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Oxidative damage of DNA induced by the reaction of methylglyoxal with lysine in the presence of ferritin

*Sung Ho An & Jung Hoon Kang**
Department of Genetic Engineering, Cheongju University, Cheongju 360-764, Korea

Methylglyoxal (MG) is an endogenous metabolite which is present in increased concentrations in diabetics and reacts with amino acids to form advanced glycation end products. In this study, we investigated whether ferritin enhances DNA cleavage by the reaction of MG with lysine. When plasmid DNA was incubated with MG and lysine in the presence of ferritin, DNA strand breakage was increased in a dose-dependent manner. The ferritin/MG/lysine system-mediated DNA cleavage was significantly inhibited by reactive oxygen species (ROS) scavengers. These results indicated that ROS might participate in the ferritin/MG/lysine system-mediated DNA cleavage. Incubation of ferritin with MG and lysine resulted in a time-dependent release of iron ions from the protein molecules. Our data suggest that DNA cleavage caused by the ferritin/MG/lysine system via the generation of ROS by the Fenton-like reaction of free iron ions released from oxidatively damaged ferritin. [BMB Reports 2013; 46(4): 225-229]

INTRODUCTION

The three-carbon α-dicarbonyl compound, methylglyoxal (MG), is an endogenous metabolite and product of triose spontaneous oxidation and of acetone and aminoacetone metabolism. MG has been implicated in secondary diabetic complications as it promotes the formation of advanced glycation end products (AGE) (1, 2). In addition, MG readily reacts with protein lysine and arginine residues to produce cross-linked products (3). It has also been reported that the glycation reaction of amino acids with MG generates reactive oxygen species (ROS) (4, 5).

Ferritins are a family of iron storage and detoxification proteins in animals, plants and microbes. Ferritin is a 24 subunit protein composed of two subunit types, termed H (heavy) and L (light), which perform complementary functions in the protein. Subunits of type L contribute to the nucleation of the iron core, but lack the ferroxidase activity necessary for uptake of ferrous (Fe^{2+}) iron. Subunits of type H possess ferroxidase activity and promote rapid uptake and oxidation of ferrous iron (6). Iron is an essential element for mammalian cell growth. It is a required constituent of numerous enzymes, including iron-sulfur and heme proteins of the respiratory chain (7). However, free iron has the capacity to participate in ROS formation via Fenton reaction (8). The significance of ROS in the damage of many biological molecules, including DNA, has drawn much attention. Cleavage of plasmid DNA has been efficiently induced by direct treatment with several kinds of metals plus H_2O_2 (6, 9). Moreover, DNA cleavage and fragmentation induced by alloxan in pancreatic islets cells seem to play an important role in the development of diabetes (10). This fragmentation is also thought to result from the accumulation of ROS produced directly by alloxan (11). Balancing the deleterious and beneficial effects of iron thus emerges as an essential aspect of cell survival.

Carnosine (β-alanine-L-histidine) is an endogenous dipeptide, which is accumulated in the brain, heart and skeletal muscle. Water-soluble functions in the cytosol, where the oxidation mediators (metals and oxygen radicals) are abundantly located. It has an antioxidant property, due to its biological function of scavenging ROS. It has been demonstrated that carnosine has a protective effect, based on its scavenging effect on hydroxyl radicals and superoxide anions, in the liver, kidneys and brain (12-14). However, the involvement of carnosine and related compounds in ferritin/MG/lysine-mediated DNA damage has not been reported.

In this study, we examined DNA cleavage caused by the ferritin/MG/lysine system. Our results suggest that the DNA cleavage induced by the ferritin/MG/lysine system and MG is due to the oxidative damage resulting from ROS generated by the Fenton-like reaction of free iron ions released from oxidatively damaged ferritin. We also investigated the protective effects of carnosine and anserine against DNA cleavage by the ferritin/MG/lysine system.

RESULTS AND DISCUSSION

We previously reported that DNA cleavage was induced by the reaction of MG with lysine in the presence of iron (15). In

*Corresponding author. Tel: +82-43-229-8562; Fax: +82-43-229-8432; E-mail: jhkang@cju.ac.kr
http://dx.doi.org/10.5483/BMBRep.2013.46.4.225

Received 5 November 2012, Revised 19 November 2012, Accepted 10 December 2012

Keywords: DNA, Fenton reaction, Ferritin, Methylglyoxal

ISSN: 1976-6696 (print edition) / 1976-670X (electronic edition)
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addition, we have also reported that the modification of ferritin, iron containing protein, was induced by MG (16). To further examine whether the modification of ferritin causes DNA damage, we exposed the plasmid pUC19 to the ferritin/MG/lysine system. As shown in Fig. 1A, the amount of supercoiled (SC) DNA was slightly decreased by MG/lysine. The loss of SC-DNA induced by MG/lysine was remarkably stimulated by the addition of ferritin. The effect of ferritin concentrations on the production of DNA cleavage was studied. When DNA was incubated with MG/lysine and various concentrations of ferritin, DNA cleavage increased up to 1 μM ferritin (Fig. 1B). Free amino groups in protein react with the carbonyl groups of reducing sugars or α-ketoaldehyde, which has been implicated as the onset of glycation. Previous investigations have shown that free radicals are produced in the reaction between MG and proteins or amino acids (4, 5). Furthermore, it has been shown that the reaction of ferritin with MG generates ROS that oxidizes amino acid residues at or near that cation-binding site, which then introduces carbonyl groups. Such an oxidative modification is an indicator of oxidative stress and may be significant in several physiological and pathological processes (17, 18).

The participation of ROS in the DNA damage by the ferritin/MG/lysine system was studied by examining the protective effect of ROS scavengers. When plasmid DNA was incubated with ferritin, MG and lysine in the presence of urate, N-acetyl cysteine and thiourea at 37°C for 2 h, all scavengers significantly prevented DNA cleavage (Fig. 2A). It has been reported that iron ions could stimulate the Fenton-like reaction to produce ROS, which mediates DNA cleavage (19). Attack of ROS on the sugar 2-deoxyribose produces a variety of different products, some of which are mutagenic in bacterial systems. Some of the fragmentation products, which can be detected by adding thiobarbituric acid (TBA) to the reaction mixture, results in the formation of a pink $(TBA)_2$-MDA chromogen (20). Fig. 2B demonstrated that the oxidative damage of deoxyribose was induced by the ferritin/MG/lysine system. Therefore, our results suggest that ROS may participate in the mechanism of DNA cleavage produced by the ferritin/MG/lysine system. Cellular metabolism has been shown to generate

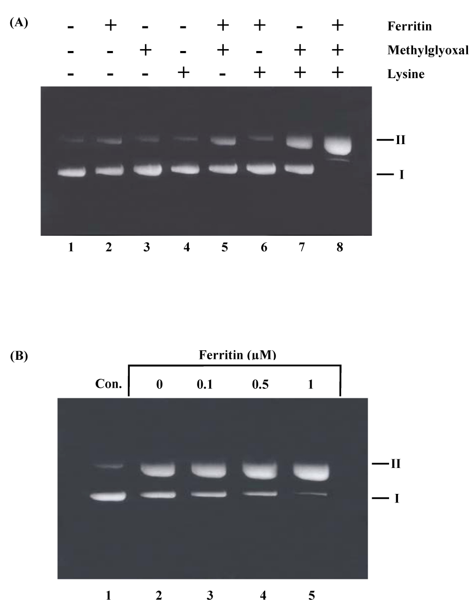

Fig. 1. DNA strand breakage by the ferritin/MG/lysine system. (A) pUC19 (2 μg) was incubated at 37°C for 2 h : lane 1, pUC19 DNA control; lane 2, pUC19 DNA plus 1 μM ferritin; lane 3, pUC19 DNA plus 20 mM MG; lane 4, pUC19 DNA plus 20 mM lysine; lane 5, pUC19 DNA plus 1 μM ferritin plus 20 mM MG; lane 6, pUC19 DNA plus 1 μM ferritin plus 20 mM lysine; lane 7, pUC19 DNA plus 20 mM MG plus 20 mM lysine; lane 8, pUC19 DNA plus 1 μM ferritin plus 20 mM MG plus 20 mM lysine. (B) pUC19 (2 μg) was incubated with 0-1 μM ferritin and 20 mM MG and 20 mM lysine at 37°C for 2 h. The reaction was stopped by freezing at −80°C. The loading buffer was added to samples and analyzed by electrophoresis on 1% agarose gel. I and II indicate the position of the supercoiled and nicked circular DNA plasmid forms, respectively.

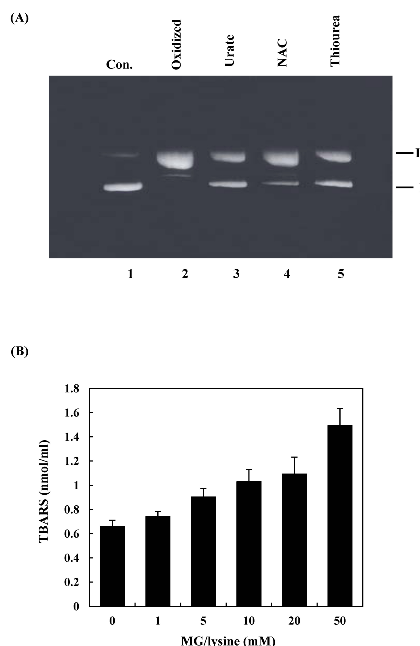

Fig. 2. Effects of ROS scavengers on DNA strand breakage and the generation of ROS in the ferritin/MG/lysine system. (A) pUC19 (2 μg) was incubated with 1 μM ferritin, 20 mM MG and 20 mM lysine in the presence of the ROS scavenger. Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 20 mM urate; lane 4, 20 mM N-acetyl-L-cysteine (NAC); lane 5, 20 mM thiourea. (B) Ferritin (1 mg/ml) was incubated with various concentrations of MG/lysine in a 10 mM potassium phosphate buffer (pH 7.4) at 37°C for 72 h. After incubation, the detection of ROS formation was determined by measuring thiobarbituric acid reactive 2-deoxy-D-ribose oxidation products.

oxygen species such as hydrogen peroxide, hydroxyl radical, and superoxide radical (21). Trace metals such as copper and iron which are present in biological systems may interact with active oxygen species, ionizing radiation, or microwaves to damage macromolecules (6, 22-26). The participation of iron ions in the production of DNA cleavage was studied by examining the protective effect of the iron chelator, DFX. The DNA cleavage was effectively inhibited by 1 mM DFX (Fig. 3A). The results indicate that free iron ions are involved in the DNA cleavage by the ferritin/MG/lysine system. The cleavage of the metalloproteins by oxidative damage may lead to a higher level of metal ions in biological cells. We investigated the release of iron in the reaction of ferritin with MG and lysine. When ferritin was incubated with various concentrations of MG/lysine, free iron ions gradually increased in a concentration-dependent manner with regard to MG/lysine (Fig. 3B). Therefore, the released iron ions could then enhance the Fenton-like reaction to produce ROS and play a critical role in DNA cleavage.

The ferritin/MG/lysine system-mediated DNA damage is expected to increase when the rate of MG formation increases. A chemical mechanism of cellular DNA damage by glyoxal and methylglyoxal has been elucidated using an analogues plasmid cleavage assay *in vitro* and comet single cell DNA electrophoresis analysis (27). Oxidative modification of DNA may contribute to mutagenesis, apoptosis, and aging. Therefore, the results presented here suggest that the oxidative damage of DNA induced by the glycation reaction of MG with amino acids in the presence of ferritin may cause the irreversible deterioration seen in mutagenesis, aging and diabetic complications.

Carnosine is antioxidant and antiglycating agent that inhibits sugar-mediated protein crosslinking (28-33). The effects of carnosine and anserine on ferritin/MG/lysine-mediated DNA cleavage was investigated. Both compounds showed a dose-dependent inhibition of DNA cleavage induced by ferritin/MG/lysine (Fig. 4). Anserine inhibited DNA cleavage more effectively than carnosine. 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 anserine have been shown to be efficient copper-chelating agents, and it has been suggested

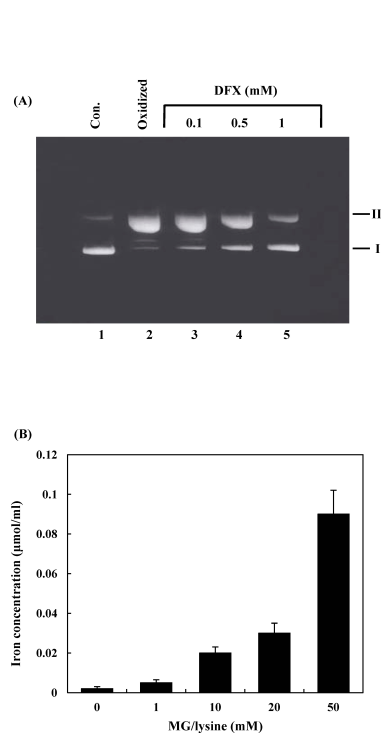

Fig. 3. Effects of deferoxamine (DFX) on DNA strand breakage and the release of iron from ferritin. (A) pUC19 (2 μg) was incubated with 1 μM ferritin, 20 mM MG and 20 mM lysine in the presence of various concentrations of DFX. Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 0.1 mM DFX; lane 4, 0.5 mM DFX; lane 5, 1 mM DFX. (B) Ferritin (1 mg/ml) was incubated with various concentrations of MG/lysine in a 10 mM potassium phosphate buffer (pH 7.4) at 37°C for 72 h.

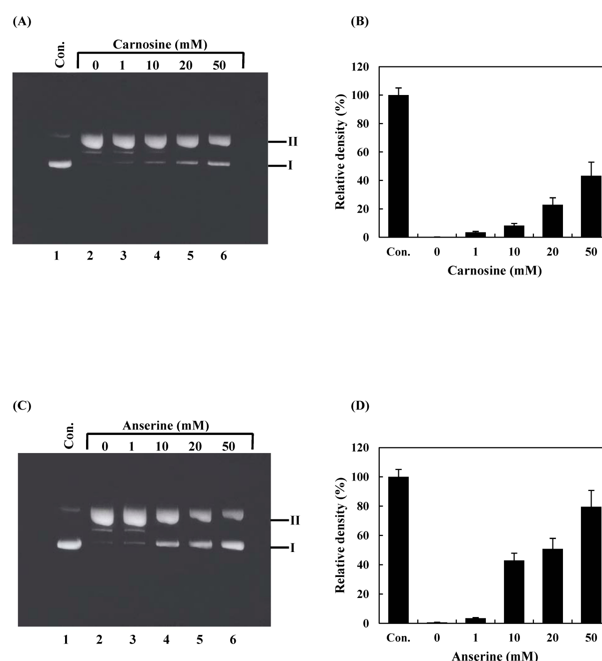

Fig. 4. Effects of carnosine and anserine on DNA cleavage induced by the ferritin/MG/lysine system. pUC19 DNA was incubated with 1 μM ferritin, 20 mM MG and 20 mM lysine in the presence of carnosine or anserine. (A) Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 1 mM carnosine; lane 4, 10 mM carnosine; lane 5, 20 mM carnosine; lane 6, 50 mM carnosine. (C) Lane 1, DNA alone; lane 2, no addition of effector; lane 3, 1 mM anserine; lane 4, 10 mM anserine; lane 5, 20 mM anserine; lane 6, 50 mM anserine. (B and D) The relative staining intensity of agarose gel was analyzed by densitometric scanning. I and II indicate the position of the supercoiled and nicked circular DNA plasmid forms, respectively.

that they may play a role in copper metabolism *in vivo* (34, 35). However, carnosine and related compounds have not been found to chelate iron in a manner that reduces its prooxidant activity (36). It has been reported that carnosine and related compounds quench 50-95% of hydroxyl radicals produced in the Fenton reaction (37). Thus, the result indicates that the abilities of carnosine and anserine to inhibit iron-promoted oxidation is likely due to free radical scavenging activity. Carnosine can react with methylglyoxal (38, 39) and it has been described as glyoxalase mimetic (40).

In conclusion, the present study indicates that the ferritin/MG/lysine system may lead to oxidative DNA damage. Moreover, the formation of ROS and the release of iron were detected during this reaction. In this study, our results may provide a partial explanation for the deterioration of organs in diabetes. Although the present results were obtained from in vitro experiments, carnosine and anserine should be explored as potential therapeutic agents for the pathological processes of oxidative stress associated with many diseases.

MATERIALS AND METHODS

Materials

pUC19 plasmid DNA was prepared and purified from *E. coli* cultures by using the QIAGEN plasmid kit (Santa Clarita, USA). Equine spleen ferritin (Calbiochem) was further purified by gel filtration chromatography. Lysine, 2-deoxy-D-ribose, thiobarbituric acid, bathophenanthroline sulfonate and deferoxamine (DFX) 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 a 10 mM potassium phosphate buffer (pH 7.4) was incubated for 3 h at 37°C with ferritin, MG and lysine in a total volume of 20 μl. The samples were 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 ROS formation

Detection of ROS was done by measuring thiobarbituric acid reactive 2-deoxy-D-ribose oxidation products (20). The assay mixture contained a 10 mM potassium phosphate buffer (pH 7.4), 10 mM 2-deoxy-D-ribose, ferritin, MG and lysine in a total volume of 100 μl. The reaction was stopped by the addition of 2.8 % trichloroacetic acid (200 μl), PBS (200 μl), and 1% thiobarbituric acid (200 μl), and boiled at 100°C for 15 min. Afterwards, 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.

Determination of free iron ion concentration

The concentration of iron ions were released from oxidatively damaged ferritin using a bathophenanthroline sulfonate by the method described previously (41). The reaction mixture contained ferritin, MG and lysine in a 10 mM potassium phosphate buffer (pH 7.4). The reaction was incubated for 72 h at 37°C and then the mixture was placed into an Ultrafree-MC filter and centrifuged at 13,000 rpm for 1 h. The colorimetic reagent was added for analysis by a uv/vis spectrophotometer at 535 nm. The final concentrations of the color reagent were 1% ascorbate, 0.02% bathophenanthroline sulfonate and a 1% acetic acid-acetate buffer (pH 4.5).

Statistical analysis

Values are expressed as the means ± S.D of three to five separate experiments. The statistical differences between the means were determined by the Student *t*-test.

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