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Salsolinol, a Tetrahydroisoquinoline Catechol Neurotoxin, Induces Human Cu,Zn-superoxidie Dismutase Modificaiton

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The endogenous neurotoxin, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol), has been considered a potential causative factor for the pathogenesis of Parkinson's disease (PD). In the present study, we examined the pattern of human Cu,Zn-superoxide dismutase (SOD) modification elicited by salsolinol. When Cu,Zn-SOD was incubated with salsolinol, some protein fragmentation and some higher molecular weight aggregates were occurred. Salsolinol led to inactivation of Cu,Zn-SOD in a concentration-dependent manner. Free radical scavengers and catalase inhibited the salsolinol-mediated Cu,Zn-SOD modificaiton. Exposure of Cu,Zn-SOD to salsolinol led also to the generation of protein carbonyl compounds. The deoxyribose assay showed that hydroxyl radicals were generated during the oxidation of salsolinol in the presence of Cu,Zn-SOD. Therefore, the results indicate that free radical may play a role in the modification and inactivation of Cu,Zn-SOD by salsolinol.

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

Introduction

The endogenous neurotoxin, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol), has been considered a potential causative factor for Parkinsons disease (PD) (Ohta *et al*., 1987; Niwa *et al*., 1991; Moser and Kompf, 1992; Ikeda *et al*., 1993). Salsolinol can be synthesized from dopamine and acetaldehyde by the enzyme salsolinol synthase. Alternatively, it can also be synthesized from dopamine and pyruvate by forming an intermediate metabolite, salsolinol-1-carboxylic acid. Salsolinol-1-carboxylic acid can be directly metabolized by an unknown enzyme to salsolinol or at first to 1,2-dehydrosalsolinol and then to salsolinol (Naoi *et al*., 1996; 2002). Several studies indicated that salsolinol is toxic to dopaminergic neurons *in vitro* as well as *in vivo*. Salsolinol is known to inhibit tyrosine hydroxylase and monoamine oxidase (Bembenek *et al*., 1983) as well as mitochondrial complex-I and complex-II enzyme activities (Moser and Kompf, 1992; McNaught *et al*., 1995; Morikawa *et al*., 1998). However, the precise biochemical and molecular mechanisms underlying the oxidative stress-mediated neurotoxicity of salsolinol is still poorly understood.

The accumulation of reactive oxygen species (ROS) has been proposed to be a critical factor of the pathogenesis of various neurodegenerative disorders (Jenner *et al*., 1992; Bowling *et al*., 1993; Hensley *et al*., 1994). The autoxidation of catecholamines, such as dopamine and 6-hydroxydopamine, led to the generation of ROS during the pathogenic processes of neurodegenerative diseases including PD (Montine *et al*., 1995; Holtz *et al*., 2006; Saito *et al*., 2007). Salsolinol, tetrahydroisoquinoline catechol neurotoxin, was shown to induce cell death by generation ROS (Jung and Surh, 2001; Kim *et al*., 2001) in a manner similar to 6-hydroxydopamine (Wu *et al*., 1996; Choi *et al*., 2003).

Cu,Zn-superoxide dismutase (SOD) is an enzyme catalyzing the disproportion of superoxide radicals to dioxygen and hydrogen peroxide. Cu,Zn-SOD has great physiological significance and therapeutic potential. This enzyme requires Cu and Zn for its biological activity, and loss of Cu, results in its complete inactivation, leading in many cases to the development of human disease (Kim *et al*., 2002; Ihara *et al*., 2005). The role of ROS and Cu,Zn-SOD has been investigated in neuronal injury (Facchinctti *et al*., 1999). ROS may play an important role in several pathological conditions of the central nervous system, where the directly injure tissue, and where their formation may also be a consequence of tissue injury. ROS produce tissue damage through multiple mechanisms and can worsen acute neurodegenerative disorders including PD. A correlation between ·OH and Cu,Zn-SOD activity in PD has also been suggested by the studies of Ihara *et al.*

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(1999). The higher ·OH level and lower Cu,Zn-SOD activity may play a role in the onset of progression of PD, and pergolide may act neuroprotectively by inducing Cu,Zn-SOD. It has been reported that salsolinol in conjugation with copper ion undergoes redox cycling to produce ROS such as hydroxyl radicals that cause DNA strand scssion and cell death (Jung and Surh, 2001; Kim *et al*., 2001).

In the present study, the effect of salsolinol on the modification of human Cu,Zn-SOD was investigated. Present results revealed that the aggregation and fragmentation of Cu,Zn-SOD were induced by salsolinol via the generation of free radicals. The modification of Cu,Zn-SOD by salsolinol led to the loss of enzyme activity.

Materials and Methods

Materials. Salsolinol, sodium azide, N-acetyl-$_L$-cysteine, catalase, 2-deoxy-D-ribose, glutathione, thiourea, thiobarbituric acid, diethylenetiaminepentaacetic acid (DTPA), diethyldithiocarbamic acid (DDC) and penicillamine were purchased from Sigma. Chelex 100 resin (sodium form) was obtained from Bio-Rad. All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

Preparation of proteins. Using the plasmid vector containing human Cu,Zn-SOD cDNA (pET-wtSOD) (Kang *et al*., 1997), the protein was expressed in *Escherichia coli* strain BL21. Bacteria were grown in Luria broth supplemented with 0.4 mM IPTG beginning at an $OD_{600\ nm}$ 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. Induced bacterial cells (2 L cultures) were suspended 50 mM potassium phosphate (pH 7.8), 0.1 mM EDTA and disrupted by lysozyme. The lysate was centrifuged at 50,000 × 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 × g for 30 min, and additional ammonium sulfate was added to the supernatant fraction to 95% saturation. The precipitate was collected after 20 h by centrifugation at 30,000 × g for 1 h and was dissolved in a minimal volume of 5 mM potassium phosphate (pH 7.8), 0.1 mM EDTA (buffer I) and loaded onto Sephacryl S-100 (2.5 × 100 cm) column. Proteins were eluted with buffer I and then active fractions were absorbed onto 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 5 to 50 mM. Active fractions were concentrated to 5 ml by Amicon YM-10 ultrafilter. This material was dialyzed against 10 mM potassium phosphate (pH 7.8), 0.1 mM EDTA containing Chelex 100. Apoproteins and remetallated enzymes were prepared by published procedures (Crow *et al*., 1997). 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). Remetallated enzymes were made by adding 2 equiv of Zn^{2+} and adding 2 equivalents of Cu^{2+} to the apoproteins.

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 (1969).

Protein modification. Protein concentration was determined by the BCA method (Smith *et al*., 1985). Modification of Cu,Zn-SOD (0.5 mg/ml) was carried out by incubation of the enzyme in 10 mM potassium phosphate buffer (pH 7.4) both in the presence and absence of salsolinol at 37°C. After the incubation of the reaction mixtures, the mixtures were then placed into Microcon filter (Amicon) and centrifuged at 13,000 rpm for 1 h to remove salsolinol. The mixture was then washed with Chelex 100 treated water and centrifuged for 1 h at same speed to further remove salsolinol. This was repeated four times. The filtrate was dried by freeze dryer and dissolved with 10 mM potassium phosphate buffer (pH 7.4). The protection by free radical scavengers against salsolinol-mediated Cu,Zn-SOD modification was performed by preincubation of the enzyme in the presence of free radical scavengers for 5 min at room temperature and the reaction of mixture with salsolinol for 24 h at 37°C. The unreacted reagent was washed by using Microcon filter (Amicon).

Analysis of Cu,Zn-SOD modification. Cu,Zn-SOD (0.5 mg/ml) in 10 mM potassium phosphate buffer, pH 7.4, was incubated at 37°C for 24 h with different concentrations of salsolinol in a total volume of 20 ml. The samples were treated with seven ml of 4 X concentrated sample buffer (0.25 M Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenolblue) and were boiled at 100°C for 10 min before electrophoresis. Each sample was subjected to SDS-PAGE as described by Laemmli (Laemmli, 1970), using a 18% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Measurement of hydroxyl radical. Detection of hydroxyl radicals was determined by measuring thiobarbituric acid reactive 2-deoxy-D-ribose oxidation products (Kim and Kang, 2006). Reaction mixtures contained various concentrations of salsolinol and Cu,Zn-SOD (0.5 mg/ml). Mixtures were incubated at 37°C for 24 h. The degradation of 2-deoxy-D-ribose was measured by adding 200 ml of PBS, 200 ml of 2.8% (w/v) trichloroacetic acid, 200 ml 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).

Detection of protein carbonyl compound. The carbonyl content of proteins was determined by immunoblotting with anti-DNP antibody as described elsewhere (Levine *et al*., 1994). Both native and oxidized proteins were incubated with 20 mM 2,4-DNPH in 10% (v/v) trifluoroacetic acid at room temperature for 1 h. After incubation, a neutralization solution (2 M Tris) was added at room temperature for 15 min. After SDS-PAGE of the derivatized protein with 18% polyacrylamide gel, the proteins were transferred onto a nitrocellulose sheet and then probed with rabbit anti-DNP sera, used a dilution of 1 : 1000. The detection method used alkaline phosphatase-labelled goat anti rabbit IgG with the BCIP/NBT detecting system (Bio-Rad).

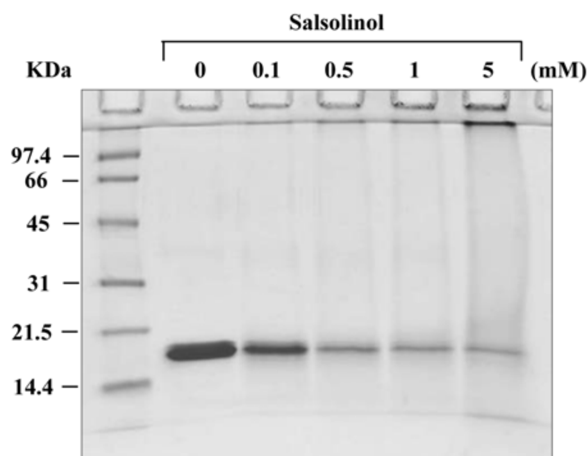

Fig. 1. Modification of Cu,Zn-SOD by salsolinol. Cu,Zn-SOD (0.5 mg/ml) was incubated in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h under various conditions. Lane 1, Cu,Zn-SOD control; lane 2-5, with 0.1, 0.5, 1 and 5 mM salsolinol. The positions of molecular weight markers (kDa) are indicated on the left.

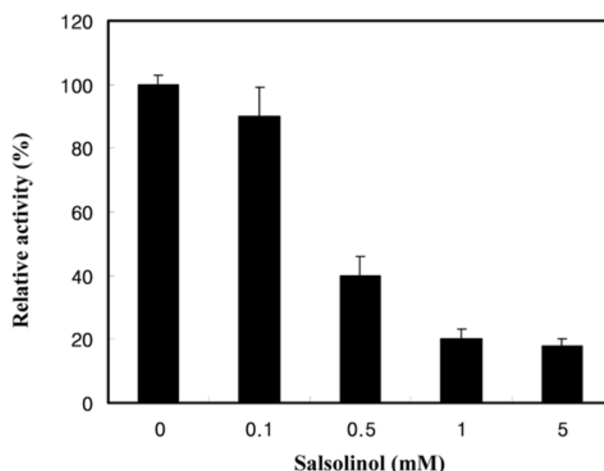

Fig. 2. Inactivation of Cu,Zn-SOD by salsolinol. After the reactions of Cu,Zn-SOD with 0.1, 0.5, 1 and 5 mM salsolinol, the activities were measured by monitoring their capacities to inhibit the reduction of ferricytochrome *c* by xanthine/xanthine oxidase. Data represent the means ± S.D. (n = 3-5).

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

Results

To investigation if the salsolinol-mediated modification of Cu,Zn-SOD is associated with either protein aggregation or fragmentation, the reaction mixtures were subjected to SDS-PAGE analysis. As shown in Fig. 1, there was a salsolinol concentration-dependent increase in the formation of protein aggregates. When Cu,Zn-SOD was incubated with 5 mM salsolinol, most of the protein was at the top of the gel. In addition to aggregation, the treatment of Cu,Zn-SOD with salsolinol led to lower molecular weight fragments. During incubation of Cu,Zn-SOD with salsolinol, dismutation activity was gradually decreased as a concentration of salsolinol (Fig. 2). The results suggested that the protein modification by salsolinol was associated with the inactivation of Cu,Zn-SOD. It has been shown that protein oxidation leads to the conversion of some amino acid residues to carbonyl derivatives (Levine *et al*., 1994). 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 anti-DNP sera. Result obtained from the immunoblotting analysis of salsolinol-treated Cu,Zn-SOD was shown in Fig. 3. Carbonyl compound was detected in major band and aggregates of protein.

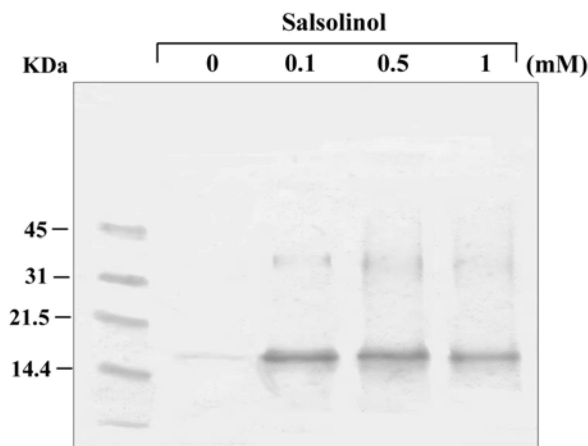

Fig. 3. Detection of carbonyl compounds after the incubation of Cu,Zn-SOD with salsolinol. Cu,Zn-SOD (0.5 mg/ml) was incubated with various concentration of salsolinol in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for 24 h. Reaction mixtures were derivatized DNPH as described under Materials and Methods. DNPH-derivatized proteins were subjected to SDS-PAGE for immunoblot with anti-DNP sera.

The participation of free radicals in the modification of Cu,Zn-SOD by salsolinol was studied examining the inhibition of free radical scavengers during the reaction of Cu,Zn-SOD with slasolinol. The modification of Cu,Zn-SOD was slightly suppressed in the presence of azide, mannitol and ethanol, whereas the protein modification was significantly inhibited by glutathione, *N*-acetyl-$_L$-cysteine and thiourea (Fig. 4). Catalase also inhibited the modification of Cu,Zn-SOD by salsolinol (Fig. 5). These results suggest that the salsolinol may lead to the generation of hydrogen peroxide and produce free radicals via transitional metal-catalyzed reaction (Fenton reaction). Evidence that copper chelators, DTPA, DDC and penicillamine protected the Cu,Zn-SOD against salsolinol supported this mechanism (Fig. 6). To investigation the mechanism of hydroxyl radical generation, hydroxyl radicals in the reaction of salsolinol with Cu,Zn-SOD was measured with

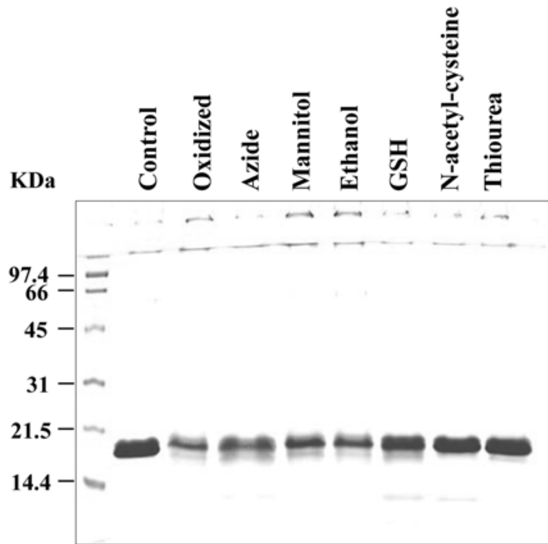

Fig. 4. Effect of free radical scavengers on the modification of Cu,Zn-SOD by salsoinol. Cu,Zn-SOD (0.5 mg/ml) was incubated with 1 mM salsolinol in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of free radical scavengers. Lane 1, Cu,Zn-SOD control; lane 2, oxidized Cu,Zn-SOD (without free radical scavenger); lane 3, 200 mM azide; lane 4, 200 mM mannitol; lane 5, 200 mM ethanol; lane 6, 20 mM glutathione; lane 7, 20 mM N-acetyl cysteine; lane 8, 20 mM thiourea.

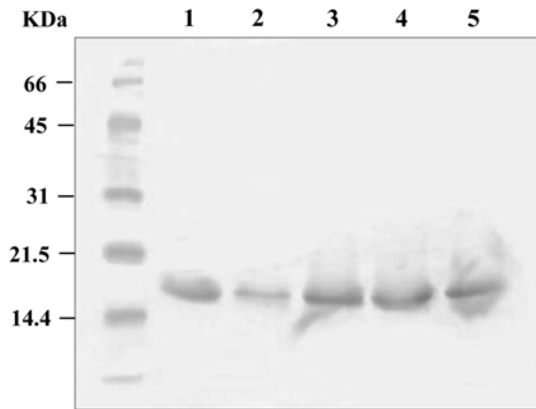

Fig. 5. Effect of catalase on the modification of Cu,Zn-SOD by salsoinol. Cu,Zn-SOD (0.5 mg/ml) was incubated with 1 mM salsolinol in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of catalase. Lane 1, Cu,Zn-SOD control; lane 2, oxidized Cu,Zn-SOD (without catalase); lane 3, 20 μg catalase; lane 4, 40 μg catalase; lane 5, 80 μg catalase.

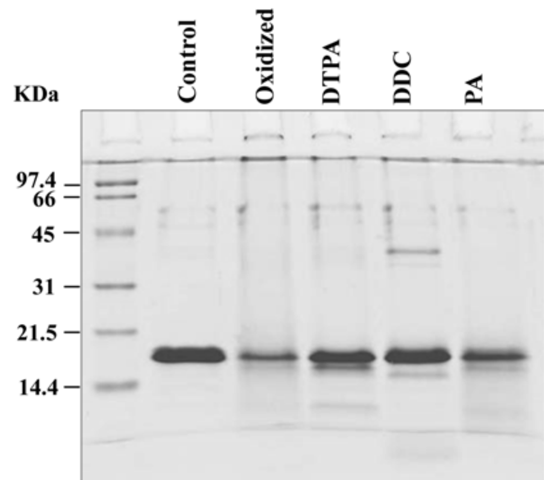

Fig. 6. Effect of copper chelators on the aggregation of Cu,Zn-SOD by salsolinol. Cu,Zn-SOD (0.5 mg/ml) was incubated with 1 mM salsolinol in 10 mM phosphate buffer (pH 7.4) at 37°C for 24 h in the presence of radical scavengers. Lane 1, Cu,Zn-SOD control; lane 2, oxidized Cu,Zn-SOD (without chelator); lane 3, 10 mM DTPA; lane 4, 10 mM DDC; lane 5, 10 mM penicillamine (PA).

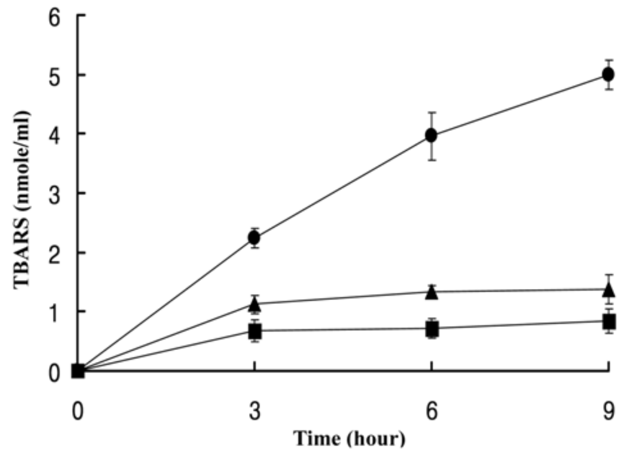

Fig. 7. Generation of hydroxyl radical during the oxidation reaction of salsolinol. The reaction mixtures contained 1 mM salsolinol or Cu,Zn-SOD (0.5 mg/ml) and 10 mM 2-deoxy-D-ribose in 10 mM phosphate buffer at pH 7.4 and the following: salsolinol (▲), Cu,Zn-SOD (■), salsolinol and Cu,Zn-SOD (●). Data represent the means ± S.D. ($n = 3$-5).

thiobarbituric acid-reactive substance (TBARS). Hydroxyl radicals was slightly increased as a function of time after the incubation of 2-deoxy-D-ribose with salsolinol or Cu,Zn-SOD alone (Fig. 7). However, when 2-deoxy-D-ribose was incubated in a mixture of salsolinol and Cu,Zn-SOD, the amount of hydroxyl radical was rapidly increased as a function of time.

Discussion

The present study investigated the potential role of salsolinol in modification of human Cu,Zn-SOD. The toxicity of salsolinol may be augmented by its free radical-generating function in neurodegenerative disorder. Since the level of free radicals was reported to be increase in PD patients (Ilic *et al.*, 1998; 1999), the oxidative modification of Cu,Zn-SOD might have pathological significance. Present result showed that the

aggregation and fragmentation of Cu,Zn-SOD was induced by salsolinol. It has been reported that salsolinol induces DNA strand breaks in PC12 cells and neurons in the presence of copper or iron (Jung and Surh, 2001; Surh *et al*., 2002). It was suggested that salsolinol might induce ROS which led to DNA fragmentation. Recent report has been revealed that salsolinol increased the production of ROS and significantly decreased glutathione levels in SH-SY5Y cells (Wanpen *et al*., 2004). In the present study, free radical scavengers and catalase inhibited the salsolinol-mediated Cu,Zn-SOD modification. Thus, it was suggested that free radicals and hydrogen peroxide might be involved in the modification of Cu,Zn-SOD by salsolinol.

Trace metal such as iron and copper, which are variously present in biological systems, were generated ·OH through Fnton reaction and then led to damages of macromolecules (Sagripanti *et al*., 1987; Imlay *et al*., 1988; Sagripanti and Kraemer, 1989; Halliwell and Gutteridge 1992). The cleavage of the metalloproteins by oxidative damage may lead to increase in the levels of metal ions in some biological cells (O'Connell and Peters, 1987). It has been reported that copper concentration was significantly increased in the cerebrospinal-fluid of PD patients (Pall *et al*., 1987) and that in the cerebrospinal-fluid copper concentration was increased 2.2-fold in Alzherimer's Disease patients (Multhaup *et al*., 1996). These reports suggested that iron or copper-catalyzed oxidative reaction might contribute to the pathogenesis of PD. In the present study, the modification of Cu,Zn-SOD was significantly inhibited by copper chelators. These results suggested that copper ions might be involved in salsolinol-mediated Cu,Zn-SOD modification.

The present results indicate that the inactivation of Cu,Zn-SOD by salsolinol may be closely associated with the modification of Cu,Zn-SOD. X-ray crystallographic studies on Cu,Zn-SOD have shown three histidine side chains (His-46, His-48, His-120) to coordinated to the copper and two histidine (His-74, His-80) and one aspartic acid (Asp-83) residues to the zinc ion (Tainer *et al*., 1983). It has been reported that Cu,Zn-SOD was inactivated by oxidation *in vitro* leading to the loss of one histidine residue (Maria *et al*., 1995). Thus, it was suggested that copper binding sites might be modified during the reaction of Cu,Zn-SOD with salsolinol. Consequently, copper became almost free form the ligand and was released from the oxidatively damaged enzyme, which resulted in the loss of activity.

In conclusion, the present results suggested that the modification of Cu,Zn-SOD was induced by salsolinol, involving ·OH generation from H_2O_2. The salsolinol-mediated Cu,Zn-SOD modification might be associated with the pathogenesis of PD and related disorders.

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