DNA Cleavage Induced by the Reaction of Salsolinol with Cu,Zn-Superoxide Dismutase

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Salsolinol, endogenous neurotoxin, is known to be involved in the pathogenesis of Parkinson's disease (PD). In the present study, we have investigated the oxidative damage of DNA induced by the reaction of salsolinol with Cu,Zn-SOD. When plasmid DNA incubated with salsolinol and Cu,Zn-SOD, DNA cleavage was proportional to the concentrations of salsolinol and Cu,Zn-SOD. The salsolinol/Cu,Zn-SOD system-mediated DNA cleavage was significantly inhibited by radical scavengers such as mannitol, ethanol and thiourea. These results indicated that free radicals might participate in DNA cleavage by the salsolinol/Cu,Zn-SOD system. Spectrophotometric study using a thiobarbituric acid showed that hydroxyl radical formation was proportional to the concentration of salsolinol and was inhibited by radical scavengers. These results indicated that hydroxyl radical generated in the reaction of salsolinol with Cu,Zn-SOD was implicated in the DNA cleavage. Catalase and copper chelators inhibited DNA cleavage and the production of hydroxyl radicals. These results suggest that DNA cleavage is mediated in the reaction of salsolinol with Cu,Zn-SOD via the generation of hydroxyl radical by a combination of the oxidation reaction of salsolinol and Fenton-like reaction of free copper ions released from oxidatively damaged SOD.

Key Words : Salsolinol, Copper,zinc-superoxide dismutase, DNA cleavage

Introduction

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydro-isoquinoline, Figure 1) is an endogenous catechol-derived neurotoxin supposedly related to the development of Parkinsons disease (PD).[1-4] Several studies indicated that salsolinol is toxic to dopaminergic neurons *in vitro* as well as *in vivo*. Salsolinol increased the production of reactive oxygen species (ROS) and significantly decreased glutathione levels in SH-SY5Y cells.[5,6] Among several causative factors, oxidative stress is known to be a major contributing factor to the biological cascade leading to dopamine cell degeneration in PD.[7-9] Furthermore, oxidative damage of biological macromolecules has been proposed to be a critical factor of the pathogenesis of various neurodegenerative disorders.[10-12]

A group of metalloproteins known as Cu,Zn-superoxide dismutase (SOD) catalyzes the dismutation of two super-oxide anions into one oxygen and one hydrogen peroxide and thus is involved in protecting the cell from oxygen toxicity. 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.[13,14] Previous reports have shown that several oxidants lead to Cu,Zn-SOD fragmentation and the release of copper ions from the enzyme.[15-17] Trace metal such as iron and copper, which are variously present in biological systems, may interact with ROS, ionizing radiation, or microwave radiation to damage macromolecules.[18-23] ROS may play an important role in several pathological conditions of the central nervous system, where the directly injure tissue. ROS produce tissue damage through multiple mechanisms and can worsen acute neurodegenerative disorders including PD. 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 scission and cell death.[5,6] Recently, we have shown that salsolinol led to the fragmentation and aggregation of Cu,Zn-SOD.[24] In this context, we hypothesize that Cu,Zn-SOD could be a source of copper and oxidative stress that might trigger the oxidative damage of DNA induced by salsolinol. Although salsolinol-dependent DNA damage has been known previously, the oxidative damage of DNA by the reaction of salsolinol with Cu,Zn-SOD has not been reported.

In this study, we examined the DNA cleavage caused by salsolinol and Cu,Zn-SOD. Our results indicate that salsolinol in the presence of Cu,Zn-SOD can cleave DNA through a mechanism that involves hydroxyl radical.

Materials and Methods

Materials. Recombinant human Cu,Zn-SOD was over-produced and purified as described previously.[25] pUC19 plasmid DNA was prepared and purified from *E. coli* cul-

Figure 1. Chemical structure of salsolinol.

2330   *Bull. Korean Chem. Soc.* **2007**, Vol. 28, No. 12

Jung Hoon Kang

tures by using QIAGEN plasmid kit (Santa Clarita, USA). Catalase, thiourea, diethyldithiocarbamic acid (DDC), and thiobarbituric acid 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.

Analysis of DNA cleavage. Supercoiled plasmid pUC19 DNA (0.5-1.0 μg) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 2 h at 37°C with different concentrations of salsolinol and Cu,Zn-SOD in a total volume of 20 μL. The reaction was stopped at −80 °C. The loading buffer (0.25% bromophenolblue, 40% sucrose) was added and samples analyzed by electrophoresis in 0.8% agarose in TBE buffer (2 mM EDTA, 89 mM boric acid and 89 mM Tris at pH 8.3). The gel was stained with ethidium bromide. Bands of DNA were detected and photographed under UV light in a dark room.

Measurement of hydroxyl radical. Detection of hydroxyl radicals was determined by measuring thiobarbituric acid reactive substance (TBARS) by a modification of the method previously described.[25] An assay mixture contained 10 mM potassium phosphate buffer (pH 7.4), 10 mM 2-deoxy-D-ribose, various concentrations of Cu,Zn-SOD and salsolinol in a total volume of 100 μL. Reaction mixtures were incubated at 37 °C for 12 h. The degradation of 2-deoxy-D-ribose was measured by addition of 2.8% trichloroacetic acid (200 μL), PBS (200 μL), and 1% thiobarbituric acid (200 μL), followed by heating at 100 °C for 15 min. After the samples were cooled to room temperature and centrifuged at 15,000 rpm for 10 min. Results were read by a uv/vis spectrophotometer (Shimadzu, UV-1601) at 532 nm. All solutions used in the present experiments were treated with Chelex 100.

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

Results and Discussion

When DNA was incubated in a mixture of salsolinol and Cu,Zn-SOD, DNA cleavage became apparent at 0.1 mM salsolinol and 1 μM Cu,Zn-SOD; the cleavage increased up to 1 mM salsolinol and 15 μM Cu,Zn-SOD (Fig. 2). Plasmid DNA remained intact after incubation with 15 μM Cu,Zn-SOD or 1 mM salsolinol alone (data not shown). The participation of free radicals in the DNA damage by the salsolinol/Cu,Zn-SOD system was studied by examining the protective effect of radical scavengers. When plasmid DNA was incubated with salsolinol and Cu,Zn-SOD in the presence of mannitol, ethanol and thiourea at 37 °C for 2 h, all scavengers significantly prevented DNA cleavage (Fig. 3, lane 3-5). The ability of radical scavengers to protect DNA from damage indicates that the free radicals participate in the mechanism of DNA strand break produced by salsolinol and Cu,Zn-SOD.

Cellular metabolism has been shown to generate oxygen species such as hydrogen peroxide, hydroxyl radical, and
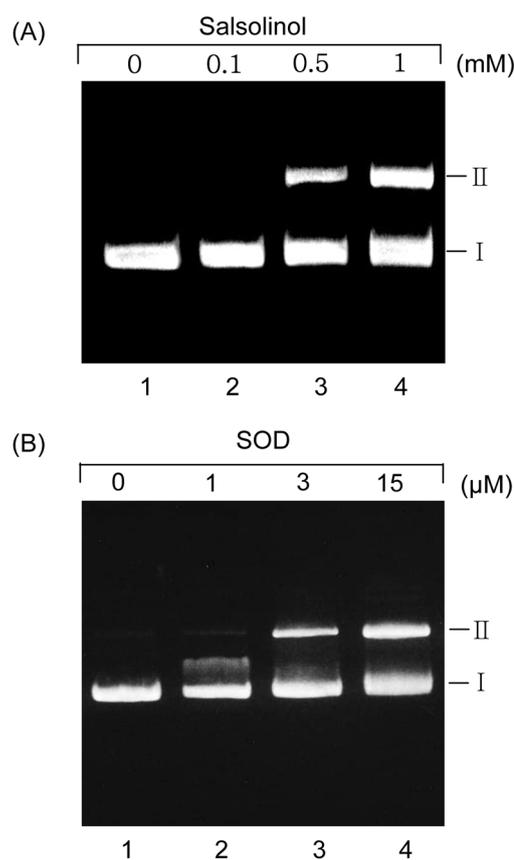

Figure 2. DNA cleavage after incubation with Cu,Zn-SOD and salsolinol. pUC 19 DNA (2 μg) was incubated with various concentrations of Cu,Zn-SOD and salsolinol in 10 mM potassium phosphate buffer (pH 7.4) at 37 °C for 2 h. (A) Plasmid DNA was incubated with 0.1-1 mM salsolinol, 15 μM Cu,Zn-SOD. Lane 1, no addition; lane 2, 0.1 mM; lane 3, 0.5 mM; lane 4, 1 mM salsolinol. (B) Plasmid DNA was incubated with 1 mM salsolinol, 1-15 μM Cu,Zn-SOD. Lane 1, no addition; lane 2, 1 μM; lane 3, 3 μM; lane 4, 15 μM Cu,Zn-SOD. Reaction was stopped at −80 °C, loading buffer was added, and the sample analyzed by electrophoresis in 0.8% agarose. I and II indicate the position of the supercoiled and circular DNA plasmid forms, respectively.

superoxide radical.[26] Trace metals such as copper and iron which are present in biological systems may interact with hydrogen peroxide, to damage biological molecules including DNA.[18-23] Mixtures of copper ions and H_2O_2 have been shown to produce extensive strand breakage in DNA.[22,27] Strand breakage often occurs near guanine residues, and it has been suggested that copper ions bind to DNA at these sites.[22] Several authors have suggested that copper ions could stimulate the Fenton-like reaction to produce hydroxyl radicals, which mediates DNA strand breakage.[29-31] We have investigated the participation of copper ions in the salsolinol/Cu,Zn-SOD-mediated DNA strand breaks. When DNA was incubated with salsolinol and Cu,Zn-SOD in the presence of copper specific chelator, DDC, DNA cleavage was significantly inhibited (Fig. 3, lane 6). The result indicated that copper ions was involved in the DNA cleavage by the salsolinol/Cu,Zn-SOD system.

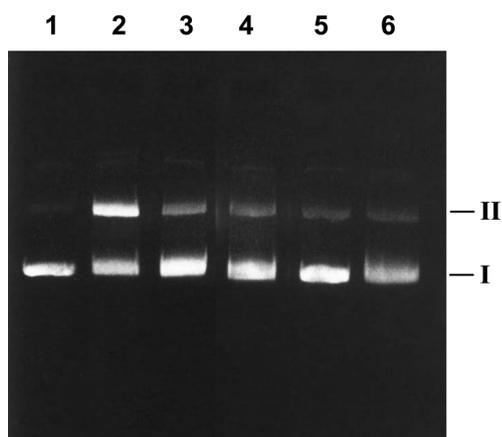Autoxidation of a neurotoxin, 6-hydroxydopamine, leads

Figure 3. Effect of radical scavengers and copper chelators on the DNA cleavage by the reaction of Cu,Zn-SOD with salsolinol. pUC 19 DNA was incubated with Cu,Zn-SOD (15 μM) and 1 mM salsolinol in potassium phosphate buffer (pH 7.4) at 37 °C for 2 h, in the absence and the presence of 100 mM scavengers or 5 mM copper chelator at 37 °C. Lane 1, plasmid alone; lane 2, no addition of scavenger; lane 3, mannitol; lane 4, ethanol; lane 5, thiourea; lane 6, DDC. Agarose gel electrophoresis was performed in 0.8% agarose. I and II indicate the position of the supercoiled and circular DNA plasmid forms, respectively.

to produce hydrogen peroxide and hydroxyl radicals.[32] Hydroxyl radical is the most powerful oxidant and is able to damage macromolecules.[20,22] Recently, we reported that the fragmentation of Cu,Zn-SOD was induced by salsolinol and might lead to the releasing of copper ions from the protein.[24] The previous results have been suggested that the copper releasing of Cu,Zn-SOD by salsolinol might 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.[33] 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. It has been reported that the induction of DNA damage by salsolinol and copper ion might be due to the generations of hydrogen peroxide and hydroxyl radicals.[5] Therefore, the present results suggested that early oxidation of salsolinol is participated in the releasing of copper ions from Cu,Zn-SOD and the generation of hydrogen peroxide. The subsequent interaction of free copper ions and hydrogen peroxide in the Fenton-like reaction will yield hydroxyl radical. Evidence that catalase protected the DNA cleavage induced by the salsolinol/Cu,Zn-SOD system supports this mechanism (Fig. 4).

The generation of hydroxyl radicals in the salsolinol/Cu,Zn-SOD system was measured with thiobarbituric acid-reactive substance (TBARS). The level of hydroxyl radicals were increased in a salsolinol concentration-dependent manner (Fig. 5). Radical scavengers inhibited the formation of hydroxyl radicals in salsolinol/Cu,Zn-SOD system (Table
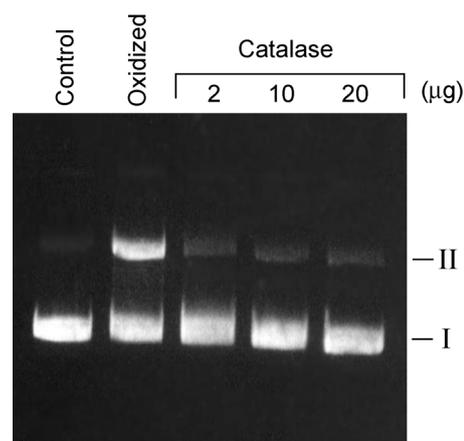

Figure 4. Effect of catalase on the DNA cleavage by the reaction of Cu,Zn-SOD with salsolinol. pUC 19 DNA was incubated with Cu,Zn-SOD (15 μM) and 1 mM salsolinol potassium phosphate buffer (pH 7.4) at 37 °C for 2 h in various concentrations of catalase. Lane 1, plasmid alone; lane 2, no addition of catalase; lane 3, 2 μg catalase; lane 3, 10 μg catalase; lane 4, 20 μg catalase. Agarose gel electrophoresis was performed in 0.8% agarose. I and II indicate the position of the supercoiled and circular DNA plasmid forms, respectively.
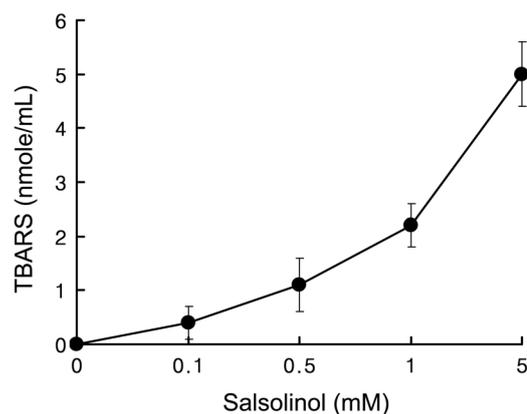

Figure 5. Generation of hydroxyl radical during the reaction of Cu,Zn-SOD with salsolinol. The reaction mixtures contained 10 mM 2-deoxy-D-ribose, 15 μM Cu,Zn-SDOD and various concentration of salsolinol in 10 mM phosphate buffer at pH 7.4 for 12 h. The degradation of 2-deoxy-D-ribose was measured by 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. Data represent the means ± S.D. (n = 4-5).

1). The result supports our finding that free radicals may be involved in salsolinol/Cu,Zn-SOD-mediated DNA cleavage. It has been reported that copper concentration was significantly increased in the cerebrospinal-fluid of Parkinson's disease and Alzheimer's disease.[34,35] These results suggested that copper-catalyzed oxidative reaction might contribute to the pathogenesis of neurodegenerative disorders. Copper chelator significantly inhibited the generation of hydroxyl radicals by the salsolinol/Cu,Zn-SOD system (Table 1). The result suggests that redox reactions of copper may facilitate the generation of hydroxyl radical.

Table 1. Effect of radical scavengers and copper chelator on the formation of hydroxyl radicals by salsolinol and Cu,Zn-SOD

Scavenger/chelator (mM)	TBARS (nmole/mL)	nhibition of formation of TBARS (%)
No addition	2.18 ± 0.2	
Mannitol (100)	0.32 ± 0.1	85 ± 4.6
Ethanol (100)	1.20 ± 0.2	52 ± 9.2
Thiourea (20)	0.36 ± 0.1	84 ± 5.1
DDC (5)	0.56 ± 0.1	74 ± 5.9

Reaction mixture containing 15 μM Cu,Zn-SOD, 1 mM salsolinol in 10 mM potassium phosphate buffer at pH 7.4, in the presence of each scavenger and copper chelator.

In conclusion, the present results indicated that DNA cleavage was induced by the reaction of salsolinol/Cu,Zn-SOD involving hydroxyl radical generation from hydrogen peroxide. The hydroxyl radicals were generated by a combination of the autoxidation of salsolinol and the Fenton-like reaction of free copper ions released from oxidatively damaged SOD. It would be expected that the cellular radical detoxification system, including SOD, would be capable of handling increased radical levels. Interestingly, SOD activity is increased in discrete brain regions in different neurodegenerative disorders; in PD, it is characteristically increased in the subtantia nigra.[36,37] Therefore, DNA damage by the salsolinol/Cu,Zn-SOD system could be relatively favored in diseases where Cu,Zn-SOD concentration is elevated such as PD and other neurodegenerative disorders.

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