Epalrestat, an Aldose Reductase Inhibitor, Reduces the Levels of N^{ε}-(Carboxymethyl)lysine Protein Adducts and Their Precursors in Erythrocytes From Diabetic Patients

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OBJECTIVE — To clarify the role of the polyol pathway in the intracellular formation of advanced glycation end products in human tissues, we examined the effects of epalrestat, an aldose reductase inhibitor, on the level of N^{ε}-(carboxymethyl)lysine (CML) along with 3-deoxyglucosone (3-DG) and triosephosphates in erythrocytes from diabetic patients. Plasma thiobarbituric acid–reactive substances (TBARS) were also determined as indicators of oxidative stress.

RESEARCH DESIGN AND METHODS — Blood samples were collected from 12 nondiabetic volunteers, 38 untreated type 2 diabetic patients, and 16 type 2 diabetic patients who had been treated with 150 mg epalrestat/day. Blood samples were also collected from 14 of the untreated type 2 diabetic patients before and after the administration of epalrestat for 2 months. The amount of erythrocyte CML was determined by a competitive enzyme-linked immunosorbent assay, and 3-DG was measured by high-performance liquid chromatography.

RESULTS — In diabetic patients not treated with epalrestat, the erythrocyte CML level was significantly elevated above levels seen in nondiabetic individuals (49.9 ± 5.0 vs. 31.0 ± 5.2 U/g protein, $P < 0.05$) and was significantly lower in patients receiving epalrestat (33.1 ± 3.8 U/g protein, $P < 0.05$). Similar results were observed with 3-DG. The treatment of patients with epalrestat for 2 months significantly lowered the level of erythrocyte CML (46.2 ± 5.6 at baseline vs. 34.4 ± 5.0 U/g protein, $P < 0.01$) along with erythrocyte 3-DG ($P < 0.05$), triosephosphates ($P < 0.05$), fructose ($P < 0.05$), sorbitol ($P < 0.05$), and plasma TBARS ($P < 0.05$) without changes in plasma glucose and HbA_{1c} levels. A positive correlation was evident between the erythrocyte CML and sorbitol ($r = 0.49$, $P < 0.01$) or fructose ($r = 0.40$, $P < 0.05$) levels in diabetic patients.

CONCLUSIONS — The results indicate that epalrestat administration lowers CML and associated variables and that polyol metabolites are correlated with CML in the erythrocytes of diabetic patients. The observed results suggest that aldose reductase activity may play a substantial role in the intracellular formation of CML in the mediation of reactive intermediate metabolites and oxidative stress.

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Abbreviations: 3-DG, 3-deoxyglucosone; ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); AGE, advanced glycation end product; BSA, bovine serum albumin; CML, N^{ε}-(carboxymethyl)lysine; MDA, malonedialdehyde; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid–reactive substances.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

The enhanced formation of advanced glycation end products (AGEs) induced by hyperglycemia has been implicated in the pathogenesis of diabetic complications (1). Increased levels of plasma glucose are known to accelerate the formation of AGEs with extracellular proteins such as collagens (2,3) or serum proteins (4). These AGE-modified extracellular proteins have been shown to play important roles in a sequence of reactions that leads to tissue damage and eventually to diabetic complications (5–8). Results from the latest prospective study have provided strong support for this hypothesis (9). However, the extracellular AGE accumulation may not be a predominant contributor to diabetic microangiopathy given the fact that elderly nondiabetic people never develop microangiopathy despite accumulated AGEs in their tissue collagen (2).

In addition to extracellular AGEs, the role of intracellular AGE formation in the pathogenesis of diabetic complications has recently attracted attention. Superoxide dismutase is known to lose its activity as a consequence of reacting with reducing sugars or other intermediate metabolites, which in turn augments radical species (10,11). Fibroblast growth factor modified by glycosylation may result in impaired mitogen activity (12). Nucleic acids could also be vulnerable targets of these reactive metabolites (13,14), which may initiate apoptosis (15,16). The association between diabetic retinopathy and the lymphocyte N^{ε}-(carboxymethyl)lysine (CML) level (17) clearly indicates important roles for intracellular AGEs in the etiology of diabetic microangiopathy.

Interestingly, AGEs are produced rather rapidly from various intracellular precursors that are increased in diabetic subjects (18). Thus, high levels of intermediate metabolites likely cause an increase in intracellular AGEs in people with diabetes. Among various metabolic routes that are accelerated by hyperglycemia, the polyol pathway has

Table 1—*Clinical characteristics of nondiabetic individuals and type 2 diabetic patients treated or not treated with epalrestat*

Group	*n*	Age (years)	Duration of diabetes (years)	Sex (M/F)	Fasting plasma glucose (mmol/l)	HbA_{1c} (%)	Retinopathy	Neuropathy	Treatment (diet/ oral hypoglycemic agents/insulin)
Nondiabetic	12	45 ± 5	—	6/6	5.1 ± 0.2	5.2 ± 0.1	—	—	—
Untreated type 2 diabetic	38	52 ± 2	9.4 ± 7.7	23/15	10.6 ± 0.8*	9.7 ± 0.4*	11 (28)	14 (37)	5/20/13
Type 2 diabetic treated with epalrestat	16	57 ± 3†	11.8 ± 6.6	11/5	10.1 ± 0.8*	9.1 ± 0.4*	8 (50)	11 (69)	0/9/7

Data are means ± SEM, *n* (%), or *n*. *$P < 0.001$ and †$P < 0.05$ vs. the nondiabetic group.

been intensively investigated as an important mechanism underlying the development of diabetic complications (19). This pathway is known to play a role in the synthesis of reactive intermediates such as fructose-3-phosphate (20), which indicates the potential influence of the pathway on intracellular AGEs. Enhanced oxidative stress associated with accentuated polyol pathway flux (21,22) may also mediate AGE formation. This hypothesis may be supported by previous observations that long-term treatment with an inhibitor of aldose reductase (the first enzyme of the pathway) prevented the accumulation of AGEs in lenses from galactosemic rats (23) or in skin collagen from diabetic rats (24,25). However, whether short-term treatment with an aldose reductase inhibitor is able to suppress the formation of AGEs from intracellular proteins in diabetic human tissues remains to be determined.

In the present study, the level of CML, a dominant AGE epitope in tissue proteins (26,27), was determined in erythrocytes from type 2 diabetic patients, and the effects of epalrestat, an aldose reductase inhibitor, on CML were observed. We also examined the effects of the inhibitor on erythrocyte 3-deoxyglucosone (3-DG) and triosephosphates along with plasma thiobarbituric acid–reactive substances (TBARS) to clarify mechanisms that may link the polyol pathway and AGE formation.

RESEARCH DESIGN AND METHODS

Samples

Fasting blood samples were collected from 12 healthy volunteers and 54 type 2 diabetic patients after subjects gave informed consent according to the principles in the Declaration of Helsinki. Patients were recruited in the outpatient clinic of Nagoya University Hospital (Nagoya, Japan). Patients whose serum creatinine levels were above the normal limit were excluded from the study to avoid the influence of renal dysfunction on CML and related substances. A total of 16 patients had been treated with 150 mg epalrestat/day for at least 2 months before the examination, and 38 patients were free of the compound (control subjects). After collecting blood, 14 of the untreated patients were administered epalrestat (150 mg/day) for 2 months, after which blood samples were again collected.

Approximately 5 ml blood was drawn into heparin vacutainers and was centrifuged at 1,000*g* for 15 min at 4°C. After plasma was separated and a buffy coat was removed, erythrocytes were suspended in phosphate-buffered saline (PBS) and were centrifuged again. Obtained packed erythrocytes and the separated plasma were stored at −70°C until use.

Determination of CML

Thawed erythrocytes were lysed in 200 vol PBS and were centrifuged at 1,000*g* for 15 min at 4°C. The supernatants were diluted with PBS to a protein concentration of 1 mg/ml. Proteins were quantified by the procedure described previously (28). The amount of CML in the solution was determined by a slight modification of the competitive enzyme-linked immunosorbent assay described previously (29). In brief, each well of an immunoplate was incubated with 0.1 ml 5.0 μg/ml AGE–bovine serum albumin (BSA) in 50 mmol/l carbonate buffer (pH 9.7) at room temperature for 2 h. The wells were washed 3 times with PBS containing 0.05% (vol/vol) Tween 20 (buffer A) and were blocked with 0.25 ml of 0.5% (wt/vol) gelatin in 5 mmol/l carbonate buffer (pH 9.7) for 1 h. The wells were washed with buffer A, and 50 μl of sample premixed with 50 μl biotinylated anti-CML antibody (6D12) solution (0.5 μg/ml) was placed in each well. The wells were incubated for 1 h at room temperature and were washed again, and then 0.1 ml avidin-biotin horseradish peroxidase conjugate was added to each well. After incubation for 1 h at room temperature, the wells were washed, and then 0.1 ml substrate solution containing 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was added. The wells were incubated for 20–30 min at room temperature in a dark place, and absorbance at 405 nm was measured on a Bio-Rad 3550 UV microplate reader (Richmond, CA). We defined 1 U CML protein adducts as the amount that showed immunoreactivity equal to 1 μg CML-BSA. The interassay and intra-assay coefficients of variation were 7.1 and 8.2%, respectively.

Determination of 3-DG

The 3-DG was determined by the methods described previously (30). In brief, 1 ml erythrocytes was deproteinized with 6% perchloric acid and was centrifuged at 3,000*g* at 4°C for 20 min. The supernatant was neutralized with bicarbonate buffer and reacted with 0.1 ml 0.1% 2,3-diaminonaphthalene in methanol with 0.025 ml 1 part per million 3,4-hexanedione in methanol as an internal standard. After incubation at 4°C overnight, the resultant product was extracted by 4 ml ethyl acetate and evaporated at 30°C for 12 min. The product was dissolved in 50% methanol solution and was filtered using Millipore Ultrafree C3-LG ultrafiltration units (Bedford, MA).

The obtained solution was applied to a high-performance liquid chromatography system using a model CCPM computer-controlled pump (Tosoh, Tokyo), a TSK-GEL ODS-80Ts column (Tosoh) with a solvent buffer of 50 mmol/l phosphate buffer, and methanol and acetonitrile (7:2:1 and 4:3:3, respectively). The fluorescence (excitation 271 nm, emission 503 nm) of the effluent was monitored by a model FS-8010 fluorophotometer (Tosoh), and the 3-DG content was calculated from the peak height of the derivative using an SC-8010 chromatointegrator (Tosoh).

Table 2—*Erythrocyte CML, sorbitol, fructose, triosephosphate, 3-DG, plasma TBARS, and triglyceride levels in nondiabetic individuals and diabetic patients treated or not treated with epalrestat*

Group	CML (U/g protein)	Sorbitol (nmol/g Hb)	Fructose (nmol/g Hb)	Triosephosphates (nmol/g Hb)	3-DG (nmol/g Hb)	TBARS (nmol MDA equivalents/ml)	Triglycerides (mmol/l)
Nondiabetic	31.0 ± 5.2	14.4 ± 2.2	27.3 ± 5.9	182.6 ± 38.8	3.23 ± 0.24	2.20 ± 0.16	1.05 ± 0.14
Untreated type 2 diabetic	49.9 ± 5.0*	29.0 ± 3.4*	84.5 ± 9.6†	253.2 ± 24.0	5.46 ± 0.57†	2.70 ± 0.14*	1.56 ± 0.17
Type 2 diabetic treated with epalrestat	33.1 ± 3.8‡	14.4 ± 1.1‡	32.7 ± 7.7‡	188.9 ± 17.4	4.12 ± 0.33‡	2.53 ± 0.10	1.61 ± 0.18

Data are means ± SEM. *$P < 0.05$ and †$P < 0.01$ vs. the nondiabetic group; ‡$P < 0.05$ vs. the untreated type 2 diabetic group.

Measurement of erythrocyte sorbitol, fructose, and triosephosphates

The erythrocyte sorbitol level was determined by the enzyme fluorometric method (31). Fructose and triosephosphates were measured with the enzymatic assay as reported previously (32,33).

Determination of plasma TBARS

TBARS were measured as thiobarbituric acid–reactive species by the methods described previously (34).

Materials

The protein assay kit was obtained from Bio-Rad. Immunoplates (No. 3590) were purchased from Costar (Cambridge, MA). Avidin, biotinylated horseradish peroxidase, and an ABTS substrate kit were purchased from Vector (Burlingame, CA). Other chemicals were obtained from Sigma (St. Louis, MO).

Statistical analysis

The erythrocyte CML levels among groups of nondiabetic and diabetic individuals were assessed by analysis of variance (Fisher's protected least significant differences). The alterations in the levels of erythrocyte CML, erythrocyte sorbitol, plasma glucose, or HbA_{1c} after treatment with epalrestat were evaluated by the Wilcoxon's signed-rank test. Simple linear regression analysis was performed to assess the relationship between HbA_{1c} and erythrocyte CML levels.

RESULTS

Table 1 shows the clinical characteristics of the nondiabetic individuals and diabetic patients. Plasma glucose and HbA_{1c} levels were significantly higher in diabetic patients than in nondiabetic control subjects. No differences were evident regarding plasma glucose, HbA_{1c} levels, age, duration of diabetes, and sex between the 2 diabetic groups. The prevalence rates of retinopathy and neuropathy were higher in the patients receiving epalrestat than in the untreated patients because the drug was used for the treatment of those complications.

As shown in Table 2, in diabetic patients not treated with epalrestat, the erythrocyte CML level was significantly elevated above levels seen in nondiabetic individuals (49.9 ± 5.0 vs. 31.0 ± 5.2 U/g protein, respectively, $P < 0.05$) and was significantly lower in patients receiving epalrestat (33.1 ± 3.8 U/g protein, $P < 0.05$), which was close to the level in nondiabetic control subjects. Similar results were evident in erythrocyte 3-DG and erythrocyte sorbitol and fructose levels. An elevation and a partial restoration by epalrestat were also observed in triosephosphates and TBARS in diabetic individuals, although the differences were not statistically significant.

The treatment of patients with epalrestat for 2 months significantly lowered the level of erythrocyte CML (46.2 ± 5.6 vs. 34.4 ± 5.0 U/g protein, $P < 0.01$) and the levels of erythrocyte 3-DG, triosephosphates, sorbitol, and fructose without changes in plasma glucose and HbA_{1c} levels (Table 3). Epalrestat also reduced plasma TBARS in the same patients with no effect on plasma triglycerides (Table 3).

Erythrocyte CML levels differed among diabetic patients by ~8-fold, and a positive correlation was evident between the CML levels and the erythrocyte sorbitol ($r = 0.49$, $P < 0.01$) or fructose ($r = 0.40$, $P < 0.05$) levels in diabetic patients. The CML levels also showed a weak correlation with current HbA_{1c} levels in diabetic individuals ($r = 0.35$, $P < 0.05$). Age and duration of diabetes were not correlated with the erythrocyte CML level in the patients observed in this study ($r = 0.04$ and $r = 0.06$, respectively). A significant correlation was also evident between erythrocyte 3-DG and sorbitol ($r = 0.687$, $P < 0.01$) or fructose ($r = 0.58$, $P < 0.05$). No correlation was found between plasma TBARS and erythrocyte sorbitol or fructose.

The erythrocyte CML levels were not significantly different between patients with or without overt diabetic retinopathy (62.0 ± 13.1 vs. 44.9 ± 4.4 U/g protein, respectively) or neuropathy (43.3 ± 7.1 vs. 53.8 ± 6.7 U/g protein, respectively) in this small number of subjects.

CONCLUSIONS

Our data show that the erythrocyte CML levels were elevated in diabetic patients, which supports previous observations in lymphocytes (17). The elevated CML level was at least partially restored by an aldose reductase inhibitor

Table 3—*Effects of treatment with epalrestat on the levels of erythrocyte CML, sorbitol, fructose, triosephosphates, 3-DG, plasma TBARS, and triglycerides in diabetic patients*

	Fasting plasma glucose (mmol/l)	HbA_{1c} (%)	CML (U/g protein)	Sorbitol (nmol/g Hb)	Fructose (nmol/g Hb)	Triosephosphates (nmol/g Hb)	3-DG (nmol/g Hb)	TBARS (nmol MDA equivalents/ml)	Triglycerides (nmol/ml)
Baseline	10.4 ± 1.5	8.6 ± 0.6	46.2 ± 5.6	22.8 ± 2.4	92.5 ± 8.4	280.3 ± 26.3	4.70 ± 0.45	2.87 ± 0.13	1.77 ± 0.32
After treatment	10.4 ± 1.4	8.7 ± 0.6	34.4 ± 5.0*	11.3 ± 1.4†	36.8 ± 6.4†	179.2 ± 19.4†	4.14 ± 0.45†	2.51 ± 0.10†	1.66 ± 0.27

Data are means ± SEM. *$P < 0.01$ and †$P < 0.05$ vs. baseline values.

independent of glycemic control. In addition, a significant correlation was evident between CML and polyol pathway metabolites. The results suggest that the polyol pathway plays a substantial role in the intracellular formation of CML from short-lived proteins in human cells, which is relevant to the observations of pentosidine in tissues from galactosemic or diabetic rats (23–25). Although hyperglycemia has been thought to accelerate AGE formation through mediation of Amadori adducts (35) or glucose-derived autoxidation products (36), unrevealed factors related to the polyol pathway are likely to contribute to the increased AGEs in diabetic tissues.

Enhanced catabolism of glucose via the polyol pathway is known to augment reactive oxygen species by mechanisms such as glutathione depletion or increased prostaglandin synthesis (21,22). The accentuated oxidative stress has been linked to CML production by several lines of evidence (37). The oxidative stress may accelerate autoxidation of glucose to dicarbonyl compounds (e.g., glyoxal) that are known to be precursors of CML (36). The oxidative condition has also been shown to boost glycoxidation of Amadori products to CML (38). Recent investigations have revealed that lipid peroxidation, which is enhanced by oxidative stress as well, could be another route through which CML is produced (39). Of more interest, the latest investigation provided direct evidence for the link between intracellular CML production and oxidative stress (17). The present study has successfully shown that an aldose reductase inhibitor is able to suppress lipid peroxidation, which suggests that the inhibitor may suppress CML formation in part by its antioxidant effects (40).

Another potential explanation is the production of CML from reactive intermediate metabolites related to the polyol pathway. We previously observed that triosephosphates rapidly reacted with proteins to exhibit the high immunoreactivity to the same antibodies that were used in the present study (18), which means that triosephosphates are favorable precursors of CML. Methylglyoxal is known to arise from triosephosphates (41) and potentially mediates the formation of Nε-(carboxyethyl)lysine, which is a homolog of CML (42). Triosephosphates were shown to be elevated in diabetes and decreased by aldose reductase inhibitors (43,44) as a result of redox changes (44), and the suppressive effects of the inhibitor were also reported with methylglyoxal (45). Fructose-3-phosphate and 3-DG are other reactive metabolites originating from fructose (46), which is a product of the polyol pathway. Both metabolites are also increased in a diabetic state (20,47) and are reduced by aldose reductase inhibitors as shown by the present study and previous reports (20,48). Present data also revealed a positive correlation between 3-DG and polyol metabolites, which indicates the close link of 3-DG with the polyol pathway. Although these metabolites are thought to be potential precursors of pentosidine (49), our previous experiments showed that products from fructose-3-phosphate and 3-DG reacted with antibodies to CML (18), which suggests that these agents are possible (although not likely principal) sources of CML as well. The increased polyol pathway flux may further accentuate the pentose phosphate pathway as a consequence of a reduced NADPH/$NADP^+$ ratio (50), which leads to marked elevation in the level of sedoheptulose-7-phosphate, which is another reactive intermediate (51). We found that products from sedoheptulose-7-phosphate and BSA were also definitely reactive to the anti-CML antibodies (Y.H., et al., unpublished data). These metabolites related to the polyol pathway may account in part for the preventive effects of the drug on the accumulation of AGEs in diabetic tissues. Also noteworthy is that the potential roles of the intermediate metabolites are not excluded by their concentrations which are orders of magnitude lower than glucose, given the remarkably higher reactivity of these intermediates compared with glucose (18).

Whether the present results observed in erythrocytes are applicable to other tissues may be controversial. Several lines of evidence indicate that polyol pathway activity in erythrocytes can be a surrogate of that in other tissues. The polyol metabolite levels were correlated between erythrocytes and lens or nerve (52), and a significant correlation was evident in aldose reductase protein levels between erythrocytes and sciatic nerve (53). The association of the enzyme activity or content of erythrocyte aldose reductase with diabetic microangiopathy was also clarified (53,54).

Researchers have noticed that aldose reductase inhibition may have paradoxical aspects in AGE formation. Aldose reductase is known to favor methylglyoxal as one of the substrates (55). Most aldose reductase inhibitors at high concentrations may partially suppress the activity of aldehyde reductase (56), which has been identified as a degradation enzyme for 3-DG (57). In terms of these facts, the inhibition of the enzyme may act adversely on the levels of these reactive metabolites and on those of AGEs. However, our data and previous observations have clarified that the overall effect of aldose reductase inhibitors suppresses the formation of AGEs, which provides a novel explanation for the beneficial effects of the drugs in the prevention of diabetic complications.

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