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Research Article

Prevention of peroxynitrite-dependent damage by carnosine and related sulphonamido pseudodipeptides

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Abstract. The naturally occurring dipeptides carnosine and anserine have been proposed to act as antioxidants in vivo. We investigated whether these compounds can act as protective agents able to counteract peroxynitrite-dependent reactions. The results showed that the dipeptides efficiently protect tyrosine against nitration, α_1-antiproteinase against inactivation and human low-density lipoprotein against modification by peroxynitrite. Carnosine exerts its protective effect at concentrations similar to those found in human tissues. In addition, some synthetic pseudodipeptides, stucturally related to carnosine but stable to hydrolytic enzymes, possess protective properties against peroxynitrite-dependent damage similar to the natural dipeptides. These pseudodipeptides may represent stable mimics of the biologically active carnosine suitable for pharmacological applications.

Key words. Carnosine; anserine; pseudodipeptides; scavengers; peroxynitrite; free radicals; antioxidants.

Carnosine (β-alanyl-L-histidine) and related compounds such as anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine) are naturally occurring histidine-containing dipeptides, present in several mammalian tissues, including skeletal muscle, heart and brain, at high concentrations (up to 20 mM in human muscle) [1, 2]. Many proposals have been made regarding the biological action of carnosine, including a role as a physiological intracellular buffer [3], a neurotransmitter [4], a metal chelator [5] and an antiglycating agent [6]. In addition, many biochemical studies have demonstrated that carnosine possesses antioxidant and oxygen-free radical-scavenging properties [7–12]. Numerous pharmacological properties have been related with these findings, attributing to this natural compound and its analogues a role against inflammation, cancer, atherosclerosis, ageing and cataracts [13–16]. However, carnosine and related compounds are substrates of a specific dipeptidase (carnosinase) present in human tissues [17]. This results in high effective doses in vivo which are not suitable for a pharmacological agent [18]. Therefore, in recent years, interest has been increasing in the structural modification of these biologically active peptides in order to optimize potency, selectivity and metabolic stability [19]. Recent studies reported the synthesis and properties of pseudodipeptides containing the residue taurine (2-aminoethansulphonic acid) at the amino terminal, tauryl-histidine, tauryl-3-methyl-histidine and tauryl-1-methyl-histidine, structurally related to carnosine, anserine and isoanserine, respectively (fig. 1). These tauryl peptides are characterized by the presence of a sulphonamide junction which confers stability to carnosinase activity [20] and thus they may represent interesting stable mimics of the biologically active carnosine and its analogues.

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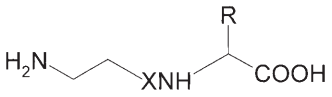


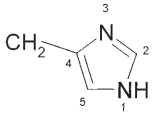
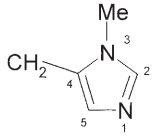
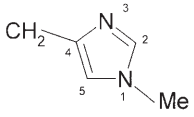
R	X = CO	X = SO_2
	Carnosine	Tauryl-histidine
	Anserine	Tauryl-3-methyl-histidine
	Isoanserine	Tauryl-1-methyl-histidine

Figure 1. β-alanyl-containing dipeptides (X = CO) and tauryl-pseudodipeptides (X = SO_2). Numbering of imidazole ring follows the IUPAC-IUB recommendations.

Oxidative species that might mediate cellular damage include reactive nitrogen species such as peroxynitrite ($ONOO^-$). This is a highly toxic oxidizing and nitrating agent which can be produced in vivo by the reaction of nitric oxide ($^{\bullet}NO$) and superoxide anion ($O_2^{\bullet-}$) [21]. Peroxynitrite, once protonated ($pK_a = 6.8$), decomposes rapidly with a half-life of less than 1 s at physiological pH and 37 °C, generating reactive species which react with a variety of biological molecules including thiols, lipids, amino acids, antioxidants and nucleic acids [22–28]. In particular, tyrosine is susceptible to an $ONOO^-$-dependent nitration reaction to yield 3-nitrotyrosine [29]. In addition, $ONOO^-$ can inactivate important cellular targets such as α_1-antiproteinase (α_1AP), where oxidation of critical methionine residues destroys the antiproteinase activity [30]. $ONOO^-$ has also been implicated in the oxidative modification of low-density lipoprotein (LDL), which suggests that $ONOO^-$ may play a significant role in the development of atherosclerotic lesions [31]. Recent studies have shown that in vivo, $ONOO^-$ predominantly reacts with CO_2 to yield the nitrosoperoxycarboxylate anion ($ONOOCO_2^-$) that can participate in the oxidation and nitration process [32–34]. Increased generation of $ONOO^-$ is being implicated in a wide range of human diseases [35–39], hence agents able to scavenge $ONOO^-$ and/or $ONOO^-$-derived species have been the object of several studies in view of possible pharmacological applications.

In the present study, the ability of the natural dipeptides carnosine and anserine to prevent $ONOO^-$-dependent damage was investigated using three in vitro assays: decrease in the nitration of tyrosine, protection of α_1AP against inactivation, and the ability to limit oxidative modification of LDL induced by $ONOO^-$. Moreover, we extended these studies to the three stable sulphonamido pseudodipeptides, tauryl-histidine, tauryl-3-methyl-histidine and tauryl-1-methyl-histidine.

Materials and methods

Reagents

Sulphonamido pseudopeptides were synthesized as described in Calcagni et al. [20]. Carnosine[1], anserine, N-succinyl-$(Ala)_3$ p-nitroanilide (SANA), L-tyrosine and 3-nitro-L-tyrosine were from Fluka (Buchs, Switzerland); elastase (E0258) and α_1-AP (A9024) were from Sigma (St. Louis, Mo.).

Peroxynitrite synthesis

Peroxynitrite was synthesized essentially as described by Beckmann et al. [41]. Five millilitres of an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with 5 ml of KNO_2 (0.6 M) on ice for 1 s and the reaction quenched with 5 ml of ice-cold NaOH (1.2 M). Excess hydrogen peroxide was removed by passing the solution through a manganese dioxide column. The solution was then frozen overnight (–20 °C) and the yellow liquid layer on top of the ice crystals collected for the experiments. The concentration of $ONOO^-$ was determined spectrophotometrically at 302 nm using a molar absorption coefficient of 1670 M^{-1} cm^{-1}.

Tyrosine nitration assay

Peroxynitrite (final concentration 100 µM) was added to a solution containing L-tyrosine (100 µM) in the presence of varying concentrations (0.5–15 mM) of carnosine or analogues in 0.5 M K-phosphate buffer, pH 7.4, giving a final volume of 1 ml. The tubes vortexed for 15 s were incubated for 10 min at 25 °C. The pH was measured after the addition of $ONOO^-$ and found to be 7.4–7.5. Appropriate controls, without the carnosine, were carried out to estimate levels of tyrosine nitration.

[1] Hydrazine, a powerful reducing agent present as a contaminant in some commercial preparations of synthetic carnosine, has been reported to affect the analyses used by researchers to investigate the antioxidant properties of carnosine [40]. We have found that in this preparation of carnosine, the concentration of hydrazine was 0.1% (w/w). However, control experiments showed that hydrazine, at concentrations equivalent to those found with synthetic carnosine, did not interfere with the assays used in this study. Therefore, this preparation of carnosine was used without further purification.

The samples were then analysed for 3-nitrotyrosine formation by high-performance liquid chromatography. Analyses were carried out with a Waters Chromatograph equipped with a model 600 pump, a model 600 gradient controller and a model 996 photodiode array detector linked to a Millenium 32 workstation. The column was a Nova-pak C18 (3.9 × 150 mm), 4 µm. The mobile phase was: A, 50 mM K-phosphate/H_3PO_4, pH 3.0; B, acetonitrile:water (50:50, v/v). A linear gradient from A to 33% B for 10 min was used at a flow rate of 1 ml/min. Tyrosine and 3-nitrotyrosine were monitored at 274 and 360 nm, respectively, and concentrations were calculated from standard curves. The detection limit for 3-nitrotyrosine was 10 pmol. Carnosine and related dipeptides did not interfere with the analyses of tyrosine and 3-nitrotyrosine.

Prevention of α_1-AP inactivation

Elastase and α_1AP activity were measured as described in Moreno and Pryor [30]. Briefly, 60 µl α_1AP (4 mg/ml) was incubated without or with carnosine (1–20 mM) in 0.5 M K-phosphate buffer, pH 7.4, to give a volume of 0.945 ml, and incubated at 37°C for 15 min, when $ONOO^-$ (typically 5 µl) was added at a final concentration of 0.5 mM. After 10 s vortexing and incubation for 5 min, 50 µl elastase (5 mg/ml) was added and the sample further incubated for 15 min followed by addition of 2.0 ml buffer (0.5 M K-phosphate, pH 7.4). Then, after 15 min, 0.1 ml of 1 mM elastase substrate (SANA) was added and the reaction followed at 410 nm for 30 s. Appropriate control experiments showed that carnosine did not affect the elastase activity or the α_1AP capacity to inhibit elastase.

Isolation of LDL

LDL was isolated from human plasma by sequential ultracentrifugation through a potassium bromide density gradient [42]. LDL protein concentration was measured by the Lowry assay [43]. The freshly prepared LDL was stored under nitrogen at 4°C in 0.01 M K-phosphate buffer, containing 0.15 M NaCl, pH 7.4.

Gel electrophoresis of LDL

Modification of LDL by $ONOO^-$ was assessed by measuring changes in electrophoretic mobility by using agarose gel electrophoresis. Varying concentrations of carnosine (0.1–10 mM) were added to LDL (125 µg protein/ml) in 0.2 M K-phosphate buffer, pH 7.4 and incubated for 15 min at 37°C, before $ONOO^-$ (0.6 mM final concentration) addition. A control sample without carnosine and a sample without $ONOO^-$ were included for each experiment. Samples were incubated for 2 h at 37°C prior to gel electrophoresis. An aliquot (5 µl) of each sample was subsequently applied to the 0.5% agarose gels at 100 V for 50 min in 50 mM barbital buffer, pH 8.6 (Beckman Paragon Lipo Gel electrophoresis system). Lipoproteins were visualized by staining with Sudan Black B.

Results

Inhibition of tyrosine nitration

Peroxynitrite mediates nitration of tyrosine in phosphate buffer, pH 7.4, with the formation of 3-nitrotyrosine [29]. In our experimental conditions, exposure of tyrosine (100 µM) to $ONOO^-$ (100 µM) resulted in the production of 8.4 ± 0.4 µM 3-nitrotyrosine. As shown in figure 2A, carnosine and anserine decreased the $ONOO^-$-dependent nitration of tyrosine dose-dependently in the range 0.5–15 mM, with anserine being more efficient than carnosine. Figure 2B shows that the pseudodipeptides tauryl-histidine, tauryl-1-methyl-histidine, and tauryl-3-methyl-histidine exert a protective effect comparable to that of natural dipeptides. Moreover, the finding that histidine but not β-alanine or taurine protects tyrosine from nitration by $ONOO^-$ (fig. 2A, B) suggests that the imidazole moiety, present in all the dipeptides under study,

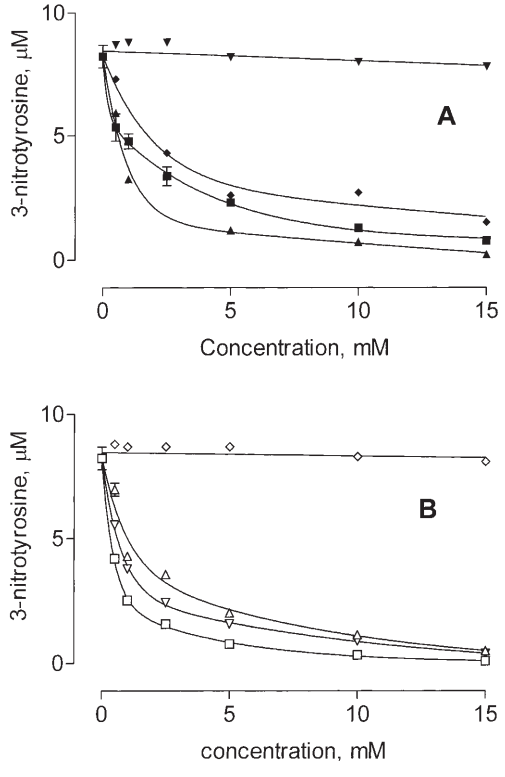


Figure 2. Prevention of $ONOO^-$-dependent nitration of tyrosine. The assay conditions were as described in Materials and methods. Compounds, at the indicated concentrations, were mixed with tyrosine (100 µM) before $ONOO^-$ addition (100 µM). Results are the mean ± SE of three or more experiments. (*A*) Concentration dependence of the protective effect of carnosine (■), anserine (▲), β-alanine (▼) and histidine (◆). (*B*) Concentration dependence of the protective effect of tauryl-histidine (△), tauryl-3-methyl-histidine (▽), tauryl-1-methyl-histidine (□) and taurine (◇).

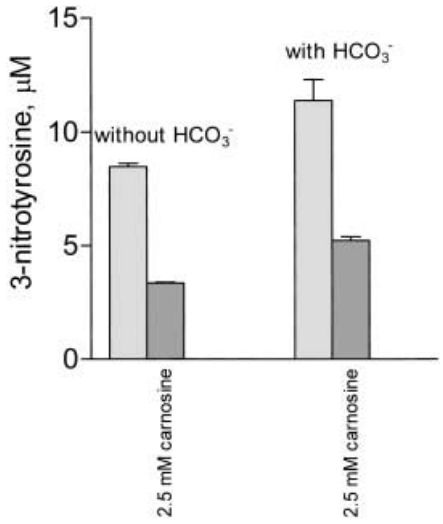


Figure 3. Inhibition of $ONOO^-$-dependent tyrosine nitration by 2.5 mM carnosine in the absence and presence of added 25 mM bicarbonate. The assay conditions are as in the legend for figure 2. Results are the mean ± SE of three experiments.

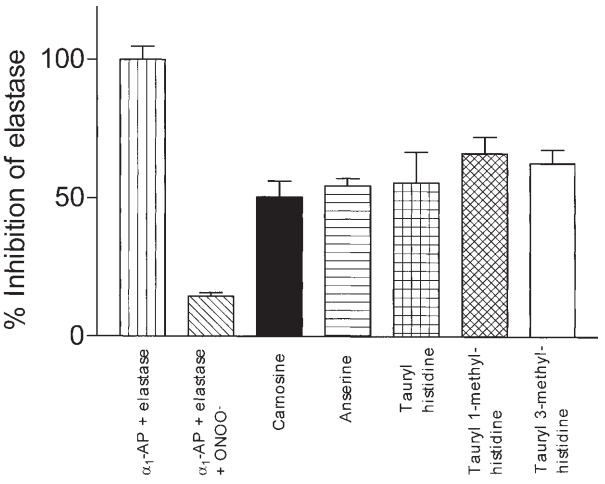


Figure 5. Protection of carnosine and related compounds against $ONOO^-$-dependent α_1AP inactivation. The compounds at a final concentration of 5 mM were mixed with α_1AP before addition of $ONOO^-$ (final concentration 0.5 mM). For further experimental details see Materials and methods. Results are the mean ± SE of three experiments.

could be the main contributor to the mechanism of protection. As previously reported, in the presence of added bicarbonate, the $ONOO^-$-mediated nitration of tyrosine is usually enhanced because of the nitrating and oxidizing properties of the peroxynitrite-CO_2 adduct ($ONOOCO_2^-$) [32–34]. The results reported in figure 3 demonstrate that carnosine is equally effective in counteracting the nitrating properties of $ONOO^-$ in the absence and presence of 25 mM bicarbonate (about 60% of inhibition by carnosine at 2.5 mM concentration).

Prevention of α_1-AP inactivation

Treatment of α_1AP with $ONOO^-$ leads to a strong inactivation of its elastase inhibitory ability [30]. Figure 4 shows the concentration dependence of the protection of α_1AP by carnosine. The compound was not protective if added to α_1AP preincubated with $ONOO^-$ for 5 min, indicating that it does not reverse but prevents α_1AP inactivation by scavenging $ONOO^-$ and/or $ONOO^-$-derived products. Compared with carnosine, anserine and tauryl peptides showed similar protective effects against α_1AP inactivation by $ONOO^-$ (fig. 5). None of these compounds had any direct effects on elastase or on the ability of α_1AP to inhibit elastase (data not shown).

Inhibition of human LDL modification

$ONOO^-$ has previously been reported to oxidatively modify LDL, resulting in a significant increase in electrophoretic mobility [31]. In our experimental conditions, when human LDL was incubated with 0.6 mM $ONOO^-$, a

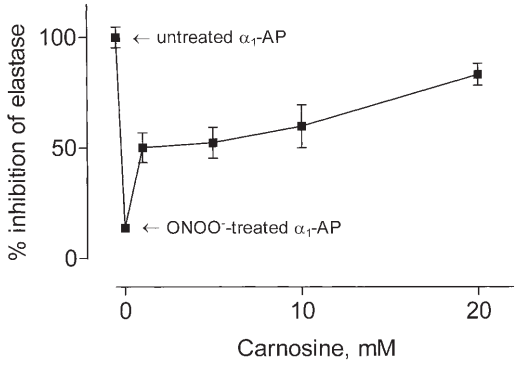


Figure 4. Concentration dependence of the protective effect of carnosine against inactivation of α_1AP by $ONOO^-$. The assay conditions were as described in Materials and methods. Carnosine was added to give the final concentrations indicated and incubated with α_1AP before $ONOO^-$ addition (final concentration 0.5 mM). Results are the mean ± SE of three experiments.

Table 1. Effect of carnosine and related dipeptides on the relative electrophoretic mobility (REM) of human LDL (125 μg protein/ml) treated with 0.6 mM peroxynitrite.

	REM[a]	Percent reduction in REM[b]
Peroxynitrite-treated LDL	1.91	
+ carnosine 0.1 mM	1.45	50.5
+ carnosine 0.5 mM	1.32	64.8
+ carnosine 5 mM	1.09	90.1
+ carnosine 10 mM	1.05	94.5
+ anserine 5 mM	1.10	89.0
+ tauryl-histidine 5 mM	1.14	84.6
+ tauryl-1methylhistidine 5 mM	1.18	80.2
+ tauryl-3methylhistidine 5 mM	1.10	89.0

[a] The REM of LDL samples was determined setting the electrophoretic mobility of untreated LDL as 1.0.

[b] Calculated as: $(REM_{ONOO^- treated} - REM_{sample})/(REM_{ONOO^- treated} - 1.0) \times 100$.

relative electrophoretic mobility (REM) of 1.91 was observed for LDL (the electrophoretic mobility of the untreated LDL was set as 1.00). Control experiments showed that carnosine and related pseudopeptides at 5 mM concentration did not affect the electrophoretic mobility of LDL not treated with $ONOO^-$. The effectiveness of carnosine in the range 0.1–10 mM, and anserine and related pseudopetides at 5 mM in lowering $ONOO^-$-mediated LDL oxidation, as measured by the REM, is shown in table 1. The relative potencies of the compounds at 5 mM concentration were carnosine ≥ anserine = tauryl-3-methyl-histidine > tauryl-histidine > tauryl-1-methyl-histidine.

Discussion

Previous studies have proposed many functions for the natural histidine-containing dipeptides, carnosine and anserine, including antioxidant and oxygen-derived-free-radical-scavenging properties [7–12]. The present results indicate that these dipeptides may also act as protective agents able to prevent $ONOO^-$-mediated reactions such as tyrosine nitration, α_1AP inactivation and LDL modification. These in vitro assays have been used in several studies to investigate the ability of various compounds to act as $ONOO^-$ antagonists [44]. Moreover, damage to these important biological targets has been shown to occur in various forms of tissue injury where the generation of peroxynitrite has been implicated [35–39].

Recent studies have shown that $ONOO^-$ reacts rapidly with CO_2 producing reactive species which influence peroxynitrite-mediated reactions [32–34] such as, for example, increased tyrosine nitration. The finding that carnosine is also able to counteract the nitrating properties of $ONOO^-$ in the presence of added bicarbonate indicates that the dipeptide could play an important role as protective agent under physiological conditions where the reaction of peroxynitrite with CO_2 precedes those with other biological targets. Since directly scavenging peroxynitrite is difficult because of its fast reaction with CO_2, the protective effect of carnosine must involve trapping the reactive species generated from the $ONOO^-/CO_2$ reaction.

Experiments, performed to find out which part of the dipeptides is involved in the mechanism of protection, indicate that the $ONOO^-$-scavenging activity of carnosine and anserine may be attributable to the imidazole moieties of the molecules, since histidine but not β-alanine is an effective inhibitor of $ONOO^-$-induced tyrosine nitration. Accordingly the synthetic pseudodipeptides, which share the same histidine residue as the natural dipeptides, inhibit nitration with the same efficiency as the physiological parents, whereas taurine substituting for β-alanine, does not. Although free histidine does not react with peroxynitrite [45], this amino acid is an efficient antioxidant and free radical scavenger [7, 10, 46]. Moreover, despite the controversy surrounding the mechanisms of peroxynitrite reactions, recent studies have presented evidence identifying the free radicals $^{\bullet}OH$ and $^{\bullet}NO_2$ as the reactive intermediates of peroxynitrous acid degradation [47–49] and the radicals $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ as the reactive species generated from the $ONOO^-/CO_2$ reaction [32–34]. Thus, consistent with these observations, any protection from $ONOO^-$-mediated damage by carnosine and related dipeptides may be linked to the ability of the imidazole ring of the molecules to scavenge $ONOO^-$-derived radicals which participate in the oxidation and nitration process.

In the experimental conditions used in the present study, carnosine was able to prevent the damaging effect of peroxynitrite at concentrations similar to those found in mammalian tissues (up to 20 mM in muscle). However, the half-life in the body and thus the beneficial effect of carnosine is limited due to the activity of carnosinase, a peptidase present in blood, liver and kidney [17, 50]. In this respect, the striking result of this work is that the tauryl-pseudodipeptides, characterized by the presence of a sulphonamide junction stable to carnosinase activity [20], possess protective properties against peroxynitrite-dependent damage rather similar to the corresponding natural dipeptides. These synthetic pseudopeptides may thus represent therapeutically useful agents which maintain the antioxidant properties of carnosine and related dipeptides, while being resistant to cleavage by serum dipeptidases.

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