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Carnosine and Related Compounds Protect Against HOCl-Induced Damage of Biomolecules

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ABSTRACT : *The antioxidant activity of carnosine and related compounds such as anserine, homocarnosine, histidine, and β-alanine which are found in most mammalian tissues, was investigated using hypochlorite (HOCl)-induced oxidant systems. Carnosine and related compounds were protective against HOCl-induced ascorbic acid oxidation, as determined by UV absorbance at 265 nm. L-histidine was the most effective among them. The inhibitory effect of these compounds was strongly associated with a decrease in HOCl. It was also found that carnosine and related compounds significantly protected against the HOCl-mediated erythrocyte damage, as determined by hemoglobin release and hemolysis ($p<0.05$). Carnosine and anserine also inhibited inactivation of α-antiprotease (α-AP) by HOCl, thereby inactivating porcine elastase. The inhibitory effect of carnosine on inactivation of α-AP by HOCl depended on the concentration of carnosine and on the time preincubated with HOCl. Homocarnosine, histidine, and β-alanine did not inhibit the reaction. These results indicate that carnosine and related compounds can neutralize or scavenge HOCl. Thus, these compounds may play an important role in protecting against HOCl-mediated damage of biomolecules in vivo.*

Key Words : *Antioxidant, Carnosine, Anserine, Homocarnosine, Histidine, β-alanine, HOCl, Ascorbic acid, Hemolysis, Elastase*

I. INTRODUCTION

Histidine-containing dipeptides such as carnosine (β-alanyl-L-histidine), anserine (β-alanyl-3-methyl-L-histidine), and homocarnosine (γ-aminobutyric-L-histidine) are present in considerable amounts in several tissues of vertebrates, especially in the skeletal and cardiac muscle, olfactory epithelium and bulbs, brain, and eye (Crush, 1970; Flancbaum *et al.*, 1990). At physiological concentrations these dipeptides inhibit lipid peroxidation (Dupin *et al.*, 1987). Their antioxidant mechanisms may result from chelation of transition metals, enzyme-like activity such as superoxide dismutase, and/or their free radical scavenging ability (Babizhayev *et al.*, 1994; Brown, 1981; Chan *et al.*, 1994; Kohen *et al.*, 1988; Lee and Hendricks, 1997). Carnosine is known to form a stable chloramine complex with hypochlorite (Formazyuk *et al.*, 1992).

Hypochlorite (HOCl) is a form of reactive oxidant produced by myeloperoxidase (EC 1.11.1.7) from H_2O_2 and chloride in activated neutrophils (Zgliczynski *et al.*, 1971). It is responsible for killing bacteria and has been implicated in neutrophil-mediated tissue injury associated with the inflammatory process (Bernfosky, 1991; Weiss *et al.*, 1982). HOCl oxidizes and chlorinates a wide variety of biological molecules. The formation of chloramine by the reaction of HOCl with proteins and amino acids has been considered as primary mechanism of the cytotoxicity of HOCl. Scavenging of HOCl by physiological agents or drugs may be important for protection against neutrophil-mediated tissue injury.

The most important biological targets that can be attacked by HOCl include α-antiprotease pro-

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Abbreviations used: α-antiprotease, α-AP; glutathione, GSH; low density lipoprotein, LDL; methemoglobin, metHb; oxyhemoglobin, oxyHb; red blood cells, RBC.

tein, brain Na-K-ATPase, ascorbate, low density lipoprotein (LDL), and erythrocytes (Boldyrev *et al.*, 1995; Chesney *et al.*, 1991; Hazell *et al.*, 1996; Wasil *et al.*, 1987). HOCl inactivates α-antiprotease protein, leading to permit uncontrolled activity of protease, especially elastase (Anderson and Theron, 1990). HOCl can easily oxidize ascorbic acid (Chesney *et al.*, 1991). Red blood cells (RBC) or their membranes have been used in many oxidant-mediated studies. A variety oxidizing agents can induce changes in normal RBC structure and function, thereby leading to the destruction of RBC. We examined the ability of carnosine and related compounds to protect against HOCl-mediated ascorbic acid oxidation, erythrocyte damage, and α-antiprotease inactivation.

II. MATERIALS AND METHODS

1. Materials

L-Carnosine, homocarnosine, L-anserine, L-histidine, β-alanine, glutathione (GSH), taurine, ascorbic acid, α-1-antitrypsin (α-1-antiprotease), porcine elastase (EC 3.4.21.36), and succinyl trialanyl p-nitroanilide were purchased from Sigma Chemical Co. (St. Louis, MO). HOCl solution was obtained from Mallinckrodt Inc. (Paris, KY). Heparinized bovine blood was obtained from the animal physiological laboratory at Utah State University.

2. Ascorbic acid measurement

The reaction mixture included 0.1 M potassium phosphate buffer (pH 7.2), 100 μM ascorbic acid, 40 μM HOCl, and 1 mM test solution. Concentration of ascorbic acid was monitored by reading absorbance of the reaction mixture at 265 nm.

3. Chloramine and HOCl measurement

The reaction mixtures containing 0.1 M potassium phosphate buffer (pH 7.4), 4 mM HOCl, and test solution were monitored spectrophotometrically at 250 nm for chloramine (Formazyuk *et al.*, 1992) and at 290 nm for HOCl (extinction coefficient 317 M^{-1} cm^{-1}) (Chesney *et al.*, 1991).

4. Hemolysis, hemoglobin, and metHb/oxyHb

Bovine blood was centrifuged for 15 min at 1,000× g and the supernatant including buffy coat was discarded by aspiration. The erythrocytes were washed three times with 5 volumes of 10 mM sodium phosphate buffered saline (pH 7.4). Reaction mixtures containing 10% (v/v) bovine erythrocyte suspension in 10 mM sodium phosphate buffered saline (pH 7.4), 400 μM HOCl, and 1 mM test solution were incubated for 2 h at 37°C, and then 0.1 m*l* of each incubation mixture was taken, hemolyzed with 5 m*l* of deionized water, vortexed vigorously, and measured spectrophotometrically at 575 nm for 100% hemolyzed control. After the remaining 0.9 m*l* of the incubation mixture was centrifuged at 1,000× g for 10 min at 5°C, 0.1 m*l* of the supernatant was diluted with 5 m*l* of deionized water and the absorbance was measured at 575 nm. Twenty microliters of the supernatant was used for the determination of hemoglobin using the Sigma commercial kit for plasma hemoglobin. The precipitate was then hemolyzed with 8 m*l* of deionized water, and the absorbance was measured at 540 nm for oxyhemoglobin (oxyHb) and 630 nm for methemoglobin (metHb) as described by Shinar *et al.* (1989).

5. Elastase activity

Reaction mixtures (0.9 m*l*) in 10 mM sodium phosphate buffered saline (pH 7.4) containing 50 μM HOCl and 5 mM test solutions were pre-incubated for 20 min at room temperature (25°C). One tenth milliliter of α-1-antitrypsin (2 mg/m*l*) was then added to the solutions, followed by incubation for 30 min at room temperature. The incubation solutions were diluted with 3 m*l* of 10 mM sodium phosphate buffered saline (pH 7.4), 50 μ*l* of 0.1% (w/v) porcine elastase (EC 3.4.21.36) was added, and then allowed to stand for 20 min at room temperature. After 100 μ*l* of 5 mM elastase substrate (succinyl trialanyl p-nitroanilide) and 1 m*l* of the incubation solution were mixed in a cuvette, the change of absorbance at 410 nm for 1 min was recorded and calculated as an elastase activity by the increased absorbance per second as described

by Wasil *et al.* (1987).

6. Statistical analysis

Data were analyzed using SAS (1985) program on duplicate samples with three replications. If overall F-test was significant, the least significant difference procedure was used to determine significant differences at the level of $p<0.05$ between the means of control and those of treatment groups.

III. RESULTS

1. Protection against HOCl-induced ascorbic acid oxidation

When ascorbic acid concentrations were monitored by UV absorbance at 265 nm, 100 μM ascorbic acid was completely oxidized within 1 min in the presence of 40 μM HOCl at room temperature (Fig. 1). At the concentration of 1 mM, carnosine, anserine and histidine inhibited the HOCl-induced ascorbic acid oxidation by about 80% and homocarnosine and β-alanine inhibited the reaction by 55%. One millimolar GSH almost completely blocked the HOCl-induced ascorbic acid oxidation. With increasing reaction time, ascorbic acid was further oxidized even in the presence of 1 mM carnosine and related compounds. In the presence of GSH, the reduced form of ascorbic acid was increased slightly after incubation for 2 min. After incubation of the reaction mixture for 10 min, glutathione, histidine, carnosine, anserine, homocarnosine, and β-alanine inhibited ascorbic acid oxidation by 95, 70, 45, 42, 24, and 19%, respectively.

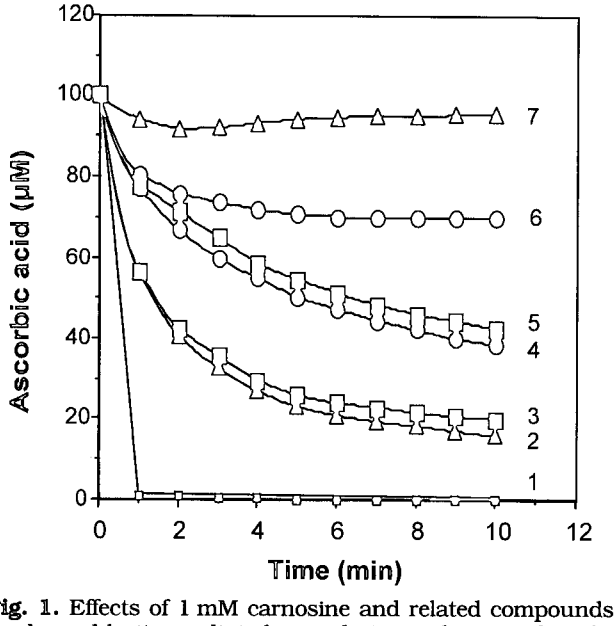

Fig. 1. Effects of 1 mM carnosine and related compounds on hypochlorite-mediated ascorbate oxidation. The absorbance of reaction mixture (pH 7.2) containing 0.1 M potassium phosphate buffer, 100 μM ascorbic acid, 40 μM HOCl, and 1 mM test solutions was monitored at 265 nm. 1: control (HOCl only), 2: homocarnosine, 3: β-alanine, 4: carnosine, 5: anserine, 6: histidine, 7: glutathione. Data points represent the mean of three determinations.

2. Activity of Scavenging HOCl

The inhibitory effect of carnosine and related compounds and GSH on HOCl-induced ascorbic acid oxidation was related to their scavenging activity on hypochlorite (Table 1). These compounds decreased hypochlorite concentration, as monitored by UV absorbance at 290 nm. Glutathione most rapidly scavenged the sodium hypochlorite. The disappearance rates of HOCl by glutathione, histidine, carnosine, anserine, homocarnosine, and β-alanine were 88.9, 66.9, 87.7, 149.8, 57.1, 230.9 mM/ml/sec, respectively (Table 1). The decrease in hypochlorite corresponded to an increase in chloramine formation during short-time incubation, as monitored by UV absorbance at 250 nm. With increasing incubation time, however, carnosine and anserine continuously increased the absorbance at 250 nm (Fig. 2). Taurine and homocarnosine formed a stable chloramine which was not degraded

Table 1. Effects of 1 mM carnosine and related compounds on decrease in 4 mM hypochlorite[a]

Compounds	Decrease in HOCl (ΔA290)[b]	Disappearance Rate of HOCl (mM/ml/sec)
Carnosine	0.282+0.028[p]	88.9[p]
Homocarnosine	0.212+0.023[q]	66.9[q]
Anserine	0.278+0.020[p]	87.7[p]
L-Histidine	0.475+0.045[r]	149.8[r]
β-Alanine	0.181+0.024[q]	57.1[q]
Glutathione	0.732+0.050[s]	230.9[s]

[a]After HOCl was finally added to reacton mixture, the absorbance of the reaction mixture was monitored for 10 seconds at 290 nm (Initial absorbance of 4 mM HOCl was 1.268 at 290 nm).
[b]ΔA290=1.268-absorbance at 290 nm.
[p,q,r,s]Means in each column with different superscripts are significantly different ($p<0.05$).

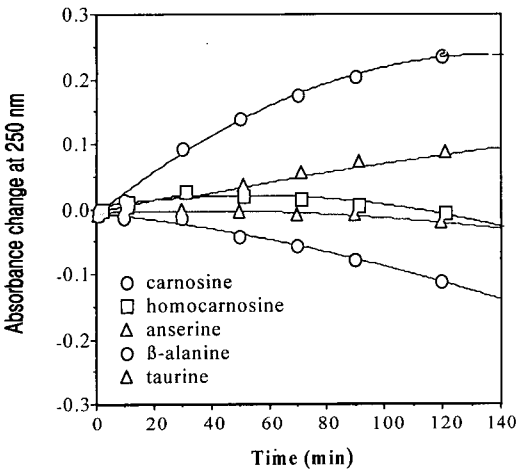

Fig. 2. Stability of chloramine (change in absorbance at 250 nm). Absorbance of the reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.4), 4 mM HOCl, and 5 mM test solution was monitored at 250 nm from 1 min to 2 h, after HOCl was finally added to the reaction mixture. Data points represent the mean of three determinations.

within 2 h, but the β-alanine chloramine was not stable over time.

3. Protection against HOCl-induced hemolysis

In the presence of 400 μM HOCl, bovine erythrocytes were hemolyzed by 85% after incubation for 2 h at 37°C (Table 2). Carnosine, homocarnosine, anserine, L-histidine, and β-alanine almost completely protected against the HOCl-induced erythrocyte hemolysis. The hemoglobin release was also measured as an indication of hemolysis. All the test compounds strongly inhibited hemoglobin release into reaction solution ($p<0.05$). Carnosine was the most effective for protection against HOCl-induced damage of erythrocytes. HOCl also increased formation of metHb, but carnosine and related compounds significantly inhibited metHb formation ($p<0.05$).

Table 2. Effects of 1 mM carnosine and related compounds on HOCl-induced hemolysis, hemoglobin release, and methemoglobin (MetHb) formation

Compounds	Hemolysis (%)	Hemoglobin (mg/dl)	Met Hb/ OxyHb (%)
Control (buffer)	84.6[b]	1343.4+93.5[b]	4.7[b]
Carnosine	1.3[a]	15.6+2.3[a]	2.7[a]
Homocarnosine	1.8[a]	20.8+5.6[a]	2.7[a]
Anserine	1.8[a]	20.3+4.2[a]	3.0[a]
L-Histidine	1.8[a]	21.1+4.0[a]	3.1[a]
β-Alanine	2.2[a]	23.8+7.5[a]	2.9[a]

[a,b]Means in each column with different superscripts are significantly different ($p<0.05$).

4. Protection against HOCl-induced inactivation of α-antitrypsin

α-Antitrypsin (α-AP) was inactivated in the presence of 50 μM HOCl as indicated by maintenance of elastase activity (Table 3). Carnosine and anserine significantly inhibited HOCl-induced inactivation of α-AP ($p<0.05$), but homocarnosine, histidine and β-alanine did not. The inhibitory effect of carnosine and anserine was dependent on the preincubation time with HOCl. Without preincubation, 5 mM carnosine and anserine inhibited the HOCl-mediated inactivation of α-AP by 20 and 12%, respectively. Preincubation for 20 min by 5 mM carnosine and 5 mM anserine significantly inhibited the HOCl-mediated inactivation of α-AP by 66 and 19%, respectively ($p<0.05$). The inhibitory effect of carnosine was also dose-dependent

Table 3. Protection by 5 mM carnosine and related compounds against inactivation of α-antitrypsin (α-AP) by HOCl

Compounds	Porcine elastase activity (A_{410}/sec)	
	Not pre-incubated	Pre-incubated for 20 min
Without HOCl		
Buffer (without α-AP)	7.25×10^{-3}	7.31×10^{-3}
(with α-AP)	0.65×10^{-3}	0.60×10^{-3}
With HOCl		
Buffer (control)	7.17×10^{-3}	7.24×10^{-3}
Carnosine	5.74×10^{-3}*	2.46×10^{-3}*
Homocarnosine	7.11×10^{-3}	7.12×10^{-3}
Anserine	6.34×10^{-3}*	5.87×10^{-3}*
L-Histidine	7.16×10^{-3}	7.21×10^{-3}
β-Alanine	7.18×10^{-3}	7.19×10^{-3}

*Significantly different from the control ($p<0.05$).

Table 4. Protection by carnosine against inactivation of α-antitrypsin (α-AP) by HOCl

Compounds	Porcine elastase activity (A_{410}/sec)	% Inhibition
Without HOCl		
Buffer (without α-AP)	7.07×10^{-3}	–
(with α-AP)	0.58×10^{-3}	91.8
With HOCl		
Buffer (control)	6.95×10^{-3}	1.7
Carnosine, 1 mM	6.10×10^{-3}	13.7
2 mM	5.27×10^{-3}	25.5
4 mM	3.39×10^{-3}	52.1
6 mM	1.71×10^{-3}	75.8
8 mM	0.69×10^{-3}	90.2

Data represent the mean of three determinations.

(Table 4). When preincubated with 50 μM HOCl for 20 min, 1, 2, 4, 6, and 8 mM carnosine inhibited the HOCl-induced inactivation of α-AP by 14, 26, 52, 76, and 90%, respectively. In the presence of 8 mM carnosine, the α-AP was almost completely protected, thereby, resulting in the inhibition of elastase activity.

IV. DISCUSSION

Biological oxidations/reductions are indispensable for human leucocytes and tissue cell functions. The respiratory burst in phagocytes is characterized by enhanced oxygen consumption, resulting in the generation of superoxide anion by NADPH-oxidase (Anderson and Theron, 1990). Superoxide anions dismute spontaneously or enzymatically to form H_2O_2.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The oxidizing potential of H_2O_2 is potentiated by the primary granule enzyme myeloperoxidase (MPO) in combination with a halide. The MPO/H_2O_2/chloride system generates the potent oxidant hypochlous acid by oxidation of chloride (Anderson and Theron, 1990).

$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_2O$$

Reactive oxidants generated by phagocytes are primary antimicrobial and are essential for the intracellular destruction of ingested microorganisms. However, the generation and activity of these phagocyte-derived reactive oxidants is not restricted to the intracellular milieu and extracellular release of reactive oxidants occurs during phagocyte activation. The antimicrobial oxidant released extracellularly can be toxic to a variety of biomolecules.

In human atherosclerotic lesions, HOCl can modify LDL, thereby resulting in transformation of LDL into a high-uptake form (Hazell *et al.*, 1996). One of the most important targets attacked by HOCl *in vivo* is α-AP, the major circulating inhibitor of serine proteases such as elastase (Anderson and Theron, 1990). α-AP is crucial to the biological defense of tissue from protease enzymes, primarily protecting against phagocyte-derived elastase. However, α-AP is particularly vulnerable to oxidative inactivation with consequent loss of elastase inhibitory capacity. α-AP is rapidly inactivated by HOCl, and loses its ability to inhibit elastase. The activity of scavenging HOCl by a compound is, therefore, related to its ability to protect against α-AP inactivation by HOCl (Wasil *et al.*, 1987). Endogenous N-compounds of the neutrophils trap HOCl and thus prevent HOCl from directly attacking neutrophils or target cells (Grisham *et al.*, 1984). The inhibitory effect of carnosine on HOCl-induced inactivation of α-AP may be related to its scavenging activity on HOCl, corresponding to an increase in chloramine formation. However, carnosine appeared to react with HOCl more slowly than with α-AP, as indicated by longer preincubation of carnosine with HOCl required to inactivate α-AP. Carnosine chloramine may also have less oxidizing activity than the other related compound chloramines. Among carnosine and related compounds, histidine showed the strongest scavenging activity on HOCl. However, histidine did not protect against inactivation of α-AP by HOCl. These results may indicate that the secondary products of histidine and HOCl has the stronger oxidizing activity on α-AP than carnosine chloramine. The protection of α-AP by carnosine or anserine may maintain the elastase inhibitory capacity, thereby protecting against uncontrolled tissue proteolysis.

Ascorbate, present in high concentrations in mammalian tissues, has biochemical and physiological functions including collagen formation, cancer prevention, iron absorption, immune function, cardiovascular disease prevention, and bone metabolism (Davies *et al.*, 1991; Graby and Singh, 1991). Ascorbic acid can not only scavenge active and stable oxygen radicals but can also regenerate vitamin E (Winkler *et al.*, 1994; Niki, 1991). At pH 7.2, 60 μM HOCl rapidly oxidizes ascorbic acid within a few seconds; 60 μM monochloramine and taurine chloramine oxidize only a small fraction of the ascorbic acid even after 5 min, indicating that the chloramines have less oxidizing activity than HOCl (Chesney *et al.*, 1991). Carnosine scavenges OCl^-, resulting in the formation of stable chloramine complexes when directly measured by UV absorbance at 251 nm (Babizhayev *et al.*, 1994). Carnosine also inhibits Cu (II)-catalyzed oxidation of

ascorbic acid. The protective effect of carnosine against ascorbic acid oxidation may preserve the antioxidant potential of ascorbic acid. It was found that in the eye lens ascorbic acid protected against cataract formation and high concentration of carnosine was found (Boldyrev *et al.*, 1987; Meister, 1994).

The erythrocyte has unique biological structure containing high concentrations of polyunsaturated fatty acids, cellular oxygens, and ferrous ions in the ligand state. These conditions might be expected to make it highly susceptible to oxidative damage. HOCl, as an potential initiator of membrane lipid peroxidation, can not only modify membrane structure but it can also cause loss of adenine nucleotides from target cells (Bernfosky, 1991). These actions of HOCl might damage the bovine erythrocytes, resulting in hemolysis. The activity of scavenging HOCl by carnosine and related compound may contribute to the protection against HOCl-mediated erythrocyte damage.

Our results suggest that carnosine and related compounds, which can be endogenously synthesized or supplied by a diet, may protect against HOCl-mediated damage of biomolecules thereby preserving their biochemical and physiological functions in biological systems.

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