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Short communication

Oxidative Modification of Human Ceruloplasmin by Methylglyoxal: An *in vitro* study

Jung Hoon Kang*
Department of Genetic Engineering, Cheongju University, Cheongju 360-764, Korea

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Methylglyoxal (MG) is an endogenous physiological metabolite which is present in increased concentrations in diabetics. MG reacts with the amino acids of proteins to form advanced glycation end products. In this *in vitro* study, we investigated the effect of MG on the structure and function of ceruloplasmin (CP) a serum oxidase carrier of copper ions in the human. When CP was incubated with MG, the protein showed increased electrophoretic mobility which represented the aggregates at a high concentration of MG (100 mM). MG-mediated CP aggregation led to the loss of enzymatic activity and the release of copper ions from the protein. Radical scavengers and copper ion chelators significantly prevented CP aggregation. CP is an important protein that circulates in plasma as a major copper transport protein. It is suggested that oxidative damage of CP by MG may induce perturbations of the copper transport system and subsequently lead to harmful intracellular condition. The proposed mechanism, in part, may provide an explanation for the deterioration of organs in the diabetic patient.

Keywords: Aggregation, Ceruloplasmin, Methylglyoxal

Introduction

The non-enzymatic glycation of proteins, an early stage of the Maillard reaction, is a post-translation modification process between free reducing sugars and the free amino groups of proteins (Monnier *et al*., 1992). This process is initiated by the condensation reaction of reducing sugars with free amino group to form Schiff bases, which undergo rearrangement to form the relative stable Amadori products (Reynolds, 1965). It has been suggested that α-oxoaldehydes, glyoxal, methylglyoxal and 3-deoxyglucosone are formed before and after Amadori product formation (Thornalley *et al*., 1999). These compounds are more reactive than the parent sugar in their ability to react with amino groups of proteins to form cross-links, stable end products called advanced glycation end products (AGEs) (Brownlee *et al*., 1988). AGEs are irreversibly formed and found to accumulate with aging, atherosclerosis, and diabetes mellitus, and they are especially associated with long-lived proteins such as collagens (Monnier *et al*., 1986) and lens crystallins (Monnier and Cerami, 1981). The three-carbon α-dicarbonyl compound, methylglyoxal (MG), is an endogenous metabolite and product of triose spontaneous oxidation and acetone and aminoacetone metabolism. MG has been implicated in secondary diabetic complications by promoting formation of AGEs (Shipanova *et al*., 1997). It was reported that glycation reaction of amino acids by MG generated reactive oxygen species (ROS) (Yim *et al*., 1995). MG can generate ROS in the absence of lysine residues and also in the presence of N-acetyl-arginine (Ortwerth *et al*., 1998).

The damage of cellular proteins induced by oxidative stresses has been described under many pathological conditions (Berlett and Stadtman, 1997). Ceruloplasmin (CP) is an important protein that circulates in plasma as a major copper ion transporter and accounts for more than 95% of the copper found in serum. The functions of CP include copper transport, iron metabolism, antioxidant defense, tissue angiogenesis and blood coagulation (Goldstein *et al*., 1979). It has been reported that CP catalyzed the oxidation of Fe (II) to Fe (III), the catalytic cycle involving four of the six coppers associated with CP that employ dioxygen as the terminal electron acceptor without the intermediacy of a partially reduced oxygen species such as $O_2^{-\bullet}$ or H_2O_2 (Ryden, 1984). This oxidase activity increases during inflammation, infection, and injury which suggests that serum CP possibly acts as an antioxidant and as an acute phase protein (Fleming *et al*., 1991). It has been implied that during exposure to oxidative stress, substantial CP inactivation may occur and free copper ions may be released (Swain *et al*., 1994; Choi *et al*., 2000). Therefore, damaged CP may cause the augmentation of free

*To whom correspondence should be addressed.
Tel: 82-43-229-8562; Fax: 82-43-229-8432
E-mail: jhkang@chongju.ac.kr

radical-mediated damage to other macromolecules upon exposure to oxidative stress. The mechanism by which MG induces the generation of ROS is well known, however, the modification of CP induced by MG has not been reported.

The modification and inactivation of human CP by MG is described in this report. The results showed that exposure of CP to MG led to protein aggregation and that aggregation of CP induced the inactivation of feroxidase and the release of copper ions.

Materials and Methods

Materials. Methylglyoxal (MG), sodium azide, formate, bathocuproine, and diethyldithio carbamic acid (DDC) were purchased from Sigma. Chelex 100 resin was purchased from Bio-Rad. The commercial human CP obtained from Calbiochem was subjected to gel filtration chromatography using a Superose 6 FPLC column (Pharmacia) to enable further purification. All solutions were treated with the Chelex 100 resin to remove traces of transition metal ions.

Protein (CP) modification. Protein concentrations were determined by the BCA method (Smith *et al*., 1985). Modification of the purified CP (0.25 mg/mL) was carried out by incubation in 10 mM potassium phosphate buffer (pH 7.4) either in the presence or absence (control) of MG at 37°C. After incubation of the reaction mixtures, the mixtures were placed into a Microcon filter (Amicon) and centrifuged at 13,000 rpm for 1 h to remove the MG. The mixture was then washed with Chelex 100 resin treated water and centrifuged for 1 h at 13,000 rpm to further remove MG. This procedure was repeated four times. The combined filtrates were lyophilized and thereafter dissolved with 10 mM potassium phosphate buffer (pH 7.4). Protection by radical scavengers against MG-mediated CP modification was performed by preincubating the enzyme in the presence of a radical scavenger for 5 min at room temperature and the reaction of the mixture with MG for 10 h at 37°C. The unreacted reagent was washed pit using Microcon filter (Amicon).

Characterization of MG-modified proteins. After treatment with various concentrations of MG for 10 h at 37°C, samples of the reaction mixture were diluted with concentrated sample buffer (0.25 mM Tris, 40% glycerol, 0.01% bromophenol blue). An aliquot of each sample was subjected to native polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970), using a 10% acrylamide slab gel. The gels were stained with 0.15% Coomassie Brilliant Blue R-250.

Measurement of feroxidase activities. The feroxidase activities of CP were measured as described previously (Eum *et al*., 2005). CP was incubated in 0.1 mM sodium acetate buffer (pH 5.7) containing 0.5 mg/mL of *p*-phenylenediamine for 1 h at 37°C and the absorption measured at 540 nm.

Determination of free copper ion concentration. Protein samples (0.25 mg/mL) were incubated with various concentrations of MG for 10 h and then subjected to ultra filtration using a Millipore Ultrafree-MC filter with a molecular mass cut-off of 3 kDa. The concentration of copper ion in the filtrate was determined by atomic absorption spectrophotometry (Shimadzu, AA-6601F).

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

Results and Discussion

CP was incubated with various concentrations of MG in 10 mM Chelex-treated phosphate buffer (pH 7.4) at 37°C for 10 h. The native PAGE of the reaction products demonstrated that the electrophoretic mobility of MG-CP increases with increasing concentration of MG used for glycation (Fig. 1). These results indicate the progressive loss of the positive charge of the MG-CP during the glycation reaction. MG readily reacts with the lysine and arginine residues of the protein to produce cross-linked products (Nagaraj *et al*., 1996). Thus, the net charge of MG-CP adducts may shift to more negative values. When a high concentration of MG (100 mM) was used for glycation, additional bands appeared with lower electrophoretic mobilities. The molecular species with the lowest mobility may represent the larger aggregates of MG-CP and the aggregate-formed precipitates (Fig. 1, lane 6). These results suggest that methylglyoxal cross-links the inter- or intramolecular lysine residues of a protein to form cross-linked Schiff bases which lead to the formation of oligomers. During the incubation of CP with MG, ferroxidase activity decreased and the release of free copper ions gradually increased with increasing MG concentrations (Fig. 2). These results indicate that the inactivation of CP by MG is

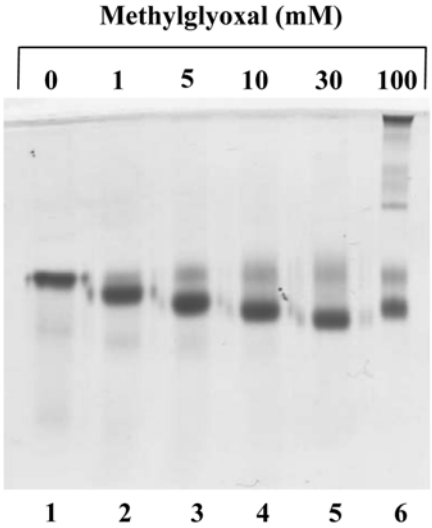

Fig. 1. CP aggregation during incubation with MG. CP (0.25 mg/mL) was incubated with various concentrations of MG in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for 10 h. Reaction mixtures were analyzed by native PAGE.

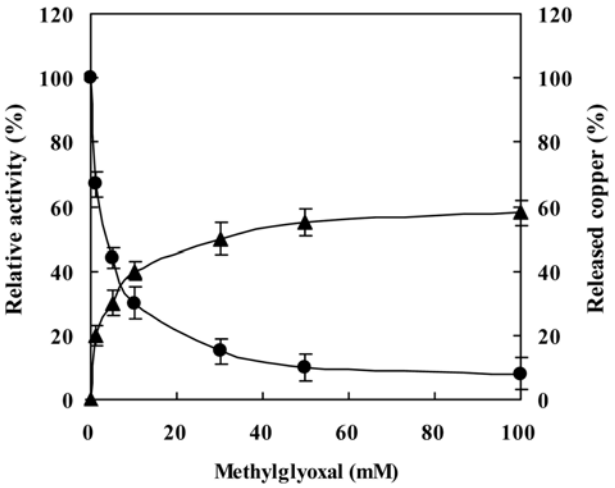

Fig. 2. Inactivation of feroxidase and the release of copper ions from CP during incubation with various concentrations of MG. CP (0.25 mg/mL) was incubated with MG in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for 10 h. Closed circles, relative CP feroxidase activity, closed triangles, the content of released copper ions.

associated with protein modification. The modification of the metalloproteins as a result of oxidative damage may induce the higher level of intracellular metal ions (Kang, 2004; Kim and Kang, 1997). It has been reported that the modification of Cu, Zn-SOD induced by oxidative damage could lead to the release of copper ions from the enzyme (Choi *et al*., 1999). Trace metals such as copper and iron, which are present in biological systems, may interact with ROS leading to the damage of macromolecules (Halliwell and Gutteridge, 1999). Therefore, copper ions released by MG-modified CP may lead to a pro-oxidant condition.

The effects of radical scavengers on CP aggregation by MG has been investigated in this study. We were able to demonstrate that azide and formate protected CP against protein aggregation (Fig. 3A). Although radical scavengers could not completely protect CP (lane 3 and 4), the aggregation of CP was effectively inhibited by these compounds at the level utilized. Glycation is believed to be modulated by oxidative stress (Monnier and Cerami, 1981) and it has been demonstrated that reducing sugars can undergo oxidation in the presence of oxygen and transition metal ions, which generate H_2O_2, oxygen radicals, and a-ketoaldehydes (Jiang *et al*., 1990). Such a reaction leads to protein "browning", conformational changes, and fragmentation. Therefore, AGEs *in vivo* are the products of the combined processes of glycation and oxidative modification.

The participation of copper ions in MG-mediated CP aggregation was investigated by examining the protective effects of the copper chelators, bathocuproine and diethyldithiocarbamic acid. These chelators significantly prevented CP aggregation (Fig. 3B) and while these agents could not completely protect CP (lanes 3 and 4) they effectively prevented the aggregation

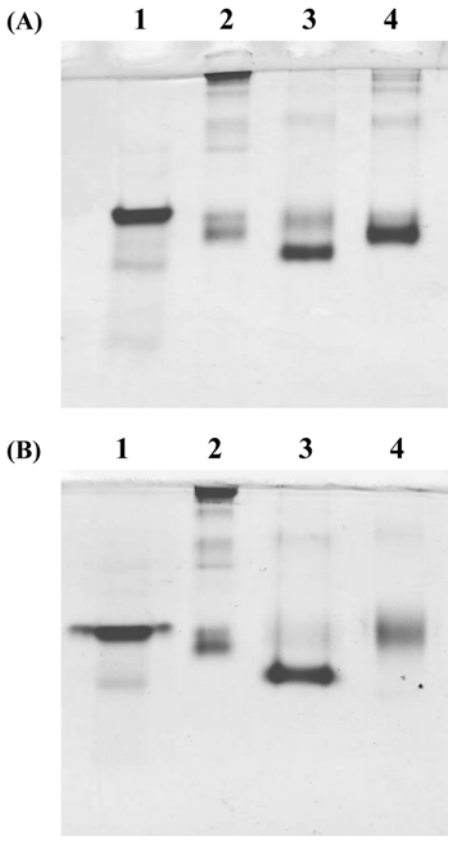

Fig. 3. Effects of radical scavengers and copper chelators on CP aggregation by MG. CP was incubated with 100 mM MG in the presence of effectors. (A) Lane 1, CP control; lane 2, incubation with MG; lane 3, lane 2 plus 100 mM azide; lane 4, lane 2 plus 100 mM formate. (B) Lane 1, CP control; lane 2, incubation with MG; lane 3, lane 2 plus 20 mM bathocuproine; lane 4, lane 2 plus 20 mM DDC. Reaction mixtures were analyzed by native PAGE.

of CP. These findings suggest that copper ions may be associated with the MG modification of CP.

CP is a blue-copper oxidase found in the sera of all vertebrate species where it accounts for greater that 95% of the circulating plasma copper and may participate in the antioxidant defense system because of its feroxidase and thiol-linked peroxidase activities. Thus, CP is a very important component of the cellular defense mechanism against oxygen toxicity. The inactivation of CP by MG may lead to perturbations of the antioxidant system. In addition, the copper ions released from the oxidative-damaged CP by these radicals can enhance metal-catalyzed reactions to produce ROS which induces the oxidative damage to the macromolecules. The proposed mechanism, therefore, may contribute to the increased peroxidation of lipids, as when glycated proteins were added *in vitro,* and may also contribute to accelerating oxidative modifications of the vascular wall lipids in the diabetic patient.

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