Journal of Biochemistry and Molecular Biology, Vol. 37, No. 3, May 2004, pp. 325-329

JOURNAL OF Biochemistry and Molecular Biology

Modification of Cu,Zn-Superoxide Dismutase by Oxidized Catecholamines

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Received 8 August 2003, Accepted 3 September 2003

Oxidation of catecholamines may contribute to the pathogenesis of Parkinson's disease (PD). The effect of the oxidized products of catecholamines on the modification of Cu,Zn-superoxide dismutase (SOD) was investigated. When Cu,Zn-SOD was incubated with the oxidized 3,4-dihydroxyphenylalanine (DOPA) or dopamine, the protein was induced to be aggregated. The deoxyribose assay showed that hydroxyl radicals were generated during the oxidation of catecholamines in the presence of copper ion. Radical scavengers, azide, N-acetylcysteine, and catalase inhibited the oxidized catecholamine-mediated Cu,Zn-SOD aggregation. Therefore, the results indicate that free radicals may play a role in the aggregation of Cu,Zn-SOD. When Cu,Zn-SOD that had been exposed to catecholamines was subsequently analyzed by an amino acid analysis, the glycine and histidine residues were particularly sensitive. These results suggest that the modification of Cu,Zn-SOD by oxidized catecholamines might induce the perturbation of cellular antioxidant systems and led to a deleterious cell condition.

Keywords: Catecholamine, Free radical, Cu,Zn-superoxide dismutase

Introduction

A variety of markers and indices in PD patients and animal models indicate the involvement of oxygen-free radicals and oxidative stress in the pathogenesis of PD. These include lipid peroxidation (Dexter *et al*., 1989; Jenner, 1996), reduced glutathione (Sian *et al*., 1994), increased levels of iron, and reduction of ferritin concentrations in the substantia nigra pars compacta of PD (Dexter *et al*., 1992; Jellinger *et al*., 1993). Faults with the respiratory chain and dopamine metabolism have also been theorized to contribute to free radical production (Jenner, 1996). Catechols are hydroquinones that may undergo oxidation to electrophilic semiquinones and quinines. Enzymatic oxidation of catechols, followed by polymerization, is a common form of pigmentation in animals. Also, protein-protein crosslinking by enzymatically-oxidized catechols is a known mechanism of post-translational modification that widely occurs in nature (Waite, 1990). Although this enzymatic activity is not present in the human brain, endogenous brain catechols do oxidize spontaneously to quinoid compounds that polymerize to form neuromelanin in the substantia nigra and locus ceruleus (Graham, 1978; Miller *et al*., 1990). Other quinone-mediated reactions, beside polymerization, may contribute to neurodegeneration in PD [e.g. catalytic transfer of reducing equivalents to molecular oxygen (redox cycling) and adduction to macromolecules (Graham *et al*., 1978)]. DOPA and dopamine can oxidize *in vitro* to generate semiquinones, $O_2^{-\cdot}$ and H_2O_2 (Pileblad *et al*., 1988), a process greatly facilitated by the presence of transition-metal ions. In the case of iron and copper, ·OH will also be generated by the Fenton reaction (Sutton and Winterbourn, 1989; Halliwell and Guteridge, 1992). ·OH is the most powerful oxidizing species among several reactive oxygen radicals, and is able to oxidize most macromolecules (like DNA, protein, lipid, and carbohydrate).

In the present study, the effect of the non-enzymatically oxidized DOPA and dopamine on the modification of Cu,Zn-SOD was investigated. Present results revealed that the aggregation of Cu,Zn-SOD was induced by the products of oxidized catecholamines via the generation of free radicals.

Materials and methods

Materials Sodium azide, dihydorxyphenylalanine (DOPA), dopamine, 5, 5-dimethyl 1-pyrolline N-oxide (DMPO), N-acetylcysteine, catalase, diethylenetiaminepentaacetic acid (DTPA), and diethyldithiocarbamic acid (DDC) were purchased from Sigma (St. Louis, USA). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, USA). All of the solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

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Preparation of proteins Using the plasmid vector containing human Cu,Zn-SOD cDNA (pET-wtSOD) (Kang *et al*., 1997), the protein was expressed in the *Escherichia coli* strain BL21. Bacteria were grown in Luria broth that was supplemented with 0.4 mM IPTG, beginning at an OD_{600} reading of 0.6. $CuCl_2$ (0.5 mM) and $ZnCl_2$ (0.5 mM) were also added to the medium at this time. Induction was performed at 25°C for 3 h. The induced bacterial cells (2 l cultures) were suspended in 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, and disrupted by lysozyme. The lysate was centrifuged at $50,000 \times g$ for 1 h and the precipitate was discarded. The ammonium sulfate was added to this supernatant fraction to 60% of saturation. After 2-3 h, the precipitate was removed at $15,000 \times g$ for 30 min, and additional ammonium sulfate was added to the supernatant fraction to a 95% saturation. The precipitate was collected after 20 h by centrifugation at $30,000 \times g$ for 1 h and dissolved in a minimal volume of 2.5 mM potassium phosphate (pH 7.8), 0.1 mM EDTA (buffer I), and loaded onto a Sephacryl S-100 (2.5×100 cm) column. The proteins were eluted with buffer I and then the active fractions were absorbed onto a DEAE-Sephacel (2.5×20 cm) column that was preequilibrated with buffer I. After washing with 5 volumes of buffer I, the bound proteins were eluted with a linear gradient of potassium phosphate 2.5 to 50 mM. The active fractions were concentrated to 5 ml by an Amicon YM-10 ultrafilter. This material was dialyzed against 10 mM potassium phosphate (pH 7.8), 0.1 mM EDTA, containing Chelex 100. The apoproteins and remetallated enzymes were prepared by published procedures (Lu *et al*., 1993). Apoprotein was prepared by sequential dialysis against 0.5 M sodium acetate containing 100 mM EDTA (pH 3.8), 0.5 M sodium acetate containing 1 M NaCl (pH 5.5), and 0.5 M sodium acetate (pH 5.5). The remetallated enzymes were made by adding 2 equivalent of Zn^{2+} and adding 2 equivalent of Cu^{2+} to the apoproteins.

Measurement of Cu,Zn-SOD activity The Cu,Zn-SOD activity was measured by monitoring their capacities to inhibit the reduction of ferricytochrome c by xanthine/xanthine oxidase, as described by McCord and Fridovich (1969).

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 catecholamines in a total volume of 20 μl. The samples were treated with 7 μl of a 4 x concentrated sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenolblue) and boiled at 100°C for 10 min before electrophoresis (Treerattrakool *et al*., 2002). Each sample was subjected to SDS-PAGE (Laemmli, 1970), using a 15% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Measurement of hydroxyl radical Detection of the hydroxyl radicals was determined by measuring the thiobarbituric acid reactive 2-deoxy-D-ribose oxidation products (Halliwell and Gutteridge, 1981). The reaction mixtures contained 100 μM DOPA or 100 μM dopamome in the absence or presence of 100 μM Cu^{2+}. The mixtures were incubated at 37°C for 24 h. The degradation of 2-deoxy-D-ribose was measured by adding 200 μl of PBS, 200 μl of 2.8% (w/v) trichloroacetic acid, 200 μl of 1% (w/v) thiobarbituric acid, followed by heating at 100°C for 10 min. After cooling, the absorbance at 532 nm was measured by UV/VIS spectrophotometer (Shimadzu, UV-1601).

Amino acid analysis Aliquots of the 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 the oxidized and native Cu,Zn-SOD preparations was determined by means of alkaline hydrolysis, as described previously (Kim *et al*., 2002). The amino acid content of the acid and alkaline hydrolysates was determined by HPLC separation of their phenylisothiocyanate-derivatives by using Pico-tag free amino acid analysis column and 996 photodiode array detector (Waters, Milford, USA).

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

Results

Since free radicals can be generated in autoxidation of catecholamines (Pileblad *et al*., 1988; Sutton and Winterbourn, 1989; Halliwell and Gutteridge, 1992), the effects of oxidized catecholamines on the Cu,Zn-SOD modification was investigated. An SDS-PAGE analysis showed that the band shift was observed when Cu,Zn-SOD was incubated with DOPA or dopamine (Fig. 1A). When Cu,Zn-SOD was incubated with DOPA or dopamine, a time-dependent decrease of Cu,Zn-SOD activity was observed by the cytochrome c reduction assay (Fig. 1B)

The participation of free radicals in the aggregation of Cu,Zn-SOD by the oxidized catecholamines was studied by examining the generation of hydroxyl radicals during the oxidation of catecholamines. The generation of hydroxyl radicals in the oxidation of catecholamines was measured with a thiobarbituric acid-reactive substance (TBARS). The incubation of 2-deoxy-D-ribose with DOPA and dopamine in the presence of Cu^{2+} produced 13.46 nmol and 8.38 nmol when compared to 2.74 nmol and 1.34 nmol in the absence of Cu^{2+} (Fig. 2). The aggregation of Cu,Zn-SOD was significantly suppressed in the presence of azide and N-acetyl cysteine (Fig. 3). Catalase also inhibited the aggregation of Cu,Zn-SOD (Fig. 3, lanes 5 and 9). These results suggest that the autoxidation of catecholamines may lead to a generation of hydrogen peroxide and produce hydroxyl radical via the transitional metal-catalyzed reaction (Fenton reaction). Evidence that copper chelators, DTPA and DDC, protected Cu,Zn-SOD against oxidized catecholamines supported this mechanism (Fig. 4). Thus, the hydroxyl radical might play a critical role in the aggregation of Cu,Zn-SOD by the oxidized catecholamines.

In order to specify a target residue in the enzyme, Cu,Zn-SOD that was exposed to catecholamines was analyzed by an amino acid analysis following acid hydrolysis of the modified

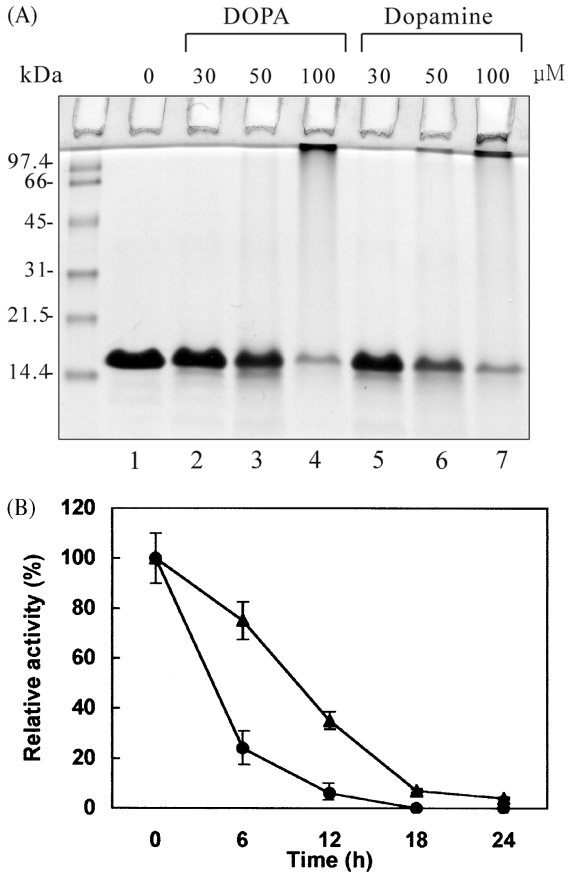

Fig. 1. Modification and inactivation of Cu,Zn-SOD by oxidized catechols. (A) Cu,Zn-SOD (0.25 mg/ml) was incubated in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h under various conditions. Lane 1, Cu,Zn-SOD control; lanes 2-4, with 30, 50, and 100 μM DOPA; lanes 5-7, with 30, 50, and 100 μM dopamine. The positions of the molecular weight markers (kDa) are indicated on the left. (B) After the reactions of Cu,Zn-SOD with 100 μM catecholamines, the activities were measured by monitoring their capacities to inhibit the reduction of ferricytochrome c by xanthine/xanthine oxidase.

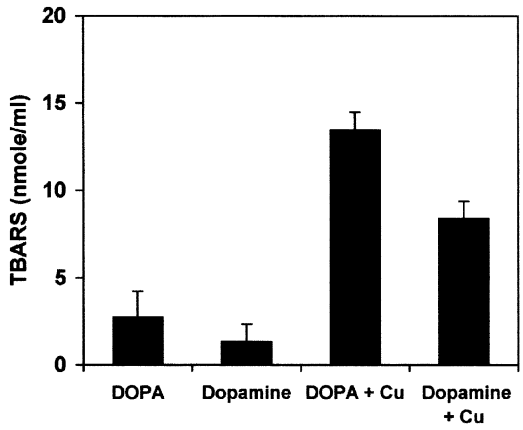

Fig. 2. Generation of hydroxyl radical during the oxidation reaction of catecholamines. The reaction mixtures contained Cu^{2+} and 10 mM 2-deoxy-D-ribose in 10 mM phosphate buffer at pH 7.4 under various conditions: DOPA, 2-deoxy-D-ribose + 100 μM DOPA; Dopamine, 2-deoxy-D-ribose + 100 μM dopamine; DOPA + Cu, 2-deoxy-D-ribose + 100 μM DOPA + 100 μM $CuSO_4$; Dopamine + Cu, 2-deoxy-D-ribose + 100 μM dopamine + 100 μM $CuSO_4$.

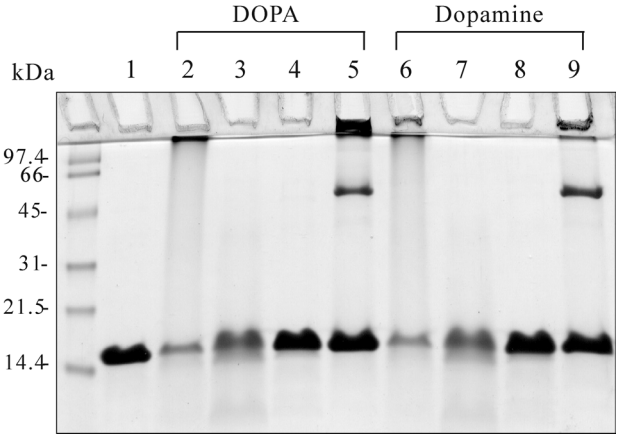

Fig. 3. Effect of radical scavengers on the aggregation of Cu,Zn-SOD by oxidized catechols. Cu,Zn-SOD (0.25 mg/ml) was incubated with 100 μM catechols in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of radical scavengers. Lane 1, Cu,Zn-SOD control; lane 2, Cu,Zn-SOD + 100 μM DOPA; lane 3, Cu,Zn-SOD + 100 μM DOPA + 200 mM azide; lane 4, Cu,Zn-SOD + 100 μM DOPA + 50 mM N-acetyl cysteine; lane 5, Cu,Zn-SOD + 100 μM DOPA + catalase (0.1 mg/ml); lane 6, Cu,Zn-SOD + 100 μM dopamine; lane 7, Cu,Zn-SOD + 100 μM dopamine + 200 mM azide; lane 8, Cu,Zn-SOD + 100 μM dopamine + 50 mM N-acetyl cysteine; lane 9, Cu,Zn-SOD + 100 μM dopamine + catalase (0.1 mg/ml).

proteins. When Cu,Zn-SOD was treated with 100 μM catechoamine for 24 h at 37°C, the glycine and histidine residues were particularly sensitive to modification by catecholamines. As shown in Fig. 5, when Cu,Zn-SOD was incubated with DOPA, then 11 of 25 glycine residues and 4 of 8 histidine residues were lost. During the incubation of dopamine with Cu,Zn-SOD, 6 of 25 glycine residues and 2 of 8 histidine residues were lost.

Discussion

The present study investigated the potential role of catechols in the modification of Cu,Zn-SOD. The toxicity of catechol may be augmented by its free radical-generating function in neurodegenerative disorder. Since the level of free radicals was reported to increase in PD patients (Ilic *et al*., 1998; 1999), then the oxidative modification of Cu,Zn-SOD might have pathological significance. In several common neurodegenerative diseases [including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD)], Cu,Zn-SOD activity decreased (Bowling and

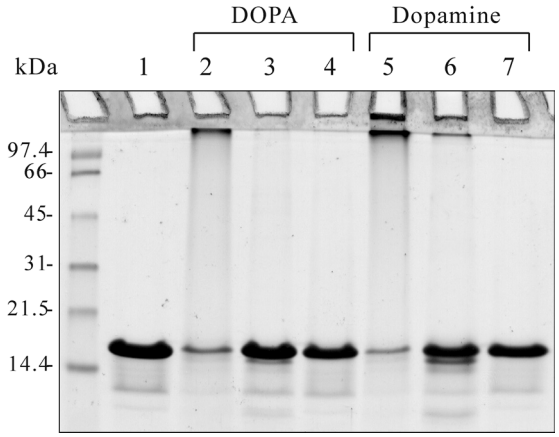

Fig. 4. Effect of copper chelators on the aggregation of Cu,Zn-SOD by oxidized catechols. Cu,Zn-SOD (0.25 mg/ml) was incubated with 100 μM catechols in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of radical scavengers. Lane 1, Cu,Zn-SOD control; lane 2, Cu,Zn-SOD + 100 μM DOPA; lane 3, Cu,Zn-SOD + 100 μM DOPA + 10 mM DTPA; lane 4, Cu,Zn-SOD + 100 μM DOPA + 10 mM DDC; lane 5, Cu,Zn-SOD + 100 μM dopamine; lane 6, Cu,Zn-SOD + 100 μM dopamine + 10 mM DTPA; lane 7, Cu,Zn-SOD + 100 μM dopamine + 10 mM DDC.

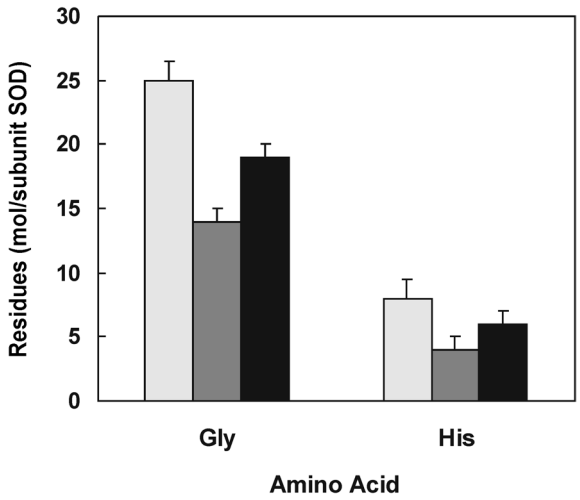

Fig. 5. Modification of amino acid residues in Cu,Zn-SOD by catecholamines. Cu,Zn-SOD was incubated with 100 μM DOPA or 100 μM dopamine in 10 mM potassium phosphate buffer (pH 7.4) at 37°C. After incubation of Cu,Zn-SOD with DOPA (gray bar) or dopamine (black bar) and without catecholamines (light gray bar), the amino acid composition of acid hydrolysates was determined as described under "Materials and methods". Data represent the means ± S.D. (n = 3).

Beal, 1995). Present results show that the modification of Cu,Zn-SOD was induced by the autoxidation of catecholamines. DOPA and dopamine can oxidize *in vitro* to generate semiquinones, $O_2^{-}\cdot$ and H_2O_2 (Pileblad *et al*., 1988), a process greatly facilitated by the presence of transition-metal ions. In the case of iron and copper, ·OH will also be generated by the Fenton reaction (Sutton and Winterbourn, 1989; Halliwel and Gutteridge 1992). In this study, the catecholamine-mediated Cu,Zn-SOD aggregation was inhibited by the hydroxyl radical scavenger and catalase. These results indicate that hydroxyl radicals and hydrogen peroxide may be involved in the aggregation of Cu,Zn-SOD.

Trace metals, such as iron and copper that are variously present in biological systems, may interact with active oxygen species, ionizing radiation, or microwave radiation to damage macromolecules (Sagripanti *et al*., 1987; Imlay *et al*., 1988; Sagripanti and Kraemer, 1989). The cleavage of the metalloproteins by oxidative damage may lead to increases in the levels of metal ions in some biological cells (O'Connell and Peters, 1987). It has been reported that the copper concentration was significantly increased in the cerebrospinal-fluid of PD patients (Pall *et al*., 1987) and that the cerebrospinal-fluid copper concentration was increased 2.2-fold in AD patients (Multhaup *et al*., 1996). These results suggest that the iron or copper-catalyzed oxidative reaction might contribute to the pathogenesis of PD. In the present study, the aggregation of Cu,Zn-SOD was significantly inhibited by copper chelators. These results suggest that copper ions are released from oxidatively-damaged Cu,Zn-SOD by oxidized catecholamines and then induce the formation of ·OH through a Fenton reaction.

In this study, when Cu,Zn-SOD was treated with 100 μM catechoamine for 24 h at 37°C, then the glycine and histidine residues were particularly sensitive to modification by catecholamines. No other statistically significant differences in the amino acid compositions were observed between the modified and control enzymes. The present results indicate that the inactivation of Cu,Zn-SOD by catecholamines may be closely associated with the loss of histidine residues because this amino acid residue is essential for Cu,Zn-SOD activity (Maria *et al*., 1995). 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, His-46, His-48, and His-120 in the active site of Cu,Zn-SOD (Tainer *et al*., 1983). Thus, it was suggested that copper binding sites were modified during the reaction of Cu,Zn-SOD with catecholamines. Consequently, copper became almost a free form of the ligand and was released from the oxidatively-damaged enzyme, which resulted in the loss of activity. Glycine residues are abundant in the Cu,Zn-SOD molecule. Therefore, it was assumed that the modification of glycine is easily the most susceptible to oxidation by the ctecholamine-generated free radicals.

In conclusion, the present results suggest that the modification of Cu,Zn-SOD was induced by the autoxidation of catecholamines, involving ·OH generation from H_2O_2. This catecholamine-mediated Cu,Zn-SOD modification might, therefore, be associated with the pathogenesis of PD and related disorders.

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