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Inactivation of Copper,Zinc Superoxide Dismutase by the Lipid Peroxidation Products Malondialdehyde and 4-Hydroxynonenal

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Membrane lipid peroxidation processes yield reactive aldehydes that may react with copper,zinc superoxide dismutase (Cu,Zn SOD), one of the key antioxidant enzymes against oxidative stress. We investigated this possibility and found that exposing Cu,Zn SOD to malondialdehyde (MDA) or 4-hydroxynonenal (HNE) caused the loss of dismutase activity, cross-linking of peptides, and an increase in protein oxidation, reflected by the increased level of carbonyl groups. When Cu,Zn SOD that had been exposed to MDA or HNE was subsequently analyzed by amino acid analysis, histidine content was found to be significantly lost. Both MDA- and HNE-treated Cu,Zn SOD were resistant to proteolysis, which may imply that damaged proteins exist *in vivo* for a longer period of time than the native enzyme. The lipid peroxidation-mediated damage to Cu,Zn SOD may result in the perturbation of cellular antioxidant defense mechanisms, and subsequently lead to a pro-oxidant condition.

Keywords: Aldehydes, Cu,Zn SOD, Lipid peroxidation.

Introduction

Oxidative modification of cellular constituents, including lipids, proteins, and nucleic acids, has been implicated in the etiology of many pathological conditions, such as diabetes, cataracts, pulmonary emphysema, arthritis, cancer, and aging (Ames, 1983). In biological membranes, lipid peroxidation is frequently a consequence of radical attack. The peroxidation of unsaturated fatty acids of cells produces many reactive species such as free radicals, hydroperoxides, and carbonyl compounds which may cause damage to proteins and DNA (Cerutti, 1985). It has also been assumed that the decomposition of hydroperoxides mediated by catalytic transition metal ions may form much more toxic breakdown products such as alkoxy radicals (RO·), peroxyl radicals (RO·), hydroxyl free radical (·OH), and reactive aldehydes including malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (Slater, 1984; Ueda *et al.*, 1996). It is possible that in complex biological systems, oxygen-free radicals and reactive aldehydes may indirectly cause protein and DNA damage by initiating lipid peroxidation, since polyunsaturated side chains of membrane lipids are especially susceptible to free radical-initiated oxidation (Fleming *et al.*, 1982; Koh *et al.*, 1997).

The damage brought about by oxidative stress is expected to be exacerbated if antioxidant enzymes are inactivated by such events. Superoxide dismutase (SOD; EC 1.15.1.1) is one of the key antioxidant enzymes which provide the essential defense against oxygen toxicity to the cell (McCord and Fridovich, 1969). It has been shown that Cu,Zn SOD, a homodimeric metalloenzyme which catalyzes the dismutation of the toxic superoxide anion to O_2 and H_2O_2, undergoes free radical damage (Hodgson and Fridovich, 1975; Blech and Borders, 1983; Park and Lee, 1995; Kwon *et al.*, 1998). The possibility of Cu,Zn SOD inactivation being mediated by lipid peroxidation was proposed and the free radicals formed by the decomposition of lipid hydroperoxides has been suggested as one of the damaging species (Lee and Park, 1995). However, an attack on Cu,Zn SOD by the stable end-products of lipid peroxidation may also cause the inactivation of the enzyme. There is increasing evidence that end-product aldehydes are causally involved in many pathological effects associated with oxidative stress in cells and tissues (Uchida *et al.*, 1997).

In this report, the inactivation of Cu,Zn SOD mediated by reactive aldehydes produced by lipid peroxidation was examined using MDA and HNE.

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Modification of Cu,Zn SOD was determined by monitoring the loss of enzyme activity, and by measuring the carbonyl content and cross-linking of the peptide as indices of damage. The results indicate that lipid peroxidation may be an intermediary event in Cu,Zn SOD inactivation through the generation of reactive aldehydes.

Materials and Methods

Materials Chemicals and enzymes were obtained from the following sources: Cu,Zn SOD (from bovine erythrocytes), chymotrypsin, 1,1,3,3-tetraethoxypropane (TEP), 2,4-dinitrophenyl-hydrazine (DNPH), o-phthaldialdehyde (OPA), and rabbit anti-dinitrophenyl (DNP) antibodies were from Sigma (St. Louis, USA); Acrylamide, ammonium persulfate, sodium dodecyl sulfate (SDS), N,N,N′,N′-tetramethylenediamine (TEMED), riboflavin, bromochloroindolyl phosphate (BCIP), and nitroblue tetrazolium (NBT) were from Bio-Rad (Hercules, USA); HNE was from Biomol (Plymouth Meetings, USA). All other chemicals used were of analytical grade. A rabbit antiserum against Cu,Zn SOD was kindly provided by Dr. Y. S. Lee (Kyungpook National University, Taegu, Korea).

Preparation of MDA TEP (1 mmol) was dissolved in 90 ml double-distilled water. Hydrochloric acid (1 ml, 1 N) was added, and then the volume was brought up to 100 ml with distilled water. A glass stopper was held firmly in place with parafilm to prevent the loss of MDA while heating. After heating in a water bath at 50°C for 60 min, the solution was cooled to room temperature, and the appropriate stock solution was made. The exact concentration of the stock solution was confirmed by measuring its absorbance at 245 nm (ε = 13,700 $M^{-1}cm^{-1}$) (Csallany *et al.*, 1984).

SDS activity assay Activity of Cu,Zn SOD was assayed by the cytochrome C reduction assay using hypoxanthine and xanthine oxidase as an $O_2^{\cdot-}$-generating system, as described previously (McCord and Fridovich, 1969). Cu,Zn SOD activity was also determined by electrophoresis in 10% nondenaturing polyacrylamide gels, and visualized as described by Beauchamp and Fridovich (1971). Briefly, each gel was placed in 2.45 mM nitroblue tetrazolium for 20 min, followed by 15 min in 28 μM riboflavin and 28 mM TEMED. The gel was then exposed to moderately intense light until the bands showed maximum resolution.

SDS-polyacrylamide gel electrophoresis and immunoblotting Polyacrylamide gel electrophoresis was used to determine the aggregation of Cu,Zn SOD (3 μg) treated with MDA or HNE in 40 mM Hepes, pH 7.0. SDS-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970), using a slab gel (stacking gel, 2.5% acrylamide; separating gel, 12.5% acrylamide). Proteins were transferred onto a nitrocellulose sheet (Towbin *et al.*, 1979), blocked with 1% non-fat milk for 1 h, and then probed with rabbit antiserum against Cu,Zn SOD (1:1000 dilution). The detection method employed alkaline phosphatase-labeled goat anti-rabbit IgG (CALTAG, 1:2000 dilution) with the BCIP/NBT detecting system (Bio-Rad, Hercules, USA).

Determination of protein carbonyl content The carbonyl content of modified and native Cu,Zn SOD was measured using either spectrophotometric analysis or immunoblotting with anti-DNP antibody according to the method of Levine *et al.* (1994). For the immunoblotting analysis, both native and aldehyde-treated Cu,Zn SOD were precipitated with 20% TCA, and then treated for 1 h with 20 mM DNPH in 10% (v/v) trifluoroacetic acid at room temperature. After incubation, a neutralization solution (2 M Tris, 30% glycerol, 19% 2-mercaptoethanol) was added and further incubated at room temperature for 15 min. After SDS-PAGE of the derivatized proteins with 12.5% polyacrylamide gel, the separated proteins were probed with rabbit anti-DNP sera, used at a dilution of 1:1000.

Amino acid analysis Cu,Zn SOD treated with either MDA or HNE was subjected to acid hydrolysis. Amino acids in the hydrolyzate were converted to their OPA derivatives, and analyzed by HPLC as described previously (Rivett and Levine, 1990). HPLC was performed on a Hewlett-Packard model 1050 instrument equipped with a fluorescence detector. Statistically significant differences were assessed after normalization.

Determination of rate of proteolysis Proteolysis of the MDA- and HNE-treated Cu,Zn SOD by chymotrypsin was carried out in 40 mM Hepes at pH 7.0 and 37°C. At different times, aliquots were removed and subjected to a 10% trichloroacetic acid treatment. After centrifugation of precipitated proteins for 10 min at 14,000 rpm in an Eppendorf microfuge, the supernatant was neutralized with a predetermined volume of 2 M potassium borate, pH 10. The amount of small peptides in the supernatant was then determined as described by Church *et al.* (1985).

Replicates Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

Results

Examination of MDA-exposed SOD solutions by polyacrylamide gel electrophoresis under nondenaturing conditions and subsequent activity staining showed the loss of Cu,Zn SOD activity. Inactivation of Cu,Zn SOD was reflected by the disappearance of the native protein band. As shown in Fig. 1, when Cu,Zn SOD was exposed to MDA in the range of 0.2–2 mM for 1 h at 37°C, a concentration-dependent decrease of activity was observed. When the activity of MDA (1 mM)-treated Cu,Zn SOD was measured by the cytochrome C reduction assay after the reaction mixture was incubated for 1 h at 37°C, approximately 58% of the original activity of the enzyme was lost. Incubation with 1–10 mM HNE for 4 h at 37°C caused the diffused Cu,Zn SOD band, which may indicate that the cross-linking of the enzyme did not cause an extensive loss of activity compared to the MDA-treated Cu,Zn SOD (data not shown). Incubation of Cu,Zn SOD with 1 mM HNE for 4 h at 37°C resulted in a loss of less than 10% of the original activity when measured by the cytochrome C assay.

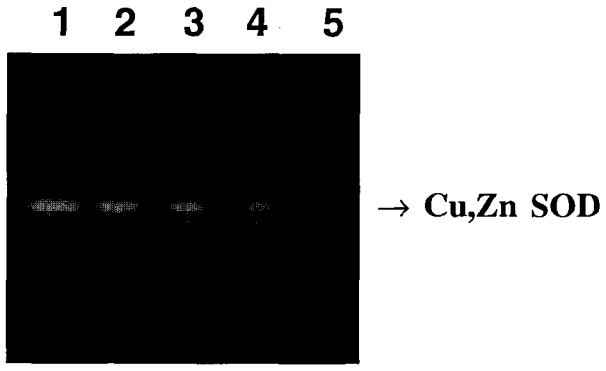

Fig. 1. Effect of MDA on the activity of Cu,Zn SOD. Reactions were carried out for 1 h at 37°C. MDA-treated Cu,Zn SOD (100 ng) samples were applied to a 10% nondenaturing polyacrylamide gel. Electrophoresed gels were stained for SOD activity as described in Materials and Methods. Concentrations of MDA were 0, 0.2, 0.5, 1, and 2 mM for lanes 1–5, respectively.

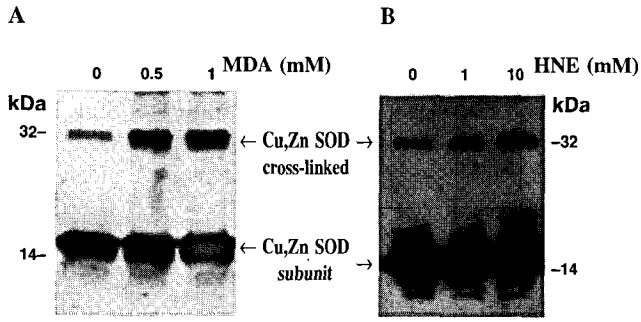

Fig. 2. Effect of MDA (**A**) or HNE (**B**) on the aggregation of Cu,Zn SOD. After incubation with MDA or HNE as described in Materials and Methods, an aliquot of the incubation mixture containing 3 μg of Cu,Zn SOD was directly loaded onto an SDS-polyacrylamide gel electrophoresis, and protein bands were visualized by immunoblotting with antiserum against Cu,Zn SOD.

Aggregation of Cu,Zn SOD by MDA was measured by the appearance of higher molecular weight peptide fragments in denaturing electrophoresis gels. The electrophoresed Cu,Zn SOD samples and the separated proteins probed with antiserum against Cu,Zn SOD revealed the presence of one major band, the size of a Cu,Zn SOD subunit (~16 kDa). As shown in Fig. 2A, incubation of Cu,Zn SOD with MDA for 1 h at 37°C resulted in the appearance of a ~32 kDa band, indicative of aggregation of the protein. These findings suggest that intermolecular cross-linking of peptides occurs during the course of the damage. Treatment of Cu,Zn SOD with HNE also resulted in an increase of aggregation as shown in Fig. 2B.

Oxidation of proteins introduces carbonyl groups into the amino acid side chain of proteins and provides a convenient assay of oxidative modification. The carbonyl groups introduced into damaged proteins react with DNPH to form a Schiff base, phenylhydrazine. The carbonyl contents of the derivatized protein can be detected using either spectrophotometric analysis or immunoblotting analysis with anti-DNP sera in a Western blot procedure (Levine *et al.*, 1994). Incubation of Cu,Zn SOD with MDA or HNE for 2 h at 37°C resulted in a concentration-dependent increase in carbonyl groups (Fig. 3). Immunoblotting results obtained from Cu,Zn SOD samples treated with MDA showed that a concentration-dependent increase in the staining intensity of a 16 kDa band reflects the increase of carbonyl adducts (Fig. 4).

In order to specify a target molecule in the enzyme, Cu,Zn SOD that had been exposed to MDA or HNE was analyzed by amino acid analysis following acid hydrolysis of the modified proteins. When bovine erythrocyte Cu,Zn SOD was treated with 1 mM MDA or 1 mM HNE in 50 mM sodium phosphate buffer (pH 7.2), histidine was significantly lost. Other amino acids were not altered significantly.

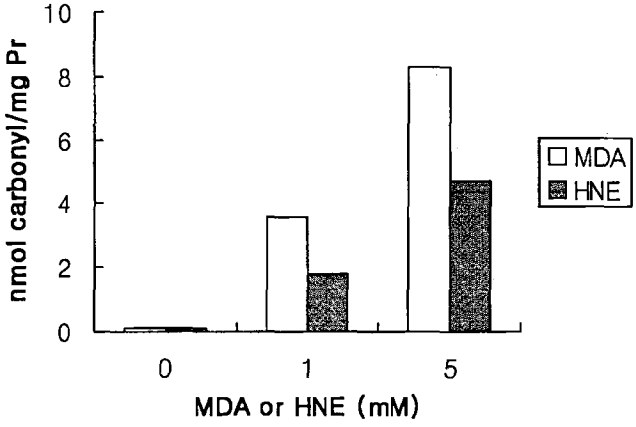

Fig. 3. Protein carbonyl content of Cu,Zn SOD exposed to MDA or HNE. Protein carbonyls were measured by the method of Levine and co-workers (1994) with the use of 2,4-dinitrophenyl hydrazine.

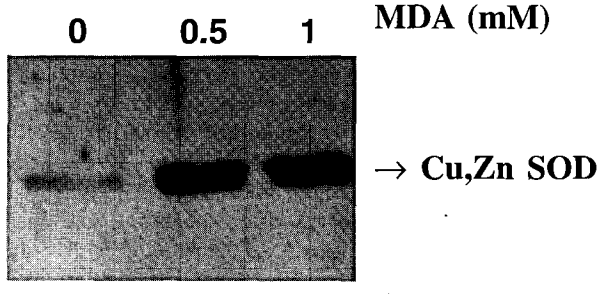

Fig. 4. Immunochemical analysis of carbonyl groups in Cu,Zn SOD after 2 h incubation with MDA at 37°C. After incubation, samples were derivatized with DNPH as described in Materials and Methods. DNPH-derivatized proteins were subjected to SDS-polyacryamide gel electrophoresis for immunoblotting with anti-DNP sera.

To test whether the damage induced the alteration in the susceptibility of Cu,Zn SODs to proteolysis, MDA- and HNE-treated Cu,Zn SOD were digested with various amounts of chymotrypsin, and then the liberated primary amines were then quantitated by reaction with OPA (Church *et al.*, 1985). It was found that MDA- and HNE-treated Cu,Zn SODs appeared to be significantly less susceptible than the native enzyme to limited proteolysis by chymotrypsin (Fig. 5).

Discussion

It has been proposed that lipid peroxidation is a continual process in living aerobic cells, is maintained at a low level, and can be prevented from entering into the autocatalytic phase by protective enzymes and antioxidants (Munkres, 1976). Chemical and physical agents that enhance membrane free-radical reactions may accelerate this process beyond the capabilities of the protective systems, and thus result in widespread lipid peroxidation (Tappel, 1975). Oxidative damage to protein has been postulated to have biological importance (Stadtman, 1992). In particular, the oxidative damage to antioxidant enzymes may lead to serious consequences. Therefore, the study of Cu,Zn SOD inactivation and its relationship to lipid peroxidation is relevant. Along with membrane-bound enzymes, soluble enzymes may also be damaged by lipid peroxidation products since it has been shown that oxidative stress induces phospholipase A_2, which may release lipid hydroperoxide from phospholipids (Sevanian and Kim, 1985). Furthermore, aldehydes are more stable than free radical species and may more readily diffuse into cellular media, where they are available for facile reaction with various macromolecules (Szweda *et al.*, 1993).

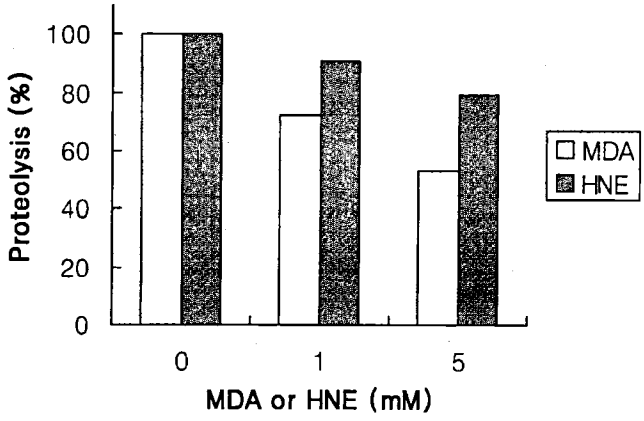

Fig. 5. Proteolytic susceptibility of Cu,Zn SOD following exposure to either MDA or HNE. The extent of proteolysis was observed when MDA- or HNE-modified Cu,Zn SOD was digested with 37.5 μg chymotrypsin in 40 mM Hepes at pH 7.0 for 1 h at 37°C, and then the extent of amine liberation was assessed using the assay of Church *et al.* (1985).

The reaction of reactive end-products of lipid peroxidation such as HNE and MDA, which are produced from the decomposition of unstable lipid hydroperoxides in the presence of transition metals, with Cu,Zn SOD is likely to be important in the overall action of these toxic species. Results presented here show that aldehydes are capable of reacting with Cu,Zn SOD causing its inactivation. The loss of Cu,Zn SOD activity by aldehydes observed in this study seems to be initiated through irreversible oxidation and aggregation. The lipid peroxidation-dependent inactivation of Cu,Zn SOD is accompanied by the oxidation of amino acids, as evidenced by the increase of reactive carbonyl moieties in the protein.

The reactions of MDA and HNE with proteins have received particular attention and were mainly found to involve the primary addition to nucleophilic residues such as Lys, His, or Cys (Burcham and Kuhan, 1997). His has been found to be essential for Cu,Zn SOD activity, but amino acids analysis of MDA- and HNE-treated Cu,Zn SOD revealed a significant loss of His. Therefore, we believe that the four His residues (His44, His46, His61, and His118) located at the active site of Cu,Zn SOD (Tainer *et al.*, 1983) are modified by aldehyde.

The increase of susceptibility of modified proteins to proteases is considered to be a form of secondary defense (Rivett, 1993). However, both MDA- and HNE-modified Cu,Zn SOD are resistant to proteolytic degradation, indicating that damaged Cu,Zn SOD is turned over less rapidly *in vivo*. It can be assumed that the diminished proteolytic susceptibility of 4-HNE or MDA-modified Cu,Zn SOD may likely be due to the formation of cross-linked protein aggregates. Under normal circumstances, a minor loss of Cu,Zn SOD to oxidative inactivation may not be of physiological significance. During exposure to oxidative stress, however, substantial Cu,Zn SOD inactivation may occur. With Cu,Zn SOD considered to play an important role in aging and inflammation, as well as carcinogenic processes (Fridovich, 1986), its inactivation by lipid peroxidation products may play an important role in the aging of various cells and tissues.

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