

Notes

Carnosine and *N*-Acetyl-carnosine Inhibit Salsolinol/Cu,Zn-superoxide Dismutase-mediated DNA Cleavage

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Salsolinol is an endogenous tetrahydroisoquinoline derivative, which is known to be involved in the pathogenesis of Parkinson disease (PD).¹⁻⁴ Salsolinol was detected in urine of PD patients administered with L-DOPA.⁵ The properties of salsolinol, as a neurotoxin, are intensively studied. Salsolinol and/or its methylated derivatives have been suggested to act as endogenous dopaminergic neurotoxins, inducing selective neuronal cell death and eliciting symptoms almost identical to idiopathic Parkinson disease.⁶ It has been reported that salsolinol in conjugation with cupric ion or ferric ion undergoes redox cycling to produce reactive oxygen species (ROS) such as hydroxyl radicals that cause DNA strand scission and cell death.⁷ ROS can lead to damage of macromolecules in cells, an event which is implicated in the development of many human disease and aging. To protect against such damages, organisms have developed a variety of antioxidant defenses including, such as superoxide dismutase (SOD). SOD is an enzyme catalyzing the disproportion of superoxide radicals to dioxygen and hydrogen peroxide. Recently, it was reported that salsolinol induced the fragmentation of Cu,Zn-SOD.⁹ This phenomenon might be due to the generation of free radicals by autoxidation of salsolinol. Although Cu,Zn-SOD has great physiological significance and therapeutic potential, this enzyme could be a source of copper and oxidative stress that may trigger the oxidative damage of macromolecules.

Carnosine (β -alanyl-L-histidine) and the derivative *N*-acetyl-carnosine (Fig. 1) perform important biological functions: in particular, it exhibits antioxidant properties directed at suppression of free radical reactions.^{10,11} Carnosine can delay senescence and extend the life-span of cultured human

fibroblasts, kill transformed cells, and protect cells against aldehydes and an amyloid peptide fragment.^{12,13} The imidazolium group of histidine or carnosine stabilizes adducts formed at the primary amino group and may play an important role for an anti-crosslinking agent.¹⁴ Many biochemical studies have suggested that carnosine possesses antioxidant and free radical-scavenging function which may partly explain its apparent homeostatic function.^{15,16} However, the inhibitory action of carnosine and related compounds against salsolinol-mediated DNA damage has not been reported.

In the present study, it was found that the salsolinol/Cu,Zn-SOD system led to DNA strand breakage. The results suggested that Cu,Zn-SOD as a source of copper, might be related to the oxidative damage of DNA. Carnosine and *N*-acetyl-carnosine inhibited the salsolinol/Cu,Zn-SOD system-mediated DNA strand breakage. These compounds also inhibited the generation of hydroxyl radicals induced by the salsolinol/Cu,Zn-SOD system. The results suggest that carnosine and *N*-acetyl-carnosine inhibited the salsolinol/Cu,Zn-SOD system-mediated DNA strand breakage through the scavenging of hydroxyl radicals.

Experimental Section

Materials. Recombinant human Cu,Zn-SOD was over-produced and purified as described previously.³⁴ pUC19 plasmid DNA was prepared and purified from *E. coli* cultures by using QIAGEN plasmid kit (Santa Clarita, USA). Carnosine and thiobarbituric acid were purchased from Sigma. *N*-Acetyl-carnosine was synthesized by Pepton Inc (Daejeon, Korea). 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 (1 μ g) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 2 h at 37 °C with 1 mM salsolinol and 15 μ M Cu,Zn-SOD in a total volume of 20 μ L. The reactions were stopped by freezing at -80 °C. 4 μ L of 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

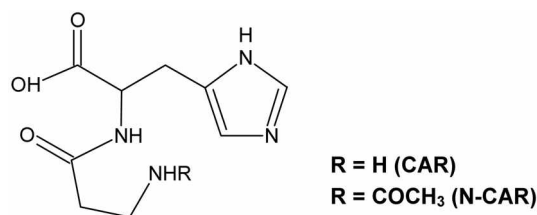


Figure 1. Structures of carnosine and related dipeptide: carnosine (CAR), *N*-acetyl-carnosine (N-CAR).

Tris at pH 8.3).³¹ The gel was stained with ethidium bromide. Bands of DNA was 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 2-deoxy-D-ribose oxidation products.¹⁷ The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4), 10 mM 2-deoxy-D-ribose, 1 mM salsolinol and 15 μ M Cu,Zn-SOD in a total volume of 100 μ L. Reaction mixtures were incubated at 37 $^{\circ}$ C for 12 h. Reaction was stopped by addition of 2.8% trichloroacetic acid (200 μ L), PBS (200 μ L), and 1% thiobarbituric acid (200 μ L), and boiled at 100 $^{\circ}$ C for 15 min. After the samples were cooled and centrifuged at 15,000 rpm for 10 min. Results were read by a uv/vis spectrophotometer (Shimadzu, UV-1601) at 532 nm.

Mutagenicity assay. Plasmid DNA was transformed in *Escherichia coli* DH5 α competent cells. The treated plasmid pUC19 carrying the *lacZ* gene with or without 1 mM salsolinol and 15 μ M Cu,Zn-SOD was incubated with 50 μ L of competent cells at 0 $^{\circ}$ C for 10 min. SOC medium was added, and the cells were incubated at 37 $^{\circ}$ C for 1 h. Transformed cells were spread on LB agar plates containing 0.8 mg of X-gal, 2 mg of IPTG and 0.01% ampicillin. The plates were inverted and incubated for 24 h at 37 $^{\circ}$ C before counting colonies.

Results and Discussion

Previous studies have been shown that ROS induces DNA strand breakage and site-specific modification at guanosine.³⁵⁻³⁷ Strand breakage can be measured ROS by gel electrophoresis method using supercoiled plasmid DNA. Strand breakage causes 'relaxation' to open circle and linear forms, measured as different migration on agarose gel. As

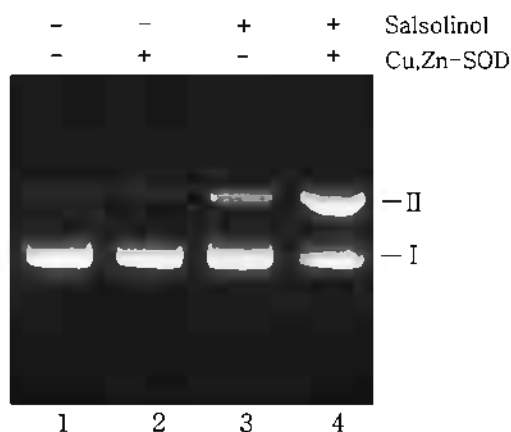


Figure 2. DNA strand breakage after the incubation with salsolinol and Cu,Zn-SOD. pUC 19 DNA (1 μ g) was incubated at 37 $^{\circ}$ C for 2 h with following: Lane 1, control DNA; lane 2, 15 μ M Cu,Zn-SOD; lane 3, 1 mM salsolinol; lane 4, 1 mM salsolinol and 15 μ M Cu,Zn-SOD. Reaction was stopped by freezing at -80 $^{\circ}$ C. Loading buffer was added to the samples and analyzed by electrophoresis on 0.8% agarose gel. I and II indicate the positions of the supercoiled and nicked circular DNA plasmid forms, respectively.

shown in Figure 2, the plasmid DNA remained intact after incubation with 15 μ M Cu,Zn-SOD alone, whereas DNA was slightly cleaved by 1 mM salsolinol. The non-enzymatic oxidation of *N*-methyl salsolinol results in the formation of hydroxyl radical.¹⁸ The present result indicates that hydroxyl radicals may be involved in DNA strand breakage by salsolinol. When DNA was incubated in a mixture of salsolinol and Cu,Zn-SOD, DNA was significantly cleaved (Fig. 2, lane 4). It has been reported that salsolinol induces DNA strand breakage in PC12 cells and neurons in the presence of copper or iron.^{7,8,15} Previous study suggested that autoxidation of salsolinol was anticipated to be mediated through reduction of Cu(II) to Cu(I) with concomitant generation of hydrogen peroxide.²⁰ Therefore, our results suggested that early oxidation of salsolinol led to the releasing of copper ions from oxidatively damaged Cu,Zn-SOD and the generation of hydrogen peroxide. The subsequent reaction of free copper ions with hydrogen peroxide will yield hydroxyl radical.

It has been shown that several types of damages induced in DNA by free radicals are mutagenic.²⁷ The biological consequences of DNA damage induced by Cu,Zn-SOD/salsolinol was investigated. pUC19 plasmid DNA was treated with or without Cu,Zn-SOD and salsolinol. *E. coli* (DH5 α) competent cells were transformed with treated DNA. *E. coli* encodes a β -galactosidase that hydrolyzes the lactose into glucose and galactose. The activity of the enzyme can be assayed with a chromogenic substrate such as X-gal, which is converted by β -galactosidase into an insoluble dense blue compound.²⁸ *E. coli* that carry active β -galactosidase produce dense blue colonies, whereas mutation within the *lacZ* β segment of pUC19 plasmid DNA resulted in the expression of much less active β -galactosidase and yields light blue or white colonies. The result showed that Cu,Zn-SOD enhanced the mutagenicity of salsolinol by about two fold (Table 1). Cu,Zn-SOD alone did not cause any increase of mutagenicity. The toxicity and mutagenicity of catechols *in vitro* are documented and the possible role of Fenton chemistry was implicated.^{29,30} Our result suggested that the reactivity of salsolinol may be accelerated by the increasing level of transition metals available for Fenton-type reactions.

Carnosine has a protective effect in many diseases such as Alzheimer disease (AD),³² because of its abundant functions

Table 1. Mutagenicity of salsolinol/Cu,Zn-SOD treated pUC19 plasmid in *E. coli*

Treatment	Relative mutation frequency (fold) ^a
pUC19	1.0
pUC19 + salsolinol	1.9 \pm 0.4
pUC19 + Cu,Zn-SOD	1.4 \pm 0.3
pUC19 + salsolinol/Cu,Zn-SOD	4.3 \pm 0.6

^aMutation frequency was calculated as the ratio between the mutant colonies and total colonies. The results are expressed as a relative mutation frequency and represent the mean \pm S.D. from triplicate measurements.

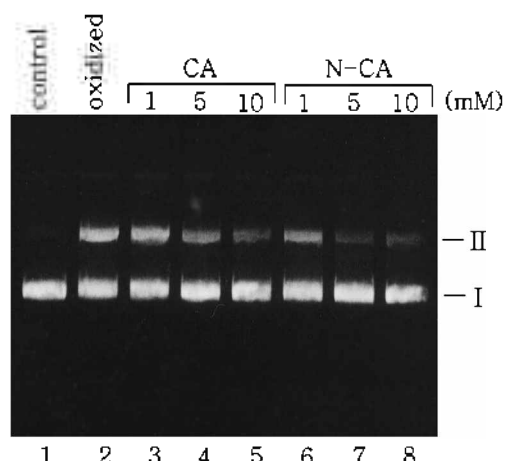


Figure 3. Effects of carnosine and *N*-acetyl-carnosine on DNA strand breakage induced by the salsolinol and Cu,Zn-SOD system. pUC 19 DNA was incubated with 15 μM Cu,Zn-SOD + 1 mM salsolinol in the presence of various concentrations of carnosine and *N*-acetyl-carnosine at 37 for 2 h. Lane 1, control DNA; lane 2, salsolinol + Cu,Zn-SOD; lane 3, lane 2 + 1 mM carnosine; lane 4, lane 2 + 5 mM carnosine; lane 5, lane 2 + 10 mM carnosine; lane 6, lane 2 + 1 mM *N*-acetyl-carnosine; lane 7, lane 2 + 5 mM *N*-acetyl-carnosine; lane 8, lane 2 + 10 mM *N*-acetyl-carnosine.

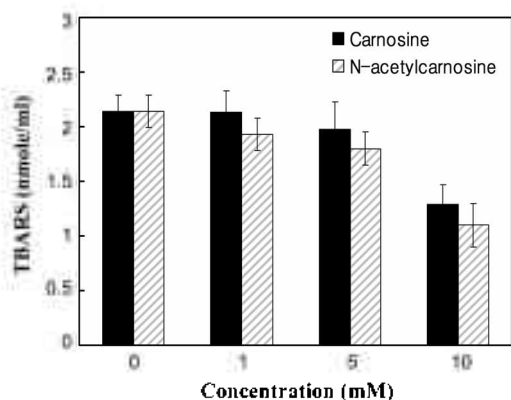


Figure 4. Effects of carnosine and *N*-acetyl-carnosine on hydroxyl radical formation induced by the salsolinol and Cu,Zn-SOD system. The reaction mixtures contained 10 mM 2-deoxy-D-ribose, 15 μM Cu,Zn-SOD and 1 mM salsolinol in the presence of carnosine and *N*-acetyl-carnosine at pH 7.4 for 12 h.

as a free radical scavenger, physiological buffer, neurotransmitter, radioprotectant, metal chelator, wound healing agent, anti-oxidant, anti-glycation and anti-aging.^{10,21-23,33} Therefore, it is of interest to know how carnosine works in the protection against disease. In the present study, it was found that carnosine and *N*-acetyl-carnosine significantly inhibited DNA strand breakage induced by the salsolinol/Cu,Zn-SOD system (Fig. 3). It has been reported that carnosine and related compounds quench 50-95% of hydroxyl radicals produced in the Fenton reaction.²⁴ Therefore, the result suggested that protection of DNA might be due to the scavenging of hydroxyl radical. When salsolinol was incubated with Cu,Zn-SOD in the presence of carnosine and *N*-acetyl-carnosine, both compounds effectively inhibited the formation of hydroxyl radicals (Fig. 4). The enhancing effect

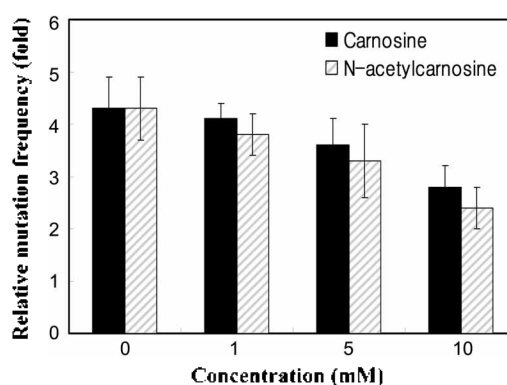


Figure 5. Effects of carnosine and *N*-acetyl-carnosine on the mutagenicity by the salsolinol and Cu,Zn-SOD system. The treated plasmid pUC19 carrying the *lacZ'* gene with 1 mM salsolinol and 15 μM Cu,Zn-SOD was incubated with 50 μL of competent cells in the presence of carnosine and *N*-acetyl-carnosine. Transformed cells were spread on LB agar plates containing 0.8 mg of X-gal, 2 mg of IPTG and 0.01% ampicillin.

of Cu,Zn-SOD on the mutagenicity of salsolinol in DNA was inhibited by carnosine and *N*-acetyl-carnosine (Fig. 5). One of the mechanisms by which antioxidants can protect their biological targets from oxidative stress is the chelation of transition metals such as copper and iron, preventing them from participating in the deleterious Fenton reaction. Carnosine and related compounds have been shown to be very efficient copper chelating agents.²¹ These compounds might be able to bind Cu²⁺ and prevent some Cu²⁺-dependent radical reaction.¹¹ Therefore, it can be assumed that carnosine and *N*-acetyl-carnosine may prevent the salsolinol/Cu,Zn-SOD-mediated DNA cleavage through a combination of scavenging of hydroxyl radicals and the chelation of copper ion. The *N*-acetyl derivatives of histidine and carnosine exist in the cardiac and skeletal mammalian muscles and the total concentration of these imidazoles may lie within the measured range of that of L-carnosine in skeletal muscle (about 10 mM).²⁵ Among several dipeptides of the carnosine family tested as potential substrates for the purified human serum carnosinase, *N*-acetyl-carnosine and few other compounds were not hydrolyzed²⁵ thus anticipating a prolongation of physiological responses to the therapeutical treatments.

From the present results, we suggest that carnosine and related compounds may be explored as potential therapeutic agents for pathogenesis that involve the oxidative damage of DNA mediated by neurotoxin, salsolinol.

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