

Hydrazine compounds inhibit glycation of low-density lipoproteins and prevent the in vitro formation of model foam cells from glycolaldehyde-modified low-density lipoproteins.

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Running Title- B. E. Brown et al.: Prevention of glycation and cell lipid loading

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None of the authors have any commercial interests with any companies that might constitute a conflict of interest with regard to the data reported in this manuscript.

Word count for abstract: 232

Word count for main body of text: 3932

Abstract

Aims/hypothesis: Previous studies have shown that glycation of low-density lipoproteins (LDL) by methylglyoxal and glycolaldehyde, in the absence of significant oxidation, results in lipid accumulation in macrophage cells. Such “foam cells” are a hallmark of atherosclerosis. In this study we examined whether LDL glycation by methylglyoxal or glycolaldehyde, and subsequent lipid loading of cells, can be inhibited by agents that scavenge reactive carbonyls; such compounds may have therapeutic potential in diabetes-associated atherosclerosis. *Methods:* LDL was glycated with methylglyoxal or glycolaldehyde in the absence or presence of metformin, aminoguanidine, Girard’s reagents P and T or hydralazine. LDL modification was characterised by changes in mobility (agarose gel electrophoresis), cross-linking (SDS-PAGE) and loss of amino acid residues (HPLC). Accumulation of cholesterol and cholesteryl esters in murine macrophages was assessed by HPLC. *Results:* Inhibition of LDL glycation was detected with equimolar or greater concentrations of the scavengers over the reactive carbonyl. This inhibition was structure dependent and accompanied by a modulation of cholesterol and cholesteryl ester accumulation. With aminoguanidine, Girard’s reagent P and hydralazine, cellular sterol levels were returned to control levels, despite incomplete inhibition of LDL modification.

Conclusions/interpretation: Inhibition of LDL glycation by interception of the reactive aldehydes that induce LDL modification prevents lipid-loading and model foam cell formation in murine macrophage cells. Carbonyl-scavenging reagents, such as hydrazines, may therefore be of use in inhibiting LDL glycation *in vivo* and the prevention of diabetes-induced atherosclerosis.

Keywords Atherosclerosis · low-density lipoproteins · glycation · hydrazines · macrophages · foam cells · apolipoprotein B · cholesterol esters · aldehydes · protein modification.

Abbreviations ApoB: apolipoprotein B-100 · AG: aminoguanidine · GA: glycolaldehyde · GP: Girard's reagent P (1-(carboxy)pyridinium chloride hydrazide) · GT: Girard's reagent T ((carboxymethyl)trimethylammonium chloride hydrazide) · H: hydralazine · LDH: lactate dehydrogenase · LPDS: lipoprotein-deficient serum · MF: metformin · MG: methylglyoxal · OPA: *o*-phthaldialdehyde · REM: relative electrophoretic mobility

Introduction

Diabetic complications are the major cause of mortality and morbidity in patients with diabetes. These complications affect both the micro- and macro-vascular systems, with the former inducing damage to the retina, nephrons and peripheral nerves, whereas macrovascular disease is associated with accelerated atherosclerosis in the coronary, peripheral and carotid arteries [1]. Cardiovascular disease is responsible for approximately 50% of deaths in people with diabetes [2].

A number of features of diabetes mellitus have been proposed as contributing factors to atherosclerosis, including chronic hyperglycaemia and insulin resistance, dyslipidaemias, and abnormalities of haemostasis [3, 4]. Macrovascular disease appears in most patients with type 2 diabetes at, or near, the time of first diagnosis, consistent with a shared underlying pathogenesis [3].

An early and persistent feature of atherosclerotic lesions is the presence of lipid-laden (foam) cells, arising from cholesterol and cholesteryl ester accumulation in macrophages in the intima of the artery wall [5]. Low-density lipoproteins (LDL) are the likely source of this lipid. The expression of native LDL receptors on arterial cells types is tightly controlled and feedback regulated [6]. In contrast, the uncontrolled uptake of modified LDL, which results in foam cell formation, occurs via alternative receptors including CD36, CD68 and Scavenger Receptors [6, 7]. A number of LDL modifications including oxidation, aggregation, chemical modification and formation of immune complexes [6, 8] are recognized by these receptors.

Of particular relevance to diabetes-associated atherosclerosis is the role of glucose (or species derived from glucose) in inducing LDL modification [9, 10]. Previous studies have proposed multiple mechanisms including glycation and glycoxidation reactions [4, 9]. Glycation of LDL involves the covalent binding of aldehyde groups of glucose, or low-molecular-mass compounds derived from glucose or metabolic processes, to reactive amines (e.g. Lys and Arg

side chains, N-terminus), and thiol groups (Cys) on the single apolipoprotein B-100 (apoB) protein molecule of the particle [11-13]. The initial product of such reactions is an unstable Schiff base, which undergoes subsequent rearrangement to yield Amadori products, such as fructose-lysine. Glycooxidation consists of two related processes – oxidation of protein-bound sugars (e.g. those from glycation) and oxidation of free glucose and its products. Each mechanism can generate free radicals that can modify LDL, and hence potentially play a role in the enhanced cellular uptake of modified LDL [12, 14-16].

The species arising from glycation and glycooxidation reactions can undergo further reactions to give complex end products often termed advanced glycation end products (AGE); many of these are incompletely characterised [9, 12]. Elevated levels of AGE have been reported in patients with diabetes [17]. Some AGE, such as N^ε-carboxymethyl- and N^ε-carboxyethyl-lysines and pentosidine, accumulate with age on tissue proteins, and at an increased rate in LDL and atherosclerotic lesions from patients with diabetes [16, 18-20]. N^ε-carboxymethyllysine and N^ε-carboxyethyllysine are formed on reaction of Lys residues with low-molecular-mass aldehydes (glyoxal / glycolaldehyde and methylglyoxal respectively), arising from glucose and metabolic pathways [21], providing evidence for a role of such aldehydes in diabetes-induced complications. These aldehydes are present at elevated levels in the plasma of patients with diabetes [22, 23].

We have shown previously that glycation of LDL by reactive aldehydes such as glycolaldehyde, in the absence of (glyc)oxidation, is sufficient for rapid lipid accumulation by macrophage cells [24]. This aldehyde-mediated ‘carbonyl stress’ [16] may therefore facilitate lipid accumulation in cells in the artery wall, and promote atherosclerosis in patients with diabetes. Hydrazine compounds (of general structure R-NH-NH₂) react rapidly with carbonyl compounds (aldehydes and ketones), and may therefore inhibit glycation reactions [25]. We show here that a number of these compounds inhibit modification of LDL induced by

methylglyoxal and glycolaldehyde, and thereby inhibit lipid accumulation in cultured macrophage cells (i.e. model foam cell formation). Complete inhibition is observed, in some cases, with equimolar concentrations of inhibitor to carbonyl compound, suggesting that these species are highly efficacious protective agents.

Materials and methods

Materials Methylglyoxal, glycolaldehyde, metformin (1,1-dimethylbiguanide hydrochloride), hydralazine (1-hydrazinophthalazine), aminoguanidine (guanylhiazine hydrochloride), Girard's reagent P (1-(carboxy)pyridinium chloride hydrazide), Girard's reagent T ((carboxymethyl)trimethylammonium chloride hydrazide), fatty acid-free BSA, EDTA, Dulbecco's phosphate buffered saline (PBS, pH 7.4), methanesulfonic acid, deoxycholic acid, trichloroacetic acid, sodium borohydride, β -mercaptoethanol, *o*-phthaldialdehyde (OPA), L-homoarginine, amino acid standards and tryptamine were obtained from Sigma-Aldrich (Castle Hill, NSW, Australia). Pre-cast 4-15% SDS-PAGE Gels and Chelex-100 resin were from Bio-Rad (Regents Park, NSW, Australia). PD10 columns were from Amersham Biosciences (Castle Hill, NSW, Australia). Pre-cast 1% agarose gels were from Helena Laboratories (Mt. Waverly, VIC, Australia). All other chemicals were of analytical grade, and all solvents were of HPLC grade. Solutions were prepared using Nanopure water (Milli Q system, Millipore-Waters, Lane Cove, NSW, Australia) pre-treated with washed Chelex-100 resin to remove transition metal ions, with the exception of tissue culture reagents where Baxter (Old Toongabbie, NSW, Australia) sterile water or PBS were used.

LDL modification LDL was isolated from multiple healthy male and female donors and glycated as described previously [24]. Briefly, sterile LDL (1 mg protein/mL) was incubated with 10 mmol/L glycolaldehyde or methylglyoxal in chelexed PBS (pH 7.4) at 37 °C, under 5% (v/v) humidified CO₂ for 7 days. 50 μ M EDTA was added to the incubation controls (to prevent

artificial oxidation), but not the aldehyde-treated samples, as rapid reaction occurs between EDTA and these aldehydes. We have previously shown that oxidation is an insignificant process under the conditions employed [24]. Hydrazine compounds (0 - 50 mmol/L) were added prior to the aldehyde as indicated, and were present throughout the modification period. Excess reagents were removed before addition of the modified LDL to cells by elution through PD10 columns, as per the manufacturer's instructions, using chelexed PBS.

Characterisation of LDL modifications Changes in charge (agarose gels) and cross-linking (reducing SDS-PAGE) of the modified and control LDL were quantified as described previously [24]. Amino acid analysis was performed by HPLC analysis using OPA derivatization after delipidation and methanesulfonic acid hydrolysis [26]. LDL samples (250 µg protein) were prepared for hydrolysis by addition of sodium borohydride (10 µL, 10 mg/mL), deoxycholic acid (50 µL, 0.3% w/v), and trichloroacetic acid (100 µL, 50% w/v), followed by centrifugation at 4300 g for 2 min. Samples were then washed twice with 500 µL ice-cold acetone, re-pelleted by centrifugation at 7000 g for 2 min and dried by vacuum centrifugation. The protein pellets were hydrolysed with methanesulfonic acid (150 µL) containing tryptamine (0.2% w/v). L-Homoarginine (80 nmoles) was added as an internal standard. Vials were placed in Pico-Tag vessels (Alltec Associates, Baulkham Hills, NSW, Australia), subjected to 3 cycles of purging (N₂ gas) and evacuation, then incubated (20 h, 110 °C). The samples were subsequently neutralized (150 µL, 4 mol/L NaOH), filtered, diluted 25-fold and stored at 4 °C. Analysis was performed within 48 h by reverse-phase HPLC using a Zorbax ODS 5 µm 4.6 x 250 mm column (Agilent, Forest Hill, NSW, Australia) fitted with a 2 cm Supelco LC-18 guard cartridge (Sigma-Aldrich, Castle Hill, NSW, Australia). Pre-column derivatization of samples was performed using a Shimadzu (Shimadzu Oceania, Rydalmere, NSW, Australia) SIL-10A auto-injector that mixed 20 µL of an OPA/β-mercaptoethanol solution (produced by mixing 1 mL of the former with 5 µL of the latter) with 40 µL of sample. After 1 minute, 15 µL of derivatized samples was

injected. Samples were separated (flow rate of 1 mL/min) using a gradient of buffer A (20% methanol, 2.5% tetrahydrofuran in 20 mmol/L sodium acetate, pH 5.4) with 5% buffer B (80% methanol, 2.5% tetrahydrofuran in 20 mmol/L sodium acetate, pH 5.4) for 7 min; 5-25% B over 10 min; 25-45% B over 2 min; 45-50% B over 8 min; 50-58% B over 8 min; 58-100% B over 5 min; 100% B for 5 min; 100-5% B over 1 min; and re-equilibration at 5% buffer B for 9 min. Buffers were degassed by vacuum sonication and sparged with helium during chromatography. Derivatized amino acids were detected by fluorescence (λ_{ex} 340 nm, λ_{em} 440 nm; Shimadzu RF-10AXL detector) and quantified using amino acid standards with added L-homoarginine and methionine sulfoxide.

Cell studies J774A.1 mouse macrophages (TIB-67, ATCC, Manassas, VA, USA) were cultured as described previously [24]. Cells were harvested when confluent, replated (12-well plates, 0.5×10^6 cells/mL at 1 mL per well) and incubated overnight before exposure to modified LDL or controls (200 μg apoB/mL), for 24 h in media containing 10% lipoprotein-deficient serum (LPDS; prepared as described previously [24]). After exposure, cells were equilibrated (2 h) using lipoprotein-free media containing 1 mg/mL BSA instead of serum. Media samples were then collected, and cells washed before lysis in water. Cell viability was determined by lactate dehydrogenase (LDH) release as described previously [24]. Cell lysates were extracted for analysis of sterols as described previously [24]. Cholesterol and cholesteryl esters were separated by reverse-phase HPLC using isocratic elution (70% isopropanol / 30% acetonitrile, filtered and degassed) at 1 mL/min [27]. Eluted materials were detected by UV absorption at 205 nm. Absolute levels of cholesterol and cholesteryl docosahexaenoate, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl palmitate, cholesteryl oleate, and cholesteryl stearate were determined by use of standards (Sigma, Castle Hill, NSW, Australia).

Protein Assay LDL and cell lysate protein concentrations were quantified (60 min incubation at 60 °C) using the Bicinchoninic Acid (BCA) assay (Pierce, Rockford, IL, USA), with BSA as the standard.

Statistical Analysis Data are expressed as mean \pm SEM from ≥ 3 separate experiments with triplicate samples, unless noted otherwise. Statistical analysis was performed with one-way analysis of variance (ANOVA) and Tukey's *post hoc* analysis. P values of < 0.05 were taken as statistically significant.

Results

LDL characterization Incubations of LDL with glycolaldehyde and methylglyoxal were carried out under conditions that give minimal oxidation of the protein, lipids, cholesterol or α -tocopherol, but extensive glycation of the apoB protein as determined previously [24, 28]. These conditions also do not affect the relative apoB, cholesterol and cholesteryl ester [24, 28], triglyceride and phospholipid composition of the particles (Brown and Davies, unpublished data). Glycation of the apoB molecule was quantified by changes in overall particle charge (relative electrophoretic mobility, REM, on agarose gels), cross-linking (reducing SDS-PAGE) and consumption of amino acid residues (HPLC). These changes were subsequently compared to those induced by glycolaldehyde and methylglyoxal in the presence of equimolar, or greater, concentrations of metformin, aminoguanidine, Girard's reagent P, Girard's reagent T and hydralazine (Fig. 1).

The REM data from agarose gels provides data on the overall charge of the LDL particles, and hence the extent of modification of Arg, Lys, His residues and the N-terminal amine group of apoB. As observed previously [24] exposure of LDL to glycolaldehyde or methylglyoxal resulted in significantly larger REM values than incubation controls and native LDL (Fig. 2, Fig. 3a, b). Each of the compounds tested (metformin, aminoguanidine, Girard's

reagent P, Girard's reagent T, hydralazine), when included during the LDL modification period at equimolar concentrations to the modifying agent, inhibited these increases (Fig. 3a, b). Aminoguanidine, and Girard's reagents P and T, were significantly more effective in preventing the changes induced by methylglyoxal than metformin, with the REM of LDL in the presence of aminoguanidine, Girard's reagents P and T not significantly different from the incubation control. Inclusion of hydralazine in incubations with methylglyoxal resulted in precipitation of the LDL. With glycolaldehyde, hydralazine was the most effective at preventing the increases in REM, with the REM under these conditions not significantly different to the incubation control. Aminoguanidine, Girard's reagents P and T were again more effective ($P < 0.05$) than metformin in preventing the increases induced by glycolaldehyde. Higher concentrations of metformin (Fig. 3c), aminoguanidine (Fig. 3d) and Girard's reagent P (Fig. 3e) resulted in more marked inhibition of the aldehyde-induced changes.

We also examined whether these compounds inhibited aldehyde-induced cross-linking of LDL using SDS-PAGE. Both glycolaldehyde (Fig. 4) and methylglyoxal (data not shown) induce significant cross-linking of LDL when compared to controls [24]. With methylglyoxal-mediated cross-linking, greater than equimolar concentrations of metformin and aminoguanidine were required to prevent cross-linking, whereas equimolar concentrations of Girard's reagent P and T were effective (data not shown). With glycolaldehyde-treated LDL, aminoguanidine (10 mmol/L), Girard's reagent P (10 mmol/L) and hydralazine (3 mmol/L) did not prevent apoB cross-linking (Fig. 4, lanes 5, 8 and 9 respectively), whereas higher concentrations of aminoguanidine (25 or 50 mmol/L) were effective (Fig. 4, lanes 6 and 7 respectively). Metformin (≤ 40 mmol/L) and hydralazine (≤ 10 mmol/L) were ineffective (data not shown).

Changes in the apoB content of Ser, His, Gly, Thr, Arg, Ala, Tyr, Met, Trp, Val, Phe, Ile, Leu, Lys residues, as well as the generation of methionine sulfoxide, were assessed by HPLC. Previous studies have reported changes in Arg, Lys and Trp levels [24, 28]. Data for these

residues are presented in Fig. 5. Data for other residues, which were not affected, are not shown. Recovery of the internal standard L-homoarginine was ca. 90%, and no significant differences were observed for the recovery of other amino acid residues (data not shown). With LDL treated with glycolaldehyde, only Lys residues were depleted relative to controls, with 10 mmol/L glycolaldehyde giving 60% loss (Fig. 5b). Addition of 10, 25 or 50 mmol/L aminoguanidine to such reactions totally prevented the loss of Lys residues; 3 mmol/L hydralazine was partially protective. The trend towards protection observed for Girard's reagent P failed to reach statistical significance.

Model foam cell formation The tested compounds at concentrations utilised in the above LDL studies did not significantly affect J774A.1 cell viability, as determined by intracellular LDH release, or total cell protein levels (BCA assay, data not shown). Cells exposed to glycolaldehyde-modified LDL, in the absence or presence of these agents, did not have significantly different cell viability or protein levels when compared to controls (data not shown).

Previous studies have shown that incubation of J774A.1 cells with glycolaldehyde- or methylglyoxal-modified LDL (prepared as outlined above, with excess aldehyde removed by column chromatography of the LDL before addition to cells) results in significant cellular accumulation of cholesterol and cholesterol esters [24]. This observation was confirmed, with J774A.1 cells exposed to glycolaldehyde-treated LDL having ca. 37% of their total sterol content present as cholesteryl esters (Fig. 6). Cells exposed to control LDL had < 7% of total sterols present as cholesteryl esters. Aminoguanidine (10 mmol/L), Girard's reagent P (10 mmol/L) or hydralazine (3 mmol/L) present during the LDL modification phase, prevented the subsequent intracellular accumulation of free cholesterol (Fig. 6a) and cholesteryl esters (Fig. 6b). As these modified LDL preparations were subject to column chromatography before addition to the cells, direct effects of excess hydrazine drug on the cells can be discounted. The levels of free

cholesterol and cholesteryl esters in these cells were not significantly different to cells exposed to incubation control LDL or no LDL (i.e. cholesterol ester content < 7% of total cholesterol present). These data are consistent with these compounds being highly efficient protective agents.

Discussion

Previously we have shown that methylglyoxal and glycolaldehyde can modify LDL by glycation, in the absence of significant oxidation (as assessed by the levels of protein side-chain oxidation products, parent lipids, lipid peroxides and alcohols, parent cholesterol, the cholesterol oxidation product 7-ketocholesterol, and α -tocopherol [24, 28]). In the current study we have confirmed that the apoB protein of glycated LDL exhibits increased mobility on agarose gels (i.e. increased REM, as a result of charged residue modification), cross-linking, and loss of Lys residues (as assessed by HPLC). Previous studies have also provided evidence for Arg and Trp modification [24, 28]. No significant changes in lipid composition (triglyceride, phospholipid, cholesterol and cholesteryl esters) or the lipid : protein ratio were detected (Brown and Davies, unpublished data). These glycated LDL are recognised by scavenger receptors (other than the LDL receptor) on J774A.1 murine macrophages [29] and primary human monocyte-derived macrophages (Brown, Rashid, van Reyk and Davies, unpublished data) giving rise to intracellular accumulation of cholesterol and cholesterol esters. Such lipid-laden cells are models of the foam cells that play a critical role in the development and progression of atherosclerosis. These lipid-laden cells contain a similar percentage of cholesterol esters to foam cells isolated from lesions (i.e. ca. 40% of total cholesterol present as esters [24]). It has been proposed that glycation of LDL, and subsequent unregulated cellular uptake of the modified particles, may contribute to foam cell formation and thus to the increased atherosclerosis observed in people with diabetes. Strategies that prevent LDL glycation may therefore have therapeutic potential.

Hydrazine compounds, which contain the RNHNH₂ functional group (Fig. 1), react rapidly with aldehydes and ketones, and this chemistry has found extensive use in the characterization of carbonyl-containing compounds [30]. Such reactions prevent glycation of LDL induced by reactive aldehydes, glucose and other low-molecular-mass carbonyl compounds [25, 31].

Aminoguanidine has been studied intensively as an anti-glycation agent, and reacts rapidly with aldehydes, such as methylglyoxal, thereby preventing AGE formation [32]. Aminoguanidine can also prevent cross-linking of soluble proteins to matrix, AGE-mediated cross-linking of collagen, increases in AGE matrix content of aortas, and the quantity of plasma proteins cross-linked to matrix [33]. Clinical trials (under the name Pimagedine) have however, provided evidence of complications that make it unsuitable for clinical use [32]. Metformin, used clinically as an antihyperglycaemic agent, is structurally related to aminoguanidine and may therefore exhibit some protective actions [25, 31]. Metformin partially inhibits the increase in AGE-related fluorescence detected on reaction of methylglyoxal with albumin [34]. A previous study has reported a 40-50% reduction in coronary events with metformin treatment, though glycaemic control was not as effective as intensive treatment with sulfonylureas or insulin [35]. The increased methylglyoxal concentrations detected in people with diabetes is significantly reduced by metformin [36], perhaps due to carbonyl trapping reactions [31, 37]. Hydralazine has been employed clinically as an anti-hypertensive agent, but is known to react with carbonyls [38, 39] consistent with a potential protective effect against glycation [25]. Girard's reagents P and T were examined here as they contain a hydrazine group that is known to be highly reactive with aldehydes and ketones [30]; these materials have not been used therapeutically to our knowledge.

Inclusion of these agents in LDL incubations concurrently with methylglyoxal or glycolaldehyde reduced glycation-associated changes to LDL. With methylglyoxal, the presence

of equimolar concentrations of aminoguanidine, or Girard's reagents P and T, prevented the observed increases in REM of the modified LDL. Higher concentrations of metformin were needed to produce a comparable effect. In LDL cross-linking experiments greater than equimolar concentrations of metformin and aminoguanidine were required to prevent cross-linking of apoB, whereas equimolar concentrations of Girard's reagents P and T were sufficient. These results suggest that hydrazine compounds efficiently prevent the modification of apoB protein residues induced by methylglyoxal [28], thereby preventing the observed increase in net negative charge of the LDL particle and protein cross-linking.

When glycolaldehyde was used as the LDL modifying agent, hydralazine was the most effective agent in preventing the observed REM increases. Aminoguanidine and Girard's reagents P and T provided partial protection against the REM increases, but none of these compounds prevented LDL cross-linking at equimolar concentrations to the aldehyde. Metformin prevented some of the REM increases, but was the least effective of the compounds tested. Aminoguanidine completely prevented, and hydralazine offered partial protection against, the loss of Lys residues. Girard's reagent P was ineffective. With 10 mM glycolaldehyde, ca. 60% of the Lys residues of apoB protein were modified. This level of modification has been reported to be sufficient for recognition of glycolaldehyde-modified LDL by macrophage scavenger receptors [24, 40]. The observed cellular accumulation of cholesteryl esters from such glycolaldehyde-modified LDL is consistent with this earlier data. Interestingly, none of the compounds tested provided complete inhibition of the changes in LDL particle properties induced by glycation, yet these agents provided virtually complete protection against lipid accumulation in J774A.1 cells. Even in cases where 40% of Lys residues remained modified, no significant intracellular sterol accumulation was detected, suggesting that there is a dramatic cut-off in scavenger receptor recognition in the region between 40 and 60% Lys modification (i.e. that there is a threshold value of modification required for recognition of the modified LDL by

the scavenger receptors). This has potentially important therapeutic ramifications, as relatively small changes in the extent of LDL modification may give profound effects on the extent of intracellular lipid accumulation by macrophage cells exposed to modified particles.

These studies also demonstrate that apoB cross-linking does not appear to be a key determinant of receptor recognition and intracellular lipid accumulation, as equimolar concentrations (with regard to glycolaldehyde) of aminoguanidine, hydralazine and Girard's reagent P did not prevent cross-linking of the apoB protein, but did prevent model foam cell formation.

The observed changes in the extent of Lys modification do not correlate well with the other two measures of glycation examined (REM and cross-linking). Aminoguanidine (at 10 mmol/L) completely prevented Lys residue loss induced by glycolaldehyde, whereas the REM and cross-linking changes were incompletely inhibited. The REM and cross-linking changes may therefore be dependent on the nature of the products formed from the Lys residues, rather than the loss of parent Lys *per se*. Furthermore, the changes in Arg, Lys and Trp residues on apoB measured by total amino acid analysis (i.e. after acid hydrolysis of the protein) differ significantly to those reported previously using fluorescence assays on "intact" particles. With the latter technique a greater loss of Lys and Trp residues was detected under identical conditions [28]. The reasons for this discrepancy are not fully understood, but it is possible that the extent of loss detected by direct fluorescence is misleading as a result of contributions from other fluorophores or changes in environment. It is well established that fluorescent products are generated during glycation [12] and this may result in an overestimation of loss. Alternatively it is possible that the HPLC and direct fluorescent assays are measuring different populations - for example - both reversibly and irreversibly modified species in the case of the fluorescence assays, but only irreversibly damaged materials in the total amino acid analysis. Thus some of the initial (reversible) products may be converted back to the parent amino acid by the strong

acid used in the protein hydrolysis. The discrepancies between these methods need to be investigated further to determine which is the more accurate method of assessing amino acid modification.

The concentrations of aldehydes utilised in this study are much higher than those reported clinically [22, 23, 41]. The *in vivo* values, which are in the nano- to micro-molar range, are however potentially misleading as they (presumably) represent unreacted, steady-state, levels of highly reactive species, and the true flux of these compounds may be higher. Subendothelial entrapment of LDL [42-44] may also allow greater extents of LDL modification than observed in plasma, as a result of chronic exposure to low levels of carbonyl compounds. However, the key observation in this study is that equimolar concentrations of the hydrazine compounds, when compared to the reactive aldehyde, are sufficient to completely prevent intracellular lipid accumulation (i.e. model foam cell formation). Furthermore, as incomplete inhibition of LDL modification appears to be sufficient to prevent scavenger receptor recognition and particle uptake, it is possible that even low levels of these carbonyl-scavenging compounds may be beneficial. Hydralazine and metformin can reach micromolar, and aminoguanidine tens of micromoles, after oral dosing in humans [32, 45-47], and these concentrations are considerably in excess of those of the reactive aldehydes (see above), so it is conceivable that these compounds may act as efficient scavengers of reactive aldehydes *in vivo*, especially as it has been demonstrated that equimolar levels of these drugs are sufficient to induce significant protection against aldehyde-mediated LDL modification. Thus hydrazine compounds and analogues may be worthy of further study as agents to inhibit diabetes-induced atherosclerosis.

Acknowledgements The authors thank the Australian Research Council and Diabetes Australia Research Trust for financial support. Ms. B.E. Brown acknowledges receipt of an Australian

Postgraduate Award via the University of Sydney. The authors thank Dr. Clare Hawkins and Mr Philip Morgan for assistance with the amino acid analysis.

Conflict of Interest

None of the authors have any commercial interests with any companies that might constitute a conflict of interest with regard to the data reported in this manuscript.

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FIGURE LEGENDS

Fig. 1 Structures of compounds studied.

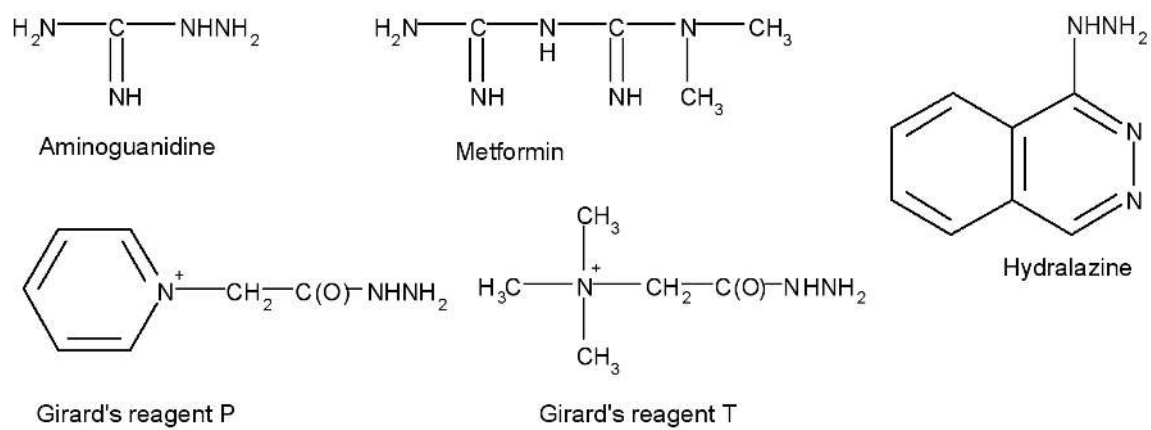


Fig. 2 Agarose gel of native and modified LDL. Lane 1: native LDL; lane 2: LDL plus 50 $\mu\text{mol/L}$ EDTA (incubation control); lane 3: LDL modified with glycolaldehyde (10 mmol/L for 7 days); lanes 4-8: As lane 3 except modification carried out in the presence of aminoguanidine (10 mmol/L, lane 4; 25 mmol/L, lane 5; 50 mmol/L, lane 6), Girard's reagent P (10 mmol/L, lane 7), and hydralazine (3 mmol/L, lane 8). Representative gel of 3.

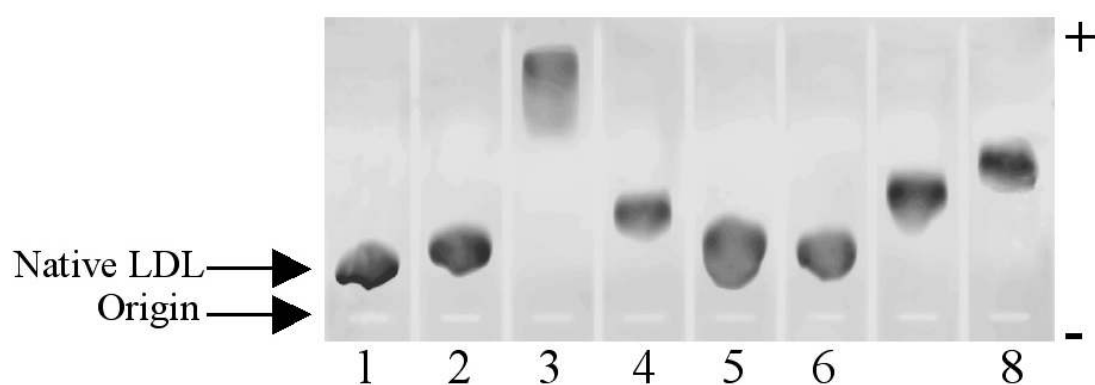


Fig. 3 Relative electrophoretic mobility (REM) of native LDL and LDL modified with 10 mmol/L methylglyoxal (MG; panel a) or glycolaldehyde (GA; panel b) for 7 days, compared to native LDL (N; REM set at 1), incubation control (LDL incubated with EDTA), and LDL incubated with MG or GA and 10 mM of each of metformin (MF), aminoguanidine (AG), Girard's reagent P (GP), Girard's reagent T (GT) or hydralazine (H). The effect of various concentrations of metformin (panel c), aminoguanidine (panel d), Girard's reagent P (panel e) and hydralazine (panel f) on the REM of LDL modified by 10 mmol/L methylglyoxal (open symbols) or glycolaldehyde (closed symbols). Columns with different superscript letters are significantly different by one-way ANOVA.

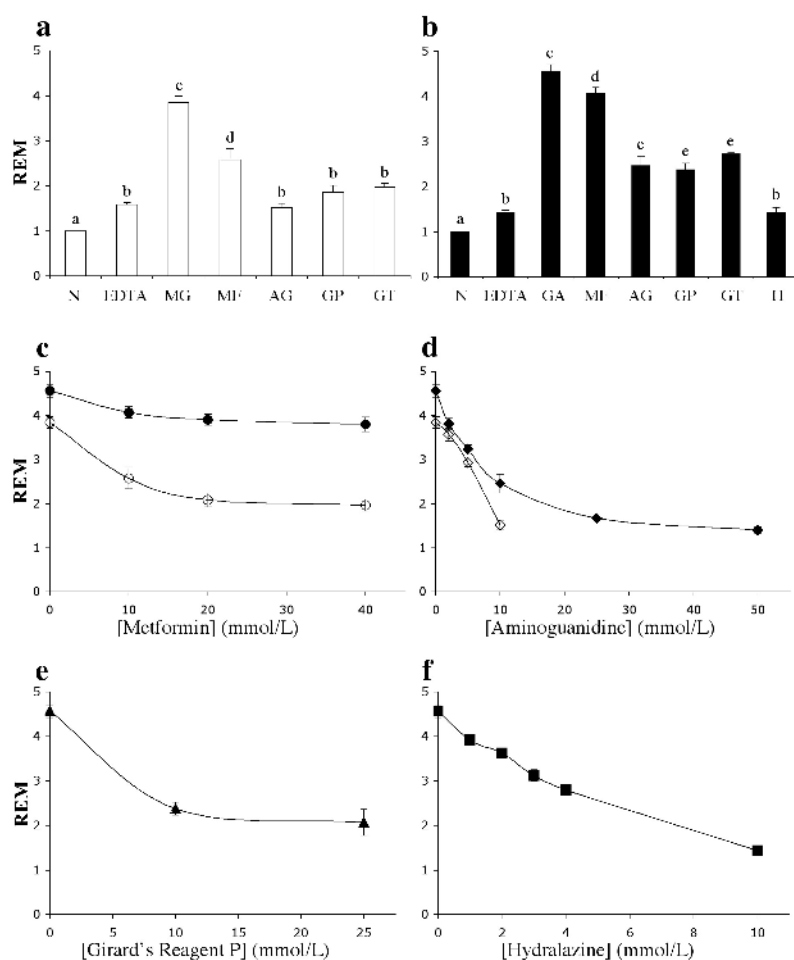


Fig. 4 SDS-PAGE of LDL modified by 10 mmol/L glycolaldehyde for 7 days, in the absence or presence of hydrazine compounds. Lane 1: molecular weight markers (kD); lane 2: native LDL; lane 3: LDL plus 50 μ mol/L EDTA (incubation control); lane 4: LDL modified with glycolaldehyde; lanes 5-9: as lane 4 except in the presence of aminoguanidine (10 mmol/L, lane 5; 25 mmol/L, lane 6; 50 mmol/L, lane 7), Girard's reagent P (10 mmol/L, lane 8), or hydralazine (3 mmol/L, lane 9). Representative gel of 3.

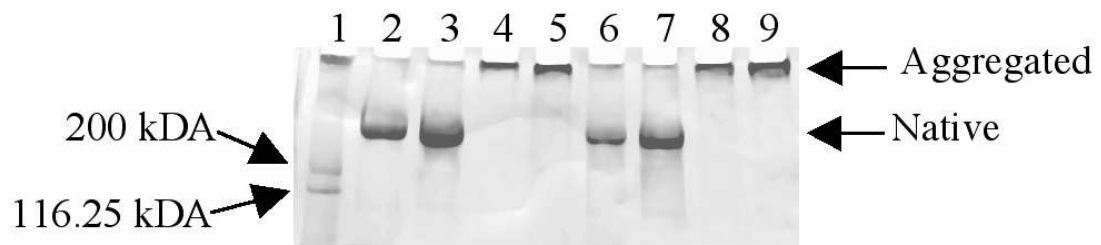


Fig. 5 Arg (panel a), Lys (panel b) and Trp (panel c) levels of apoB modified by 10 mmol/L glycolaldehyde in the absence or presence of 10, 25 and 50 mmol/L aminoguanidine (AG), 10 mmol/L Girard's reagent P (GP) and 3 mmol/L hydralazine (H) as detected by HPLC. Data are expressed relative to native LDL (N) that contains 148 Arg, 356 Lys and 37 Trp residues [48]; these values are set as 100%. Columns with different superscript letters are significantly different to each other by one-way ANOVA ($P < 0.05$).

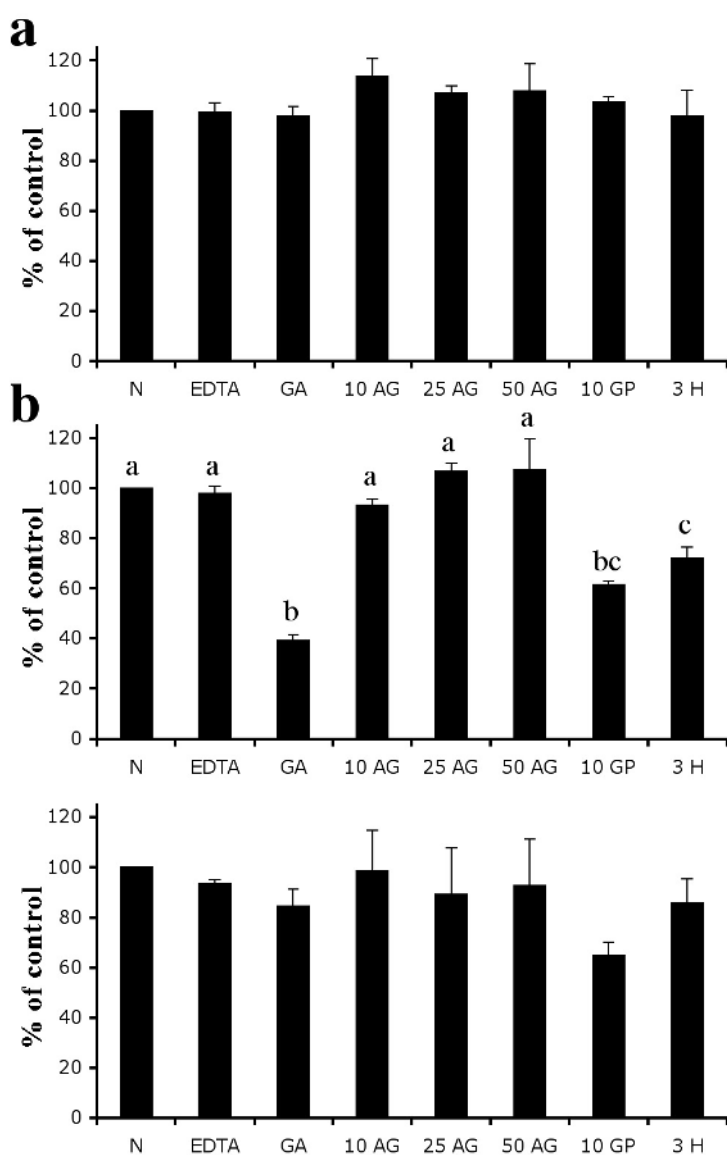


Fig. 6 Free cholesterol (panel a), total cholesteryl esters (panel b) and percent cholesteryl esters of total cholesterol (panel c) present in J774A.1 cells, after exposure to 200 $\mu\text{g/mL}$ of LDL for 24 h that had been previously modified for 7 days by 10 mmol/L glycolaldehyde (GA) compared to cells exposed to no LDL for the same time periods (Control), incubation control LDL (LDL plus EDTA), or LDL incubated with aminoguanidine (AG, 10 mmol/L), Girard's reagent P (GP, 10 mmol/L), or hydralazine (H, 3 mmol/L). Total cholesterol values are the sum of free cholesterol plus total cholesteryl ester levels. Columns with different superscript letters are significantly different by one-way ANOVA ($P < 0.05$).

