Carnosine and its constituents inhibit glycation of low-density lipoproteins that promotes foam cell formation in vitro

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Abstract Glycation of low-density lipoprotein (LDL) by reactive aldehydes, such as glycolaldehyde, can result in the cellular accumulation of cholesterol in macrophages. In this study, it is shown that carnosine, or its constituent amino acids \(\beta\)-alanine and L-histidine, can inhibit the modification of LDL by glycolaldehyde when present at equimolar concentrations to the modifying agent. This protective effect was accompanied by inhibition of cholesterol and cholesteryl ester accumulation in human monocyte-derived macrophages incubated with the glycated LDL. Thus, carnosine and its constituent amino acids may have therapeutic potential in preventing diabetes-induced atherosclerosis.

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1. Introduction

People with diabetes have a 2-fold or greater risk of cardiovascular disease compared to control populations [1], with diabetes identified as an independent risk factor for cardiovascular-associated mortality [2]. There is convincing evidence that the hyperglycaemia characteristic of diabetes contributes to the increased incidence of atherosclerosis. In the recent follow up to the DCCT [3], it was shown that a period of intensive diabetes therapy directed towards attaining normoglycaemia was associated a significant reduction in cardiovascular disease risk. A significant reduction in Hb\textsubscript{A1c} was identified as a principal contributor to the reduction, although notably the levels achieved were still the above current targets of \(<7\%\). Thus, the development of therapies targeted at hyperglycaemia-induced damage remains a key treatment objective.

Glycation of proteins involves non-enzymatic reaction of a sugar, or reactive aldehyde derived from these, with a nucleophilic group on the protein. The rate and extent of reaction is dependent on the nature, concentration, and duration of exposure to the modifying species. Aldehydes (e.g. methylglyoxal, glyoxal and glycolaldehyde), formed via glucose autoxidation, amino acid and lipid oxidation, and cellular metabolism, are particularly potent glycating agents [4]. The plasma levels of these aldehydes are increased in people with diabetes [5–7]. Glycation of amino acids leads to the generation of a heterogeneous group of adducts known as advanced glycation end products (AGE) [8]. Glucose and other aldehydes, both free and protein-bound, can also undergo autoxidation (glycoxidation) reactions that contribute to AGE formation [8,9].

Glycation reactions have been linked with the development of diabetes-associated cardiovascular diseases [10]. We have shown that ex vivo glycation of low-density lipoprotein (LDL) induces intracellular cholesterol accumulation in macrophages [11,12]. Such cholesterol-laden “foam cells” are a hallmark of atherosclerosis and play a critical role in disease development [13]. We therefore hypothesised that compounds that inhibit protein glycation may prevent foam cell generation and thus the development of diabetes-associated atherosclerosis.

Carnosine (\(\beta\)-alanyl-L-histidine) is an endogenous dipeptide that can also be derived from the diet [14] or supplements [15]. It reacts rapidly with aldehydes, and can detoxify aldehyde-modified proteins (reviewed [16,17]). Recently, Janssen and colleagues [18] have identified an allele of the \(CDNPI\) gene for serum carnosinase, which hydrolyses carnosine, associated with both a reduced serum enzyme activity and a reduced susceptibility to diabetic nephropathy. These findings support a protective role for carnosine in the complications of diabetes.

The aim of the present study was to examine whether carnosine and its constituents inhibit LDL modification induced by glycolaldehyde, and whether this decreases the in vitro accumulation of cholesterol and cholesterol esters in human monocyte-derived macrophages (HMDM).

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources. Sigma-Aldrich (Castle Hill, NSW): \(\beta\)-alanine (\(\beta\)-Ala), BSA (fatty acid-free), carnosine, EDTA, glycolaldehyde, Hanks Balanced Salt Solution (with phenol red and 0.01% EDTA, but without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}), histidine (His), PenStrep; Bio-Rad (Regents Park, NSW): Chelex-100 resin; Amer-sham Biosciences (Castle Hill, NSW): PD10 columns; Helena Laboratories (Mt. Waverly, Vic): pre-cast 1% agarose gels. Axis-Shield (Oslo, Norway): Lymphoprep; JRH Biosciences/CSL (North Ryde, NSW) Trace Scientific (Melbourne, Vic): L-glutamine. All other chemicals were of analytical grade, and all solvents were of HPLC grade.
2.2. LDL modifications
LDL was isolated from normoglycaemic and normolipidaemic volunteers [12] and glycated with 10 mM glycolaldehyde under conditions previously described [12,19]. Incubation controls contained 50 μM EDTA in place of glycolaldehyde. Carnosine, His, β-Ala or His + β-Ala (all 10 mM) were added, prior to glycolaldehyde, and were present throughout the modification period. The added reagents (aldehydes or EDTA, carnosine, β-Ala or histidine) were removed, by PD10 chromatography, before addition of the modified LDL to cells.

2.3. Assessment of modification of LDL
Changes in overall LDL particle charge were quantified by electrophoretic mobility on agarose gels [12].

2.4. Isolation and culture of human monocyte-derived macrophages (HMDM)
Monocytes were isolated by counter-current elutriation from white cell concentrates [20], then added (1 × 10⁶/mL) to 12-well plates (Costar, Corning, NY) in serum-free RPMI 1640, and left to adhere for 1–2 h. The cells were then washed and incubated (5% CO₂ and 37 °C) for 2 h. The cells were then washed and incubated (5% CO₂ and 37 °C) in RPMI for 9–11 days to give HMDM [20].

2.5. Cellular cholesterol-loading studies
HMDM were exposed to modified or native LDL (100 μg/mL in media containing 10% lipoprotein-deficient serum [21]). After 48 h, cell media samples were collected, the cells washed with PBS, and lysed in water. Cholesterol and cholesteryl ester concentrations were quantified using the Pierce (Rockford, IL, USA) bicinchoninic acid (BCA) assay [12]. Cell viability was determined by lactate dehydrogenase release [12].

2.6. Protein assay
Protein concentrations were quantified using the Pierce (Rockford, IL, USA) bicinchoninic acid (BCA) assay [12].

2.7. Data analysis
Data was performed using one-way analysis of variance using the Newman–Keuls multiple comparison test. P ≤ 0.05 was taken as significant.

3. Results

3.1. Characterisation and inhibition of LDL modification
Incubation of LDL with 10 mM glycolaldehyde resulted in a significant increase in relative electrophoretic mobility (REM), and thus an overall decrease in positive charge (Table 1). These incubation conditions do not lead to a significant oxidation of the LDL as determined by consumption of α-tocopherol, cholesterol or cholesteryl esters, or formation of lipid or protein oxidation products [11,12,19]. The presence of equimolar concentrations of carnosine (CN), His, or His + β-Ala, but not β-Ala alone, decreased particle modification with the efficacy of these agents decreasing in the order CN > His + β-Ala > His > β-Ala.

### Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>REM (n = 4–5)</th>
<th>S.D.</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation control (EDTA)</td>
<td>1.5 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM glycolaldehyde (GA)</td>
<td>4.3 ± 0.2*</td>
<td></td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>GA + 10 mM carnosine</td>
<td>3.1 ± 0.4**</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GA + 10 mM histidine</td>
<td>3.7 ± 0.4**</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GA + 10 mM β-alanine</td>
<td>4.8 ± 0.4**</td>
<td></td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GA + 10 mM histidine + 10 mM β-alanine</td>
<td>3.4 ± 0.7**</td>
<td></td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Data (n = 4–5) are means ± S.D. *P < 0.05 compared with incubation control. **P < 0.01 compared with incubation control.

3.2. Characterisation and inhibition of foam cell formation
Incubation of HMDM with native or modified LDL particles did not significantly affect cell viability or cell protein levels (data not shown). With HMDM incubated for 48 h in the absence of LDL, the total cholesterol levels have been determined as being between 64 and 74 nmoles/mg cell protein with less than 3% present as cholesteryl esters [31]. Exposure of HMDM to 100 μg/mL glycolaldehyde-modified LDL for 48 h, resulted in a significant accumulation of cellular cholesterol and cholesteryl esters; the latter account for ca. 50% of the measured sterol (Fig. 1). The presence of each of the potential inhibitors during the LDL modification phase significantly inhibited subsequent cholesterol and cholesteryl ester accumulation by the HMDMs on exposure to the modified LDL particles (Fig. 1). There were no significant differences between the...
different inhibitor treatments, though the greatest decrease in cholesterol ester levels was observed with CN. Carnosine, unlike the other treatments, reduced the percentage of cholesterol esters present in the cells exposed to glycoaldehyde-modified LDL, to levels that were not significantly different to the controls (cells exposed to non-modified LDL).

4. Discussion

Previous studies have shown that ex vivo treatment of LDL with reactive aldehydes such as methylglyoxal and glycoaldehyde results in LDL modifications that result in these particles being taken up, in an unregulated manner, by both murine and human macrophages, with a resultant intracellular accumulation of cholesterol and cholesteryl esters [11,12,31]. A number of hydrazine compounds can inhibit these effects of glycoaldehyde and methylglyoxal, at concentrations less than or equal to that of the modifying aldehyde [11]. In the current study, we investigated whether the dipeptide carnosine, which is present in many diets and can be safely taken as a supplement, is effective in preventing cellular cholesterol and cholesteryl esters in human macrophages.

Carnosine efficiently protects LDL against modification, as demonstrated by the abrogation of the change of overall particle charge induced by glycoaldehyde. This decrease in LDL modification results in complete protection against the proatherogenic (promotion of intracellular cholesterol and cholesteryl ester accumulation) effects of the aldehyde-modified LDL; this is in accord with the data obtained with other carboxyl scavengers such as hydrazines [11]. Carnosine has been reported previously to both react with aldehydes, and detoxify aldehyde-modified proteins (reviewed in [16,17]). Recently, it has also been shown [22] that low micromolar concentrations of carnosine (and one of its constituents, histidine) can inhibit LDL glycation induced by 6-day exposure to 100 mM glucose.

In plasma, carnosine is rapidly hydrolysed by carnosinase [23] to its constituent amino acids, His and β-Ala; we therefore compared the effects of carnosine with these amino acids. All three compounds were effective in preventing cellular cholesterol and cholesteryl ester accumulation induced by glycoaldehyde-modified LDL in vitro. The effectiveness of both carnosine and its constituents is of interest in the light of the action of carnosinase, and contrasts with previous ex vivo studies. Thus, His and β-Ala have been reported to have negligible carboxyl scavenging activity over a 5 h period [17,24–26]. However, over 24 h, His but not β-Ala prevented modification [27]. Over even longer periods (2–17 days), all three compounds afforded significant protection against aldehyde-mediated protein cross-linking and loss of enzyme activity [26,28]. The last of these studies is in agreement with the results obtained here. One interpretation of this apparently conflicting data is that carnosine reacts more rapidly with the modifying aldehydes than His and β-Ala, but over longer periods these differences become insignificant. Thus, both carnosine and its constituents may offer protection against protein glycation/glycoxidation, if the rate of modification is slow. This has potential biological ramifications for the protection of short-lived proteins against modification by highly reactive aldehydes, where only carnosine may be effective, whereas each of these components may offer protection against slower glycation reactions (e.g. those induced by glucose).

Interestingly, whilst all three compounds significantly inhibited cholesterol and cholesterol ester accumulation, β-Ala did not induce a significant reduction in REM. The greater shift in REM observed with β-Ala compared with the other inhibitor treatments may represent the formation of an adduct that contributes to charge modification. Notably, the REM values observed for all the treatments were still significantly higher than for the incubation control. Similar behavior has been observed with hydrazines [11]. These observations suggest that complete inhibition of lipid loading can be achieved without complete prevention of LDL modification, and that all that is required to prevent lipid loading is reduction of damage below a key threshold related to modifications of key residues on apolipoprotein B-100 [29]. This has potentially important therapeutic ramifications.

The concentration of glycoaldehyde used in this study is higher than that detected in vivo, with experimental values for plasma varying from submicromolar to hundreds of micromolar, depending on the technique employed [5–7]. However, irrespective of the absolute levels of these aldehydes, the key finding of the current study is that carnosine and its constituents are effective at equimolar concentrations to the modifying agent. As plasma levels of up to hundreds of micromolar have been detected in people taking oral doses of carnosine (as a supplement or in food or drink) [14,30], our data suggests that carnosine and its constituents may serve as effective scavengers of carbonyl compounds and inhibitors of protein glycation in vivo, and as potential therapeutic agents to inhibit diabetes-induced atherosclerosis.

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References