Modification and inactivation of Cu,Zn-superoxide dismutase by the lipid peroxidation product, acrolein

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INTRODUCTION

The modification of biological macromolecules by reactive oxygen species (ROS) has been regarded to play a potential role in neurodegenerative diseases, including Alzheimer’s disease (AD) and Parkinson’s disease (PD). Previous studies have shown an increase in the amount of redox active metals (1-3) and in the levels of lipid peroxidation (4), as well as a decline in membrane polyunsaturated fatty acids (5, 6) and increased protein oxidation (7, 8) in the brain in patients with AD. Lipid peroxidation is one of the major sources of ROS-mediated injury that directly damages neuronal membranes, and yields a number of secondary products responsible for extensive cellular damage. ROS attacks on polyunsaturated fatty acids lead to the formation of highly reactive electrophilic aldehydes, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and acrolein.

Acrolein is the most reactive aldehyde product of lipid peroxidation and is found to be elevated in the brain when oxidative stress is high. The effects of acrolein on the structure and function of human Cu,Zn-superoxide dismutase (SOD) were examined. When Cu,Zn-SOD was incubated with acrolein, the covalent crosslinking of the protein was increased, and the loss of enzymatic activity was increased in a dose-dependent manner. Reactive oxygen species (ROS) scavengers and copper chelators inhibited the acrolein-mediated Cu,Zn-SOD modification and the formation of carbonyl compound. The present study shows that ROS may play a critical role in acrolein-induced Cu,Zn-SOD modification and inactivation. When Cu,Zn-SOD that has been exposed to acrolein was subsequently analyzed by amino acid analysis, serine, histidine, arginine, threonine and lysine residues were particularly sensitive. It is suggested that the modification and inactivation of Cu,Zn-SOD by acrolein could be produced by more oxidative cell environments. [BMB Reports 2013; 46(11): 555-560]

RESULTS

As shown in Fig. 1A, acrolein led to a concentration-dependent increase in the cross-linking of Cu,Zn-SOD subunits, and a decrease in the intensity of the original band. The kinetics of the decrease of the intact Cu,Zn-SOD was assessed by densito...
Modification of Cu,Zn-SOD by acrolein
Jung Hoon Kang

Acrolein irreversibly inhibited Cu,Zn-SOD activity in a concentration-dependent manner (Fig. 1B). The decrease of intact Cu,Zn-SOD was paralleled with the loss of enzyme activity. The participation of ROS in the modification of Cu,Zn-SOD by acrolein was studied by examining the protective effects of ROS scavengers. When Cu,Zn-SOD was incubated with acrolein in the presence of ROS scavengers, the modification of Cu,Zn-SOD was prevented by azide, mannitol, ethanol, N-acetyl-L-cysteine (NAC), and glutathione (Fig. 2A). Copper-specific chelators DTPA, DDC, and penicillamine significantly inhibited the modification of Cu,Zn-SOD (Fig. 2B). It has been shown that protein oxidation is accompanied by the conversion of some amino acid residues into carbonyl derivatives (20). The carbonyl content of protein can be measured using phenylhydrazine formation reaction. The method for detecting carbonyl-containing proteins employs derivatization with 2,4-DNPH followed by analysis with a spectrophotometer (21). The incubation of Cu,Zn-SOD with acrolein for 24 h at 37°C resulted in a concentration-dependent increase in carbonyl groups (Fig. 3A). ROS scavengers also prevented the formation of carbonyl compounds (Fig. 3B). The results suggest that ROS may participate in the mechanism of acrolein-mediated Cu,Zn-SOD modification.

In order to investigate the possible mechanism underlying the loss of Cu,Zn-SOD activity during the modification by acrolein, amino acid residues were analyzed by HPLC. When Cu,Zn-SOD was treated with 10 mM acrolein for 24 h at 37°C, serine, histidine, arginine, threonine, and lysine residues were particularly sensitive to modification by acrolein. 3 of 8 serine residues, 4 of 8 histidine residues, 2 of 4 arginine residues, 7 of 12 threonine residues, and 5 of 11 lysine residues were lost.
Modification of Cu,Zn-SOD by acrolein
Jung Hoon Kang

Fig. 3. Effects of ROS scavengers and copper chelators on acrolein-mediated carbonyl compound formation. Cu,Zn-SOD (0.25 mg/ml) was incubated in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h under various concentrations of acrolein. (A) The formation of carbonyl compounds was determined by spectrophotometry. (B) Cu,Zn-SOD (0.25 mg/ml) was incubated with 10 mM acrolein in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of ROS scavengers and copper chelators. Oxidized: no addition of effectors; Azide: 200 mM azide; Mannitol: 200 mM mannitol; Ethanol: 200 mM ethanol; NAC: 20 mM N-acetyl-L-cysteine; GSH: 20 mM glutathione; DTPA: 1 mM DTPA; DDC: 1 mM DDC; PA: 1 mM penicillamine.

Fig. 4. Modification of amino acid residues in Cu,Zn-SOD by acrolein. Cu,Zn-SOD was incubated with 10 mM acrolein in 10 mM potassium phosphate buffer (pH 7.4) at 37°C. After incubation of Cu,Zn-SOD without acrolein (black bar) and with acrolein (gray bar), the amino acid composition of acid hydrolysates was determined as described under "Materials and methods".

as shown in Fig. 4. A previous study reported that the inactivation of Cu,Zn-SOD was accompanied by the loss of one or more histidine residues (22). Thus, the result suggested that the inactivation of Cu,Zn-SOD by acrolein could be due to a decrease in the histidine number in Cu,Zn-SOD.

DISCUSSION

This study is the first to investigate the effects of acrolein on the structure and function of Cu,Zn-SOD. Acrolein is a neurotoxic aldehyde that is produced during the peroxidation of polyunsaturated fatty acids. This aldehyde has been observed by immunohistochemistry in neurofibril tangles and senile plaques as a hallmark pathological feature of AD (23, 24). Several studies demonstrated that acrolein is highly reactive, and disrupts normal cellular processes and activities through the modification of key amino acids such as lysine, cysteine, and histidine (25). In addition, acrolein treatment leads to increased production of ROS in Sertoli cells (26-28), which interferes with the tissue antioxidant defense system (29). The present results showed that acrolein led to covalent cross-linking of proteins and the inactivation of enzymes (Fig. 1). It is suggested that the inactivation of Cu,Zn-SOD by acrolein is associated with protein modification. ROS scavengers and copper chelators were able to protect Cu,Zn-SOD against the cross-linking (Fig. 2). It has been shown that protein oxidation leads to the conversion of some amino acid residues to carbonyl derivatives (20). The carbonyl content of protein can be measured using a phenylhydrazine formation reaction. The method for detecting carbonyl-containing proteins employs derivatization with 2,4-DNPH followed by analysis with a spectrophotometer. The present results show that acrolein led to the formation of carbonyl compound in a concentration-dependent manner (Fig. 3A). ROS scavengers and copper chelators inhibited the formation of carbonyl compound (Fig. 3B). DDC is an effective chelating agent for copper ions, and has been used in its colorimetric estimation (30, 31) and to study the ligation of DDC to copper ions attached at the active sites of Cu,Zn-SOD (32). The present data demonstrates that ROS may participate in the acrolein-mediated Cu,Zn-SOD modification and inactivation.

Cellular metabolism has been shown to generate ROS such as hydrogen peroxide, hydroxyl radicals, and superoxide anions. Trace metals such as copper and iron, which are present in biological systems, may interact with ROS, ionizing radiation, or microwaves and damage macromolecules (33). The cleavage of metalloproteins by oxidative damage may lead to increases in the levels of metal ions in some biological cells (34). It has been reported that the copper concentration in the cerebrospinal fluid was increased 2.2-fold in AD patients (35). One of the hallmarks of AD within the postmortem brain of
disease victims is the existence of amyloid plaques, which are composed primarily of Aβ peptide in a fibrillar conformation. The Aβ peptide will coordinate copper to induce Aβ aggregation through the generation of ROS. Therefore, the interactions of Aβ with copper potentially explain the Aβ deposition and oxidative stress in AD, which are two of the prominent pathological features of the disease (36). In the present study, Cu,Zn-SOD modification was significantly affected by copper chelators. These results suggest that copper ions may participate in the acrolein-mediated Cu,Zn-SOD modification.

It is assumed that the inactivation of many enzymes during oxidative modification is due to the alteration of amino acid residues. To test this possibility, amino acids were analyzed in acrolein-treated Cu,Zn-SOD. The results show that serine, histidine, arginine, threonine, and lysine residues are particularly sensitive to modification by acrolein. The reaction of acrolein with lysine residues in protein yields theimidined with His-63, His-46, His-48, and His-120 in the active site of Cu,Zn-SOD (39). Thus, it was suggested that the ROS generated by acrolein might react with lysine residues, leading to the covalent cross-linking of Cu,Zn-SOD. The results indicate that the inactivation of Cu,Zn-SOD by acrolein may be closely associated with the loss of histidine residues, because this amino acid residue is essential for Cu,Zn-SOD activity (38). Cu,Zn-SOD contains a binuclear cluster, with the active copper and zinc bridged by a common ligand (His-63). Copper is bound to the ligands coordinated with His-63, Hist-46, His-48, and His-120 in the active site of Cu,Zn-SOD (39). Thus, it was suggested that the inactivation of Cu,Zn-SOD by acrolein could be produced by the loss of histidine residues in the protein with acrolein. Consequently, copper became almost free from the ligand, and was released from the oxidatively damaged enzyme, which resulted in the loss of activity. The result that copper ion chelators inhibited the acrolein-mediated Cu,Zn-SOD modification supports this mechanism. Serine and threonine residues may be difficult to react with acrolein, because these residues do not have amino and thiol groups in the side chain. Therefore, it was assumed that the modification of serine and threonine residues might be due to oxidative damage by ROS.

Cu,Zn-SOD is a metalloenzyme that is essential to the dismutation of O2- to H2O2. Thus, Cu,Zn-SOD is a very important component of the cellular defense mechanism against oxygen toxicity. The inactivation of Cu,Zn-SOD by acrolein is able to induce the perturbation of the antioxidant system. In addition, the copper ions released from the Cu,Zn-SOD oxidatively damaged by acrolein can enhance the metal-catalyzed reaction to produce ROS, which induce oxidative damage to the other macromolecules. Further research is required to determine if acrolein modifies Cu,Zn-SOD in vivo.

In summary, acrolein modified Cu,Zn-SOD, which resulted in protein cross-links. Acrolein irreversibly inhibited Cu,Zn-SOD activity in a concentration-dependent manner. This mechanism occurred through the modification of amino acids by ROS.

**MATERIALS AND METHODS**

**Materials**

Acrolein, sodium azide, N-acetyl-L-cysteine, diethylenetriaminepentaacetic acid (DTPA), diethyldithiocarbamic acid (DDC), and penicilamine were purchased from Sigma (St. Louis, MO, USA). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA, USA). All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

**Preparation of proteins**

Using a plasmid vector containing human Cu,Zn-SOD cDNA (pET-wtSOD) (40), the protein was expressed in *Escherichia coli* strain BL21. Bacteria were grown in Luria broth supplemented with 0.4 mM IPTG beginning at an OD600 nm reading of 0.6. CuCl2 (0.5 mM) and ZnCl2 (0.5 mM) were also added to the medium at this time. Induction was performed at 25°C for 3 h. Induced bacterial cells (2-L cultures) were suspended in 50 mM potassium phosphate (pH 7.8) and 0.1 mM EDTA, and disrupted by lysozyme. The lysate was centrifuged at 50,000 × g for 1 hour, and the precipitate was discarded. Ammonium sulfate was added to this supernatant fraction to 60% of saturation. After 2-3 hours, the precipitate was removed at 15,000 × g for 30 minutes, and additional ammonium sulfate was added to the supernatant fraction to 95% saturation. The precipitate was collected after 20 hours by centrifugation at 30,000 × g for 1 hour and dissolved in a minimal volume of 2.5 mM potassium phosphate (pH 7.8) and 0.1 mM EDTA (buffer I), and was loaded onto a Sephacryl S-100 (2.5 × 100 cm) column. Proteins were eluted with buffer I, and then active fractions were absorbed onto a DEAE-Sephacel (2.5 × 20 cm) column pre-equilibrated with buffer I. After washing with 5 volumes of buffer I, bound proteins were eluted with a linear gradient of potassium phosphate of 2.5 to 50 mM. Active fractions were concentrated to 5 ml with an Amicon YM-10 ultrafilter. This material was dialyzed against 10 mM potassium phosphate (pH 7.8) and 0.1 mM EDTA containing Chelex 100.

**Measurement of Cu,Zn-SOD activity**

The activity of Cu,Zn-SOD was measured by monitoring their capacities to inhibit the reduction of ferricytochrome c by xanthine/xanthine oxidase, as described by McCord and Fridovich (41).

**Analysis of Cu,Zn-SOD modification**

Cu,Zn-SOD (0.25 mg/ml) in 10 mM potassium phosphate buffer, pH 7.4, was incubated at 37°C for 24 h with different concentrations of acrolein in a total volume of 20 µl. The samples
were treated with 5 μl of 4X concentrated sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenol blue) and boiled at 100°C for 10 min before electrophoresis. Each sample was subjected to SDS-PAGE as described by a previous method (42), using a 15% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Detection of carbonyl compounds
The carbonyl contents of the proteins were determined via spectrophotometric assays, as described elsewhere (20). Both native and oxidized proteins were incubated with 10 mM 2,4-DNPH in 2.5 M HCl for 1 h at room temperature. After incubation, 20% TCA was added to the sample, and the tubes were left in an ice bucket for 10 min and centrifuged for 5 min with a tabletop centrifuge to collect the protein precipitates, after which the supernatants were discarded. Another wash was performed using 10% TCA, and the protein pellets were mechanically broken using a pipette tip. Finally, the pellets were washed 3 times with ethanol-ethyl acetate (1:1 v/v) to remove any free DNPH. The final precipitates were dissolved in 2 ml of 6 M guanidine hydrochloride solution and left for 10 min at 37°C with general vortex mixing. Carbonyl contents were calculated from the absorbance (370 nm) with an absorption coefficient ε of 22,000 M⁻¹ cm⁻¹.

Amino acid analysis
Aliquots of modified and native Cu,Zn-SOD preparations were hydrolyzed at 110°C for 24 h after the addition of 6 N HCl. Since acid hydrolysis destroys tryptophan, the tryptophan content of oxidized and native Cu,Zn-SOD preparations was determined by means of alkaline hydrolysis as described previously (43). The amino acid content of acid and alkaline hydrolysates was determined by HPLC separation of their phenylisothiocyanate derivatives by using a Pico-tag free amino acid analysis column and a 996 photodiode array detector (Waters, USA).

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

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Modification of Cu,Zn-SOD by acrolein
Jung Hoon Kang

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