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# Effect of Carnosine and Related Compounds on Glucose Oxidation and Protein Glycation *In Vitro*

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**The effects of carnosine and related compounds (CRC) including anserine, homocarnosine, histidine, and β-alanine, found in most mammalian tissues, were investigated on *in vitro* glucose oxidation and glycation of human serum albumin (HSA). Carnosine and anserine were more reactive with D-glucose than with L-lysine. In the presence of 10 μM Cu (II), although carnosine and anserine at low concentrations effectively inhibited formation of α-ketoaldehyde from D-glucose, they increased generation of $H_2O_2$ in a dose-dependent manner. Carnosine, homocarnosine, anserine, and histidine effectively inhibited hydroxylation of salicylate and deoxyribose degradation in the presence of glucose and 10 μM Cu (II). In the presence of 25 mM D-glucose, copper and ascorbic acid stimulated carbonyl formation from HSA. Except for β-alanine, CRC effectively inhibited the copper-catalyzed carbonyl formation from HSA. The addition of 25 mM D-glucose and/or 10 μM Cu (II) to low density lipoprotein (LDL) increased formation of conjugated dienes. CRC effectively inhibited the glucose and/or copper-catalyzed LDL oxidation. CRC also inhibited glycation of HSA as determined by hydroxymethyl furfural and lysine with free ε-amino group. These results suggest that CRC may play an important role in protecting against diabetic complications by reacting with sugars, chelating copper, and scavenging free radicals.**

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## Introduction

Histidine-containing dipeptides such as carnosine, anserine, and homocarnosine are present in considerable amounts in several tissues of vertebrates including skeletal and cardiac muscles, eye lens, and brains (Crush, 1970; Flancbaum *et al.*, 1990). At physiological concentrations, these depeptides can act as antioxidants by chelating transition metals and/or scavenging free radicals (Brown, 1981; Kohen *et al.*, 1988; Chan *et al.*, 1994). In the presence of copper, these compounds can dismute superoxide radicals released by activated neutrophils (Kohen *et al.*, 1991). Carnosine and anserine can not only inhibit the process of lipid peroxidation but they also lower the content of its products already accumulated (Dupin *et al.*, 1987). These effects of histidine-containing dipeptides may be implicated as a protective role against oxidative damage to biomolecules (Lee *et al.*, 1999).

Metal ions may play an important role in the increase of oxidative stress associated with diabetic complications (Mateo *et al.*, 1978; Noto *et al.*, 1983, Koh *et al.*, 1997; Kim *et al.*, 1998). The oxidation of glucose is catalyzed by trace amounts of transition metals, generating free radicals, hydrogen peroxide, and reactive ketoaldehydes (Wolff and Dean, 1986; 1987; Hunt *et al.*, 1988; Jiang *et al.*, 1990). Proteins such as low density lipoprotein (LDL), albumin, lens crystalline, collagen, and hemoglobin, for example, undergo structural alterations in the presence of glucose by the process of 'glycosylation', the nonenzymatic attachment of glucose to amino acid groups of the proteins (Kennedy *et al.*, 1982; Hunt *et al.*, 1988; Lyons *et al.*, 1991). A metal chelating agent such as diethylenetriamine-

pentaacetic acid or ethylenediamine-tetraacetic acid (EDTA) inhibits the glycosylation of BSA and protein browning induced by glucose *in vitro* (Wolff and Dean, 1987). The hydroxyl radical scavenger, sorbitol, also inhibits glucose-mediated fragmentation and benzoate hydroxylation. $H_2O_2$ is presumably the precursor of the proximal protein oxidant since catalase inhibits glucose-stimulated protein fragmentation. The manifold positive effects of carnosine can be employed in clinical practice for the treatment of many pathological conditions such as breast cancer, atherosclerosis, gastritis, ischemia-reperfusion injury, and cataracts (Babizhayev, 1989; Cho *et al.*, 1991; Borgadus *et al.*, 1993; Boissonneault *et al.*, 1994). Carnosine and related compounds (CRC) may protect against protein glycation and glucose oxidation by chelating metal ions, scavenging free radicals, and supplying an amino sink which would compete with the lysine group of proteins. These actions of CRC were investigated in this study.

## Materials and Methods

**Materials** D-Glucose, glyoxal, L-carnosine, homocarnosine, L-anserine, L-histidine, ß-alanine, glutathione, human serum albumin (HSA), and human plasma low density lipoprotein [solution (1.0 ml) in 0.15 M NaCl with 0.01% (w/v) EDTA, pH 7.4] were purchased from Sigma (St. Louis, USA). All solutions were prepared in chelax-treated phosphate buffer (pH 7.4).

**UV spectroscopy** Reaction mixtures prepared in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM D-glucose and 5 mM CRC in the absence/presence of 10 $\mu$M Cu (II) were incubated at 37°C, and aliquots were withdrawn at appropriate intervals. Ultraviolet absorption of reaction mixtures at 283 nm was measured using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan).

**Determination of $\alpha$-ketoaldehyde** The reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 $\mu$M D-glucose, 10 $\mu$M Cu (II), and CRC solutions were incubated for 6 days at 37°C. An aliquot (0.3 ml) of the above mixture was further incubated with 0.1 ml of 0.5 N sodium formate (pH 2.9) and 0.1 ml of 0.1 N Girard T-reagent for 10 min at 30°C after which 0.5 ml of 0.1 N sodium formate (pH 2.9) was added to the mixtures. The absorbance at 295 nm was read against appropriate blanks [test solution + Cu (II)], and the concentrations of $\alpha$-ketoaldehyde were determined using glyoxal as a standard (Mitchel and Birnboim, 1977).

**Determination of hydrogen peroxide** The reaction mixtures in 10 mM sodium phosphate buffered saline (pH 7.4) containing 40 mM D-glucose, 10 $\mu$M Cu (II), and various concentrations of test solutions were incubated at 37°C for various time periods, and 0.1 ml of the reaction mixture was then mixed with 0.9 ml of the color reagent (0.1 mM xylenol orange, 0.25 mM Fe (II), and 0.1 M sorbitol in 25 mM $H_2SO_4$). After standing for 30 min at room temperature, the absorbance at 560 nm was measured, and the concentration of hydrogen peroxide was calculated using a known molar absorption coefficient ($1.5 \times 10^4$ $M^{-1}cm^{-1}$) (Wolff, 1994). Addition of catalase (140 IU) and desferrioxamine (0.2 mM) to the incubation mixture completely abolished hydrogen peroxide formation.

**Aromatic hydroxylation determination** Reaction mixtures (1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM glucose, 5 mM sodium salicylate, 50 $\mu$M cupric chloride, and 5 mM CRC solutions were incubated for 5 days at 37°C. The reaction was terminated by addition of 30 $\mu$l of 11.6 M HCl, followed by 4 ml of ethyl ether. The contents were mixed vigorously for 10 s, and organic layer was then pipetted off and evaporated using a water bath at 40°C. The residue was dissolved in 0.25 ml distilled deionized water to which the following was added; 0.125 ml of 10% (w/v) trichloroacetic acid in 0.5 N HCl, 0.25 ml of 10% (w/v) sodium tungstate, and 0.25 ml of 0.5% (w/v) $NaNO_2$. After the mixture was allowed to stand for 5 min, 0.5 ml of 0.5 M KOH was added, and the absorbance was read at 510 nm after 1 min (Kaur and Halliwell, 1994). The reference standards were prepared using 2,3-dihydroxybezoate (10–100 nmole/ml).

**Thiobarbituric acid reactive substance (TBARS) determination** Reaction mixtures (1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 $\mu$M glucose, 10 mM deoxyribose, 10 $\mu$M cupric chloride, and 5 mM CRC solutions were incubated for 3.5 h at 37°C, and 0.9 ml of the incubated mixtures was subjected to TBARS determination after adding 50 $\mu$l of 1 mM Cu (II) and 50 $\mu$l of 2 mM ascorbic acid, followed by subsequent incubation for 30 min at 37°C. One ml thiobarbituric acid (TBA) stock solution [1% (w/v) TBA in 50 mM NaOH + 2.8% (w/v) TCA] was added to the final incubation mixture, heated in a boiling waterbath for 10 min, and the absorbance at 532 nm was then read (Halliwell and Gutteridge, 1981).

**LDL oxidation** The human plasma LDL solution (5.0 mg protein/ml) was diluted to 200 $\mu$g protein/ml with 10 mM sodium phosphate buffered saline. The reaction mixtures containing 100 $\mu$g protein/ml, 10 $\mu$M Cu (II), 25 mM D-glucose, and 1 mM CRC were incubated for 12 h at 37°C. To extract lipid, 0.3 ml of the incubated mixture was mixed with 0.7 ml of a mixture of chloroform and methanol (2:1). After mixing, the test tube was centrifuged for 10 min at 250 × *g*. The lower layer (0.1 ml) was carefully taken and evaporated in a waterbath (45°C). The residue was then dissolved in 1 ml of cyclohexane (reagent grade) and the absorbance was read at 234 nm (Phul *et al.*, 1994).

**BSA oxidation** The reaction mixtures (1.0 ml) containing 2 mg HSA/ml, 50 $\mu$M cupric ion, 150 $\mu$M ascorbic acid, 0.8 mM $H_2O_2$, and 10 mM test solutions were incubated for 60 min at 37°C. After 1 ml of 20% (w/v) TCA was added to the reaction mixture, the solution was centrifuged for 10 min at 1000 × *g*, and the supernatant was discarded. After adding 1 ml of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2.5 M HCl, the pellet was gently broken with a glass rod and allowed to stand for 60 min at room temperature, with vortexing every 10–15 min. Then, 1 ml of 20% TCA was added and centrifuged for 10 min at 1000 × *g*. The resulting pellet was washed with 2 ml of 10% TCA twice and with 1 ml of ethanol-ethyl acetate (1:1) three times to remove the

free DNPH. The protein precipitates were dissolved in 1 ml of 6 M guanidine hydrochloride solution and incubated for 10 min at 37°C in a waterbath with shaking. Absorbance at 370 nm was read against the blank of guanidine solution. Protein carbonyl content was calculated using a molar absorption coefficient of 22,000 $M^{-1}$ $cm^{-1}$ (Reznick and Packer, 1994).

**Glycation of HSA** Reaction mixtures (vol 1 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mg HSA, 25 mM D-glucose, and CRC solutions were incubated for 7 days at 37°C. The incubation mixtures (0.7 ml) were diluted with 3 ml of deionized distilled water (DDW) after which 0.5 ml of 40% (w/v) TCA was added to the diluted reaction mixtures. After centrifugation at 1000 × *g* for 10 min at 5°C, the supernatants were discarded, and 2 ml of DDW and 1 ml of 1 M oxalic acid were added to the pellet and vortexed for 20 sec. The protein solutions were hydrolyzed for 4 h in an oven (110°C) after which 0.5 ml of 40% TCA was added to the hydrolysate, followed by centrifuging at 1000 × *g* for 10 min. The supernatant (1.0 ml) was mixed with 0.5 ml of 0.05 M TBA and incubated for 30 min at 40°C. The absorbance was measured at 443 nm, and the concentration of hydroxymethyl furfural (HMF) was determined using a HMF standard curve (Ma *et al.*, 1981).

For determination of free ε-amino group of lysine, sodium bicarbonate buffer [4% (w/v), pH 8.5, 0.8 ml] was mixed with the 0.2 ml of the above incubated mixture and pre-incubated for 10 min at 40°C after which 1 ml of freshly prepared 0.1% (w/v) trinitrobenzenesulfonic acid (TNBS) was added. After incubation of the mixture for 2 h at 40°C, 3 ml of concentrated HCl was added to the test tubes, and they were then heated at 110°C for 100 min. The hydrolysate (5 ml) was diluted with 3 ml DDW and washed twice with 20 ml of diethyl ether to remove the N-amino-TNP (trinitrophenol) complex. The residual diethyl ether was removed from the bottom layer by evaporation in a hot water-bath. The absorbance at 346 nm was read, and the concentrations were calculated using a molar absorption coefficient, 1.5 × $10^4$ $M^{-1}cm^{-1}$ for ε-TNP-L-lysine (Kakade and Leiner, 1969).

## Results

The nonenzymatic reaction of proteins, peptides, and amino acids with sugars has been studied extensively. The pathway initially involves the production of a Schiff's base, followed by an Amadori rearrangement and, eventually, the formation of advanced glycation end products. In the presence of 25 μM D-glucose, 5 mM test compounds (carnosine, homocarnosine, anserine, and lysine) had a typical UV absorbance maximum at 283 nm regardless of the presence of 10 μM Cu (II). With increasing incubation time, the absorbance at 283 nm was increased. In the absence of Cu (II), carnosine and anserine were very reactive with D-glucose, and L-lysine also reacted with D-glucose (Fig 1A). Among the test compounds, homocarnosine had the lowest reactivity with D-glucose. Carnosine and anserine were less reactive with D-glucose in the presence of 10 μM Cu (II) than in the absence of Cu (II) (Fig. 1B). However, the reactivity of lysine and homocarnosine with glucose increased in the presence of 10 μM Cu (II). These results suggest that carnosine and anserine can act as an amino sink by competing with protein lysine residues for reactive carbonyl compounds (Amadori products) formed as intermediates in the Maillard reaction.

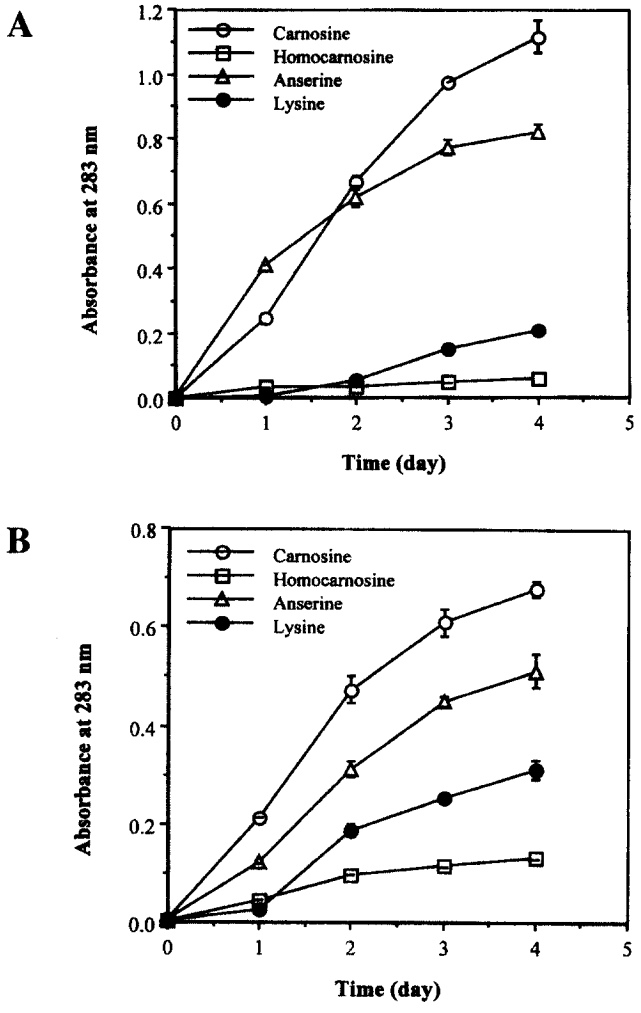



**Fig. 1.** Reaction of dipeptides with glucose (A) in the absence of 10 μM Cu (II) or (B) in the presence of 10 μM Cu (II). Reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM glucose and 5 mM test solutions in the absence/presence of 10 μM Cu (II) were incubated at 37°C for various time periods. Appropriate blanks (test solutions or test solutions + 10 μM Cu (II)) were used for measurement of absorbance at 283 nm. Data represent the mean ± SD of three determinations.

Metal ions can catalyze the formation of α-ketoaldehyde from glucose oxidation. The effect of CRC on the formation of α-ketoaldehyde was studied in the presence of copper. The formation of α-ketoaldehyde increased with increasing incubation time and glucose concentration (data not shown). In the presence of 10 μM Cu (II), 0.1 mM and 1 mM carnosine significantly

inhibited α-ketoaldehyde formation ($p < 0.05$), but 5 mM carnosine did not, indicating a biphasic inhibitory effect of carnosine on α-ketoaldehyde formation (Fig. 2). Homocarnosine and anserine also effectively inhibited α-ketoaldehyde formation at the concentrations tested in this study. Histidine and β-alanine significantly inhibited α-ketoaldehyde formation at concentrations of 1 mM and 5 mM ($p < 0.05$). By increasing the concentrations of carnosine, anserine, and homocarnosine, their inhibitiory effects on α-ketoaldehyde formation decreased, whereas histidine and β-alanine inhibited the reaction more effectively with increasing concentrations of the amino acids.

The presence of metal ions such as iron and copper catalyzes glucose oxidation, resulting in the generation of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals. In the absence of Cu (II), a small amount of $H_2O_2$ was produced and even Cu,Zn-SOD could not increase $H_2O_2$ formation, indicating that the presence of metal ions is very important in production of superoxide anion. In the presence of 10 μM Cu (II), incubation of glucose in 0.1 M phosphate buffer (pH 7.4) increased the production of hydrogen peroxide in a dose-dependent manner. Cu,Zn-SOD (290 units) increased the production of $H_2O_2$ from oxidation of glucose in the presence of 10 μM Cu (II), but catalase (280 units) did not prevent the accumulation of $H_2O_2$. Carnosine, anserine, and homocarnosine increased the production of $H_2O_2$ in the presence of 10 μM Cu (II) compared with the control; β-alanine had no effect on the $H_2O_2$ production, but histidine actually inhibited the production of $H_2O_2$ (Table 1). In the absence of Cu (II), the effect of these compounds on the production of $H_2O_2$ was negligible. This result may be due to the SOD-like activity of carnosine and anserine in the presence of Cu (II). The production of $H_2O_2$ by carnosine and anserine from the oxidation of glucose increased in a dose-dependent manner (Fig. 3). In the presence of metal ions, $H_2O_2$ produced from glucose oxidation may break down to hydroxyl radicals which may be most reactive and dangerous in the biological system. In the presence of glucose and Cu (II), carnosine, homocarnosine, anserine, and histidine inhibited aromatic hydroxylation by 49, 52, 52, and 45% and deoxyribose degradation by 46, 49, 45, and 26%, respectively, perhaps indicating that they have a hydroxyl radical scavenging activity (Table 2). Catalase also inhibited the reaction by 59%, but β-alanine did not. These results indicate that although carnosine and anserine increase production of $H_2O_2$ in the presence of Cu (II), they may also protect against cellular damage by hydroxyl radicals.

**Table 1.** Effect of 5 mM carnosine and related compounds on the production of hydrogen peroxide in the presence/absence of Cu (II).

| Test compounds | Hydrogen peroxide (μM) | |
|---|---|---|
| | with Cu (II) | without Cu (II) |
| Buffer | 4.0 ± 0.3 | 1.1 ± 0.3 |
| Carnosine | 25.3 ± 1.1* | 1.6 ± 0.2 |
| Homocarnosine | 5.3 ± 0.3* | 0.9 ± 0.2 |
| Anserine | 16.6 ± 0.7* | 1.4 ± 0.2 |
| Histidine | 1.8 ± 0.2* | 0.3 ± 0.1* |
| β-Alanine | 4.3 ± 0.5 | 1.0 ± 0.2 |
| Catalase (280 units) | 0.2 ± 0.1* | 0 |
| SOD (290 units) | 11.3 ± 0.6* | 1.5 ± 0.3 |

Reaction mixtures (vol, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM glucose and 5 mM test solutions in the presence/absence of 10 μM cupric chloride were incubated for 5 h at 37°C. Data represent mean ± SD of three determinations. *Significant difference ($p<0.05$) from the control (buffer).

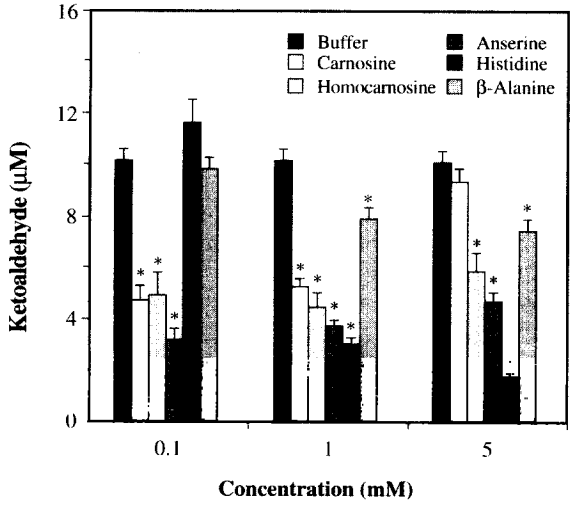



**Fig. 2.** Effect of carnosine and related compounds on α-ketoaldehyde formation from glucose autoxidation in the presence of 10 μM Cu (II). Data represent mean ± SD of three determinations. *Significantly different ($p<0.05$) from the control (buffer) by student's *t*-test.

Carbonyl content has been used as a marker for the oxidative modification of protein. Metal ions can catalyze protein oxidation. In the presence of 50 μM Cu (II), 150 μM ascorbic acid, 0.8 mM $H_2O_2$, and 25 mM glucose, carbonyl formation from HSA increased by two-fold compared to the control (Table 3). In the presence of 25 mM D-glucose, 2 mM carnosine, anserine, homocarnosine, histidine, and glutathione inhibited the Cu (II)-catalyzed oxidation of HSA by 44, 23, 28, 42, and 28%, respectively. Carnosine was the most protective against copper-catalyzed protein modification, but β-alanine did not protect against protein damage.

In the presence of 6.9 μM EDTA, 25 mM D-glucose, and/or 10 μM Cu (II), the antioxidant effects of CRC were investigated using human serum LDL (Fig. 4). Glucose

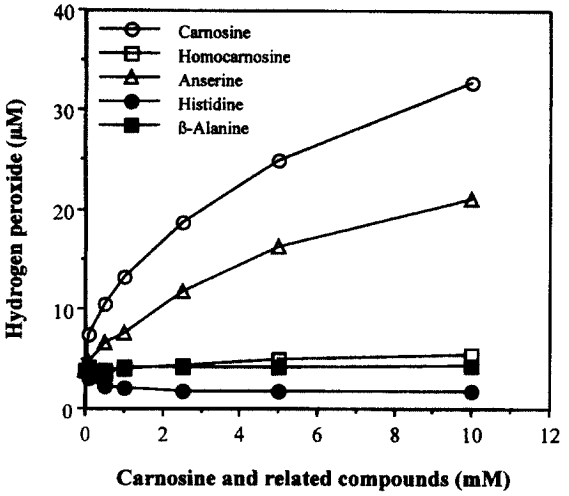



**Fig. 3.** Effect of carnosine and related compounds on formation of hydrogen peroxide from glucose autoxidation in the presence of copper. Reaction mixtures (vol, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM glucose in the presence of 10 $\mu$M cupric chloride were incubated for 5 h at 37°C. Catalase (280 units) abolished the production of hydroperoxide and SOD (290 units) produced 10.6 $\mu$M hydroperoxide in the same reaction mixtures. Data represent mean of three determinations.

**Table 2.** Effect of 5 mM carnosine and related compounds on aromatic hydroxylation and TBARS formation by glucose and Cu (II).

| Test compounds | Dihydroxybenzoic acid (nM) | TBARS ($A_{532}$) |
|---|---|---|
| Buffer | 34.5 ± 2.1 | 0.147 |
| Carnosine | 17.5 ± 0.6* | 0.080* |
| Homocarnosine | 16.5 ± 0.5* | 0.075* |
| Anserine | 16.4 ± 0.6* | 0.081* |
| Histidine | 19.0 ± 1.1* | 0.109* |
| $\beta$-Alanine | 32.5 ± 0.6 | 0.143 |
| Catalase (280 units) | 14.1 ± 1.5* | 0.060* |

Values are the mean ± SD of three determinations.
*Significant difference from the control (buffer) ($p$<0.05).

stimulated LDL oxidation as determined by conjugated dienes. Copper also catalyzed LDL oxidation. The combination of glucose and copper strongly stimulated conjugated diene formation in the LDL solution. When no Cu (II) and glucose were added, carnosine, anserine, and homocarnosine weakly inhibited the LDL oxidation. EDTA present in LDL solution might delay the LDL oxidation. In the presence of 25 mM glucose and/or 10 $\mu$M Cu (II), CRC significantly inhibited LDL oxidation by the following order: histidine > carnosine > anserine > homocarnosine > $\beta$-alanine ($p$ < 0.05) (Fig. 4). These results indicate that

**Table 3.** Protection of 2 mM carnosine and related compounds against oxidative damage to human serum albumin in the presence of 25 mM glucose.

| Reaction mixtures | Carbonyl (nmol/2 mg HSA protein) | % vs control |
|---|---|---|
| Glucose + HSA | 14.7 ± 0.1 | 47.0 |
| + Cu (II) + AA + $H_2O_2$ + buffer | 31.3 ± 2.8 | – |
| + Carnosine | 17.4 ± 1.2 | 55.6 |
| + Homocarnosine | 22.5 ± 1.2 | 71.9 |
| + Anserine | 24.1 ± 0.1 | 77.0 |
| + L-Histidine | 18.2 ± 1.7 | 58.1 |
| + $\beta$-Alanine | 31.6 ± 0.9 | 101.0 |
| + Glutathione | 22.4 ± 0.1 | 71.6 |

Reaction mixtures containing 2 mg HSA/ml, 25 mM glucose, 50 $\mu$M cupric ion, 150 $\mu$M ascorbic acid, 0.8 mM $H_2O_2$, and test solutions were incubated for 60 min at 37°C and then were assigned to the carbonyl content assay. Data represent the mean ± SD of three determinations.

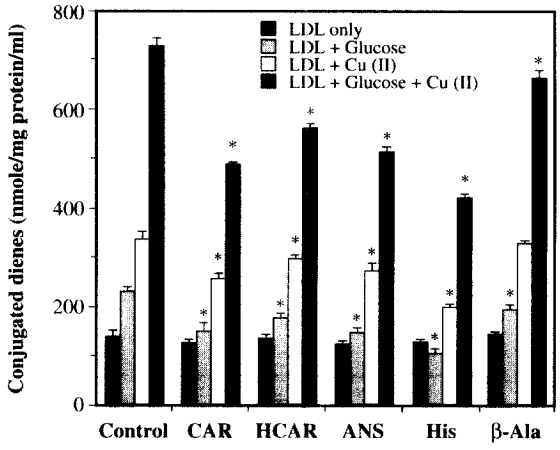



**Fig. 4.** Effect of 1 mM carnosine and related compounds on LDL oxidation. Reaction mixtures containing 100 $\mu$g protein/ml LDL solution in 10 mM sodium phosphate buffered saline (pH 7.4), 6.9 $\mu$M EDTA, 1 mM test solutions, in the presence of 10 $\mu$M Cu (II) and/or 25 mM glucose were incubated for 12 h at 37°C and conjugated dienes in hexane were determined spectrophotometrically at 234 nm after lipid extraction by a mixture of methanol:chloroform (1:2). Bars represent the mean ± SD of three determinations. *Significant difference from the control ($p$<0.05).

glucose autoxidation generated free radicals which could participate in the cascade of LDL lipid peroxidation, thereby stimulating LDL oxidation. CRC might inhibit the reaction by chelating copper and/or scavenging the free radicals produced by glucose autoxidation.

As glycated proteins can release HMF by acid hydrolysis, HMF has been used as a marker for protein

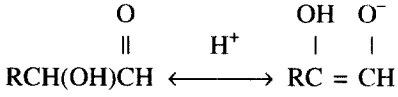
glycation or diabetes. Lysine and cysteine residues of proteins are very reactive with glucose. The effect of CRC on glycation of HSA was studied by determining the release of hydroxymethyl furfural (HMF) and the disappearance of the ε-amino group of lysine in the presence of 25 mM D-glucose and 10 mg/ml HSA (Table 4). CRC significantly inhibited the release of HMF from HSA ($p < 0.05$), indicating that CRC protects against glycation by glucose. Carnosine, anserine, and histidine also abolished a significant decrease in free lysine residues of HSA by glycosylation. Homocarnosine and β-alanine slightly increased the free lysine groups compared to the control (buffer). The protective effects of CRC might be due to the reaction of the CRC with glucose, resulting from competing with lysine residues as an amino sink. CRC inhibited the reaction of lysine of HSA with glucose, resulting in protection against glycation of HSA by glucose. Carnosine and anserine were more protective than was homocarnosine, compared with the control.

## Discussion

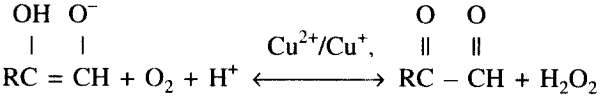
There is considerable evidence suggesting that oxidative stress contributes to development of the diabetic complications (Baynes, 1991; Wolff *et al.*, 1991). Antioxidants such as ascorbic acid, vitamin E, uric acid, and glutathione (GSH) are all decreased in diabetes (Oberley, 1988). There are also increased levels of plasma lipid peroxidation products, as determined by the formation of TBARS. Although biological systems are replete with antioxidants such as vitamin E, there is some evidence for abnormality in levels of catalytic transition metals in individuals with diabetes mellitus (Noto *et al.*, 1983). Together with hyperglycemia these could contribute to oxidative stress which is associated with diabetic complications such as cataract and atherosclerosis (Oberley, 1988; Wolff *et al.*, 1991). In diabetic complications, copper may play a role since total plasma copper concentration is higher in diabetes than in normal subjects (Mateo *et al.*, 1978; Noto *et al.*, 1983). Free copper ions are also detected in human atherosclerotic lesions (Smith *et al.*, 1992). These observations suggest that the copper ion-mediated oxidative damage to protein may be an important process *in vivo*. In our *in vitro* study, copper was used as a catalyst for the production of ketoaldehydes and free radicals, thereby resulting in oxidative damage or glycation to HSA and LDL.

Glyceraldehyde and other simple monosaccharides oxidize under physiological conditions generating α-ketoaldehyde (dicarbonyl) and intermediates of dioxygen reduction: superoxides, hydrogen peroxides, and hydroxyl radicals (Thornalley *et al.*, 1984). Enolization of monosaccharides is prerequisite to the oxidative process (reaction 1). Transition metal ions can catalyze ene-diol oxidation (reaction 2); metal chelators can retard ene-diol oxidation.

(1) Enolization



(2) Ene-diol oxidation and hydroxyl radical formation



$$H_2O_2 + Cu^+ \longleftrightarrow {}^{\bullet}OH + OH^- + Cu^{2+} \text{ (Fenton reaction)}$$

The production of superoxide during monosaccharide oxidation has been probed by ESR technique using the spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Thornalley *et al.*, 1984). SOD increased DMPO-R and DMPO-OH production from oxidizing glyceraldehyde/DMPO incubations, resulting from enhancement of $H_2O_2$ formation. Carnosine is known to be able to interact with high affinity with the superoxide anions and to decrease the reactivity of active hydroxyls formed via the radiolysis of water (Boldyrev and Severin, 1990). In addition, complexes of copper:carnosine and copper:anserine can dismute superoxide radicals. This action of carnosine and anserine might contribute to the increase in $H_2O_2$ formation from the glucose solutions in our study. However, CRC protected against deoxyribose degradation and aromatic hydroxylation, presumably by scavenging hydroxyl radicals produced from $H_2O_2$ via the Fenton reaction. Chan *et al.* (1994), using the EPR technique of spin trapping, were able to show that CRC

**Table 4.** Effect of 1 mM carnosine and related compounds on glycation of human serum albumin by glucose.

| Test compounds | HMF (μM) | Lysine with free ε-amino group (μM) |
|---|---|---|
| HSA only | 11.2 ± 1.2 | 96.1 + 3.2 |
| Control (buffer) | 40.0 ± 2.6** | 78.9 ± 2.3** |
| Carnosine | 31.6 ± 1.4* | 95.0 ± 4.2* |
| Homocarnosine | 31.8 ± 1.8* | 84.3 ± 2.5 |
| Anserine | 31.4 ± 2.0* | 95.1 ± 3.9* |
| L-Histidine | 30.8 ± 2.2* | 92.8 ± 3.1* |
| β-Alanine | 32.4 ± 1.9* | 83.1 ± 4.0 |

Reaction mixtures (vol, 1 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mg human serum albumin (HSA), 25 mM D-glucose, and 1 mM test solutions were incubated for 7 days at 37°C. Data represent the mean ± SD of three determinations. **Significantly different from the HSA only ($p<0.05$). *Significantly different from the control (buffer) ($p<0.05$).

quench 50–95% of hydroxyl radicals produced by ferrous ion and hydrogen peroxide. The hydroxyl radical scavenging property is related to the presence of the peptide bond and the amino acid composition of dipeptides.

α-Ketoaldehydes (dicarbonyl) are produced from monosaccharide autoxidation (Wolff *et al.*, 1991). In addition, these can be produced by degradation of the Amadori product which is formed from rearrangement of a Schiff base. These secondary products are more protein-reactive than the parent monsaccharides and can react with protein to form cross-links as well as advanced glycation endproducts (Njorge *et al.*, 1986). Many proteins are modified adversely when incubated with glucose *in vitro*. When exposed to glucose *in vitro*, albumin shows diminished ligand binding capacity due to conformational alterations, SOD loses activity, and lens crystallins aggregate due to glycation (Shaklia *et al.*, 1984; Arai *et al.*, 1987; Monnier *et al.*, 1992). Higher levels of glycated erythrocyte SOD are found in aged erythrocyte and in diabetes (Arai *et al.*, 1987). In our study, CRC effectively inhibited the formation of α-ketoaldehyde from glucose autoxidation. In addition, carnosine and anserine were much more reactive with glucose than lysine was. These results suggest that the CRC compounds can protect glycation of protein by reducing the formation of α-ketoaldehyde as well as by acting as an amino sink. Monnier *et al.* (1991) proposed that amino acids would be primary targets of Maillard reactants and protect DNA and key proteins from damage. In our study, CRC inhibited glycation of HSA by glucose as measured by release of HMF and free lysine groups of HSA, indicating that amino acids such as histidine and β-alanine as well as dipeptides are protective against diabetic complications. In addition, glycated carnosine and alanine are known to be non-mutagenic as determined by the Ames test, whereas, glycated lysine is mutagenic (Hipkiss *et al.*, 1995).

When LDL is incubated with glucose *in vitro*, it becomes preferentially accumulated by macrophages (Lopes-virella *et al.*, 1988). This suggests that glycated or oxidized LDL contributes to atherosclerosis in diabetes. In our study, copper and glucose were used as catalysts for LDL oxidation. The free radicals such as superoxide, hydrogen peroxide, and hydroxyl radicals produced during glucose autoxidation can participate in LDL oxidation. CRC effectively inhibited LDL oxidation in the Cu (II) and/or glucose-dependent systems. Several mechanisms are involved in the inhibition by these compounds. Histidine derivatives including carnosine, homocarnosine, and anserine, showed hydroxyl radical- and peroxyl radical-trapping ability (Kohen *et al.*, 1988; Chan *et al.*, 1994). In addition, carnosine and anserine are very effective copper chelating agents (Brown and Antholin, 1979; Brown, 1981). The chelating activity and/or free radical scavenging activity of CRC may be related to their antioxidant activity. Carnosine cannot inhibit lipid peroxidation but it also diminished the amount of the already formed lipid peroxidation products (Babizhayev *et al.*, 1994)

Oxygen radicals have been implicated as an important cause of oxidative modification of proteins, which may lead to their rapid degradation (Davies, 1987; Reznick and Packer, 1994; Kim *et al.*, 1998). Among the various oxidative modifications of amino acid in proteins, carbonyl formation may be an early marker for protein oxidation. The most likely amino acid residues to form carbonyl derivatives are lysine, arginine, proline, and histidine (Davies, 1987; Stadtman and Oliver, 1991). The accumulation of oxidized proteins is associated with some oxygen radical-mediated diseases such as inflammation, atherosclerosis, and ischemia-reperfusion injury (Stadtman and Oliver, 1991). In addition, oxidized proteins accumulate during aging. In our study, carnosine and related compounds also inhibited the copper-catalyzed HSA oxidation in the presence of glucose, as indicated by a decrease in carbonyl formation. This result suggests that CRC may play an important role in increasing life-span or retarding aging by protecting key proteins against oxidation. MacFarland and Holliday (1994) reported that carnosine retards the senescence of human diploid fibroblast and increases the life-span of fibroblast cells.

The results from our *in vitro* studies suggest that carnosine and related compounds, which can be endogenously synthesized or supplied by diet, may be physiological antioxidants capable of effectively protecting against free radical-mediated damage of biomolecules thereby preserving their biochemical and physiological functions in biological systems. These compounds also protect against sugar-induced glycation of proteins, suggesting a possibility of use as therapeutic agents to minimizing diabetic complications. This *in vitro* study documents another property of carnosine and related compounds, whereby they act as an amino sink for glycation. *In vivo* studies are further required to illustrate the anti-glycating activity of these compounds in detail.

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