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Glycation of Copper, Zinc-Superoxide Dismutase and its Effect on the Thiol-Metal Catalyzed Oxidation Mediated DNA Damage

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Abstract: The nonenzymatic glycation of copper, zinc-superoxide dismutase (Cu,Zn-SOD) led to inactivation and fragmentation of the enzyme. The glycated Cu,Zn-SOD was isolated by boronate affinity chromatography. The formation of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) in calf thymus DNA and the generation of strand breaks in pBluescript plasmid DNA by a metal-catalyzed oxidation (MCO) system composed of Fe^{3+}, O_2, and glutathione (GSH) as an electron donor was enhanced more effectively by the glycated Cu,Zn-SOD than by the nonglycated enzyme. The capacity of glycated Cu,Zn-SOD to enhance damage to DNA was inhibited by diethylenetriaminepentaacetic acid (DETAPAC), azide, mannitol, and catalase. These results indicated that incubation of glycated Cu,Zn-SOD with GSH-MCO may result in a release of Cu^{2+} from the enzyme. The released Cu^{2+} then likely participated in a Fenton-type reaction to produce hydroxyl radicals, which may cause the enhancement of DNA damage.

Key words: copper, DNA damage, glycation, Zinc-superoxide.

Autoxidation of sugars, and of the products of nonenzymatic glycosylation (glycation) of proteins (i.e., Amadori products) is a free radical mediated reaction that occurs under aerobic conditions. The glycation proceeds through the formation of a Schiff base between glucose and an α- or ε-amino group in a protein together with Amadori rearrangement to yield a relatively stable ketamine (Wolff and Dean, 1987; Azeredo *et al.*, 1988). Such a reaction has been expected to occur in various kinds of proteins under physiological conditions.

Cu,Zn-SOD, a homodimeric metalloenzyme, is an essential antioxidant enzyme for superoxide dismutation *in vivo*. It has been shown that Cu,Zn-SOD undergoes glycation and that the enzyme is inactivated by glycation *in vitro* as well as *in vivo* (Arai *et al.*, 1987; Ookawara *et al.*, 1992). The level of glycated Cu,Zn-SOD is increased in the erythrocytes of patients with diabetes mellitus, as well as patients with Werner's syndrome, an age-accelerated disease (Arai *et al.*, 1987). Therefore, involvement of the glycation reaction in the pathogenesis of diabetic complications and aging was suggested. It has been implied that the release of free copper ion resulted in the modification of Cu,Zn-SOD including glycation (Ookawara *et al.*, 1992). The released Cu^{2+} then likely participated in a Fenton-type reaction to produce hydroxyl radicals.

In this regard, it can be proposed that the glycation of Cu,Zn-SOD may result in the decrease of the antioxidant defense systems by inactivating the enzyme as well as the exacerbation of oxidative damage of cells by releasing free copper.

In this report, we present results obtained when investigating the glycation of Cu,Zn-SOD and the enhancing effect of glycated Cu,Zn-SOD compared to nonglycated Cu,Zn-SOD on DNA damage caused by a thiol MCO system.

Materials and Methods

Materials

Cu,Zn-SOD from bovine erythrocytes, D-glucose, D-fructose, nitroblue tetrazolium (NBT), calf thymus DNA, DETAPAC, GSH, and catalase were obtained from Sigma Chemical Co. (St. Louis, USA) Agarose, glycerol, bromophenol blue, 2-mercaptoethanol, riboflavin, N,N,N′,N′-tetramethylethylenediamine (TEMED), and a silver staining kit were obtained from Bio-Rad (Hercules, USA). Deoxyribonuclease I, phosphodiesterase, endonuclease, and alkaline phosphatase were from Boehringer Mannheim (Mannheim, Germany). A boronate af-

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finity column (Glyco-Gel B) was purchased from Pierce Chemical Co. (Rockford, USA) pBluescript KS plasmid DNA was purified from *E. coli* cultures by using PROMEGA Magic Minipreps (Madison, USA). 8-OH-dG was synthesized by the method of Kasai and Nishimura (1984).

Glycation of Cu,Zn-SOD

Cu,Zn-SOD was incubated with 0.1 M glucose or 0.1 M fructose in 50 mM sodium phosphate buffer, pH 7.4/150 mM NaCl/0.025% NaN_3. After incubation at 37℃ for the indicated duration, the reaction was stopped by freezing the mixture. Each sample was stored at −70℃ until use.

Separation of glycated Cu,Zn-SOD

The glycated Cu,Zn-SOD was purified as described by Arai *et al.* (1987). The Cu,Zn-SOD treated with glucose was applied to a Glyco-Gel B column (1×7 cm), which had been equilibrated with 0.25 M ammonium acetate buffer, pH 8.5, containing 0.05 M $MgCl_2$ (buffer A). The nonglycated enzyme was washed through the column with the above buffer and the glycated enzyme was eluted with 0.1 M potassium phosphate buffer, pH 8.5, containing 0.2 M sorbitol (buffer B).

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was carried out as described by Laemmli (1970) after the sample was reduced with 2.5% 2-mercaptoethanol. Fifteen percent slab gels (90×55-cm) were used. The gels were stained with a Bio-Rad silver stain kit.

Activity staining of Cu,Zn-SOD

The activity of Cu,Zn-SOD in native (non-denaturing) 10% polyacrylamide gel (Davis, 1964) was visualized as described by Beauchamp and Fridovich (1971). Briefly, gel was placed in 2.45 mM NBT for 20 min followed by 15 min in 28 mM riboflavin and 28 mM TEMED. Gel was then exposed to moderately intense light until the bands showed maximum resolution.

Detection of DNA single strand breaks

DNA single strand breaks were assayed by measuring the conversion of covalently circular double-stranded supercoiled DNA (form I) to open (relaxed) circular double-stranded DNA (form II). The pBluescript plasmid DNA (200 ng) was incubated in 40 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.0 under various conditions, as described in the figure legends, in a microfuge tube. The final volume was 10 μl. The reactions were terminated by the addition of 2 μl six-fold strength agarose gel loading solution (30% glycerol, 0.01% bromophenol blue). DNA samples were applied to 1% agarose gels in a TAE buffer system, and electrophoresis was performed usually at 5 V/cm for 2 h at room temperature. Following electrophoresis, gels were stained with ethidium bromide irradiated from below with a UV transilluminator box and photographed.

Analysis of 8-OH-dG level in DNA

Quantitation of the amount of 8-OH-dG in DNA was determined as described previously (Floyd *et al.*, 1986). Calf thymus DNA (1 mg/ml) was incubated under various conditions, as described in the figure legends, in 100 ml of 40 mM HEPES, pH 7.0 at 37℃ for 2 h. After incubation, the DNA was separated by ethanol precipitation, dissolved in 10 mM TE buffer (pH 7.4) and digested to the nucleoside level by incubation with deoxyribonuclease I, phosphodiesterase, endonuclease, and alkaline phosphatase for 2 h at 37℃. The resulting mixture was filtered and injected into an HPLC apparatus (Waters) (Milford, USA) equipped with both a UV detector and an electrochemical detector. The column was a Beckman (Fullerton, USA) Ultrasphere ODS (0.46×25 cm) and the eluent was 10% aqueous methanol containing 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, and 10 mM acetic acid. The flow rate was 1 ml/min. The molar ratio of 8-OH-dG to deoxyguanosine in each DNA sample was measured, based on the peak height of authentic 8-OH-dG, with an electrochemical detector and the UV absorbance at 254 nm for dG. All results, presented as means of at least duplicate separate experiments, yielded results with only minor variations from each other.

Results and Discussion

During the incubation of Cu,Zn-SOD with 0.1 M glucose or fructose for 0~7 days at 37℃, the enzyme activity gradually decreased and fragmentation simultaneously increased. As shown in Fig. 1, the activity band of Cu,Zn-SOD decreased in proportion to the increase of incubation time. The intensity of native Cu,Zn-SOD bands almost completely disappeared after incubation with 0.1 M fructose for 7 days. SDS-PAGE visualized with silver staining (Fig. 2) showed a gradual increase in the intensity of the lower molecular weight band and the simultaneous appearance of a large molecular weight fragment ~40-kDa which may have been caused by cross-linking. The effect of fructose on the fragmentation and inactivation was much more significant than that of glucose. It has been shown that fructose has a greater reducing capacity than glucose in the Maillard reaction (Kashimura *et al.*, 1979). Antioxidant

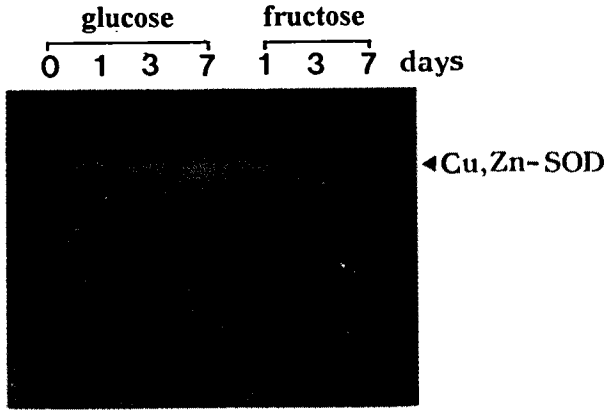

Fig. 1. Effect of glycation on the activity of Cu,Zn-SOD. After incubation with 0.1 M glucose or fructose for 1, 3, and 7 days as described under "Materials and Methods", an aliquot of the incubation mixture containing 0.5 μg of Cu,Zn-SOD was directly loaded onto a 10% nondenaturing polyacrylamide gel electrophoresis and stained for superoxide dismutase activity.

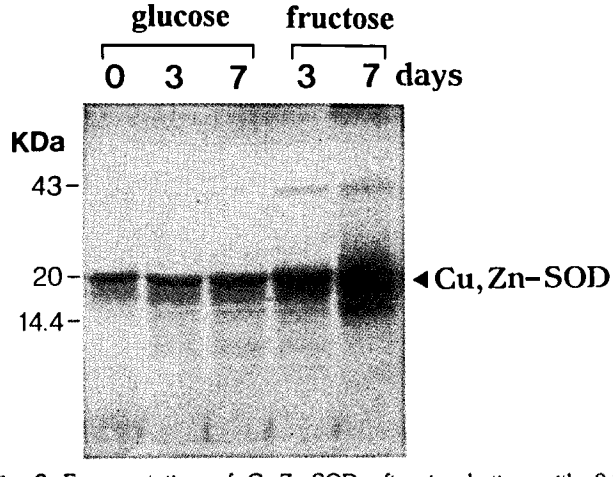

Fig. 2. Fragmentation of Cu,Zn-SOD after incubation with 0.1 M glucose or fructose for 3 and 7 days. The incubation mixtures were then analyzed using a 15% SDS-polyacrylamide gel electrophoresis. Gel was visualized with a silver staining.

enzymes, SOD and catalase, as well as glutathione peroxidase, provide a substantial defense network against the accumulation of reactive oxygen intermediates. These enzymes normally act in concert; thus SOD protects catalase and glutathione peroxidase against inhibition by O_2^-, while catalase and glutathione peroxidase protect SOD against inhibition by H_2O_2 (Hodgson and Fridovich, 1975; Kono and Fridovich, 1982; Blum and Fridovich, 1985). Therefore it is implied that the inactivation of superoxide dismutase by glycation may play an important role in the aging of various cells and tissues as well as in diabetes via the development of a pro-oxidant state.

The Cu,Zn-SOD, exposed to 0.1 M glucose for 7 days, was loaded on a Glyco-Gel B column. A typical

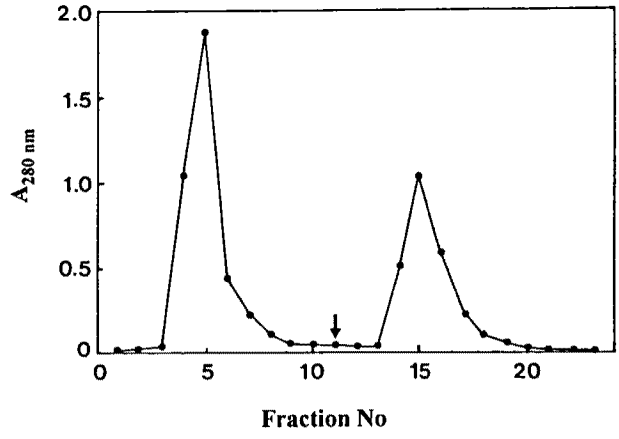

Fig. 3. Separation of the Cu,Zn-SOD in glucosylated and non-glucosylated components by affinity chromatography on a Glyco-Gel B column. Samples in buffer A was applied to a column of Glyco-Gel B which had been equilibrated with the buffer A, and washed with 10 volumes of the starting buffer. Elution was started at the point indicated by an arrow, using buffer B. Each fraction was 2 ml.

elution pattern is shown in Fig. 3. The result of the boronate column experiment indicates that about 45% of the glucosylated Cu,Zn-SOD was obtained. The eluate of glucosylated Cu,Zn-SOD was dialyzed and employed to estimate the effect on the DNA damage induced by GSH-MCO. It has been shown that the autoxidation of thiol in the presence of iron generates a reactive oxygen species as well as sulfur radicals which cause damage to many cellular components including lipids, proteins, and DNA (Tien *et al.*, 1982; Stadtman, 1988; Kwon *et al.*, 1993). Cu,Zn-SOD not only does not have an inhibitory effect but, in fact, enhanced the effect of the thiol-MCO system on mediating both DNA strand breaks and 8-OH-dG formation (Park and Floyd, 1994).

DNA lesions resulting from exposure to reactive oxygen species have been shown to be modified bases and strand breaks. DNA damage, measured as single strand breaks, leads to the loss of biological activity and to mutagenicity (Wallace, 1987). The modified base, 8-OH-dG, is considered to be one of the oxidative DNA products induced by oxygen radicals that can be easily measured by HPLC-EC (Floyd *et al.*, 1986). Therefore, it has been used as an important biological marker of oxidative DNA damage *in vivo* and *in vitro*. Because 8-OH-dG causes misreplication of DNA (Shibutani *et al.*, 1991), it has been implicated as a possible cause of mutation and cancer.

Strand breaks were manifested by a decrease in the amount of supercoiled (form I) DNA present and an increase in the amount of the relaxed (form II) DNA as shown by agarose gel electrophoresis. It has been

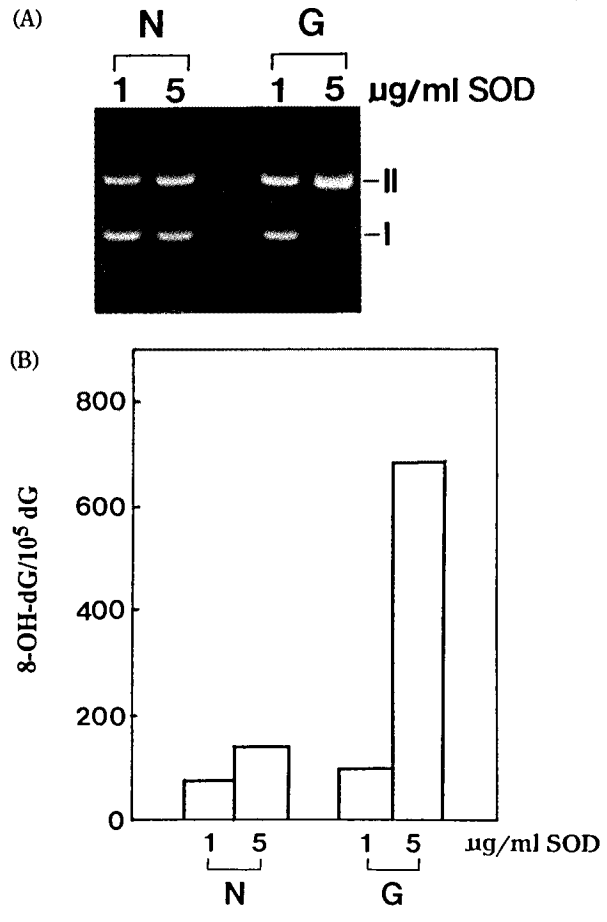

Fig. 4. Effect of Cu,Zn-SOD on strand breaks in pBluescript DNA (A) and 8-OH-dG formation in calf thymus DNA (B) by the GSH-MCO system. (A) The reaction mixture contained 200 ng pBluescript DNA, 3 μM $FeCl_3$, 10 mM GSH, and glycated or nonglycated Cu,Zn-SOD in 40 mM HEPES, pH 7.0. Incubations were carried out for 2 h at 37℃. B) The reaction mixture contained 0.1 mg calf thymus DNA, 3 μM $FeCl_3$, 10 mM GSH, and glycated or nonglycated Cu,Zn-SOD in 100 μl 40 mM HEPES, pH 7.0. N, nonglycated Cu,Zn-SOD; G, glycated Cu,Zn-SOD.

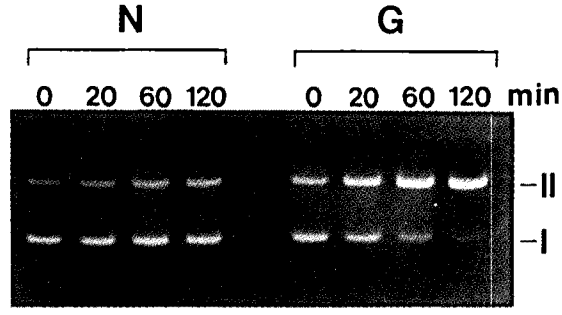

Fig. 5. Time-dependent increase of strand breaks in pBluescript DNA with 5 μg/ml non-glycated Cu,Zn-SOD and 5 μg/ml glycated Cu,Zn-SOD by the GSH-MCO system. Reaction conditions were same as described in Fig. 4A. N, nonglycated Cu,Zn-SOD; G, glycated Cu,Zn-SOD.

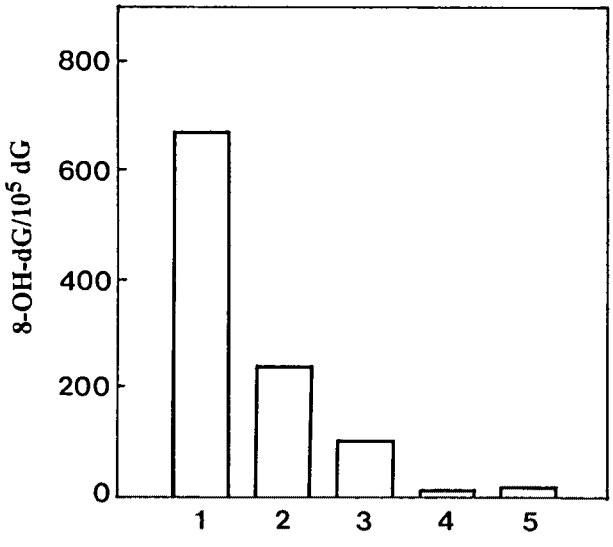

Fig. 6. Effects of scavengers, the metal chlator, and catalase on the GSH-MCO along with 5 μg/ml glycated Cu,Zn-SOD induced formation of 8-OH-dG in DNA. Conditions for the incubation mixture were the same as Fig. 4B. 1. No addition, 2. 100 mM mannitol, 3. 25 mM azide, 4. 1 mM DETAPAC, 5. 100 μg/ml catalase.

shown that GSH-MCO failed to induce significant strand breaks and 8-OH-dG formation in DNA (Park and Floyd, 1994). However, DNA damage by a GSH-MCO system was increased by the addition of Cu,Zn-SOD. Whereas 5 μg/ml of nonglycated Cu,Zn-SOD was not effective in enhancing cleavage of the DNA induced by GSH-MCO, the glycated Cu,Zn-SOD at the same concentration caused almost complete conversion of supercoiled DNA into the relaxed form as shown in Fig. 4. A similar degree of strand breaks was obtained with about a fivefold higher concentration of nonglycated Cu,Zn-SOD. The formation of 8-OH-dG in DNA by GSH-MCO was also substantially increased with the addition of glycated Cu,Zn-SOD (Fig. 4). A time-dependent increase of strand breaks was observed with 5 μg/ml of glycated Cu,Zn-SOD on the GSH-MCO mediated DNA damage as shown in Fig. 5.

To gain insight into the enhancement mechanism of DNA strand breaks and 8-OH-dG formation by GSH-MCO with glycated Cu,Zn-SOD, the effect of a chelating agent and free radical scavengers were examined. The data in Fig. 6 demonstrates that hydroxyl free radical scavengers such as mannitol and azide inhibited reaction. Sodium azide (25 mM) and mannitol (100 mM) decreased the amount of 8-OH-dG formed by 85% and 64%, respectively. Catalase at 0.1 mg/ml and a copper chelator, DETAPAC (1 mM), almost completely prevented the formation of 8-OH-dG. These results suggest that GSH-MCO may lead to a release of Cu^{2+} ions from the Cu,Zn-SOD molecule. It has been shown that Cu^{2+} became essentially free from the ligand and was released from the oxidatively damaged protein (Yim *et al.*, 1990; Ookawara, 1992). The

released copper ions could then enhance the Fenton-type reaction to produce hydroxyl radicals from the H_2O_2 generated by the GSH-MCO system and play a major role in the 8-OH-dG formation and strand breaks. Although iron is more abundant than copper in living organisms, copper is more soluble at physiological pH and it is thought that free Cu^{2+} is a much more effective catalyst than free Fe^{3+} in Fenton-type reaction (Chevion, 1988). The following lines of evidence support the notion that Cu,Zn-SOD is a source of Cu^{2+} for the Fenton-type reaction in the GSH-MCO system. i) A copper chelator, DETAPAC, inhibits damage to DNA. ii) Exogenoulsy added free Cu^{2+} argumented oxidative damage to DNA similar to that observed with the glycated Cu,Zn-SOD. Although GSH-MCO failed to induce a considerable amount of 8-OH-dG, a 14-fold increase of 8-OH-dG formation in DNA in the presence of 1 μM Cu^{2+} was observed. iii) GSH-MCO led to a release of free copper ions from the glycated Cu,Zn-SOD molecule. Analysis of weakly bound or free copper in Cu,Zn-SOD samples was carried out using the bathocuproin reagents as described previously (Jewett *et al.*, 1989). While Cu,Zn-SOD with GSH-MCO released 14% of bound copper after incubation for 2 h at 37℃, glycated Cu,Zn-SOD under the same conditions released 67% of bound copper. Therefore it can be supposed that glycated Cu,Zn-SOD has a structure which is more susceptible to oxidative damage such as that caused by the GSH-MCO system. It has been suggested that Amadori rearrangement of lysine residues might result in the conformational changes of Cu,Zn-SOD. The conformational integrity of the enzyme molecule is destroyed and thus an altered copper ligand (which is normally concealed within the tertiary structure of an enzyme) binding may precede the increase in copper release.

The discovery that Cu,Zn-SOD is capable of argumentation of oxidative damage via glycation has important implications when it is considered along with its protective effects. Since antioxidant enzymes in a cell work together as a team, it is possible that once Cu,Zn-SOD is inactivated and then begins to release Cu^{2+}, it may aggravate oxidative damage.

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