

Protective Effects of Carnosine and Relative Compounds on DNA Cleavage by Advanced Glycation End Products

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Three-carbon α -dicarbonyl compound, methylglyoxal (MG), is endogenous metabolite and product of triose spontaneous oxidation and acetone and aminoacetone metabolism. MG has been implicated in secondary diabetic complications promoting formation of advanced glycation end products (AGEs).^{1,2} MG readily reacts with protein lysine and arginine residues to produce cross-linked products.³ It was reported that the glycation reaction of amino acids by MG generated reactive oxygen species (ROS).⁴ The significance of ROS in damage to 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 .^{5,6} DNA strand breaks and DNA fragmentation induced by alloxan in pancreatic islet cells seem to play an important role in the development of diabetes.⁷ This fragmentation is also thought to result from the accumulation of ROS produced directly from alloxan.⁸

Carnosine (β -alanyl-L-histidine) and related compounds such as anserine (β -alanyl-1-methyl-L-histidine) and homocarnosine (γ -amino-butyryl-L-histidine) are present in several mammalian tissues, including skeletal muscle and brain at high concentrations (up to 20 mM in humans).^{9,10} Carnosine and anserine have been shown to be efficient copper-chelating agents, and it has been suggested that they may play a role in copper metabolism *in vivo*.¹¹ Carnosine also protects rabbit heart from reperfusion injury after ischemia.¹² Many biochemical studies have suggested that carnosine possesses antioxidant and free radical-scavenging function which may partly explain its apparent homeostatic function.¹³⁻¹⁵ Recently, it has been reported that carnosine could act as an antiglycation agent.¹⁶ However, the inhibitory action of carnosine and related compounds against oxidative DNA cleavage by glycation reaction has not been studied.

In the present study, the protective effects of carnosine and homocarnosine on DNA cleavage induced by the glycation reaction of lysine with MG in the presence of Fe^{3+} was investigated.

Experimental Section

Materials. Methylglyoxal (MG), L-lysine, catalase from bovine liver, sodium formate, 2-deoxy-D-ribose and thiobarbituric acid were purchased from Sigma. Chelex 100 resin was purchased from Bio-Rad. All solutions were treated with Chelex 100 to remove traces of transition metal ions.

pUC 19 plasmid DNA was purified from *E. coli* cultures by using Qiagen Plasmid Midi Kit.

Analysis of DNA strand breaks. The pUC 19 plasmid DNA (1 μ g) in 10 mM potassium phosphate buffer at pH 7.4 was incubated for 2 h at 37 °C with 20 mM lysine and 20 mM MG in the absence or presence of Fe^{3+} . The reaction was stopped by freezing at -80 °C. Four μ L of loading buffer (0.25% bromophenol blue, 40% sucrose) was added and samples analyzed by electrophoresis in 0.8% agarose in TBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0).¹⁷ The gel was stained with ethidium bromide, photographed.

Measurement of hydroxyl radical. Detection of hydroxyl radicals was determined by measuring thiobarbituric acid reactive 2-deoxy-D-ribose oxidation products.¹⁸ Reaction mixtures contained 20 mM lysine and 20 mM MG in the absence or presence of 100 μ M Fe^{3+} . Mixtures were incubated at 37 °C for 2 h. The degradation of 2-deoxy-D-ribose was measured by adding 200 μ L of PBS, 200 μ L of 2.8% (w/v) trichloroacetic acid, 200 μ L 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 a UV/Vis spectrophotometer (Shimadzu, UV-1601). Unless otherwise indicated, each result described in this paper is representative of at least three separate experiments.

Results and Discussion

Free amino groups in protein react with carbonyl groups of reducing sugars or α -ketoaldehyde, which has been implicated as the onset of glycation. Previous investigations have shown that free radicals were produced in the reaction of MG with proteins and amino acids.⁴ In this study, DNA strand breakage induced by the reaction of MG with amino acids in the presence of transition metal, iron was investigated. Untreated plasmid DNA showed a major band corresponding to the supercoiled form (form I) (Fig. 1, lane 1). Plasmid DNA remained nearly intact after incubation with 20 mM lysine or 20 mM MG or the combination of lysine and MG (Fig. 1, lane 2-5). However, when DNA was incubated with lysine and MG in the presence of 100 μ M Fe^{3+} , DNA strand breakage was occurred as shown by the decrease in the amount of form I molecules and concomitant increase in nicked circular form (form II) (Fig. 1, lane 6). This indicates that Fe^{3+} may facilitate the production of

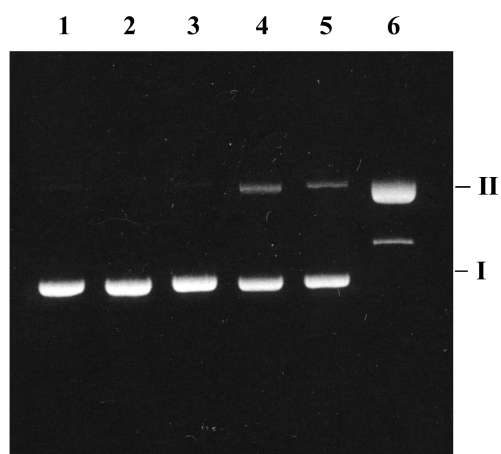


Figure 1. DNA cleavage by the glycation reaction of amino acid with MG in the absence or presence of Fe^{3+} . pUC19 DNA ($1 \mu\text{g}$) was incubated with the following: lane 1, DNA alone; lane 2, 20 mM lysine; lane 3, 20 mM MG; lane 4, $100 \mu\text{M}$ FeCl_3 ; lane 5, 20 mM lysine + 20 mM MG; lane 6, lane 5 + $100 \mu\text{M}$ FeCl_3 . The reactions were stopped by freezing at -80°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 circular DNA plasmid forms, respectively.

DNA strand breaks in the glycation reaction of lysine with MG

Oxidative DNA damage from ROS has been hypothesized to play a critical role in several diverse biological processes including mutagenesis, aging, carcinogenesis.¹⁹ Trace metals such as copper and iron which are present in biological systems may react with H_2O_2 to produce hydroxyl radical and then induce DNA strand breakage.^{5,6} The participation of H_2O_2 and hydroxyl radical in DNA breaks by the

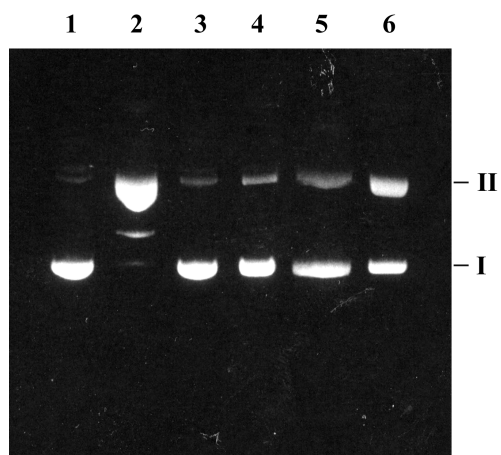


Figure 2. Effect of catalase and hydroxyl radical scavengers on DNA strand breakage. DNA was incubated with 20 mM lysine, 20 mM MG, and $100 \mu\text{M}$ Fe^{3+} in the presence of catalase (0.2 mg/mL) or 100 mM hydroxyl radical scavengers. Lane 1, DNA alone; lane 2, no addition of effector; lane 3, catalase; lane 4, formate; lane 5, mannitol; lane 6, ethanol. The reactions were stopped by freezing at -80°C . The samples were analyzed by electrophoresis on 0.8% agarose gel. I and II indicate the positions of the supercoiled and circular DNA plasmid forms, respectively.

glycation reaction of lysine with MG in the presence of Fe^{3+} was studied by examining the inhibitory effect of catalase and radical scavengers. DNA strand breakage was significantly inhibited by the addition of catalase in the reaction mixture (Fig. 2, lane 3). This implies that H_2O_2 may be involved in the DNA strand breakage. When plasmid DNA was incubated with lysine, MG and Fe^{3+} in the presence of hydroxyl radical scavengers such as formate, mannitol and ethanol at 37°C for 2 h, all tested scavengers prevented DNA strand breaks (Fig. 2, lane 4-6). It can be assumed that DNA cleavage is due to hydroxyl radicals generated in the reaction of Fe^{3+} with H_2O_2 .

Carnosine and related compounds are reported to be the active free radical scavenger.^{20,21} Effect of carnosine, homocarnosine and anserine on MG/lysine/ Fe^{3+} -mediated DNA

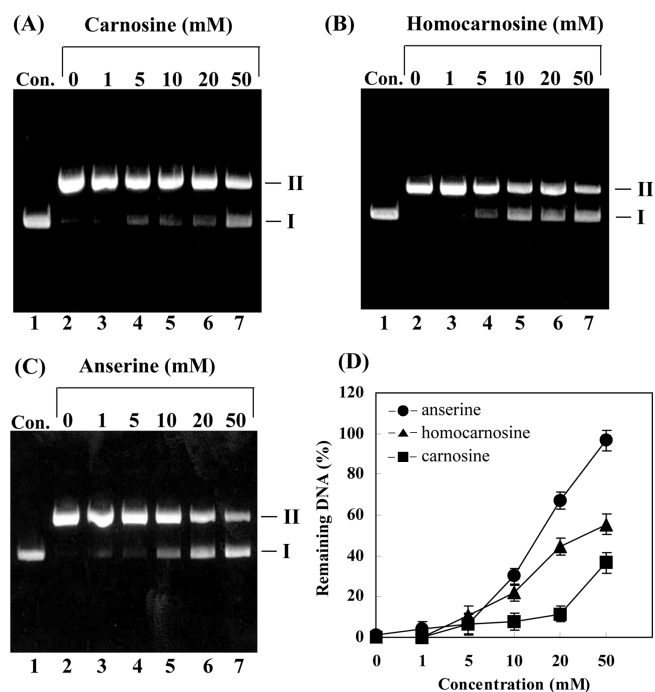


Figure 3. Effect of carnosine and homocarnosine on DNA cleavage. Control reaction mixture containing DNA ($1 \mu\text{g}$), 20 mM lysine, 20 mM MG, and $100 \mu\text{M}$ Fe^{3+} in 10 mM potassium phosphate buffer (pH 7.4). Various concentrations of carnosine, homocarnosine and anserine were added into the control reaction mixture. Reaction mixtures were incubated at 37°C for 2 h and an aliquot was analyzed by agarose gel electrophoresis. (A) Lane 1, DNA control; lane 2, incubated with 20 mM lysine, 20 mM MG, and $100 \mu\text{M}$ Fe^{3+} ; lane 3, lane 2 plus 5 mM carnosine; lane 4, lane 2 plus 10 mM carnosine; lane 5, lane 2 plus 20 mM carnosine; lane 6, lane 2 plus 50 mM carnosine. (B) Lane 1, DNA control; lane 2, incubated with 20 mM lysine, 20 mM MG, and $100 \mu\text{M}$ Fe^{3+} ; lane 3, lane 2 plus 5 mM homocarnosine; lane 4, lane 2 plus 10 mM homocarnosine; lane 5, lane 2 plus 20 mM homocarnosine; lane 6, lane 2 plus 50 mM homocarnosine. (C) Lane 1, DNA control; lane 2, incubated with 20 mM lysine, 20 mM MG, and $100 \mu\text{M}$ Fe^{3+} ; lane 3, lane 2 plus 5 mM anserine; lane 4, lane 2 plus 10 mM anserine; lane 5, lane 2 plus 20 mM anserine; lane 6, lane 2 plus 50 mM anserine. (D) Relative staining intensity of agarose gels was analyzed by densitometric scanning. I and II indicate the positions of the supercoiled and circular DNA plasmid forms, respectively. Values in (D) represent the mean \pm S.D. ($n = 3-5$).

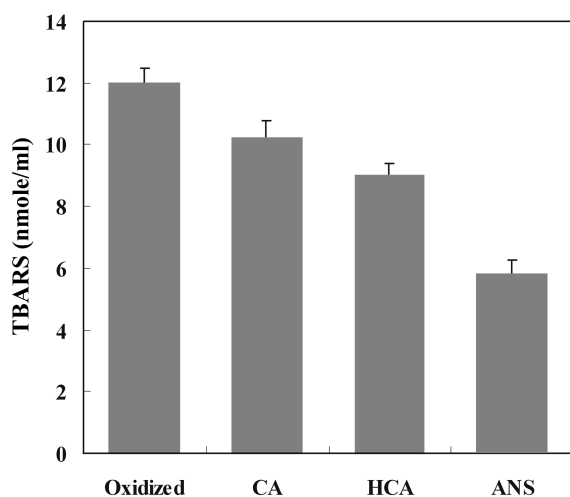


Figure 4. Effects of carnosine, homocarnosine and anserine on hydroxyl radical generation by the MG/lysine/Fe³⁺ system. The reaction mixtures contained 10 mM 2-deoxy-D-ribose and 20 mM lysine, 20 mM MG, and 100 μ M Fe³⁺ without (**Oxidized**) and with 20 mM carnosine (**CA**) or 20 mM homocarnosine (**HCA**) or 20 mM anserine (**ANS**). Reaction mixtures were incubated at 37 °C for 2 h. The degradation of 2-deoxy-D-ribose was measured by adding 200 μ L of PBS, 200 μ L of 2.8% (w/v) trichloroacetic acid, 200 μ L of 1% (w/v) thiobarbituric acid, followed by heating at 100 °C for 10 min. After cooling, the absorbance at 532 nm was measured. Data represent the means \pm S.D. ($n = 4-5$).

cleavage was investigated. These compounds showed a concentration-dependent inhibition of DNA cleavage induced by MG/lysine/Fe³⁺ (Fig. 3A, B, and C). The kinetics of the change in concentration of the intact DNA was assessed by densitometric scanning of intensity on agarose gels (Fig. 3D). Anserine inhibited DNA cleavage more effectively than carnosine and homocarnosine. Figure 4 shows the effect of carnosine and related compounds on the formation of hydroxyl radicals in MG/lysine/Fe³⁺ system. The generation of hydroxyl radicals in MG/lysine/Fe³⁺ system was measured with thiobarbituric acid-reactive substance (TBARS). The inhibitory activity of anserine was enhanced relative to those of carnosine and homocarnosine. Carnosine and related compounds have not been found to chelate iron in a manner that reduces its prooxidant activity.⁹ It has been reported that carnosine and related compounds quench 50-95% of hydroxyl radicals produced in the Fenton reaction.²² Therefore, it was suggested that the ability of carnosine and related compounds to inhibit iron-promoted oxidation was likely due to free radical scavenging activity.

MG is an endogenous metabolite which is present in increased concentrations in diabetics and secondary diabetic complications. The results presented here suggest that under hyperglycemic conditions, oxidative DNA damage may be induced by advanced glycation end products in the presence of transition metal ion, and may cause the irreversible deterioration seen in diabetes and diabetic complications. Although the present results were obtained from *in vitro* experiments, carnosine, homocarnosine and anserine may play an important role in the maintenance of the antioxidant system under hyperglycemic conditions.

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