

The impact of glycation on apolipoprotein A-I structure and its ability to activate lecithin:cholesterol acyltransferase

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Abstract

Aims/hypothesis Hyperglycaemia, one of the main features of diabetes, results in non-enzymatic glycation of plasma proteins, including apolipoprotein A-I (apoA-I), the most abundant apolipoprotein in HDL. The aim of this study was to determine how glycation affects the structure of apoA-I and its ability to activate lecithin:cholesterol acyltransferase (LCAT), a key enzyme in reverse cholesterol transport.

Materials and methods Discoidal reconstituted HDL (rHDL) containing phosphatidylcholine and apoA-I ([A-I] rHDL) were prepared by the cholate dialysis method and glycated by incubation with methylglyoxal. Glycation of apoA-I was quantified as the reduction in detectable arginine, lysine and tryptophan residues. Methylglyoxal-AGE adduct formation in apoA-I was assessed by immu-

noblotting. (A-I)rHDL size and surface charge were determined by non-denaturing gradient gel electrophoresis and agarose gel electrophoresis, respectively. The kinetics of the LCAT reaction was investigated by incubating varying concentrations of discoidal (A-I)rHDL with a constant amount of purified enzyme. The conformation of apoA-I was assessed by surface plasmon resonance.

Results Methylglyoxal-mediated modifications of the arginine, lysine and tryptophan residues in lipid-free and lipid-associated apoA-I were time- and concentration-dependent. These modifications altered the conformation of apoA-I in regions critical for LCAT activation and lipid binding. They also decreased (A-I)rHDL size and surface charge. The rate of LCAT-mediated cholesterol esterification in (A-I)rHDL varied according to the level of apoA-I glycation and progressively decreased as the extent of apoA-I glycation increased.

Conclusions/interpretation It is concluded that glycation of apoA-I may adversely affect reverse cholesterol transport in subjects with diabetes.

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Abbreviations

(A-I) rHDL	reconstituted HDL containing phosphatidylcholine and apoA-I
(A-I) rHDL _{glyc}	reconstituted HDL containing non-enzymatically glycated apoA-I
apoA-I	apolipoprotein A-I
CE	cholesteryl ester
DOPA	3,4-dihydroxy phenylalanine
k_{arg}	rate constant for MG-mediated modification of apoA-I arginine

k_{lys}	rate constant for MG-mediated modification of apoA-I lysine
k_{trp}	rate constant for MG-mediated modification of apoA-I tryptophan
LCAT	lecithin:cholesterol acyltransferase
mAb	monoclonal antibody
MG	methylglyoxal
POPC	1-palmitoyl-2-oleoyl phosphatidylcholine
rHDL	reconstituted HDL
UC	unesterified cholesterol
[^3H]UC	1 α ,2 α - ^3H -labelled unesterified cholesterol

Introduction

Macrovascular complications are responsible for up to 80% of the deaths in subjects with diabetes [1]. There is a large body of evidence to suggest that this is partly due to non-enzymatic protein glycation and AGE formation. AGE have been implicated in the development of premature atherosclerosis in diabetic subjects by virtue of their ability to increase endothelial dysfunction and decrease endothelial cell viability and smooth muscle cell proliferation [2, 3].

Chronic hyperglycaemia, a feature of both type 1 and type 2 diabetes, generates highly reactive and toxic α -oxoaldehydes, such as methylglyoxal (MG), glyoxal and 3-deoxyglucosone [4]. Covalent binding of these oxoaldehydes to reactive lysine, arginine and cysteine residues as well as to N-terminal amino groups in plasma proteins and apolipoproteins, including apolipoprotein A-I (apoA-I), the major apolipoprotein of HDL, generates early glycation adducts and AGE [5–8]. These modifications are associated with protein cross-linking and aggregation [9]. It has been suggested that MG-derived, as well as other AGE, not only accelerate the development of coronary artery disease in humans [10], but are also responsible for the macro- and microvascular complications frequently observed in diabetes.

The impact of apoA-I glycation on HDL function is poorly understood. Some evidence suggests that glycation adversely affects HDL metabolism by diminishing the binding of HDL to fibroblasts and macrophages [11, 12] and enhancing HDL catabolism [13]. Glycation also increases cholesteryl ester (CE) transfer protein-mediated transfers of CEs from HDL to apoB-containing lipoproteins [14] and alters the ability of lecithin:cholesterol acyltransferase (LCAT) to esterify cholesterol [15].

LCAT is the enzyme that generates almost all of the CEs in plasma. It plays a key role in reverse cholesterol transport and is activated by the apoA-I in HDL [16]. The impact of diabetes on the ability of HDL to act as a substrate for LCAT

is unclear, with reports suggesting that it either diminishes [17], does not alter [18] or increases the rate of LCAT-mediated cholesterol esterification [19]. These discrepancies are probably due to variations in the study populations and the methods used to measure cholesterol esterification [15]. Although there is evidence suggesting that glycation of apoA-I in HDL impairs the LCAT reaction in vivo and in vitro [20–22], the underlying mechanism of these observations are not understood. These issues are addressed in the present study using preparations of discoidal reconstituted HDL (rHDL) containing apoA-I and phosphatidylcholine ([A-I]rHDL). These preparations are structurally and functionally indistinguishable from the discoidal HDL that are the main LCAT substrates in plasma.

Materials and methods

Isolation of apolipoprotein A-I xHDL were isolated from pooled samples of autologously donated human plasma (Gribbles Pathology, Adelaide, SA, Australia) by sequential ultracentrifugation ($1.063 < d < 1.21$ g/ml). The HDL were delipidated and apoA-I was isolated by anion exchange chromatography on a Q sepharose fast flow column (GE Healthcare, Uppsala, Sweden) attached to a fast protein liquid chromatography system [23–25]. The apoA-I appeared as a single band when subjected to SDS-Phastgel electrophoresis (GE Healthcare) and Coomassie staining.

Preparation of discoidal (A-I)rHDL labelled with unesterified [^3H]cholesterol Discoidal (A-I)rHDL containing 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) (Avanti Polar Lipids, Alabaster, AL, USA), unesterified cholesterol (UC) (Sigma-Aldrich, St Louis, MO, USA), a trace amount of 1 α ,2 α - ^3H -labelled UC ([^3H]UC; GE Healthcare) and apoA-I (initial POPC:UC:apoA-I molar ratio 100:10:1) were prepared by the cholate dialysis method [26]. The (A-I)rHDL were dialysed against PBS (pH 7.4) containing 0.006% (w/v) NaN_3 and 0.005% (w/v) EDTA- Na_2 before use.

Isolation of LCAT LCAT was isolated from pooled samples of human plasma as described [23] and appeared as a single band following SDS-Phastgel electrophoresis and silver staining. LCAT activity was assessed using [^3H]UC-labelled discoidal (A-I)rHDL as the substrate [27]. The LCAT preparations used in this study generated 346–1,600 nmol CE ml LCAT $^{-1}$ h $^{-1}$.

Glycation of lipid-free apoA-I by glucose Lipid-free apoA-I (final concentration 1 mg/ml) was incubated at 37°C under 5% CO_2 for 3 days to 3 weeks with 5, 25 or 100 mmol/l glucose

(Sigma). Unreacted glucose was removed by dialysis against PBS.

Glycation of lipid-free apoA-I and discoidal (A-I)rHDL by methylglyoxal Lipid-free apoA-I and discoidal (A-I)rHDL (final apoA-I concentration 1 mg/ml) were incubated individually at 37°C under 5% CO₂ for 0.5–24 h with either 0, 0.5, 1.5, 3, 15 or 30 mmol/l MG (Sigma). When the incubations were complete, unreacted MG was removed by dialysis against PBS.

ApoA-I arginine, lysine and tryptophan modification MG-mediated modification of apoA-I arginine, lysine and tryptophan residues was assessed fluorometrically and expressed as a percentage of the values obtained for unmodified samples incubated with PBS [28]. Rate constants for MG-mediated modification of apoA-I arginine (k_{arg}), lysine (k_{lys}) and tryptophan (k_{trp}) residues in discoidal (A-I)rHDL were derived by non-linear regression analysis (GraphPad Prism version 4.0; GraphPadSoftware, San Diego, CA, USA) using the equation:

$$\% \text{ of unmodified residues} = 100 \times e^{-k_{\text{amino acid}} \times t} \quad (1)$$

where t is the duration of the incubation with MG. The results are expressed as h^{-1} .

Determination of discoidal (A-I)rHDL surface charge Discoidal (A-I)rHDL containing non-enzymatically glycosylated apoA-I, (A-I)rHDL_{glyc}, were electrophoresed at 90 V for 45 min on 1% agarose gels (Helena Laboratories, Mt Waverly, VIC, Australia), fixed in methanol, stained with Coomassie Blue R and destained with water::acetic acid::isopropanol (77.5::10::12.5, v::v::v). Electrophoretic mobilities were calculated as described [29]

$$\text{Mobility} = \frac{\text{Migration distance } (\mu\text{m})/\text{time(s)}}{\text{Voltage(V)}/\text{Length of gel(cm)}} \quad (2)$$

and corrected for pI-dependent retardation effects according to the following relationship:

$$\text{Mobility}_{\text{corrected}} = \left(\text{Mobility}_{\text{agarose}} - 0.136 \right) / 1.21 \quad (3)$$

Conformation of unmodified and glycosylated apoA-I in the lipid-free form and in discoidal (A-I)rHDL A Biacore 2000 biosensor (GE Healthcare, Uppsala, Sweden) was used to measure the association rate constant (k_a) of seven unique apoA-I-specific monoclonal antibodies (mAbs) for rHDL. Saturating amounts of rabbit anti-mouse Fc were immobilised on all four flow cells of a CM5 chip using amine coupling [30]. Injected isotype control or specific mAbs were captured by the rabbit anti-mouse Fc at dilutions predetermined to give 400 response units. This was followed by injection of the rHDL (analyte). The rHDL injections were made on the basis of apoA-I molarity.

Data were collected at a high (5 Hz) data collection rate. Evaluation of the data began by synchronising the injection time and zeroing the baselines of the sensorgram. Control antibody sensorgrams were subtracted from each specific antibody sensorgram. Each antibody and rHDL combination was evaluated with a 1:1 (Langmuir) model.

Kinetic studies Various concentrations of discoidal (A-I)rHDL and discoidal (A-I)rHDL_{glyc} containing unlabelled UC (final UC concentration 0.5–14.0 $\mu\text{mol/l}$) and a tracer amount of [³H]UC were incubated at 37°C for 1 h in a shaking water-bath with a constant amount of LCAT, fatty acid-free BSA (final concentration 3.7 mg/ml), β -mercaptoethanol (final concentration 5.3 mmol/l) and Tris-buffered saline (pH 7.4). The final volume of the incubation mixtures was 135 μl . The incubations were carried out in triplicate on three separate occasions. Cholesterol esterification was quantitated as described [27]. The kinetic parameters, $K_{\text{m(app)}}$ and V_{max} , were determined by non-linear regression analysis (GraphPad Prism) using the equation

$$V = (V_{\text{max}} \times [\text{UC}]) / (K_{\text{m(app)}} + [\text{UC}]) \quad (4)$$

where V is the rate of cholesterol esterification. Results are expressed as $\mu\text{mol CE formed ml LCAT}^{-1} \text{ h}^{-1}$.

Competition studies [³H]UC-labelled discoidal (A-I)rHDL (final UC concentration 0.5–5 $\mu\text{mol/l}$) was mixed with varying amounts of unlabelled discoidal (A-I)rHDL_{glyc} (final UC concentration 0, 1.5 or 3 $\mu\text{mol/l}$) and incubated with a fixed amount of LCAT. The rate of cholesterol esterification in the [³H]UC-labelled discoidal (A-I)rHDL was determined according to equation 5 [31].

$$\nu = \frac{V_{\text{max}}[{}^3\text{H}]\text{UC} - (\text{A-I})\text{rHDL}}{K_{\text{m(app)}}(1 + [(\text{A-I})\text{rHDL}_{\text{glyc}}]/K_i) + [{}^3\text{H}]\text{UC} - (\text{A-I})\text{rHDL}(1 + [(\text{A-I})\text{rHDL}_{\text{glyc}}]/K_i)} \quad (5)$$

where: (1) v is the predicted rate of cholesterol esterification ($\mu\text{mol CE formed ml LCAT}^{-1} \text{ h}^{-1}$); (2) V_{max} and $K_{\text{m(app)}}$ are the kinetic parameters for [^3H]UC-labelled discoidal (A-I)rHDL incubated with LCAT in absence of unlabelled (A-I)rHDL_{glyc}; and (3) [^3H]UC-labelled (A-I)rHDL and [(A-I)rHDL_{glyc}] represent the UC concentrations of the unmodified (A-I)rHDL and (A-I)rHDL_{glyc}, respectively. K_i is the inhibitor constant for [^3H]UC-labelled discoidal (A-I)rHDL in presence of unlabelled (A-I)rHDL_{glyc}, and was calculated as follows:

$$K_i = \frac{[(\text{A-I})\text{rHDL}_{\text{glyc}}]}{(V_{\text{max}}/V_{\text{max (app)}}) - 1} \quad (6)$$

where $V_{\text{max(app)}}$ is the V_{max} for the unmodified (A-I)rHDL incubated with LCAT in presence of different concentrations of (A-I)rHDL_{glyc}.

Other techniques All chemical analyses were performed in triplicate on an automatic analyser (Hitachi 902; Roche Diagnostics, Mannheim, Germany). UC and POPC concentrations were measured enzymatically [32, 33]. ApoA-I concentrations were determined by the bicinchoninic assay using BSA as a standard [34].

Protein oxidation was determined by HPLC as the formation of the protein-bound tyrosine and phenylalanine oxidation products 3,4-dihydroxy phenylalanine (DOPA), *o-o'*-di-tyrosine and *o*-tyrosine [28]. Lipid hydroperoxides were quantified using a lipid hydroperoxide assay kit (Cayman Chemicals, Ann Arbor, MI, USA). Protein cross-linking was assessed by SDS-PAGE. Autofluorescence (excitation 370 nm, emission 440 nm) of cross-linked proteins was determined with a Cytofluor II fluorimeter (Perspective Biosystems, Framingham, MA, USA). Results are expressed in arbitrary fluorescent units (FU) after blank subtraction.

Discoidal (A-I)rHDL diameters were determined by 3–40% non-denaturing polyacrylamide gradient gel electrophoresis and staining with Coomassie Blue R [35]. The stained gels were scanned with a laser densitometer. Particle sizes were determined by reference to high-molecular mass standards of known diameter.

MG-AGE adducts were detected by incubating apoA-I with MG, removing unreacted MG by dialysis against PBS, electrophoresing the incubation mixture on a 20% homogeneous SDS Phastgel (GE Healthcare) and immunoblotting with a 1:1,000 dilution of an anti-MG-AGE mAb (Biologo, Kronshagen, Germany). The blots were developed by electrochemoluminescence (GE Healthcare). Carboxyethyllysine and carboxymethyllysine were quantified by gas chromatography/mass spectrometry and pentosidine levels were quantitated by HPLC as described [36–38].

Statistical analyses Significant differences in apoA-I residue modifications were determined by one-way ANOVA with Tukey's multiple comparison post-test analysis. Two-way ANOVA was used to assess differences in cholesterol esterification rates. Surface plasmon resonance data were compared with a two-tailed, unpaired *t* test. Graphpad Prism (GraphPad Prism Software Inc., San Diego, CA, USA) was used for all analyses. Significance was set at $p < 0.05$.

Results

Modification of lipid-free apoA-I arginine, lysine and tryptophan residues by glucose Compared with lipid-free apoA-I incubated with a physiological, non-pathological concentration of glucose (5 mmol/l), 1 week of incubation with either 25 or 100 mmol/l glucose did not modify apoA-I significantly. After 3 weeks of incubation with 25 mmol/l glucose, 6% of the apoA-I lysine residues were modified, but the arginine and tryptophan residues were not affected (data not shown). After 2 weeks of incubation with 100 mmol/l glucose, 10% of the apoA-I lysine residues were modified, while 30% of the lysine residues and approximately 8% of the arginine and 6% of the tryptophan residues were modified at 3 weeks. Given that the half-life of apoA-I in plasma is 3–4 days [39], it was concluded that these modifications are unlikely to be of physiological or pathological significance.

Modification of lipid-free apoA-I and the apoA-I in discoidal (A-I)rHDL by MG: time and concentration dependence Additional experiments were carried out to determine if the reactive dicarbonyl derivative of glucose, MG, modifies apoA-I arginine, lysine and tryptophan residues more rapidly than glucose. When lipid-free apoA-I was incubated for 24 h with 1.5 mmol/l MG, the arginine, lysine and tryptophan residues were significantly modified ($p < 0.001$) (Table 1). More extensive modifications were apparent when lipid-free apoA-I was incubated with 15 mmol/l MG ($p < 0.001$ compared with 1.5 mmol/l MG). The apoA-I lysine and tryptophan, but not the arginine residues, were modified even further by incubation with 30 mmol/l MG ($p < 0.001$ compared with 15 mmol/l MG). As these modifications impaired the ability of lipid-free apoA-I to associate with phospholipids, these preparations could not be used to generate discoidal rHDL.

To investigate the effects of apoA-I glycation on HDL structure and function, it was therefore necessary to incorporate apoA-I into discoidal rHDL prior to incubation with MG. When discoidal (A-I)rHDL were incubated for 1 h with 3 mmol/l MG, the apoA-I arginine, lysine and

Table 1 Methylglyoxal-mediated modification of lipid-free apoA-I

Incubation conditions	Unmodified residues (%)		
	Arginine	Lysine	Tryptophan
0 mmol/l MG, 37°C, 24 h	100	100	100
1.5 mmol/l MG, 37°C, 24 h	62.8±1.4*	84.7±0.6*	90.8±1.2*
15 mmol/l MG, 37°C, 24 h	48.5±3.3**	66.8±0.4**	70.7±0.6**
30 mmol/l MG, 37°C, 24 h	45.6±2.0	45.4±1.3***	56.1±0.0***

Lipid-free apoA-I (final concentration 1 mg/ml) was incubated at 37°C for 24 h in absence or presence of 1.5, 15 or 30 mmol/l MG. The final volume of the incubation mixtures was 4 ml. All data represent the mean±SEM ($n=3$).

* $p<0.001$ compared with unmodified, lipid-free apoA-I; ** $p<0.001$ compared with incubation with 1.5 mmol/l MG; *** $p<0.001$ compared with incubation with 15 mmol/l MG.

tryptophan residues were extensively modified ($p<0.05$ for lysine, $p<0.01$ for arginine and $p<0.001$ for tryptophan compared with [A-I]rHDL incubated for 1 h in the absence of MG) (Table 2). Further lysine and tryptophan, but not arginine, modifications were observed in incubations carried out for 1 h with 30 mmol/l MG.

The apoA-I arginine, lysine and tryptophan residues were not significantly modified when discoidal (A-I)rHDL were incubated with 0.5 mmol/l MG for 24 h (Table 2). By contrast, significant apoA-I modifications were apparent when the rHDL were incubated for 24 h with 1.5 mmol/l MG ($p<0.01$ for the arginine, lysine and tryptophan residues in [A-I]rHDL), 3 mmol/l MG ($p<0.01$ for the lysine residues in [A-I]rHDL; $p<0.001$ for the arginine and tryptophan residues in [A-I]rHDL) or 30 mmol/l MG ($p<0.001$ for the arginine, lysine and tryptophan residues in [A-I]rHDL). Comparison of the results in Tables 1 and 2 shows that, during a 24-h incubation, twice as much MG was required to achieve comparable modification of the arginine, lysine and tryptophan residues in the apoA-I in discoidal (A-I)rHDL as in lipid-free apoA-I.

The rate of modification of apoA-I arginine, lysine and tryptophan residues was determined by incubating discoidal rHDL in the absence or presence of 3 mmol/l MG. The arginine residues were modified more rapidly ($k_{\text{arg}}=0.34\pm 0.05 \text{ h}^{-1}$) than the lysine ($k_{\text{lys}}=0.11\pm 0.02 \text{ h}^{-1}$) ($p<0.001$) and tryptophan residues ($k_{\text{trp}}=0.045\pm 0.02 \text{ h}^{-1}$) ($p<0.01$) (Fig. 1).

Effects of glycation on the physical properties of apoA-I in discoidal (A-I)rHDL Incubation with MG did not affect discoidal (A-I)rHDL stoichiometry (Table 3). However, the diameter of the particles decreased progressively from 9.7 to 9.0 nm (not shown), probably as a consequence of alterations in the conformation of apoA-I (see below). As judged by agarose gel electrophoresis, the rHDL surface also became more negatively charged as the apoA-I became progressively glycated (Table 3).

To ensure that the changes in rHDL surface charge and particle size were not due to glycooxidation, POPC and apoA-I oxidation was assessed as the formation of lipid hydroperoxides and protein-bound DOPA, *o-o'*-di-tyrosine

Table 2 Methylglyoxal-mediated modification of discoidal (A-I)rHDL: time and concentration dependence

Incubation conditions	Unmodified residues (%)		
	Arginine	Lysine	Tryptophan
0 mmol/l MG, 37°C, 1 h	100	100	100
3 mmol/l MG, 37°C, 1 h	80.3±2.5 ^c	92.7±1.9 ^a	97.3±0.6 ^b
30 mmol/l MG, 37°C, 1 h	77.1±0.1 ^c	85.7±1.4 ^{c,h}	91.5±0.2 ^{c,j}
0 mmol/l MG, 37°C, 24 h	100	100	100
0.5 mmol/l MG, 37°C, 24 h	106.7±5.5	93.3±1.7	102.7±1.2
1.5 mmol/l MG, 37°C, 24 h	82.9±2.1 ^{b,e}	84.4±3.9 ^b	92.2±1.3 ^{b,e}
3 mmol/l MG, 37°C, 24 h	67.5±0.8 ^{c,e,f}	83.5±0.6 ^{b,d}	87.9±0.4 ^{c,e}
30 mmol/l MG, 37°C, 24 h	48.4±1.5 ^{c,e,g,i}	62.7±1.9 ^{c,e,g,j}	65.9±0.4 ^{c,e,g,j}

Discoidal (A-I)rHDL (final apoA-I concentration 1 mg/ml) were incubated at 37°C for 1 or 24 h in absence or presence of 0.5, 1.5, 3 or 30 mmol/l MG. The final volume of the incubation mixtures was 4 ml. All data represent the mean±SEM ($n=3$).

^a $p<0.05$, ^b $p<0.01$, ^c $p<0.001$ compared with unmodified (A-I)rHDL.

^d $p<0.05$, ^e $p<0.001$ compared with (A-I)rHDL incubated with 0.5 mmol/l MG

^f $p<0.05$, ^g $p<0.001$ compared with (A-I)rHDL incubated with 1.5 mmol/l MG

^h $p<0.05$, ⁱ $p<0.01$, ^j $p<0.001$ compared with (A-I)rHDL incubated with 3 mmol/l MG

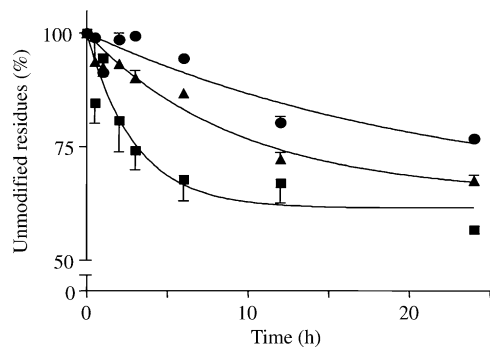


Fig. 1 Methylglyoxal-mediated modification of discoidal (A-I)rHDL: time dependence. Discoidal (A-I)rHDL (final apoA-I concentration 1 mg/ml) were incubated at 37°C for 0.5–24 h with 3 mmol/l MG (final volume 4 ml). The percentage of unmodified arginine (*square*), lysine (*triangle*) and tryptophan (*circle*) residues was determined as described in **Materials and methods**. All data represent the mean±SEM ($n=3$)

and *o*-tyrosine, respectively. The results for the unmodified and glycosylated particles were comparable, indicating that the changes in (A-I)rHDL size and surface charge were due to glycation and not to glycooxidation (data not shown).

To determine whether incubation with MG produced cross-linked apoA-I, discoidal (A-I)rHDL were incubated in absence or presence of 3 mmol/l MG for 1, 2, 6, 12 or 24 h (Fig. 2). As judged by SDS-PAGE, incubation with MG progressively decreased the concentration of monomeric apoA-I in association with a concomitant increase in oligomer formation (Fig. 2). The increase in discoidal (A-I)rHDL autofluorescence (Table 3), indicated that the oligomers represent cross-linked, rather than aggregated apoA-I. Comparable results were obtained when lipid-free apoA-I was incubated with MG, indicating that the cross-linking could not be attributed to the lipid content of the particles (data not shown).

To determine whether AGE adducts were formed when apoA-I was incubated with MG, lipid-free apoA-I was incubated for 24 h in the presence or absence of

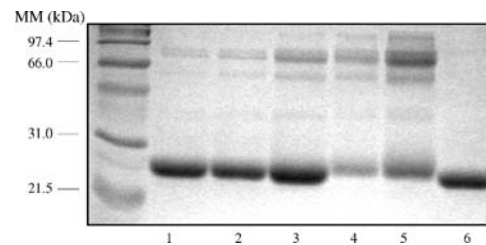


Fig. 2 Methylglyoxal-mediated cross-linking of apoA-I in discoidal (A-I)rHDL. Discoidal (A-I)rHDL (final apoA-I concentration 1 mg/ml) were incubated at 37°C in the absence (Track 6) or presence of 3 mmol/l MG for 1, 2, 6, 12 or 24 h (tracks 1–5 respectively). A track containing low molecular mass standards is shown on the left (unnumbered track). The samples were delipidated, subjected to SDS-PAGE and stained with Coomassie Blue R. A scan of a representative stained gel is shown. MM, molecular mass

1.5 mmol/l MG, subjected to SDS-PAGE and immunoblotted with an mAb that recognises MG-AGE adducts (Fig. 3). Adducts were evident in the apoA-I that had been incubated in the presence, but not in that incubated in the absence of MG. As judged by gas chromatography/mass spectrometry (for carboxymethyllysine and carboxyethyllysine) and HPLC (for pentosidine), the apoA-I that had been incubated with MG contained 0.19 mmol carboxyethyllysine/mol lysine, 0.07 mmol carboxymethyllysine/mol lysine and 417 pmol pentosidine/mg apoA-I.

Effects of glycation on the conformation of apoA-I in the lipid-free form and in discoidal (A-I)rHDL Surface plasmon resonance was used to assess the binding of a panel of seven well defined, epitope-specific apoA-I mAbs to discoidal (A-I)rHDL that had been incubated for 24 h in the absence or presence of 30 mmol/l MG (Fig. 4a). Lipid-free apoA-I was incubated for 24 h in the absence or presence of 15 mmol/l MG (Fig. 4b). The discoidal (A-I)rHDL were incubated with twice as much MG as the lipid-free apoA-I to ensure that the arginine, lysine and tryptophan residues in both of the samples were modified

Table 3 Physical characterisation of discoidal (A-I)rHDL after incubation in the presence and absence of MG

Incubation conditions	Stoichiometry POPC:UC:apoA-I (mol:mol:mol)			Electrophoretic mobility ($\mu\text{m s}^{-1} \text{cm V}^{-1}$)	Autofluorescence (FU)
No MG, 37°C, 24 h	109	8.7	1	-0.29	0
MG, 37°C, 0.5 h	109	8.8	1	-0.33	1
MG, 37°C, 1 h	85	9.7	1	n.d.	2
MG, 37°C, 2 h	111	8.6	1	-0.34	n.d.
MG, 37°C, 3 h	112	8.9	1	-0.42	15
MG, 37°C, 6 h	111	8.9	1	-0.51	n.d.
MG, 37°C, 12 h	106	8.5	1	-0.55	17
MG, 37°C, 24 h	102	9.7	1	n.d.	36

Discoidal (A-I)rHDL (final apoA-I concentration 1.0 mg/ml) were incubated at 37°C for 0.5–24 h with 3 mmol/l MG, or for 24 h in the absence of MG. The final volume of the incubation mixtures was 4 ml.

n.d., not determined; FU, arbitrary fluorescence units

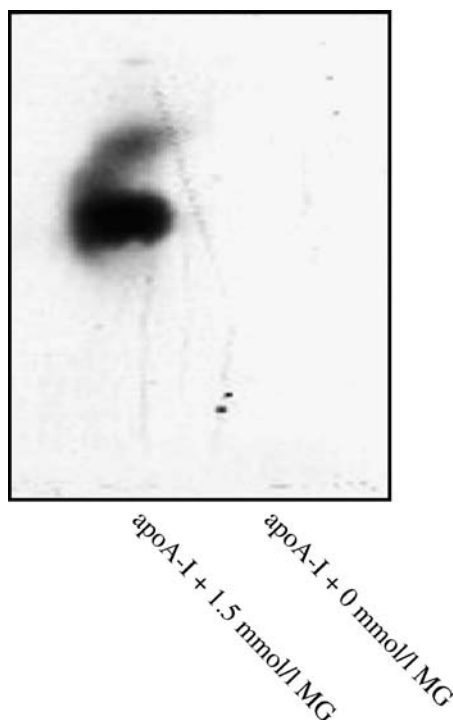


Fig. 3 Immunoblot of methylglyoxal-modified lipid-free apoA-I. Lipid-free apoA-I (final concentration 1 mg/ml) was incubated for 24 h at 37°C in the presence or absence of MG (final concentration 1.5 mmol/l). When the incubations were complete the samples were subjected to SDS-PAGE and immunoblotted for MG-AGE adducts as described under [Materials and methods](#)

to the same extent (see [Tables 1 and 2](#)). Incubation with MG dramatically altered the affinity of all the mAbs for their epitopes in lipid-associated apoA-I. With the exception of mAb AI-11, this was also the case for lipid-free apoA-I. The affinity of all seven mAbs for their epitopes in lipid-free apoA-I differed from the finding for lipid-associated apoA-I, confirming that association with lipid alters the conformation of apoA-I.

Effect of glycation on the ability of apoA-I to activate LCAT: time and concentration dependence Aliquots of discoidal (A-I)rHDL were incubated for up to 24 h with 3 mmol/l MG. Increasing amounts of the resulting (A-I)rHDL_{glyc} were then incubated for 1 h with a constant amount of LCAT ([Fig. 5](#)). The (A-I)rHDL_{glyc} that had been incubated for 1 h with MG exhibited a slight, non-statistically significant increase in the rate of cholesterol esterification compared with unmodified (A-I)rHDL ([Fig. 5](#)). When (A-I)rHDL that had been incubated with MG for 2, 3 and 6 h were subsequently incubated with LCAT, the rate of cholesterol esterification progressively decreased relative to the control rHDL ($p < 0.0001$) ([Fig. 5](#)).

The V_{\max} for the (A-I)rHDL_{glyc} that were incubated with MG for 3 or 6 h was significantly decreased compared with the control (A-I)rHDL and the (A-I)rHDL_{glyc} that were

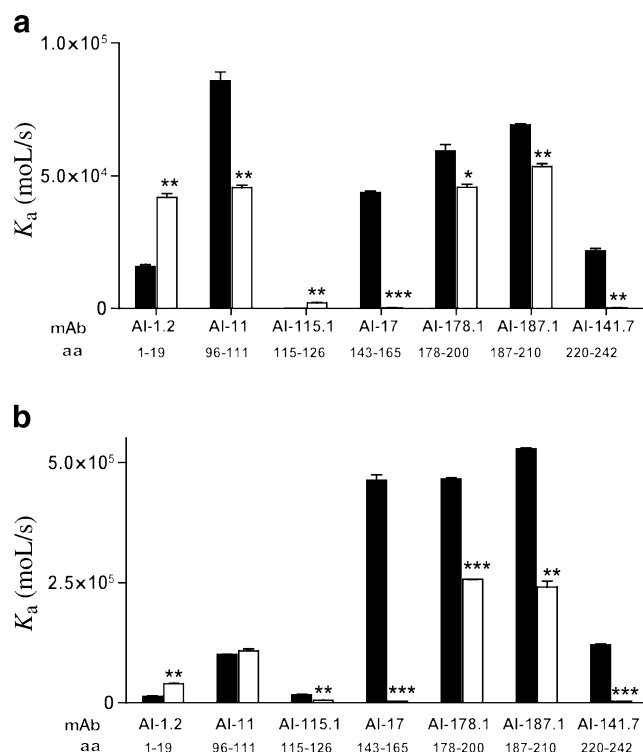


Fig. 4 Effect of glycation on the conformation of lipid-free and lipid-associated apoA-I. Discoidal (A-I)rHDL (**a**) and lipid-free apoA-I (**b**) were incubated at 37°C for 24 h in the absence (*closed bars*) or presence (*open bars*) of 30 and 15 mmol/l MG, respectively. Surface plasmon resonance was used to determine association rate constants (k_a) of seven unique, apoA-I-specific mAbs for the lipid-free and lipid-associated apoA-I. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus controls incubated in the absence of MG. aa, amino acid

incubated with MG for 1 h ([Table 4](#)). The affinity, $K_{m(\text{app})}$, of LCAT for (A-I)rHDL was independent of the duration of the incubation with 3 mmol/l MG. Kinetic parameters could not be determined for rHDL incubated with MG for more than 12 h because the rate of cholesterol esterification was very low. The ability of the discoidal (A-I)rHDL that had been incubated with a more physiologically relevant MG concentration (0.5 mmol/l) to esterify cholesterol was also significantly impaired compared with control (A-I)rHDL ([Table 5](#)).

Competitive inhibition of cholesterol esterification in unmodified (A-I)rHDL by (A-I)rHDL_{glyc} The preceding kinetic studies suggested that discoidal (A-I)rHDL_{glyc} may competitively inhibit LCAT-mediated cholesterol esterification in unmodified discoidal (A-I)rHDL. This possibility was investigated by incubating discoidal (A-I)rHDL with 3 mmol/l MG for 24 h. When the resulting (A-I)rHDL_{glyc} (final UC concentration 1.5 or 3 μmol/l) were incubated with unmodified [³H]UC-labelled (A-I)rHDL (final UC concentration 0.5–5 μmol/l) and a constant amount of LCAT, the rate of cholesterol esterification in the [³H]UC-labelled (A-I)rHDL decreased as the concentration of

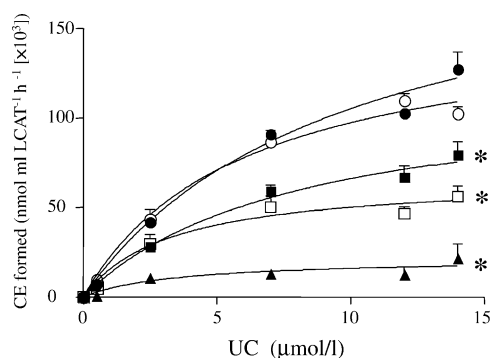


Fig. 5 LCAT-mediated cholesterol esterification in discoidal (A-I)rHDL and discoidal (A-I)rHDL_{glyc}. Discoidal (A-I)rHDL were incubated at 37°C in the absence (open circles) or presence of 3 mmol/l MG for 1 (closed circles), 2 (closed squares), 3 (open squares) and 6 h (closed triangles). The rHDL (0.5–14 μmol/l UC) were then incubated at 37°C for 1 h with LCAT (5 μl of 1/50 dilution of a preparation that esterified 1,600 nmol CE ml LCAT⁻¹ h⁻¹) and the rate of cholesterol esterification was determined. Values represent the mean±SEM of triplicate determinations. **p*<0.0001 versus (A-I)rHDL incubated in the absence of MG

unlabelled (A-I)rHDL_{glyc} in the incubation mixture increased (Table 6).

The values obtained from Eqs. (4) and (5), which predicted the ability of (A-I)rHDL_{glyc} to decrease the rate of cholesterol esterification in the unmodified [³H]UC-labelled (A-I)rHDL, agreed closely with the experimentally determined values (Table 6). This indicates that (A-I)rHDL_{glyc} competitively inhibits LCAT-mediated cholesterol esterification in unmodified, [³H]UC-labelled (A-I)rHDL.

Discussion

Many plasma proteins and apolipoproteins, including apoA-I, are non-enzymatically glycosylated in both type 1 and type 2 diabetes [7]. Given that protein glycation is closely associated with adverse metabolic outcomes, as evidenced

by the strong association of HbA_{1c} concentrations with cardiovascular disease and mortality in the EPIC-Norfolk study [40], it is important to understand the underlying mechanisms of these modifications and to identify exactly which metabolic processes are adversely affected.

A key aim of the present study was to determine how glycation with MG influences the ability of apoA-I to activate LCAT, a plasma factor that plays a key role in HDL metabolism and reverse cholesterol transport [41]. Plasma MG levels as high as 0.4–0.5 mmol/l have been reported in diabetic subjects, irrespective of the level of glycaemic control [42, 43]. The present studies show that, although incubation of discoidal (A-I)rHDL with 0.5 mmol/l MG, does not appreciably modify the apoA-I arginine, lysine and tryptophan residues, it does markedly impair the ability of the discs to act as substrates for LCAT (Tables 2 and 5). These observations raise the possibility that minor modifications of apolipoproteins that are mediated by α-oxoaldehyde could have a major impact on HDL function in vivo.

In order to investigate LCAT activation, it was important to use apoA-I-containing discoidal HDL, the primary substrates for LCAT in human plasma [16]. As sufficient amounts of these particles cannot be isolated readily from normolipidaemic plasma, well characterised preparations of discoidal (A-I)rHDL that are structurally and functionally comparable to their plasma counterparts were used for the study [44]. As judged by agarose gel electrophoresis and surface plasmon resonance, incubation with MG altered both the surface charge, and the conformation of apoA-I in the discoidal rHDL. The change in surface charge was probably due to the modification of lysine and arginine residues reducing the overall positive charge of apoA-I. Similar changes in surface charge have been reported for MG-modified LDL [28, 45].

It is likely that changes in rHDL surface charge as well as the conformation of apoA-I both contributed to the decreased ability of discoidal (A-I)rHDL_{glyc} to activate LCAT. Alexander et al. have shown that mutation of negatively charged residues in apoA-I significantly reduces

Table 4 Kinetic parameters for LCAT-mediated cholesterol esterification in discoidal (A-I)rHDL and discoidal (A-I)rHDL_{glyc}: time dependence

	0 mmol/l MG	3 mmol/l MG	3 mmol/l MG	3 mmol/l MG	3 mmol/l MG
Incubation conditions	37°C, 24 h	37°C, 1 h	37°C, 2 h	37°C, 3 h	37°C, 6 h
<i>V</i> _{max} (nmol CE ml LCAT ⁻¹ h ⁻¹)	1615.0±149.9	2069.0±256.9	1185.0±155.0	668.0±77.0 [†]	346.7±133.3 [§]
<i>K</i> _{m(app)} (μmol UC)	6.4±1.4	9.7±2.4	8.0±2.3	3.1±1.3	6.4±5.8

Discoidal (A-I)rHDL (final apoA-I concentration 1 mg/ml) were incubated at 37°C for times as shown with 3 mmol/l MG or for 24 h in the absence of MG then dialysed against PBS. The rHDL (final UC concentration 0.5–14 μmol/l) were then incubated for 1 h with 5 μl of 1/50 dilution of a preparation of LCAT that generated 1600 nmol CE ml LCAT⁻¹ h⁻¹. Kinetic parameters were determined by non-linear regression analysis of the rate of cholesterol esterification versus the concentration of substrate.

* *p*<0.05 compared with unmodified (A-I)rHDL.

[†] *p*<0.05 compared with (A-I)rHDL incubated for 1 h with 3 mmol/l MG

[§] *p*<0.01 compared with (A-I)rHDL incubated for 1 h with 3 mmol/l MG

Table 5 Kinetic parameters for LCAT-mediated cholesterol esterification in discoidal (A-I)rHDL and discoidal (A-I)rHDL_{glyc}: concentration dependence

	0 mmol/l MG	0.5 mmol/l MG	1.5 mmol/l MG
Incubation conditions	37°C, 24 h	37°C, 24 h	37°C, 24 h
V_{max} (nmol CE ml LCAT ⁻¹ h ⁻¹)	2893.0±191.8	1589.0±125.4*	806.6±191* [†]
$K_{m(app)}$ (μmol UC)	26.3±3.6	19.2±3.6	38.4±16.6

Discoidal (A-I)rHDL (final apoA-I concentration 1 mg/ml) were incubated at 37°C for 24 h in the absence of MG or in the presence of MG (final concentration 0.5 or 1.5 mmol/l), then dialysed against PBS. The rHDL (final UC concentration 1–25 μmol/l) were incubated for 1 h with 5 μl of a 1/10 dilution of an LCAT preparation that generated 346 nmol CE ml LCAT⁻¹ h⁻¹. Kinetic parameters were determined by non-linear regression analysis of the rate of cholesterol esterification versus the concentration of substrate.

* $p < 0.001$ compared with unmodified (A-I)rHDL.

[†] $p < 0.01$ compared with (A-I)rHDL incubated with 0.5 mmol/l MG

LCAT activation [46]. The present results suggest that this may also be the case when positively charged residues are modified. Additional evidence that alterations in the conformation of apoA-I affect LCAT activation comes from studies of the apoA-I in human interstitial fluid, which activates LCAT poorly compared with plasma apoA-I [47]. That result was attributed to a change in the conformation of an epitope of apoA-I that is recognised by the mAb A1–11, one of the antibodies used in the present study.

Incubation with MG also dramatically altered the exposure of the epitope that is recognised by mAb AI-17, which spans residues 143–165 of apoA-I and is known to activate LCAT [48]. It is also noteworthy that other regions of apoA-I that changed in conformation during incubation with MG, including the C-terminal domain, where the epitopes for mAbs AI-178.1, AI-187.1 and AI-141.7 are located, regulate additional functional properties of HDL such as lipid binding, cholesterol efflux and apoA-I stability [49].

The observation that LCAT binds to discoidal rHDL in which the apoA-I is extensively glycosylated, but is unable to esterify cholesterol in these particles, suggests that rHDL which contain glycosylated apoA-I may competitively inhibit the LCAT reaction. The results in Table 6 confirm that this

is the case. The possibility that this result reflects the transfer of radiolabelled UC from unmodified (A-I)rHDL to rHDL that contain glycosylated apoA-I was considered to be unlikely because the transfer of cholesterol from a donor to an acceptor is independent of the acceptor concentration [50]. In other words, if significant transfer of radiolabelled UC between the unmodified (A-I)rHDL and the (A-I)rHDL_{glyc} had occurred, any reduction in the experimentally determined rate of cholesterol esterification would have been independent of how much glycosylated rHDL was present in the incubation mixture. The results in Table 6 show clearly that this is not the case. The close agreement between the calculated and experimentally determined rates of cholesterol esterification in the unmodified (A-I)rHDL also argues against significant transfer of radiolabelled UC between the two types of particles.

In conclusion, this study explains previous conflicting results where glycosylation of apoA-I either enhances, inhibits or has no effect on LCAT-mediated cholesterol esterification. As the LCAT-mediated esterification of cholesterol in HDL is one of the key events in the reverse cholesterol transport pathway, the present findings indicate that, depending on the extent to which it is glycosylated, apoA-I may either impair or enhance

Table 6 Inhibition of LCAT mediated cholesterol esterification in [³H]UC-labelled discoidal (A-I)rHDL by discoidal (A-I)rHDL_{glyc}

Unmodified [³ H]UC-labelled (A-I)rHDL, UC (μmol/l)	Rate of cholesterol esterification (nmol CE ml LCAT ⁻¹ h ⁻¹)				
	Unmodified [³ H]UC-labelled (A-I)rHDL	Unmodified [³ H]UC-labelled (A-I)rHDL + 1.5 μmol/l (A-I)rHDL _{glyc}		Unmodified [³ H]UC-labelled (A-I)rHDL + 3 μmol/l (A-I)rHDL _{glyc}	
		Observed	Predicted	Observed	Predicted
5	1.19±0.23	0.81±0.04	0.80	0.52±0.05	0.56
2.5	0.89±0.20	0.68±0.09	0.55	0.40±0.01	0.38
1.5	0.53±0.05	0.36±0.02	0.37	0.22±0.02	0.25
0.5	0.17±0.09	0.09±0.03	0.14	0.07±0.03	0.10

Discoidal (A-I)rHDL were incubated at 37°C for 24 h with 3 mmol/l MG to generate (A-I)rHDL_{glyc}. Unmodified ³H-labelled UC-discoidal (A-I)rHDL (final UC concentration 0.5–5 μmol/l) were incubated with LCAT (5 μl of a 1/50 dilution of a preparation that generated 1,600 nmol CE ml LCAT⁻¹ h⁻¹) in the absence or presence of (A-I)rHDL_{glyc} (final UC concentration 1.5 or 3 μmol/l). The observed rates of cholesterol esterification are the mean±SEM of triplicate determinations. Predicted rates of cholesterol esterification were obtained from Eqs. (4) and (5).

removal of cholesterol from peripheral tissues and macrophages. The impaired removal of cholesterol from tissues and macrophages in poorly controlled diabetic subjects, in whom the extent of apoA-I glycation is likely to be considerable, may contribute directly to the premature atherosclerosis that is frequently observed in these subjects. It remains to be seen if glycation of apoA-I also affects other processes involved in cholesterol transport and HDL metabolism.

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