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Protective effects of carnosine and homocarnosine on ferritin and hydrogen peroxide-mediated DNA damage

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Previous studies have shown that one of the primary causes of increased iron content in the brain may be the release of excess iron from intracellular iron storage molecules such as ferritin. Free iron generates ROS that cause oxidative cell damage. Carnosine and related compounds such as endogenous histidine dipetides have antioxidant activities. We have investigated the protective effects of carnosine and homocarnosine against oxidative damage of DNA induced by reaction of ferritin with H_2O_2. The results show that carnosine and homocarnosine prevented ferritin/H_2O_2-mediated DNA strand breakage. These compounds effectively inhibited ferritin/H_2O_2-mediated hydroxyl radical generation and decreased the mutagenicity of DNA induced by the ferritin/H_2O_2 reaction. Our results suggest that carnosine and related compounds might have antioxidant effects on DNA under pathophysiological conditions leading to degenerative damage such as neurodegenerative disorders. [BMB reports 2010; 43(10): 683-687]

INTRODUCTION

Excess free iron promotes the generation of reactive oxygen species (ROS) based on the Fenton reaction, which leads to oxidative stress. Hence, inappropriate regulation of iron metabolism and abnormal release of iron from ferritin will provoke oxidative cell damage. Ferritin is composed of 24 subunits, which form a cavity that can store up to 4,500 atoms of ferric ions (1). Mammalian ferritin complexes are heteropolymers composed of two types of subunits, termed H (heavy) and L (light), which are present in various ratios in different tissues. 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.

It has been reported that iron can be released from ferritin by various exogenous (2-5) and endogenous substances via reductive mechanisms (6, 7). If iron is released from ferritin, low molecular weight iron complexes may undergo redox reactions, resulting in cytotoxic damage to macromolecules (8, 9). Oxidants, including H_2O_2, are considered mostly as damaging entities that mediate pathogenic processes. H_2O_2 has been implicated in ischemia and reperfusion within the brain (10), in cancer (11) and in neurodegenerative disease (12).

Carnosine, a naturally occurring dipeptide (β-alanyl-L-histidine), is found predominantly in long-lived tissues, including the brain, muscle and stomach, in high amounts (13). Carnosine has been demonstrated to play a number of biological roles as an anti-inflammatory agent, free radical scavenger and protein glycosylation inhibitor (14, 15). More recently, it has been suggested that carnosine and homocarnosine protect neuronal cells against glutamate-induced toxicity (16). In addition, carnosine has been demonstrated to protect PC12 cells from Aβ42-induced neurotoxicity via regulation of glutamate release (17) However, no information is yet available on the effect of carnosine and related compounds on ferritin-mediated DNA damage. In the current study, we examined the protective effects of carnosine and homocarnosine on ferritin/H_2O_2 system-mediated DNA damage.

RESULTS AND DISCUSSION

DNA strand breakage can be detected by a gel electrophoresis method involving supercoiled plasmid DNA. Strand breakage causes 'relaxation' or opening of circular and linear forms of DNA, which are observed as different bands on agarose gel. As shown in Fig. 1A, the plasmid DNA remained intact after incubation with either 10 μM ferritin or 1 mM H_2O_2, whereas DNA was cleaved by a mixture of ferritin and H_2O_2. This indicates that both ferritin and H_2O_2 were required to produce strand breaks in DNA. When DNA was incubated in a mixture of H_2O_2 and ferritin, a substantial increase in the proportion of nicked circular DNA (form II) and linear DNA (form III) occurred with concomitant loss of supercoiled DNA (form I) in a concentration of ferritin-dependent manner (Fig. 1B). It has been shown that the reaction of ferritin with H_2O_2 generates free radicals that oxidize amino acid residues at or near the

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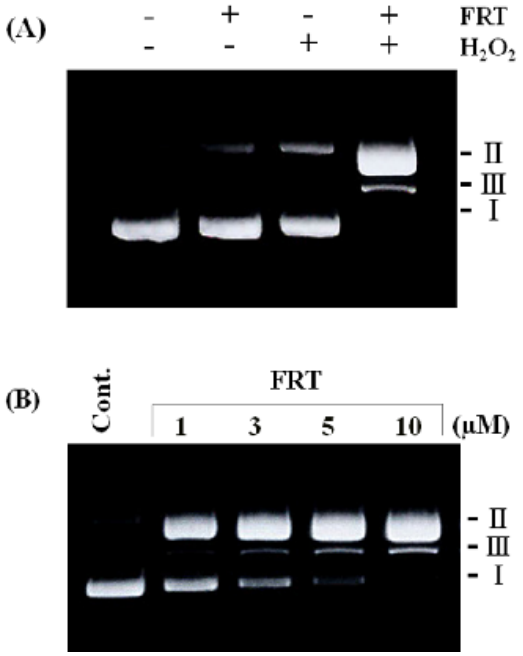

Fig. 1. DNA cleavage is induced by the ferritin and H_2O_2 system. (A) pUC 19 DNA (1 μg) was incubated with ferritin and H_2O_2 in 10 mM potassium phosphate buffer (pH 7.4) at 37°C for 3 h with the following: Lane 1, control DNA; lane 2, 10 μM ferritin; lane 3, 1 mM H_2O_2; lane 4, 10 μM ferritin + 1 mM H_2O_2 (B) pUC 19 DNA was incubated with 1-10 μM ferritin and 1 mM H_2O_2 at 37°C for 3 h. Reaction was stopped by freezing at −80°C. Loading buffer was added to the samples, followed by electrophoresis on 0.8% agarose gel. I, II and III indicate the positions of the supercoiled, nicked circular and linear DNA plasmid forms, respectively.

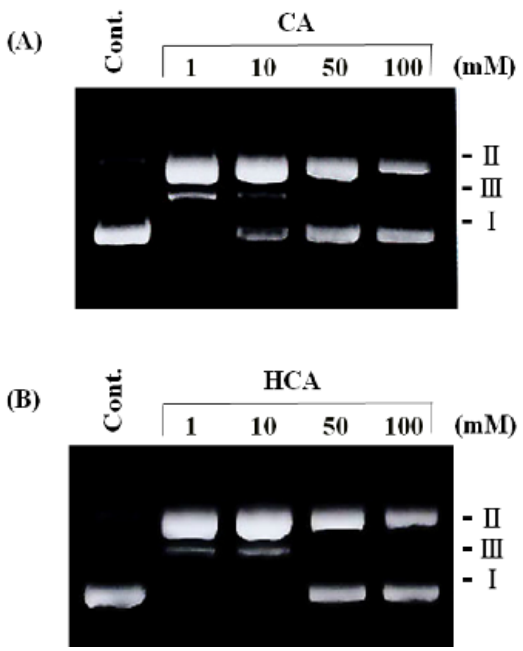

Fig. 2. Effects of carnosine and homocarnosine on DNA strand breakage induced by the ferritin and H_2O_2 system. pUC19 DNA was incubated with 10 μM ferritin + 1 mM H_2O_2 in the presence of various concentrations of carnosine (A) and homocarnosine (B) at 37°C for 3 h. Agarose gel electrophoresis was performed in 0.8% agarose. I, II and III indicate the positions of the supercoiled, nicked circular and linear DNA plasmid forms, respectively.

cation-binding site, which results in introduction of carbonyl groups (18). In cultured cells, raising the level of iron in the culture medium leads to increases in the steady-state levels of oxidative DNA damage (19). Previous studies have suggested that iron ions are able to stimulate the Fenton-like reaction for production of hydroxyl radicals, which mediates DNA strand breakage (20). The present result suggests that free radicals might be involved in ferritin/H_2O_2-induced DNA strand breakage.

Many functions have previously been proposed for carnosine, including as an antioxidant and free radical scavenger, physiological buffer, neurotransmitter, radioprotectant, metal chelator and wound healing agent (21-24). In the present study, it was found that carnosine and homocarnosine significantly inhibited DNA strand breakage induced by the ferritin/H_2O_2 system (Fig. 2). It has been reported that imidazole-containing peptides, such as carnosine and related compounds, may react with di- or mono-aldehydes, powerful cross-linking agents, which are released during the oxidative breakdown of unsaturated lipids (25). Our data suggest that the imidazolium group of carnosine inhibits the formation of the oxoferryl derivative. Attack of .OH on the 2-deoxyribose sugar produces a huge variety of different products, some of which are mutagenic in bacterial systems. Some of the fragmentation products, when can be detected by the addition of thiobarbituric acid (TBA) to the reaction mixture, result in formation of a pink $(TBA)_2$-MDA chromogen (26). This can then be used to detect .OH production, although it is unclear whether or not some other ROS can also degrade deoxyribose. Our results show damage to deoxyribose induced by the ferritin and H_2O_2 system did occur (Fig. 3A). Therefore, the released iron ions could have enhanced the Fenton-like reaction to produce .OH and play a critical role in DNA cleavage.

We next investigated whether or not carnosine and homocarnosine can inhibit the formation of hydroxyl radicals in the ferritin/H_2O_2 system. When ferritin was incubated with H_2O_2 in the presence of carnosine and homocarnosine at 37°C, all compounds effectively inhibited the formation of hydroxyl radicals (Fig. 3B). The results suggest that carnosine and homocarnosine may protect DNA against oxidative damage induced by the ferritin/H_2O_2 system through the scavenging of free radicals.

H_2O_2 produced *in vivo* is probably a direct product of O_2^-.

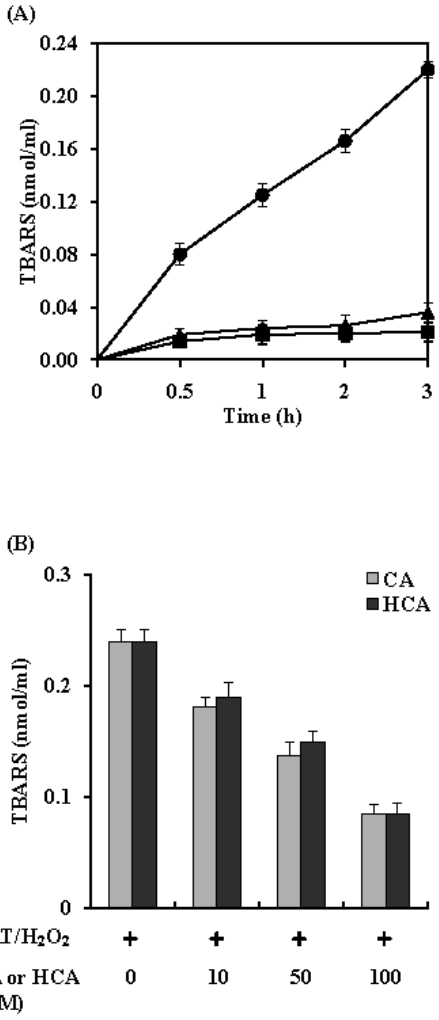

Fig. 3. Effects of carnosine and homocarnosine on hydroxyl radical formation induced by the ferritin and H_2O_2 system. (A) 10 mM 2-deoxy-D-ribose was incubated at 37°C for various incubation periods with the following: 10 μM ferritin alone (▲); 1 mM H_2O_2 alone (■); 10 μM ferritin + 1 mM H_2O_2 (●). (B) The reaction mixtures contained 10 mM 2-deoxy-D-ribose, 10 μM ferritin and 1 mM H_2O_2 in the presence of carnosine (CA) and homocarnosine (HCA) at pH 7.4 for 3 h. Hydroxyl radicals were determined by measuring TBARS as described in Materials and Methods.

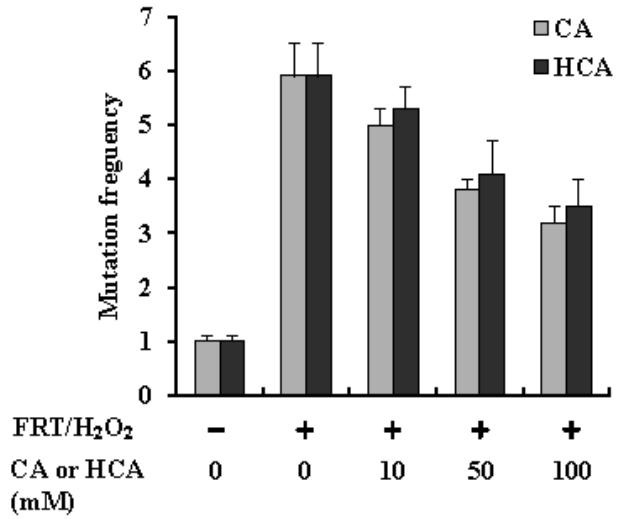

Fig. 4. Effects of carnosine and homocarnosine on DNA mutagenicity induced by the ferritin and H_2O_2 system system. Plasmid pUC19 carrying the *lacZ′* gene was treated with 10 μM ferritin and 1 mM H_2O_2 in the presence of carnosine (CA) and homocarnosine (HCA) and then used to transform *Escherchia coli* DH5α competent cells. Transformed cells were spread on LB agar plates containing 0.8 mg of X-gal, 2 mg of IPTG and 0.01% ampicillin.

dismutation and various other oxidase reactions. The rates of H_2O_2 and O_2^-. formation under physiological conditions have been measured. Considering the volume actually used and the amount of products formed, a rates of 90 μM H_2O_2/min in liver (27) and 340 μM O_2^-./min in glucose-fed *Escherichia coli* cells have been obtained (28). However, the local concentration of H_2O_2 in the immediate vicinity of SOD will be much larger than these values. Further, the production of O_2^-. will be much larger as the concentration of oxygen increases during exposure to hyperoxia or when the respiratory chain becomes inhibited, leading to an increased concentration of reducing equivalents (29). Recently it was reported that hyperoxia-induced H_2O_2 production is increased in human U87 glioblastoma cells (30). Thus, H_2O_2 will be produced continuously at a rate of at least 0.1 mM/min under physiological conditions and at a much higher rate under adverse conditions, such as hyperoxia or ischemia and reperfusion.

It was previously reported that the mutagenic spectrum of oxygen free radicals is produced by the aerobic incubation of single-strand M13mp2 DNA with iron (31). Therefore, the effects of carnosine and homocarnosien on the mutation of DNA induced by ferritin/H_2O_2 were investigated. pUC19 plasmid DNA was treated with or without ferritin and H_2O_2. Damaged DNA was transfected into *E. coli* (DH5α) competent cells, and mutant cells within the nonessential *lacZ′*α-gene for β-galactosidase were identified by observing decreased α-complementation. *E. coli* harboring active β-galactosidase produced dense blue colonies, whereas mutation within the *lacZ′* α segment of pUC19 plasmid DNA induced the expression of substantially less active β-galactosidase, yielding light blue or white colonies. The frequency of mutants obtained with ferritin/H_2O_2 was approximately six-fold greater than that obtained with untreated DNA. The mutagenicity of DNA by the ferritin/H_2O_2 system was inhibited by carnosine and homocarnosine (Fig. 4). 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, which

prevents them from participating in the deleterious Fenton reaction. However, carnosine and related compounds have not been found to chelate iron in a manner that reduces its prooxidant activity (32). It has been reported that carnosine and related compounds quench 50-95% of hydroxyl radicals produced in the Fenton reaction (33). Therefore, it was suggested that the ability of carnosine and related compounds to inhibit ferritin/H_2O_2-mediated DNA damage was likely due to free radical scavenging activity.

In conclusion, the data presented in this paper are consistent with the protective actions of carnosine and homocarnosine against oxidative damage of DNA by the ferritin and H_2O_2 system. Therefore, these compounds should be explored as potential therapeutic agents of oxidative stress associated with neurodegenerative disorders.

MATERIALS AND METHODS

Materials

pUC19 plasmid DNA was prepared and purified from *E. coli* cultures using a QIAGEN plasmid kit (Santa Clarita, USA). Ampicillin, bathophenanthroline sulfonate, deferoxamine and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Ferritin was purchased from Cabiochem (Darmstadt, German). Commercial equine spleen ferritin was performed by gel filtration chromatography using a Superose 6 FPLC column (Pharmacia, Sweden) for further purification. 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

DNA single strand breakages were assayed by measuring the conversion of supercoiled plasmid DNA (form I) into nicked circular DNA (form II) and linear DNA (form III). pUC19 DNA (0.5-1.0 μg) in 10 mM potassium phosphate buffer (pH 7.4) was incubated for 3 h at 37°C with different concentrations of ferritin and 1 mM H_2O_2 in a total volume of 20 μl. The reaction was stopped at −80°C. The 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 were detected and photographed under UV light in a dark room.

Measurement of hydroxyl radical

Hydroxyl radicals were detected by measuring thiobarbituric acid reactive substance (TBARS) according to a previously described method with some modifications (26). The assay mixture contained 10 mM potassium phosphate buffer (pH 7.4), 10 mM 2-deoxy-D-ribose, 10 μM ferritin and 1 mM H_2O_2 in a total volume of 100 μl. Reaction mixtures were incubated at 37°C for 3 h. The degradation of 2-deoxy-D-ribose was measured by addition of 2.8% trichloroacetic acid (200 μl), PBS (200 μl) and 1% thiobarbituric acid (200 μl), followed by heating at 100°C for 15 min. After the samples were cooled to room temperature and centrifuged at 15,000 rpm for 10 min, results were read at 532 nm by a UV/vis spectrophotometer (Shimadzu, UV-1601). All solutions used in the present experiments were treated with Chelex 100.

Mutagenecity assay

Plasmid DNA was transformed in *Escherchia coli* DH5α competent cells. The treated plasmid pUC19 carrying the *lacZ′* gene was incubated with or without 10 μM ferritin and 1 mM H_2O_2 with 50 μl of competent cells at 0°C for 10 min. SOC medium was added and the cells incubated at 37°C for 1 h. Transformed cells were spread on LB agar plates containing 0.8 mg of X-gal, 2 mg of IPTG and 0.01% ampicillin. The plates were inverted and incubated for 24 h at 37°C before counting colonies.

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 Student's *t*-test.

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