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Detoxification of aldehydes by histidine-containing dipeptides: From chemistry to clinical implications

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ABSTRACT

Aldehydes are generated by oxidized lipids and carbohydrates at increased levels under conditions of metabolic imbalance and oxidative stress during atherosclerosis, myocardial and cerebral ischemia, diabetes, neurodegenerative diseases and trauma. In most tissues, aldehydes are detoxified by oxidoreductases that catalyze the oxidation or the reduction of aldehydes or enzymatic and nonenzymatic conjugation with low molecular weight thiols and amines, such as glutathione and histidine dipeptides. Histidine dipeptides are present in micromolar to millimolar range in the tissues of vertebrates, where they are involved in a variety of physiological functions such as pH buffering, metal chelation, oxidant and aldehyde scavenging. Histidine dipeptides such as carnosine form Michael adducts with lipid-derived unsaturated aldehydes, and react with carbohydrate-derived oxo- and hydroxy-aldehydes forming products of unknown structure. Although these peptides react with electrophilic molecules at lower rate than glutathione, they can protect glutathione from modification by oxidant and they may be important for aldehyde quenching in glutathione-depleted cells or extracellular space where glutathione is scarce. Consistent with *in vitro* findings, treatment with carnosine has been shown to diminish ischemic injury, improve glucose control, ameliorate the development of complications in animal models of diabetes and obesity, promote wound healing and decrease atherosclerosis. The protective effects of carnosine have been linked to its anti-oxidant properties, its ability to promote glycolysis, detoxify reactive aldehydes and enhance histamine levels. Thus, treatment with carnosine and related histidine dipeptides may be a promising strategy for the prevention and treatment of diseases associated with high carbonyl load.

1. Aldehydes: occurrence and association with disease

Biological aldehydes are ubiquitous products of normal metabolism and are generated during intermediary metabolism and lipid and nucleotide synthesis. In addition, they are present in several types of food and are produced to high levels during the processes of uncontrolled lipid and carbohydrate oxidation [1]. Accumulation of aldehydes due to excessive production has been suggested to contribute to the etiology of several diseases characterized by the presence of oxidative stress such as atherosclerosis, diabetes or Alzheimer's disease. Because of the presence of an electrophilic carbonyl group, aldehydes are highly reactive and they react readily with nucleophilic functional groups present on the macromolecules such as proteins, lipids and DNA [2]. In addition, recent evidence suggests that aldehydes also act as signaling molecules mediating inflammation and endoplasmic reticulum stress [3].

Although the aldehyde group by itself is only moderately reactive, its reactivity increases dramatically by the presence of other neighboring electron-withdrawing groups. Aldehydes associated with cellular oxidative stress are derived from two major classes of biological reactions: oxidation of polyunsaturated fatty acids and enzymatic or nonenzymatic metabolism of carbohydrates [4]. Aldehydes derived from the oxidation of lipids (lipid-derived aldehydes) are often characterized by the presence of an α,β double

Abbreviations: HNE, 4-hydroxy-trans-2-nonenal; HHE, 4-hydroxy-trans-2-hexenal; ONE, 4-oxo-trans-2-nonenal; AGEs, advanced glycation end-products; STZ, streptozotocin; CN, carnosinase; MS, mass spectrometry; MS/MS, tandem MS; NMR, nuclear magnetic resonance; MRS, magnetic resonance spectroscopy; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis; POVPC, 1-palmitoyl-2-(5′-oxo-valeroyl)-phosphatidylcholine; GSH, glutathione; ROS, reactive oxygen species.

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bond, or other oxo-or hydroxyl-groups present on the molecule. Major aldehydes derived from lipid peroxidation are: 4-hydroxy-trans-2-nonenal (HNE), 4-hydroxy-trans-2-hexenal (HHE), 4-oxo-trans-2-nonenal (ONE), nonenal, hexenal, etc. These aldehydes are electrophilic in nature and they form Michael adducts with cellular nucleophiles, such as amine and sulfhydryl compounds. The carbohydrate-derived aldehydes, i.e., methylglyoxal, glyoxal, glycolaldehyde, 3-deoxyglucosone, usually contain a keto or hydroxyl group in the α-position relative to the aldehyde group. This structural feature activates the aldehyde group and allows it to attack amine and guanidinium groups in proteins to undergo Schiff base formation, decarboxylation, and α-aminoketone condensation leading to the formation of advanced glycation end-products (AGEs) [5]. Several diseases such as atherosclerosis, diabetes, stroke, myocardial ischemia, Alzheimer's disease, schizophrenia, neurodegenerative diseases, and even aging are associated with increased oxidative stress and aldehyde accumulation. Products of oxidized lipoproteins accumulate in the atherosclerotic lesions of humans and atherosclerosis-prone animals and the circulating levels of lipid oxidation-derived aldehydes correlate strongly with angiographically documented coronary artery disease in humans [6–9]. In support of a causative role of aldehydes in atherogenesis, it has been shown that deletion of enzymes that promote the removal of lipid peroxidation products, such as paraoxonase [10] or aldose reductase [11] increases atherogenesis. Products of glycation of amino acid residues with α-dialdehydes (AGEs) have been shown to accumulate and contribute to the etiology of diabetes [12–14]. Hydroimidazolones, arising from arginine modification with aldehydes, often the major AGEs in proteins of tissues and body fluids, increase in diabetes and associated vascular complications, renal failure, cirrhosis, Alzheimer's disease, arthritis, Parkinson's disease and aging [15]. Because several AGE inhibitors such as pyridoxamine and benfotiamine inhibit the development of retinopathy and neuropathy in streptozotocin (STZ)-induced diabetic rats, it has been suggested that AGEs play a key role in the development of secondary diabetic complications [13,14].

It has been proposed that aldehydes contribute significantly to the pathological damage in high-metabolic-rate organs, such as liver, heart, and skeletal muscle. This suggestion is based on the observation that injury and dysfunction in several tissues is associated with aldehyde accumulation. For example, HNE adducts are frequently detected in liver samples from patients with non-alcoholic fatty liver disease, but they are absent in control livers [16]. Similarly, urine from subjects with inflammatory cancer-prone liver diseases caused by alcohol abuse or viral infection contains massively higher level of 1,N^6-etheno-2-deoxyadenosine, a miscoding etheno-modified DNA adduct formed by reaction of HNE with DNA-bases [17]. Increased levels of HNE or HNE-modified proteins have also been detected in the skeletal muscle of type 2 diabetic patients [18,19] and adipose tissue of HF fed mice [20].

Like other tissues, the brain is also susceptible to oxidative damage due to its high lipid content and oxygen consumption. Increased levels of lipid peroxidation products malondialdehyde, 4-hydroxynonenal and acrolein have been found in neurodegenerative diseases such as Alzheimer's, amyotrophic lateral sclerosis, bipolar disorder, epilepsy, Parkinson's disease, schizophrenia and central nervous system after trauma. Increased levels of specific histidine-HNE and glutathione-HNE Michael adducts have been detected in the brain tissue of Alzheimer's disease patients [21,22]. In addition, it has been suggested that increased oxidative stress may be relevant to the pathophysiology of schizophrenia, which is also associated with an increase in lipid-derived aldehydes such as malondialdehyde and HNE [23,24].

2. Aldehyde metabolism

The toxicity of aldehydes under normal physiological conditions and during disease and injury is likely to depend upon the ability of the biological processes involved in their detoxification. Such detoxification pathways include a combination of enzymatic and nonenzymatic reactions. The major enzymatic pathways of aldehyde detoxification are oxidation by aldehyde dehydrogenases, reduction by aldo–keto reductases and conjugation with glutathione (catalyzed by glutathione-*S*-transferases) (Fig. 1). Non-enzymatic pathways include conjugation with cellular sulfhydryl and amine-based nucleophiles, such as glutathione and carnosine. As conjugation of unsaturated aldehydes involves the formation of a Michael adduct at the double bond, the aldehyde group remains intact in the conjugate and can be further oxidized or reduced. Indeed, it has been shown that glutathione conjugates of HNE and acrolein are excellent substrates of aldose reductase, AKR1B1, which catalyzes their reduction into chemically inert glutathionyl-dihhydroxynonanol (GS-DHN) and glutathionyl-propanol (GS-propanol) [25,26]. Glutathione reacts with unsaturated aldehydes much faster than amine-based agents; however, the latter may be important under the conditions that lead to glutathione depletion or in removing aldehydes from extracellular spaces where the concentration of glutathione is two to three orders of magnitude lower than in the cells [27,28]. For the carbohydrate-derived aldehydes, it has been shown that the interaction with amine-containing compounds plays a major role in their biological disposition [29]. Hydrazine-based antiglycating agents such as aminoguanidine, hydralazine, and pyridoxamine, have been demonstrated to scavenge carbohydrate-derived oxo-aldehydes [30] and lipid-derived α,β-unsaturated aldehydes with high efficiency [31]. Therefore, recent interest has been directed towards the use of endogenous nitrogen-based compounds for aldehyde detoxification. These naturally occurring histidine-containing dipeptides have the potential to reduce the formation of aldehydes, detoxify pre-formed aldehydes, prevent generation of modified proteins and possibly even repair proteins that are covalently adducted to aldehydes and related carbonyls [32].

3. Histidine dipeptides

Histidine-containing dipeptides are a family of soluble peptides that consist of a histidine (or a histidine-like amino acid) containing an imidazole ring and an atypical amino acid (e.g. β-alanine or γ-hydroxybutyric acid) at the N-terminus of the peptide [33]. The imidazole ring appears to be important for several of the putative biological roles of this family of dipeptides. Carnosine is a "founding member" of this family and it consists of β-alanine bound to

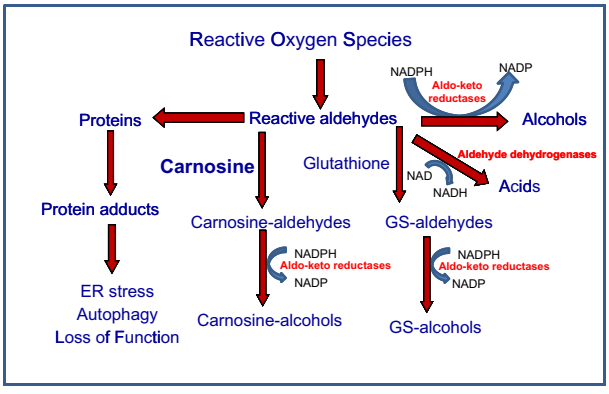

Fig. 1. Major pathways of aldehyde detoxification.

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histidine (Fig. 2). Carnosine was first discovered in meat by the Russian scientist Gulewitsch in 1900, hence its name carnosine (Latin *carnos* = meat) [34]. Variations in the structure of histidine dipeptides include methylation on either π (anserine), or τ (balenine) nitrogen on the imidazole ring of histidine, substitution of γ-aminobutyric acid for β-alanine (homocarnosine), or acetylation of the terminal amino group of β-alanine (*N*-acetylcarnosine). These modifications are species and tissue-specific and may provide means of fine-tuning the biological function of these peptides according to biological need and their biological roles that vary in a tissue-specific manner. For instance, only carnosine and N-acetylcarnosine are believed to be produced in human heart and skeletal muscle [35], whereas anserine is abundant in the pectoral muscle of birds and the leg muscle of rabbits [36]. Balenine is found mainly in muscles of murine diving mammals such as whales and dolphins [37]. Homocarnosine, which contains γ-hydroxybutyric acid in place of β-alanine, is predominantly present in the central nervous system and olfactory bulb [38,39]. However, the specific function of each of these dipeptides is still not fully understood.

Histidine dipeptides are present at the highest level in fast twitch glycolytic muscle fibers, and their levels may depend upon the metabolic needs of the tissue. For instance, higher levels of

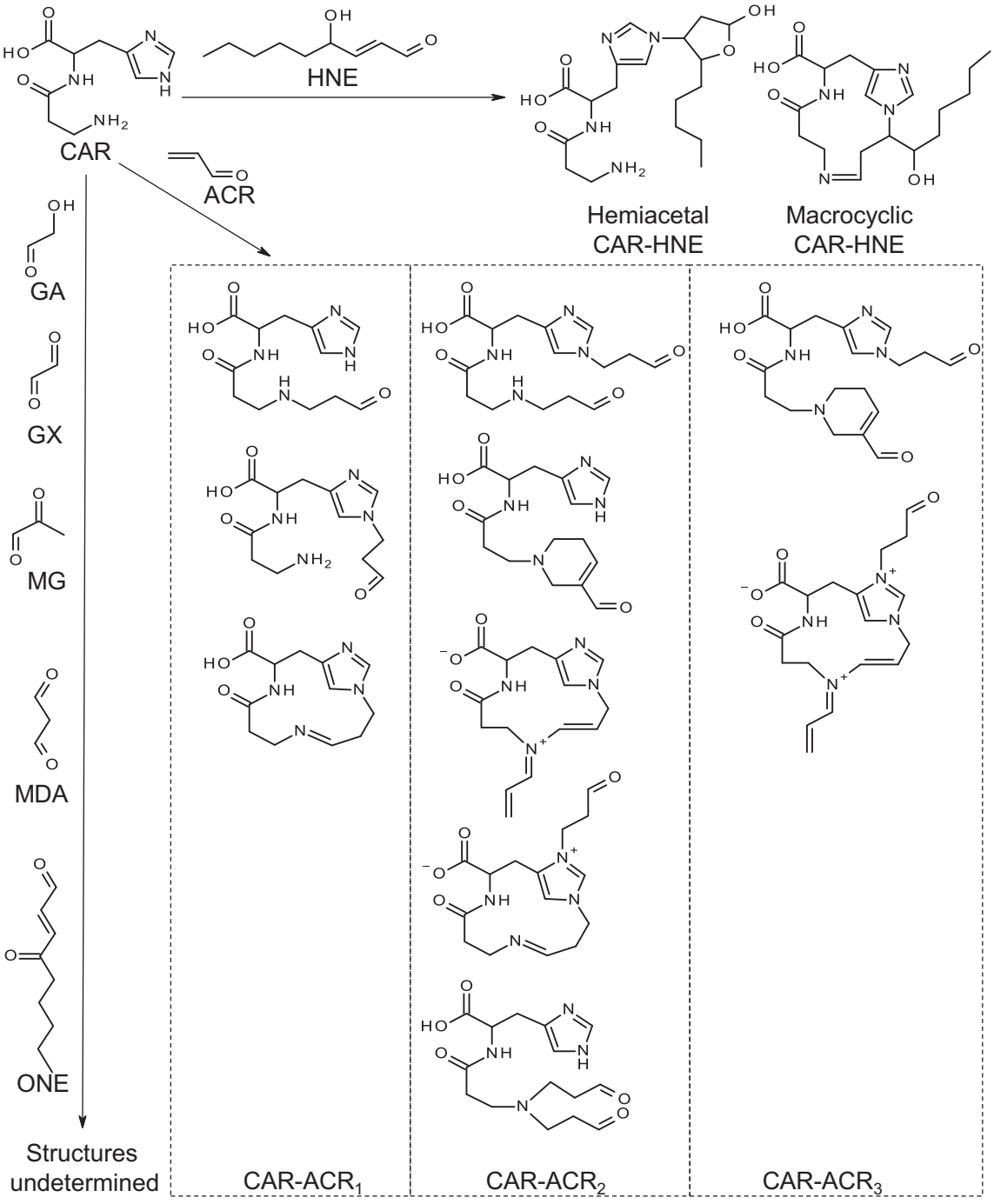

Fig. 2. Structures of carnosine - aldehydes reaction products. CAR, carnosine; HNE, 4-hydroxy-trans-2-nonenal; ACR, acrolein; GA, glycolaldehyde; GX, glyoxal; MG, methylglyoxal; MDA, malondialdehyde; ONE, 4-oxo-trans-2-nonenal, CAR-ACR$_1$, conjugates and dehydration products with 1:1 carnosine-acrolein ratio; CAR-ACR$_2$, conjugates and dehydration products with 1:2 carnosine-acrolein ratio; CAR-ACR$_3$, conjugates and dehydration products with 1:3 carnosine-acrolein ratio.

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histidine dipeptides are present in the pectoral muscle of pheasant, chicken and turkey (80–100 mM), species that are involved in explosive flight behaviors where rapid beating of wings for short periods is required, verses endurance flyers such as geese and pigeons [37]. Higher levels of histidine dipeptides have also been found in animals where frequent sprints and prolonged hypoxic dives are important for survival, such as the whale [40,41]. High levels of these peptides have also been detected in animals bred for athletic competition, such as quarter horses, greyhound dogs, and camels [41]. The highest level of histidine dipeptides (150 mM) has been found in the little piked whale which makes long hypoxic dives [37]. Such wide distribution of these dipeptides among species, their high concentration, and their specific localization attest to the importance of their biological role.

4. Metabolism of carnosine

Carnosine is synthesized *in vivo* from its constituent amino-acids histidine and β-alanine by the enzyme carnosine synthase (ATPGD1). The availability of β-alanine, which can be derived from panthothenic acid, a ubiquitous grain constituent or synthesized in liver through the degradation of uracil [42], is considered to be a limiting factor in the biosynthesis of carnosine [43–45]. In humans, carnosine is hydrolyzed back into its constituent amino acids by a specific peptidase – carnosinase (CN). Carnosinase is present in two isoforms, CN1 and CN2. While CN1 is secreted into the plasma from the liver and kidneys, CN2 is more ubiquitously expressed and remains in the cytosol [46–48]. The carnosinase gene is subject to polymorphisms in the human population, which determines the efficiency of secretion and plasma activity level. Higher levels of CN1 activity are associated with lower bioavailability of carnosine [49] and progression or susceptibility to diabetic nephropathy.

In addition to synthesis and degradation, carnosine disposition in tissues is determined by its transport in and out of cells. Carnosine is a substrate of the proton-coupled oligopeptide transporter Pept2 [50]. Studies with the Pept2-knockout mice have shown that Pept2 plays a major role in determining the systemic clearance and reabsorption of carnosine in the kidney tubules [51]. In Pept2-knockout mice, higher levels of carnosine are present in skeletal muscle, compared to lower levels in the choroid plexus, olfactory bulb and spleen. However, the uptake of carnosine was severely diminished in the skeletal muscle of these mice. Therefore, it appears an endogenous system exists to maintain carnosine homeostasis in tissues with high and low synthetic capacity.

5. *In vivo* measurement of carnosine

Carnosine and other histidine dipeptides can be measured using high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). Two challenges exist for HPLC analysis of histidine-containing dipeptides like carnosine: (1) carnosine is highly hydrophilic and therefore is poorly retained on the reverse-phase C18 HPLC columns, and (2) it lacks sufficient absorbance in the UV–Visible range. The first issue is addressed by chromatographic separation of carnosine on reverse phase column with addition of surfactant [52] or ion pair agent [53,54] into the mobile phase. The second issue can be overcome by using detection methods such as amperometric detection [55], or post-column derivatization [56]. Alternatively, pre-column derivatization can be used to improve both chromatographic behavior and detection, but it requires additional steps during sample preparation [57–59].

The combination of mass analysis capabilities of mass spectrometry (MS) or tandem MS (MS/MS) with physical separation capabilities of HPLC offers exceptional sensitivity and specificity, which is especially useful for simultaneous determination of multiple small molecules in a biological sample in "metabolomics" studies [60–64]. Along with HPLC, capillary electrophoresis (CE) offers higher separation efficiency, requires a smaller sample size, and is suitable for separation of peptides [65,66]. Similar to HPLC-MS, CE-MS has also been used for metabolomics research, and histidine-containing dipeptides were detected in these studies [67–72]

Histidine-containing dipeptides like carnosine can be quantified in a non-invasive manner using nuclear magnetic resonance (NMR) or magnetic resonance spectroscopy (MRS), spectroscopy in human skeletal muscles [73–76], or in intact animals [77]. MRS method has recently been used to show that carnosine is depleted in the gastrocnemius muscle of type 2 diabetic patients [78]. The NMR signal of carnosine has been used to investigate the dynamics of intracellular pH in working muscles [79–81]. Like the MS based methods, NMR is also a method of choice for metabolomics studies, and levels of carnosine measured by this method have been reported [82,83].

6. Carnosine in normal physiology

Histidine dipeptides are particularly abundant in skeletal muscle (8–50 mM), especially muscles that undergo anaerobic activity and maintain a high level of glycolysis. It has been suggested that in skeletal muscle the physiological function of these dipeptides is to maintain pH during strenuous exercise characterized by increased production of lactic acid. It has been estimated that carnosine provides ~10% of buffering capacity in vastus lateralis of humans [41]. Carnosine content was found higher in glycolytic type II fast-twitch muscle, than in more oxidative type I fibers, and its benefits in exercise performance have been specifically associated with muscle subjected to anaerobic activity and thus high level of glycolysis [40]. It has also been demonstrated that the levels of carnosine in the muscle are increased through training, and trained athletes in general have higher levels of carnosine in their muscle than untrained individuals [84]. Men have 36% to 82% higher muscle levels of carnosine than women. Vegetarians have lower levels of carnosine, suggesting that diet is an important determinant of carnosine content [85]. It has been shown that carnosine content of the soleus muscle decreases with age both in male and female omnivores [85]. In contrast, polymorphisms in the carnosinase gene, which affect plasma carnosine concentration after ingestion of carnosine, do not affect muscle carnosine content [85]. Supplementation with an isomolar amount of carnosine and its precursor β-alanine have been reported to increase muscle carnosine content by as much as 80% accompanied by improvements in ergometric performance [41,86,87].

7. Histidine dipeptides as antioxidants

Histidine dipeptides are capable of binding transition metal ions, including Cu^{2+} [88], Co^{2+} [89], Mn^{2+} [90], Zn^{2+} [91], Cd^{2+} [91] and Fe^{2+} [90]. The chemical character and biological relevance of this chelation capability has been summarized in several reviews [92,93]. Metal binding affinity of carnosine is utilized in the carnosine-zinc complex, which has membrane-stabilizing [94], antioxidant [95], and wound healing [96] effects, and has been approved in Japan to treat gastric ulcer under the name Polaprezinc [97].

In addition to pH buffering and metal chelation, it has been suggested that carnosine and other histidine dipeptides can also act as antioxidants. A number of early studies indicated that carnosine is a scavenger of hydroxyl, peroxyl, superoxide radicals and singlet oxygen. In several *in vitro* oxidizing systems, including iron-ascorbate and linoleic acid hydroperoxide – hemoglobin systems, which generate superoxide and lipid peroxyl radicals, respectively,

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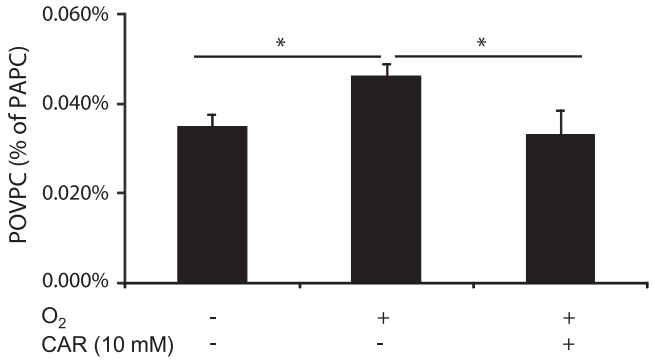

Fig. 3. Carnosine protects phospholipid from oxidation by molecular oxygen. Oxygen was bubbled at a rate of 30 ml/min through a solution containing 1-palmitoyl-2-arachidonyl phosphatidylcholine (PAPC; 25 μg/ml) in 3 ml of phosphate-buffered saline (KH_2PO_4 1.06 mM, NaCl 155.17 mM, Na_2HPO_4 2.97 mM, pH 7.4) for 1 h at 37 °C in the presence and absence of 10 mM carnosine. Formation of PAPC oxidation product 1-palmitoyl-2-(5′-oxo-valeroyl)-phosphatidylcholine (POVPC) was determined by ESI mass-spectrometry and POVPC concentration is expressed as the percentage of PAPC ion. $^*P < 0.05$.

carnosine inhibits oxidation of linoleic acid and phosphatidylcholine liposomes [98]. Carnosine also inhibits iron-dependent hydroxylation of deoxyribose, suggesting that it might be an efficient hydroxyl radical scavenger [98]. Carnosine also inhibits oxidative hydroxylation of deoxyguanosine induced by an ascorbic acid/copper mixture; however, this effect may be due to its ability to chelate copper [99]. In addition to these properties, carnosine has also been shown to enhance ferric-reducing capacity of human plasma and to protect the DNA from damage induced by ferric and copper ascorbate systems to an extent much higher than that of histidine and β-alanine alone or in combination [100].

Several early studies suggested that carnosine quenches singlet molecular oxygen. In some studies it has been shown that carnosine protects bacteriophage DNA against damage induced by γ-irradiation and inhibits singlet oxygen phosphorescence [101,102]. In a more biologically relevant setting carnosine has been shown to improve functional recovery of ischemic hearts by singlet oxygen quenching, as established by electron paramagnetic resonance measurements [103]. To determine whether histidine dipeptides have the ability to protect biological lipids from oxidation in metal-free systems, we bubbled oxygen through a suspension of 1-palmitoyl-2-arachidonyl phosphatidylcholine, a naturally occurring phospholipid containing polyunsaturated fatty acid. The formation of an aldehyde product 1-palmitoyl-2-(5′-oxo-valeroyl)-phosphatidylcholine (POVPC) was monitored using electrospray ionization MS. Carnosine reduced the formation of POVPC to the level observed without oxygen bubbling indicating that it protects phospholipids from oxidation (Fig. 3). Hence, a combination of metal chelating and radical scavenging properties makes these dipeptides important and universal antioxidants *in vivo*.

8. Reaction of histidine dipeptides with aldehydes

In addition to radical quenching, carnosine has also been found to protect proteins from attack by biologically reactive aldehydes, e.g. aldose sugars [104,105], malondialdehyde [106], methylglyoxal [107,108], glycolaldehyde [105], HNE [109], acrolein [110], and ONE [111] *in vitro*. In the early literature, carnosine was presumed to act as a sacrificial sink due to its structural similarity with liable proteins [104]. This view is supported by the studies of Zhou and Decker, which showed that carnosine quenches α,β-unsaturated aldehydes, such as HNE and *trans*-2-hexenal [112], and that the quenching ability of carnosine is much higher than its constituent amino acids separately. Products of the reaction of carnosine with aldehydes, such as HNE and acrolein, have been identified (Fig. 2).

The reaction of HNE and carnosine has been investigated using both mass-spectrometry and NMR [109,113–115] and summarized by A. Guiotto et. al. [116]. Briefly, the reaction between carnosine and HNE follows two pathways and leads to the formation of two major products (Fig. 2). The first pathway starts with Schiff base formation between the aldehyde functional group and the amino group of the β-alanyl residue, followed by the formation of a 13-member cyclic adduct (macrocyclic CAR-HNE) by ring closure. In the second pathway the reactive C3 of the aldehyde links with N^{τ}-imidazole nitrogen of the histidine residue by Michael adduction, and then a 5-member ring derivative (hemiacetal CAR-HNE) is formed. The two pathways could occur sequentially, i.e. the second pathway may follow the first one [113]. Due to the lack of a hydroxyl group, the second pathway is not possible for *trans*-2-nonenal, and therefore this aldehyde reacts with carnosine to form the 13-member cyclic adduct but not the hemi-acetal derivative [113].

In the first pathway (starting with Schiff base formation), the amino group of the β-alanyl residue acts as a catalyst, which explains the higher quenching ability of carnosine than histidine [113]. This catalyzing role is supported by the observation that *N*-acetyl-carnosine, which lacks free amino group of the β-alanyl residue, has significantly lower quenching ability than carnosine; but quenching ability of anserine, the N^{π}-methyl derivative of carnosine, is similar to that of carnosine [113]. Molecular modeling of the Schiff base intermediate shows that the "folded" conformer is the reactive conformation, and aldehyde quenching ability can be optimized by structural modification of carnosine to increase the proportion of this reactive conformation [117].

Among carnosine-HNE reaction products, the hemiacetal CAR-HNE has drawn great interest because it is the only carnosine-aldehyde reaction product that has been detected and quantitatively measured in biological samples. This conjugate has been detected in HNE-spiked rat skeletal muscle [118], urine of Zucker obese rat [119], and apoE-null mice [120] suggesting that hemiacetal CAR-HNE could be used as a specific biomarker of lipid peroxidation.

The reaction between acrolein and carnosine or homocarnosine has been investigated using mass spectrometry [121]. One, two, or three acrolein molecules can be linked to nitrogens of one carnosine molecule (Fig. 2), and different reaction products are generated after different reaction times. In addition to Michael addition and Schiff base formation, aldol condensation and dehydration are also involved in the reaction between carnosine and acrolein. The reaction of homocarnosine with acrolein also follows the same pathway as the carnosine-acrolein reaction [121].

Although reactions between α,β-unsaturated aldehydes and histidine dipeptides have been studied intensively, reactions between histidine dipeptides and carbohydrate-derived aldehydes, such as methylglyoxal, glycolaldehyde, and glyoxal have attracted less attention. However, the reactions between histidine dipeptides and these aldehydes are implied in studies showing that carnosine protects proteins from attack by methylglyoxal [107,108] and glycolaldehyde [105]. Due to the biological importance of carbohydrate-derived aldehydes, it is important to identify the products and elucidate reaction mechanisms of histidine dipeptides and these aldehydes. To study the chemical reaction between methylglyoxal and carnosine, we monitored the UV spectra of a reaction mixture containing methylglyoxal and carnosine. Incubation of carnosine with methylglyoxal led to a large increase in the absorbance at 280 nm. However, incubation of methylglyoxal or carnosine alone did not produce this peak, indicating that the peak at 280 nm is characteristic of the product of the reaction between carnosine and methylglyoxal (Fig. 4). A similar spectroscopic change

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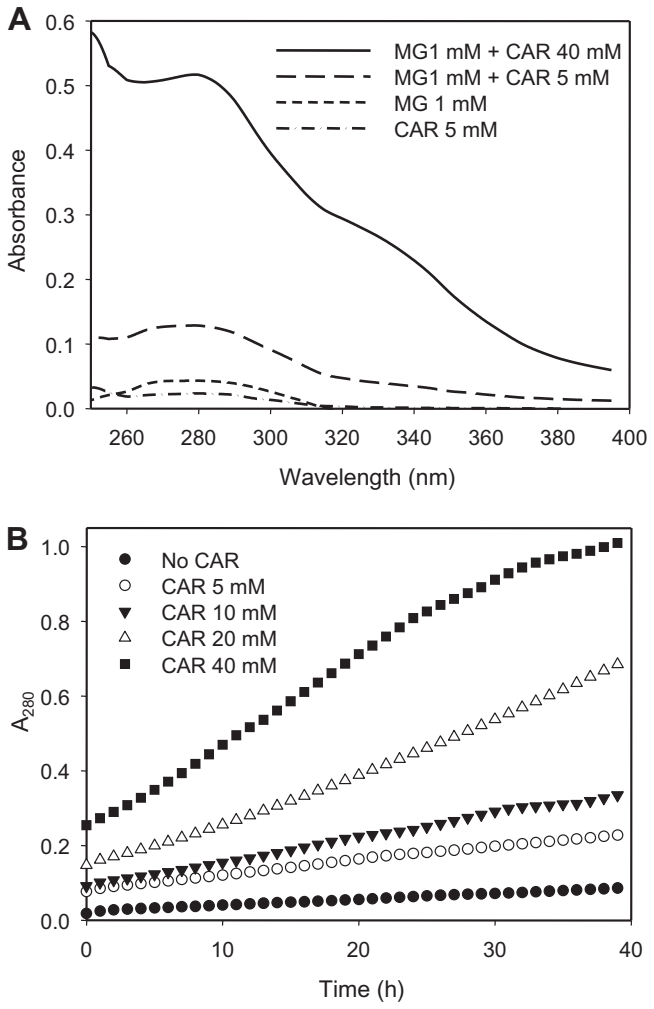

Fig. 4. Reaction of carnosine with methylglyoxal. A, UV spectra of a reaction mixture containing methylglyoxal (1 mM) and carnosine (5 and 40 mM) after 12 h of incubation. B, Time course of the reaction between methylglyoxal and carnosine monitored at 280 nm. Carnosine at several concentrations was incubated with 1 mM methylglyoxal in 150 mM potassium phosphate buffer, pH 7.4, at 37 °C. Change in absorbance was acquired every 60 min.

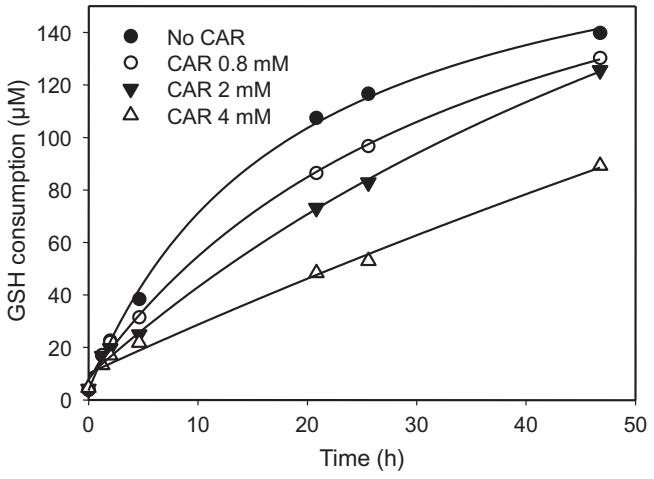

Fig. 5. Carnosine protects glutathione from methylglyoxal-mediated depletion. Carnosine at indicated concentrations was incubated with 0.2 mM GSH and 0.2 mM methylglyoxal in 150 mM potassium phosphate buffer, pH 7.4, at 37 °C. Time-dependent consumption of free GSH was monitored spectrophotometrically after the addition of DTNB.

was observed when carnosine was incubated with glyoxal and glycolaldehyde (not shown), indicating a common structural motif is present in the product formed by these reactions. In a separate experiment, we incubated methylglyoxal with glutathione (GSH) in the absence and presence of carnosine and monitored the consumption of GSH using thiol-specific Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) or DTNB) [122]. In the absence of carnosine, GSH was consumed due to reaction with methylglyoxal. However, addition of carnosine slowed down GSH consumption in a dose-dependent manner (Fig. 5). These results indicate that within its physiological concentration range carnosine could prevent cellular GSH from depletion by carbohydrate-derived aldehydes.

9. Mechanisms of *in vivo* action of carnosine: pre-clinical and clinical studies

As described above, increased generation of reactive oxygen and carbonyl species has been implicated in the comorbidity of numerous pathological conditions including ischemia, lung injury, diabetes, atherosclerosis, and cardiac dysfunction. A unique nucleophilic structure of carnosine allows this dipeptide to alleviate oxidative stress by scavenging singlet oxygen, hydroxyl and superoxide radicals and bind reactive aldehydes. Given this rationale, carnosine has been investigated in multiple disease states to determine potential benefits due to its antioxidant properties and its function in scavenging free radicals and aldehydes.

Carnosine has been shown to improve functional recovery of rat hearts after ischemia–reperfusion injury, where its positive effect has been attributed to its capacity to avidly intercept singlet oxygen formation generated upon post ischemic reperfusion [103]. Also, studies with the murine models of cerebral ischemia have demonstrated that pre and post-treatment with carnosine reduced infarct size when administered both before and after induction of ischemia. The reduction in the infarct size was accompanied by decreased reactive oxygen species (ROS) levels in the ischemic brain and preservation of normal glutathione levels, suggesting that antioxidant properties of carnosine are at least partly responsible for its neuroprotective effect against cerebral ischemia [123].

Administration of carnosine to obese Zucker rats attenuated the development of hypertension, weight gain and dyslipidemia, which were accompanied by reduced indices of oxidative burden, namely, reduced urinary content of oxidative stress markers 8-epi-PGF2α and AGEs, and protein–carbonyl content in kidney [124]. Zucker rats had a 6-fold higher level of carnosine-HNE adduct in urine compared to lean counterparts, suggesting that endogenous carnosine plays a role in elimination of this lipid oxidation-derived aldehyde. Despite the combined level of carnosine and histidine conjugates of HNE was 10-fold lower than the level of its glutathione-derived metabolite, 1,4-dihydroxynonane-mercapturic acid; therefore, conjugation with carnosine may play an important role in specific niches, where glutathione is unavailable. The observation prompted the authors to propose that HNE adducts with histidine-containing peptides might be biomarkers of lipid-derived carbonyl stress.

Similarly, the non-hydrolyzable and membrane-permeable analog of carnosine, octyl-D-carnosine, has also been shown to attenuate the development of atherosclerosis in apoE-null mice fed a Western diet. Supplementation with octyl-D-carnosine was associated with the reduction in protein carbonylation, circulating and tissue ALEs, expression of receptors for these products, and systemic and tissue oxidative stress. Importantly, octyl-D-carnosine feeding led to a 2.5-fold increase of CAR-HNE conjugate in the urine, indicating that the beneficial effect of carnosine could at least in part be attributed to the removal of toxic carbonyl species [120]. These studies raise an interesting possibility that treatment with

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carnosine or its analogs could prevent or retard the development of atherosclerotic lesions in humans.

On the other hand, in STZ-induced type 1 diabetes in rats, carnosine prevented podocyte loss and apoptosis of glomerular cells but failed to prevent accumulation of AGEs (N^{ϵ}-(carboxymethyl)-lysine and methylglyoxal-modified proteins) and nitrotyrosine-containing proteins in kidney cortex [125]. Similarly, oral carnosine treatment prevented retinal vascular damage after 6 months of experimental hyperglycemia; however, the protection was not accompanied by ROS- or AGE-inhibition, but associated with significant induction of Hsp27 in activated glial cells and normalization of increased Ang-2 levels in diabetic retinas [126]. These results indicate that the beneficial effects of carnosine may not be limited to its antioxidant and carbonyl-scavenging properties, but may be attributed to gene-induction effects, e.g., induction of protective Hsp27 in activated glial cells and normalization of hyperglycemia-induced Ang-2, or potentially its pro-histaminic properties.

As described in the previous sections, carnosine is hydrolyzed by carnosinase present in plasma and different tissues, and the liberated histidine may be converted to histamine [127]. Because carnosine can act a reservoir of histidine, several investigators suggested that carnosine might control glucose and lipid metabolism through the facilitation of sympathetic nervous system driven by histamine neurons through the H1 receptor activity. Whether this mechanism contributes to the effect of carnosine may be condition and tissue-dependent. Carnosine-induced protection of PC-12 cells from neurotoxicity of *N*-methyl-D-aspartate was diminished by H1 histamine receptor antagonist pyrilamine and by histidine decarboxylase inhibition [128], implicating histamine generation as part of the mechanism of the neuroprotective effect of carnosine. It has been suggested that histamine generated from endogenous carnosine released from muscle during exercise reduces sympathetic nerve activity, which leads to decrease in blood pressure and blood glucose [129]. Similarly, the protective effect of carnosine against ischemia/reperfusion-induced renal injury in rats could be blocked by histamine H_3 receptor antagonist thioperamide and corroborated by agonist R-α-methylhistamine. These observations support the hypothesis that metabolic conversion to histamine plays a significant role in the action of carnosine [130].

The data supporting the involvement of histamine as a mediator of carnosine effect are counteracted by experimental evidence showing that the pro-histaminic pathway could not be the only mechanism for the protective effect of carnosine. Anti-obeseogeneic and anti-atherogenic effects of D-carnosine (β-alanine-D-histidine, a non-hydrolyzable enantiomer) or octyl-D-carnosine, which are not precursors of histamine, argue in favor of the carbonyl quenching mechanism of action of carnosine. In addition, the pro-histaminic pathway is tissue-dependent. Indeed, carnosine is an inhibitor of histidine decarboxylase [131], and it has been shown that histamine release from mast cells is decreased after carnosine treatment [132]. Therefore, antioxidant and carbonyl-scavenging activity, as well as pro-histaminic properties may contribute to the effect of carnosine in different tissues under a variety of physiological and pathological conditions. Clearly, additional studies are necessary in order to understand the complexity and multitude of biological effects of a multifaceted peptide such as carnosine.

In a number of human and animal studies, the beneficial effects of carnosine supplementation or genetic association between polymorphisms in the carnosinase gene have been demonstrated; however, the mechanism remains unknown or incompletely understood. These include type 2 diabetes and its complications, and mental disorders, such as autism [133]. In human diabetic patients, it was found that carnosine levels were decreased in the gastrocnemius muscles of type 2, but not type 1, diabetics compared with healthy subjects [134] providing a rationale for using carnosine in the treatment or prevention of diabetic complications. Administration of local and intraperitoneal carnosine has been shown to enhance wound healing and promote granulation, increase tensile strength, collagen deposition and hyroxyproline content in the wound area in diabetic mice [135]. Supplementation of type 2 diabetic db/db mice with carnosine delayed the development of hyperglycemia, increased fasting insulin levels and preserved β-cell mass. Conversely, increased levels of carnosine-degrading enzyme carnosinase correlated with increased fasting plasma glucose, increased HbA1C levels, and lower fasting insulin levels [136].

These findings are consistent with the results of genetic association studies on the role of carnosinase in the development of diabetic complications in humans. Two independent groups have found that polymorphisms in the carnosinase gene are associated with lower serum carnosinase activity as well as the absence of diabetic nephropathy in European Caucasians, Arabs and European Americans [137,138].

In summary, carnosine supplementation has been found to be beneficial in multiple conditions in human and animal studies including diabetes and its complications, obesity, atherosclerosis, and neurodegenerative and psychological disorders. In addition, polymorphisms in the carnosinase gene (CDNP1), leading to higher enzyme activity in plasma and by inference, higher carnosine turnover, are associated with protection from diabetic nephropathy [139]. Whether these effects are due to antioxidant or aldehyde-scavenging functions of carnosine, its pro-histaminic properties, or other functions, such as regulation of gene expression is not clear in all cases. Further studies are required to connect *in vitro* observations with *in vivo* data to establish the mechanistic basis of carnosine action.

10. Conclusion

Histidine dipeptides are present in millimolar concentrations in skeletal muscle and other tissues such as brain, heart, spleen and kidney. These peptides could be involved in pH buffering in the muscle, antioxidant defense, chelation of copper ions, and scavenging of aldehydes. Antioxidant and aldehyde-scavenging properties of these peptides make them efficient agents for combating oxidative stress. Carnosine supplementation has been shown to prevent atherosclerosis, delay the development of diabetic complications and enhance wound healing. Hence, carnosine and other histidine dipeptides promise to be potentially useful therapeutic agents for conditions characterized by carbonyl overload. Several clinical and preclinical trials testing the efficacy of these compounds are under way and additional trials are warranted to assess fully the efficacy of these peptides in prevention or treatment of human diseases associated with high rates of carbohydrate metabolism or oxidative stress.

Conflict of Interest statement

The authors declare that there are no conflicts of interest.

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