Modification and Inactivation of Human Ceruloplasmin by Oxidized DOPA

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Ceruloplasmin (CP), the blue oxidase present in all vertebrates, is the major copper-containing protein of plasma. It has been proposed that oxidation of L-3,4-dihydroxyphenylalanine (DOPA) may contribute to the pathogenesis of neurodegenerative disorders. The effect of the oxidized products of DOPA on the modification of human CP was investigated. When CP was incubated with the oxidized L-DOPA, the protein was induced to be aggregated and ferroxidase activity was decreased in a time-dependent manner. Radical scavengers and catalase significantly inhibited the oxidized DOPA-mediated CP aggregation. Copper chelatrors, Diethylenetriaminepenta acetic acid (DTPA) and Diethyldithiocarbamic acid (DDC), also inhibited the oxidative modification of CP. The results suggested that DOPA oxidation led to the formation of free radical and induced the CP aggregation.

Key Words : Ceruloplasmin, DOPA, Oxidation, Free radical

Introduction

Ceruloplasmin (CP) is an important protein that circulates in plasma as a major copper transport protein and contains greater than 95% of copper found in serum. The protein is a α-2 glycoprotein, which is synthesized in hepatocytes and is secreted into the plasma with six atoms of tightly bound copper/molecule.[1] The functions of CP are copper transporter, iron metabolism, antioxidant defense, tissue angiogenesis, and coagulation.[2-5] It has been reported that CP catalyzed the oxidation of Fe(II) to Fe(III), the catalytic cycle involving four of the six copper associated with CP employs dioxygen as the terminal electron acceptor without the intermediacy of partially reduced oxygen species such as $O_2^{-\cdot}$ or H_2O_2.[6,7] This oxidase activity increases during inflammation, infection, and injury, suggesting that serum CP acts possibly as an antioxidant and as an acute phase protein.[8,9] Several functional activities of CP can be inactivated by exposure to a flux of oxygen free radicals generated by the hypoxanthine/xanthine oxidase system.[10] During exposure to oxidative stress, it has been implicated that substantial CP inactivation may occur and free copper ions could be released.[11-13] Therefore, damaged CP may cause the augmentation of free radical-mediated damage to other macromolecules upon exposure to oxidative stress.

Oxygen-free radicals were associated with pathogenic processes in neurodegenerative disorders, such as Parkinson's Disease (PD) and Alzheimer's Disease (AD). Faults with the respiratory chain and dopamine metabolism have also been theorized to contribute to free radical production.[14] Catechols are hydroquinones that may undergo oxidation to electrophilic semiquinones and quinines. It has been proposed that the 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.[15] L-DOPA and dopamine can generate semiquinones, $O_2^{-\cdot}$ and H_2O_2 [16] in the presence of transition-metal ions. Iron and copper are able to generate ·OH through non-enzymatic reaction, Fenton reaction.[17,18] ·OH is the most powerful oxidizing species among several reactive oxygen radicals, and is able to oxidize most macromolecules. The oxidation of cellular proteins has been described under many pathological conditions.[19-22]

In the present study, the effect of the non-enzymatically oxidized L-DOPA on the modification of CP was investigated. The results revealed that the aggregation of CP was induced by the products of oxidized DOPA *via* the generation of free radicals. Present results suggest that the oxidation of DOPA may be involved in the oxidative stress-induced aggregation of CP in neurodegenerative disorders.

Materials and Methods

Materials. Sodium azide, mannitol, Dihydorxyphenylalanine (DOPA), dopamine and catalase were purchased from Sigma (St. Louis, MO, USA). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA, USA). All solutions were treated with Chelex 100 resin to remove traces of transition metal ions.

Protein oxidation. Protein concentration was determined by the method of Smith *et al.*[23] and Ahn *et al.*[24] Oxidation of CP was carried out by incubation of the enzyme (0.2 mg/mL) in 10 mM potassium phosphate buffer (pH 7.4) both in the presence or absence of 1 mM DOPA at 37 °C. After 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 DOPA. The mixture was then washed with Chelex 100 treated water and centrifuged for 1 h at same speed to further remove DOPA. This was repeated four times. The filtrate was dried by freeze drier and dissolved with phosphate buffer.

Measurement of ferroxidase activity. The ferroxidase of CP was measured by the method of Sunderman and

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Nomoto.[25] CP (0.2 mg/mL) was incubated in 0.1 M sodium acetate buffer (pH 5.7) containing 500 μg/mL of *p*-phenylenediamine for 1 h at 37 °C and the absorption was measured at 540 nm.

Analysis of CP aggregation. After treatment with various concentrations of DOPA for various periods of time, samples of the reaction mixtures were diluted with a concentrated sample buffer (0.25 M Tris, 40% glycerol, 0.01% bromophenol blue). An aliquot of each sample was subjected to native PAGE as described by Laemmli,[26] using a 10% 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.[27] Reaction mixtures contained various concentrations of DOPA in the absence or presence of 10 μM Cu^{2+}. 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).

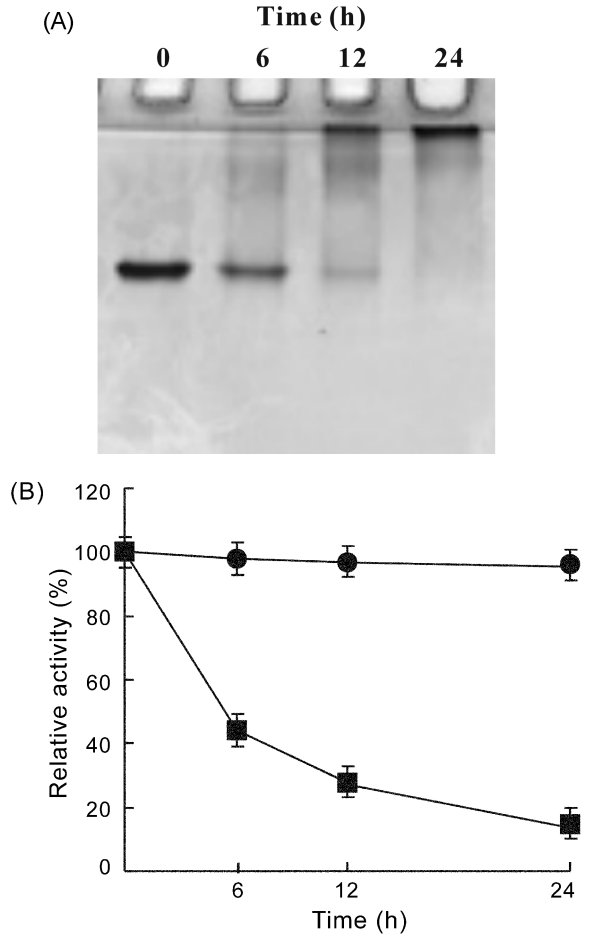

Figure 1. Modification and inactivation of CP after the incubation with DOPA. (A) CP was incubated with 1 mM DOPA in 10 mM potassium phosphate buffer (pH 7.4) at 37 °C for various incubation periods. Reactions were stopped at the time indicated by freezing at -80 °C and an aliquot was analyzed by native PAGE. (B) CP (0.2 mg/mL) was incubated with (■) or without (●) 1 mM DOPA in 10 mM potassium phosphate buffer (pH 7.4) at 37 °C for various incubation periods and the ferroxidase activity was measured by using *p*-pheylenediamine. Data represent the means ± S.D. (n = 5).

Results

Since free radicals can be generated in autoxidation of catecholamines,[16,18] the effect of DOPA on the CP modification was investigated. When CP was incubated with 1 mM DOPA at 37 °C, the frequency of the protein aggregation increased in a time-dependent manner (Fig. 1A). During the incubation of CP with DOPA, ferroxidase

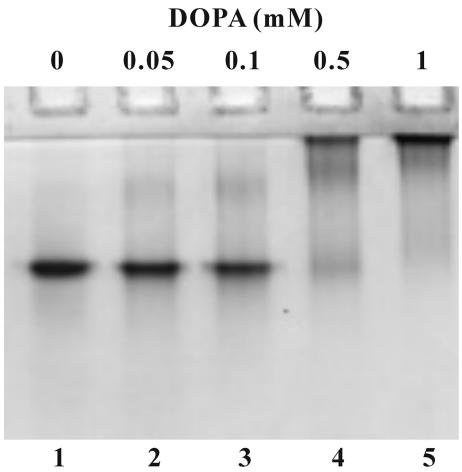

Figure 2. Effect of DOPA concentrations on modification of CP. CP (0.2 mg/mL) was incubated in 10 mM phosphate buffer (pH 7.4) at 37 °C for 24 h under various concentrations of DOPA. Lane 1, CP control; lane 2-5, with 0.05, 0.1, 0.5 and 1 mM DOPA.

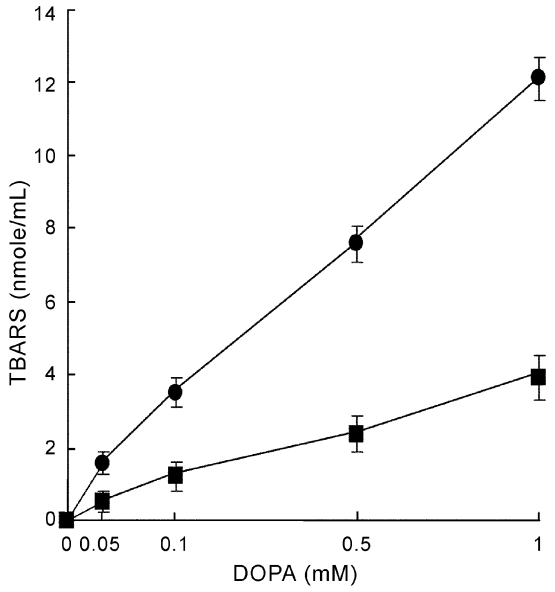

Figure 3. Generation of hydroxyl radical during the oxidation reaction of DOPA. The reaction mixtures contained 10 mM 2-deoxy-D-ribose and various concentrations of DOPA without (■) or with 100 μM $CuSO_4$ (●) in 10 mM phosphate buffer at pH 7.4.

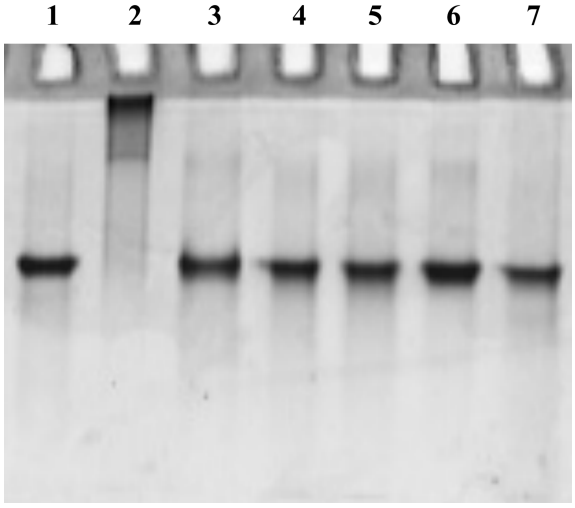

Figure 4. Effect of radical scavengers, catalase and copper chelators on the aggregation of CP by oxidized DOPA. CP (0.2 mg/mL) was incubated with 1 mM DOPA in 10 mM phosphate buffer (pH 7.4) at 37 °C for 24 h in the presence of radical scavengers and copper chelators. Lane 1, CP control; lane 2, no addition of effectors; lane 3, 200 mM azide; lane 4, 200 mM mannitol; lane 5, catalase (0.2 mg/mL); lane 6, 10 mM DTPA; lane 7, 10 mM DDC.

activity was gradually decreased as a function of time (Fig. 1B). The results suggest that the protein aggregation by DOPA is associated with the inactivation of ferroxidase activity of CP. The aggregation of CP became apparent at 500 μM DOPA; the aggregation increased up to 1 mM DOPA (Fig. 2).

The participation of hydroxyl radical in the aggregation of CP by the oxidized DOPA was investigated. It has been shown that the reaction of hydroxyl radical with deoxyribose causes formation of a product that reacts with thiobarbituric acid to produce a chromogen. Thus the generation of hydroxyl radicals in the oxidation of DOPA was measured with thiobarbituric acid-reactive substance (TBARS). When 2-deoxy-D-ribose was incubated with various concentrations of DOPA in the presence of Cu^{2+}, the rate of TBARS formation was increased up to 12.1 nmole. However, in the absence of Cu^{2+}, the maximum rate of TBARS formation was 3.9 nmole (Fig. 3). The aggregation of CP was significantly suppressed in the presence of azide, mannitol and catalase (Fig. 4, lane 3-5). These results suggest that the autoxidation of DOPA may lead to the generation of hydrogen peroxide and produce hydroxyl radical via transitional metal-catalyzed reaction (Fenton reaction). Evidence that copper chelators, DTPA and DDC protected the CP against oxidized DOPA supported this mechanism (Fig. 4, lane 6 and 7). Thus, hydroxyl radical might play a critical role in the aggregation of CP by the oxidized DOPA.

Discussion

The present study investigated the potential role of DOPA in the modification of CP. The toxicity of catechol may be augmented by its free radical-generating function in neurodegenerative disorders. Since the level of free radicals was reported to be increased in the patients of neurodegenerative disorders, such as Parkinson's Disease (PD) and Alzheimer's Disease (AD),[28,29] the oxidative modification of CP by free radicals might have pathological significance. The present results showed that modification of CP was induced by the autoxidation of DOPA. L-DOPA and dopamine can oxidize *in vitro* to generate semiquinones, $O_2^{-\cdot}$ and H_2O_2,[16] a process greatly facilitated by the presence of transition-metal ions. In the case of iron and copper, ·OH will also be generated by Fenton reaction.[18,27] In this study, the DOPA-mediated CP aggregation was inhibited by hydroxyl radical scavengers and catalase. These results indicate that hydroxyl radicals and hydrogen peroxide may involve in the aggregation of CP.

Trace metal such as iron and copper, which are variously present in biological systems, may interact with active oxygen species, ionizing radiation, or microwave radiation to damage macromolecules.[30-32] The cleavage of the metalloproteins by oxidative damage may lead to increases in the levels of metal ions in some biological cell.[33] It has been reported that copper concentration was significantly increased in the cerebrospinal-fluid of PD and AD patients.[34,35] These results suggested that iron or copper-catalyzed oxidative reaction might contribute to the pathogenesis of neurodegenerative disorders. In the present study, copper ions led to enhancement of the hydroxyl radical formation. In addition to, copper chelators, DTPA and DDC, also inhibited DOPA-mediated CP aggregation. These results indicate that copper ions of CP may contribute to DOPA-mediated CP aggregation.

In conclusion, the present results suggest that the modification of CP was induced by the autoxidation of DOPA, involving ·OH generation from H_2O_2. Therefore, DOPA-mediated CP modification might be involved in the pathogenesis of neurodegenerative disorders.

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