Inhibition of Low-Density Lipoprotein Oxidation by Carnosine and Histidine

Eric A. Decker,*,† Vadim Ivanov,‡ Ben-Zhan Zhu,‡ and Balz Frei‡

Department of Food Science, Chenoweth Laboratory, University of Massachusetts, Amherst, Massachusetts 01003, and Linus Pauling Institute, 571 Weniger Hall, Oregon State University, Corvallis, Oregon 97331-6512

Carnosine is a β-alanylhistidine dipeptide found in skeletal muscle and nervous tissue that has been reported to possess antioxidant activity. Carnosine is a potential dietary antioxidant because it is absorbed into plasma intact. This research investigated the ability of carnosine to inhibit the oxidation of low-density lipoprotein (LDL) in comparison to its constituent amino acid, histidine. Carnosine (3 μM) inhibited Cu^{2+}-promoted LDL (20 μg of protein/mL) oxidation at carnosine/copper ratios as low as 1:1, as determined by loss of tryptophan fluorescence and formation of conjugated dienes. Carnosine (6 μM) lost its ability to inhibit conjugated diene formation and tryptophan oxidation after 2 and 4 h of incubation, respectively, of LDL with 3 μM Cu^{2+}. Compared to controls, histidine (3 μM) inhibited tryptophan oxidation and conjugated diene formation 36 and 58%, respectively, compared to 21 and 0% for carnosine (3 μM) after 3 h of oxidation. Histidine was more effective at inhibiting copper-promoted formation of carbonyls on bovine serum albumin than carnosine, but carnosine was more effective at inhibiting copper-induced ascorbic acid oxidation than histidine. Neither carnosine nor histidine was a strong inhibitor of 2,2′-azobis(2-amidinopropane) dihydrochloride-promoted oxidation of LDL, indicating that their main antioxidant mechanism is through copper chelation.

Keywords: *Carnosine; histidine; low-density lipoprotein; lipid oxidation; protein carbonyls; ascorbic acid; copper; peroxyl radicals; antioxidant; dipeptide*

INTRODUCTION

The modification of low-density lipoprotein (LDL) by lipid oxidation products may be involved in the pathogenesis of atherosclerosis. LDL modified by aldehydic lipid oxidation products is taken up by macrophages and smooth muscle cells by scavenger−receptor mediated pathways in a seemingly unregulated manner that leads to the formation of lipid-laden foam cells (*1*−*3*). Antibodies to LDL−aldehyde adducts have been found in plasma (*4*) and in material from atherosclerotic lesions (*5*).

If modification of LDL by secondary lipid oxidation products is associated with the development of atherosclerosis, it is possible that transition metals are involved in this process (*6*, *7*). A potential pathway by which transition metals could promote the oxidative modification of LDL is through interactions with lipid hydroperoxides. During this reaction, reduced and oxidized states of transition metals decompose fatty acid hydroperoxides to alkoxyl and peroxyl radicals, respectively. Alkoxyl radicals can undergo further modification via β-scission reactions resulting in the cleavage of fatty acids to produce many secondary oxidation products including low molecular weight carbonyls and hydrocarbons (*8*). These fatty acid hydroperoxide breakdown products can be cytotoxic, in part due to their ability to modify proteins and nucleic acids via Schiff base and Michael addition reactions (*9*−*11*).

Cupric ions (Cu^{2+}) are the most widely studied transition metals in LDL oxidation models. Direct binding of cupric ions to LDL is important for copper reactivity because modification of histidine will decrease the binding of copper to LDL and will decrease copper-promoted oxidation rates (*12*, *13*). In addition, physical removal of copper from the surface of LDL under conditions that do not alter copper redox cycling (e.g., histidine) also decreases copper-promoted LDL oxidation (*14*, *15*). The necessity for copper to be bound to LDL to be able to promote the formation of secondary lipid oxidation products is likely due to the relatively slow reactivity between cupric ions and hydroperoxides. Binding to LDL would increase Cu^{2+} concentrations near other components that can reduce copper to the more reactive cuprous state. Potential cupric ion reducing components include hydroperoxides (*16*, *17*) and α-tocopherol (*18*).

Inhibition of cupric ion-dependent LDL oxidation can occur by free radical scavenging and/or copper chelation. Carnosine is a β-alanylhistidine dipeptide found in skeletal muscle at millimolar concentrations (*19*). Carnosine is absorbed intact into plasma, suggesting that it may be a bioactive food component (*20*, *21*). One potential biological role of carnosine has been postulated to involve its ability to act as an antioxidant (*22*, *23*). Unfortunately, many of these early antioxidant studies were performed with commercially available sources of carnosine that were contaminated with hydrazine, a strong reducing agent with antioxidant activity (*24*). A

* Author to whom correspondence should be addressed [telephone (413) 545-1026; fax (413) 545-1262; e-mail edecker@foodsci.umass.edu].

† University of Massachusetts.

‡ Linus Pauling Institute.

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reevaluation of the antioxidant activity of purified carnosine has shown that carnosine is a more effective antioxidant against copper than iron in a phosphatidylcholine liposome model (*25*), a property that is likely due to the ability of the histidine moiety of carnosine to chelate copper (*23, 26*). However, millimolar concentrations of purified carnosine can also inactivate peroxyl radicals originating from 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), suggesting that it may also inhibit lipid oxidation by scavenging free radicals (*25*).

The objective of this research was to evaluate the potential of carnosine as an antioxidant against LDL oxidation. The ability of carnosine to inhibit the metal-dependent and metal-independent oxidation of LDL was evaluated and compared to that of histidine to determine if the dipeptide possessed antioxidant activity superior to that of histidine alone.

MATERIALS AND METHODS

Materials. Water was deionized prior to use, and both water and buffers used in all experiments were treated with Chelex 100 (2 g/L; Bio-Rad, Richmond, CA) to remove contaminant metals. AAPH was from Wako Chemical Co. (Richmond, VA). Ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), histidine, and carnosine were obtained from Sigma Chemical Co. (St. Louis, MO). Contaminating hydrazine was removed from carnosine by formation of a hydrazine−hexanal complex that was removed by methylene chloride (*24*). Purified carnosine was lyophilized, and carnosine concentrations were determined by HPLC (*24*). Purified carnosine was stored at −20 °C until use.

LDL was prepared from blood plasma of healthy volunteers by a two-step ultracentifugation method previously described (*27*). LDL was desalted by two passes through PD-10 gel filtration columns (Pharmacia Biotech, Uppsala, Sweden) and was stored in phosphate-buffered saline (PBS; 10 mM phosphate/0.15 M NaCl, pH 7.4) containing 100 μM diethylenetriaminpentaacetic acid (DTPA) under nitrogen at 4 °C for up to 1 week. LDL protein concentrations were determined using a modification of the Lowry method using BSA as a standard (*28*). Immediately prior to use of LDL in oxidation experiments, DTPA was removed by desalting through a 10 mL Bio-Rad EconoPack P6 column pre-equilibrated with PBS.

Methods. *LDL Oxidation.* Oxidation of LDL (20 μg of protein/mL of PBS) was promoted by either Cu^{2+} or AAPH. AAPH (3.0 mM)-promoted oxidation as performed in PBS containing 0.1 mM EDTA to minimize metal-promoted oxidation. Oxidation promoted by $CuSO_4$ (3 μM) was monitored by measuring the formation of conjugated dienes (234 nm) and the loss of tryptophan fluorescence (280 nm excitation/331 nm emission) (*29*). AAPH (3 mM) oxidation was monitored by tryptophan fluorescence and thiobarbituric acid reactive substances (TBARS) (*29*). LDL was diluted in PBS containing 0.1 mM EDTA to 5 μg/mL for measurement of tryptophan fluorescence. Measurement of TBARS was performed in a system containing 125 μg of LDL/mL of PBS. Comparisons of the inhibitory effects of carnosine and histidine were determined within the same experiment to minimize variations that may occur between batches of LDL.

BSA Oxidation. Protein carbonyls were assayed as described by Levine et al. (*30*). Briefly, BSA (1 mg/mL) was oxidized either by Cu^{2+} (0.1 mM) and ascorbate (1.0 mM) or by Cu^{2+} (0.1 mM) and hydrogen peroxide (1.0 mM), in 0.1 M phosphate buffer (pH 7.4) at 37 °C for 1 h. The reaction was terminated by addition of the metal chelator DTPA (1 mM). BSA was then precipitated by trichloroacetic acid (TCA; 10%). To each sample was added 0.5 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 N HCl. The mixture was incubated at 37 °C for 1 h, with vortexing every 10−15 min, followed by the addition of 0.5 mL of 20% TCA. The samples were then incubated on ice for 10 min and centrifuged in a benchtop centrifuge at 10000 rpm for 5 min. Protein pellets were washed three times with 3 mL of ethanol/ethyl acetate (1:1, v/v) and dissolved in 6 M guanidine (pH 2.3). The peak absorbance of the protein-bound hydrazone at 370 nm was used to quantitate protein carbonyls, and the final data were expressed as nanomoles of carbonyl groups per milligram of protein, using a molar absorption coefficient of 22000 M^{-1} cm^{-1} for the DNPH derivatives.

Ascorbic Acid Oxidation. The ability of carnosine and histidine to alter the redox cycling of copper was determined by monitoring changes in Cu^{2+}-promoted ascorbic acid oxidation rates (*15*). Ascorbic acid (60 μM), cupric sulfate (3 μM), and carnosine or histidine (0−48 μM) were incubated in 10 mM HEPES buffer (pH 7.4) at 37 °C. Ascorbic acid oxidation was monitored at 265 nm for 10 min. The oxidation rate (micromolar per minute) was calculated using the extinction coefficient for ascorbic acid (14000 M^{-1} cm^{-1}).

Statistics. All LDL experiments were performed in triplicate and were repeated a minimum of two times. BSA oxidation experiments were performed in duplicate. Data are expressed as means ± standard deviations (SD). Differences between means were determined using the Student *t* test (Microsoft Excel) with a level of significance of $p \leq 0.05$.

RESULTS AND DISCUSSION

The major objective of this research was to evaluate the potential of physiological plasma carnosine concentrations to inhibit LDL oxidation. However, very little is known about the plasma levels of carnosine following ingestion of muscle foods. Perry et al. (*20*) observed plasma carnosine concentrations of 1.0−1.8 μM in two individuals, 2 h after ingestion of chicken breast (4.2−5.2 g of meat/kg of body weight) or beef (4.1−4.4 g of meat/kg of body weight). Using typical carnosine concentrations in beef and chicken breast, the amount of dietary carnosine in the study of Perry et al. (*20*) would range from 12 to 15 μg of carnosine/kg of body weight for chicken breast from 6.2 to 6.6 μg of carnosine/kg of body weight for beef. Gardner et al. (*21*) fed 4 g of purified carnosine to a single individual and reported plasma carnosine concentrations of 81.1, 44.4, and 4.6 μM at 0.5, 1.0, and 1.5 h after ingestion, respectively. These plasma carnosine levels would be expected to be high because 4 g of carnosine would be equivalent to the ingestion of approximately 2.7 kg of beef or 1.4 kg of chicken. Although the carnosine levels in plasma reported in these studies are quite limited, it seems likely that plasma carnosine concentration following ingestion of normal levels of muscle foods would be in the low micromolar range. On the other hand, plasma histidine concentrations in humans are well characterized and range from 10 to 100 μM (*31, 32*).

We initially determined the inhibitory effects of carnosine and histidine on LDL oxidation over the concentration range of 0.18−96 μM, which represents a carnosine/copper molar ratio of 1:8 to 16:1. These experiments showed that the major differences in the ability of carnosine and histidine to inhibit LDL oxidation could be observed over the concentration range of 3.0−24.0 μM; thus, this concentration range was used in the majority of subsequent experiments. Figure 1 shows the ability of 3.0−24.0 μM carnosine (A) and histidine (B) to inhibit Cu^{2+}-promoted oxidation of LDL as determined by the loss of tryptophan fluorescence. None of the carnosine concentrations tested inhibited Cu^{2+}-promoted tryptophan oxidation after 2 h of incubation (Figure 1A). After 3 h of oxidation, all carnosine concentrations significantly ($p \leq 0.05$) decreased tryptophan oxidation compared to the control, with inhibition ranging from 22 to 50%. As oxidation proceeded,

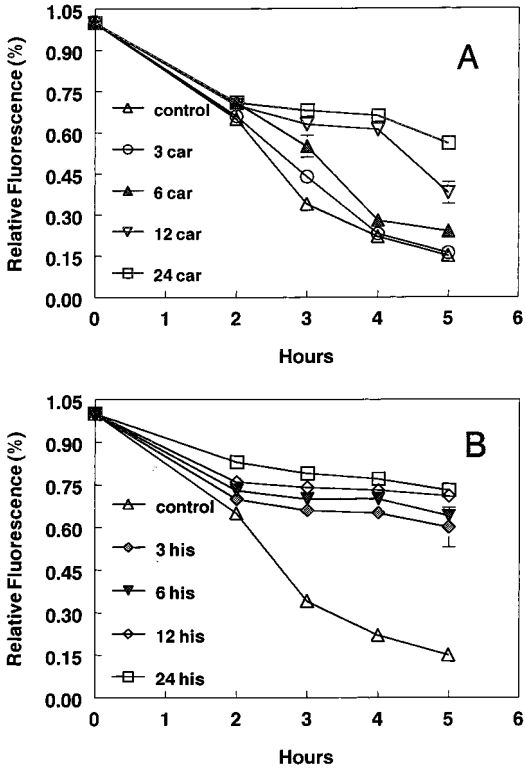

Figure 1. Cu^{2+}-promoted oxidation of LDL tryptophan fluorescence in the presence of various concentrations of carnosine (A) and histidine (B). LDL (20 μg of protein/mL of PBS, pH 7.4) was oxidized by Cu^{2+} (3 μM) at 37 °C, and tryptophan fluorescence was monitored at excitation and emission wavelengths of 280 and 331 nm, respectively.

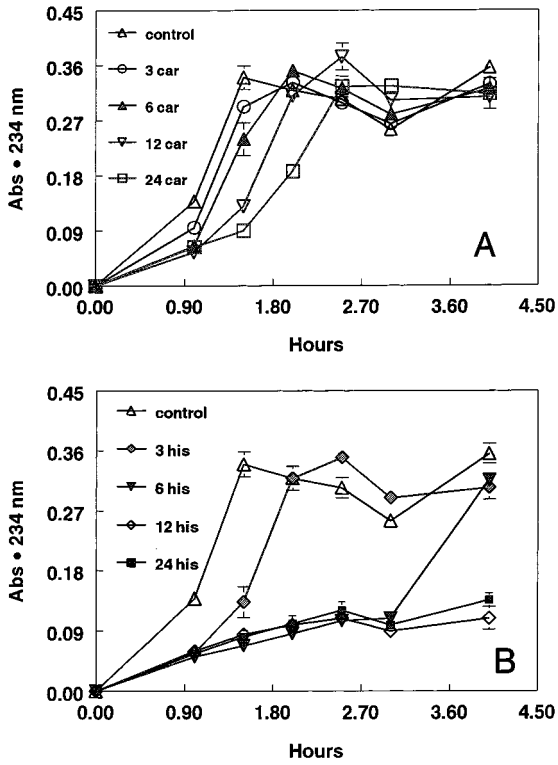

Figure 2. Cu^{2+}-promoted oxidation of LDL in the presence of various concentrations of carnosine (A) and histidine (B) as measured by the formation of conjugated dienes. LDL (20 μg of protein/mL of PBS, pH 7.4) was oxidized by Cu^{2+} (3 μM) at 37 °C, and conjugated dienes were monitored at 234 nm.

carnosine concentrations of 3.0 and 6.0 μM lost their inhibitory effect, with no significant differences ($p > 0.05$) being observed compared to controls after 4 and 5 h of incubation. Inhibition of Cu^{2+}-promoted tryptophan oxidation in LDL was similar in the presence of 12.0 and 24.0 μM carnosine until 5 h of oxidation, when inhibition of oxidation by 12.0 μM carnosine began to decrease. Histidine was more effective than carnosine at inhibiting the oxidation of LDL tryptophan residues, with all histidine concentrations (3.0−24.0 μM) providing significant protection for up to 5 h of incubation (Figure 1B).

The ability of carnosine and histidine to inhibit Cu^{2+}-promoted oxidation of LDL was confirmed by measuring the formation of conjugated dienes (Figure 2). After 1 and 1.5 h of oxidation, all carnosine concentrations tested (3.0−24.0 μM) inhibited conjugated diene formation. However, the ability of carnosine to inhibit conjugated diene formation was lost as oxidation proceeded, with 3.0−12.0 and 24.0 μM carnosine being no different ($p > 0.05$) from controls after 2 and 2.5 h of incubation, respectively (Figure 2A). Histidine was more effective than carnosine in its ability of inhibit Cu^{2+}-promoted oxidation of LDL as determined by formation of conjugated dienes (Figure 2B). For example, after 1.5 h of oxidation, 3.0 μM histidine decreased conjugated dienes 61% compared to 15% for an equivalent carnosine concentration. In addition, the ability of 12.0 and 24.0 μM histidine to inhibit conjugated diene formation was not lost during the 4 h incubation period, whereas the inhibitory effect of 12.0 and 24.0 μM carnosine was lost after 2 and 2.5 h of oxidation, respectively. Histidine was able to inhibit both loss of tryptophan fluorescence and conjugated diene formation in LDL at histidine/copper ratios ≥1:1. Inhibition was not observed at 1.5 μM histidine (data not shown). The histidine/copper ratios that inhibited Cu^{2+}-promoted LDL oxidation in this research are lower than those reported by Retsky et al. (*15*), who observed inhibition at histidine/copper ratios ≥2:1. These differences could be due to the longer oxidation time (6 h) used by Retsky et al. (*15*) because the inhibitory activity of histidine is lost as oxidation proceeds (Figure 2B).

Differences in the abilities of carnosine and histidine to inhibit copper-promoted oxidation reactions were also determined in a protein oxidation model using BSA and a histidine/ or carnosine/copper ratio of 1:2 to 8:1. Transition metal mediated oxidation of proteins results in the formation of carbonyl groups in quantities that reflect the level of oxidative damage (*30*). Thus, carbonyl formation has been frequently used as a biomarker for protein oxidation. Oxidation of BSA was promoted by either Cu^{2+}/ascorbate (Figure 3A) or Cu^{2+}/H_2O_2 (Figure 3B). Carnosine and histidine were able to significantly inhibit carbonyl formation in both oxidation systems at concentrations ≥0.2 mM (carnosine/ or histidine/copper ratios >2:1). As in the LDL oxidation system, histidine (18−75% inhibition for Cu^{2+}/ascorbate and 10−87% inhibition for Cu^{2+}/H_2O_2) was a more effective inhibitor of copper-promoted oxidation than carnosine (10−46% inhibition for Cu^{2+}/ascorbate and 9−54% inhibition for Cu^{2+}/H_2O_2).

The ability of carnosine and histidine to alter the redox activity of copper was determined by measuring changes in copper-promoted ascorbic acid oxidation rates (Table 1). Carnosine was capable of decreasing copper-promoted ascorbic acid oxidation 31−66% with significant inhibition being observed at carnosine/copper ratios as low as 1:8. Baran et al. (*33*) also observed a

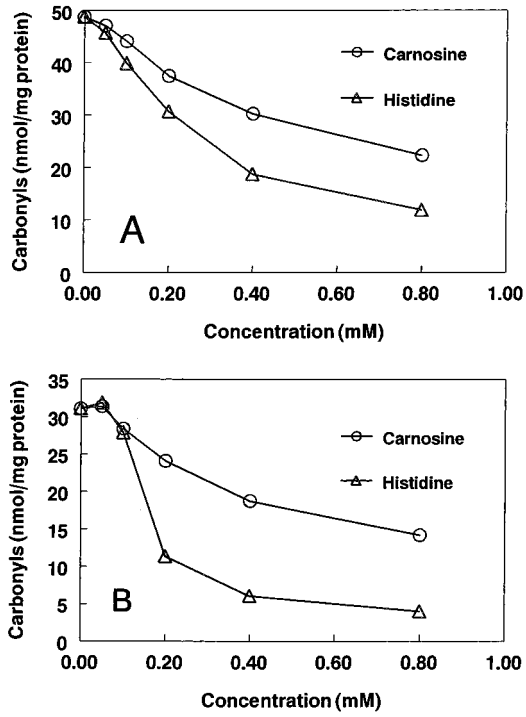

Figure 3. Inhibition of copper-promoted oxidation of BSA by carnosine or histidine. BSA was incubated with Cu^{2+} (0.1 mM) in combination with either ascorbate (1.0 mM; A) or hydrogen peroxide (1.0 mM; B) for 1 h, and protein oxidation was monitored by the formation of carbonyls.

Table 1. Ability of Carnosine and Histidine To Alter the Cu^{2+}-Promoted Oxidation of Ascorbic Acid[a]

treatment	histidine/ or carnosine/Cu ratio	ascorbic acid oxidation rate (μM/min)	% of control
control		2.07 ± 0.23	100
histidine			
3.0 μM	1:1	2.95 ± 0.11[b]	143
6.0 μM	2:1	1.64 ± 0.15	79
12 μM	4:1	1.64 ± 0.18	79
24 μM	8:1	1.14 ± 0[b]	55
48 μM	16:1	1.24 ± 0.04	60
carnosine			
0.18 μM	1:16	1.95 ± 0.18	94
0.38 μM	1:8	1.40 ± 0.15[b]	68
0.75 μM	1:4	1.14 ± 0.14[b]	55
1.5 μM	1:2	1.05 ± 0.15[b]	51
3.0 μM	1:1	0.90 ± 0.04[b]	34
6.0 μM	2:1	1.02 ± 0.11[b]	49
12 μM	4:1	1.05 ± 0.18[b]	51
24 μM	8:1	1.43 ± 0.26[b]	69
48 μM	16:1	1.24 ± 0.15[b]	60

[a] Ascorbic acid (60 μM) oxidation was monitored at 265 nm at 37 °C in the presence of 3 μM cupric sulfate and 10 mM HEPES buffer (pH 7.4). Oxidation rates were calculated using an extinction coefficient of 14000 M^{-1} cm^{-1}. [b] Statistically different from control ($p \leq 0.05$).

decrease in the redox activity of copper by carnosine as determined by cyclic voltammetry. At a carnosine/copper ratio of 1:1, reduction of copper occurred at −0.7 V compared to −0.05 V in the absence of carnosine, indicating a high redox stability of the Cu^{2+}−caronsine complex. In our study maximal inhibition of the redox activity of copper by carnosine occurred at a copper/carnosine ratio of 4:1, after which no major changes in redox activity were observed. The inability of carnosine to completely inhibit copper-promoted ascorbic acid oxidation at carnosine/copper ratios as high as 16:1

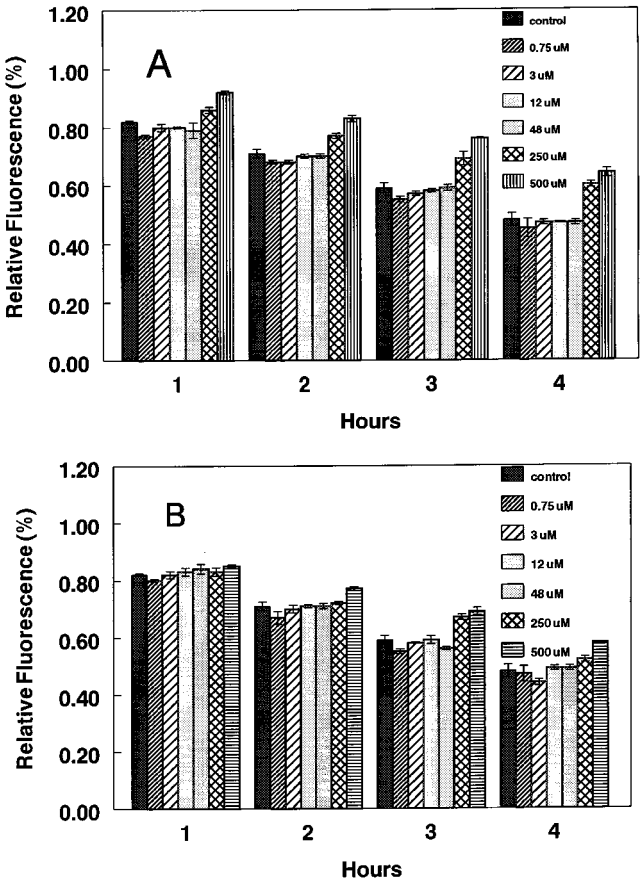

Figure 4. AAPH-promoted oxidation of LDL tryptophan fluorescence in the presence of various concentrations of carnosine (A) and histidine (B). LDL (20 μg of protein/mL of PBS, pH 7.4) was oxidized by AAPH (3 mM) at 37 °C, and tryptophan fluorescence was monitored at excitation and emission wavelengths of 280 and 331 nm, respectively.

suggests that the copper/carnosine complex maintains some redox activity. Interestingly, the ability of carnosine to decrease the redox activity of copper is greater than that of histidine (Table 1). Histidine increased copper-promoted ascorbic acid oxidation at a histidine/copper ratio of 1:1 and did not significantly decrease ascorbic acid oxidation rates until histidine/copper ratios were ≥8:1. Retsky et al. (*15*) also observed a decrease in copper redox cycling as histidine/copper ratios increased, with significant inhibition of copper-promoted ascorbic acid oxidation occurring at a histidine/copper ratio of 12:1. The ability of histidine to more effectively decrease copper-promoted LDL and BSA oxidation than carnosine (Figures 1−3) is likely due to the more efficient removal of copper from the surface of LDL and BSA by histidine.

The ability of carnosine and histidine to inhibit LDL oxidation by inactivating free radicals was determined by oxidizing LDL with peroxyl radicals generated from AAPH. In these experiments TBARS were used to monitor lipid oxidation instead of conjugated dienes due to strong interference by AAPH at 234 nm. Neither carnosine nor histidine was able to inhibit AAPH-promoted LDL oxidation as determined by loss of tryptophan fluorescence (0.75−48.0 μM carnosine or histidine; Figure 4) or TBARS formation (Figure 5; 48.0 μM carnosine or histidine). At higher concentrations (250 μM) both purified carnosine and histidine have been found to inhibit the peroxyl radical (generated by 5 mM AAPH) promoted decay of β-phycoerythrin, with

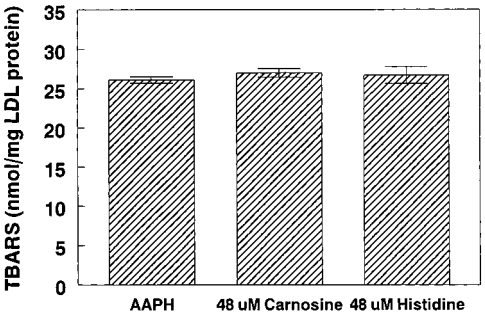

Figure 5. AAPH-promoted oxidation of LDL in the presence of 48 μM carnosine and histidine as determined by the formation of TBARS. LDL (20 μg of protein/mL of PBS, pH 7.4) was oxidized by AAPH (3 mM) at 37 °C, and TBARS were measured at 532 nm.

no differences in activity being detected between carnosine and histidine (*25*). However, at 250 and 500 μM, carnosine and histidine had only small inhibitory effects against AAPH-induced oxidation of tryptophan residues in LDL (Figure 4). These data suggest that plasma concentrations of neither carnosine nor histidine are effective at inactivating peroxyl radicals.

Carnosine is capable of inhibiting copper-promoted oxidation reactions. However, carnosine's inhibitory activity against copper is less than that of histidine as determined in both LDL and BSA models, presumably due to its lower ability to remove copper from the surface of the macromolecules. Because plasma carnosine concentrations are likely to be lower than plasma histidine concentrations and because carnosine is rapidly removed from plasma within several hours by plasma and renal carnosinase (*21*), it is likely that histidine would be the more important inhibitor of the prooxidant activity of copper. Dietary carnosine could increase the copper chelating potential of plasma. However, until the relationship between dietary and plasma carnosine concentrations is better understood, the role of copper chelation by carnosine compared to other plasma components (e.g., albumin and ceruloplasmin) cannot be determined.

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