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Glycated Cu,Zn-Superoxide Dismutase in Rat Lenses: Evidence for the Presence of Fragmentation *in Vivo*

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Cu,Zn-superoxide dismutase (Cu,Zn-SOD) exists in tissues of rats as both glycated and non-glycated forms when separated by boronate acid column chromatography. Glycated Cu,Zn-SOD is most abundant in rat lenses compared to other tissues. In normal rats lens levels of glycated Cu,Zn-SOD showed a gradual increase with age, whereas in diabetic rats substantial increases were observed. Immunoblotting analyses, using anti-hexitol lysine IgG, indicated that glycated Cu,Zn-SOD contains Amadori products. Moreover, Cu,Zn-SOD in lenses was site-specifically fragmented probably because of glycation. This is the first report of a fragmented protein, such as Cu,Zn-SOD, occurring *in vivo.*

Cu,Zn-superoxide dismutase (Cu,Zn-SOD) is an anti-oxidant enzyme that converts superoxide radicals to hydrogen peroxide and oxygen. Previous studies in our laboratory indicated that human Cu,Zn-SOD undergoes nonenzymatic glycation at specific lysine residues, such as Lys-122 and Lys-128, resulting in inactivation of the enzyme (1). We also reported site-specific (between Pro-62 and His-63) and random fragmentations during the glycation reaction (2). Levels of glycated Cu,Zn-SOD were increased in the erythrocytes of patients with diabetes mellitus (3,4), and in Werner's syndrome, which is an accelerated aging disease (5). The present study was undertaken to investigate how widely glycated Cu,Zn-SOD occurs in other tissues, and to identify the fragmentation products *in vivo.* We found that levels of glycated Cu,Zn-SOD increased in various rat tissues, especially lenses during normal aging, as well as in diabetes mellitus. Moreover, the glycated Cu,Zn-SOD is fragmented to a 14.3 kDa product *in vivo* as previously reported in our *in vitro* studies (2).

MATERIALS AND METHODS

Animals

The rats were maintained at the Institute of Experimental Animal Sciences of Osaka University Medical School and the Tokyo Metropolitan Institute of Gerontology. Streptozotocin-induced diabetic rats were obtained by injecting 100 mg/ml of streptozotocin (Sigma) into Sprague-Dawley rats (130–150 g weight and 1 month old) via a tail vein.

Procedure for a Boronate Affinity Column and ELISA of Cu,Zn-SOD

The glycated and nonglycated forms of the enzyme were separated by a boronate column as previously described (3). In brief, 1 ml of the Tsuchihashi's extract was loaded on a boronate column (PBA-60; Amicon.) and the glycated Cu,Zn-SOD was eluted with 100 mM sorbitol. Rat Cu,Zn-SOD was purified from normal rat erythrocytes according to the method described (6). ELISA was carried out according to the method described (6). The ELISA technique was able to measure 0.05–5.0 ng Cu,Zn-SOD/ml.

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Abbreviations: SOD, superoxide dismutase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, tris-buffered saline; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Immunoblot Detection with a Polyclonal Hexitol-Lysine Antibody

A polyclonal hexitol-lysine antibody was produced using the method previously described (7). Each lens was homogenized in 2 parts of 50 mM PBS(pH 7.4), containing 1 mM APMSF (*p*-amidinophenyl methanesulfonyl fluoride hydrochloride), 10mM benzamidine 10 mg of antipain and 1 mM EDTA. The homogenates were centrifuged at 100,000 × g at 4°C for 1 hr. Supernatants of homogenates were subjected to reduction by 0.1M $NaBH_4$ for immunoblot detection with a polyclonal hexitol-lysine antibody (7).

Specific Detection of Glycated Cu,Zn-SOD by Immunoprecipitation

Reduced samples were incubated with 10 μg of rabbit anti-Cu,Zn-SOD IgG for 2 hr at 4°C, followed by incubation with 20 μl of Protein-A trisacryl (50% suspension) for 2 hr at 4°C. The samples were dissolved in SDS-PAGE sample buffer and fractionated by 12.5% SDS-PAGE. Proteins in the gels were transferred to PVDF (Millipore) membranes and incubated with anti-hexitol-lysine IgG as previously indicated (7). After washing, the membranes were incubated with second antibody (HRP-conjugated goat anti-rabbit IgG). HRP was detected by ECL kit (Amersham). The membranes were exposed to a Kodak scientific imaging film.

RESULTS

Glycated Cu,Zn-SOD Levels in Several Tissues of Diabetic Rats

Tissue extracts from diabetic rats were analyzed by ELISA. As shown in Table 1, the levels of glycated Cu,Zn-SOD in rat lenses were significantly higher than these of other tissues examined.

Progressive Changes of Glycated Cu,Zn-SOD in Diabetic and Normal Rat Lenses

The percentages of glycated Cu,Zn-SOD in rat lenses at 4,8,16 weeks after the development of diabetes were 37.0 ± 5.3%, 55.9 ± 4.8%, 61.3 ± 4.1%, respectively. In normal rat lenses, however, the percentage of the glycated form was about half that seen in diabetic rat lenses. (Fig. 1)

Increase of Glycated Cu,Zn-SOD Levels in Rat Lenses during the Normal Aging Process

In normal rats lenses the levels of glycated Cu,Zn-SOD showed a gradual age-related increase, whereas in diabetic rats substantial increases were observed. In the lenses of 2 year-old normal rats, the percentage of the glycated form was 51.4 ± 1.9% (Fig. 2). These data indicate that, in the hyperglycemic state, the level of lens glycated Cu,Zn-SOD increased within 2 months to the same levels seen in 2 years-old normal rat lenses.

Immunoblot Detection of Glycated Cu,Zn-SOD with a Polyclonal Hextol-Lysine Antibody, and Evidence for the Presence of fragmentation in Vivo

Fig. 3-A shows immunoblot analysis of lens extracts from diabetic and normal rats with anti-hexitol-lysine IgG. In the hyperglycemic state, glycated proteins were obviously increased. The two major bands were crystallins as expected (7). After immunoprecipitation using anti-Cu,Zn-SOD IgG, two components of Cu,Zn-SOD were detected in diabetic and normal rat lenses by the

TABLE 1
Percentages of the Glycated Cu,Zn-SOD in Several Tissues of Streptozotocin Diabetic Rats (Diabetic Duration 12 Weeks, n = 4)

Tissue	Glycated form (%)
Lens	60.0 ± 5.8
Blood	37.1 ± 5.6
Pancreas	17.2 ± 4.1
Kidney	21.8 ± 1.7
Liver	22.3 ± 2.8

Values represent means ± S.D.

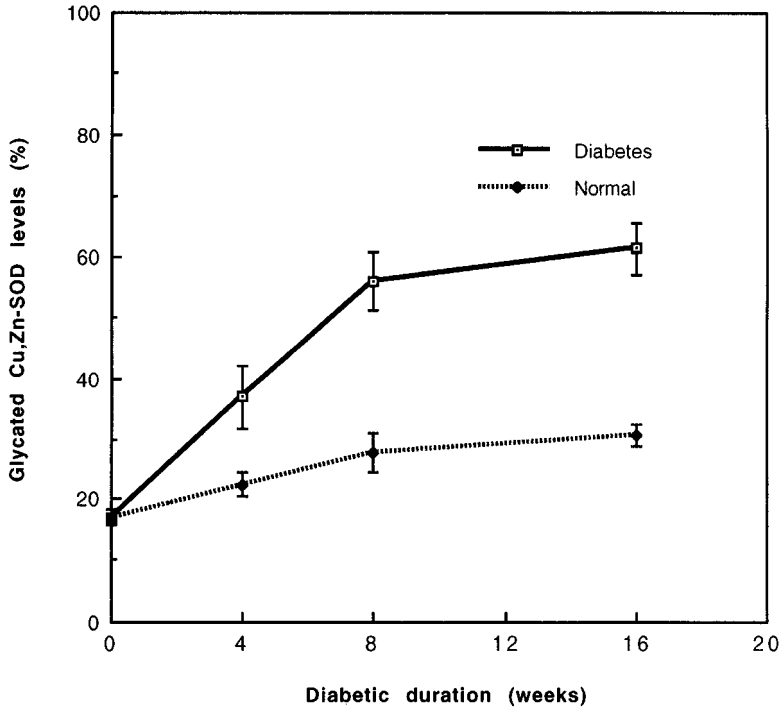

FIG. 1. Levels of glycated Cu,Zn-SOD in diabetic and normal rat lenses. The glycated and nonglycated forms of Cu,Zn-SOD in diabetic and normal rat lenses were separated by a boronate acid column (1.0 × 6 cm). Then glycated Cu,Zn-SOD levels were measured by specific ELISA methods using polyclonal antibodies. Means ± SD; n = 5.

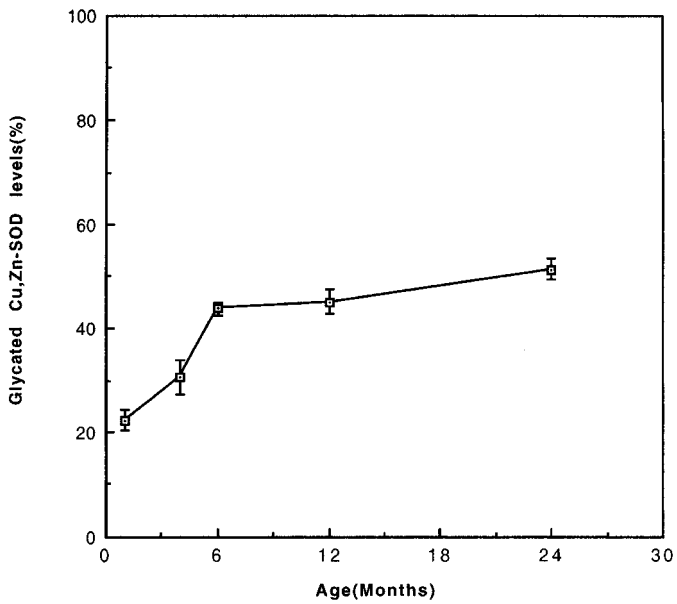

FIG. 2. Increase of glycated Cu,Zn-SOD levels in normal rat lenses with aging process. In normal rat lenses, glycated Cu,Zn-SOD was measured by ELISA at various ages. Means ± SD; n = 5.

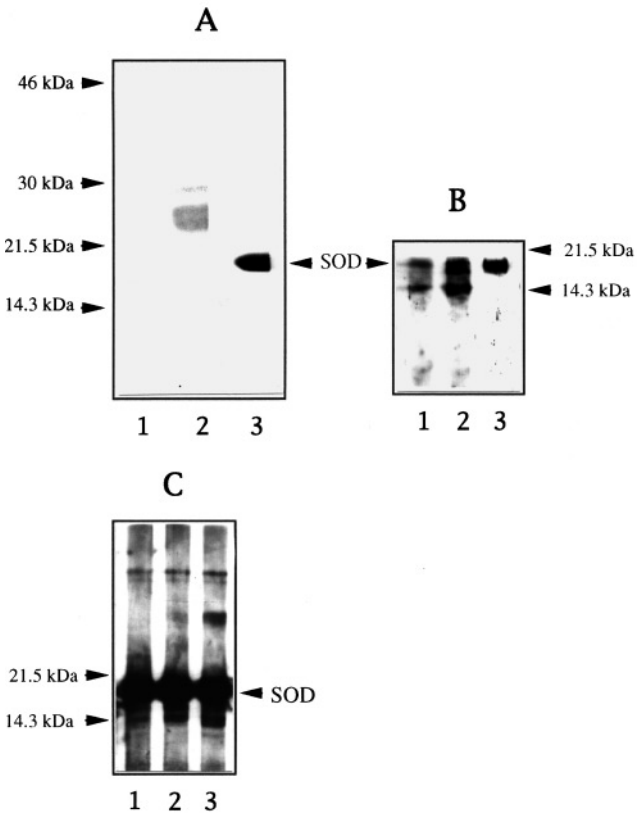

FIG. 3. Detection of glycated proteins (A) and glycated and fragmented Cu,Zn-SOD (B) of diabetic and normal rat lenses. (A): Immunoblot analysis of lens extracts from pooled lenses of 5 each diabetic and normal rats with anti-hexitol-lysine IgG. 100mg of each protein was reduced and subjected to 12.5% SDS-PAGE. After electrophoresis, immunoblotting was performed as described. Peroxidase activity was detected in a solution of 4-chloro, 1-naphthol containing H_2O_2 in 10mM TBS. Lane 1, normal rat lens. Anti-hexitol-lysine IgG was used as 1st antibody. Lane 2, diabetic rat lens. Anti-hexitol-lysine IgG was used as 1st antibody. Lane 3, purified rat Cu,Zn-SOD. Anti-Cu,Zn-SOD IgG was used as 1st antibody. (B): After immunoprecipitation using anti-Cu,Zn-SOD IgG, immunoblot analysis of lens extracts from diabetic and normal rat lens with anti-hexitol-lysine IgG. Peroxidase activity was detected by the ECL method. Lanes 1, 2 and 3 are the same as (A). (C): After incubation with 100 mM glucose, an aliquot of the incubation mixture of Cu,Zn-SOD was directly loaded onto SDS-PAGE. After electrophoresis, immunoblotting was performed. Anti-Cu,Zn-SOD IgG was used as 1st antibody. Peroxidase activity was detected by the ECL method. Lane 1, purified rat Cu,Zn-SOD. Lane 2, incubated for 3 days. Lane 3, incubated for 5 days.

immunoblot analysis with anti-hexitol-lysine IgG (Fig 3-B). One clear band was a monomer of Cu,Zn-SOD with a molecular mass of 20 kDa and the other band was a 14.3 kDa fragment. The membrane was then stripped off and reconfirmed with anti-Cu,Zn-SOD IgG. The both bands were visible indicating that a band with 14.3kDa is a specific fragment of Cu,Zn-SOD. This is the same band as observed during *in vitro* glycation of Cu,Zn-SOD (see C-lane2,3). The molecular mass of a site-specific fragment was also reported using sequence analysis as 14.3 kDa (2). This suggests that the 14.3 kDa species *in vivo* are probably an fragmented Cu,Zn-SOD. Moreover, the contents of fragmented protein in diabetic rat lenses (B-lane2) are much higher than those of normal rat lenses.

DISCUSSION

Our previous studies indicate that, in human diabetic patients, the glycated form of erythrocyte Cu,Zn-SOD is increased, and the activity of glycated Cu,Zn-SOD is decreased (3,4). In strepto-

zotocin-induced diabetic rats, we have demonstrated, by using anti-hexitol-lysine IgG, that glycated proteins increase in several tissues (7). We also know that human Cu,Zn-SOD undergoes glycation *in vitro* at specific lysine residues, such as Lys-122 and Lys-128, which are located at the active site binding loop region (3).

It has previously been reported that glycated proteins can produce superoxide anion in the presence of transition metal ions (8,9). Ookawara et al. reported that glycated Cu,Zn-SOD produces superoxide which is converted to hydrogen peroxide and hydroxyl radicals by a Fenton type reaction. A similar fragmentation sequence occurred in the copper-containing protein, ceruloplasmin (10). In order for reactive oxygen species to be produced, Cu,Zn-SOD was first cleaved at a peptide between Pro-62 and His-63, and then in the second step, a random fragmentation occurred (7).

In the present study, we demonstrate that the levels of glycated Cu,Zn-SOD in lenses are significantly higher than those found in other tissues. The lens is a tissue that does not turn over, and also where the sorbitol pathway is active. The conversion of glucose to fructose leads to a rise in the level of fructose, making this sugars twice as concentrated as glucose (11). In the Maillard reaction, fructose has a greater reducing capacity compared to glucose (12). Our previous studies also indicate that the effect of fructose on the fragmentation reaction is more pronounced than that of glucose (2,13). Therefore, the fragmentation of Cu,Zn-SOD may occur at a much higher rate in the lens than it does in other tissues. In the present study, using antibodies against Cu,Zn-SOD and hexitol lysine, a distinct band with a molecular mass of 14.3 kDa was detected. This strongly suggests that Cu,Zn-SOD is also fragmented *in vivo* by glycation.

Several reports concerning the role of glycation in cataractogenesis have already been published (14). Current opinion favors the postulate that the etiology of cataract formation is related to the progressive glycation and aggregation of lens crystallins. In streptozotocin-diabetic rats, the glycation of crystallin increased time-dependently (15), and glycation has been observed to lead to inter-crystallin cross-links (16). In diabetic human lenses, glycated crystallins have also been identified (17). These studies support the protein glycation theory, and suggest that the primary cause of cataractogenesis is related to the glycation of crystallin. Under these conditions, oxidative damage may be the primary cause of sugar induced cataractogenesis (18). There is however, an argument against these glycation theories. Chiou et al. (19) demonstrated that treatment of galactosemic rats with an aldose reductase inhibitor, sorbinil, prevented cataractogenesis but had no effect on the non-enzymatic glycation. At present each theory is incomplete and cannot further explain the etiopathogenesis of cataractogenesis.

Our study shows that, the lenses of diabetic and aging rats, (I) accumulate large amounts of glycated Cu,Zn-SOD, that is mainly localized in epithelial cells. As the protein undergoes glycation, the lens is prone to undergo exogenous oxidative stress. (II) The glycation of Cu,Zn-SOD eventually brings about fragmentation and leads to the formation of superoxide and hydroxyl radicals *in vivo*. This may further increase oxidative stress to the lens.

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