Available online at www.sciencedirect.com
SCIENCE DIRECT®
Life Sciences 78 (2006) 2343 – 2357
Life Sciences
www.elsevier.com/locate/lifescie

Biological activities of the natural imidazole-containing peptidomimetics *n*-acetylcarnosine, carcinine and L-carnosine in ophthalmic and skin care products

Mark A. Babizhayev *

Innovative Vision Products, Inc., 3511 Silverside Road, Suite 105, County of New Castle, Delaware 19810, USA
Moscow Helmholtz Research Institute of Eye Diseases, 14/19, Sadovaya-Chernogryazskaya, 103064 Russian Federation Moscow

Received 3 June 2005; accepted 21 September 2005

Abstract

Apart from genetically programmed cell aging, different external aggressors related to oxidative stress and lipid peroxidation (LPO) can accelerate the skin aging phenomenon. Oxidative stress associated with the formation of lipid peroxides is suggested to contribute to pathological processes in aging and systemic diseases known as the risk factors for cataract. Despite the fact that L-carnosine-related peptidomimetics *N*-acetylcarnosine (*N*-acetyl-β-alanyl-L-histidine) (NAC) and carcinine (β-alanylhistamine) are metabolically related to L-carnosine and have been demonstrated to occur in tissues of many vertebrates, including humans, these compounds were shown resistant toward enzymatic hydrolysis. A series of related biocompatible imidazole-containing peptidomimetics were synthesized in order to confer resistance to enzymatic hydrolysis and ex vivo improvement of protective antioxidative properties related to L-carnosine. The included findings revealed a greater role of *N*-acetylcarnosine (NAC) and carcinine ex vivo in the prolongation and potentiation of physiological responses to the therapeutical and cosmetics treatments with L-carnosine as antioxidant. 3-D molecular conformation studies proposed the antioxidant activity of peptidomimetics (carcinine, L-prolylhistamine, *N*-acetylcarnosine, L-carnosine) for metal ion binding, quenching of a number free radicals, and binding of hydroperoxide or aldehyde (including dialdehyde LPO products) in an imidazole-peroxide adducts. NAC can act as a time release (carrier) stable version of L-carnosine during application in ophthalmic pharmaceutical and cosmetics formulations which include lubricants. Carcinine, L-prolylhistamine show efficient deactivation of lipid hydroperoxides monitored by HPLC and protection of membrane phospholipids and water soluble proteins from the lipid peroxides-induced damages. This activity is superior over the lipophilic antioxidant vitamin E. The biologically significant applications of carnosine mimetics were patented by Dr. Babizhayev and the alliance Groups (WO 2004/028536 A1; WO 94/19325; WO 95/12581; WO 2004/064866 A1).

Keywords: Natural peptidomimetics; Carcinine; *N*-acetylcarnosine; L-prolylhistamine; L-carnosine; Ophthalmic and skin care; Cataract; Oxidative stress; Lipid peroxidation; Lipid hydroperoxides; Protein cross-linking; SOD-like activity; Peptidase hydrolysis; Universal antioxidants

"There is nothing stronger in all the armies in the world.....than an idea whose time has come!!"

Introduction

Multilateral biological activity of histidine-containing dipeptides in combination with their high content in human and animal tissues have long been a serious challenge to biologists, pharmacologists, physiologists and clinicians. There is presently a large body of literature on the variety of biological effects of carnosine (β-alanyl-L-histidine) in various pathological states in experimental animals and in clinics.

Carnosine and related dipeptides have been postulated to have numerous biological roles including pH buffering, regulation of enzyme activity and inhibition of oxidative reactions. Among antioxidant mechanisms reported for carnosine are its ability to inactivate reactive oxygen species, scavenge free radicals, and chelate prooxidative metals (Boldyrev et al., 1988; Kohen et al., 1988; Decker et al.,

* Innovative Vision Products, Inc., Moscow Division, Ivanovskaya 20, Suite 74, 127434 Russian Federation Moscow.
E-mail addresses: markbabizhayev@mail.ru, markbabizhayev2004@yahoo.com.

0024-3205/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.lfs.2005.09.054

1992; Aruoma et al., 1989). The dipeptide L-carnosine has beneficial effects on cultured human fibroblasts. The continuous growth of these cells in carnosine not only increases their lifespan in population doublings and period of growth, but also can reverse the normal features of senescent cells (McFarland and Holliday, 1994, 1999). A rather unusual reported antioxidant property of carnosine was its ability to reduce concentrations of thiobarbituric acid reactive substances (TBARS) when added to previously oxidized lipids (Boldyrev et al., 1988; Aruoma et al., 1989). A potential mechanism for this observation would be the ability of carnosine to interact with aldehydic lipid oxidation products through Schiff base or Michael addition-type reactions (Aldini et al., 2002). The adduction chemistry of carnosine to 4-hydroxy-nonenal (HNE) thus appears to start with the formation of a reversible alpha,beta-unsaturated imine, followed by ring closure through an intra-molecular Michael addition. If carnosine could interact with aldehydic lipid oxidation products, this could potentially help protect biological tissues from oxidation, since aldehydes can form adducts with DNA, proteins, enzymes, and lipoproteins, causing alterations in their biological activity. Previously published data suggest that L-carnosine has excellent potential to act as a natural antioxidant with hydroxyl-radical- and singlet oxygen-scavenging and lipid peroxidase activities (Babizhayev et al., 1994; Babizhayev, 1989; Dahl et al., 1988). However, 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, a dipeptidase present in blood plasma, liver, kidney and other tissues, except muscle and, probably, lens (Jackson et al., 1991; Lenney et al., 1985; Babizhayev et al., 1996).

The *N*-acetyl derivatives of histidine, carnosine and anserine exist in the cardiac and skeletal mammalian muscles and the total concentration of these imidazoles may lie within the measured range of that of L-carnosine in skeletal muscle (i.e. ~10 mM) (O'Dowd et al., 1988). The level of carnosine in tissues is controlled by a number of enzymes transforming carnosine into other carnosine related compounds, such as carcinine, *N*-acetylcarnosine (NAC), anserine or ophidine (by decarboxylation, acetylation or methylation, respectively) or its cleavage into the amino acids, histidine and β-alanine. Hydrolysis is mainly due to tissue carnosinase (EC 3.4.13.3) which is widely distributed among different subjects (Jackson et al., 1991; Lenney, 1976) or serum carnosinase (EC 3.4.13.20), obtained in brain and blood plasma of primates and humans (Kunze et al., 1986; Lenney, 1990). Both carnosinases are characterized by higher activity toward carnosine compared with anserine or homocarnosine (Murphey et al., 1972; Lenney et al., 1982). Comparative study of hydrolysis of carnosine and a number of its natural derivatives by human serum and rat kidney carnosinase was carried out (Pegova et al., 2000). The rate of carnosine hydrolysis was 3–4-fold higher then for anserine and ophidine. The rate of homocarnosine, *N*-acetylcarnosine and carcinine hydrolysis was negligible by either of the enzymes used. Thus despite the fact that carcinine and *N*-acetylcarnosine are metabolically related to carnosine, they have not observed to be a substrate for carnosinase or other dipeptidases (Pegova et al., 2000; Fitzpatrick et al., 1989). Therefore, both carcinine and *N*-acetylcarnosine may play a greater role in the prolongation and potentiation of physiological responses to the therapeutical treatments with carcinine and *N*-acetylcarnosine as antioxidant. It has been shown during the ophthalmic applications that due to its relative hydrophobicity compared to L- carnosine, *N*-acetylated form of carnosine might cross the cornea of the treated eye gradually and maintain longer the concentration of the active principle (carnosine) reaching the aqueous humor (Babizhayev et al., 1996). We propose that *N*-acetylcarnosine can act as a time release version of L-carnosine during its external topical application to the ocular and probably skin tissues. In the present study we have examined the prospects of applications of the bioactive natural imidazole-containing compounds carcinine, L-carnosine and *N*-acetylcarnosine against phospholipid hydroperoxides and toxic aldehydes involved in the development of cataracts and cutaneous ageing. In the same way, it was also necessary to design new experimental models for the evaluation of the protective efficiency of carnosine related compounds.

Materials and methods

Carcinine (Decarboxy carnosine˙2HCl), L-prolylhistamine and *N*-acetyl-β-alanylhistamine were synthesized by Exsymol S.A.M. (Monaco, Principaute de Monaco). L-Carnosine and *N*-acetylcarnosine were synthesized by Hamari Chemicals Ltd (Japan) per specifications proposed by Innovative Vision Products, Inc.

Molecular modeling

Low-energy 3-D conformations of carnosine, carcinine and *N*-acetylcarnosine were derived using the PM_3 method of the MOPAC 6.0 program (Stewart MOPAC Air Force Academy: Boulder, CO 80840). The precise energy minima conformations were determined by semi-empirical Quantum mechanics. This technique structures a pool of energetically accessible shapes especially suitable for dipeptides comparative to large protein molecules. The program is supplemented with ZINDO/1 computer software for estimation of chelating properties of dipeptides and related compounds. The conformational geometry optimization was carried out using the revised computer program (Stewart, 1989a,b).

Pharmacokinetics of topical *N*-acetylcarnosine application

Formulations and animals

Grey Chinchilla rabbits (male) aged 3–4 months weighing 2–3 kg were used. Animal experiments conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research. Thirty minutes prior to the ocular incision right eyes of rabbits were instilled with 80 μl of formulation A containing 1% *N*-acetylcarnosine (NAC) and the control right eyes of the

separate rabbits were similarly instilled with their vehicles (placebo) solutions. Formulation A contained the following ingredients:

Deionized water	970 g
Glycerine, 1.0%	13 g
NAC, 1.0%	10 g
Carboxymethylcellulose, 0.3%	3 g
Benzyl alcohol, 0.3%	3 g
Potassium borate	7.91 g*
Potassium bicarbonate	3.47 g**Or what is necessary to bring the solution up to around a pH of 6.3–6.5, total dosage of Formulation A 1000 *g*.

Formulation A was presented in the final ophthalmic tubes (per volume of 2.5 ml) and in the moiety of the plastic bottles. Placebo solution contained the same ingredients without NAC.

Surgical procedure

Topical anaesthesia of the rabbit eyes was performed after 25 min of instillation of the formula ophthalmic solutions with instillations of 4% lidocaine hydrochloride solution eye drops (three times with 1 drop at 1.5–2.0 min intervals). The eye drops of 4% lidocaine hydrochloride contain benzaltonium chloride preservative. When ocular anaesthesia was achieved, the lids were extended and fixed with the lid-holder and the ocular bulb was fixed by tweezers in the area of the inferior rectus muscle. A stab incision was performed transcorneally 1.0–2.0 mm from the limbus in the temporal superior quadrant. Aqueous humor (0.1–0.2 ml) was aspirated from the anterior chamber of a rabbit eye with 25-gauge needle connected to an insulin syringe and immediately introduced into an Eppendorf tube with addition of ethanol (0.2 ml), keeping the sample on ice before extraction.

Extraction of imidazoles from aqueous humor

Extractions of imidazole-containing compounds from the aqueous humor aliquots were performed according to Babizhayev et al., 1996. The published data showed that all the desired imidazole-containing compounds in the aqueous humor thus obtained could be of good purity and recovery (Babizhayev et al., 1996). Portions of aqueous humor were added to ethanol as above and thoroughly mixed (20 °C, 15 min). Extracts were centrifuged ($2000 \times g$, 15 min) and the supernatants removed. Samples were frozen in the gradient of temperatures to −70 °C and lyophilized using the apparatus JOAN (France). The lyophilized residue was dissolved in 1 ml of 0.1 M Na_2 HPO_4 (pH 2.1 adjusted with 85% phosphoric acid) and filtrated through the membrane filter with the dimensions of pores 0.22 μm directly prior the analysis.

Analytical HPLC for detection of L-carnosine and N-acetylcarnosine

Reverse phase analytical HPLC was performed using a Breeze chromatography system (USA), detector Waters 2487 Dual λ Absorbance Detector, column (250 × 4.6 mm) Symmetry 300 C_{18} 5 μm (Waters), loop 20 μl. The column was eluted isocratically at 30 °C with the cited phosphate buffer 0.1 M Na_2 HPO_4 (pH 2.1) over 25 min at a flow rate of 1.0 ml/min. Eluates were monitored for absorbance at 210 nm. The standards of L-carnosine and *N*-acetylcarnosine were prepared by weighing of the dry material using the analytical balance Mettler Toledo (accuracy 0.00004) and were further dissolved in the phosphate buffer 0.1 M Na_2 HPO_4 (pH 2.1). The quantitative determination of L-carnosine and *N*-acetylcarnosine in the samples was undertaken using the technique of external standard according to the area of the peak and linear extrapolation. The standards of eye drops were prepared by dissolution of initial solutions of eye drops by 100 fold using the phosphate buffer 0.1 M Na_2 HPO_4 (pH 2.1). Statistical significance was evaluated by the unpaired Student's *t*-test and $P = 0.05$ was taken as the upper limit of significance.

Peroxidation reaction system

The techniques for phospholipid extraction, purification and preparation of liposomes (reverse-phase evaporation technique) have been described previously (Babizhayev, 1989; Babizhayev and Bozzo Costa, 1994). Peroxidation of phosphatidylcholine (PC, derived from egg yolks) was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbic acid to the suspension of liposomes (1 mg/ml) in 0.1 M Tris-HCl buffer (pH 7.4). The incubations were performed at 37 °C. The tested compounds, NAC and L-carnosine, were added at 10–20 mM concentration to the system of iron-ascorbate-induced liposome PC peroxidation. The kinetics of accumulation of lipid peroxidation (LPO) products in the oxidized liposomes were measured by reaction with thiobarbituric acid (TBA). The peroxidation reaction was arrested by adding EDTA to a final concentration of 50 μM or by the addition of 2.0 ml of ice-cold 0.25 M HCl containing 15% (w/v) three-chloroacetic acid (TCA). TBA (0.125% w/v) was then added to the mixture and followed by boiling for 15 min. The TBA assay was described previously (Babizhayev and Bozzo Costa, 1994). The differential absorbance of the condensation product, malonyl dialdehyde (MDA), at 535 and 600 nm was measured spectrophotometrically ($\varepsilon_{535} = 1.56 \times 10^5$ M^{-1} cm^{-1}). The TBA reaction itself was not affected by the components of the radical generators or scavengers used in the study. To determine conjugated dienes the lipid residue of the samples was partitioned through chloroform during the extraction procedure (Babizhayev and Bozzo Costa, 1994). This protocol removes any water-soluble secondary oxidation products, leaving them in the methanol-aqueous phase. Correlation of the extracted lipid concentrations to the measured phosphorus was done by means of characteristic absorption at 206–210 nm of the lipid sample (redissolved in 2–3 ml of methanol/heptane mixture 5:1, v/v). Accumulation of net diene conjugates corresponding to the level of lipid hydroperoxides was assessed from characteristic absorbance of diene conjugates at ~230 nm ($\eta CD = 2.8 \times 10^4$ M^{-1} cm^{-1}), in a Shimadzu UV−260 spectrophotometer (Japan) (Babizhayev and Bozzo Costa, 1994). Absorbance of the secondary LPO products at ~274 nm, corresponding to the concentration of

conjugated trienes and ketodienes, was also measured spectrophotometrically from the lipid spectra (Babizhayev and Bozzo Costa, 1994). An average MW of phospholipid was assumed to be ~730 Da. Statistical significance was evaluated by the unpaired Student's *t*-test, and $P=0.01$ was taken as the upper limit of significance.

Reactivity of carcinine and carnosine to hydroperoxide of linoleic acid

Standard hydroperoxide of linoleic acid (LOOH) and its alcohol form (LOH) were obtained as described by Iacazio et al. (Iacazio et al., 1993). The reaction conditions of pure 13(*S*) linoleic acid hydroperoxide with imidazole-containing peptidomimetic compounds (carnosine, carcinine) were described earlier (Babizhayev et al., 1994). The results of experiments demonstrating the lipid peroxidase activity of L-carnosine and carcinine were carefully described (Babizhayev et al., 1994).

Electrophoresis assays

One millimole of 13(*S*) linoleic acid hydroperoxide were incubated in phosphate buffer solution (PBS) (0.1 M, pH 7.4) with a bovine serum albumin (BSA) solution (0.5 mg ml^{-1}) at 37 °C with 3 mM of several antioxidants (carcinine (β-alanylhistamine), L-carnosine (β-alanyl-L-histidine), L-prolylhistamine, *N*-acetyl-β-alanylhistamine or vitamin E).

In another experiment, liposomes made from phospholipids containing unsaturated fatty acids were peroxidized during 2 days by contact with copper (Babizhayev et al., 2000). In a second step, the imidazole-containing antioxidants were introduced in the liposome mixture. The representative protein BSA was then added, and incubated for 2 days.

After 2 days incubation, a SDS-PAGE electrophoresis (7.5% polyacrilamide gel containing 0.1% SDS) was made according to Laemmli, 1970 and stained with the normal silver technique (Sammons et al., 1981). The analytical scanner and the appropriate software used to realize figures were purchased from Advanced American Biotechnology.

HPLC analysis for detection of lipid hydroperoxide

Following different incubation times, a fraction of the solution was processed, which contains the fatty acid hydroperoxide, BSA and the antioxidant compound. After the addition of 100 ml HCl (1N) to the same volume of reaction mixture and a centrifugation (10000 *g*; 10 min), a HPLC analysis was also made. The supernatant of each sample was diluted 3 times in methanol;

40 μl were used for the following reverse phase HPLC analysis technique.

- Column C18 Macherey–Nagel, 4.6 mm, 5 mm, 12.5 cm.
- Elution: 50/50 acetonitrile-acetic acid 0.01%.
- Controls: retention time of 13(*S*) linoleic acid hydroperoxide=15 min
- Retention time of 13(*S*) linoleic acid alcohol=12.8 min (obtained after $NaBH_4$ reduction (Iacazio et al., 1993)).
- Spectrophotometer Hewlett Packard HP 1050.

Hydrolysis studies of L-carnosine and carcinine in the skin

The specific carnosinase activity was not reported in the epidermic cells and in the overall homogenate of the epidermic tissue (Jackson et al., 1991; Lenney et al., 1985). If a metabolism of carnosine and carcinine is observed as a mix of hydrolytic pathway and oxidative process, this is assigned to the semi-specific degradation of peptidomimetic compounds. The kinetics of cumulative degradative effect of carnosine and carcinine in the skin were determined in the microsome fractions of the epidermal tissues equipped with the enzymatic heterogeneous systems of peptide metabolism.

Microsome assay

Ventral skin of hairless male rats was collected. After epidermic tissue homogenization with UltraTurrax treatment, the mixture was centrifuged at 105,000 *g* (1 h) for the microsomal pellet preparation. Microsome fraction was collected and suspended in a Tris-HCl buffer (pH 7.2). Concentration of total proteins was evaluated according to Lowry method (Langrand et al., 1999). A Tris-HCl mixture (pH 7.2) containing keratinocytes' microsome fraction was incubated with (0.5 mM) carnosine or carcinine pseudodipeptide at 37 °C. The reaction was stopped by addition of 1 ml of dansyl chloride dissolved in acetonitrile (15 mg/ml). Dansylation was realized by heating for 15 min at 50 °C. After cooling, the two phases were separated, and substrate concentrations were analyzed by HPLC (Supercosil column type LC 18 DB, length 30 cm, diameter 4 mm, diameter of particles 5 μm, volume of injection 20 μl) with diode array detector (190–600 nm). The kinetics were evaluated by the decrease of the area under the curve of the initial HPLC signal of carnosine and carcinine after dansylation. The activation of microsomes (by the addition of NADP) influences the kinetics of pseudodipeptides degradation. $NADPH/H^+$-dependent or catalyzing a cycle of $NADPH/H^+$-dependent reactions enzymes are involved. The microsomal fractions obtained contain different enzymes such as diamine oxidase, histaminase, monoamine oxidase and cytochrome P450.

Protection of skin Superoxide Dismutase (SOD) activity with an imidazole-containing peptidomimetic

Skin treatment with carcinine during UV irradiation

Porcine ears were heated at 70 °C for 70 s. The epidermderm fraction was removed with a mechanical treatment. Skin fragments were treated with creams containing 0%, 0.5%, 1% and 2% carcinine during 5 min. Skins were then washed with 1% Triton X-100 solution in phosphate buffer to take emulsion off the skin surface. After UVA-UVB irradiation (0.8 J/cm^2), skin fragments were cut, suspended (30 g/l) in phosphate buffer and crushed with ultra turrax (0 °C for 2 min). Extracts obtained

were diluted (1/3) in Triton X-100 (1%) and kept 2 h at 0 °C. The mixtures were then centrifuged at 10,000 *g* for 10 min. The SOD-like activity was measured in the supernatant fraction.

Measurement of SOD-like activity

Anion superoxides, produced by the hypoxanthine/xanthine oxidase system, react with cytochrome *c*. This reaction induces a ferrous cytochrome formation which absorbs at 550 nm. SOD is able to dismute a part of anion superoxides. Due to the SOD activity, the level of the anion superoxides decreases. Thus, the cytochrome *c* is less reduced, and the OD values (550 nm) decrease.

Six hundred microliters of phosphate buffer or of epiderm extract (obtained from 10 g of skin/l of phosphate buffer) were added to 400 µl of solution A. Solution A contained 45 µM of cytochrome *c*, 540 µM of hypoxanthine and 1250 units of catalase. Xanthine oxidase was solubilized in a phosphate buffer solution (0.06 units/ml). The reaction was initiated by the addition of 100 µl of xanthine oxidase. The kinetics were realized at 25 °C for 2–3 min (550 nm) (see Fig. 7A). The rate of cytochrome c reduction (delta OD/min) at 25 °C was assessed. The protective effect of carcinine was obtained with the following formula.

$$\frac{\text{Irradiation } 0\% - \text{Irraditation } X\%}{\text{Irradiation } 0\% - \text{No irradiation}} \times 100$$

- No irraditation: kinetics obtained from non-irradiated skin fraction. It represents the natural SOD-like activity of the skin extract.
- Irradiation 0%: kinetics obtained with irradiated skin fractions treated with the cream containing 0% carcinine. It represents the maximum impact of UV irradiation on the SOD-like activity in the extract.
- Irradiation X%: kinetics obtained with irradiated skin fractions treated with creams (oil/water) containing 0.1%, 0.5%, 1% or 2% carcinine. It represents the SOD-like activity of the extracts after irradiation and treatment with carcinine.

Results

Chemical and 3-D chemical structures of N-acetylcarnosine, L*-carnosine and carcinine*

Fig. 1 shows the formula structures and the energy-minimized 3-D conformations of *N*-acetylcarnosine, L-carnosine and carcinine derived from their chemical structures using ball-and-stick model. Due to energy differences determined by molecular mechanics, PM_3 semi-empirical quantum mechanics among different conformations of the natural imidazole-containing peptidomimetics, a dynamic equilibrium of energetically permissible C-linked and N-linked analogs of rotamers exists in aqueous solution. The resulting minimized structures indicate that a common characteristic for all the calculated conformations for peptidomimetics is that a claw-

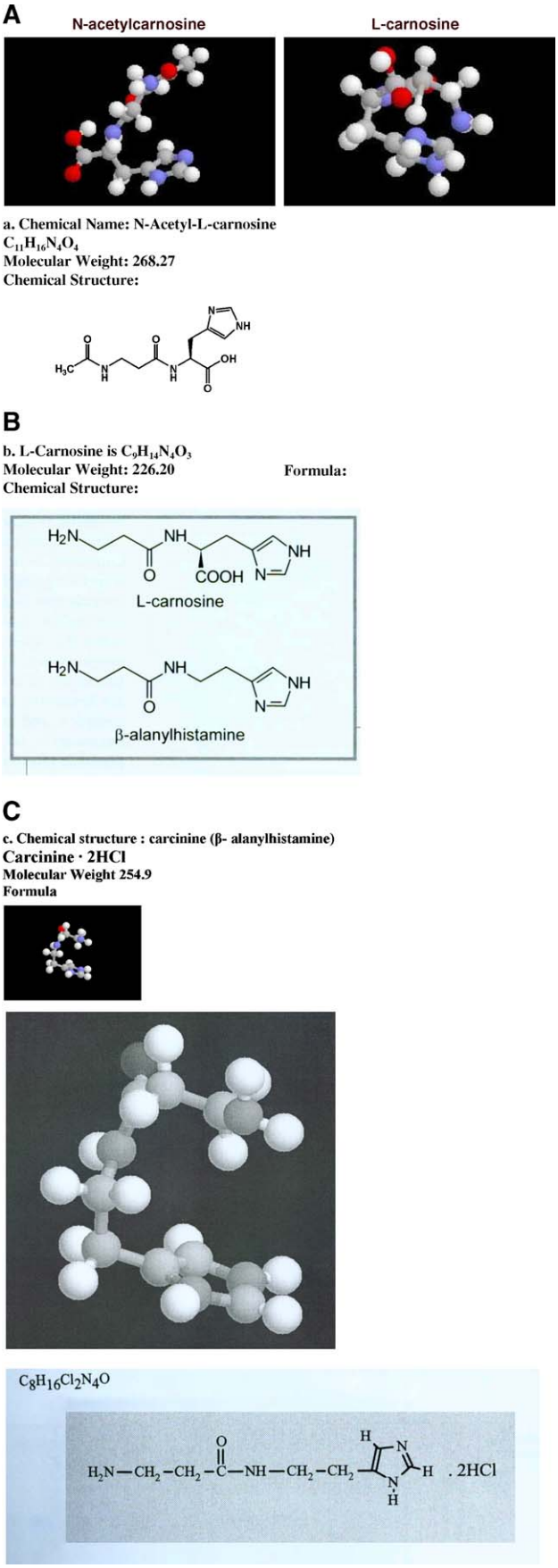


Fig. 1. Structures of *N*-acetylcarnosine, carnosine and carcinine shown as chemical structures and energy-minimized structures (ball-and-stick model).

like structure of every compound results in proper stabilization and for the possible metal chelating such as when iron (Fe^{2+})-natural imidazole containing compound complex is obtained. The data provide the hypothesis supported by 3-D molecular conformational studies that Fe^{2+} can be enveloped inside the natural peptidomimetic (see ball-and-stick model for each peptidomimetic compound). This activity can lie in the basis of the antioxidant properties of the studied imidazole-containing compounds. The calculated lowest energy conformations and their metal-bound conformations are considered at neutral pH in this study and result in significant adverse steric interactions on changes of pH or temperature or during competition with high-affinity metal chelator (such as EDTA). The results are expected to be applicable to physiological pH and temperature conditions. Our study proposed that the antioxidant activity of the natural imidazole-containing peptidomimetic is cancelled at addition of EDTA (Babizhayev et al., 1994). The claw-like structure of the peptidomimetic compounds can provide specificity for metal ion binding, quenching of a number of free radicals, and binding of hydroperoxide or aldehyde (including dialdehyde LPO products) in an imidazole-peroxide adducts (Aldini et al., 2002). This structure can be related to imidazole moiety for structure-activity explorations. The presented on the Fig. 1 claw-like structures allow to visualize the "favoured" conformations of peptidomimetics which are "folded up" and create a "pocket" in which an adduct may insert. The stability of the claw-like structure to form a chelate with an atom of iron (Fe^{2+}) decreases from carcinine to L-carnosine. Computer-assisted calculations allowed to visualize the conformation and gave also theoretical indications on the strength of the interaction forces between the tested molecule and the atom of iron which decreases according to bound energy for different chelates (eV): carcinine–Fe^{2+}(−7725 eV)<L-carnosine-Fe^{2+}(−883 eV). The structural coincidence of the carnosine-related moieties with histamine may have effects on regulatory proteins in cells and on DNA and genes involved in normal growth and metabolism. *N*-acetylation or decarboxylation of carnosine molecule can lead to the stabilization of the *N*-acetylcarnosine and carcinine peptidomimetics to hydrolysis with dipeptidases (including carnosinase activity) (Pegova et al., 2000).

N-acetylcarnosine as a time release (carrier) version of L-carnosine in the topical external biological applications

An important chemical difference between carnosine and *N*-acetylcarnosine is that carnosine is relatively insoluble in lipids (fats and fatty compounds), whereas *N*-acetylcarnosine is relatively soluble in lipids (as well as in water) (Babizhayev et al., 2004). This means that *N*-acetylcarnosine may pass through the lipid membranes of the corneal and skin cells more easily than carnosine, and may thereby gain access more readily to the cells' interior, which is primarily aqueous. *N*-acetylcarnosine can be gradually broken down to carnosine which then exerts its beneficial effects (Babizhayev et al., 1996).

N-acetylcarnosine-containing eye drops have demonstrated efficacy in treating a variety of ophthalmic conditions, including corneal diseases, cataracts, glaucoma (Babizhayev et al., 2001, 2002, 2000). The clinical trials included two randomized, double-blind, placebo-controlled trials of 6-months and 24 months duration, with eye drops consisting of a 1% solution of NAC administered as two drops twice daily (Babizhayev et al., 2001, 2002). Due to its relative hydrophobicity compared to L-carnosine, NAC might cross the cornea of the treated eye gradually and maintain longer the concentration of the active principle (L-carnosine) reaching the aqueous humor. In the present section of the study, we considered whether NAC acts in the ophthalmic formulation with lubricants and preservatives when topically administered to the eye as a time release carrier (prodrug) of L-carnosine. The HPLC pattern of an extract of the aqueous humor obtained 30 min after instillation to the rabbit eye of ophthalmic formulation containing 1% NAC, lubricants carboxymethylcellulose, glycerine and preservative benzyl alcohol in the borate buffer confirms that the peak characteristic of L-carnosine has a concentration and a retention time (3.225 min) clearly distinct from *N*-acetylcarnosine (6.0 min) and basically different from the dead time of the column (3.0 min) (Fig. 2). This identified peak of L-carnosine quantified and integrated by the data processor showed that virtually all *N*-acetylcarnosine after the overall extraction efficiency is converted into the L-carnosine compound with a retention time of 3.225 min (Fig. 2). The data on the L-carnosine-related product were blanked with the control placebo data applied to the paired eyes of the animals. The mean ratio of L-carnosine (C)/(NAC) relevant to the L-carnosine release in the aqueous humor 30 min after instillation of Formulation A with 1% *N*-acetylcarnosine into the rabbit eye corresponded to C/NAC = 6.64 ± 0.06 (n = 8, where n = number of the examined treated rabbit eyes; only right eyes were treated). In the control placebo formulation-treated eyes the same indices could not be quantified at statistically significant rate. Concentrations of imidazole products in the aqueous humor corresponded to those of intact rabbit eyes and refer to baseline values of L-carnosine 0.19 ± 0.10 μg/ml related products variously detected in extracts from normal animals. Our data demonstrate that topical administration of pure L-carnosine (1% solution) to the rabbit eye (instillation, subconjunctival injection) does not lead to accumulation of this natural compound in the aqueous humor over 30 min in concentration exceeding that in the placebo-treated matched eyes, and its effective concentration is exhausted more rapidly (Babizhayev et al., 1996, 2000).

In another aspect, the data presented in Results sections demonstrate the prospects of applications of an ophthalmic (cosmetic) composition comprising NAC, or its pharmacologically acceptable salt in combination with a cellulose compound or its pharmaceutically acceptable salt which are effective to treat the eye (skin) complex of symptoms. This complex of symptoms may have an oxidative component in their genesis, such as inflammation. The intraocular (or transepidermal) absorption of L-carnosine are increased in a mammal upon the ophthalmic or cosmetic applications of the above described composition(s).

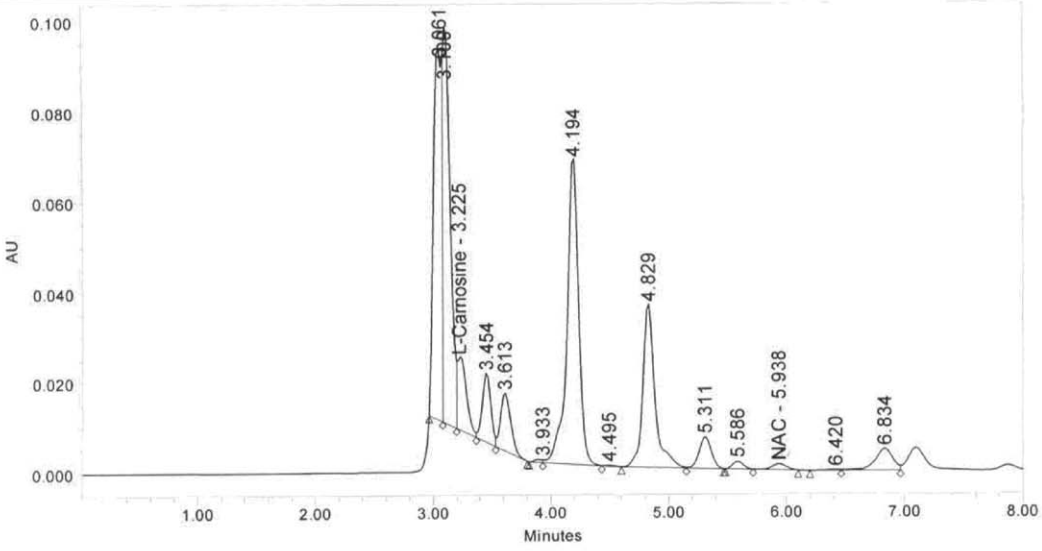
MMA

Project Name: Peptides
Reported by User: Makarov

Breeze

SAMPLE INFORMATION

Sample Name:	No.2 OD	Acquired By:	Makarov
Sample Type:	Narrow Unknown	Date Acquired:	7/25/02 12:21:47 PM
Vial:	1	Acq. Method:	Rabbit
Injection #:	4	Date Processed:	7/25/02 12:32:01 PM
Injection Volume:	20.00 ul	Channel Name:	2487Channel 1
Run Time:	8.00 Minutes	Sample Set Name	
Column Type:			



	Peak Name	RT (min)	Area (μV*sec)	% Area	Height (μV)	% Height	Amount	Units
1		3.061	402670	21.41	83190	24.86		
2		3.100	389886	20.73	87965	26.28		
3	L-Carnosine	3.225	89479	4.76	16173	4.83	3.392	mkg/ml
4		3.454	76173	4.05	15081	4.51		
5		3.613	76279	4.06	12690	3.79		
6		3.933	2892	0.15	625	0.19		
7		4.194	463529	24.65	67930	20.30		
8		4.495	1150	0.06	174	0.05		
9		4.829	257552	13.69	36153	10.80		

Fig. 2. HPLC of extract of aqueous humor aspirated 30 min after the instillation of ophthalmic formulation with 1% NAC and lubricants into the rabbit eye. The integrated concentration of the carnosine related product (3.392 μg/ml, 3.225 min) is attributed to accumulation of carnosine in the ophthalmic formulation-treated eye. Chromatograms of solutions of L-carnosine and its putative *N*-acetyl derivative show that these compounds are well separated. The elution order of the compounds was compared to a predicted order based upon their relative hydrophobicities and the chromatographic system was shown to be suitable to monitor the behaviour of other histidine containing derivatives of L-carnosine. The calibrating chromatograms showed the predicted elution order and the average elution times for each standard of L-carnosine and *N*-acetylcarnosine in mixtures. Peaks were unequivocally identified by comparison of their retention times to those of the authentic standard compounds or of putative acetylated compound run singly. Tests for specific chemical reactivity provided additional evidence for the identification of L-carnosine and *N*-acetyl-carnosine (Babizhayev et al., 1996).

Antioxidant activities of L-carnosine, N-acetylcarnosine and carcinine in the liposome peroxidation system

The comparative antioxidant activity of NAC and L-carnosine was assessed in the liposome peroxidation system catalyzed by Fe^{2+}+ascorbate (Fig. 3). The accumulation kinetics of molecular LPO products such as MDA and liposomal conjugated dienes and trienes are shown in Fig. 3 (A–C). The results demonstrate that the LPO reactions in the model system of lipid membranes are markedly inhibited by L-carnosine. The effective concentrations of L-carnosine are 10 and 20 mM. Data on the biological effectiveness of L-carnosine and carcinine as antioxidants preventing PC liposome or linoleic acid peroxidation in physiological concentration ranges

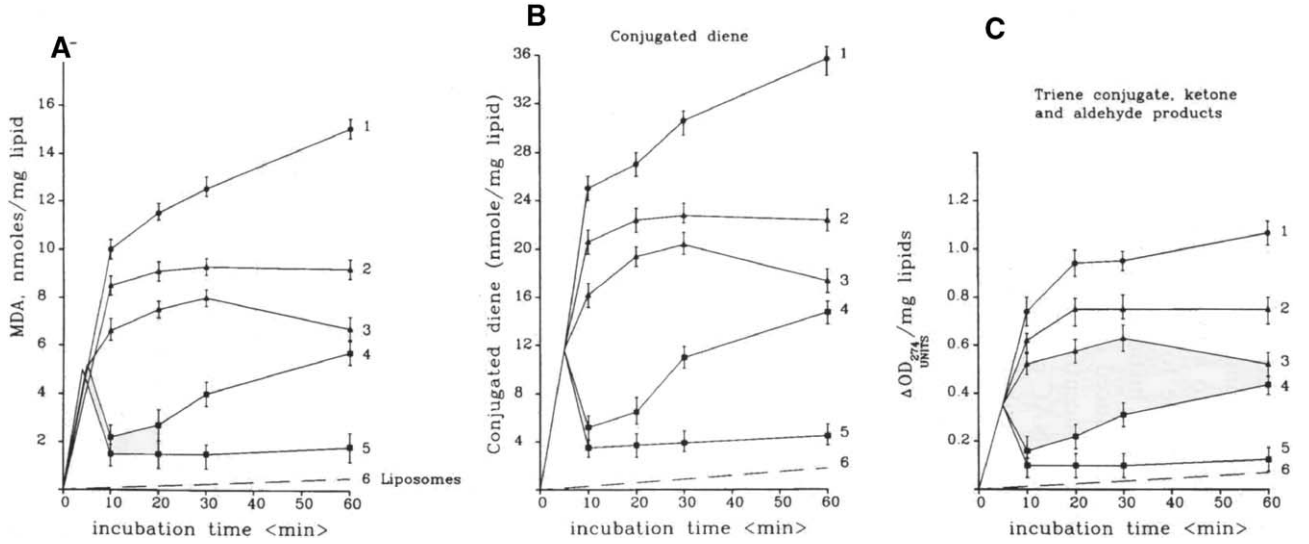


Fig. 3. Accumulation of lipid peroxidation products (TBARS, measured as MDA) (A), diene conjugates (B), triene conjugates and ketone and aldehyde products (274 nm absorbing material) (C) in liposomes (1 mg/ml) incubated for 60 min alone (6, dotted line) and with addition of the peroxidation-inducing system of Fe^{2+}+ascorbate (1). Antioxidants *N*-acetylcarnosine (NAC) (10 or 20 mM) (2, 3) or L-carnosine (10 or 20 mM) (4, 5) were added at the fifth minute of the incubation period to the system containing the peroxidation inducers. Samples were taken at zero time and at time intervals indicated in the figures and were used immediately for measurement of TBARS (see "Materials and methods"). A similar amount of sample was partitioned through chloroform and used for detection of conjugated dienes and trienes dissolved in 2–3 ml of methanol–heptane mixture (5:1 v/v).

of 5–25 mM have already been published (Babizhayev et al., 1994, 1989; Babizhayev and Bozzo Costa, 1994). The scavenging of lipoperoxide-derived free radicals with L-carnosine and carcinine during the peroxidation of linoleic acid and PC liposomes in the peroxidizing system Fe^{2+}/ascorbate was documented (Tables 1 and 2). Fig. 3A shows that the level of TBA reactive substances (TBARS) reached at 5-min incubation decreases in the presence of L-carnosine (10 or 20 mM) at 10 min and at later time points (20 mM), 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. The ability of the histidine-containing compound NAC to inhibit the (Fe^{2+}+ascorbate)-induced oxidation of PC liposomes was compared with that of equimolar concentrations of L-carnosine. The antioxidant activity of 10 and 20 mM NAC corresponded to 38% and 55% inhibition of LPO for the two concentrations after 60-min incubation. NAC exhibited less antioxidant protection than L-carnosine, corresponding to 60% and 87% of the equimolar (10 or 20 mM) L-carnosine inhibition percentage. However, since NAC can act as a time release version metabolized into L-carnosine during its topical and external application to the tissues (but not oral use), the antioxidant activity of NAC in vivo application is significantly increased. Once released from NAC in tissues, L-carnosine might act against peroxidation during its target pharmaceutical or cosmetic use.

Table 1
Percentage of inhibition obtained by comparison with a control experiment with no antioxidant

Compound tested at concentration 25 mM	% Inhibition of MDA release from oxidative degradation of linoleic acid
L-Carnosine (β-alanyl-L-histidine)	59
Carcinine (β-alanylhistamine)	47

Detailed experimental procedures are described in Ref. 8. Each result represents the mean of 5 experiments.

Reactivity of the actives L-carnosine and carcinine with lipid hydroperoxide. Lipid peroxidase-like activity of peptidomimetics

The lipid peroxidase-like effect of carnosine and carcinine was preliminary demonstrated (Babizhayev et al., 1994). The lipid peroxidase-like activity was described as a reduction activity of fatty acid hydroperoxide into the alcohol form that was assayed by TLC analysis. The same reducing effect (alcohol formation from hydroperoxides) was found now in a biphasic model system in which the oxidative stress was generated by the 13(*S*) linoleic acid hydroperoxide (liposoluble), and the target of the oxidation was a sample water soluble protein (bovine serum albumin, BSA). The in vitro model system described in Material and methods shows the reaction of linoleic acid hydroperoxide (LOOH) with BSA. The reaction products were analyzed by HPLC (Fig. 4A–C). Fig. 4A,B show representative chromatograms in quantitative analysis of lipid linoleic acid hydroperoxide and its reduced with $NaBH_4$ alcohol (LOH) product. The incubation of BSA with a lipid hydroperoxide would result in the formation of

Table 2
Percentage of inhibition obtained by comparison with a control experiment with no antioxidant

Compound tested at concentration 10 mM	% Inhibition of MDA release from oxidative degradation of PC liposomes
L-Carnosine (β-alanyl-L-histidine)	53
Carcinine (β-alanylhistamine)	42

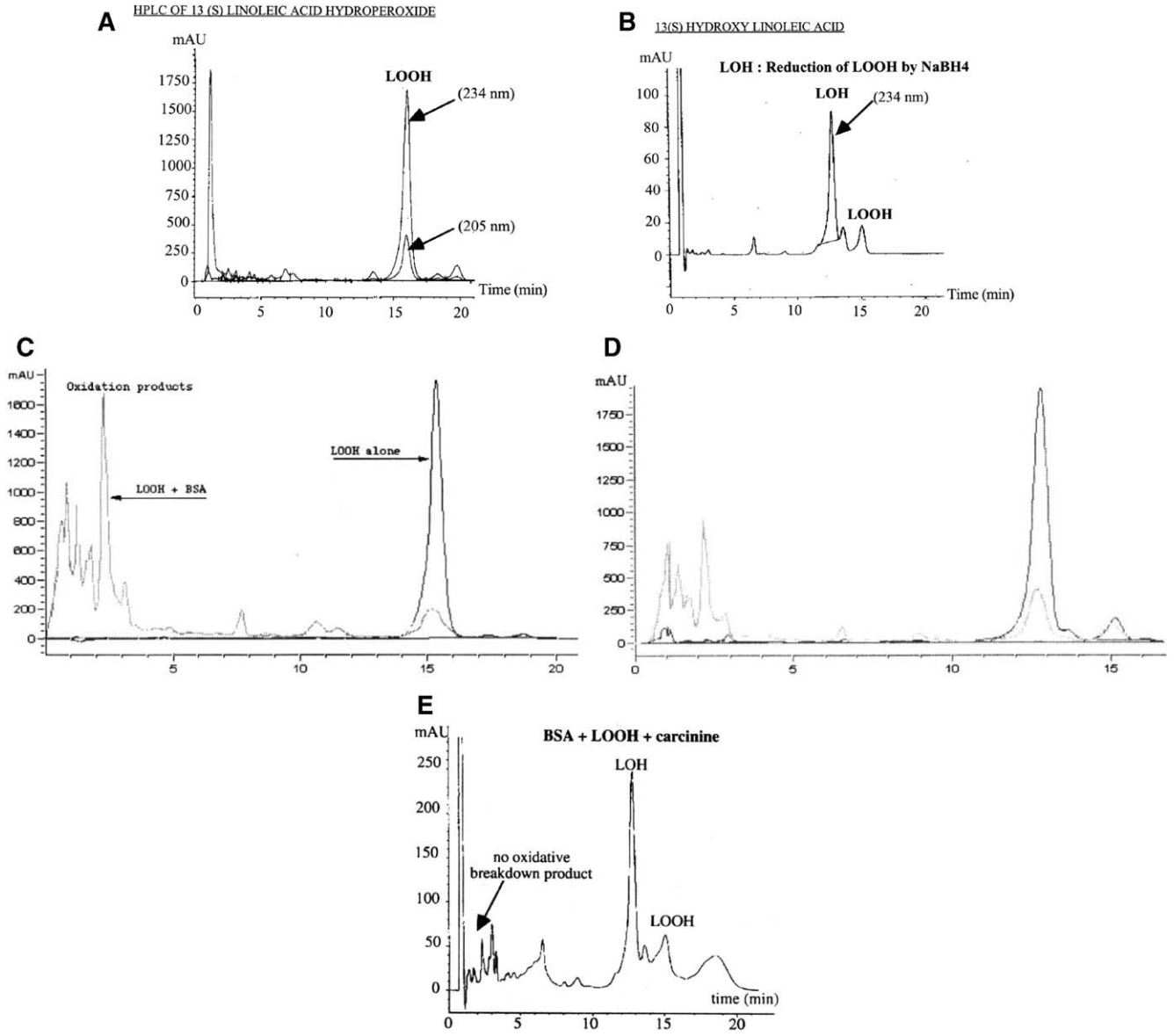


Fig. 4. (A) HPLC spectrum of 13(*S*) linoleic acid hydroperoxide in a phosphate buffer solution (0.1 M; pH 7.3) after 15 min of incubation at 37 °C. Absorbance wavelengths used: 234 and 205 nm. (B) HPLC spectrum of 13(*S*) hydroxy linoleic acid phosphate buffer solution (0.1 M; pH 7.3). Monitoring absorbance wavelength used: 234 nm. (C) HPLC monitoring of protein (BSA) oxidation degradation by linoleic acid hydroperoxide (LOOH). (D) Correlation of the natural imidazole-containing peptidomimetic protective effect with linoleic acid hydroperoxide (LOOH) reduction. (E) HPLC spectra recorded at 234 nm wavelength. BSA (0.33 g/l) in 0.1 M phosphate buffer, pH=7.3 was incubated with 1.5 mM 13(*S*)-linoleic acid hydroperoxide and 5 mM carcinine during 60 h at 37 °C.

characteristic peaks, and, indeed, numerous polar, low-molecular weight degradation products, which would not appear when the BSA protein or the peroxide were incubated alone, could be detected at 205 nm (Fig. 4C). The formation of the reduced product LOH when linoleic hydroperoxide alone was incubated with the imidazole-containing peptidomimetic was also monitored with the HPLC technique. The HPLC spectra revealed that carcinine would avoid the formation of low-molecular-weight degradation products of BSA and that concomitantly LOH was formed (see Fig. 4D,E). It was verified that LOH is harmless for the protein: no breakdown products were observed when BSA was incubated during an extended period of time (12 days) with the reduced form. The HPLC analysis substantiates the ability of the naturally occurring imidazole-containing peptidomimetics to reduce (LOOH) into non-toxic alcohols (LOH). The reduction of various lipid hydroperoxides may result from the cleavage of lipid hydroperoxide with a transition metal complex of L-carnosine (carcinine) and supplement with electrons for the reductive reaction LOOH¬LOH (Babizhayev et al., 1994). The commonly used lipophilic antioxidant vitamin E, being only capable of free radical scavenging, is therefore ineffective once hydroperoxides are formed.

This unique lipoperoxidase activity is correlated with the protection of protein against oxidative cross-linking induced by these toxic lipid peroxides. This was demonstrated using SDS-

PAGE electrophoresis (Fig. 5A). For this purpose, the representative protein BSA was incubated in the presence of the chemically well-defined 13(*S*)-linoleic acid hydroperoxide and, in a similar fashion as before, the protein's cross-linking was observed after 2 days of incubation (Fig. 5A, lane 2). Here again, carcinine and L-prolylhistamine (endowed with lipid peroxidase activities and being both strong aldehyde quenchers) (lanes 3 and 6) were able to protect the protein, while at the same concentrations L-carnosine, *N*-acetyl-β- alanylhistamine or vitamin E were uneffective (lanes 4, 5 and 7). Vitamin E cannot act with lipid peroxidase activity and is not an aldehyde quencher in the conditions used.

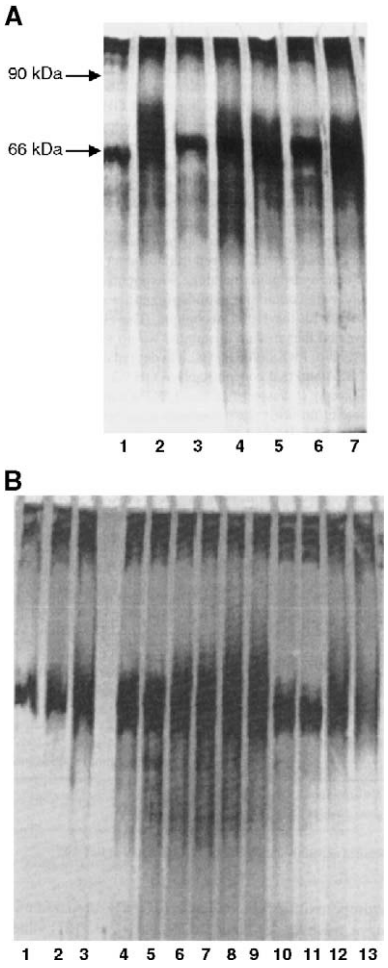


Fig. 5. (A) SDS-PAGE of BSA exposed to 13(*S*)-linoleic acid hydroperoxide. 1: BSA control; 2: BSA+LOOH; 3: BSA+LOOH+carcinine; 4: BSA+LOOH+L-carnosine; 5: BSA+LOOH+*N*-acetyl-β-alanylhistamine; 6: BSA+LOOH+L-prolylhistamine; 7: BSA+LOOH+vitamin E. Gel silver stain method. (B) SDS-PAGE of BSA exposed to peroxidized liposomes after treatment with different imidazole-containing antioxidants. 1: BSA control; 2: BSA and non-oxidized liposomes; 3: BSA and oxidized liposomes; 4: BSA, oxidized liposomes and 1 equiv (versus ROOH) of carcinine; 5: BSA, oxidized liposomes and 2 equiv of carcinine; 6: BSA, oxidized liposomes and 1 equiv of L-carnosine; 7: BSA, oxidized liposomes and 2 equiv of L-carnosine; 8: BSA, oxidized liposomes and 1 equiv of *N*-acetyl-β-alanylhistamine; 9: BSA, oxidized liposomes and 2 equiv of *N*-acetyl-β-alanylhistamine; 10: BSA, oxidized liposomes and 1 equiv of L-prolylhistamine; 11: BSA, oxidized liposomes and 2 equiv of L-prolylhistamine; 12: BSA, oxidized liposomes and 1 equiv of vitamin E; 13: BSA, oxidized liposomes and 2 equiv of vitamin E. Gel stained with silver.

In another experiment, the imidazole-containing antioxidants were introduced in the peroxidized liposome mixture. The representative protein BSA was then added, and incubated for 2 days. The protective effect was illustrated by electrophoretic monitoring of the protein molecular weight (Fig. 5B). After 2 days of incubation, phospholipid peroxides (Fig. 5B, lane 3) induced protein cross-linking (and to some extent degradation), as indicated by the formation of a multimolecular weight diffuse band around 66 kDa. Interestingly, carcinine's (lanes 4 and 5) protective effect was far superior to L-carnosine's (lanes 6 and 7), which gave very poor results with this experiment. L-Prolylhistamine was the most effective peptidomimetic, while *N*-acetyl-β-alanylhistamine was almost uneffective. In these experimental conditions, the reference lipophilic antioxidant vitamin E was also completely unable to protect BSA from this kind of cross-linking.

This test shows that lipid peroxides break down into free radicals and toxic amphiphilic aldehydes, resulting in the spread of the oxidative stress from the oily phase (lipid hydroperoxides) to the water phase, leading to the oxidation of surrounding proteins (e.g. collagen, BSA, SOD etc.).

Catabolism of L-carnosine and carcinine in the skin microsome fraction

As expected, decarboxylation of L-carnosine resulted in a higher resistance of peptidomimetic to the hydrolytic enzyme (Pegova et al., 2000). This result was confirmed using a multi-enzymatic microsomal fraction obtained from epidermic cells. Microsomes are identified as enzymatic heterogeneous systems. In biochemical studies, hepatocyte microsomal mixtures (S9 or S9 mix) are used to study different metabolic effects related to toxicity released by the testing compounds. In order to study the peptidase metabolism of carnosine and carcinine in the epidermal cells, we have carried out the detection of the compounds in a microsomal mixture derived from the epidermic cells. Skin microsomes contain heterogeneous kind of proteases, including tissue peptidases, transferase activity, oxidase etc. and are more representative for the proteolysis capability of cells. The mixture containing the fraction of microsomes was incubated with natural imidazole containing peptidomimetics. The kinetics was evaluated by HPLC after dansyl chloride reaction. As illustrated in Fig. 6A, carcinine demonstrates a good resistance to the deactivation in microsomes. L-Carnosine is subjected to hydrolysis at a significantly higher rate than carcinine. The catabolism of the cited two compounds in the microsome fraction does not reveal the appearance of their proteolysis degradation products. The activation of microsomes influences the kinetics of natural imidazole-containing compounds and their degradation rates. $NADPH/H^+$-dependent enzymes catalyzing a cycle of $NADPH/H^+$-dependent reactions are involved in catabolism of L-carnosine and carcinine in the skin microsome fraction (Fig. 6B). The use of single step ultracentrifugation spins down besides microsomes, also cellular organelles. That is probably why carnosine is metabolized rapidly without NADPH.

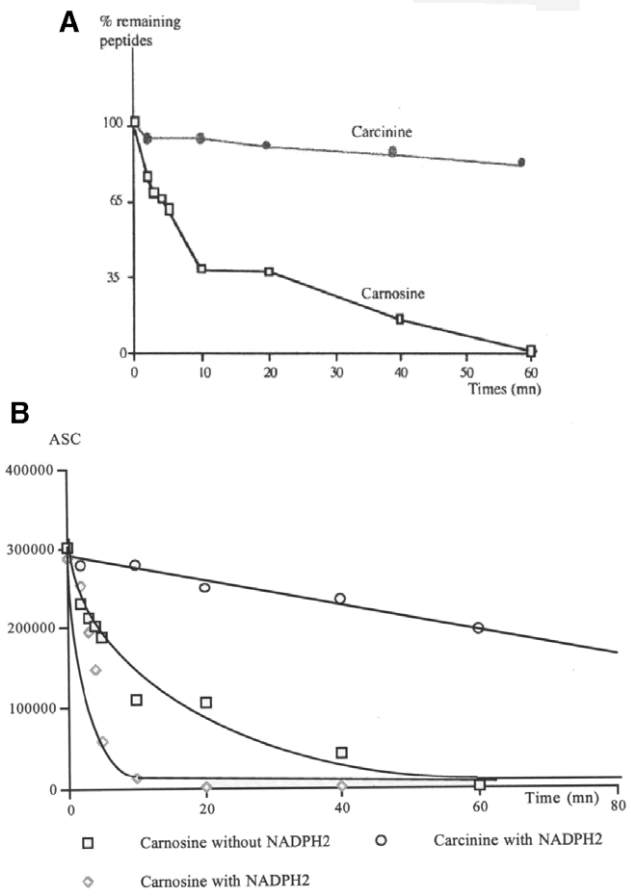


Fig. 6. (A) Microsome (4 mg/ml of total proteins) metabolism kinetic of (0.5 mM) carcinine and L-carnosine (0.5 mM) measured by HPLC after dansyl chloride reaction. Each point represents the average of 3 experiments. (B) Kinetics of L-carnosine and carcinine catabolism in the skin microsome fraction (4 mg/ml of total proteins) during the activation of $NADPH_2$-dependent enzymes. Microsome metabolism kinetic at addition of (0.5 mM) $NADPH_2$, carcinine and L-carnosine measured by HPLC after dansyl chloride reaction. Each point represents the average of 3 experiments. ASC = area under the curve.

Protection of SOD-like activity with carcinine treatment of the skin after UVA-UVB irradiation

The effectiveness of natural imidazole-containing peptidomimetics in vivo was demonstrated with an ex vivo study performed on a porcine dermis-epidermis fraction. Skin tissues were UV-irradiated (UVA-UVB) and the resulting inactivation of SOD (Miyachi et al., 1987) was monitored. The oxidative deactivation of SOD in cutaneous cells during a UV irradiation represents both the decrease of a part of the skin's natural antioxidant defenses and the increase of the oxidative stress impact. Results obtained with a carcinine treatment are shown in Fig. 7A. The protective effect of carcinine demonstrated as example on Fig. 7A is about 43% ($p < 0.001$, $n = 10$). The antioxidants were applied as a cream on the tissues prior to irradiation. The protective effect was evaluated by measuring the catalytic activity of the SOD after extraction from the cells (Fig. 7B). According to the method described in the Materials and methods section, a SOD-like activity was measured from the extracts and a pure commercial SOD was used as the reference for quantitation. In the ex vivo test, the treatment with carcinine containing creams, confers to the skin a significant protection against the oxidative stress induced by UVA-UVB irradiation. Carcinine in applied creams do not absorb in UVA (320–400 nm) or UVB (280–320 nm) regions and the action is different from the UV filters. The protection of natural skin defenses, such as SOD activity provides the facility of the skin to withstand the oxidative stress, such as UV irradiation and aging.

Discussion

L-Carnosine (β-alanyl-L-histidine) was originally isolated by V. S. Gulewitsch, a Russian scientist as a component of compounds extracted from muscle tissue at the beginning of the XXth century (Gulewitsch and Amiradzibi, 1900). It has been established that L-carnosine, a natural L-histidine-containing dipeptide performs important biological functions; in

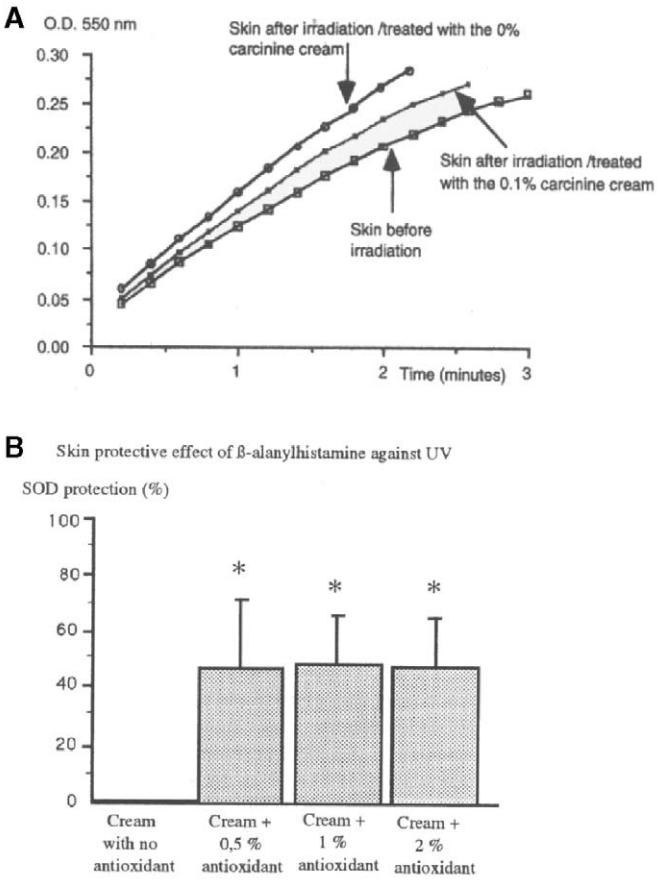


Fig. 7. (A) Kinetics of SOD-like activity in extracts from non-irradiated or irradiated skin previously treated with creams containing 0% or 0.1% of carcinine. The slope obtained with the non-irradiated skin is 0.1 OD units/min. The slope obtained with the irradiated skin treated with 0% carcinine is 0.17 OD units/min. The slope obtained with the irradiated skin treated with 0.1% carcinine is 0.14 OD units/min. (B) Protection of the SOD activity of isolated porcine ear dermis-epidermis treated with various concentrations of an imidazole-containing peptidomimetic. Average ± SEM from 10 independent experiments are given. *: significant differences ($p < 0.001$) with control (Student's t-test). Percent of protection is calculated by comparing with the SOD activity of a non-irradiated skin.

particular, it exhibits antioxidative properties (Boldyrev et al., 1988, 1987; Kohen et al., 1988) directed at suppression of free-radical reactions.

Study of the antioxidative action of L-carnosine has shown that its effects are realized not only owing to binding of lipid oxidation products in the course of free-radical reactions, but also through interaction with active oxygen species (Babizhayev et al., 1994). L-Carnosine may serve as a scavenger of peroxyl and hydroxyl radicals (Babizhayev et al., 1994), for singlet oxygen (Dahl et al., 1988), and can neutralize hypochlorite anion by forming with it stable chloramines complexes (Boldyrev et al., 1988). The antioxidative properties of L-carnosine provide its successful application in the form of an ophthalmic prodrug *N*-acetylcarnosine for treatment of cataracts (Babizhayev et al., 1996, 2004, 2001, 2002, 2000), application of L-carnosine for the treatment of superficial burns of epiderma, wound healing and various inflammatory processes developing on the background of cellular membrane damage (Nagai and Suda, 1988). However, the exact molecular mechanism of L-carnosine action and the biological significance of its natural imidazole-containing derivatives are not clear.

According to the data obtained in the present and previous (Jackson et al., 1991; Lenney et al., 1985; Lenney, 1976) studies, the natural dipeptide L-carnosine is a substrate for specific cutaneous proteolytic enzymes and other specific dipeptidases including carnosinases. In the present study this result was confirmed using a multi-enzymatic microsomal fraction obtained from epidermic cells. According to our data, a rapid deactivation of the L-carnosine dipeptide occurred when deposited on the skin and topically to the eye (see Results of the present study; Refs. Babizhayev et al., 1996, 2000). Looking for the minimal structural modification likely to improve resistance against dipeptidases, we performed decarboxylation and *N*-acetylation of the L-carnosine molecule. This led us to synthesis of the natural imidazole-containing peptidomimetics of L-carnosine, i.e. carcinine and *N*-acetylcarnosine which combine certain advantages of L-carnosine (including the verification of the antioxidant properties which were maintained) with the unique resistance to enzymatic hydrolysis (see Results of the present study; Ref. Pegova et al., 2000). In addition, in vivo and in vitro experiments related to metabolism of *N*-acetylcarnosine and carcinine have demonstrated another important properties for the natural imidazole-containing peptidomimetics (derivatives of L-carnosine) which are described in the Results section of this study. In vivo studies have confirmed the improvement of *N*-acetylcarnosine and carcinine properties towards the natural product of origin, L-carnosine.

The present study related to the suitability of the 1% *N*-acetylated form of L-carnosine to act as a time release version of L-carnosine resistant to hydrolysis by human serum carnosinase in pharmaceutical compositions for ophthalmic application which include lubricants (such as carboxymethylcellulose and glycerine) and preservatives approved for the ocular application. The application of a lubricant (carboxymethylcellulose) can increase the intraocular absorption of the active principle (L-carnosine). In different animals or tissues, there may be other transporters. Carnosine was reported to be transported by $PEPT_2$ (Teuscher et al., 2004).

This result is also related to the skin care and cosmetic compositions, which include solutions, creams, emulsions, gels etc. specific formulations applied ex vivo. Because of the physiological and antioxidant activities of L-carnosine, other forms such as *N*-acetylated time release version of L-carnosine is suggested in order to prolong and even to increase the inherent antioxidant characteristics of this dipeptide. However, the ability of the putative carrier to penetrate through the skin tissue and accumulate in the skin layers for any reasonable time is still not clear and may include additionally the use of liposomal (nanosphere) vehicle or addition of specific hydroglycolic solutions during formulation studies. The application of *N*-acetylcarnosine would be beneficial during the application in the peeling systems used for dermo-esthetique and in the skin anti-aging therapeutical treatments.

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 briefly involves enzymic removal of lipid-derived hydroperoxide intermediates typically catalyzed by glutathione-requiring enzymes (Se-dependent GSH peroxidases and certain Se-independent enzymes, such as GSH-*S*-transferase B) (Ursini and Bindoli, 1987). During lipid peroxidation, reactive lipid and fatty acid hydroperoxides are formed, and these contribute to ongoing autooxidation (Gunstone, 1996). Lipid hydroperoxides play a key role in the development of eye cataracts and skin disorders, and they act as substrates for pathways that yield leukotrienes and lipoxins as part of the inflammatory response. Fatty acid hydroperoxides have been shown to be more toxic than phospholipid hydroperoxides to endothelial cells (Kaneko et al., 1994), and it has been shown that phospholipid hydroperoxides are broken down to the fatty acid hydroperoxide moiety to exert their toxic effects (Kaneko et al., 1996).Under certain conditions, linoleic acid hydroperoxide can form a delocalized lipid radical (L') which self-reacts to form dienoic dimers (L"L) or reacts with another hydroperoxide to form the peroxy radical (LOO') (Garssen et al., 1971). High levels of lipid hydroperoxides are also found in plants following environmental stress or physical injury, and these compounds are important in cosmetics industry (Beeor-Tzahar et al., 1995). Total spectrum of enzymes involved in the breakdown of lipid hydroperoxides may include phospholipases, glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase, which is specific to phospholipid hydroperoxides (Marinho et al., 1997), thioredoxin reductase (Bjornstedt et al., 1995), and cytochrome P-450 enzymes (Thompson et al., 1995).

The lipoxygenase enzyme, which is used to generate lipid hydroperoxides in vitro, can also use them as a substrate (Garssen et al., 1971). In continuation to our previous work showing preliminary that carnosine and carcinine were able to react directly with lipid peroxides and reduce them into the

corresponding alcohol, thus inhibiting the chain reaction of LPO (Babizhayev et al., 1994), we now present the specific evidence based on the characteristic HPLC assay for the transformation of the model reagent 13(*S*)-hydroperoxide of linoleic acid (LOOH) into its reduced form LOH assigned to lipid peroxidase-like activity of natural imidazole-containing peptidomimetics. Particularly for carcinine, a peptidomimetic with a higher resistance to enzymatic hydrolysis, an improved antioxidant efficiency against the oxidative stress induced by lipid hydroperoxides and phospholipid hydroperoxides is provided. 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 and carcinine are active electrochemically as a reducing agent in cycle voltammetric measurements, donating a hydrogen atom to the peroxyl radical (Babizhayev et al., 1994 and references thereof).

The advantage of carcinine, *N*-acetylcarnosine and L-carnosine as universal antioxidants relates to their ability to give efficient protection against LPO both in the lipid phase of biological membranes and in an aqueous environment. Various protective antioxidant enzymes such as SOD or catalase can only react with their substrates in the aqueous environment. In the present study deactivation of lipid hydroperoxides was monitored by HPLC and protection of membrane phospholipids and water soluble proteins (BSA, SOD) by natural imidazole-containing peptidomimetics was demonstrated. L-Prolylhistamine and carcinine could protect almost completely the protein from cross-linking by lipid hydroperoxide and by peroxidized PC-containing liposomes, while L-carnosine was unable to do so.

The surprising result was perhaps the demonstration of the complete uneffectiveness of the reference lipophilic antioxidant vitamin E in this experimental situation. What could explain such important differences and the advantages of antioxidant activities of natural imidazole-containing peptidomimetics over vitamin E, despite the fact that the lipophilic vitamin E can scavenge the hydrophobic radicals LOO·, LO·, and over L-carnosine (canonic molecule) which can per se protect, in the aqueous phase, the protein from the free radical attack?

The advantages of natural imidazole-containing peptidomimetcs supported by the created in this study 3-D chemical structures can be formulated as follows:

- Peptidomimetics may form adducts with the lipoperoxide, thus decreasing its reactivity or increasing the kinetics of reduction. This interaction was confirmed in our laboratories, and it was separately reported furthermore that L-carnosine could bind hydrogen peroxide (Schubert et al., 1969).
- Imidazole-containing peptides may react with di- or monoaldehydes, powerful cross-linking agents, released during the oxidative breakdown of unsaturated lipids (Aldini et al., 2002; Hipkiss, 2002; Hipkiss et al., 1998). These are water-soluble, non-radical compounds that cannot be deactivated by vitamin E. It was claimed in the literature (Hipkiss et al., 1995) that L-carnosine could react with glucose and prevent glycation.
- Natural imidazole-containing peptidomimetics may fight more efficiently against site-specific, transition metal ion-mediated degradation of LOOH at the surface of the protein (proteins can easily bind transition metal ions). It is known that in such a situation scavengers with no chelating properties are useless. Our results (partially not shown) have demonstrated that the most effective peptidomimetics (L-prolylhistamine and carcinine) were the strongest metal chelators (superior to L-carnosine), according to our 3-D structural study. In addition, some preliminary experimental results from our laboratories suggest that imidazole-containg peptidomimetics may interact also with the protein's surface (non-specific interaction with hydrophobic pockets of the protein).

The protective effect of the carnosine-related compounds against the inactivation of Cu,Zn-SOD and ceruloplasmin in conditions of oxidative stress and glycation was described in the recent in vitro experiments (Ukeda et al., 2002; Kang et al., 2002a,b,c). In one of these studies (Kang et al., 2002a) the protective effects of carnosine and related compounds against the oxidative damage of human Cu,Zn-superoxide dismutase (SOD) by peroxyl radicals generated from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) were examined. The oxidative damage to Cu,Zn-SOD by AAPH-derived radicals led to protein fragmentation, which is associated with the inactivation of enzyme. Carnosine, homocarnosine and anserine significantly inhibited the fragmentation and inactivation of Cu,Zn-SOD by AAPH. The results suggest that carnosine and related compounds act as the copper chelator and peroxyl radical scavenger to protect the protein fragmentation. Skin is a complex biological medium, and even if cosmetic chemists relate to the major biological function of the skin, the barrier function, they must take into consideration what happens to the product when applied on the skin. Thus, "tailoring" a molecule provides structural features more suitable with the important aspects of the biology of the skin. Carcinine, the active molecule of cosmetic cream, demonstrated the protection of the skin SOD and cutaneous cells protection under the conditions of oxidative stress induced by UVA-UVB irradiations. So, one of the effectiveness routes of antioxidant action characteristic for the natural imidazole-containing peptidomimetics is mediated via the protection of the proprietary tissue antioxidant enzymes, such as SOD, which is a witness of the internal cellular integrity. This result is important when one considers the therapeutic use of carnosine related compounds applied to the skin in vivo.

To conclude, in this study the unique 'universal' antioxidant protective effect of natural imidazole-containing peptidomimetics carcinine, *N*-acetylcarnosine and L-carnosine was revealed. The products were effective in protecting both hydrophilic biological molecules (such as proteins, enzymes) as well as the lipophilic ones (unsaturated fatty acids, membrane phospholipids). These biocompatible mimetic actives, being well accepted ex vivo conditions in terms of

the resistance to enzymatic deactivation present the effective tool and active ingredients with unusual anti-aging properties during the novel biological skin and ophthalmic treatments. These actives will give a growing place to anti-aging formulations with specific protective and selective activities that fulfill professional and common demand.

Acknowledgement

Dr. Mark A. Babizhayev is thankful to Exsymol SAM (Principaute de Monaco) and Hamari Chemicals Ltd (Japan) for the provided cooperation in this study. The biologically significant applications of carnosine mimetics were patented by Dr. Babizhayev and the alliance Groups (WO 2004/028536 A1; WO 94/19325; WO 95/12581; WO 2004/064866 A1).

References

Aldini, G., Carini, M., Beretta, G., Bradamante, S., Facino, R.M., 2002. Carnosine is a quencher of 4-hydroxy-nonenal: through what mechanism of reaction? Biochemical Biophysical Research Communications 298, 699–706.

Aruoma, O.I., Laughton, M.J., Halliwell, B., 1989. Carnosine, homocarnosine and anserine: could they act as antioxidants in vivo? Biochemical Journal 264, 863–869.

Babizhayev, M.A., 1989. Antioxidant activity of L-carnosine, a natural histidine-containing dipeptide in crystalline lens. Biochimica et Biophysica Acta 1004, 363–371.

Babizhayev, M.A., Bozzo Costa, E., 1994. Lipid peroxide and reactive oxygen species generating systems of the crystalline lens. Biochimica et Biophysica Acta 1225, 326–337.

Babizhayev, M.A., Seguin, M.C., Gueyne, J., Evstigneeva, R.P., Ageyeva, E.A., Zheltukhina, G.A., 1994. L-carnosine (beta-alanyl-L-histidine) and carcinine (beta-alanylhistamine) act as natural antioxidants with hydroxyl-radical-scavenging and lipid-peroxidase activities. Biochemical Journal 304, 509–516.

Babizhayev, M.A., Yermakova, V.N., Sakina, N.L., Evstigneeva, R.P., Rozhkova, E.A., Zheltukhina, G.A., 1996. *N*-acetylcarnosine is a prodrug of L-carnosine in ophthalmic application as antioxidant. Clinica Chimica Acta 254, 1–21.

Babizhayev, M.A., Yermakova, V.N., Semiletov, Yu.A., Deyev, A.I., 2000. The natural histidine-containing dipeptide *N*-acetylcarnosine as an antioxidant for ophthalmic use. Biochemistry (Moscow) 65, 588–598.

Babizhayev, M.A., Deyev, A.I., Yermakova, V.N., Semiletov, Yu.A., Davydova, N.G., Kurysheva, N.I., Zhukotskii, A.V., Goldman, I.M., 2001. *N*-acetylcarnosine, a natural histidine-containing dipeptide, as a potent ophthalmic drug in treatment of human cataracts. Peptides 22, 979–994.

Babizhayev, M.A., Deyev, A.I., Yermakova, V.N., Semiletov, Yu.A., Davydova, N.G., Doroshenko, V.S., Zhukotskii, A.V., Goldman, I.M., 2002. Efficacy of *N*-acetylcarnosine in the treatment of cataracts. Drugs in Research and Development 3, 87–103.

Babizhayev, M.A., Deyev, A.I., Yermakova, V.N., Brikman, I.V., Bours, J., 2004. Lipid peroxidation and cataracts. *N*-acetylcarnosine as a therapeutic tool to manage age-related cataracts in human and in canine eyes. Drugs in Research and Development 5, 125–139.

Beeor-Tzahar, T., Ben-Hayyim, G., Holland, D., Faltin, Z., Eshdat, Y., 1995. A stress-associated citrus protein is a distinct plant phospholipid hydroperoxide glutathione peroxidase. FEBS Letters 366, 151–155.

Bjornstedt, M., Hamberg, M., Kumar, S., Xue, J., Holmgren, A., 1995. Human thioredoxin reductase directly reduces lipid hydroperoxides by NADPH and selenocystine strongly stimulates the reaction via catalytically generated selenols. Journal of Biological Chemistry 270, 11761–11764.

Boldyrev, A.A., Dupin, A.M., Babizhayev, M.A., Bunin, A.Ya., Severin, S.E., 1987. The antioxidative properties of carnosine, a natural histidine containing dipeptide. Biochemical International 15, 1105–1113.

Boldyrev, A.A., Dupin, A.M., Pindel, E.V., Severin, S.E., 1988. Antioxidative properties of histidine-containing dipeptides from skeletal muscles of vertebrates. Comparative Biochemistry Physiology 89B, 245–250.

Dahl, T.A., Midden, W.R., Hartman, P.E., 1988. Some prevalent biomolecules as defenses against singlet oxygen damage. Photochemistry Photobiology 47, 357–362.

Decker, E.A., Crum, A.D., Calvert, J.T., 1992. Differences in the antioxidant mechanism of carnosine in the presence of copper and iron. Journal of Agriculture Food Chemistry 40, 756–759.

Fitzpatrick, J.C., Brotman, D.N., Fisher, H., Flancbaum, L., 1989. Involvement of carcinine (β-alanylhistamine) in the carnosine-histamine pathway. FASEB Journal 3, 1028.

Garssen, G.J., Vliegenthart, J.F.G., Boldingh, J., 1971. An anaerobic reaction between lipoxygenase, linoleic acid and its hydroperoxides. Biochemical Journal 122, 327–332.

Gulewitsch, V.S., Amiradzibi, S., 1900. Uber das Carnosin eine neue organische Base des Fleischextraktes. Berichte der Deutschen Chemischen Gesellschaft 33, 1902–1903.

Gunstone, F.D., 1996. Fatty Acid and Lipid Chemistry. Blackie Academic and Professional, London, United Kingdom.

Hipkiss, A.R., 2002. Could carnosine be a naturally-occurring scavenger for acrolein and other reactive aldehydes in the brain? Neurobiology Aging 23, 645–646.

Hipkiss, A.R., Michaelis, J., Syrris, P., 1995. Non-enzymatic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent. FEBS Letters 371, 81–85.

Hipkiss, A.R., Preston, J.E., Himsworth, D.T., Worthington, V.C., Keown, M., Michaelis, J., Lawrence, J., Mateen, A., Allende, L., Eagles, P.A., Abbott, N.J., 1998. Pluripotent protective effects of carnosine, a naturally occurring dipeptide. Annals of the New York Academy of Sciences 854, 37–53.

Iacazio, G., Langrand, G., Baratti, J., Buono, G., Triantaphylides, C.J., 1993. Preparative, enzymatic synthesis of linoleic acid (13*S*)-hydroperoxide using soybean lipoxygenase-1. Organic Chemistry 55, 1690.

Jackson, M.C., Kucera, C.M., Lenney, J.F., 1991. Purification and properties of human serum carnosinase. Clinica Chimica Acta 196, 193–206.

Kaneko, T., Baba, N., Matsuo, M., 1994. Phospholipid hydroperoxides are significantly less toxic to cultured endothelial cells than fatty acid hydroperoxides. Life Sciences 55, 1443–1449.

Kaneko, T., Baba, N., Matsuo, M., 1996. Cytotoxicity of phosphatidylcholine hydroperoxides is exerted through decomposition of fatty acid hydroperoxide moiety. Free Radicals in Biology and Medicine 21, 173–179.

Kang, J.H., Kim, K.S., Choi, S.Y., Kwon, H.Y., Won, M.H., Kang, T.C., 2002. Protective effects of carnosine, homocarnosine and anserine against peroxyl radical-mediated Cu, Zn-superoxide dismutase modification. Biochimica et Biophysica Acta 1570, 89–96.

Kang, J.H., Kim, K.S., Choi, S.Y., Kwon, H.Y., Won, M.H., Kang, T.C., 2002. Carnosine and related dipeptides protect human ceruloplasmin against peroxyl radical-mediated modification. Molecules Cells 13, 498–502.

Kang, J.H., Kim, K.S., Choi, S.Y., Kwon, H.Y., Won, M.H., Kang, T.C., 2002. Protection by carnosine-related dipeptides against hydrogen peroxide-mediated ceruloplasmin modification. Molecules Cells 13, 107–112.

Kohen, R., Yamamoto, Y., Cundy, K.C., Ames, B., 1988. Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. Proceedings of the National Academy of Sciences of the United States of America 85, 3175–3179.

Kunze, N., Kleinkauf, H., Bauer, K., 1986. Characterization of two carnosine-degrading enzymes from rat brain. Partial purification and characterization of a carnosinase and a beta-alanyl-arginine hydrolase. European Journal of Biochemistry 160, 605–613.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680.

Langrand, G., Courbebaisse, Y., Levrat, B., Nicolay, J.F., 1999. Design, evaluation and use of new cosmetic raw materials: pseudodipeptides. IFSCC Magazine 2, 47–57.

Lenney, J.F., 1976. Specificity and distribution of mammalian carnosinase. Biochimica et Biophysica Acta 429, 214–219.

Lenney, J.F., 1990. Separation and characterization of two carnosine-splitting cytosolic dipeptidases from hog kidney (carnosinase and non-specific dipeptidase). Biological Chemistry Hoppe-Seiler 371, 433–440.

Lenney, J.F., George, R.P., Weiss, A.M., Kucera, C.M., Chan, P.W.H., Rinzler, G.S., 1982. Human serum carnosinase: characterization, distinction from cellular carnosinase, and activation by cadmium. Clinica Chimica Acta 123, 221–231.

Lenney, J.F., Peppers, S.C., Kucera-Orallo, C.M., George, R.P., 1985. Characterization of human tissue carnosinase. Biochemical Journal 228, 653–660.

Marinho, H.S., Antunes, F., Pinto, R.E., 1997. Role of glutathione peroxidase and phospholipid hydroperoxide glutathione peroxidase in the reduction of lysophospholipid hydroperoxides. Free Radicals in Biology and Medicine 22, 871–883.

McFarland, G.A., Holliday, R., 1994. Retardation of the senescence of cultured human diploid fibroblasts by carnosine. Experimental Cell Research 212, 167–175.

McFarland, G.A., Holliday, R., 1999. Further evidence for the rejuvenating effects of the dipeptide L-carnosine on cultured human diploid fibroblasts. Experimental Gerontology 34, 35–45.

Miyachi, Y., Imamura, S., Niwa, Y., 1987. Decreased skin superoxide dismutase activity by a single exposure of ultraviolet radiation is reduced by liposomal superoxide dismutase pretreatment. Journal of Investigative Dermatology 89, 111–112.

Murphey, W.H., Patchen, L., Lindmark, D.G., 1972. Carnosinase: a fluorometric assay and demonstration of two electrophoretic forms in human tissue extracts. Clinica Chimica Acta 42, 309–314.

Nagai, K., Suda, T., 1988. Realization of spontaneous healing function by carnosine. Methods Finding Experimental Clinical Pharmacology 10, 497–507.

O'Dowd, J.J., Robins, D.J., Miller, D.J., 1988. Detection, characterisation, and quantification of carnosine and other histidyl derivatives in cardiac and skeletal muscle. Biochimica et Biophysica Acta 967, 241–249.

Pegova, A., Abe, H., Boldyrev, A., 2000. Hydrolysis of carnosine and related compounds by mammalian carnosinases. Comparative Biochemistry and Physiology, Part B 127, 443–446.

Sammons, D.W., Adams, L.D., Nishizawa, E.E., 1981. Ultra sensitive silver based color staining of polypeptides in polyacrylamide gels. Electrophoresis 2, 135.

Schubert, J., Watson, J.A., Baecker, J.M., 1969. Formation of a histidine-peroxide adduct by H_2O_2 or ionizing radiation on histidine: chemical and microbiological properties. International Journal of Radiation Biology 14, 577–583.

Stewart, J.J.P., 1989. MOPAC. F.J. Seiler Research Laboratory. Air Force Academy, Boulder, pp. 80840.

Stewart, J.J. MOPAC. Ver.6, QCPE Bull.,1989. 9,10. Revised as Ver. 6.01 by T. Hirano, University of Tokyo, for HITAC and UNIX machines (JCPE Newsletter., 1989. 1,10).

Teuscher, N.S., Shen, H., Shu, C., Xiang, J., Keep, R.F., Smith, D.E., 2004. Carnosine uptake in rat choroid plexus primary cell cultures and choroid plexus whole tissue from PEPT2 null mice. Journal of Neurochemistry 89 (2), 375–382.

Thompson, J.A., Schullek, K.M., Turnipseed, S.B., Ross, D., 1995. Role of cytochrome P450 in the metabolism and toxicity of hydroperoxides in isolated rat hepatocytes. Archives of Biochemistry and Biophysics 323, 463–470.

Ukeda, H., Hasegawa, Y., Harada, Y., Sawamura, M., 2002. Effect of carnosine and related compounds on the inactivation of human Cu,Zn-superoxide dismutase by modification of fructose and glycolaldehyde. Bioscience, Biotechnology and Biochemistry 66, 36–43.

Ursini, F., Bindoli, A., 1987. The role of selenium peroxidases in the protection against oxidative damage of membranes. Chemical Physics of Lipids 44, 255–276.