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Carnosine Protects Proteins against Methylglyoxal-Mediated Modifications

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Methylglyoxal (MG) (pyruvaldehyde) is an endogenous metabolite which is present in increased concentrations in diabetics and implicated in formation of advanced glycosylation end-products (AGEs) and secondary diabetic complications. Carnosine (β-alanyl-L-histidine) is normally present in long-lived tissues at concentrations up to 20mM in humans. Previous studies showed that carnosine can protect proteins against aldehyde-containing cross-linking agents such as aldose and ketose hexose and triose sugars, and malondialdehyde, the lipid peroxidation product. Here we examine whether carnosine can protect protein exposed to MG. Our results show that carnosine readily reacts with MG thereby inhibiting MG-mediated protein modification as revealed electrophoretically. We also investigated whether carnosine could intervene when proteins were exposed to an MG-induced AGE (i.e. lysine incubated with MG). Our results show that carnosine can inhibit protein modification induced by a lysine-MG-AGE; this suggests a second intervention site for carnosine and emphasizes its potential as a possible non-toxic modulator of diabetic complications. © 1998 Academic Press

Key Words: **glycation; aldehydes; cross-linking; AGEs; ageing; diabetes; secondary complications.**

Many aldehydes react spontaneously with protein amino groups generating products that are oxidized and cross-linked. The spontaneous modification of protein by aldehydes is probably partly causal to age-related protein oxidation and cross-linking, as well as being implicated in some age-associated pathologies (1). Unlike anti-oxidant vitamins (e.g. E and C) and protective enzymes such as superoxide dismutase, catalase and peroxidase, that scavenge or otherwise eliminate reactive oxygen species (ROS), it is uncertain whether there are general aldehyde-scavengers that perform similar functions *in vivo.* However we have recently demonstrated (2-4) that the physiological dipeptide carnosine (β-alanyl-L-histidine) can protect proteins *in vitro* and cultured cells against modifications mediated by hexoses, pentoses, trioses and the highly reactive lipid peroxidation end-product malondialdehyde (MDA).

Methylglyoxal (MG) (pyruvaldehyde) is an endogenous metabolite and product of triose spontaneous oxidation and acetone and aminoacetone metabolism. MG has been implicated in secondary diabetic complications promoting formation of advanced glycosylation end-products (AGEs) following its reaction with proteins (5-11). MG readily reacts with protein lysine (via e-amino group) and arginine (via guanidino group) residues to produce high molecular weight, cross-linked, products (7,8). MG-modified proteins undergo receptor mediated endocytosis into macrophages and monocytes prior to their destruction (6,8).

As carnosine appears to react preferentially with MDA (3), and other reactive aldehydes and ketones (e.g. deoxyribose and dihydroxyacetone) (2,12) thereby protecting polypeptides *in vitro* against aldehyde-mediated modification, we suggest that carnosine would be similarly protective against methylglyoxal. The experiments presented here test this proposal and whether carnosine can protect proteins against reaction with an MG-induced AGE.

MATERIALS AND METHODS

Materials. Carnosine was a gift from Peptide Technology, Dee Why, Sydney, NSW, Australia. Methylglyoxal, lysine, ovalbumin and a-crystallin were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

Methods. Spectra of methylglyoxal following incubation with lysine or carnosine were obtained using a Cecil C500 spectrophotometer. Proteins, normally at 1mg/ml were incubated (for the time indicated in the text) in 100mM potassium phosphate buffer pH 7 at 37°C. Methylglyoxal was used at the concentration indicated in the text. Where employed, carnosine was added at the concentration indicated in the text 30 minutes prior to addition of methylglyoxal.

Electrophoresis of proteins was carried out using 4-15% "Ready-Gels" obtained from Bio-Rad Laboratories, (Hercules, CA, USA) ac-

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0006-291X/98 $25.00

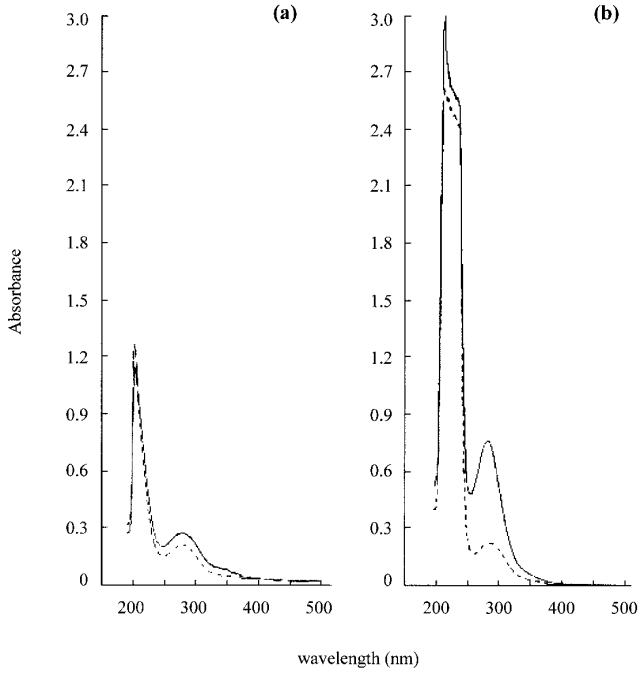

FIG. 1. The reaction of methylglyoxal with (a) lysine and (b) carnosine as revealed by spectrophotometric analysis. Methylglyoxal (5mM) was incubated with equimolar lysine (a) or carnosine (b) at pH 7 in 100mM potassium phosphate buffer for 0 (dashed line) and 2 hours (solid line).

cording to manufacturer's instructions. Both native (PAGE) and denaturing (SDS PAGE) conditions were employed. Size-exclusion chromatography chromatography of a lysine-methylglyoxal incubate was performed on a Sephadex G15 (Pharmacia) column (30×1.9cm) using 100mM phosphate buffer as eluent at 4°C.

RESULTS

Reaction of carnosine with methylglyoxal. The reaction of methylglyoxal (MG) with lysine produced a highly-coloured yellow/brown solution. The early stages of the reaction were monitored spectrophotometrically. Figure 1a shows that spectral changes around 280nm wavelength occurred within one hour. A rather larger change was observed around 280nm when carnosine was incubated with MG (Fig. 1b); the reaction mixture turned yellow/brown within two hours. This provides clear evidence for the rapid interaction between carnosine and methylglyoxal.

Protection by carnosine against methylglyoxal-mediated protein modification. MG reacts with lysine and arginine residues in proteins, which alters the charge on the modified polypeptide (7,8,9). This was demonstrated by electrophoresis of MG-treated ovalbumin under non-denaturing conditions. Figure 2 shows that exposure of ovalbumin to 10mM MG for 3 days increased the mobility of the protein toward the positive electrode (c.f. lanes 9 and 10), a change (lane 9) consistent with the loss of positive charges from ϵ-amino and guanidino groups and gain of negative charges presumably via formation of carboxyethyl derivatives (9). When carnosine was included in the incubation mixture, the presence of the dipeptide inhibited the gain in negative charge in a concentration-dependent manner; Figure 2 (lanes 2 to 8) shows that as carnosine concentration increased (5 to 500mM), the MG-induced increase in mobility diminished. The same samples were also examined electrophoretically but using denaturing conditions (SDS PAGE). Figure 3a shows that the monomeric form of MG-treated ovalbumin migrated slightly slower (lane 2) than the untreated form (lane 3). We assume this is the result of the small increase in molecular weight following the covalent attachment of MGs to the protein's lysine and arginine residues. Carnosine (10mM) prevented this change (lane 1). Using a much higher MG concentration (200mM) and 3 days incubation, additional polypeptide species migrating much slower than the original protein were observed (Fig. 3b, lane 2); we assume these represent cross-linked forms of ovalbumin. The presence of carnosine (500mM) largely prevented formation of the high molecular weight, cross-linked, protein (lane 1).

Effects of carnosine on protein modification induced by an AGE. Lysine (100mM) was incubated with equi-

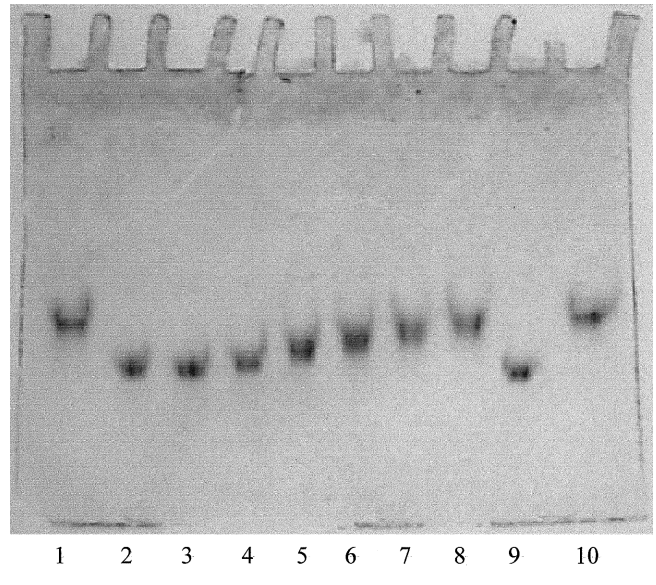

FIG. 2. The modulation of methylglyoxal-mediated protein modification by carnosine as revealed by polyacrylamide gel electrophoresis (PAGE) carried out under non-denaturing conditions. Ovalbumin (1mg/ml) was incubated with methylglyoxal (10mM) for 3 days with varying amounts of carnosine. Lane 1 ovalbumin only; lane 2 ovalbumin + MG + 5mM carnosine; lane 3 ovalbumin + MG + 10mM carnosine; lane 4 ovalbumin + MG + 20mM carnosine; lane 5 ovalbumin + MG + 50mM carnosine; lane 6 ovalbumin + MG + 100mM carnosine; lane 7 ovalbumin + MG + 200mM carnosine; lane 8 ovalbumin + MG + 500mM carnosine; lane 9 ovalbumin + MG; lane 10 ovalbumin only.

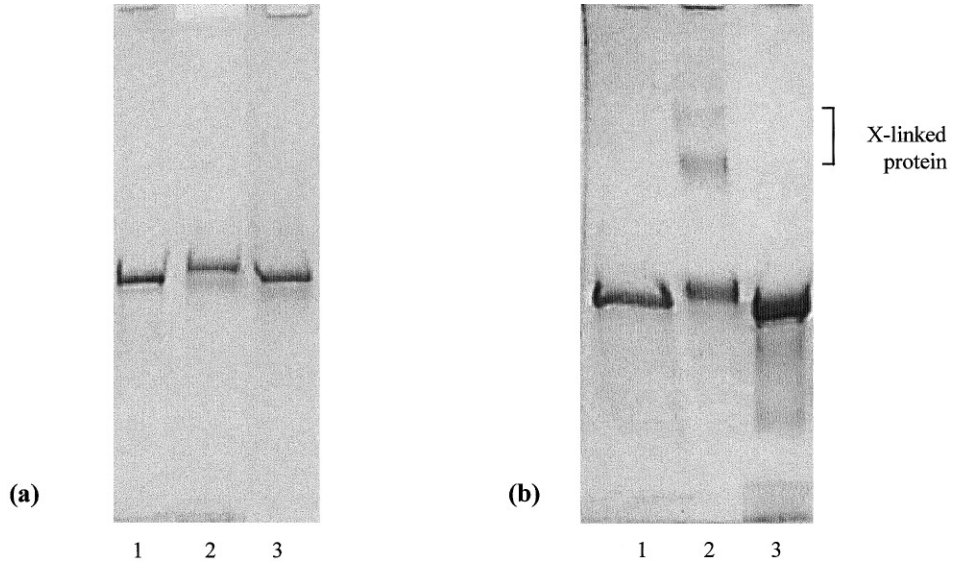

FIG. 3. The modulation of methylglyoxal-mediated protein modification as revealed by electrophoresis carried out under denaturing conditions (SDS PAGE). (a) Ovalbumin (1mg/ml) was incubated with methylglyoxal (10mM) for 5 days with/without 10mM carnosine. Lane 1 ovalbumin only; lane ovalbumin + MG; lane 3 ovalbumin + MG + carnosine. (b) Ovabumin (1mg/ml) was incubated with methylglyoxal (200mM) for 3 days with/without 500mM carnosine. Lane 1 ovalbumin only; lane 2 ovalbumin + MG; lane 3 ovalbumin + MG + carnosine.

molar MG for 19 days. The very dark brown-coloured products (presumably AGEs) were separated from MG by Sephadex G15 chromatography. The very dark material that eluted in the void volume, presumable lysine-MG-AGE, was then incubated with ovalbumin and α-crystallin for 7 days, with and without carnosine (100mM). The proteins were precipitated with 5% trichloroacetic acid and washed 3-fold. It was noted that the precipitate formed following exposure to the lysine-MG-AGE was brown, whereas if carnosine was present during the lysine-MG-AGE treatment the protein precipitate remained grey/white in colour. Upon re-dissolution of the proteins it was found that the absorbance at 450, 500 and 550nm of the lysine-MG-AGE-treated proteins was greater than the proteins exposed to the lysine-MG-AGE in the presence of carnosine (Table 1). We take this to indicate that carnosine inhibited the reaction of the proteins with the brown-coloured, MG-induced, lysine-AGE.

The lysine-MG-AGE-treated ovalbumin was also examined electrophoretically under non-denaturing conditions. Figure 4 shows that the treated protein preparations (i.e. after 7 days incubation with two separate lysine-MG-AGE fractions isolated following Sephadex G15 chromatography) was not detectable on the gel presumably because of a major change in charge (lanes 3 and 5). In contract, when the lysine-MG-AGE treatment was carried out in presence of 100mM carnosine, the protein was detectable with essentially unaltered mobility (lanes 2 and 4). SDS PAGE (i.e. electrophoresis under denaturing conditions) revealed little effect of the lysine-MG-AGE on the protein however (not shown). It should also be noted that the electrophoretic change in the protein induced by the lysine-MG-AGE was different from that observed following exposure to MG alone (c.f. Fig. 2).

Figure 4 also reveals a very rapidly migrating band (arrowed) that is present only when carnosine was added to the lysine-MG-AGE. This conceivably could be an adduct formed between carnosine and lysine-MG-AGE.

DISCUSSION

Carnosine has a long history of claims for its function. These include, physiological buffer and histidine source, anti-oxidant and free-radical scavenger, metal ion chelator, immunostimulant, anti-tumour agent and wound-healing agent (13, 14, 15,16). Our results indicate that aldehyde scavenger should also be added to the list (2,3,4,12). The dipeptide has also been shown to delay cellular senescence in cultured human fibroblasts (17), suggesting some anti-ageing activity too. Indeed as ageing is multifactorial in its causation, probably involving ROS and aldehyde-mediated macromolecular modifications, then any effective anti-ageing agent should be pluripotent in its actions, which carnosine seems to be.

During ageing, proteins become oxidized and cross-linked, modifications inducible by deleterious aldehydes ranging from glucose, fructose and deoxyribose to acetaldehyde and formaldehyde, and the lipid peroxidation products malondialdehyde and hydroxynonenal. Methylglyoxal (MG) is also thought to be promote similar protein modifications, especially associ-

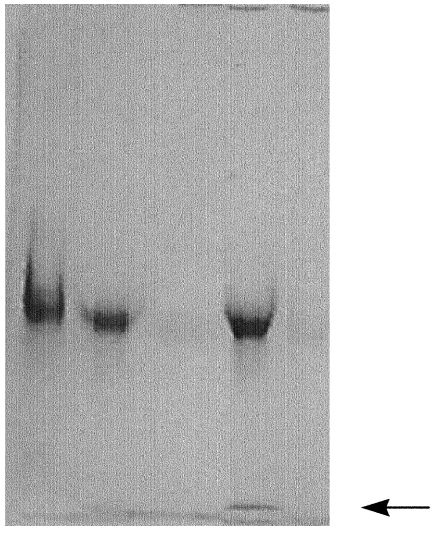

FIG. 4. The modulation of lysine-methylglyoxal-AGE-mediated protein modification by carnosine as revealed by polyacrylamide gel electrophoresis carried out under non-denaturing conditions (PAGE). Lysine (100mM) was incubated with methylglyoxal (100mM) for 19 days at 37 C. The resultant highly coloured product (putative AGE) was separated from reactants by Sephadex G15 chromatography. The first two, highly coloured, fractions (3ml) that eluted in the void volume (termed lysine-MG-AGE fractions 1 & 2) were then incubated with ovalbumin (1mg/ml) for 7 days in the presence or absence of carnosine (100mM). Lane 1 ovalbumin; lane 2 ovalbumin + lysine-MG-AGE (fraction 1) + carnosine; lane 3 ovalbumin + lysine-MG-AGE (fraction 1); lane 4 ovalbumin + lysine-MG-AGE (fraction 2) + carnosine; lane 5 ovalbumin + lysine-MG-AGE (fraction 2).

ated with secondary complications in diabetes. Our previous results have shown that carnosine readily reacts with many deleterious aldehydes (2,3,4,12,19) and the present results demonstrate that carnosine also reacts with MG.

We have previously shown (2,3,4,12,19) that carnosine protects proteins against cross-linking and oxidation induced by a range of deleterious aldehydes including aldose and ketose sugars, trioses, malondialdehyde as well as acetaldehyde and formaldehyde, presumably by the dipeptide's preferential reaction with aldehydes in general. The present results show that the dipeptide also effectively protects protein against MG-induced modification. It may not be a coincidence that the structure of carnosine resembles preferred protein glycation sites where reaction with sugar aldehydes occurs, viz. a target amino group with proximal imidazole and carboxyl groups.

Our results show that the reaction of MG with ovalbumin, and presumably other proteins too, can be separated into at least three steps. [1] Initial reaction with protein target, [2] intermolecular cross-linking of between polypeptide chains and [3] reaction of MG-AGE with an unmodified protein. Under our conditions, the MG, when present at a relatively low concentration (10mM), reacted with the protein without inducing intermolecular cross-links (Fig. 3a) as evidenced by the change in charge only. When a much higher MG concentration (200mM) was employed, evidence for cross-linked protein was obtained (Fig. 3b). It is clear that carnosine inhibited the first of these steps i.e. the initial reaction of MG with the protein. We do not know whether carnosine can also inhibit, separately, the second, cross-linking, step, although we think it possible as glycation phenomena promote the formation of protein-associated aldehydes or ketones with which, we speculate, carnosine could react.

Importantly, our results (Table 1 and Fig. 4) additionally suggest that carnosine might intervene at a third stage following sugar-mediated protein modification, namely when AGEs interact with normal, unmodified, proteins. Using a simple AGE (MG incubated with lysine) as a model compound, evidence that carnosine modulated reaction of the AGE with two proteins was obtained. Visual inspection, spectrophotometric analysis and electrophoretic separation showed that carnosine inhibited the lysine-MG-AGE-induced change in colour and electrophoretic mobility of the treated proteins. We do not know how the carnosine-mediated protection occurs, although direct reaction of the lysine-MG-AGE with carnosine is the most likely explanation. Furthermore we do not know whether carnosine is protective against AGEs generally. However, preliminary experiments have shown that carnosine protects cultured rat brain endothelial cells

TABLE 1

The Effect of Carnosine on the Lysine-MG-AGE-Induced Change in Spectrophotometric Properties of Ovalbumin and α-Crystallin

	Absorbance at		
	450 nm	500 nm	550 nm
Ovalbumin + lysine-MG-AGE	0.024	0.019	0.013
Ovalbumin + lysine-MG-AGE + carnosine	0.007	0.007	0.007
α-Crystallin + lysine-MG-AGE	0.077	0.053	0.037
α-Crystallin + lysine-MG-AGE + carnosine	0.014	0.010	0.007

Note. Lysine (100 mM) was incubated with MG (100 mM) for 19 days at 37°C in pH 7 phosphate buffer. The incubate (2 ml) was passed down a Sephadex G15 column and the most highly-coloured fraction (3 ml) that eluted in the void volume region was used (300 μl) to treat ovalbumin (0.5 mg) and α-crystallin (0.5 mg) (7 days at 37°C) in the presence or absence of carnosine (100 mM); 10 μl of 1% sodium azide solution was also added. The proteins were precipitated, washed thrice, redissolved in 1 ml of phosphate buffer and the absorbance at 450, 500 and 550 nm measured spectrophotometrically. It should be noted the colour of the thrice-washed precipitated proteins were clearly different to the naked eye; those treated with the lysine-MG-AGE in the absence of carnosine were brown in colour, whereas when carnosine also present during the treatment the precipitate was grey/white in colour.

against a bovine serum albumin-AGE (18), and that the dipeptide also protects Chinese hamster ovary (CHO) cells against lysine-deoxyribose-AGE toxicity (19). Again we do not know how carnosine protects these cells against the AGEs, but direct interaction between the dipeptide and the AGEs is one possibility. This is consistent with the present observation of a possible adduct formed between carnosine and the lysine-MG-AGE when incubated together (Fig. 4) and the reported antigenic similarity between AGEs induced by MG, glucose, fructose, ribose and glyceraldehyde (20).

Whilst these proposals are yet to be developed by, for example, characterizing putative carnosine-lysine/protein/peptide-AGE adducts, they do indicate the potential for carnosine (which is almost non-toxic) and related structures for modulating the secondary complications of diabetes and other pathologies where AGEs and their receptors (RAGEs) might be important. Protein-AGEs are well known as mediators of a number of pathological conditions associated with persistent hyperglycaemia (e.g. nephropathy, peripheral neuropathy, atherosclerosis, cataracts and basement membrane thickening) (5-11,20). Animal/human feeding experiments might reveal beneficial effects of carnosine, or structures resistant to serum carnosinase such as carcinine or acetyl-carnosine, with respect to pathologies associated with secondary diabetic complications. It may also be significant that in Alzheimer's disease the toxicity of the amyloid peptide aggregate requires the participation of an AGE receptor (RAGE) (21); our recent studies show that carnosine and related structures can modulate the toxicity of the β-amyloid peptide fragment [25-35] towards cultured cells (22).

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