Protective Effects of Carnosine and *N*-Acetylcarnosine on Salsolinol-mediated Cu,Zn-superoxide Dismutase Inactivation

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The oxidation of cellular proteins is implicated in the development of many human disease and aging. The vulnerability of various amino acid residues of protein to oxidation varies with reactive oxygen species (ROS) used. A group of metalloproteins known as Cu,Zn-superoxide dismutase (SOD) catalyzes the dismutation of two superoxide anions into one oxygen and one hydrogen peroxide and is thus involved in protecting the cell from oxygen toxicity. Previous reports showed that the reaction of Cu,Zn-SOD with H_2O_2 resulted in the oxidation of histidine [1] and the generation of protein fragmentation.[2] However, oxidation of Cu,Zn-SOD by the thiol/Fe(III)/O_2 mixed-function oxidation system did not lead to the oxidation of histidine.[3] The modification of Cu,Zn-SOD by the lipid peroxidation product, malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), lead to the exclusive modification of histidine residues and the generation of protein-protein cross-linked derivatives.[4] Finally the modification of proteins by glycation led to the modification of lysine.[5] It is therefore evident that each form of ROS elicits a different pattern of protein oxidation.

Salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) an endogenous neurotoxin, is known to be involved in the pathogenesis of Parkinsons disease (PD).[6] Salsolinol has been detected in cerebrospinal fluid of PD patients.[7] Salsolinol was also found in urine of PD patients administered with L-DOPA.[8] The properties of salsolinol, as a neurotoxin, are intensively studied. Salsolinol has a molecular structure similar to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and also to 6-hydroxy dopamine, which are known to induce loss of catecholaminergic cells. Salsolinol and its methylated dervatives have been suggested to act as endogenous dopaminergic neurotoxins, inducing selective neuronal cell death and eliciting symptoms almost identical to idiopathic Parkinson's disease.[9] It has been reported that salsolinol in conjugation with cupric ion or ferric ion undergoes redox cycling to produce ROS such as hydroxyl radicals that cause DNA strand scission and cell death.[10,11] Although neurotoxicologic effects of salsolinol have been extensively investigated, little is known about the modification of proteins mediated by salsolinol.

Carnosine (β-alanyl-L-histidine) is a potent antioxidant, antiglycating, pH-buffer and metal chelating agent.[12-14] Carnosine is accumulated in excitible tissues (brain, heart and skeletal muscles) of vertebrates in large amounts (up to 20 mM in humans).[12] The *N*-acetyl derivatives of histidine and carnosine exist in brain and heart muscle.[15] These compounds also demonstrate antioxidant, proton buffering or metal chelating abilities[16,17] but their biological function has not been clarified. In the present study, we investigated the protective effects of carnosine and *N*-acetylcarnosine on the inactivation of Cu,Zn-SOD by salsolinol. We showed that exposure of Cu,Zn-SOD to salsolinol led to the protein fragmentation and enzyme inactivation. Carnosine and *N*-acetylcarnosine effectively inhibited the fragmentation and inactivation of salsolinol-treated Cu,Zn-SOD.

Experimental Section

Materials. Salsolinol and carnosine were purchased from Sigma (St. Louis, USA). *N*-Acetylcarnosine was synthesized by Peptron Inc (Daejeon, Korea). Chelex 100 resin (sodium form) and acrylamide were obtained from Bio-Rad (Hercules, USA). Recombinant human Cu,Zn-SOD was prepared as described previously.[18] All solutions used in the present experiments were treated with Chelex 100.

Protein oxidation. Protein concentration was determined by the bicinchonic acid method.[19] Oxidation 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 (control) of salsolinol at 37 °C. After the incubation of the reaction mixtures, the mixtures were then placed into Ultrafree-MC filter 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 drier and dissolved with phosphate buffer. 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.[20]

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After treatment with 1 mM salsolinol for various periods of time, samples of the reaction mixtures were diluted with a concentrated sample buffer (0.25 mM Tris, 8% SDS, 40% glycerol, 20% β-mercaptoethanol, 0.01% bromophenol blue) and then heated at 100 °C for 5 min. An aliquot of each sample was subjected to SDS-PAGE as described by Laemmli,[21] using a 15% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Results and Discussion

Incubation of Cu,Zn-SOD with salsolinol at 37 °C resulted in a time-dependent decrease in the total protein concentration, which showed a gradual decreased in the intensity of the original band on SDS-PAGE (Figure 1A). When Cu,Zn-SOD was incubated with 1 mM salsolinol, the level of Cu,Zn-SOD activity decreased in a time-dependent manner and was accompanied by gradual loss of protein intensity (Figure 1B). The results indicate that the inactivation of Cu,Zn-SOD by salsolinol is associated with the protein fragmentation. Salsolinol was involved in the pathological processes of Parkinson's disease.[22,23] Several studies indicated that salsolinol was toxic to dopaminergic neurons *in vitro* and *in vivo*. Salsolinol is known to inhibit tyrosine hydroxylase and monoamine oxidase[24] as well as mitochondrial complex-I and complex-II enzyme activities.[25] The present study was undertaken to determine the pattern of oxidative damage and inactivation of human Cu,Zn-SOD by salsolinol in the aerobic condition. It is clear from the evidence presented here the fragmentation and inactivation of human Cu,Zn-SOD were induced by salsolinol.

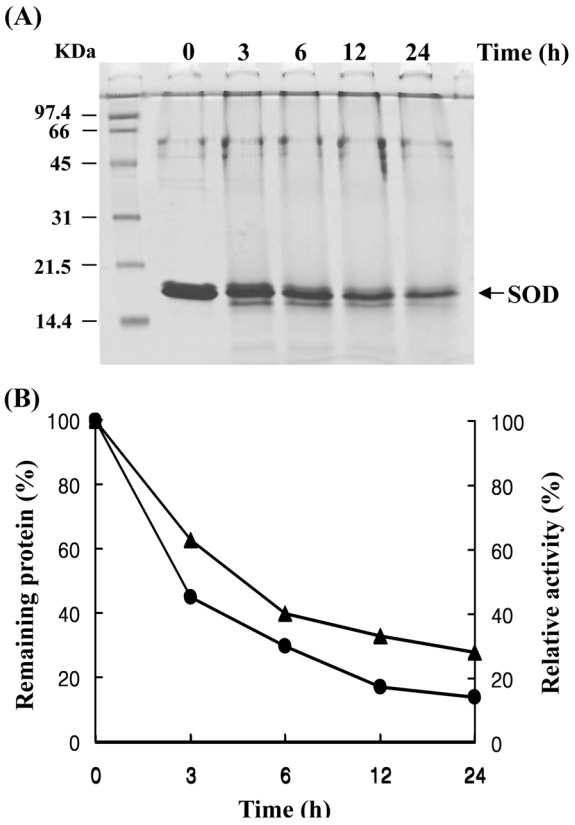

Figure 1. Fragmentation and inactivation of Cu,Zn-SOD during the incubation with salsolinol. Cu,Zn-SOD (0.5 mg/mL) was incubated with 1 mM salsolinol in 10 mM potassium phosphate buffer (pH 7.4) at 37 °C for various incubation periods and reactions were stopped at the time indicated by freezing at −80 °C. (A) An aliquot was analyzed by SDS-PAGE. (B) Relative staining density of protein band was analyzed by densitometric scanning (▲) and relative activity of Cu,Zn-SOD in the presence of salsolinol (●).

Carnosine and related compounds are reported to be the active free radical scavenger.[26-28] Carnosine is active electrochemically as a reducing agent in cyclic voltammetric measurements, donating a hydrogen atom to the peroxyl radical.[14] It has been reported that carnosine and related compounds quenched 50-95% of hydroxyl radicals produced in the Fenton reaction.[29] We investigated whether carnosine and *N*-acetylcarnosine could protect Cu,Zn-SOD against oxidative damage induced by salsolinol. These compounds showed a concentration-dependent inhibition of the Cu,Zn-SOD fragmentation induced by salsolinol (Figure 2). Carnosine and *N*-acetylcarnosine also protect the Cu,Zn-SOD activity against oxidative stress by salsolinol (Figure 3). The activity was remained 62 and 78% of native enzyme in the presence of 20 mM carnosine and 20 mM *N*-acetylcarnosine, respectively. The inactivation of Cu,Zn-SOD by salsolinol may be closely associated with the loss of histidine residues because this amino acid residue is essential for Cu,Zn-SOD activity.[30-32] Cu,Zn-SOD contains a binuclear cluster, with the active copper and zinc bridged by a

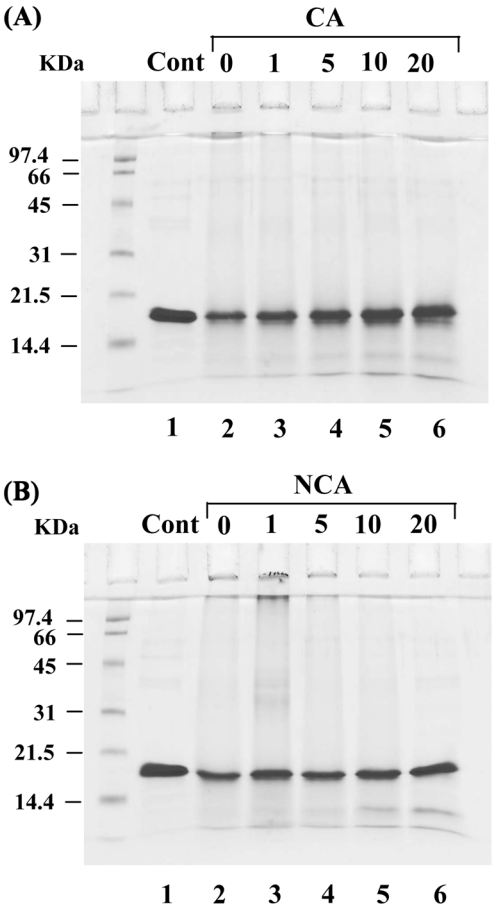

Figure 2. Effects of carnosine and *N*-acetylcarnosine on the fragmentation of Cu,Zn-SOD by salsolinol. Control reaction mixture containing Cu,Zn-SOD (0.5 mg/mL) and 1 mM salsolinol in 10 mM potassium phosphate buffer (pH 7.4). Various concentrations of carnosine (A) and *N*-acetylcarnosine (B) were added into the control reaction mixture. Reaction mixtures were incubated at 37 °C for 24 h and an aliquot was analyzed by SDS-PAGE.

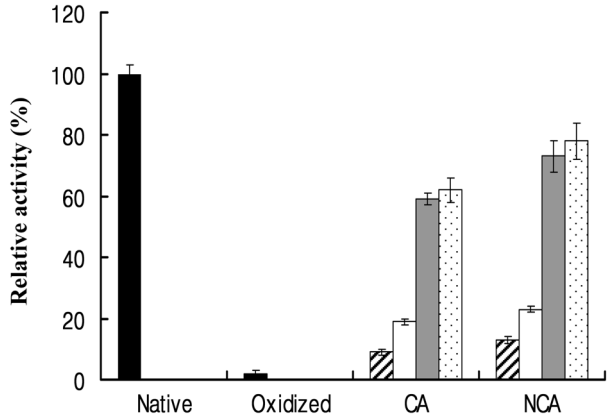

Figure 3. Protective effects of carnosine and *N*-acetylcarnosine on the loss of Cu,Zn-SOD activity during incubation with salsolinol. Cu,Zn-SOD was incubated with 1 mM salsolinol in the presence of reagents as described below at 37 °C for 24 h and an aliquot was analyzed by cytochrome *c* reduction as described under Section 2. Native, Cu,Zn-SOD control; Oxidized, incubated with 1 mM salsolinol; CA, Oxidized plus 1 mM (stripe bar), 5 mM (white bar), 10 mM (gray bar) and 20 mM (dot bar) carnosine; NCA, Oxidized plus 1 mM, (stripe bar), 5 mM (white bar), 10 mM (gray bar) and 20 mM (dot bar) *N*-acetylcarnosine.

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.[33] Thus, it is suggested that copper binding sites were 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. It was reported that Lys-120 and Lys-134 in the *Xenopus laevis* Cu,Zn-SOD[34] and Lys-136 in the bovine erythrocyte Cu,Zn-SOD[35] might play a major role in steering the superoxide anion toward the catalytic copper ion. Carnosine has been shown to delay senescence[36] and decreases production of oxidized DNA[37] in cultured human fibroblasts. It may not be coincidental that carnosine's structure strongly resembles lysine and histidine, the two most readily oxidizable amino acid residues during the protein aging.[31] Hence, it is possible that carnosine could behave as a sacrificial site for the attack by ROS, thus sparing polypeptide amino and imidazole groups.[38]

Carnosine and related compounds have been reported that have biological functions including pH buffering, antioxidant and antiglycation.[39] *In vitro* experiments, carnosine protects brain neurons[40] and non-neuronal cells[41] against oxidative stress. The *N*-acetyl derivatives of carnosine also demonstrate antioxidant and pH buffering.[16,17] The level of carnosine in tissue is controlled by a number of enzymes. The hydrolysis of carnosine is mainly dependent on tissue carnosinase (EC 3.4.13.3)[42] or serum carnosinase (EC 3.4.13.20), obtained in brain and blood plasma.[43] Comparative study of hydrolysis of carnosine and number of its derivatives by human serum and rat kidney carnosinase was carried out.[44] The rate of homocarnosine and *N*-acetylcarnosine hydrolysis was negligible by either of the enzyme used. Therefore, *N*-acetylcarnosine may play a role in the potentiality of physiological responses to the therapeutic treatment with this compound as antioxidant.

Cu,Zn-SOD is a metalloenzyme that is essential to the dismutation of $O_2^{-\cdot}$ to H_2O_2. Thus Cu,Zn-SOD is a very important component of the cellular defense mechanism against oxygen toxicity. The inactivation of Cu,Zn-SOD by salsolinol may lead to the perturbation of the antioxidant system. Although our results were obtained from *in vitro* experiments, carnosine and *N*-acetylcarnosine may play an important role in the maintenance of the antioxidant system.

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