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## Glycation of low-density lipoproteins by methylglyoxal and glycolaldehyde gives rise to the *in vitro* formation of lipid-laden cells

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**Abstract** *Aims/hypothesis:* Previous studies have implicated the glycooxidative modification of low-density lipoprotein (LDL) by glucose and aldehydes (apparently comprising both glycation and oxidation), as a causative factor in the elevated levels of atherosclerosis observed in diabetic patients. Such LDL modification can result in unregulated cellular accumulation of lipids. In previous studies we have characterized the formation of glycated, but nonoxidized, LDL by glucose and aldehydes; in this study we examine whether glycation of LDL, in the absence of oxidation, gives rise to lipid accumulation in arterial wall cell types. *Methods:* Glycated LDLs were incubated with macrophage, smooth muscle, or endothelial cells. Lipid loading was assessed by HPLC analysis of cholesterol and individual esters. Oxidation was assessed by cholesterol ester loss and 7-ketocholesterol formation. Cell viability was assessed by lactate dehydrogenase release and cell protein levels. *Results:* Glycation of LDL by glycolaldehyde and methylglyoxal, but not glucose (in either the presence or absence of copper ions), resulted in cholesterol and cholesterol ester accumulation in macrophage cells, but not smooth muscle or endothelial cells. The extent of lipid accumulation depends on the degree of glycation, with increasing aldehyde concentration or incubation time, giving rise to greater extents of particle modification and lipid accumulation. Modification of lysine residues appears to be a key determinant of cellular uptake.

*Conclusions/interpretation:* These results are consistent with LDL glycation, in the absence of oxidation, being sufficient for rapid lipid accumulation by macrophage cells. Aldehyde-mediated “carbonyl-stress” may therefore facilitate the formation of lipid-laden (foam) cells in the artery wall.

**Keywords** Aldehydes · Atherosclerosis · Carbonyl stress · Cholesterol esters · Foam cell glycation · Glycooxidation · Low-density lipoproteins · Oxidation · Protein modification

**Abbreviations** AcLDL: LDL modified by acetylation · ApoB-100: apolipoprotein B-100 · BHT: butylated hydroxytoluene · GA-LDL: LDL modified by glycolaldehyde · gLDL±Cu<sup>2+</sup>: LDL modified by glucose in the absence or presence of Cu<sup>2+</sup> · HBSS: Hanks’ balanced salt solution · HS: human serum · LDH: lactate dehydrogenase · LPDS: lipoprotein-deficient serum · MG-LDL: LDL modified by methylglyoxal · nLDL: native LDL · PPACK: *D*-phenylalanyl-*L*-arginine chloromethyl ketone · REM: relative electrophoretic mobility

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### Introduction

Diabetic complications are the major cause of mortality and morbidity in patients with diabetes. These complications include those of the microvascular and macrovascular 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]. Patients with diabetes therefore suffer from an increased risk of developing coronary heart disease, cerebrovascular disease and peripheral vascular disease and associated myocardial infarction, stroke, limb amputations, and premature death [1]. This macrovascular disease is responsible for approximately 72% of deaths in patients with diabetes.

A number of features of diabetes mellitus have been proposed as contributing factors to atherosclerosis, includ-

ing chronic hyperglycaemia and insulin resistance, dyslipidaemias, and abnormalities of haemostasis [2]. Macrovascular disease appears in most patients with type 2 diabetes, at or near the time of first diagnosis, consistent with a shared underlying pathogenesis [2]. A consistent and early feature of atherosclerotic lesions is the presence of lipid-laden (foam) cells, arising from macrophages or some types of smooth muscle cells, in the intima of the artery wall [3]. Low-density lipoproteins (LDL) are the proposed source of this lipid. The expression of native LDL receptors on arterial cells is tightly controlled and feedback regulated, consistent with the uncontrolled uptake of LDL and subsequent foam cell formation occurring via alternative receptors [4]; a number of these have been characterized, including CD36, scavenger receptors A and B1, and RAGE [5]. These receptors recognize abnormal LDLs, but not native LDLs, including those modified by oxidation, aggregation, chemical modification, and immune complex formation [4, 6].

Of particular relevance to diabetes-associated atherosclerosis is the role of glucose (or species derived from glucose) in inducing modifications to LDL [7, 8]. Previous studies have identified glycation and glycooxidation reactions as mechanisms by which such alteration can occur [7]. Glycation involves the covalent binding of the aldehyde groups of glucose (or low-molecular-mass compounds derived from glucose or other compounds) to reactive amine (e.g., Lys and Arg side chains and the N terminus), and possibly cysteine, groups on the apoB-100 protein [9, 10]. The initial product of such reactions is an unstable Schiff base, which undergoes subsequent rearrangement to yield Amadori products.

Glycooxidation consists of two related processes—oxidation of protein-bound sugars (e.g., those arising from glycation) and oxidation of free glucose and its products. Each of these mechanisms can generate free radicals that modify LDL, and hence potentially play a role in the enhanced uptake of lipids by cells in the artery wall of patients with diabetes [10–13].

The species formed by glycation and glycooxidation can undergo subsequent chemical reactions to give complex products termed advanced glycation end-products (AGE) [7, 10]. These include N<sup>ε</sup>-(carboxymethyl)lysine, N<sup>ε</sup>-(carboxyethyl)lysine, and pentosidine, which are known to accumulate with age in tissue proteins, and at an increased rate in LDL and atherosclerotic lesions from patients with diabetes [12, 14–16]. Both N<sup>ε</sup>-(carboxymethyl)lysine and N<sup>ε</sup>-(carboxyethyl)lysine are formed on reaction of lysine residues with low-molecular-mass aldehydes (glyoxal/glycolaldehyde and methylglyoxal, respectively) [17], providing evidence for a role for such aldehydes in diabetes-induced complications. The plasma concentrations of these aldehydes are elevated in patients with diabetes [18, 19], and the role of AGE products in diabetic complications has been studied extensively [20, 21].

The significance of glycation versus the two facets of glycooxidation in giving rise to LDL modification, and subsequent foam cell formation, either in vitro or in vivo are incompletely understood. Most previous studies have

employed conditions where either all of these processes have occurred, or where the nature of the LDL modification has not been adequately quantified (e.g., [11, 22]). It is therefore unclear as to what types, and extents, of modification of LDL induced by glucose or aldehydes, influence the potential formation of foam cells.

In previous studies we have developed reaction conditions which result in the formation of glycated LDL in the absence of significant (glyc)oxidation [23]. In the current work we examine whether glycation of LDL particles by glucose, or the aldehydes methylglyoxal and glycolaldehyde, in the absence of oxidation, can give rise to model lipid-laden (foam) cells on incubation with macrophage, endothelial, and smooth muscle cell lines in vitro. Furthermore, we have investigated the effectiveness of glucose (in the absence or presence of copper ions) versus low-molecular-mass aldehydes, in inducing LDL glycation and model foam cell formation.

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## Materials and methods

**Materials** Reagents were obtained from the following sources. Sigma-Aldrich (Castle Hill, NSW, Australia): methylglyoxal, glycolaldehyde, fatty acid-free BSA, chloramphenicol, BHT, EDTA, aprotinin, soybean trypsin inhibitor, thrombin, Fat Red 7B, Coomassie brilliant blue, type 1A collagenase, HBSS, PenStrep (containing 100 units/ml penicillin and 0.1 mg/ml streptomycin), and Dulbecco's phosphate-buffered saline; BDH (Merck, Kilsyth, VIC, Australia): glucose and PPACK; Bio-Rad (Regents Park, NSW, Australia): pre-cast 4–15% SDS-PAGE Gels and Chelex-100 resin; ICN (Seven Hills, NSW, Australia): CuSO<sub>4</sub>; Amersham Biosciences (Castle Hill, NSW, Australia): PD10 columns; Helena Laboratories (Mt. Waverly, VIC, Australia): pre-cast 1% agarose gels; JRH Biosciences (CSL, North Ryde, NSW, Australia): DMEM, M199, and RPMI 1640 media; Trace Scientific (Melbourne, VIC, Australia): glutamine, sodium pyruvate, and trypsin/EDTA 1:250; Gibco (Melbourne, VIC, Australia): fetal calf serum (FCS); Australian Red Cross Clarence St Blood Bank: HS (human serum); Axis-Shield (Oslo, Norway): Lymphoprep; Starrate (Bethungra, NSW, Australia): endothelial cell growth promoter. Gases (≥99.9%) were from Linde (Yennora, NSW, Australia). All other chemicals were of analytical or HPLC grade.

Solutions were prepared with nanopure water (Milli Q system; Millipore-Waters, Lane Cove, NSW, Australia) 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, endotoxin-free, water, PBS, or HBSS were used.

**LDL isolation** Plasma was isolated from blood obtained from healthy volunteers with informed consent. Blood was collected in 0.01 mmol/l EDTA, 1 μl aprotinin, 0.04 μmol/l PPACK, and 20 μg/ml soybean trypsin inhibitor per millilitre of whole blood [24]. LDL (density 1.019–1.050) was isolated by sequential density gradient ultracentrifuga-

tion (L-80 Optima; Beckman, Palo Alto, CA, USA) in degassed KBr solutions [25], using a vertical rotor (VTi50; Beckman) at 10°C for 2.5 h (206,000 g average). The LDL was washed by ultracentrifugation, density 1.064, in an angle rotor (Ti70; Beckman) for 20 h (184,000 g average) at 10°C and then dialysed overnight at 4°C, with four changes of 1 l degassed PBS containing 1 mg/ml EDTA and 0.1 mg/ml chloramphenicol. LDL was stored sterile (0.45 µm filtered) in the dark at 4°C and used within 3 weeks of isolation. Before the LDL was used, EDTA was removed by elution of the LDL through two successive PD10 columns as per the manufacturer's instructions using chelexed PBS.

*Lipoprotein-deficient serum* Lipoprotein-deficient serum (LPDS) was prepared from the LDL-deficient infranatant of healthy male donors after density ultracentrifugation as previously [26]. The LDL was adjusted to density 1.25 with KBr and ultracentrifuged (Ti70; Beckman) at 10°C for 20 h (184,000 g) to remove other lipoproteins. Extensive dialysis (5 l 0.9% w/v NaCl solution with 0.1 mg/ml chloramphenicol × 5 days) was performed to remove KBr before clotting of the LPDS with 1 ml 1 mol/l CaCl<sub>2</sub> and 55 U thrombin per 100 ml LPDS at 37°C. LPDS was then filtered to remove clots, heat inactivated, sterile filtered (0.22 µm), and stored frozen, at approximately 20 mg/ml protein concentration, at -20°C.

*LDL modifications* Acetylation of LDL was carried out by a modification of previous methods [27], with 6 µl acetic anhydride added per milligram LDL protein [28]. Modification of LDL by glycolaldehyde, methylglyoxal, or glucose was carried out as described previously [23]. Briefly, sterile LDL (1 mg/ml protein) was incubated with 1–100 mmol/l glycolaldehyde, methylglyoxal, or glucose (±1 µmol/l CuSO<sub>4</sub>) in chelexed PBS at 37°C under 5% (v/v) CO<sub>2</sub>. Incubation controls contained 50 µmol/l EDTA in place of glucose or aldehyde. Excess reagents were subsequently removed by elution of the LDL through two successive PD10 columns as per the manufacturer's instructions using chelexed PBS.

*Agarose gel electrophoresis* LDL (10–15 µl) was loaded onto 1% agarose gels and run on a Ciba-Corning (Palo Alto, CA, USA) system (45 min, 90 V, pH 8.6 barbitone buffer). Gels were fixed with 100% methanol (30 s), stained with Fat Red 7B, and destained with 70% methanol. The relative electrophoretic mobility (REM) of the LDL particles was determined relative to native LDL (REM set to 1).

*SDS-PAGE* Equal volumes were mixed of 1 mg/ml LDL and sample loading buffer containing 10% (w/v) SDS, 10% (v/v) glycerol, 2.5% (v/v) saturated bromophenol blue, and 5% (v/v) 2-mercaptoethanol in 300 mmol/l Tris-HCl (pH 6.8). Samples were heated at 100°C for 5 min, then 25 µl was loaded per well on 4–15% precast SDS-polyacrylamide gels and run (150 V, 50 min, SDS tris-glycine buffer) using a BioRad (Regents Park, NSW,

Australia) system. Bands were visualized with Coomassie brilliant blue [29, 30]. Gels were digitized using a Bio-Rad (Hercules, CA, USA) Gel Doc 1000 system.

*Cell culture* Media contained 4 mmol/l glutamine and 1% (v/v) PenStrep and 10% (v/v) heat-inactivated FCS or HS, unless specified otherwise. Cells were cultured in humidified incubators (5% CO<sub>2</sub>, 37°C) in 175-cm<sup>2</sup> flasks or 12-well (22-mm) plates (Becton Dickinson, Franklin Lakes, NJ, USA).

J774A.1 mouse macrophages (TIB-67; ATCC, Manassas, VA, USA) were grown in DMEM media with 10% FCS and supplements as described above. For experiments, confluent J774.A1 cells were harvested and replated in 12-well plates at 0.5×10<sup>6</sup> cells/ml and incubated overnight before use.

Human umbilical vein endothelial cells (HUVECs) were harvested from freshly obtained cords using type 1A collagenase (15 min, 37°C) [31], and established as primary cell cultures in M199 media containing 20% HS, glutamine, and PenStrep (as above), and 0.5% (v/v) endothelial cell growth promoter. Cells were used up to passage 4. Tissue culture flasks and plates were pretreated with Gelofusine in PBS (1:250) before replating. Confluent cells were harvested using trypsin/EDTA (1:250), diluted to 0.2×10<sup>6</sup> cells/ml, and replated in 12-well plates overnight before use.

A7r5 rat aortic smooth muscle cells (CRL-1444; ATCC, Manassas, VA, USA) were cultured in RPMI 1640 media with 10% FCS, supplements as described above, 1 mmol/l sodium pyruvate, and 3.5 g/l glucose. Confluent cells were trypsinized and replated as described above for endothelial cells. For serum-deprivation experiments, cells were plated down at 0.1×10<sup>6</sup> cells/ml in 12-well plates in 10% FCS overnight, and then incubated in 0.2% FCS for 48 h; such cells were subconfluent when used.

*Cell loading with modified LDL* Cells were exposed to 0–200 µg/ml modified LDL for 1–4 days in media containing 10% LPDS. Acetylated LDL (AcLDL) was employed as a positive control. After exposure, cells were equilibrated using media containing 1 mg/ml BSA instead of serum for 2–24 h, depending on cell type. Cells were then lysed in water, and the lysate extracted for cholesterol and cholesteryl ester analysis. Cell viability was determined by lactate dehydrogenase (LDH) release [32].

*Cholesterol and cholesteryl ester analysis* Cell lysate (800 µl) was added to 10 µl 200 mmol/l EDTA and 10 µl 0.2 mmol/l BHT, and extracted with 200 µl water (or for LDL samples: 20 µl LDL and 980 µl PBS), 2.5 ml methanol, and 5 ml hexane. Samples were mixed thoroughly and stored at -20°C until processed. Samples were centrifuged (2,060 g, 5 min) and 4 ml of the hexane layer removed and dried down under vacuum. Samples were redissolved in HPLC mobile phase (200 µl) and transferred to HPLC vials. Cholesterol and cholesteryl esters were quantified at 205 nm by reverse-phase HPLC [33]

using a Supelco (Castle Hill, NSW, Australia) ODS LC-18 column (25×0.46 cm, 5 μm particle size) and a 2-cm Pelliguard column at 25°C, on a Shimadzu HPLC system (SPD-M10AVP diode array UV detector, SIL-10ADVP auto injector and cooler, LC-10AT pump, SCL-10AVP system controller, PC running Class-VP software; Kyoto, Japan). Cholesterol and cholesteryl esters were separated isocratically at 1 ml/min with 70% isopropanol/30% acetonitrile (v/v). Solvents were filtered and degassed prior to use and flushed with helium. 7-Ketocholesterol was analysed using the same HPLC system, but with detection at 234 nm and a mobile phase consisting of 54% isopropanol, 44% acetonitrile, and 2% H<sub>2</sub>O (v/v/v) [33]. Cholesterol, cholesteryl docosahexaenoate, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl palmitate, cholesteryl oleate, cholesteryl stearate and 7-ketocholesterol levels were quantified by use of standards (Sigma, Castle Hill, NSW, Australia) and expressed as nmol/mg cell protein or nmol/mg apoB protein.

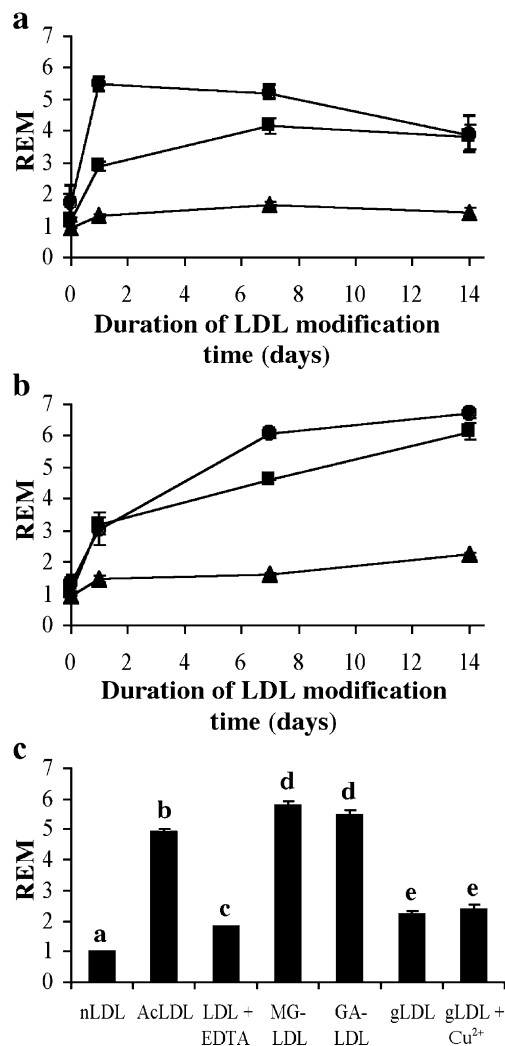
**Protein assay** LDL and cell lysate protein concentrations were quantified using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA), using a 60-min incubation at 60°C, and BSA for standards.

**Data analysis** Data are expressed as mean ± standard error of the mean (SEM) from three or more separate experiments, unless stated otherwise. Statistics were by one-way or two-way analysis of variance (ANOVA) with Tukey's post hoc analysis;  $P < 0.05$  was taken as statistically significant.

## Results

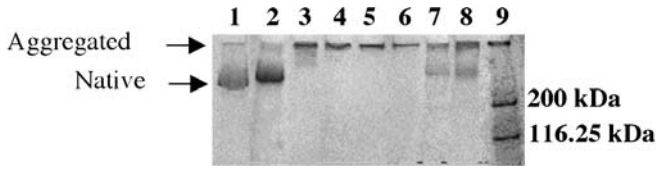
**LDL characterization** Incubations of LDL were carried out as described previously [23] using conditions which give minimal oxidation of protein, lipids, cholesterol, or antioxidants, but extensive glycation of the apoB protein. Additional experiments to characterize changes in particle charge (as assessed by relative electrophoretic mobility [REM]) and aggregation (by SDS-PAGE) were carried out in the current study. The REM assay measures the overall total charge on the LDL particle; as a result of the loss of positive charge arising from the modification of Lys and Arg residues, an increase in overall negative charge is detected with increasing extents of particle alteration. Acetylated LDL (AcLDL) was used in both assays as a positive control.

Incubation of 1 mg/ml LDL with 10 or 100 mmol/l methylglyoxal or glycolaldehyde led to significant concentration- and time-dependent changes in the REM of the LDL particles (Fig. 1). Statistically significant increases were detected by 24 h, with values for the aldehyde-treated LDL threefold to fivefold greater than those for incubation controls (LDL with 50 μmol/l EDTA; Fig. 1a, b). No significant difference was observed between 10 and 100 mmol/l methylglyoxal at 24 h (Fig. 1b), whereas 100 mmol/l glycolaldehyde generated signifi-



**Fig. 1** Relative electrophoretic mobility (REM, a measure of particle charge) of LDL modified by 0 (triangles), 10 (squares), or 100 mmol/l (circles) glycolaldehyde (a) or methylglyoxal (b) with increasing incubation time at 37°C. All REM values for the treated samples are statistically elevated compared to the 0 mmol/l controls at each time point. **c** Comparison of the REM values for normal LDL (nLDL, nonincubated), LDL modified by acetylation (AcLDL), LDL incubated with 50 μmol/l EDTA for 7 days (LDL+EDTA, incubation control), LDL modified by 100 mmol/l methylglyoxal over 7 days (MG-LDL), LDL modified by 100 mmol/l glycolaldehyde over 7 days (GA-LDL), or LDL modified with 100 mmol/l glucose (±1 μmol/l Cu<sup>2+</sup>) for 7 days. Columns with different letters above them are significantly different by one-way ANOVA ( $P < 0.05$ ).

cantly greater changes than 10 mmol/l glycolaldehyde (Fig. 1a). The rate of increase in REM values decreased at longer time points with these reaching a plateau value after 1–2 weeks for glycolaldehyde. Those for methylglyoxal continued to increase over the entire period studied (Fig. 1a, b). A significant increase in the REM value for the incubation control was detected by 7 days (Fig. 1c). Small, but statistically significant, changes were detected for glucose with or without Cu<sup>2+</sup> (gLDL±Cu<sup>2+</sup>, REM ca. 2.3 in each case versus 1.8 for LDL+EDTA incubation controls; Fig. 1c). No statistical difference was observed between the samples with or without Cu<sup>2+</sup> (Fig. 1c). The



**Fig. 2** SDS-PAGE of native LDL (lane 1), or LDL modified by 7 days of incubation with 50  $\mu\text{mol/l}$  EDTA (lane 2), 10 and 100 mmol/l methylglyoxal (lanes 3–4), 10 and 100 mmol/l glycolaldehyde (lanes 5–6), 100 mmol/l glucose (lane 7) or 100 mmol/l glucose plus 1  $\mu\text{mol/l}$   $\text{Cu}^{2+}$  (lane 8). Lane 9: molecular weight markers. Representative gel of two, with samples run in triplicate.

changes in REM for methylglyoxal-modified LDL (MG-LDL) and glycolaldehyde-modified LDL (GA-LDL) after 7 days were not significantly different from one another, but were significantly higher than for acetylated LDL (AcLDL) and incubation controls (Fig. 1c).

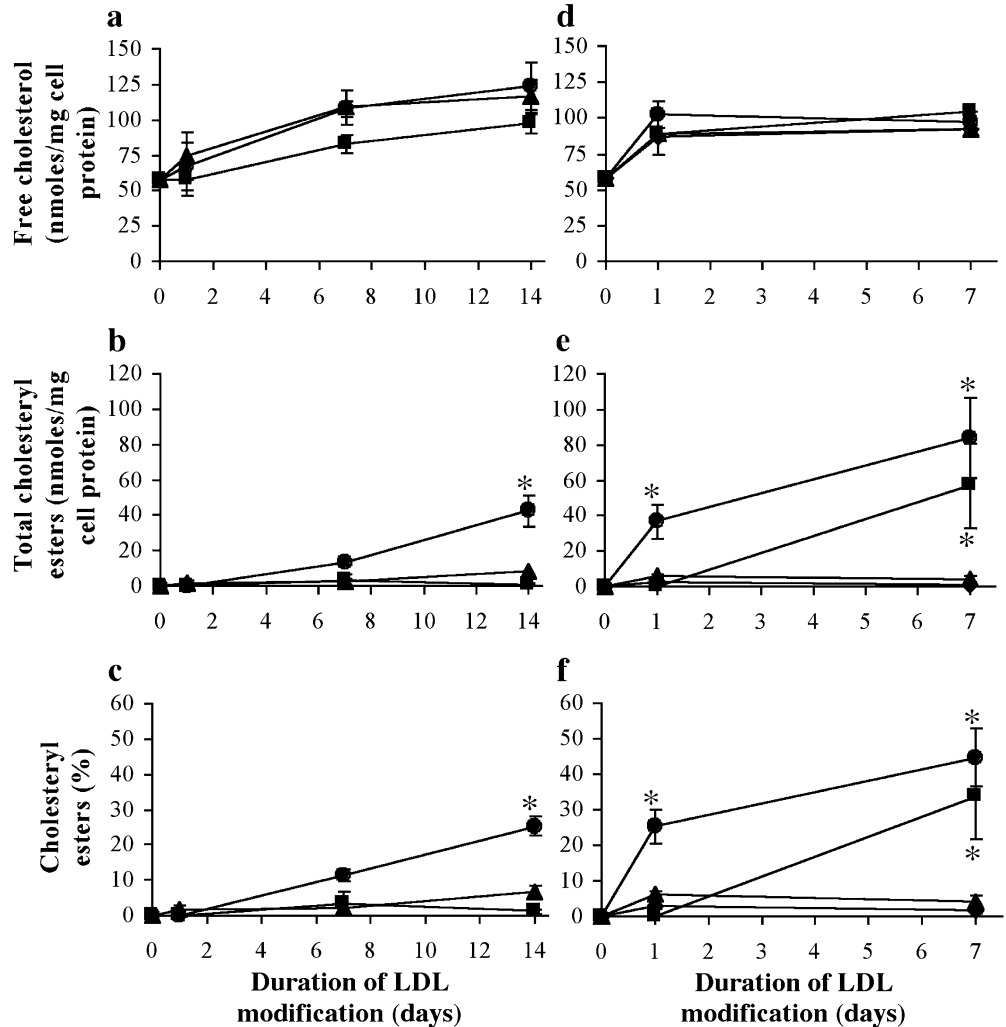
Significant aggregation was observed on SDS-PAGE reducing gels for LDL modified by methylglyoxal, glycolaldehyde, and to a lesser extent glucose (Fig. 2). This effect was concentration- and aldehyde-dependent and increased with longer incubation times for all conditions (data not shown). More extensive aggregation was ob-

served for LDL modified with 10 mmol/l glycolaldehyde than methylglyoxal (Fig. 2, lane 3 versus 5). With higher aldehyde concentrations, only low amounts of the LDL entered the gel from the loading wells (cf. Fig. 2, lane 6) consistent with extensive aggregation. For LDL incubated with 100 mmol/l glucose, with or without 1  $\mu\text{mol/l}$   $\text{Cu}^{2+}$ , aggregation was less marked (Fig. 2, lanes 7 and 8) than with either aldehyde.

Under the conditions employed, the concentrations of cholesterol, cholesteryl docosahexaenoate, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, cholesteryl palmitate and cholesteryl stearate, and 7-ketocholesterol (a lipid oxidation product) in the LDL particles did not alter significantly on treatment with methylglyoxal, glycolaldehyde, or glucose (with or without 1  $\mu\text{mol/l}$   $\text{Cu}^{2+}$ ) when compared to native LDL, or incubation controls (LDL+EDTA) (data not shown). 7-Ketocholesterol accounted for <0.1% of the total sterol in all cases (data not shown), and was <1 nmol/mg apoB in controls.

This data is consistent with rapid and extensive glycation, but minimal oxidation of the lipids in these LDL particles. The effect of incubation of arterial wall cell types with these well-characterized glycated, but essentially

**Fig. 3** Free cholesterol (a, d), total cholesteryl esters (b, e) and percentage cholesteryl esters of total cholesterol (c, f) present in J774A.1 cells, after exposure to 200  $\mu\text{g/ml}$  of LDL for 24 h that had been previously modified for the stated period by 0 (triangles), 1 (diamonds), 10 (squares), or 100 (circles) mmol/l methylglyoxal (a–c) or glycolaldehyde (d–f). Total cholesterol values are the sum of the values of free cholesterol plus total cholesteryl esters. \* Indicates statistically elevated values ( $P < 0.05$ ) compared to the 0 mmol/l controls at each time point.



unoxidized, LDL particles was subsequently examined by assessing cell viability and cellular lipid accumulation.

*Effect of glycated LDL particles on cell viability* The effect of 0–200 µg/ml glycated or control LDL on the viability (assessed by LDH release and cell protein levels) of murine macrophages (J774A.1 cell line), A7r5 rat smooth muscle cells, and human umbilical vein endothelial cells (HUVECs) was assessed after incubation for 24 h (J774A.1 and A7r5 cells), or 48 h (HUVECs). In all cases, neither LDL modification nor the concentration of LDL employed had any significant effect on either parameter of cell viability (two-way ANOVA,  $P > 0.05$ ).

*Lipid accumulation in macrophage cells* Incubation of J774A.1 murine macrophages for 24 h with 50–200 µg/ml of LDL which had been modified with 100 mmol/l glucose in the absence or presence of  $\text{Cu}^{2+}$  for up to 14 days, did not result in significantly increased cellular levels of total cholesterol or cholesterol esters, when compared to incubation controls (cells exposed to LDL which had been incubated under similar conditions with EDTA; data not shown). Experiments using 50–200 µg/ml LDL modified by 10 mmol/l methylglyoxal for 24 h, 7 or 14 days, or 100 mmol/l methylglyoxal for up to 7 days, also did not result in significant cholesterol or cholesteryl ester accumulation when compared to incubation controls (Fig. 3a–c). In contrast, LDL modified with 100 mmol/l methylglyoxal for 14 days did give rise to significant lipid accumulation (Fig. 3a–c). LDL (50–200 µg/ml) treated with 1 mmol/l glycolaldehyde, for any of the time periods examined, did not give rise to significant lipid accumulation (Fig. 3d–f), whereas LDL modified for 7 days (but not 24 h) with 10 mmol/l glycolaldehyde gave a significant increase in cellular lipid levels (Fig. 3d–f). LDL modified by 100 mmol/l glycolaldehyde for 24 h or longer, also resulted in significant cellular accumulation of lipids (Fig. 3d–f).

*Lipid accumulation in smooth muscle and endothelial cells* Free cholesterol and cholesterol ester levels in A7r5 cells and HUVECs were not significantly affected by exposure (24 h for A7r5, 48 h for HUVECs) to 0–200 µg/ml LDL, preincubated with 0–100 mmol/l methylglyoxal or glycolaldehyde for 7 days, or 100 mmol/l glucose, in the absence or presence of 1 µmol/l  $\text{Cu}^{2+}$ , for 14 days, when compared to cells incubated with control LDL as determined by two-way ANOVA (analysis of LDL concentration and modification; data not shown). Serum deprivation of A7r5 cells for 48 h, before exposure to the modified LDL for 24 h, also did not significantly affect cell viability, protein, free cholesterol, or total cholesterol ester levels.

## Discussion

Although there is strong evidence for a role for glycation and glycooxidation in the pathogenesis of diabetes-associated macrovascular disease [1, 10], the processes re-

sponsible for the formation of lipid-laden (foam) cells in the artery wall of patients with diabetes have not been fully resolved. Evidence had been presented for elevated levels of both glycooxidation (i.e., radical) and glycation (covalent addition) products on proteins, including the apolipoprotein B-100 protein of LDL, from patients with diabetes [12, 34]. While it is established that oxidation of LDL can result in recognition by macrophage cell scavenger receptors [4], and that glycooxidized LDL can give rise to enhanced lipid uptake (see e.g., [11, 35]), the role of glycation is less clear-cut. Agents that modify lysine side chains (e.g., by acetylation, succinylation, or reaction with malondialdehyde [6, 36]), can generate LDL particles that are recognized by macrophage scavenger receptors, with modification of >16% of the lysine residues required [37]. Similar data have not been obtained for glycation reactions, although use of AGE-specific antibodies (which may recognize both glycation and glycooxidation products) have identified epitopes near the LDL receptor binding site as key sites [38, 39].

We have previously determined conditions that give rise to LDL glycation in the absence of significant oxidation of protein, lipid, cholesterol, or antioxidant components [23]. Glycation of the apoB protein of LDL by methylglyoxal and glycolaldehyde has also been shown to be more rapid than with glucose [23], in line with previous studies (e.g., [40]). The current study has examined whether these glycated, but nonoxidized, LDL particles can give rise to lipid loading in murine macrophage-like (J774A.1) cells, smooth muscle (A7r5) cells, and endothelial cells (HUVECs).

The incubation conditions employed in the current study gave extensive LDL glycation in the absence of significant oxidation. Time- and concentration-dependent changes in both the charge and degree of LDL aggregation were induced by methylglyoxal and glycolaldehyde, consistent with the loss of lysine, arginine, and tryptophan side chains detected previously [23]. Slower, and less dramatic, changes in charge and aggregation were detected with 100 mmol/l glucose. The presence of  $\text{Cu}^{2+}$  did not influence these glucose reactions, though evidence has been presented for an accelerated oxidation of glycated and normal LDL by high concentrations of copper and iron at longer incubation times [23, 41, 42]. The rapid modification of LDL by methylglyoxal and glycolaldehyde (with the latter more efficient), but not glucose, is consistent with previous data for LDL [23, 43] and other proteins (e.g., [44–46]).

None of these modified LDL particles affected cell viability or protein levels. Similarly, these glycated particles, irrespective of the modifying agent or extent of modification, did not give rise to enhanced total cholesterol, total cholesterol ester, or percentage cholesterol ester levels when incubated with HUVECs or A7r5 smooth muscle cells. Serum deprivation of the A7r5 cells, to mimic a purported *in vivo* state [47, 48], also did not affect cellular viability, protein, free cholesterol, and total cholesterol ester levels.

In contrast, significant lipid accumulation was detected, in some cases, with the macrophage cell line. The extent and rate of loading was dependent on the nature and concentration of the modifying agent, and the modification time. LDL modified by glucose, with or without  $\text{Cu}^{2+}$ , did not give significant cholesterol or cholesterol ester accumulation, irrespective of the quantity of modified LDL to which the cells were exposed, or the incubation time of the LDL with the glucose. Similar behaviour was observed with LDL modified by low concentrations of methylglyoxal for any time period, or high concentrations of this agent for short periods. LDL modified by 100 mmol/l methylglyoxal for 14 days, gave rise to cholesterol ester accumulation when expressed either as nanomoles per milligram cell protein, or as a percentage of the total cholesterol present as esters. In the latter case, values of up to 25% were obtained. These positive data are in contrast to a previous study where methylglyoxal-modified LDL was found to cause significantly less cholesterol ester synthesis than native, oxidized, or acetylated LDL [43]. This may be due to the lower concentrations and shorter modification times used (<10 mmol/l for 72 h).

Significant cholesterol and cholesterol ester accumulation was detected with glycolaldehyde-modified LDL at early time points (24 h modification, 100 mmol/l aldehyde) and at lower concentrations (e.g., 10 mmol/l glycolaldehyde for >7 days). With this aldehyde, cholesterol ester levels, when expressed as a percentage of total cholesterol, reached ca. 45% using 100 mmol/l, and 34% for 10 mmol/l. These values are similar to those detected in human atherosclerotic lesions [49].

Comparison of these data with the REM and SDS-PAGE studies, and those for amino acid modification determined previously [23], allows information to be determined as to the nature and level of LDL modification required for efficient lipid accumulation in these cells. Both REM and SDS-PAGE appear to be unreliable predictors, when used in isolation, of the ability of the modified LDL to give rise to loading. Thus the REM values for LDL incubated with 10 or 100 mmol/l methylglyoxal are greater than those for the 10 and 100 mmol/l glycolaldehyde-treated LDL after 7 days, yet the methylglyoxal-treated LDL did not give rise to significant loading whereas the glycolaldehyde-treated samples did. With each preparation, significant LDL aggregation was detected, yet only the glycolaldehyde-treated samples gave rise to cholesterol and cholesterol ester accumulation. The extent of modification of some of the individual LDL amino acids (see [23]) appears to correlate more closely with lipid loading. Modification of tryptophan does not appear to correlate with loading, as significant loss of this residue is seen at short incubation times and with low methylglyoxal concentrations (e.g., 10 mmol/l) that do not result in cholesterol ester accumulation. Arginine modification also does not appear to correlate with lipid loading. Thus incubation of LDL with 100 mmol/l glucose results in the loss of 67% of these residues after 14 days—conditions where no cellular accumulation of lipid is seen—whereas LDL treated with 10 mmol/l glycolalde-

hyde after 24 h or 7 days loses only ca. 20% of its arginine, yet extensive lipid accumulation is detected.

The extent of modification of lysine residues appears to be a key factor. These residues are lost to a greater extent, and more rapidly, with glycolaldehyde than methylglyoxal at all concentrations, and no loss was detected with 100 mmol/l glucose, even after 14 days in the presence of  $\text{Cu}^{2+}$ . This incubation time is much longer than the residence time of normal LDL particles in plasma (ca. 2 days [50]), suggesting that the extent of modification induced by glucose *in vivo* is likely to be modest, though it should be noted that the residence time (and hence exposure time) of LDL in the intima of the artery wall may be longer (e.g. [51]). The residence time of LDL in plasma and/or intima may also play a key role in determining the relevance of glycation versus (glyc)oxidation reactions *in vivo*, in addition to other factors (e.g., metal ion levels which might catalyse these reactions).

This conclusion that lysine residues may be a key determinant of the recognition of modified LDL particles by the scavenger receptors of macrophage cells, and hence cellular accumulation, is in accord with studies using human monocyte-derived macrophages, where other reagents (acetic anhydride, succinic anhydride, malondialdehyde) were used to induce lysine modification [36, 37]. These previous studies concluded that >16% modification of the lysine residues was required for scavenger receptor recognition [36, 37], whereas a much higher level appears to be required for the aldehydes studied here, as ca. 40% of the lysine residues were modified by 100 mmol/l methylglyoxal after 24 h, yet cellular uptake was only detected when ca. 80% were lost after 14 days. This may indicate that it is the products of these reactions, rather than loss of the parent amino acid *per se*, which is the key determinant.

The extent of lysine modification achieved in this study is greater than that detected on LDL isolated from control or diabetic plasma, where levels of 2–5% have been reported [52]. The products of this modification are not fully elucidated. It has been reported that modified LDL isolated from diabetic plasma can stimulate cholesteryl ester synthesis in human monocyte-derived macrophages [53]—consistent with the current results. The extent of modification of lysine residues on LDL isolated from human atherosclerotic lesions has not been reported, so it is not possible to judge the extent, or type, of modification of LDL to which macrophage cells might be exposed in the intima of the artery wall.

Overall, these studies have demonstrated that LDL glycation, in the absence of significant oxidation, is sufficient to induce lipid loading in a murine macrophage cell line. Significant accumulation only occurs with LDL modified by aldehydes, and not glucose, and requires extensive modification of the lysine residues of the apoB-100 protein; the extent of lipid loading achieved is similar to that detected in atherosclerotic lesions. This process is specific to macrophages, and does not occur with the smooth muscle cell line or primary endothelial cells, and is consistent with the presence of receptors for glycated LDL only on macrophage cells. The uptake of aldehyde-modified LDL

by macrophages within lesions may play a role in the enhanced atherosclerosis observed in patients with diabetes, as the concentrations of these aldehydes are known to be elevated in such patients [18, 19]. Though the in vivo concentrations of these aldehydes are lower than those employed here, these in vivo numbers presumably reflect the low (steady-state) concentrations of these reactive carbonyls that have not undergone rapid reaction with plasma proteins; the true flux (rate of generation and removal) of these aldehydes has yet to be established.

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