# EPR Spin-Trapping Studies of the Hydroxyl Radical Scavenging Activity of Carnosine and Related Dipeptides†

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The electron paramagnetic resonance technique of spin trapping was employed to study the interaction of hydroxyl radicals with carnosine and related His-containing dipeptides. His and His-containing dipeptides were able to quench 49.1–94.9% of hydroxyl radicals produced by $Fe^{2+}$ and $H_2O_2$. The hydroxyl radical scavenging activity of dipeptides was in the order β-Ala < γ-aminobutyric acid (GABA) < Gly < His < homocarnosine < carnosine < Gly-His. Nonhydroxyl radicals spin trapped by 5,5-dimethylpyrroline *N*-oxide with similar splitting constants of $\alpha^N$ = 15.6 G and $\alpha^H_\beta$ = 18.8 G were detected from homocarnosine, carnosine, and Gly-His in the presence of the hydroxyl radical generation system. The ability of dipeptides and amino acids to inhibit hydroxyl radical catalyzed oxidation of phosphatidylcholine liposomes was in the order β-Ala < Gly = GABA < Gly-His < His < homocarnosine < carnosine. These data suggest that the hydroxyl radical scavenging and antioxidant activities of the dipeptides are related to the presence of the peptide bond and the amino acid composition of the dipeptides.

**Keywords:** *Hydroxyl radical, electron paramagnetic resonance, carnosine, homocarnosine, antioxidant*

## INTRODUCTION

Carnosine (β-alanylhistidine) is an endogenous dipeptide in animal tissues such as skeletal muscle (Davey, 1960; Harris et al., 1990; Plowman and Close, 1988). The concentration of carnosine in sketetal muscle ranges from 1.6 to 20.6 μmol/g, with higher concentrations being found in white than red muscle fibers (Davey, 1960; Plowman and Close, 1988). The high concentrations of carnosine in white fibers led investigators to postulate that carnosine's main metabolic function was as a buffering agent (Harris, 1990). However, several researchers have found carnosine contains antioxidant activity (Auroma et al., 1989; Boldyrev et al., 1989; Decker and Faraji, 1990; Kohen et al., 1988), suggesting that this dipeptide could also function to protect tissue against ischemic reperfusion injury (Yoshikawa et al., 1991).

Carnosine inhibits lipid oxidation catalyzed by iron, $H_2O_2$-activated myoglobin, lipoxidase, photoactivated riboflavin (Decker and Faraji, 1990), and peroxyl (Kohen et al., 1988) and hydroxyl radicals (Aruoma et al., 1989). The antioxidant mechanism of carnosine has been speculated to be due to chelation of transition metals and/or free radical scavenging (Kohen et al., 1988; Decker et al., 1992). Carnosine is capable of chelating copper (Decker et al., 1992), cobalt (Brown and Antholine, 1979), and zinc (Yoshikawa et al., 1991). Copper–carnosine complexes exhibit superoxide dismutase activity (Kohen et al., 1991), and carnosine can complex copper in a manner that decreases its prooxidant activity (Decker et al., 1992). Research in our laboratory indicated that physiological concentrations of carnosine and catalytic iron do not form a complex which decreases the catalytic activity of iron, yet carnosine does inhibit iron-catalyzed lipid oxidation (Decker et al., 1992).

Salim-Hanna and co-workers (Salim-Hanna et al., 1991) reported that carnosine inhibits inactivation of horseradish peroxidase and lysozyme in the presence of a peroxyl radical generation system. Carnosine was also found to inhibit hydroxyl radical oxidation of deoxyribose (Aruoma et al., 1989) and interactions between hydroxyl radicals and spin traps as measured by electron paramagnetic resonance (Rubtsov et al., 1991; Yoshikawa et al., 1991). These data plus carnosine's ability to inhibit both metallic and nonmetallic lipid oxidation catalysts (Decker and Faraji, 1990; Kohen et al., 1988) suggest that carnosine's antioxidant mechanism could be due to both chelation and free radical radical scavenging. However, observation of a carnosine radical, which would provide direct evidence of carnosine's free radical scavenging activity, has not been reported.

The objectives of this research were to use electron paramagnetic resonance (EPR) to investigate the formation of nonhydroxyl radicals after direct interaction between carnosine and related His-containing dipeptides with hydroxyl radicals. Since iron is thought to be the major lipid oxidation catalyst in muscle foods, and iron–hydrogen peroxide system was chosen to generate hydroxyl radicals. The structural relationship of carnosine and related His-containing dipeptides with hydroxyl radical scavenging and antioxidant activities was also evaluated.

## MATERIALS AND METHODS

Iron(II) sulfate, hydrogen peroxide (30%), carnosine, homocarnosine, Gly-His, β-Ala-Gly, β-Ala-Ala, γ-aminobutyric acid (GABA), His, and soybean phosphatidylcholine were purchased from Sigma Chemical Co. The spin trap, 5,5-dimethylpyrroline *N*-oxide (DMPO), was from Aldrich Chemical Co. DMPO radical

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adducts were not observed in the commercially prepared DMPO used in these experiments; therefore, no further purification was performed. Double-distilled deionized water was used in all experiments.

Hydroxyl radical generation was initiated by addition of varied concentrations of $FeSO_4$ (5 or 10 mM) to a mixture of $H_2O_2$ (5 mM) and DMPO (10 or 100 mM) in 0.1 M phosphate buffer (pH 7.4) (Kadiiska et al., 1989). All $FeSO_4$ solutions were prepared immediately prior to use. These iron concentrations were chosen to maximize hydroxyl radical signal intensity and minimize variations in hydroxyl radical production due to low levels of contaminating iron. Different amino acids or dipeptides were added to the hydroxyl radical generation system after addition of $H_2O_2$ and DMPO and prior to $FeSO_4$ addition. Iron was added to the mixture in the EPR cavity. Dipeptide concentrations used were similar to those found in skeletal muscle (Plowman and Close, 1988).

The reactive solution was mixed and transferred to a quartz flat cell which was centered in a TM EPR resonant cavity for EPR analysis. Spectra were recorded at room temperature using a Bruker 300 EPR spectrometer with computerized data acquisition and analysis capabilities. Operation conditions of the spectrometer were 7.45-mW microwave power, 0.98-G modulation amplitude, 75-G scan width, 14.3 G/s scan rate, 1.28-ms time constant, and 9.79-GHz microwave frequency. Hyperfine splitting of DMPO adducts was measured and converted to gauss on the basis of a calibration curve using well-known splitting values of Fremy's salt, and the magnetic field values were determined by use of diphenylpicrylhydrazine (Butterfield, 1974). Signal averaging of peak height was used to obtain data collected over a 3-min period. The hydroxyl radical quenching activity of amino acids or dipeptide was calculated as follows:

$$\text{OH}^{\bullet}\ \text{concn as \% of control} = \frac{\text{OH}^{\bullet}\ \text{spin adduct peak ht in the presence of additives}}{\text{OH}^{\bullet}\ \text{spin adduct peak ht in the absence of additives}} \times 100$$

The antioxidant activity of dipeptides or amino acids was determined in a multilamellar phosphatidylcholine liposome model system (Decker and Hultin, 1990) by measuring their ability to inhibit hydroxyl radical catalyzed lipid oxidation. The oxidation model system contained 0.02 mg of phosphatidylcholine/mL, 0.5 mM $H_2O_2$, and 25 mM amino acids or dipeptides (not included in control) in 0.1 M phosphate buffer (pH 7.4). Lipid oxidation was initiated by addition of $FeSO_4$ (1 mM final concentration) to generate hydroxyl radicals in the model system for 30 min. The extent of lipid oxidation was determined by measuring the thiobarbituric acid-reactive substance (TBARS) (McDonald and Hultin, 1987), and the antioxidant activity of amino acids or dipeptides was calculated as

$$\text{oxidation as \% of control} = \frac{\text{TBARS in the presence of additives}}{\text{TBARS in the absence of additives}} \times 100$$

## RESULTS

Spin trapping is an EPR technique in which a transient radical reacts with a nonparamagnetic molecule such as DMPO (the trap) to form a stable paramagnetic molecule (the spin adduct) (Janzen, 1980). A Fenton reaction system was employed to generate hydroxyl radicals which were spin trapped by DMPO. A spectrum with a splitting constant of $\alpha^N = \alpha^H_\beta = 15.0$ G was obtained in the presence of 100 mM DMPO, 5 mM $H_2O_2$, and 5 mM $Fe^{2+}$, indicating the presence of a hydroxyl radical adduct (Figure 1a; ordinate scale = $10^3$) (Janzen, 1980). Addition of 10 mM carnosine ($\beta$-alanylhistidine) to this system resulted in a decrease of the hydroxyl radical peak height (Figure 1b; ordinate scale = $10^2$). When a higher carnosine concentration (25 mM) was added to the hydroxyl radical generating system, the appearance of new peaks was observed, indicating the presence of a radical adduct

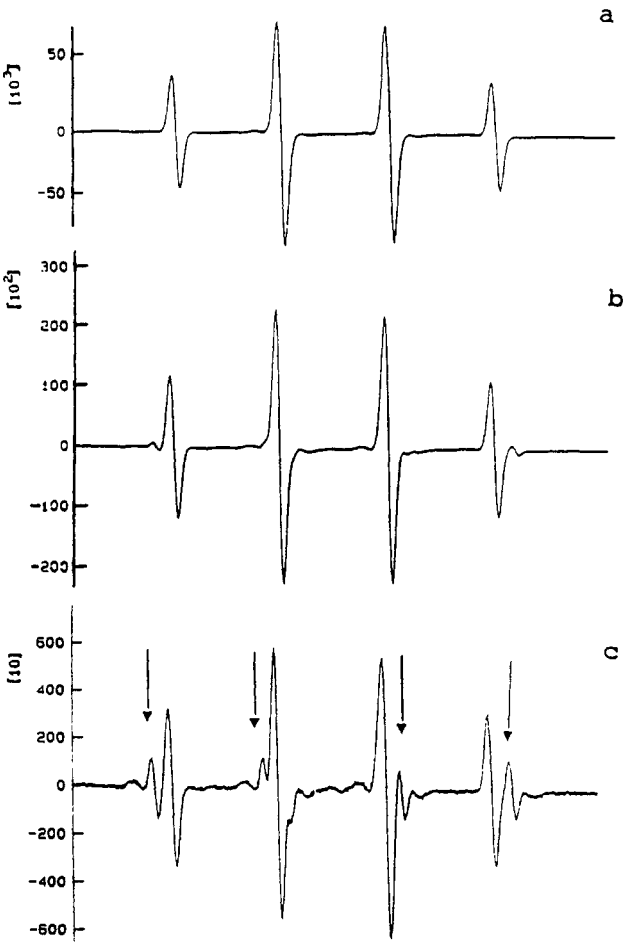


**Figure 1.** EPR spectra of (a) 5 mM $H_2O_2$, 5 mM $FeSO_4$, and 100 nM DMPO in 0.1 M phosphate buffer (pH 7.4); (b) as in (a) but with 10 mM carnosine; (c) as in (a) but with 25 mM carnosine.

different from the hydroxyl radical (Figure 1c). Controls, which included individual as well as all combinations of the additives, had either no detectable EPR spectra or hydroxyl radical spectra that were less than 3% of the hydrogen peroxide, ferrous, or DMPO spectrum (data not shown).

The spectra of the nonhydroxyl radical adduct observed in the presence of 25 mM carnosine could be enhanced by decreasing the concentration of DMPO to 10 mM (Figure 2b). The nonhydroxyl radical adduct is characterized by a six-line spectrum of equal intensity with a nitrogen coupling constant of 15.5 G, which was further split by the $\beta$-hydrogen, resulting in a hydrogen coupling constant of 18.8 G (Figure 2b). Gly-His and homocarnosine ($\gamma$-aminobutyrylhistidine) produced similar six-line spectra in the hydroxyl radical generating system (Fe 5 mM; $H_2O_2$ 5 mM; DMPO 10 mM and 25 mM dipeptides) with splitting constants of $\alpha^N = 15.6$ G, $\alpha^H_\beta = 18.6$ G (Figure 2c) and $\alpha^N = 15.7$ G, $\alpha^H_\beta = 18.9$ G (Figure 2d), respectively. The intensity of the nonhydroxyl radical adducts was in the order Gly-His > carnosine > homocarnosine (Figures 1 and 2). No nonhydroxyl radical adducts were observed for 25 mM Gly, $\beta$-Ala, GABA, or His (data not shown) in the presence of 10 mM DMPO and the hydroxyl radical generation system.

The hydroxyl radical quenching activity of individual amino acids and His- and $\beta$-Ala-containing dipeptide (25 mM) was tested in the presence of the hydroxyl radical generating system (5 mM $H_2O_2$ and 5 mM $Fe^{2+}$) and high DMPO concentrations (100 mM; Table 1). Carnosine was found to inhibit hydroxyl radical production by 74.8%.

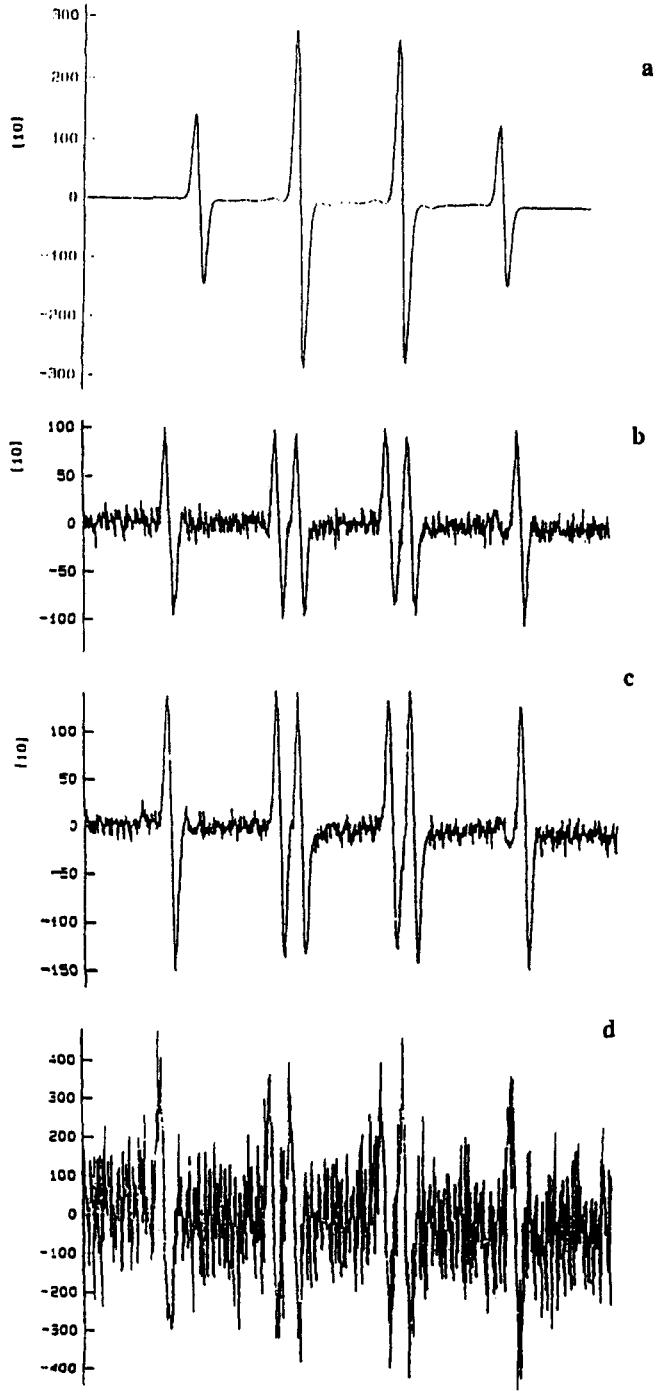


**Figure 2.** EPR spectra of (a) 5 mM $H_2O_2$, 5 mM $FeSO_4$, and 10 mM DMPO in 0.1 M phosphate buffer (pH 7.4); (b) as in (a) but with 25 mM carnosine; (c) as in (a) but with 25 mM Gly-His; (d) as in (a) but with 25 mM homocarnosine.

Replacement of β-Ala with Gly resulted in a dipeptide that had the highest observed hydroxyl radical quenching ability. Replacement of β-Ala with GABA (homocarnosine) resulted in a hydroxyl radical quenching activity lower than that of Gly-His or carnosine (Table 1). The individual constituent amino acids of the dipeptides increased hydroxyl radical production except for His, which quenched 49.1% of the hydroxyl radicals produced. His in combination with Gly, β-Ala, or GABA displayed a hydroxyl radical scavenging ability similar to that of His alone. β-Ala-Gly and β-Ala-Ala were found to promote hydroxyl radical production.

The ability of individual amino acids and dipeptides to inhibit hydroxyl radical catalyzed lipid oxidation was tested using an $Fe^{2+}/H_2O_2$ system and phosphatidylcholine liposomes. Gly-His (25 mM), carnosine (25 mM), and homocarnosine (25 mM) inhibited lipid oxidation by 19.1%, 65.3%, and 26.9%, respectively, as determined by thiobarbituric acid-reactive substances (TBARS). His itself and mixtures of His with Gly, β-Ala, or GABA showed similar antioxidant activity (21.9–23.5%). Dipeptides in which His was replaced (e.g., β-Ala-Gly and β-Ala-Ala) were found to have little or no antioxidant activity. Gly, β-Ala, and GABA showed little antioxidant activity. Controls containing individual or combinations of the additives did not exhibit prooxidant activity or reactivity toward TBA (data not shown).

**Table 1. Hydroxyl Radical (OH•) Quenching and Antioxidant Activity of Selected Amino Acids and Dipeptides**

| | OH• concn as % of control[a] | oxidation as % of control[b] |
|---|---|---|
| control | 100 | 100 |
| Gly-His | 5.1 ± 0.4 | 80.9 ± 1.0 |
| β-Ala-His | 25.2 ± 0.9 | 34.7 ± 1.4 |
| GABA-His | 31.6 ± 1.1 | 73.1 ± 0.9 |
| β-Ala-Gly | 157.3 ± 5.1 | 104.3 ± 2.0 |
| β-Ala-Ala | 108.4 ± 2.9 | 96.4 ± 4.1 |
| Gly | 157.3 ± 3.7 | 96.8 ± 1.6 |
| β-Ala | 244.6 ± 10.1 | 98.4 ± 2.5 |
| GABA | 169.1 ± 9.9 | 96.7 ± 1.4 |
| His | 50.9 ± 6.3 | 78.1 ● 6.1 |
| Gly, His | 52.5 ± 2.2 | 76.5 ± 4.5 |
| β-Ala, His | 54.4 ± 2.2 | 77.5 ± 1.9 |
| GABA, His | 48.4 ± 0.7 | 76.9 ± 2.6 |

[a] Hydroxyl radical generation system included 5 mM $H_2O_2$, 5 mM $FeSO_4$, and 100 mM DMPO in 0.1 M phosphate buffer (pH 7.4). Amino acid or dipeptide concentrations were 25 mM. [b] Antioxidant activity of dipeptides or amino acids (25 mM) was determined in a model system containing $FeSO_4$ (1 mM), $H_2O_2$ (0.5 mM), and phosphatidylcholine liposomes in 0.1 M phosphate buffer (pH 7.4). Extent of oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS). Values represent mean ± standard deviation ($n$ = 3).

## DISCUSSION

Carnosine is reported to be an active hydroxyl radical scavenger (Aruoma et al., 1989; Rubtsov et al., 1991; Yoshikawa et al., 1991). Reactions between carnosine and hydroxyl radicals result in the formation of a nonhydroxyl radical species that can be spin trapped by DMPO (Figures 1c and 2b). Detection of the nonhydroxyl radical adduct from carnosine could be observed either by increasing carnosine concentrations (Figure 1c) or decreasing DMPO concentrations (Figure 2b), suggesting that carnosine and DMPO were competing for hydroxyl radicals. Competition between DMPO and carnosine for hydroxyl radicals has also been reported by Yoshikawa et al. (1991).

Nonhydroxyl radical adducts were also observed from Gly-His (Figure 2c) and homocarnosine (Figure 2d) in the presence of a hydroxyl radical generating system but not in dipeptides having the histidine replaced with other amino acids. This suggests that formation of the nonhydroxyl radical adducts was dependent on histidine. Histidine's involvement could be through either direct formation of the radical on the imidazole ring or stabilization of side-chain radicals by the histidine moiety. Uchida and Kawakishi (1990) reported that a histidine-containing tripeptide would undergo oxidative damage in a copper-(II)/ascorbate system, resulting in the formation of a 2-imidazolone compound. Therefore, it is not clear whether the observed nonhydroxyl radicals generated by hydroxyl radicals originated from the dipeptide or from dipeptide decomposition products.

Histidine showed hydroxyl radical scavenging activity less than the histidine-containing dipeptides (Table 1), and nonhydroxyl radicals were not detected when histidine reacted with hydroxyl radicals. Individual amino acids (Gly, β-Ala, and GABA) with the exception of histidine were not found to quench hydroxyl radical production or inhibit phosphatidylcholine liposome oxidation. In addition, mixtures of histidine and Gly, β-Ala, or GABA exhibited hydroxyl radical quenching activity similar to that of histidine and less than that of the histidine-containing dipeptides (Table 1). The amino acid combinations tested did not form nonhydroxyl radicals. These results indicate that the linkage between amino acids is involved in the ability of the peptides to quench hydroxyl radicals and to form free radicals which can be spin trapped by DMPO.

Gly, β-Ala, GABA, β-Ala-Ala, and β-Ala-Gly were all found to accelerate hydroxyl radical formation (Table 1). Possible explanations for the prooxidant activity of these amino acid and dipeptides include chelation of iron in a manner which alters redox potential, chelation of iron such that iron did not donate electrons and subsequently inactivate hydroxyl radicals, and production of other sources of hydroxyl radicals during the reaction.

The detection of nonhydroxyl radicals in the presence of hydroxyl radical generating system suggests that Gly-His, carnosine, and homocarnosine can act as free radical scavengers. The ability of the dipeptides to scavenge hydroxyl radicals (as determined by EPR) was not directly related to their ability to inhibit hydroxyl radical catalyzed oxidation of phosphatidylcholine liposomes (Table 1). Hydroxyl radical scavenging activity of the dipeptides was in the order Gly-His > carnosine > homocarnosine, while antioxidant activity was in the order carnosine > homocarnosine > Gly-His. Discrepancies between hydroxyl radical scavenging and antioxidant activity could be due to several possibilities including differences in hydroxyl radical scavenging activity, stability of the dipeptide radicals, interaction of the dipeptides with lipid radicals, and rate of dipeptide radical inactivation by hydrogen donors such as ferrous ions. Further research is needed to identify dipeptide oxidation products which will allow identification of the major sites of free radical attack and evaluation of how amino acid composition influences the rate of free radical quenching.

In conclusion, similar nonhydroxyl radicals with splitting constants of $\alpha^N$ = 15.6 G and $\alpha^H_\beta$ = 18.8 G were detected when Gly-His, carnosine, and homocarnosine were incubated with a hydroxyl radical generation system. The linkage between the amino acids and His was involved in the ability of the dipeptides to quench hydroxyl radicals. The hydroxyl radical scavenging and antioxidant activity of the dipeptides could be related to the ability of dipeptides to scavenge free radicals and the stability of the resulting dipeptide radicals. This research suggests that the antioxidant activity of carnosine and related dipeptides could in part be due to free radical scavenging.

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