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Glycation of High-Density Lipoprotein Does Not Increase Its Susceptibility to Oxidation or Diminish Its Cholesterol Efflux Capacity

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In vitro oxidation of high-density lipoprotein (HDL) diminishes its capacity to mediate cholesterol efflux from J774 macrophages. To investigate the possible role of HDL glycation in the increased atherosclerotic risk in diabetes, we studied the effects of in vitro glycation of HDL on its susceptibility to oxidation and capacity to mediate cholesterol efflux. HDL isolated from normal volunteers was incubated with 25 mmol/L glucose for 70 hours, resulting in 6.1% additional derivatization of apoproteins as determined by trinitrobenzene sulfonic acid (TNBS) reactivity. Unmodified HDL and glycated HDL (glyHDL) were tested for susceptibility to oxidation by incubation with various concentrations of copper and three assays of lipid oxidation. GlyHDL produced 51% to 64% less lipid peroxide than HDL as determined by reaction with xylenol orange ($P < .02$), indicating decreased susceptibility to oxidation. However, glycation of HDL did not result in significant changes in the formation of conjugated dienes or thiobarbituric acid–reactive substances (TBARS), two other indices of oxidation. To study cholesterol efflux, J774 macrophages were labeled with ^{3}H-cholesterol followed by incubation with the various HDL preparations. HDL and glyHDL had a similar capacity to mediate efflux. The efflux mediated by oxidized HDL (oxHDL) and oxidized glyHDL was reduced to a similar extent compared with the efflux mediated by HDL and glyHDL. These data indicate that in vitro glycation of HDL does not increase its susceptibility to oxidation and does not diminish its capacity to mediate cholesterol efflux.

ACCELERATED ATHEROSCLEROSIS is a major complication of diabetes mellitus, but the role of associated risk factors such as dyslipidemia, hypertension, obesity, and hyperglycemia per se is not well delineated. Epidemiologic studies have identified an inverse relationship between the plasma level of high-density lipoprotein (HDL) and atherosclerosis,[1] ascribed at least in part to the participation of HDL in reverse cholesterol transport.[2,3] In individuals with type 2 diabetes, low levels of HDL are commonly found. However, type 1 diabetics develop equally severe atherosclerosis even though HDL levels in these individuals are normal or even elevated.[4] Thus, functional alterations of HDL resulting from hyperglycemia may contribute to accelerated atherosclerosis. Glycated low-density lipoprotein (LDL) may be atherogenic, since it is cleared from the serum more slowly than LDL, has reduced affinity for the LDL receptor, and is more avidly internalized by human monocyte-derived macrophages.[5] The effects of hyperglycemia on the function of HDL have been the subject of limited investigation. In one study using cultured fibroblasts, glycation of HDL resulted in a decrease of its capacity to stimulate the receptor-mediated efflux of intracellular sterols, while the capacity of glycated HDL (glyHDL) to accept cholesterol from the cell membrane was not diminished.[6]

The oxidative modification of LDL in vivo and its accelerated uptake by arterial wall macrophages are implicated in the development of atherosclerosis,[7] and there is evidence that glycation of LDL increases its susceptibility to oxidation.[5] Several observations suggest that HDL is also oxidized in vivo. Bowry et al[8] reported that most of the measurable lipid peroxides in plasma are found in the HDL fraction and there are particles in HDL that contain no lipophilic antioxidants. Suzukawa et al[9] found that HDL contains polyunsaturated fatty acids that are susceptible to oxidation in vitro and each HDL particle carries fewer molecules of the antioxidant vitamin E compared with LDL. Oxidized HDL (oxHDL) has been found to be less effective than HDL in abolishing cholesterol reesterification,[10] and in removing unesterified and esterified cholesterol[11] from macrophage-derived foam cells. Cholesterol efflux

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from Fu5AH rat hepatoma cells was reduced in the presence of oxHDL compared with HDL.[12] Incubation of cultured J774 macrophages with oxHDL results in intracellular accumulation of unesterified cholesterol and suppression of cholesterol synthesis, indicating internalization of oxHDL by these cells.[13] We have previously reported that oxHDL has a diminished capacity to mediate cholesterol efflux from cholesterol-loaded J774 macrophages,[14] and the decrease in efflux correlates with the extent of HDL oxidation.[14,15]

It is not known whether there is increased oxidation of HDL in individuals with diabetes. HDL isolated from subjects with diabetes did not show increased susceptibility to oxidation.[16] We determined the effect of in vitro glycation of HDL on the susceptibility of HDL to oxidation and its capacity to mediate cholesterol efflux from macrophages.

MATERIALS AND METHODS

Chemicals

Phosphate-buffered saline (PBS), Dulbecco's minimum essential medium (DMEM), bovine calf serum, tetramethoxypropane, thiobarbituric acid, trinitrobenzene sulfonic acid (TNBS), ammonium ferrous sulfate, xylenol orange, butylated hydroxytoluene, sodium cyanoborohydrate, and Hanks balanced salt solution were obtained from Sigma Chemical (St Louis, MO). Trichloroacetic acid and copper nitrate solution (1 mg/mL) were from Fisher Scientific (Springfield, NJ). Bicinchoninic acid (BCA) protein assay reagents were from Pierce (Rockford, IL).

Isolation of HDL

Serum was obtained from fasting normal volunteers and after the addition of EDTA (0.05%), gentamicin (0.02%), and sodium azide (0.01%) HDL was isolated by sequential ultracentrifugation.[17] Density adjustments were made with potassium bromide. Very–low-density lipoprotein and LDL were removed from the serum by centrifugation for 25 hours at 140,000 × *g* at density 1.063 g/mL. The density of the remaining fraction was adjusted to 1.21 g/mL. HDL was isolated by centrifugation for 50 hours at 100,000 × *g* and recentrifugation at the same density for 30 hours. HDL fractions were dialyzed against 0.02% EDTA, 0.01% sodium azide, 150 mmol/L sodium chloride, and 2 mmol/L sodium phosphate, pH 7.4, at 4°C and passed through a 0.45-μm filter after dialysis. The protein content of the fractions was estimated by the BCA method.

Glycation of HDL

HDL (2 mg protein/mL) was incubated with 25 mmol/L glucose in PBS with 0.05% EDTA and 0.01% sodium azide in the presence of 12 mg/mL sodium cyanoborohydrate for 70 hours at 30°C.[18] Sodium cyanoborohydrate was added to shorten the incubation time and minimize other modifications such as oxidation. Control incubations (without glucose) contained HDL, EDTA, azide, and sodium cyanoborohydrate in PBS. HDL and glyHDL were dialyzed for 48 hours against four changes of 2 L each of PBS to remove the glucose, EDTA, and sodium cyanoborohydrate. Reactive lysine amino groups remaining in the lipoprotein after derivatization by glucose were assessed by TNBS reactivity. Samples (50 μg protein) were incubated with 1 mL 4% sodium bicarbonate, pH 8.4, and 50 μg 0.1% TNBS for 60 minutes at 40°C. After addition of 100 μL 1N HCl and 10 μL 1% SDS, absorbency was measured at 340 nm. TNBS reactivity in the modified samples was expressed as a percent of the reactivity in unmodified HDL.[19] Incubation with glucose resulted in the modification of 6.1% ± 0.9% of reactive lysine residues on HDL apoproteins (mean ± SE, n = 7). Incubation of HDL with glucose and sodium cyanoborohydrate does not oxidize or change the composition of lipoproteins.[20]

Assay of HDL Oxidation

The susceptibility to oxidation of unmodified and glyHDL was determined after dialysis and incubation of HDL with copper (0 to 10 μmol/L) in PBS at 30°C. Samples were taken for assays of HDL oxidation and incubation with macrophages for efflux experiments. Oxidation of HDL was quantified by measuring the production of conjugated dienes, lipid peroxides, and thiobarbituric acid–reactive substances (TBARS). Conjugated dienes were determined by directly measuring the change in absorbance at 234 nm (ΔA234) of 100 μg HDL protein/mL during incubation at 30°C with 1 μmol/L copper.[21] Lipid peroxides were quantified in samples of HDL (50 μg) after incubation at room temperature for 30 minutes with 25 mmol/L ammonium ferrous sulfate dissolved in sulfuric acid combined with 125 μmol/L xylenol orange in methanol, after which absorbency was measured at 560 nm. Peroxides are expressed as nanomoles per milligram of HDL protein compared with hydrogen peroxide standards.[22] TBARS were assayed by incubating samples of HDL (100 μg protein) with 1 mL 20% trichloroacetic acid and 1 mL 1% thiobarbituric acid for 30 minutes at 90°C. The reaction tubes were cooled and centrifuged, and the absorbency of the supernatants was measured in a spectrophotometer at 532 nm. TBARS are expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram of protein compared with tetramethoxypropane standards.[23]

Measurement of Cholesterol Efflux

J774.A1 macrophages (200,000 cells per well) were loaded with radiolabeled cholesterol by incubation for 24 hours in 1 mL DMEM with serum, antibiotics, 50 μg/mL cholesterol, and 0.1 μCi/mL [^{3}H]cholesterol (final concentrations) added as a 10-mg/mL solution in 95% ethanol. The media were then removed, and Hanks balanced salt solution containing 1 mg/mL lipid-free bovine serum albumin (BSA) and antibiotics was added to the wells. After 30 minutes at room temperature, this solution was removed and this step was repeated. Radiolabeled macrophages were incubated for 24 hours with the various HDL preparations (100 μg/mL) in 1 mL serum-free DMEM that contained 1 mg/mL lipid-free BSA, insulin, transferrin, selenium, and antibiotics. After the incubations, the media were removed and centrifuged for 5 minutes at 200 × *g* to remove suspended cells. Radioactivity in aliquots of the medium was determined by scintillation spectrometry. The cells were washed with PBS, and the lipids were extracted from the cells with isopropanol:petroleum ether (1:1 vol/vol). The extracts were dried, and radioactivity in the cell extracts was determined. Efflux mediated by HDL was calculated after subtracting media radioactivity measured in the absence of HDL, and is expressed as media radioactivity as a fraction of total radioactivity in the cells.[24]

Statistics

Data were analyzed using the SAS statistical analysis system computer program (SAS Institute, Cary, NC). Comparisons were made by ANOVA. Comparisons over time were made by ANOVA with repeated measures. When significant effects were found, a Duncan's analysis was performed for individual comparisons. Results are expressed as the mean ± SE.

RESULTS

Conjugated diene formation by HDL and glyHDL determined by measuring the ΔA234 at 10-minute intervals increased with time almost linearly without exhibiting a lag phase (Fig 1). The propagation phase of oxidation extended to 270 minutes. Conjugated diene formation from HDL and glyHDL

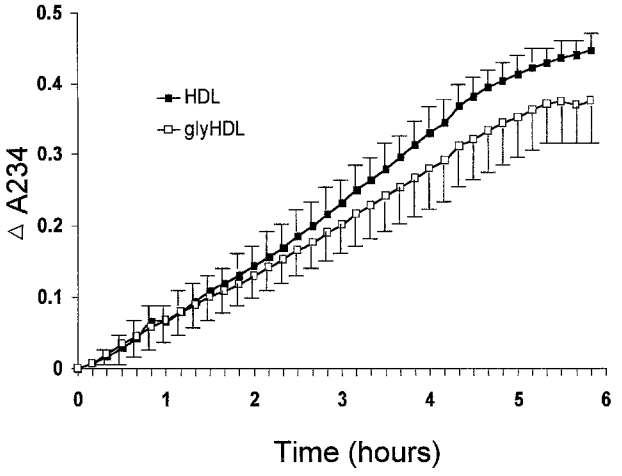

Fig 1. Time course of conjugated diene formation measured by the ΔA234 during oxidative modification of HDL. HDL (2 mg protein/mL) was incubated without and with 25 mmol/L glucose with 12 mg/mL sodium cyanoborohydrate for 70 hours at 30°C and dialyzed. HDL and glyHDL were incubated (0.1 mg protein/mL) with 1 μmol/L copper at 30°C. ΔA234 is expressed relative to unmodified HDL. Values are the mean of 7 determinations. HDL *v* glyHDL, not significant.

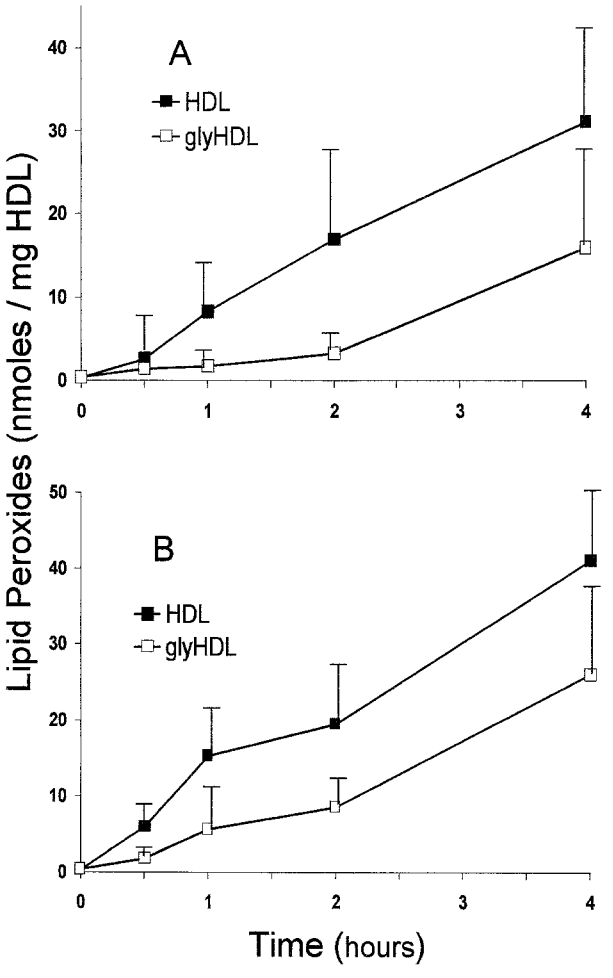

Fig 2. Time course of lipid peroxide formation after HDL oxidation. HDL and glyHDL (1.0 mg protein/mL) were incubated at 30°C with (A) 2.5 μmol/L copper or (B) 5.0 μmol/L copper. Lipid peroxide formation was determined by reaction with xylenol orange. Values are the mean of 4-6 determinations in duplicate. HDL *v* glyHDL, $P < .02$.

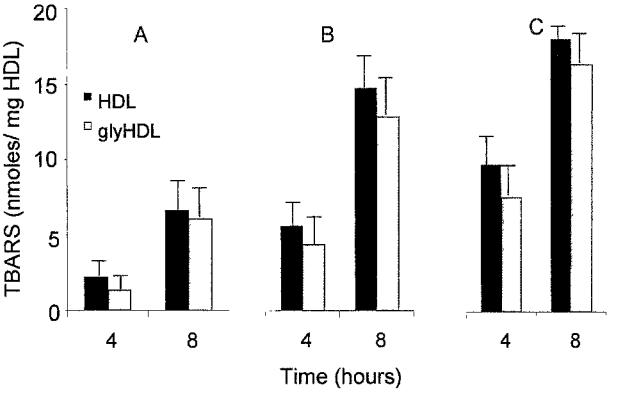

Fig 3. Time course of TBARS formation after HDL oxidation. HDL and glyHDL (1.0 mg protein/mL) were incubated at 30°C with (A) 2.5 μmol/L, (B) 5.0 μmol/L, and (C) 10.0 μmol/L copper before determination of TBARS. In experiments A, B, and C at 8 hours, values are the mean of 4-8 determinations in duplicate, and in experiment C at 4 hours, values are the mean of 15 determinations in duplicate. HDL *v* glyHDL, not significant.

was not significantly different. Lipid peroxide production in the presence of 2.5 μmol/L copper (Fig 2A) and 5.0 μmol/L copper (Fig 2B) measured by the reaction with xylenol orange increased with time up to 240 minutes at both copper concentrations. GlyHDL produced 64% (2.5 μmol/L copper) and 51% (5.0 μmol/L copper) less lipid peroxides than HDL. The percent differences were calculated from the means of data at all time points from each line shown in Fig 2A and B, respectively. There were no significant differences at any time point. However, in a retrospective analysis, it was found that when the data at all time points and copper concentrations were grouped together, the production of lipid peroxides over time was lower for glyHDL ($P < .02$). Incubation of HDL with 10 μmol/L copper did not result in a further increase in the production of lipid peroxides (data not shown). At all copper concentrations tested, glycation of HDL had no significant effect on TBARS production (Fig 3).

To determine the effects of glycation and oxidation on the capacity of HDL to mediate cholesterol efflux, unmodified HDL, glyHDL, oxHDL, and oxidized glyHDL were incubated with J774 macrophages labeled with [^{3}H]cholesterol (Table 1). There was no effect of glycation on the capacity of HDL to mediate the efflux of radiolabeled cholesterol from the cells. Data for HDL and glyHDL were combined to test the effect of oxidation. The combined data showed a 13% decrease in cholesterol efflux in the presence of oxHDL compared with HDL (0.103 ± .005 *v* 0.119 ± .005. $P < .03$). These results are comparable to the 16% decrease in efflux observed in our previous experiments for the same extent of HDL oxidation.[15]

DISCUSSION

OxHDL has a reduced capacity to mediate cholesterol efflux from macrophages in vitro,[10-14] suggesting that oxHDL could contribute to atherogenesis by being less efficient in reverse

Table 1. Effect of Glycation on HDL Oxidation and Cholesterol Efflux From Macrophages

Parameter	TBARS (nmol/mg HDL protein)	Efflux (media cpm/ cell cpm)
HDL	0	0.115 ± .005
glyHDL	0	0.122 ± .007
oxHDL	8.1 ± 1.5	0.105 ± .007
Oxidized glyHDL	6.2 ± 1.5	0.101 ± .006*

NOTE. HDL (2 mg protein/mL) was incubated for 70 hours in PBS and sodium cyanoborohydrate without or with 25 mmol/L glucose. After dialysis, unmodified HDL and glyHDL were oxidized by incubation with 10 μmol/L copper for 4 hours. The extent of oxidation was determined by measuring TBARS production. J774 macrophages were labeled with [^{3}H]cholesterol and incubated for 24 hours without or with the various HDL preparations (100 μg protein/mL). Efflux is expressed as media radioactivity as a fraction of total radioactivity in the cells. Values are the mean ± SE of 7 experiments performed with 4 replicates each.

*$P < .05$, glyHDL *v* oxidized glyHDL.

cholesterol transport. Because in vitro glycation of LDL increases its susceptibility to oxidation,[5] we determined the effects of glycation on HDL. In initial experiments (results not shown), we isolated HDL from three patients with poorly controlled diabetes and three normoglycemic controls and found no differences between the two groups in the susceptibility of HDL to oxidation and its capacity to mediate cholesterol efflux from J774 macrophages. Because of differences in the composition of HDL from the two groups of subjects, we then performed experiments using in vitro glycation wherein the same HDL preparation incubated without glucose was used as the control. Our results indicate that in vitro glycation of HDL does not increase its susceptibility to oxidation. While the measurement of the formation of lipid peroxides suggests that incubation of HDL with glucose decreases its susceptibility to oxidation. there was no significant effect when oxidation was measured using two other assays. Further studies will be needed to determine if glycation decreases the susceptibility of HDL to oxidation.

HDL apoproteins in the serum are more highly glycated in individuals with hyperglycemia than in individuals with normal glucose levels.[25] Babiy et al[16] found no differences in the in vitro susceptibility to oxidation of HDL isolated from control subjects and subjects with well-controlled type 2 diabetes. In addition, these investigators have shown that incubation in high concentrations of glucose protects HDL against irradiation-induced oxidation. This inhibition could be observed immediately after adding glucose to the incubation media, suggesting that some of the effect is due to the ability of glucose to act as an antioxidant.

In the present study, glycation of HDL did not diminish its capacity to mediate cholesterol efflux from J774 macrophages labeled with [^{3}H]cholesterol, similar to results reported by Duell et al[6] in fibroblasts. Cholesterol efflux from cells appears to have two components[24]: the first involves efflux from cell membranes mediated by HDL lipids that does not require specific binding, and the second involves efflux of intracellular cholesterol that requires specific binding of HDL apoproteins to cells. Our assay measures primarily efflux from the cell membrane. Duell et al[18] have shown that in vitro glycation of HDL results in a decrease in the specific binding of HDL to fibroblasts, but has no effect on nonspecific binding.[18] In a subsequent study, they showed that glycation enhances the non–receptor-mediated efflux of cholesterol by HDL but decreases the efflux of intracellular sterols mediated by a specific binding site, with a net effect of impaired total sterol efflux.[6] In the present study, glycation of HDL with 25 mmol/L glucose resulted in a level of modification of lysines in HDL apoproteins (6.1%) that was previously shown to inhibit binding to fibroblasts[18]; however, we did not find a significant effect on cholesterol efflux.

The relative importance of the modification of protein components compared with lipid components of HDL for its ability to mediate cholesterol efflux is still not defined, but there is evidence to suggest that the lipid component of HDL is the more important determinant of efflux. In previous studies, we observed that the degree of oxidation of HDL lipids correlates inversely with cholesterol efflux.[14,15] Similar findings were reported for the efflux from rat hepatoma cells mediated by HDL that was oxidized under conditions whereby only the lipid components were modified.[12] We found that derivatized apoproteins isolated from oxHDL are similar to unmodified apoproteins in the capacity to mediate cholesterol efflux.[14] Those data are consistent with the present results indicating that derivatization of HDL apoproteins with glucose does not inhibit efflux. The present results do not support the hypothesis that hyperglycemia promotes atherogenesis by increasing the susceptibility of HDL to oxidation and diminishing its capacity to mediate reverse cholesterol transport.

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