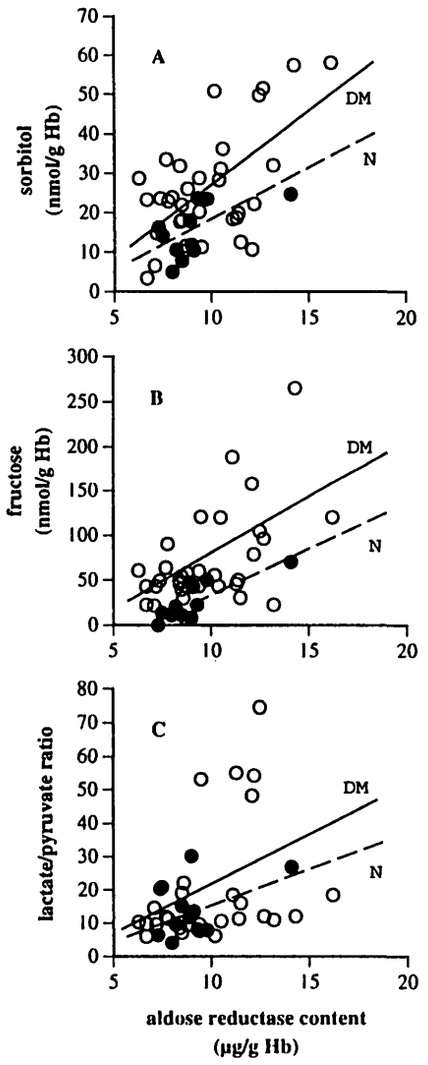

Figure 1—*Correlation of aldose reductase content with the sorbitol level* (A), *the fructose level* (B), *and the lactate-to-pyruvate ratio* (C) *in erythrocytes from diabetic* (○) *(DM) and nondiabetic* (●) *(N) individuals. A: r = 0.58, P < 0.001 (r = 0.58 in DM and r = 0.60 in N). B: r = 0.56, P < 0.001 (r = 0.54 in DM and r = 0.82 in N). C: r = 0.38, P < 0.05 (r = 0.35 in DM and r = 0.48 in N).*

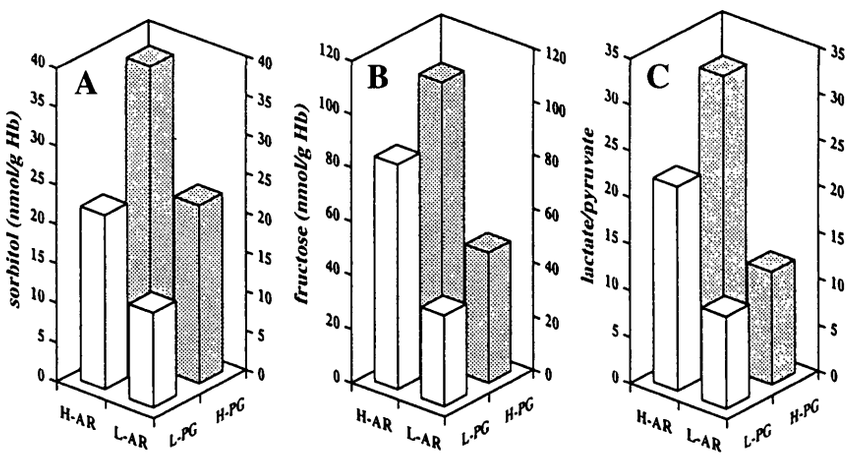

Figure 2—*Influence of aldose reductase (AR) content and the plasma glucose (PG) level on the sorbitol level* (A), *the fructose level* (B) *and the lactate-to-pyruvate ratio* (C) *in erythrocytes from diabetic patients. The high (H)-AR group had AR >9.4 μg/g Hb; the low (L)-AR group, AR <9.4 μg/g Hb; the H-PG group, PG >6.4 mmol/l; and the L-PG group, PG <6.4 mmol/l. Numbers of diabetic patients: H-AR + H-PG, n = 12; L-AR + H-PG, n = 15; H-AR + L-PG, n = 5; L-AR + L-PG, n = 3.*

high or low enzyme levels (1.32, 1.54, and 1.50 mU/μg enzyme, respectively). We have also reported that K_m values of erythrocyte aldose reductase for glucose and glyceraldehyde are similar in nondiabetic and diabetic individuals (6). These results indicate that alterations in the enzyme kinetics of aldose reductase are unlikely to play a predominant role in determining activity, and thus that the enzyme content may be usable as an indicator of enzyme activity.

Although previous data showed an increase in the aldose reductase content or activity in blood cells from patients with IDDM (6,17), the present study failed to verify the significant elevation of the enzyme content in NIDDM. One may speculatively attribute this discrepancy between IDDM and NIDDM to the heterogeneous etiology in NIDDM, because the elevation of enzyme content seen in IDDM appears to result not from hyperglycemia but from unknown mechanisms that are independent of glycemic control, according to our present findings and those of others (6,17).

We demonstrated a significant contribution of the aldose reductase content to the sorbitol level in erythrocytes from nondiabetic individuals and diabetic patients. In addition, the high level of aldose reductase was related to increased fructose concentration, along with elevated lactate-to-pyruvate ratio, which is the putative surrogate of NADH/NAD^+ ratio that is elevated as a consequence of enhanced conversion of sorbitol to fructose via sorbitol dehydrogenase activity (18). These relationships suggest a significant influence of the enzyme content in the second step of the pathway and the redox state as well. Hyperglycemia has been recognized as a crucial factor in determining the polyol-pathway metabolites, and our present analysis has shown that aldose reductase content can positively modulate the mass-action effect of hyperglycemia on the polyol-pathway flux. Thus, a combination of hyperglycemia and high aldose reductase content is likely to augment the polyol pathway–related metabolic abnormalities.

Several hypotheses have linked increased activity of the polyol pathway to the etiology of diabetic complications. The "osmotic" or "compatible-osmolyte" hypothesis (1,2) is based on the assumption that the sorbitol accumulation per se causes osmotic alterations and further metabolic abnormalities that lead to tissue damage. The "hyperglycemic pseudohypoxia" hypothesis (3) emphasizes that changes in cytoplasmic redox balance due to the enhanced oxidation of sorbitol to fructose have a predominant role in the etiology of the complications. A decrease in NADPH-to-$NADP^+$ ratio resulting from enhanced reduction of glucose to sorbitol may impair the scavenger function for oxygen free radicals (19) or may reduce nitric oxide production (20). A potential link between the polyol pathway and glycation has also been indicated by some investigations (21–23). Given that all of these theories, though still debatable, are based on the common mechanism of the enhanced polyol-pathway flux, our present data may implicate the different levels of aldose reductase in the mechanisms of diabetic complications.

The aldose reductase levels determined by current assay procedures have been observed to correspond with the prevalence or the severity of diabetic retinopathy in diabetic patients (24,25). The results are consistent with previous data that showed a link between different enzyme content (17) or activity (7,26) in blood cells and the development of diabetic microangiopathy. The latest discovery of the association of aldose reductase gene polymorphism with nephropathy in IDDM patients (27), or with retinopathy in NIDDM patients (28), may also be relevant to the results. We have lately found that the polyol pathway is involved in the functional abnormalities of erythrocytes (29), neutrophils (30), and platelets (31), with the impaired blood cell function being related to the complications (29,31). These findings are likely to account, at least in part, for the statistical association of the different aldose reductase levels in blood cells with the prevalence of diabetic complications. Correlation between the enzyme levels in blood cells and those in target tissues of microangiopathy may be considered as another explanation for the implication of the blood enzyme content in the pathogenesis of complications, yet this issue remains to be clarified.

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