Carnosine and Homocarnosine Inhibit Cytochrome *c*-Mediated DNA Strand Breakage

Jung Hoon Kang

Department of Genetic Engineering, Cheongju University, Cheongju 360-764, Korea. E-mail: jhkang@cju.ac.kr
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Peroxidases are ferric haem enzymes, which catalyses the oxidation of various organic and inorganic substrates by hydrogen peroxide or related compounds. The oxidation of substrates by classical peroxidases generally proceeds through two oxoferryl intermediates, which are two and one oxidizing equivalents, respectively, above the Fe(III) state.[1,2] It has been reported that the reaction between ferricytochrome *c* and hydrogen peroxide would initially form an oxoferryl derivative of hemeprotein.[3] Oxoferryl cytochrome *c*, similar to compound I of peroxidases, is assumed to contain two oxidizing equivalents, one in the form of oxoferryl heme ($Fe^{IV}=O$), and another as the porphyrin π radical.[3] Highly reactive ferryl-heme species are capable of oxidizing biomolecules including DNA, protein and lipid.[3,4] Oxidative damage of DNA has been hypothesized to play a critical role in several diverse biological processes including mutagenesis, aging, carcinogenesis, radiation effects and cancer chemotherapy.[5,6]

Carnosine, a naturally occurring dipeptide (β-alanyl-L-histidine), is found predominantly in long-lived tissues including the brain, innervated muscle, and the lens in high amounts (up to 20 mM in human muscle).[7] Carnosine can delay senescence and extend the life-span of cultured human fibroblasts, kill transformed cells, and protect cells against aldehydes and an amyloid peptide fragment.[7,8] The imidazolium group of histidine or carnosine stabilizes adducts formed at the primary amino group and may play an important role for an anticrosslinking agent.[9] Many biochemical studies have suggested that carnosine possesses antioxidant and free radical-scavenging function which may partly explain it apparent homeostatic function.[10,11] However, the inhibitory action of carnosine and related compounds against cytochrome *c*-mediated DNA damage has not been reported. In the current study, we examined the protective effect of carnosine and homocarnosine on the cytochrome *c*/H_2O_2 system-mediated DNA strand breakage.

Experimental Section

Materials. pUC19 plasmid DNA was prepared and purified from *E. coli* cultures by using QIAGEN plasmid kit (Santa Clarita, USA). Bovine cytochrome *c*, 4-aminoantipyrine and imidazole 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 (1 μg) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 2 h at 37 °C with 5 μM cytochrome *c* and 300 μM H_2O_2 in a total volume of 20 μL. The reactions were stopped by freezing at −80 °C. 5 μL of loading buffer (0.25% bromophenolblue, 40% sucrose) was added and samples 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 was detected and photographed under UV light in a dark room.

Measurement of peroxidase activity of cytochrome *c*. The peroxidase activity of cytochrome *c* was measured by using 4-aminoantipyrine.[3] Oxidation rate of 4-aminoantipyrine ($\varepsilon_M = 6.4 \times 10^3\ M^{-1}cm^{-1}$)[12] was followed at 505 nm. The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4) and 25 mM 4-aminoantipyrine and 1 mM hydrogen peroxide and 20 μM protein in a total volume of 1 mL. The reaction was initiated by addition of hydrogen peroxide and the increase in absorbance at 505 nm was measured by using a UV/Vis spectrophotometer (Shimazu 1601).

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

Results and Discussion

Figure 1 showed supercoiled form of plasmid DNA remained intact after incubation with 5 μM cytochrome *c* or 300 μM H_2O_2 alone. However, when DNA was incubated in a mixture of cytochrome *c* and H_2O_2, the DNA damage occurred in a time dependent manner. This indicates that both cytochrome *c* and H_2O_2 were required for the production of strand breaks in DNA. It was postulated that hydrogen peroxide oxidized cytochrome *c* to an oxoferryl derivative, compound I similar to that seen with peroxidases. The oxoferryl cytochrome *c* would then catalyze the oxidation of biomolecules.[3,4] Therefore, an oxoferryl derivative may be involved in cytochrome *c*-mediated DNA strand breakage. To test this possibility, we used imidazole, which presents the formation of the oxoferryl derivative through its binding to heme iron, replacing the axial ligand Met-80.[13] Imidazole effectively inhibited DNA strand breakage in a concentration dependent manner (Fig. 2). The ability of imidazole to protect DNA from the oxidative damage

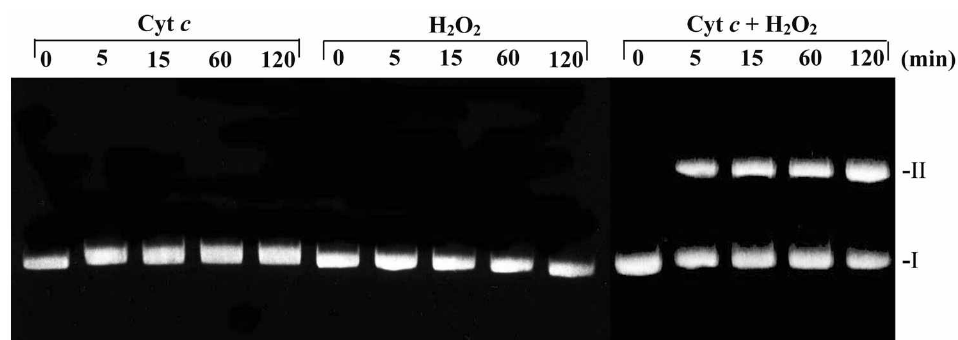

Figure 1. DNA strand breakage after the incubation with cytochrome *c* and H_2O_2. pUC 19 DNA (1 μg) was incubated at 37 °C for various incubation period with following: 5 μM cytochrome *c* (cyt *c*) alone, 0.3 mM H_2O_2 alone, 5 μM cyt *c* and 0.3 mM H_2O_2 respectively. Lane 1, 0 time control; lane 2, 5 min; lane 3, 15 min; lane 4, 60 min; lane 5, 120 min. Reaction was 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 open circular DNA plasmid forms, respectively.

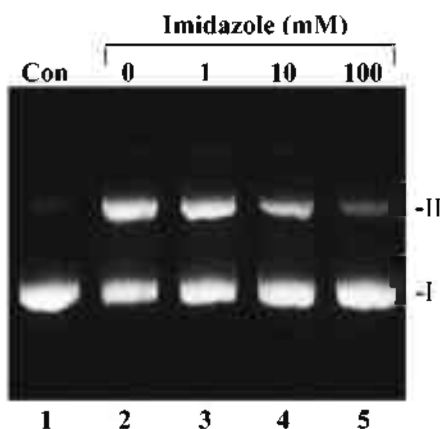

Figure 2. Effect of imidazole on DNA strand breakage induced by the cytochrome *c* and H_2O_2. pUC 19 DNA was incubated with 5 μM cytochrome *c* + 0.3 mM H_2O_2 in the presence of various concentrations of imidazole. Lane 1, DNA alone; lane 2, no addition of imidazole; lane 3, 1 mM imidazole; lane 4, 10 mM imidazole; lane 5, 100 mM imidazole.

indicates that peroxidase compound I (oxoferryl heme species) may participate in the mechanism of DNA strand break produced by the cytochrome *c* and H_2O_2 system. It has been reported that mitochondrial dysfunction may be involved in a pathogenesis of neurodegenerative disorders.[14] Therefore, oxidative damage of DNA by the cytochrome *c* and H_2O_2 system could be relatively favored in diseases where mitochondrial dysfunction is elevated such as Parkinson's disease.

Many functions have previously been proposed for carnosine; these include antioxidant and free radical scavenger, physiological buffer, neurotransmitter, radioprotectant, metal chelator, and wound healing agent.[15-18] In the present study, it was found that carnosine and homocarnosine significantly inhibited DNA strand breakage induced by the cytochrome *c*/H_2O_2 system (Fig. 3). It has been reported that imidazole-containing peptides, carnosine and related compounds, may react with di- or monoaldehydes, powerful cross-linking agents, released during the oxidative breakdown of unsaturated lipid.[19] In addition it was claimed in the literature[9] that

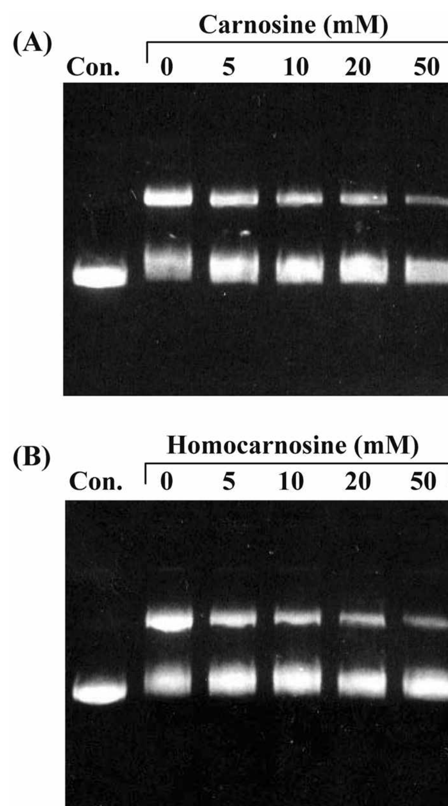

Figure 3. Effects of carnosine and homocarnosine on DNA strand breakage induced by the cytochrome *c* and H_2O_2. pUC 19 DNA was incubated with 5 μM cytochrome *c* + 0.3 mM H_2O_2 in the presence of various concentrations of carnosine (A) and homocarnosine (B) at 37 °C for 2 h.

L-carnosine could react with glucose and prevent glycation. Our data suggested that imidazolium group of carnosine might be inhibited the formation of the oxoferryl derivative. We next investigated whether carnosine and homocarnosine can inhibit the formation of peroxidase compound in the cytochrome *c*/H_2O_2 system. For these studies, the peroxidase activity of cytochrome *c* was measured by using 4-aminoantipyrine. Cytochrome *c* is electron transfer protein, and it catalyses peroxidase-like reaction *in vitro*.[20,23] It catalyses

Table 1. Effects of carnosine and homocarnosine on the oxidation of 4-aminoantipyrine by cytochrome c/H_2O_2 system

Compound	Oxidation rates of 4-aminoantipyrine	
	μmole/min	% of control
None	13.6 ± 0.6	100
Carnosine	7.1 ± 0.5	52
Homocarnosine	9.7 ± 0.8	71

several reactions such as hydroxylation and aromatic oxidation and shows peroxidase activity by oxidation of various electron donors such as 2-keto-4-thiomethyl butyric acid, 4-aminoantipyrine, luminol and 2,2'-azino-bis(3-ethyl-bezthiazoline-6-sulphonic acid).[20,21] It has been reported that the peroxidase activity of cytochrome *c* could induce the modification of biological macromolecules.[4,22] When 5 μM cytochrome *c* was incubated with 300 μM H_2O_2 in the presence of 10 mM carnosine or 10 mM homocarnosine at 37 °C, all compounds effectively inhibited the peroxidase activity of cytochrome *c* (Table 1). The results suggested that carnosine and homocarnosine may protect DNA against the oxidative damage by the cytochrome *c*/H_2O_2 system through the inhibition of the formation of peroxidase compound I in the reaction of cytochrome *c* with H_2O_2.

In conclusion, the data presented in this paper are consistent with the protective action of carnosine and homocarnosine against oxidative damage of DNA by the cytochrome *c* and H_2O_2 system. Therefore, these compounds should be explored as potential therapeutic agents for pathogenesis of oxidative stress associated to cytochrome c-mediated neurodegenerative disorders.

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