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Protective effects of carnosine against malondialdehyde-induced toxicity towards cultured rat brain endothelial cells

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Abstract

Malondialdehyde (MDA) is a deleterious end-product of lipid peroxidation. The naturally-occurring dipeptide carnosine (β-alanyl-L-histidine) is found in brain and innervated tissues at concentrations up to 20 mM. Recent studies have shown that carnosine can protect proteins against cross-linking mediated by aldehyde-containing sugars and glycolytic intermediates. Here we have investigated whether carnosine is protective against malondialdehyde-induced protein damage and cellular toxicity. The results show that carnosine can (1) protect cultured rat brain endothelial cells against MDA-induced toxicity and (2) inhibit MDA-induced protein modification (formation of cross-links and carbonyl groups). © 1997 Elsevier Science Ireland Ltd.

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Oxygen free-radicals promote the formation of a number of species that are highly deleterious to cellular macromolecules. Reactive oxygen species (ROS) have been postulated to be involved in ageing in general [5] as well as various human pathologies such as inflammatory joint diseases, atherosclerosis, diabetes [4] and Alzheimer's disease (AD) [19–21]. An end-product of ROS-induced peroxidation is malondialdehyde (MDA), itself potentially damaging to polypeptides and known to induce cross-links in protein [12]. We have previously observed [8,9] that the dipeptide carnosine can react preferentially with aldehydes and thereby protect proteins against aldehyde and ketone-containing glycating (cross-linking) agents such as fructose, deoxyribose, dihydroxyacetone and glyceraldehyde-3-phosphate. This suggests that carnosine might be protective against MDA.

Carnosine (β-alanyl-L-histidine) is found in innervated tissues, cerebrospinal fluid and the lens, sometimes at high concentrations (up to 20 mM) (for review see [2]). The proper function of this dipeptide is uncertain although many properties have been proposed including physiological buffer, wound healing agent, radioprotectant, anti-oxidant, free-radical scavenger, metal ion chelator, immunomodulator, anti-tumour agent [2] and anti-ageing compound [7,14]. This study investigates whether carnosine protects against MDA-mediated cellular and molecular damage.

Rat brain endothelial cells (RBE4), obtained from Dr. P.O. Courand (INSERM, Paris, France), were cultured as described [17]. Briefly, cells at passage 52–58 were grown to confluence in 24-well collagen-coated plates in 1 ml culture medium supplemented with 5% foetal bovine serum. The effect of MDA, prepared as described [12], was examined following its addition to confluent RBE4 cell cultures. Cell viability was determined after 24 h by assaying glucose consumption [1], intracellular lactate dehydrogenase (LDH) [23] and mitochondrial dehydrogenase function as measured by reduction of 3-[4,5-dimethylthiazol-2-yl]–2,5-diphenyltetrazolium bromide (MTT) as described [3].

Table 1 shows that MDA was toxic as indicated by

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Table 1
Effects of 2 mM MDA on cultured rat brain endothelial cells

	Control cells	cells + 2mM MDA	% Inhibition
Glucose consumption μmoles/24 h	5.59 ± 0.045	3.52 ± 0.21	37.0
Intracellular LDH μmoles/ml/min	0.657 ± 0.044	0.344 ± 0.028	47.6
MTT % control	99.9 ± 1.5	27.6 ± 1.9	72.4

Cells were cultured as described [17]. MDA (2 mM) was added to confluent cell cultures for 24 h. Glucose utilisation, cell associated LDH and mitochondria1 activities by MTT assay were measured as described [1,3,23], respectively. The presence of MDA in the culture medium prevented measurement of extracellular LDH activity. Data are the mean ± SEM (n = 16–32).

decreased glucose uptake, loss of cellular LDH and decreased mitochondrial enzyme activity. Within 24 h of the addition of the highest concentrations of MDA (4 mM) most of the cells were detached from the culture vessel surface as seen by phase-contrast microscopy (not shown).

Fig. 1 demonstrates that the effect of MDA on mitochondrial function was dose dependent; the ability to reduce MTT declined by more than 95% after addition of 3 mM MDA, while 2 mM MDA produced a 73% decline and inhibition at 1 mM MDA was more modest. Carnosine (20 mM) was protective at all MDA concentrations employed, up to 4 mM, and provided at least 50% protection (Fig. 1). Carnosine protection was concentration-dependent as measured by all three assays (Fig. 2a,b) and 20 mM carnosine provided 70–90% protection against the cellular effects of 2 mM MDA. Residual MDA in the growth medium did not inhibit the glucose assay; increased quantities of the sugar were detected in the extracellular fluid following MDA treatment (indicating decreased utilisation). Carnosine did not appear to affect glucose consumption (Fig. 2a). Given the reactivity of MDA towards proteins, etc. we did not anticipate that much of it would remain free within the cells after 24 h exposure. Nevertheless, the cells were washed prior to assay of the cell-associated activities to eliminate the remote possibility that residual MDA interference.

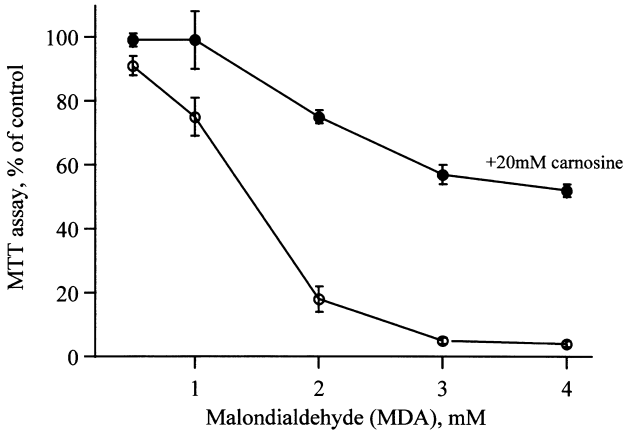

Fig. 1. Effects of MDA (0–4 mM) on cultured rat brain endothelial cell viability as measured by mitochondrial activity (MTT assay): effect of 20 mM carnosine. Cells were cultured and assayed for mitochondrial function after 24 h (see legend to Table 1) in the presence of 0–4 mM extracellular MDA. Carnosine (20 mM) was added to parallel wells 2 h prior to addition of MDA (0–4 mM). Data are the mean ± SEM (n = 3–15).

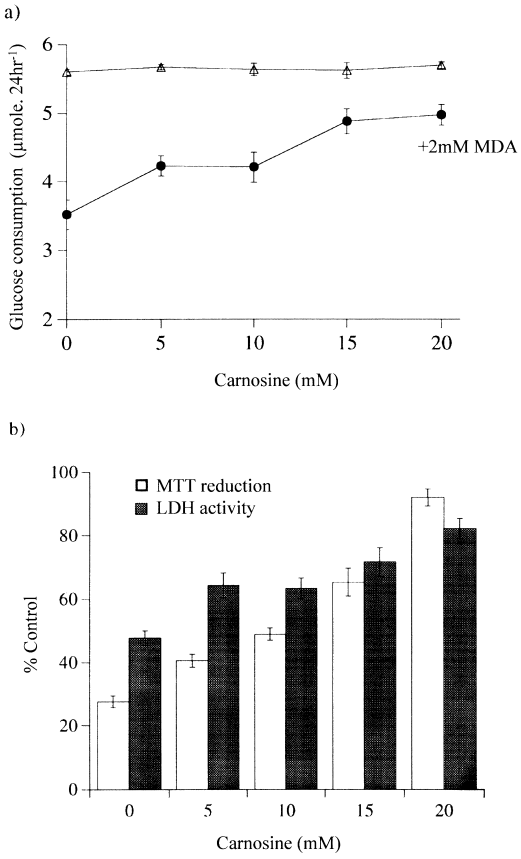

Fig. 2. Effects of carnosine on 2 mM MDA toxicity in cultured rat brain endothelial cells as measured by (a) glucose utilisation and (b) cell associated LDH and mitochondrial activity (MTT assay). Cells were cultured as described in the text. Carnosine (0–20 mM) was added to the cells. After 2 h MDA at 2 mM was added to the cells. Twenty-four hours later the cells were assayed for glucose utilisation, lactate dehydrogenase activity and mitochondrial activity as described in the text. Data are the mean ± SEM (n = 11–32).

We cannot be certain how MDA-mediated cellular toxicity is caused but deleterious interaction with proteins is one possibility. Using a model system, we found that MDA modified α-crystallin in at least two ways, (1) by induction of protein-protein cross-links, as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [8], and (2) by protein oxidation as shown by formation of carbonyl groups measured by their reaction with 2,4-dinitrophenylhydrazine [6] (Fig. 3a,b). Both modifications were inhibited by carnosine (Fig. 3a,b). Despite the high concentrations of MDA employed for this study (up to 25 times the maximum used in the cell experiments) we found that carnosine was protective at concentrations only 5–10 times those added to the cells. These results suggest

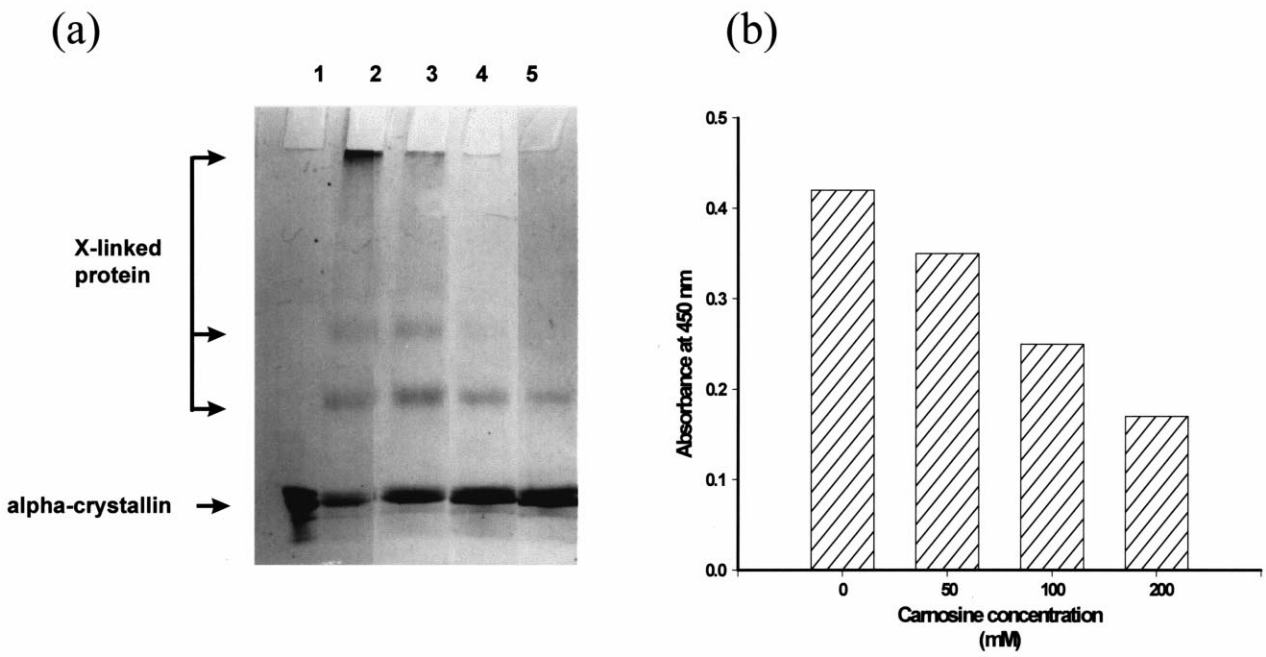

Fig. 3. Effects of carnosine on MDA-induced protein modification (a) cross-linking and (b) oxidation (formation of carbonyl groups). α-Crystallin (1 mg/ml) was treated with 100 mM MDA for 2 h in 100 mM phosphate buffer (pH 7) at 37°C. Where present, carnosine was added 30 min prior to addition of MDA. (a) Protein cross-linking was determined by SDS-PAGE as described [8]. From left to right each lane contained the following: lane 1, crystallin only; lane 2, crystallin + MDA; lane 3, crystallin + MDA + 50 mM carnosine; lane 4, crystallin + MDA + 100 mM carnosine; lane 5, crystallin + MDA + 200 mM carnosine. (b) Protein oxidation was determined by assaying for carbonyl groups (absorbance at 450 nm) following their reaction with 2,4-dinitrophenylhydrazine as described [6].

that one way that carnosine might protect endothelial cells is simply by preferential reaction with the MDA to spare cellular macromolecules, and previous studies have shown that carnosine reacts readily with other aldehydes [8]. We do not yet know anything amount the stoichiometry and kinetics of the carnosine-mediated protection, or whether prior exposure of MDA to carnosine eliminates toxicity as anticipated.

The present experiments suggest that the protective actions of carnosine and related dipeptides should be investigated further. We have recently shown that carnosine protects cultured rat brain endothelial cells against the toxic effects of an amyloid peptide [16]. The mechanisms remain to be elucidated, but an involvement of ROS [13] culminating in generation of deleterious aldehydes such as 4-hydroxynonenal and MDA which are scavenged by carnosine rather than reacting with cellular macromolecules is one possibility. Alternative or additional explanations including interference with amyloid peptide binding to receptors, stimulating peptide catabolism, effects on second messenger processes and scavenging superoxide or hydroxide radicals are not excluded. Nevertheless, the present results show that at least in our model system carnosine can prevent MDA-induced protein modifications (carbonyl groups and cross-links).

Accumulation of oxidatively-damaged and glycated proteins (as evidenced by the presence of carbonyl groups, cross-links and advanced glycosylation end-products) occurs during normal ageing [22] and in increased quantities during AD [20,21] (it is disputed whether oxidatively-damaged DNA accumulates in AD [11,13]). A number of observations suggest that carnosine is protective against age-related macromolecular damage via mechanisms additional to the dipeptide's direct involvement with oxygen free-radicals and their generation [2]. For example the amino and imidazole groups of lysine and histidine residues respectively are the most susceptible to oxidation during protein ageing [18]; carnosine possesses both targets. Similarly, carnosine may be an endogenous anti-glycating agent as it resembles preferred protein glycation sites [8,9]. Carnosine can delay senescence [14] and decrease DNA oxidation [10] in cultured human fibroblasts. The present results showing that carnosine intervenes against a major lipid peroxidation product (MDA) by modulating its toxicity and inhibiting protein damage provide additional evidence for the dipeptide's potential as a pluripotent protective agent.

Whether carnosine is protective in vivo remains to be determined, but declining lenticular carnosine levels are associated with increasingly severe cataracts containing insoluble protein modified by glucose and/or ROS [2]. Carnosine and related peptides are present (sometimes in mM amounts) in the brain, innervated muscle and the lens, and while it is not known whether carnosine concentrations change with age, a decline in CSF homocarnosine has been reported [15].

We propose that the possible relationship between carnosine, deleterious aldehydes, ageing and age-related pathologies might be worthy of further investigation, together with the potential for therapeutic activity of carnosine and related

structures (or agents that regulate the dipeptide's synthesis or degradation) with respect to pathologies that result from aldehyde-mediated macromolecular modification

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[1] Bergmeyer, H.U., Bernt, E., Schmidt, F. and Sork, H., D-glucose: determination with heoxikinase and glucose-6-phosphate dehydrogenase. In H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, Verlag Chemie, Academic Press, New York, 1974, pp. 1196–1201.
[2] Boldyrev, A.A., Formazyuls, V.E. and Sergienko, V.I., Biological significance of histidine-containing dipeptides with special reference to carnosine: chemistry, distribution, metabolism and medical application, Sov. Sci. Rev. D. Physicochem. Biol., 13 (1994) 1–60.
[3] Carmichael, J., DeGraff, W.G., Gazder, A.F., Minna, J.D. and Mitchell, J.B., Evaluation of a tetrazolium based semi-automatic colorimetic assay: assessment of radiosensitivity, Cancer Res., 47 (1987) 943–946.
[4] Halliwell, B. and Gutteridge, J.M.C., Free Radicals in Biology and Medicine, Clarenden Press, Oxford, UK, 1989.
[5] Harman, D., Aging – A Theory Based on Free Radical and Information Theory, UCRL publication 3078, University of California, 1955.
[6] Hazell, L.J., van den Berg, J.J.M. and Stocker, R., Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation, Biochem. J., 302 (1994) 297–304.
[7] Hipkiss, A.R., Holliday, R., McFarland, G. and Michaelis, J., Carnosine and senescence, Lifespan, 4 (1993) 1–3.
[8] Hipkiss, A.R., Michaelis, J. and Syrris, P., Non-enzymic glycosylation of the dipeptide L-carnosine, a potential anti-protein-cross-linking agent, FEBS Lett., 371 (1995) 81–85.
[9] Hipkiss, A.R., Michaelis, J., Syrris, P. and Dreimanis, M., Strategies for the extension of human life span, Perspect. Hum. Biol., 1 (1995) 59–70.
[10] Kantha, S.S., Wada, S., Tanaka, H., Takeuchi, M., Watabe, S. and Ochi, H., Carnosine sustains the retention of cell morphology in continuous fibroblast culture subjected to nutritional insult, Biochem. Biophys. Res. Commun., 223 (1996) 278–282.
[11] Koppele, J.M.T., Lucassen, P.J., Sakkee, A.N., Vanasten, J.G., Ravid, R., Swaab, D.F. and Vanbezooijen, C.F.A., 8-OH-deoxyguanosine levels in brain do not indicate oxidative DNA damage in Alzheimer's disease, Neurobiol. Aging, 17 (1996) 819–826.
[12] Libondi, T., Ragone, R., Vincenli, D., Stiuso, P., Auricchio, G. and Collona, G., In vitro cross-linking of calf lens alpha-crystallin by malondialdehyde, Int. J. Peptide Protein Res., 44 (1994) 342–347.
[13] Markesbery, W.R., Oxidative stress hypothesis in Alzheimer's disease, Free Radical Biol. Med., 23 (1997) 134–147.
[14] McFarland, G.A. and Holliday, R., Retardation of the senescence of cultured human fibroblasts by carnosine, Exp. Cell Res., 212 (1994) 167–175.
[15] Perry, T.L., Hansen, S., Stedman, D. and Love, D., Homocarnosine in human cerebrospinal fluid: an age-dependent phenomenon, J. Neurochem., 15 (1968) 1203–1206.
[16] Preston, J.E., Hipkiss, A.R., Himsworth, D.T.J., Ignacio, R. and Abbot, N.J., Effect of beta-amyloid (25–35) on rat brain endothelial cell line (RENE4): protection by carnosine, (submitted).
[17] Roux, F., Durieu-Trautrnann, A., Chaverot, N., Claire, M., Mailly, P., Bourre, J.-M., Strosberg, A.D. and Couroud, P.-O., Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalised rat brain microvessel endothelial cells, J. Cell Physiol., 159 (1994) 101–113.
[18] Santa Maria, C., Revilla, E., Ayala, A., de la Cruz, C.P. and Machado, A., Changes in histidine residues in Cu/Zn superoxide dismutase during aging, FEBS Lett., 374 (1995) 85–88.
[19] Schubert, D., Behl, C., Lesley, R., Brack, A., Dargusch, R., Sagara, Y. and Kimua, H., Amyloid peptides are toxic via a common oxidative mechanism, Proc. Natl. Acad. Sci. USA, 92 (1995) 1989–1993.
[20] Smith, M.A., Perry, G., Richey, P.L., Sayre, L.M., Anderson, V.E., Beal, M.F. and Kowall, N., Oxidative damage in Alzheimer's, Nature, 382 (1996) 120–121.
[21] Smith, M.A., Rudnickanawrot, M., Richey, P.L., Praprotnik, D., Mulvihill, P., Miller, C.A., Sayre, L.M. and Perry, G., Carbonyl-related post-translational modification of neurofilament protein in neurofibrillary pathology in Alzheimer's disease, J. Neurochem., 64 (1995) 2660–2666.
[22] Stadtman, E.R., Protein oxidation and aging, Science, 257 (1992) 1220–1224.
[23] Vassault, A., Lactate dehydrogenase: UV-method with pyruvate and NADH. In H.U. Bergmeyer, J. Bergmeyer, and M. Grassi (Eds.), Methods of Enzymatic Analysis, Vol. III, Verlag Chemie, Weinheim, 1983, pp. 118–126.