L-Carnosine (β-alanyl-L-histidine) and carcinine (β-alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid-peroxidase activities

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Carnosine (β-alanyl-L-histidine) and carcinine (β-alanylhistamine) are natural imidazole-containing compounds found in the non-protein fraction of mammalian tissues. Carcinine was synthesized by an original procedure and characterized. Both carnosine and carcinine (10–25 mM) are capable of inhibiting the catalysis of linoleic acid and phosphatidylcholine liposomal peroxidation (LPO) by the $O_2^{-\cdot}$-dependent iron–ascorbate and lipid-peroxyl-radical-generating linoleic acid 13-monohydroperoxide (LOOH)-activated haemoglobin systems, as measured by thiobarbituric-acid-reactive substance. Carcinine and carnosine are good scavengers of $OH^{\cdot}$ radicals, as detected by iron-dependent radical damage to the sugar deoxyribose. This suggests that carnosine and carcinine are able to scavenge free radicals or donate hydrogen ions. The iodometric, conjugated diene and t.l.c. assessments of lipid hydroperoxides (13-monohydroperoxide linoleic acid and phosphatidylcholine hydroperoxide) showed their efficient reduction and deactivation by carnosine and carcinine (10–25 mM) in the liberated and bound-to-artificial-bilayer states. This suggests that the peroxidase activity exceeded that susceptible to direct reduction with glutathione peroxidase. Imidazole, solutions of β-alanine, or their mixtures with peptide moieties did not show antioxidant potential. Free L-histidine and especially histamine stimulated iron (II) salt-dependent LPO. Due to the combination of weak metal chelating (abolished by EDTA), $OH^{\cdot}$ and lipid peroxyl radicals scavenging, reducing activities to liberated fatty acid and phospholipid hydroperoxides, carnosine and carcinine appear to be physiological antioxidants able to efficiently protect the lipid phase of biological membranes and aqueous environments.

INTRODUCTION

Carnosine (β-alanyl-L-histidine) was first identified in 1900 in beef extract, hence its name (carno, carnis: meat) [1]. It is one of the most abundant (1–20 mM) nitrogenous compounds present in the non-protein fraction of vertebrate skeletal muscle [2–4] and certain other tissues, including olfactory epithelium and bulbs (0.3–5.0 mM) [5]. Some related compounds, i.e. anserine (β-alanyl-3-methyl-L-histidine) and homocarnosine (γ-aminobutyryl-L-histidine) have been reported [6] to be present at millimolar concentrations in several mammalian tissues, including skeletal muscle and brain, although there are interesting differences in their tissue distributions [7,8]. A weak anti-inflammatory activity of carnosine and a tendency to stimulate tissue repair, particularly when used following oral procedures, has been reported [9].

A number of putative roles have been ascribed to the histidine-containing peptides, including intracellular buffering [10], regulation of glycogenolysis [11], involvement in histamine metabolism [12], neurotransmission [13], myosin activation [14] and effect on the activities of muscle calpains [15]. Carnosine was reported to protect against the clastogenicity of normobaric hyperoxia and protect rabbit hearts from reperfusion injury after ischaemia [16,17]. A striking effect of carnosine is its ability to prevent, or partly reverse, lens cataract [18,19], a disease where oxidative stress plays an important role in the genesis of the lens opacity [20–22].

Carcinine (β-alanyl-L-histamine) was discovered in cardiac tissue of the crustacean *Carcinus maenas* in 1973 [23], and has since been identified in the hearts of other crustacea. Carcinine exists in multiple-histamine-rich mammalian tissues such as heart, kidney, stomach and intestine in levels as high as, or higher than, those reported for carnosine, histamine and 3-methylhistamine, but less than those reported for free histidine [24]. The rapid incorporation (in a few minutes) of radio-isotopic tracers (^{3}H-

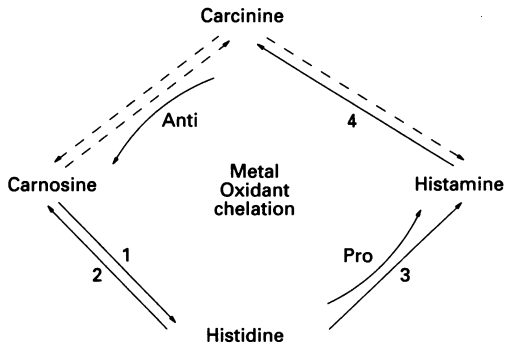

Figure 1 Involvement of carcinine in the carnosine–histidine–histamine metabolic pathway

The enzymes corresponding to the known reactions are as follows: 1, carnosinase; 2, carnosine synthetase; 3, histidine decarboxylase; and 4, carcinine synthetase. Solid lines represent known reactions while dotted lines represent potential reactions. The possible role of these compounds to act as physiological anti- or pro-oxidants in the presence of transition metals is shown.

Abbreviations used: LPO, lipid peroxidation; LOOH, linoleic acid 13-monohydroperoxide; PCOOH, phosphatidylcholine hydroperoxide; MDA, malonyl dialdehyde; PC, phosphatidylcholine; LA, linoleic acid; LOH, hydroxylinoleate; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substance; Hb, haemoglobin; SOD, superoxide dismutase; BHT, butylated hydroxytoluene.

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labelled histidine) into carcinine, carnosine and histamine in rat tissues is consistent with a metabolic link between the listed compounds and a potential role in the synthesis or degradation of histamine (Figure 1) [24]. Thus, carcinine may serve as an active intermediary in the carnosine–histidine–histamine metabolic pathway and may represent an alternative way for histamine synthesis, or may be a catabolite of histamine.

Carnosine has excellent potential to act as a natural antioxidant. It is an efficient singlet-oxygen scavenger and protects phage against γ-irradiation which gives rise to oxidative DNA damage [25,26]. Carnosine decreased the rate of oxidation of linoleic acid by peroxyl radicals and inhibited hydroxylation of deoxyguanosine by an ascorbic acid/Cu^{2+} mixture. This effect was apparently mediated by carnosine binding to the Cu^{2+} ions [27], whereas the copper·carnosine (and related histidine-containing peptides) complexes exhibited a superoxide dismutase (SOD)-like activity [28]. The mechanism by which carnosine inhibited lipid peroxidation (LPO) catalysed by iron, haemoglobin, lipoxidase and singlet oxygen did not solely comprise metal chelation [29,30]. The antioxidant properties of the Zn·carnosine chelate were established [31,32] and a potent ferroxidase activity (accelerated oxidation of the ferrous to ferric ions) was noted for 2–15 mM pure carnosine [31]. Compared with several synthetic carnosine analogues which worked as antioxidants in the Fe^{2+}–ascorbate peroxidative system of sarcoplasmic membranes and liposomes, carnosine not only inhibited the LPO reactions but also diminished the level of products accumulated during membrane LPO [19,33,34]. However, no evidence was obtained as to whether this inhibition was due to chelation, free-radical scavenging or a combination of these properties. Recently, a synergism between different histidyl dipeptides with respect to antioxidant activity was found at near physiological concentrations [35].

Some of the antioxidant properties of carnosine have been disputed. Aruoma et al. [36] argued that carnosine, homocarnosine and anserine, individually, are unable to react with $O_2^{-\cdot}$, H_2O_2 or hypochlorous acid at rates which could offer antioxidant protection *in vivo*. However, anserine and carnosine can inhibit LPO in membranes by reacting with the necessary LPO products such as aldehydes [37] rather than by inhibiting the chain reaction of LPO. Similarly, Salim-Hanna et al. [38] found that carnosine (up to 17 mM) did not protect against the free-radical damage of red-blood-cell ghost membranes or brain homogenates mediated by the 2,2′-azobis-2-amidinopropane-induced oxidation system, but inhibited in small concentrations the radiation-induced inactivation rates for lysozyme and horse radish peroxidase. Carnosine, homocarnosine and anserine were reported to cause the generation of alkyl ($R^{\cdot}$) and alkoxyl ($RO^{\cdot}$) radicals in the reaction of Ni^{2+} with organic hydroperoxides [39]. The free-radical-releasing or -scavenging activity of these peptides was suggested to be related to the formation of metal·carnosine and metal·anserine chelate complexes [40]. Recognizing that more work is required to elucidate the molecular basis of the protective effect of carnosine in biological systems, we further tested the anti- or pro-oxidant activities of carnosine and other imidazole-containing compounds (i.e. carcinine, histamine and histidine) physiologically linked to carnosine in the carnosine–histidine–histamine metabolic pathway (Figure 1).

MATERIALS AND METHODS

The techniques for phospholipid extraction, purification and preparation of liposomes (reverse-phase evaporation technique) have been described previously [19,41]. Linoleic acid and lipoxidase (EC 1.13.11.12, Type V, soybean) were obtained according to Kühn et al. [42] and were of the highest purity available. The potassium salt of linoleic acid was prepared by addition of 1.0 g (13 mmol) of KOH in 30.0 ml of methanol to 5 g (13 mmol) of linoleic acid (pH 7.0). Linoleic acid 13-monohydroperoxide (LOOH) was prepared by use of potassium linoleate (5.5 g, 17 mmol) and lipoxidase Type V from soybean according to Gardner [43]. Potassium linoleate (5.5 g, 17 mmol) in 70 ml of 50 mM phosphate buffer (pH 6.3) was exposed to 8.0 ml of lipoxidase with an activity of 10.6–11.1 μmol/min per mg of protein under stirring and aeration at 20 °C for 1 h. The reaction was arrested by addition of ~ 5.0 ml of 12 M HCl to adjust the pH of the reaction mixture to 2–3. The reaction product was extracted with ether and purified by column chromatography on a Silica Gel L 100/250 (4 cm × 30 cm) column by elution with hexane/acetone (95:5, v/v). The yield was 0.38 g (8 %). The purity of the hydroperoxide was 100 ± 5 % (mean ± S.D., $n = 5$) when estimated in methanol using $\epsilon_{234} = 2.8 \times 10^4\ M^{-1} \cdot cm^{-1}$ [44]. Linoleic hydroperoxide was quantified using an iodometric technique [45]. Peroxidation of the phosphatidylcholine (PC) liposomes (1 mg/ml) or the emulsion of linoleic acid (0.25 mg/ml) was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbic acid in 0.1 M Tris/HCl buffer (pH 7.4) to the suspension. The incubations were performed at 37 °C. The concentration of LPO products in the oxidized lipid substrates was measured by reaction with thiobarbituric acid (TBA) (0.125 %, w/v). The reaction was stopped by the addition of 2.0 ml of ice-cold 0.25 M HCl containing 15 % (w/v) trichloroacetic acid. The differential absorbance of the pink-coloured condensation product, malonyl dialdehyde (MDA), at 535 and 600 nm was measured spectrophotometrically ($\epsilon_{535} = 1.56 \times 10^5\ M^{-1} \cdot cm^{-1}$). PCOOH was prepared using egg-yolk PC and the peroxidation technique described above. The product was purified by preparative t.l.c. using Silica Gel 60H (Merck) plates with methanol/chloroform/water (10:1.5:0.5, by vol.) as a developing solvent. PCOOH showed an absorption maximum at 233 nm in methanol/heptane (5:1, v/v) and a peroxide value of 2600 ≈ 3200 meq./kg.

The lipid peroxy radicals ($-RO_2\cdot$) were generated by interaction of LOOH with a ferric complex such as haemoglobin (Hb) (Fe^{2+}). The LOOH/Hb (1 mM/30 μM) reaction mixture was added to the liposome model system to initiate peroxidation. The ability of imidazole compounds to affect LPO, inhibit lipid peroxy radicals and interact directly with LOOH (0.5 mM) or PCOOH in liposomes (1 mg/ml) was controlled. Each tested compound was mixed with hydroperoxide suspended in 0.1 M Tris/HCl buffer (pH 7.4) (or free LOOH and PCOOH were used in control without additions) and incubated at 37 °C for 60 min. The content of lipid hydroperoxide was measured after chloroform/methanol (2:1, v/v) extraction in the presence of butylated hydroxytoluene (0.5 mg/100 ml) by iodometric titration.

Conjugated dienes were determined by the absorption maximum at 233 nm after the dissolution of a dry residue in 2.5 ml of methanol/heptane (5:1, v/v). LOOH (150–190 nmol) or equidose sample aliquots were applied onto a Silica Gel 60H t.l.c. plate (Merck) after extraction and dissolution of the dry residue in 10 μl cold chloroform/methanol (1:1, v/v). The hydroxylinoleate (LOH)/LOOH ratio was determined after the t.l.c. separation on Silica Gel 60H (Merck) plates using the mixture of hexane/ethyl ether/acetic acid (8:7:0.1, by vol.) as a developing solvent. For visualization, the t.l.c. plates were sprayed with vaniline reagent to detect hydroperoxides and the generated (OH-containing) reaction products. The plates were briefly warmed at 120 °C and scanned using a t.l.c. scanner Beckman CDS-200 densitometer

equipped with data-processing accessories at 450–570 nm (reflectance mode). Identification of products was based on co-migration with a known LOOH standard. The applied measuring procedures (iodometric titration, diene conjugation studies and t.l.c. analysis) revealed similar results of kinetics and the content of lipid hydroperoxide.

The reactivity of OH· radicals with imidazole-containing compounds was detected by the release of thiobarbituric acid-reactive material from deoxyribose in the presence of the $O_2^{-\cdot}$-generating system hypoxanthine–xanthine oxidase [±EDTA (0.11 mM)] similarly as described previously [46]. The substrate molecule, i.e. deoxyribose, was present at a final concentration of 1.1 mM in PBS (pH 7.4). At the end of the incubation period, 0.5 ml of 1% (w/v) TBA in 0.05 M NaOH was added to each tube with 0.5 ml of 2.8% (w/v) trichloroacetic acid. The glass tubes were heated for 10 min at 100 °C to develop the colour. When cool, A_{532} was read against appropriate blanks.

Carcinine was synthesized according to the specification owned by Exsymol S.A.M. (Monaco, Principaute de Monaco). N.m.r. spectra were recorded on an impulsed Fourier-Transformed Spectrometer (Bruker AC 200–200 MHz). Analytical t.l.c. was performed on Silica Gel 60 F_{254} plates (Merck) using as solvent chloroform/methanol/25% ammonia (5:3:1, by vol.). The plates were developed with ninhydrin solution in *n*-butanol. I.r. spectra were monitored on a Nicolet 5-PC spectrophotometer. The melting point of the compound was determined on an Electrothermal 9100 melting-point apparatus. The product was collected to give a melting point of 195 °C and an R_F of 0.44. I.r. spectrum (KBr pellet): 3000–3300 cm^{-1}, ν(NH) and $\nu(NH_3^+)$; 1651 cm^{-1}, ν(C=O) amide I; 1622 cm^{-1}, $\delta(N-H)_{int}$; 1560 cm^{-1}, ν(C=O) amide II; 1080 cm^{-1}, ν(C–N) amine. ^{1}H-n.m.r. spectrum [200 MHz, D_2O, δ (p.p.m.)]: 2.60 (t, *J* 6.6 Hz, $-CH_2-CO-$), 2.90 (t, *J* 6.8 Hz, $-CH_2$-Im), 3.18 (t, *J* 6.6 Hz, $-CH_2-NH_2$), 3.47 (t, *J* 6.8 Hz, $-CH_2-NH-$), 7.24 (s, =CH–N–), 8.56 (s, –N–CH=N–) (Im, imidazole). ^{13}C-N.m.r. spectrum [50 MHz, D_2O, δ (p.p.m.]: 23.9 ($-CH_2$-Im), 31.8 ($-CH_2-CO-$), 35.5 ($-CH_2-NH_2$), 37.9 ($-CH_2-NH-$), 116.0 (=CH–N–), 130.6 (–C(N–)=C–), 133.0 (–N–CH=N–), 172.0 (CO). Elementary analysis: calculation for $C_8H_{14}N_4O \cdot 2HCl$ (%): C, 37.66; H, 6.32; N, 21.96; O, 6.27; Cl, 27.79. Obt.: C, 37, 18; H, 6.20; N, 21.92; O, 7.09; Cl, 27.02. U.v. spectrum (Uvikon 930, Kontron): $\lambda_{max.}$ = 225 nm, $\epsilon_{max.}$ = 2727. These data are consistent with the di-chlorhydrate form of carcinine. The analytical data for the basic form of carcinine are given below. ^{1}H-N.m.r. spectra were recorded on an Impulsed Fourier-Transformed Spectrometer (Brucker WM-250, Germany) at a working frequency of 250 MHz. Analytical t.l.c. was performed on Silica Gel 60H plates (Merck) employing two solvent systems: chloroform/methanol/25% ammonia (5:3:1, by vol.) and methanol. The plates were developed with a ninhydrin solution in acetone. Analytical reverse-phase h.p.l.c. was carried out on an LKB chromatograph (Sweden) using a column (4.0 mm × 125 mm) with Silasorb C-18 (pore size 4 μm) and elution in gradient of acetonitrile [70% in 0.05% (v/v) aqueous solution of heptafluorobutyric acid]. I.r. spectra were monitored on a Shimadzu IR-435 spectrophotometer. The melting point of the compound was determined on a thermo-melting table 'Boetius' (USA). The product was collected to give a melting point of 114–117 °C, R_{F_A} = 0.34 and R_{F_B} = 0.11. ^{1}H-n.m.r. spectrum [CD_3OD, δ (p.p.m.)]: 2.34 (t, 2 H, $\beta CH_2-\beta$-Ala), 2.87 (t, 2 H, CH_2-3-HA), 3.30 (m, 2 H, CH2-2-HA), 3.45 (t, 2 H, $\alpha CH_2-\beta$-Ala), 6.85 (s, 1 H, CH-4-Im), 7.60 [s, 1 H (CH-2-Im)] (HA, histamine; Im, imidazole; t, triplet; m, multiplet; s, singlet). Elementary analysis: calculation for $C_8H_{14}N_4O$, $0.5H_2O$ (%): C, 50.25; H, 7.91; N, 29.30; found (%): C, 51.26; H, 7.70; N, 28.31. I.r. spectrum in vaseline (cm^{-1}): 3300, amine; 1650, amide I; 1570, amide II. H.p.l.c. analysis: one peak, elution time 14.96 min.

The tested compounds were used at 1 μM–25 mM concentration to the system of LA (0.25 mg/ml) and PC liposomes (1 mg/ml) peroxidation. L-Carnosine, L-histidine and histamine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); imidazole was from Merck, and β-alanine was from Reanal (Hungary). Xanthine oxidase (grade I), hypoxanthine, 2-deoxy-D-ribose, catalase (bovine liver, thymol-free) were from Sigma Chemical Co. All other chemicals were reagent grade or purer. All experiments were performed on triplicate samples and were repeated a minimum of two times.

RESULTS

Figures 2(a) and 2(b) present the kinetic data of malonyl dialdehyde accumulation during peroxidation of linoleic acid (LA) and PC liposomes, catalysed by the Fe^{2+}–ascorbate ($O_2^{-\cdot}$-dependent) and LOOH–Hb ($O_2^{-\cdot}$-independent, generating $RO_2\cdot$ radicals) systems. The time course for the generation of the peroxidized lipid products measured with TBA-reactive sub-

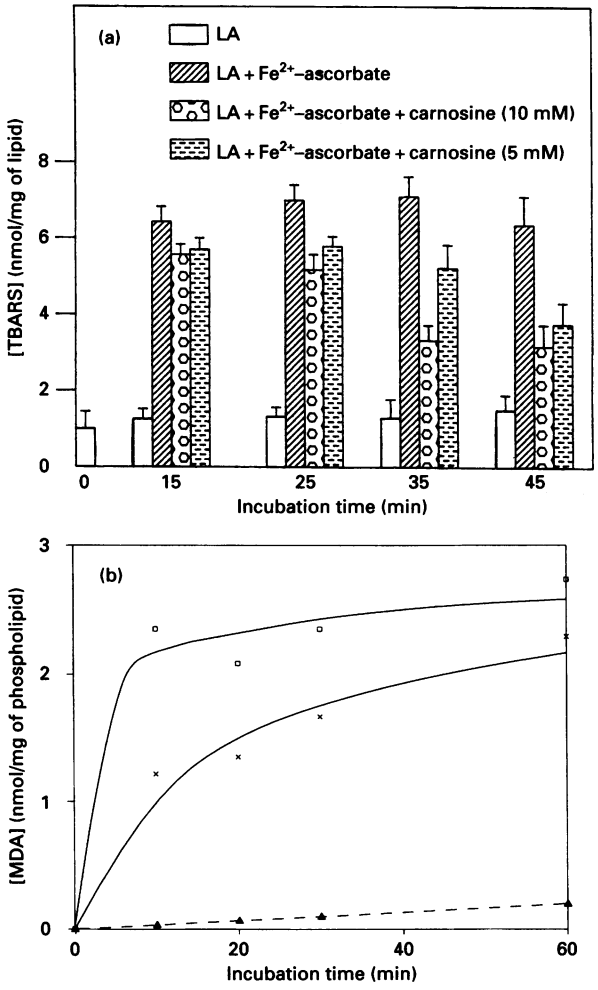

Figure 2 Oxidation of (a) LA and (b) phospholipid liposomes catalysed by the Fe^{2+}–ascorbate and LOOH–Hb systems respectively

(a) Additions of carnosine (5 and 10 mM) to the incubation mixture are indicated. Data represent means ± S.D. (n = 2). (b) Each data point is an average of two experimental values with < 10% deviation. ▲, PC only; □, PC plus Fe^{2+}–ascorbate; ×, Pc plus LOOH–Hb.

Table 1 Effect of carnosine, carcinine and related compounds on the iron–ascorbate and LOOH/haemoglobin catalysed lipid peroxidation

Peroxidation was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbate in 0.1 M Tris/HCl buffer, pH 7.4, or LOOH/Hb (1 mM/30 μM) to the reaction mixture. Data represent means ± S.D. ($n = 3$–5). Significant differences with control incubations without testing compound are indicated with asterisk(s): $^*P < 0.001$; $^{**}P < 0.05$.

Oxidation substrate	Testing compound (concentration)	Catalyst	Time of incubation at 37 °C (min)	Amount of TBARS (nmol/mg of lipid)	Percentage of control
LA	None		35	7.95 ± 0.15	100
(0.25 mg/ml)	Carcinine (25 mM)	Fe^+		4.25 ± 0.58*	53
	Carnosine (25 mM)	ascorbate		3.24 ± 0.46*	41
	Imidazole (25 mM)			6.95 ± 0.65	89
	β-Alanine (25 mM)			7.91 ± 0.10	100
	Histidine (25 mM)			10.06 ± 0.05*	126
	Histamine (25 mM)			10.15 ± 0.10*	132
	β-Alanine (25 mM) + Histidine (25 mM)			8.12 ± 0.11	103
	β-Alanine (25 mM) + histamine (25 mM)			9.06 ± 0.15*	115
PC liposomes	None			4.4 ± 0.2	100
(1 mg/ml)	Carcinine (10 mM)	Fe^+	60	2.5 ± 0.1*	58
	Histamine (10 mM)	ascorbate		6.8 ± 0.2*	163
	Histamine (1 μM)			6.4 ± 0.4*	145
	Carnosine (10 mM)			2.0 ± 0.1*	47
	Histidine (10 mM)			6.6 ± 0.3*	148
	Histidine (10 mM) + EDTA (50 μM)			2.3 ± 0.3*	51
PC liposomes	None			3.5 ± 0.4	100
(1 mg/ml)	Carcinine (10 mM)	LOOH/Hb	60	2.4 ± 0.2**	63
	Carnosine (10 mM)			1.3 ± 0.2*	48
	Histamine (10 mM)			3.0 ± 0.2	97
	Histidine (10 mM)			2.9 ± 0.5	91

stance (TBARS) in the LA was more rapid and pronounced than in the PC liposomes. This may be because the liberated LA has the relatively higher accessibility for free-radical attack than the variety of polyunsaturated fatty acids present in the phospholipid bilayer of biomembranes. Different constituents and imidazole-containing compounds were compared for their ability to inhibit the iron–ascorbate and LOOH/Hb-dependent oxidation of LA (Figure 2a) and PC liposomes (Table 1). The results demonstrate that carnosine and carcinine significantly inhibited the LPO reaction at 5–25 mM concentration. Figure 2(a) shows that maximum TBARS reached in the presence of carnosine at 15 min of incubation decreases at later time points which must be due to a loss of existing TBARS or peroxide precursors of MDA and not due to a decreased formation of peroxide compounds. A transient maximum of TBARS in the presence of carnosine results from direct interaction of carnosine or carcinine not only with free radicals, but also with primary molecular products of LPO providing the reduction of the already formed peroxides (Figures 2a and 3). The constituents histidine and histamine exhibited stimulatory peroxidative effects for free LA or PC liposomes in the Fe^{2+}–ascorbate-catalysed reaction which was abolished in the LOOH-activated Hb-peroxidizing system. The addition of 50 μM EDTA completely prevented or significantly inhibited (in excess of Fe^{2+} ions) the pro-oxidant activity of histidine and histamine (Table 1, Figure 4), indicating the role of free iron catalysts. The other tested compounds, i.e. imidazole, β-alanine and the mixtures of β-alanine + histidine or β-alanine + histamine, were inactive or showed a slight stimulatory activity to LPO (Table 1), suggesting that the redox potential of the whole β-alanyl–imidazole-containing molecule is essential for its anti-oxidant activity. Both carnosine and carcinine were effective at inhibiting the production of TBARS in the LOOH-activated Hb catalysis of LPO (not sensitive to the chelating agents such as EDTA [47]), suggesting that the antioxidant mechanism depended on the ability to scavenge lipid peroxy radicals or donate hydrogen ions and not solely on chelation.

A hypoxanthine/xanthine oxidase mixture in the presence of iron·EDTA complex forms 'free' $OH^\cdot$ radicals at pH 7.4 [46]. If iron is not added, but is present as a contaminant of all reagents used, the damage to deoxyribose could be attributed to iron binding to the 'detector' molecules catalysing a 'site-specific' production of $OH^\cdot$ which can be measured by its ability to degrade the sugar deoxyribose into fragments with the formation of TBA-reactive material [36,46]. Any 'site-specifically' produced $OH^\cdot$ is equally accessible to deoxyribose and to any added scavenger of $OH^\cdot$ [48]. Thus, the ability of a scavenger to inhibit deoxyribose degradation depends only on its concentration relative to deoxyribose and on its second-order rate constant for reaction with $OH^\cdot$ [49,46]. Degradation of deoxyribose was found to be strongly inhibited by carnosine and carcinine (Table 2). From the slopes of the competition plots and literature data, the second-order rate constants are presented for the reactions of tested compounds with $OH^\cdot$ (Table 2). Deoxyribose was present at 1.1 mM and the various test compounds at 10 mM. Because the 'site-specific' attack of the sugar molecule by $OH^\cdot$ is more damaging than attack by externally generated 'free' $OH^\cdot$ [50], high concentrations of a scavenger are necessary to scavenge $OH^\cdot$ being formed at a specific site rather than providing simple mass-action effects of the first compound to contact the extremely reactive $OH^\cdot$ radicals. The damage could be prevented by SOD and catalase. Table 2 illustrates that, at 10 mM concentration, the average ability of carcinine to inhibit deoxyribose degradation

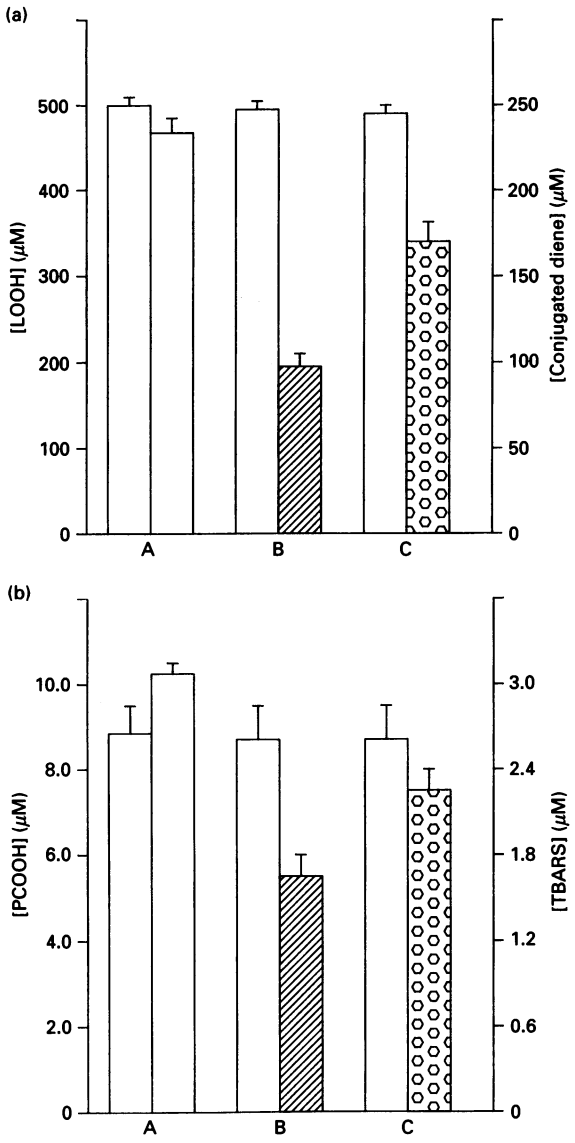

Figure 3 Reducing activities of carnosine and carcinine to (a) LOOH and (b) liposomal hydroperoxides measured by iodometric, conjugated diene and TBARS assays

(A) Background incubations of hydroperoxides without testing compounds: 0.5 mM LOOH or PCOOH in PC liposomes (1 mg/ml) (▭). (B) LOOH or PCOOH in the lipid vesicles mixed with 10 mM carnosine (▨) (C) LOOH or PCOOH in the liposomes mixed with 10 mM carcinine (▨). In each case, vesicles were suspended in 0.1 M Tris/HCl buffer, pH 7.4, and incubated at 37 °C for 60 min. At the end of incubation, samples were aspirated and the content of lipid hydroperoxide was determined (see Materials and methods section). Initial levels of peroxide in the reaction mixture and at the end of incubation are shown. Data represent means ± S.D. (n = 3–5).

was comparable with or greater than those of carnosine and related histidine-containing peptides, i.e. anserine and homocarnosine. Damage of deoxyribose in this system was also inhibitable by imidazole, L-histidine and histamine.

Reducibility of LOOH or membrane PC hydroperoxide (PCOOH) was studied by spectrophotometric, iodometric or t.l.c. assays of residual LOOH (PCOOH) during treatment with different concentrations of carnosine or carcinine (Figure 3, Tables 3 and 4). Results of a typical experiment are shown in Figures 3(a) and 3(b). The starting level of LOOH in the incubation medium was 0.5 mM and that of PCOOH was 1 μmol per 112 μmol of membrane phospholipid. Incubation with carnosine (10–25 mM) or carcinine (10–20 mM) for 1 h caused a

Table 2 Effect of OH· imidazole-containing scavengers on damage to deoxyribose by an $O_2^{-\cdot}$-generating system

Results are the means of three or more determinations, which differed by no more than 10%. The final reaction concentration was the same (10 mM) for all compounds tested. Inhibition (%) was calculated after the subtraction of appropriate blanks. The reaction was started by the addition of hypoxanthine and xanthine oxidase, as described in the text. The rate constants were determined from the slopes of the lines obtained in the presence of EDTA (k = slope · k_{dr}[dr]/A) where $k_{dr} = 3.1 \times 10^9$ $M^{-1} \cdot s^{-1}$, [dr] = 1.1 mM and A = absorbance obtained in the absence of scavenger [36]. TS, this study.

Compound tested	10^9 × Second-order rate constant ($M^{-1} \cdot s^{-1}$)	Reference	Inhibition of TBA-reactivity (%)
Carnosine	4.0	[36], TS	37
Carcinine	5.3	TS	49
Homocarnosine	2.6	[36]	25
Anserine	5.2	[36]	47
L-Histidine	7.1	[36]	65
Imidazole	4.8	[36]	44
Histamine	5.0	[36]	46

Table 3 Activity of carnosine, carcinine and sodium borohydride for reduction of lipid hydroperoxides of LOOH and PCOOH measured at 37 °C after 1 h of incubation

The incubations were performed in 0.1 M Tris/HCl buffer, pH 7.4. The molecular mass of a phospholipid molecule was assumed to be 730 Da. Reducibility of total LOOH and PCOOH contents measured on the basis of its ϵ_{233} in absorption spectra agreed closely with the value obtained by iodometric titration and by t.l.c. analysis (see Table 4). For incubations, LOOH (0.5 mM) was dissolved in methanol/water (9:1, v/v) mixture and suspended in 0.1 M Tris/HCl buffer, pH 7.4. For peroxidase activity towards LOOH, LOOH loss was determined by iodometric titration and extinction measurements, and is expressed in terms of units (nmol/ml) and as a percentage of the peroxide loss. For peroxidase activity towards PCOOH (PCOOH level 1 μmol per 112 μmol of phospholipids), PCOOH reduction was determined by iodometric titration and extinction measurements, and is expressed in terms of units (μmol of PCOOH/μmol of phospholipids) and as a percentage of the peroxide loss. Data represent means ± S.D. (n = 3–5).

Oxidation substrate	Testing compound (concentration)	Peroxidase activity (units)	(%)
LOOH (0.5 mM)	None	37 ± 15	7
	Carcinine (10 mM)	138 ± 19	28
	Carnosine (10 mM)	282 ± 33	56
	Carcinine (20 mM)	188 ± 16	38
	Carcinine (25 mM) + EDTA (0.5 mM)	0	0
	Carnosine (25 mM) + EDTA (0.5 mM)	0	0
	Carnosine (1 mM)	0	0
	Carnosine (20 mM)	291 ± 17	58
	Carnosine (25 mM)	327 ± 17	65
	$NaBH_4$ (10 mM)	313 ± 15	62
PCOOH in liposomes (0.5 mg/ml)	Carcinine (10 mM)	0.77×10^{-3}	9
	Carcinine (25 mM)	1.56×10^{-3}	17
	Carnosine (25 mM)	3.65×10^{-3}	41
	Carnosine (10 mM)	3.16×10^{-3}	35
	Carcinine (25 mM) + EDTA (0.5 mM)	0	0
	Carnosine (25 mM) + EDTA (0.5 mM)	0	0

Table 4 T.l.c. data on processing with LOOH and the reaction products generated by carnosine, carcinine and sodium borohydride

Data represent means ± S.D. of at least three measurements. Each tested compound was mixed with free LOOH (0.5 mM) sample and incubated for 1 h at 37 °C in 0.1 M Tris/HCl buffer, pH 7.4. A.u., arbitrary units.

		Scanning densitometric measurements				
		LOOH		LOH		
Contents of incubation sample	R_f of the reaction product	Peak area (a.u.)	Contents in the sample (%)	Peak area (a.u.)	Contents in the sample (%)	LOH/LOOH
LOOH (150–190 nmol), non-incubated standard	0.45	5247 ± 521	45.25 ± 7.00	1068 ± 19	9.15 ± 0.65	1/5.14
LOOH (background incubation)	0.45 (LOOH)	5281 ± 529	49.20 ± 2.00			
	0.20 (LOH)			921 ± 323	8.25 ± 1.85	1/7.50
LOOH + carnosine (20 mM)	0.45 (LOOH)	8279 ± 435	55.20 ± 2.80			
	0.20 (LOH)			2103 ± 109	13.30 ± 0.68	1/4.16
LOOH + carcinine (20 mM)	0.45 (LOOH)	7268 ± 360	66.10 ± 4.80			
	0.20 (LOH)			962 ± 68	8.80 ± 0.75	1/7.00
LOOH + $NaBH_4$ (10 mM)	0.45 (LOOH)	3959 ± 493	42.10 ± 2.90			
	0.20 (LOH)			1017 ± 71	10.80 ± 0.65	1/3.80

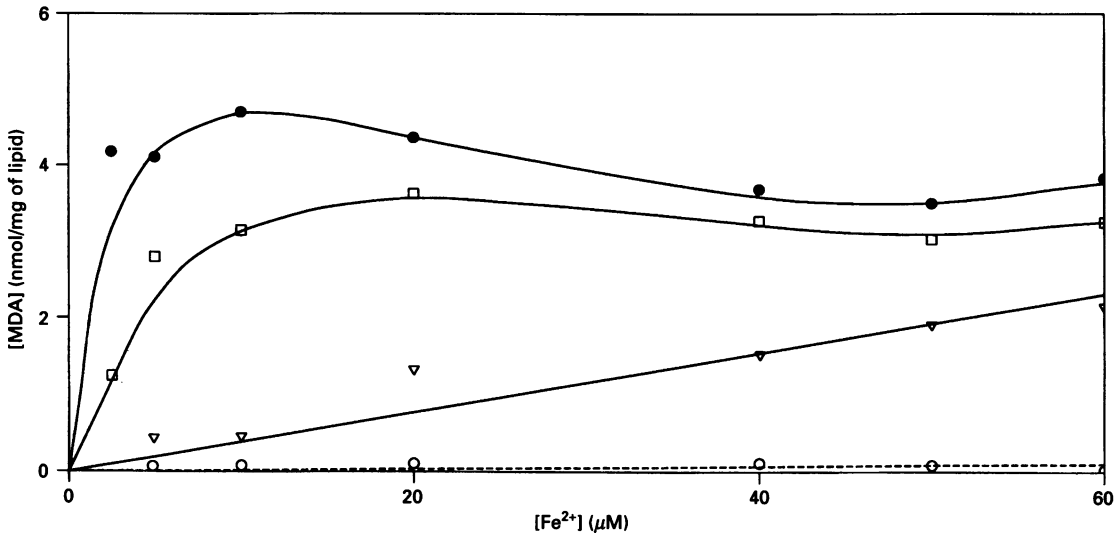

Figure 4 Effect of histamine on lipid peroxidation of liposomes

Mean data of at least three measurements in each series are presented. The data represent dependence of the lipid peroxidation reaction on the Fe^{2+} concentration. Details of the peroxidation technique are presented in the Materials and methods section. ●, Liposomes (1 mg/ml) + Fe^{2+} + ascorbate (0.2 mM) + histamine (10 mM); □, liposomes + Fe^{2+} + ascorbate; ▽, liposomes + Fe^{2+} + ascorbate + histamine + EDTA (50 μM); ○, liposomes + Fe^{2+} + ascorbate + EDTA.

decrease of total LOOH to 282–327 nmol and 138–188 nmol respectively. Within 1 h of incubation with 10–25 mM carnosine, the liposome membranes were subjected to a total loss of PCOOH and the background level of phospholipid hydroperoxides decayed to 35–40% of its value, and to 8–17% for the equitime incubation with 10–25 mM carcinine (Figure 3b). Incubation with 0.5 mM EDTA produced no net reducing effect on the LOOH (PCOOH) level over that observed in the control incubations (LOOH or PCOOH alone) (results not shown). The reducing agent, 10 mM $NaBH_4$, showed the significant reactivity for the measurable LOOH reduction relative to background decay (Table 3). The concentration of transition metals in buffers used was not estimated for reduction of lipid hydroperoxides, but the complete inhibition of the lipid peroxidase activity of carnosine and carcinine with 0.5 mM EDTA indicates the dependence on the adventitious transition metals when complexed with these natural molecules. Lipid extracts were also analysed by t.l.c. (Table 4). Based on the chromatographic migration of the hydroperoxide standards of LA and the main reduction product of 13-hydroperoxy LA generated by $NaBH_4$, the spots of the main reaction products of LOOH with carnosine or carcinine were identified on t.l.c. as hydroxy linoleate (LOH). The reduction product of PCOOH in the content of liposome vesicles gave a similar pattern of transformation, in agreement with spectrophotometric and iodometric determinations after 1 h of incubation with carnosine or carcinine (10–25 mM) (Table 3). The incubations caused a perceptible change in the spot intensity of LOOH t.l.c. product measured quantitatively by densitometric

scanning of the plates and expressed as disappearance of the free and phospholipid-derived hydroperoxides with concomitant formation of their reduction (hydroxy) products.

In the TBARS assay, histamine exhibited a pro-oxidant effect in the Fe^{2+}–ascorbate-dependent LPO. LPO of PC liposomes is inhibitable with an increase in the ferrous sulphate concentration beyond 40 μM [51], as similarly observed in the liposome model system (Figure 4). The pro-oxidant effect of histamine is most likely due to the formation of the chelate Fe(II)·histamine, with a redox activity more powerful as LPO promoter than of free ferrous ions. In the presence of 10 mM histamine, the dependence of LPO on the Fe^{2+} concentration remains unchanged (Figure 4) despite the concentration of histamine being $\sim$ 1000-fold the concentration of ions of oxygen-activating metal. Ascorbate acts as a redox mediator of such a Fe(II)·HA complex, inducing LPO under these conditions. The incubation (liposomes + iron + ascorbate + EDTA) showed the inhibition of TBARS accumulation due to Fe^{2+} chelation by the EDTA and prevention of radical generation. In the presence of the stronger ferrous ion chelator EDTA (50 μM), only slight accumulation of TBARS occurred in the 10 mM histamine-containing medium in the presence of excess ferrous ions (60 μM), illustrating a competitive mechanism for Fe^{2+} binding in the catalytically active pro-oxidant Fe(II)·HA complex.

DISCUSSION

LPO has been proposed to contribute to various pathophysiological cell and tissue abnormalities. Most known biological antioxidants that prevent oxidative damage to lipids, proteins and other essential macromolecules show some specificity in their mechanism of action, providing only one general type of protection (i.e. blocking free-radical initiation; removing oxidants from the biological targets; reacting with the reactive species, thus sparing the biological target; transforming a reactive oxygen species to a non-reactive species; stabilizing membranes; and/or acting indirectly by removal of mediators that can catalyse free-radical damage [52]). Primary defence against free radicals is based on prevention of initiating reactions achieved by agents such as enzyme scavengers of reactive oxygen species (SOD, catalase and peroxidases), chemical antioxidants (α-tocopherol, β-carotene and ascorbate) and iron-sequestering proteins (apoferritin and apolactoferrin). A secondary protection involves enzymic removal of lipid-derived hydroperoxide intermediates typically catalysed by glutathione-requiring enzymes (Se-dependent GSH peroxidases and certain Se-independent enzymes, such as GSH-S-transferase B) [53]. Recently, the reduction of oleate hydroperoxide into hydroxy oleate was demonstrated for ferrocytochrome *c*, both in aqueous medium and in a phospholipid system [54].

Among the compounds tested, a unique property of carnosine and carcinine is their water solubility combined with their ability to trap LPO products. Carnosine and carcinine exhibit an ability to inhibit LPO catalysts besides inhibiting free metals, scavenging $OH^{\cdot}$ and lipid peroxyl ($RO_2\cdot$) radicals or donating hydrogen ions. In addition to inhibiting the generation of lipid peroxyl radicals, carnosine and carcinine catabolize fatty acid hydroperoxides to their alcohols both in aqueous medium and in a phospholipid system. This differs from the native peroxidase which uses GSH as a cofactor and prior hydrolysis by Ca^{2+}/phospholipase A_2 to eliminate the phospholipid-derived hydroperoxides [55,56,57,58,59].

The antioxidative activity of carnosine and carcinine was analysed in the Fe^{2+}–ascorbate and LOOH/Hb LPO-catalysing systems. There is abundant literature on the free-radical pitfalls in these systems mainly since complexing agents differently modify their oxygen reducing and the lipid-endoperoxide degrading potentials [47,60–62]. The natural molecules used reduce more than 1 mol of phospholipid-derived hydroperoxide per 650 mol of phospholipid after 1 h of incubation and inhibit LPO to 42–53% in the lipid phase and to 47–59% in the aqueous medium at physiological concentrations. Chemically, the antioxidant activity of carnosine and carcinine is not solely due to the imidazole moieties of the molecules, since imidazole itself did not show the antioxidant activity, and histamine exhibited a pro-oxidant action dependent on the concentration of catalytically active free iron ions (Figure 4). The antioxidant activity of these molecules may involve reduction of oxidative potential or stabilization of the imidazole radical, probably due to the peptide bond: free β-alanine molecule and β-alanine in admixture with histidine or histamine did not show the inhibition of LPO (Table 1). The carboxyl group of histidine is essential for antioxidant activity of carnosine compared with the weaker antioxidant β-alanylhistamine (carcinine). Chelation of transition metals such as iron or copper is also important for antioxidant activity of carnosine and carcinine. Carnosine, carcinine and histamine have been shown to be effective chelating agents for transition metals [28,63,64], forming complexes possessing antioxidant properties [28,64]. The reduction of various lipid hydroperoxides may result from the cleavage of a lipid hydroperoxide with a transition metal complex and supplement with electrons for the reductive reaction LOOH→LOH. Carnosine is active electrochemically as a reducing agent in cyclic voltammetric measurements, donating a hydrogen atom to the peroxyl radical [27]. The peroxidase activity of carnosine and carcinine is abolished when iron is complexed with 0.5 mM EDTA (Table 3). The antioxidant protection in the free iron ion-independent (LOOH/Hb) system is most probably explained by other properties of carnosine and carcinine ($OH^{\cdot}$ and lipid-peroxyl-radical-scavenging activities and binding of hydroperoxide in an imidazole·peroxide adduct [65]). The discrepancies between histamine and β-alanylhistamine, and histidine and β-alanylhistidine in their action to the iron-catalysed LPO are consistent with a metabolic link of carcinine, histamine, histidine and carnosine and their roles in metabolism of lipid-derived peroxide intermediates. Because histamine is thought to play a central role in growth, wound healing and various types of shock [3,66–68], as well as having influences on mammalian cardiac, renal, pulmonary, gastric, neurological and immunological physiology [69–72], a burst of free-radical generation in the presence of iron and ascorbate and interactions with the antioxidants carcinine and carnosine can modulate its metabolic function.

Exogenous carnosine entering the organism intravenously, intraperitoneally, with food or topically to the eye, is not accumulated by the tissues but is excreted in the urine or destroyed by carnosinase, an enzyme that is present in blood plasma, liver, kidney, and other tissues except muscle [73,74]. Despite the fact that carcinine is metabolically related to carnosine, it has not been shown to be a substrate for carnosinase or other dipeptidases [75]. Therefore, carcinine may play a greater role in the prolongation and potentiation of physiological responses to the therapeutical treatments with carnosine as antioxidant.

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Received 5 November 1993/19 May 1994; accepted,27 May 1994