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Reaction of ferritin with hydrogen peroxide induces lipid peroxidation

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Lipid peroxidation is known to be an important factor in the pathologies of many diseases associated with oxidative stress. We assessed the lipid peroxidation induced by the reaction of ferritin with H_2O_2. When linoleic acid micelles or phosphatidyl choline liposomes were incubated with ferritin and H_2O_2, lipid peroxidation increased in the presence of ferritin and H_2O_2 in a concentration-dependent manner. The hydroxyl radical scavengers, azide and thiourea, prevented lipid peroxidation induced by the ferritin/H_2O_2 system. The iron specific chelator desferoxamine also prevented ferritin/H_2O_2 system-mediated lipid peroxidation. These results demonstrate the possible role of iron in ferritin/H_2O_2 system-mediated lipid peroxidation. Carnosine is involved in many cellular defense processes, including free radical detoxification. In this study, carnosine, homocarnosine, and anserine were shown to significantly prevent ferritin/H_2O_2 system-mediated lipid peroxidation and also inhibited the free radical-generation activity of ferritin. These results indicated that carnosine and related compounds may prevent ferritin/H_2O_2 system-mediated lipid peroxidation via free radical scavenging. [BMB reports 2010; 43(3): 219-224]

INTRODUCTION

Iron is an essential element in a variety of vital processes, including respiratory electron transfer, oxygen transport, and drug metabolism (1). However, when iron reacts with H_2O_2, hydroxyl radicals are produced via a Fenton-type reaction. The hydroxyl radical is the most powerful oxidizing species among several reactive-oxygen radicals, and is capable of oxidizing most macromolecules including nucleic acids, lipids, and proteins.

The principal function of ferritin is the storage and detoxification of intracellular iron (2). Ferritin consists of a protein shell of 24 subunits arranged in a large cavity, which can accommodate a core of up to 4500 atoms of hydrous ferric oxide polymers (3). Mammalian ferritins are constructed of different proportions of heavy chain (H-chain) and light chain (L-chain) components. It has been demonstrated that the presence of both subunits in hybrids renders the molecule more efficient in iron incorporation, owing to the combined action of the ferroxidase centers on the H-chain and the effective nucleation centers on the L-chain (4). As long as iron is bound to ferritin, cytotoxic reactions are not expected to occur. However, iron can be released from ferritin by a variety of exogenous (5, 6) and endogenous substances (7, 8). When iron is released from ferritin, low molecular iron complexes may undergo redox reactions, thus inflicting cytotoxic damage upon macromolecules (9-11).

Iron ions are strong catalysts for the peroxidation of membrane lipids, and give rise to membrane damage (12-14). It has become increasingly evident that this type of peroxidative damage to low-density lipoprotein plays a pivotal role in the etiology of atherosclerosis (15, 16). Lipid peroxidation may also contribute to other human diseases, including certain types of cancer, rheumatoid arthritis, and myocardial reoxygenation injuries, and may also play an important role in degenerative processes associated with aging (11, 17).

In this study, we assessed the lipid peroxidation induced by the reaction of ferritin with H_2O_2. Lipid peroxidation was shown to increase in the presence of ferritin and H_2O_2 in a concentration-dependent manner. Hydroxyl radical scavengers and an iron specific chelator prevented ferritin/H_2O_2 system-mediated lipid peroxidation.

RESULTS AND DISCUSSION

Lipid peroxidation by the ferritin/H_2O_2 system

In order to determine whether the ferritin/H_2O_2 system induces the peroxidation of fatty acid and phospholipid. The peroxidation of linoleic acid micelles did not occur after incubation with 0.5 μM ferritin or 1 mM H_2O_2 alone. However, when the fatty acid micelles were incubated in a mixture of ferritin and H_2O_2, lipid peroxidation increased for 4 h in a time-dependent manner (Fig. 1A). Similar results were noted when the phosphatidylcholine liposomes were incubated in the ferritin/H_2O_2 system (Fig. 1B). This revealed that both ferritin and H_2O_2 were required for lipid peroxidation. Reactive carbonyl compounds

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formed endogenously during lipid peroxidation are precursors of advanced lipid peroxidation end products, which form cross-links on cellular proteins (carbonyl stress), and accumulate during aging or in association with chronic diseases (18). Carbonyl stress induces progressive protein dysfunction and damage to all tissues, with pathological consequences including inflammation and apoptosis, which in turn contribute to the progression of diseases (18, 19). Therefore, the results of this study indicate that ferritin-mediated lipid peroxidation may play a pivotal role in aging, as well as in the pathogenesis of a variety of diseases.

The peroxidation of both the linoleic acid micelles and the phosphatidylcholine liposomes were increased by concentrations up to 5 mM H_2O_2 (Fig. 1C) and 1 μM ferritin (Fig. 1D), respectively. Our results revealed that ferritin/H_2O_2 system-iduced lipid peroxidation depends on the concentrations of ferritin and H_2O_2.

Generation of hydroxyl radicals in the ferritin/H_2O_2 system

We also evaluated hydroxyl radical production in the ferritin/H_2O_2 system by measuring the fluorescence emission spectrum in the region between 350 and 500 nm with an excitation of 305 nm. The reactions were conducted using benzoate in the presence of ferritin and H_2O_2. As the reactions proceeded, the emission peak at 410 nm (due to hydroxybenzoate formation) increased (Fig. 2A).

Effect of hydroxyl radical scavengers on the generation of hydroxyl radicals in the ferritin/H_2O_2 system

We assessed the participation of the hydroxyl radical in the ferritin/H_2O_2 system-induced lipid peroxidation by examining the protective effects of the hydroxyl radical scavengers, azide and thiourea. When the linoleic acid micelles or the phosphatidylcholine liposomes were incubated with ferritin and H_2O_2 in the presence of hydroxyl radical scavengers, all of the scavengers were shown to exert a significant protective effect against lipid peroxidation (Table 1). These results indicate that hydroxyl radicals may participate in the critical mechanisms of ferritin/H_2O_2 system-induced lipid peroxidation.

Participation of iron ions in the ferritin/H_2O_2-mediated lipid peroxidation

Trace metal such as iron and copper, which are present in a variety of biological systems, may interact with reactive oxygen species, thus inflicting damage upon macromolecules (12, 20, 21). The cleavage of the metalloproteins by oxidative dam-

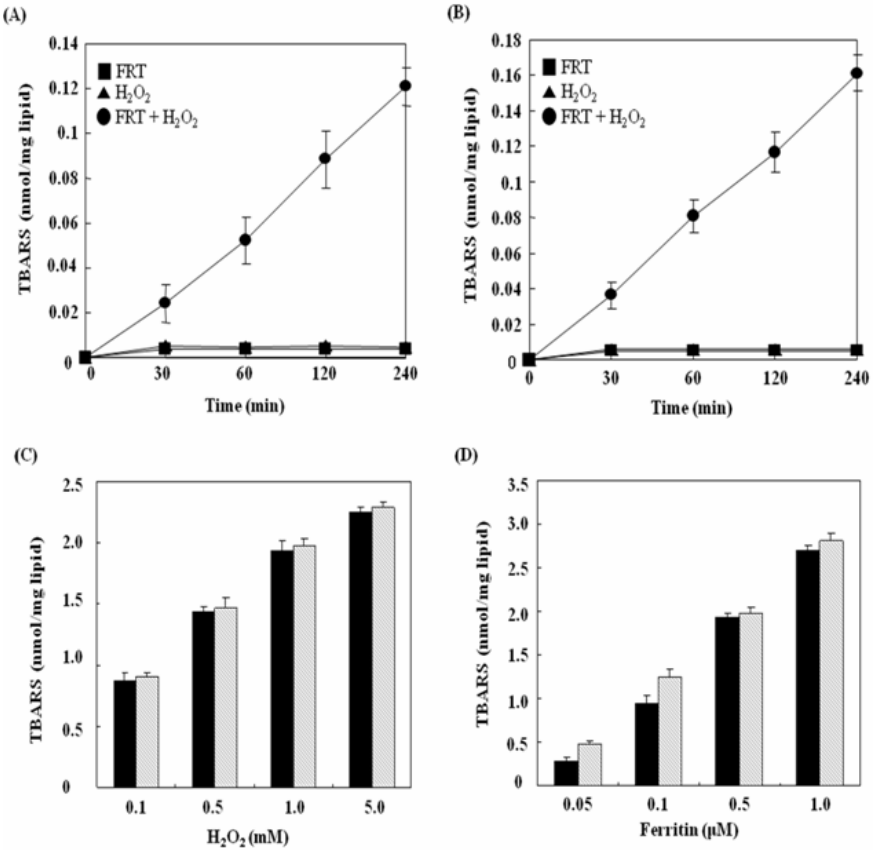

Fig. 1. Lipid peroxidation induced by incubation with ferritin and H_2O_2. Linoleic acid micelles (A) or phosphatidylcholine liposomes (B) were incubated at 37°C for various incubation periods with the following; 0.5 μM ferritin alone (■); 1 mM H_2O_2 alone (▲); 0.5 μM ferritin plus 1 mM H_2O_2 (●). (C) Linoleic acid micelles (■) or phosphatidylcholine liposomes (■) were incubated with 0.5 μM ferritin and 0.5-1 mM H_2O_2 at 37°C for 4 h. (D) Linoleic acid micelles (■) or phosphatidylcholine liposome (■) were incubated with 0.05-1 μM ferritin and 1 mM H_2O_2. Data are expressed as the means ± S.D. (n = 3-5).

age may result in increases in the levels of metal ions in some biological cells (22). The participation of iron ions in ferritin/H_2O_2-mediated lipid peroxidation was assessed via an examination of the protective effects of the iron chelator, deferoxamine (DFX). The results demonstrated that DFX prevented the peroxidation of both the linoleic acid micelles and phosphatidylcholine liposomes induced by the ferritin/H_2O_2 system (Fig. 2B). It has been previously reported that iron ions are released from the reaction of ferritin with H_2O_2. Because iron can be stimulated by a Fenton reaction to amplify hydroxyl radicals, the ferritin/H_2O_2 system may function to accelerate lipid peroxidation.

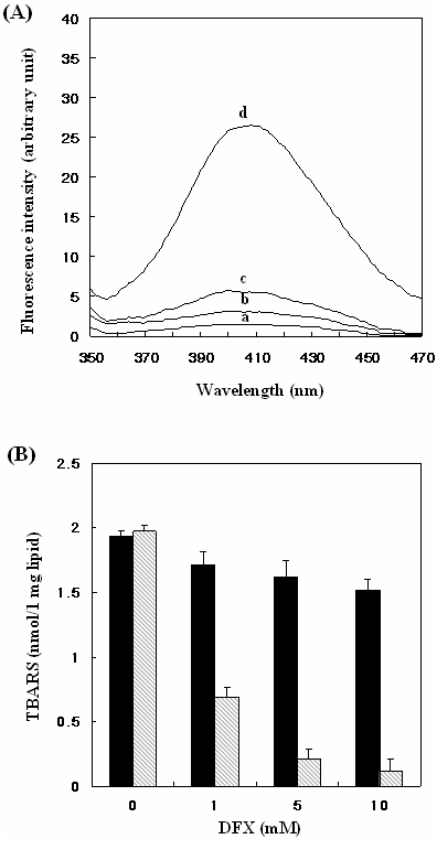

Fig. 2. Fluorescence scans of benzoate during the reaction of ferritin with H_2O_2 and effect of DFX on lipid peroxidation. (A) The fluorescence spectra of the formation of 4-hydroxybenzoate were observed when benzoate was incubated with or without 30 μM EDTA, 0.5 μM ferritin and 1 mM H_2O_2. (a) benzoate (b) ferritin (c) H_2O_2 (d) ferritin + H_2O_2. (B) Linoleic acid micelles (■) or phosphatidylcholine liposomes (▒) were incubated with 0.5 μM ferritin and 1 mM H_2O_2 at 37°C for 4 h. in the absence of DFX or in the presence of various concentrations of DFX. Data are expressed as the means ± S.D. (n = 3-5).

Lipid peroxidation frequently occurs in response to oxidative stresses, and a broad variety of aldehydes are formed upon the breakdown of lipid peroxides in biological systems. Of these, certain aldehydes are highly reactive and may be considered as secondary toxic compounds, which can themselves disseminate and augment initial free radical events (23). These aldehydes can also function as bioactive molecules under physiological and/or pathological conditions (24). The central nervous system is particularly vulnerable to oxidative stress, owing to its high rate of oxygen utilization and abundance of unsaturated lipids. Thus, a great deal of attention has been focussed on the possible roles of lipid peroxidation-derived aldehydes in terms of their contributions to neuronal dysfunction in oxidative stress-associated neurodegenerative disorders (25).

Effects of carnosine and related compounds on the ferritin/H_2O_2-mediated lipid peroxidation

Carnosine and its related compounds, homocarnosine and anserine, are present at relatively high concentrations (1-20 mM) in the muscle and brain tissues of humans and other vertebrates (26, 27). These compounds have been shown to be extremely efficient antioxidant molecules (28). We also determined that carnosine and related compounds significantly inhibited ferritin/H_2O_2 system-induced lipid peroxidation (Fig. 3 A, B). It was previously reported that carnosine and its related compounds quench hydroxyl radicals generated in the Fenton reaction (29, 30). As the effects of carnosine and its related compounds on free radical generation by the ferritin/H_2O_2 system were examined in this study, when ferritin was incubated with H_2O_2 in the presence of carnosine, homocarnosine, or anserine at 37°C, all compounds were shown to inhibit hydroxyl radical generation (Fig. 3C). These compounds may be able to bind Cu^{2+} and prevent some Cu^{2+}-dependent radical

Table 1. Effect of radical scavengers on lipid peroxidation induced by the ferritin and hydrogen peroxide system

Addition	Concentration	Linoleic acid		Phosphatidylcholine	
		Amont of TBARS (nmol/mg Lipid)	(%)	Amont of TBARS (nmol/mg Lipid)	(%)
None		1.935 ± 0.032	100	1.976 ± 0.044	100
Azide	20 mM	1.556 ± 0.051	80.4	1.689 ± 0.045	85.4
	100 mM	1.383 ± 0.047	71.4	1.475 ± 0.021	74.6
Thiourea	20 mM	1.560 ± 0.064	80.6	0.895 ± 0.038	45.3
	100 mM	1.262 ± 0.036	65.2	0.512 ± 0.059	25.9

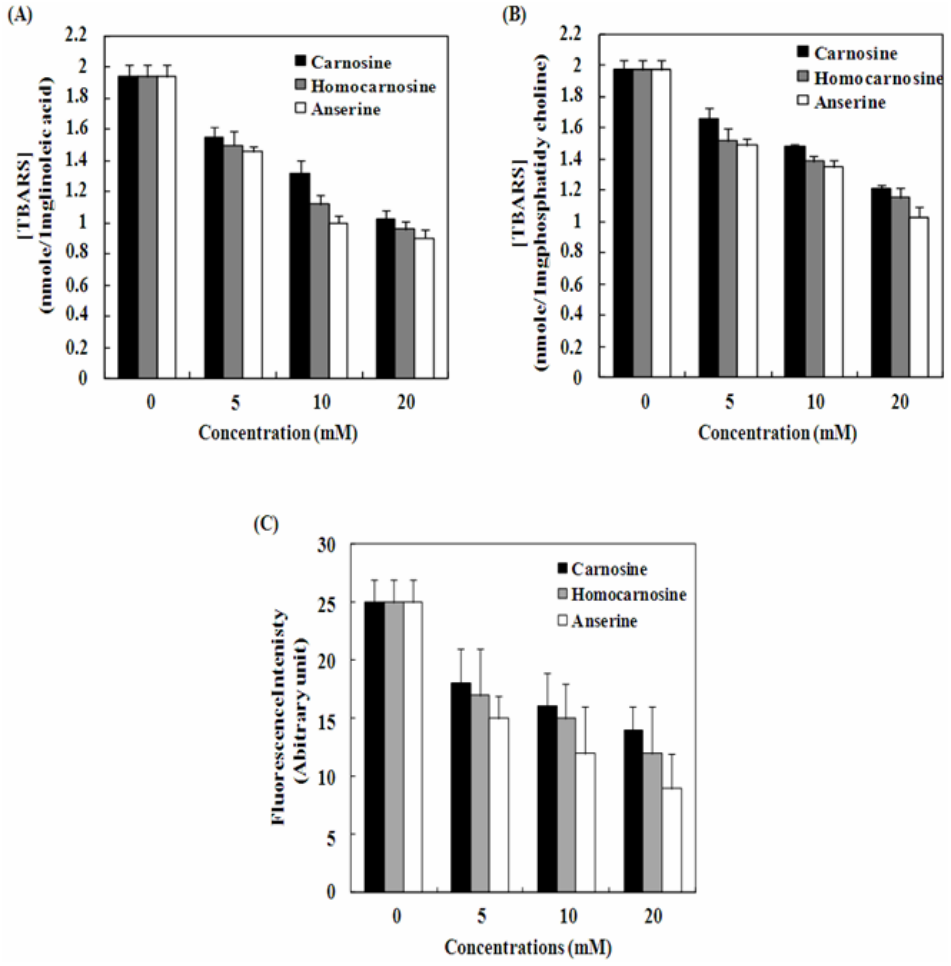

Fig. 3. Effects of carnosine and related compounds on lipid peroxidation and generation of hydroxyl radicals in the ferritin and H_2O_2 system. Linoleic acid micelles (A) or phosphatidylcholine liposomes (B) were incubated with 0.5 μM ferritin and 1 mM H_2O_2 at 37°C for 4 h in the presence of various concentrations of carnosine (■), homocarnosine (▩) and anserine (□). (C) The fluorescence intensity of 4-hydroxybenzoate was noted when benzoate was incubated with 0.5 μM ferritin and 1 mM H_2O_2 at 37°C for 4 h in the presence of various concentrations of carnosine (■), homocarnosine (▩) and anserine (□). Data are expressed as the means ± S.D. (n = 3-5).

reactions. However, they have not been shown to chelate iron in a manner in which the pro-oxidant activity of iron would be ameliorated (26). Thus, the ability of carnosine to prevent ferritin/H_2O_2-mediated lipid peroxidation is likely attributable to a free radical scavenging effect.

Lower levels of endogenous H_2O_2 are essential for normal physiological functioning and signaling, whereas higher levels are associated with a variety of diseases. Lacy et al. previously reported that normal human plasma H_2O_2 concentration ranges from 1 to 8 μM, with an average of approximately 3 μM (31, 32). Phagocytes generate and secrete higher levels of H_2O_2 than other cell types. Activated neutrophils generate up to 6×10^{-14} mol H_2O_2/h/cell, generating a local concentration of 10-1002 μM (33). Elevated H_2O_2 concentrations are detected in end-stage renal disease (34) and essential hypertension (34), ischemia and reperfusion in the brain (35), cancer (36) and Alzheimer's disease, in which β-amyloid stimulates threefold increases in ROS including H_2O_2 (37). Hence, under such abnormal conditions, the ferritin/H_2O_2 system may cause lipid peroxidation *in vivo*.

In conclusion, our results indicate that lipid peroxidation can indeed be induced by the reaction of ferritin with H_2O_2, which involves the generation of hydroxyl radicals. Lipid peroxidation can lead to the formation of several reactive carbonyl compounds that play roles in aging and oxidative stress-associated diseases, including atherosclerosis, diabetes, and many neurodegenerative disorders (38). Therefore, ferritin/H_2O_2-mediated lipid peroxidation may, in part, explain the association between the pathogenesis of oxidative stress-related diseases and ferritin. It can also be surmised that carnosine, homocarnosine, and anserine may protect against ferrritin/H_2O_2-mediated lipid peroxidation via a free radical scavenging mechanism.

MATERIALS AND METHODS

Materials

Deferoxamine (DFX), linoleic acid, soybean phosphatidylcho-

line, thiobarbituric acid and trichloroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Equine spleen ferritin was obtained from Calbiochem (Darmstadt, German). Commercial ferritin was purified further via gel filtration chromatography using a Superose 6 FPLC column (Pharmacia, Sweden). Chelex 100 resin (sodium form) was acquired from Bio-Rad (Hercules, CA, USA).

Preparation of phospholipid liposomes and fatty acid micelles

The phospholipid liposomes were prepared in accordance with a previously described method (39). Soybean phosphatidylcholine dissolved in chloroform-methanol was placed into a round-bottomed flask, and chloroform-methanol was removed with a rotary vacuum evaporator, leaving a thin lipid film on the wall of the flask. A quantity of 10 mM potassium phosphate (pH 7.4) was added, and the mixture was vigorously shaken to yield a milky suspension. The suspension was then sonicated 3 times at 30 watts for 20 s at 4°C to obtain unilamellar liposomes. A linoleic acid stock solution of 10 mg/ml was prepared in ethanol, and stored at −20°C under nitrogen. Immediately prior to use, an aliquot of the stock solution was dried with nitrogen, diluted with 10 mM potassium phosphate (pH 7.4) to a concentration of 10 mg fatty acid/ml, and subsequently sonicated 3 times at 30 watts for 5 s at 4°C to induce the formation of fatty acid micelles.

Assay for lipid peroxidation

As an index of lipid peroxidation, thiobarbituric acid-reactive substance (TBARS) was determined via a modified version of previously described method (40). Linoleic acid micelles (1 mg/ml) or phosphatidylcholine liposomes (1 mg/ml) were incubated with different concentrations of ferritin and H_2O_2 in 10 mM potassium phosphate buffer (pH 7.4) in a total volume of 100 μl. The incubations were conducted at 37°C for various periods. The reaction was halted via the addition of 200 μl of 6% trichloroacetic acid. The reaction mixture was then mixed with phosphate buffered saline (200 μl), and 1% thiobarbituric acid (200 μl), and boiled for 15 min at 100°C. The samples were then cooled and centrifuged for 10 min at 15,000 rpm, and the differential absorbance of the supernatant fractions at 532 nm was spectrophotometrically measured. The TBARS level was assessed by comparison to a standard curve constructed using malondialdehyde (MDA). All solutions were treated with Chelex 100 resin to remove any traces of transition metal ions.

Analysis of hydroxyl radical formation

Presence and concentration of the hydroxyl radical generated in the reaction of ferritin with H_2O_2 was evaluated using a benzoate (41). The reactions were carried out with 200 μM benzoate, 0.5 μM ferritin, and 1 mM H_2O_2 in a total volume of 200 μl. The samples were diluted with 2.8 ml of Chelex 100-treated water and transferred to a cuvette (3 ml). The fluorescence emission spectrum of the sample was monitored in the 340-500 region (excitation, 305 nm) using an SMF-25 fluorescent spectrometer (Bio-Tek Instruments).

Statistical analysis

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

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