In Vivo Glycated Low-Density Lipoprotein Is Not More Susceptible to Oxidation Than Nonglycated Low-Density Lipoprotein in Type 1 Diabetes

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It has been suggested that low-density lipoprotein (LDL) modified by glycation may be more susceptible to oxidation and thus, enhance its atherogenicity. Using affinity chromatography, LDL glycated in vivo (G-LDL) and relatively nonglycated. (N-LDL) subfractions can be isolated from the same individual. The extent of and susceptibility to oxidation of N-LDL compared with G-LDL was determined in 15 type 1 diabetic patients. Total LDL was isolated and separated by boronate affinity chromatography into relatively glycated (G-) and nonglycated (N-) subfractions. The extent of glycation, glycoxidation, and lipoxidation, lipid soluble antioxidant content, susceptibility to in vitro oxidation, and nuclear magnetic resonance (NMR)-determined particle size and subclass distribution were determined for each subfraction. Glycation, (fructose-lysine) was higher in G-LDL versus N-LDL, (0.28 ± 0.08 *v* 0.13 ± 0.04 mmol/mol lysine, *P* < .0001). However, levels of glycoxidation/ lipoxidation products and of antioxidants were similar or lower in G-LDL compared with N-LDL and were inversely correlated with fructose-lysine (FL) concentrations in G-LDL, but positively correlated in N-LDL. In vitro LDL ($CuCl_2$) oxidation demonstrated a longer lag time for oxidation of G-LDL than N-LDL (50 ± 0.16 *v* 37 ± 0.15 min, *P* < .01), but there was no difference in the rate or extent of lipid oxidation, nor in any aspect of protein oxidation. Mean LDL particle size and subclass distribution did not differ between G-LDL and N-LDL. Thus, G-LDL from well-controlled type 1 diabetic patients is not more modified by oxidation, more susceptible to oxidation, or smaller than relatively N-LDL, suggesting alternative factors may contribute to the atherogenicity of LDL from type 1 diabetic patients.

ATHEROSCLEROSIS IS a major cause of morbidity and causes 70% to 80% of mortality in people with diabetes.[1] Qualitative changes such as nonenzymatic glycation and oxidation may potentiate the atherogenicity of low-density lipoprotein (LDL), despite normal plasma levels in most people with type 1 diabetes.[2,3] Glycation of LDL occurs from the onset of diabetes and correlates with other indices of glycemic control, including glycosylated hemoglobin (HbA_{1c}).[4]

We have previously demonstrated that, compared with LDL from control subjects, LDL from patients with type I diabetes causes greater cholesteryl ester accumulation in macrophages.[5] Also, the in vivo glycated LDL (G-LDL) subfraction (obtained by boronate affinity chromatography) from either nondiabetic subjects or type 1 diabetic patients causes greater cholesteryl ester accumulation in macrophages than corresponding relatively nonglycated LDL (N-LDL) subfractions.[6] It has been suggested that LDL modified by glycation may be more susceptible to oxidation, enhancing its atherogenicity (reviewed in Lyons and Jenkins[7]). Previous studies have used LDL isolated from diabetic patients[8] or in vitro G-LDL and N-LDL from nondiabetic subjects,[9] and compared their responses to in vitro oxidative stress (eg, exposure to Cu^{2+}) using end-points including lag times for conjugated diene formation or the level of thiobarbituric acid reacting substances (TBARS). If carefully prepared in an antioxidant environment with appropriate controls, these G-LDL and N-LDL would differ predominantly in the extent of nonenzymatic glycation of apolipoprotein B. In contrast, using boronate affinity chromatography to prepare in vivo G-LDL and N-LDL subfractions from the same individual, we found differences not only in the extent of glycation, but also in chemical composition, with G-LDL being triglyceride enriched.[6] Higher triglyceride content and associated increases in oxidation-prone polyunsaturated fatty acids may increase susceptibility of G-LDL to oxidative damage. Since G-LDL has a longer circulating half-life,[10] G-LDL may also represent an older LDL subfraction, which might be of smaller diameter as a result of longer exposure to lipoprotein lipase. Previous studies have suggested that small dense LDL is more susceptible to in vitro oxidation.[11]

To determine associations between in vivo glycation of LDL and other potentially atherogenic LDL characteristics in diabetic patients, we prepared and evaluated in vivo G-LDL and N-LDL subfractions from subjects with type 1 diabetes. We measured levels of lipid soluble antioxidants and levels of specific glycation, glycoxidation, and lipoxidation products as markers of oxidative stress and damage occurring in vivo. As protective antioxidants are abundant in plasma and oxidative damage to LDL most likely occurs in the subendothelial space, we also exposed the in vivo G-LDL and N-LDL subfractions to

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Table 1. Clinical and Biochemical Characteristics of the Type 1 Diabetic Patients Studied

Parameter	Reference Range	Value
No. (M/F)	—	15 (13/2)
Age (yr)	—	31.9 ± 2.2
Diabetes duration (yr)	—	13.3 ± 3.4
BMI (kg/m^2)	<25	22.7 ± 0.6
Systolic blood pressure (mm Hg)	<140	123 ± 13
Diastolic blood pressure (mm Hg)	<85	77 ± 9
Macrovascular disease (n)	—	0
Albumin excretion rate (mg/24 h)	≤40 mg/d	19 ± 7
Proliferative diabetic retinopathy (n)	—	1
Fasting glucose (mg/dL)	<126	159 ± 23
HbA_{1c} (%)	<6.0	6.6 ± 0.4
Total plasma cholesterol (mg/dL)	<200	175 ± 10
Total plasma triglyceride (mg/dL)	<200	88 ± 14
VLDL-cholesterol (mg/dL)	<40	18 ± 3
LDL-cholesterol (mg/dL)	≤100	108 ± 9
HDL-cholesterol (mg/dL)	>45 (M), >55 (F)	50 ± 4
Plasma Lp(a) (mg/dL)	<25.0	19.1 ± 5.0
Serum uric acid (mg/dL)	2.5-8.5	5.9 ± 0.4
Plasma fibrinogen (mg/dL)	150-406	322 ± 33

oxidative stress induced in vitro by copper to determine the relative susceptibility to oxidation of the subfractions. We used nuclear magnetic reasonance (NMR) analysis to determine mean LDL size and LDL subclass distribution for each subfraction.

RESEARCH DESIGN AND METHODS

Subjects

The study was approved by the Medical University of South Carolina (MUSC) Institutional Review Board. Fifteen patients with type 1 diabetes diagnosed according to the criteria established by the National Diabetes Data Group[12] were recruited from the MUSC Private Diagnostic Clinic. Based on history, physical examination, and urine analysis, all subjects were clinically free of macrovascular disease, hypertension, and increased urinary albumin loss. All but 1 patient were free from proliferative diabetic retinopathy as determined by examination by an ophthalmologist (L.P.K.) after pupil dilation. No subject was taking antihypertensive or lipid-lowering therapy or supplemental vitamins. Written informed consent was obtained. Three hundred milliliters of venous blood was taken after an overnight fast, prior to administration of insulin. LDL was isolated from plasma on the day of collection, and blood, plasma, and serum were stored at −70°C for clinical characterization tests as summarized in Table 1.

General Biochemical Methods

Plasma glucose was assayed by the glucose oxidase method as adapted for use in the Beckman glucose analyzer (Beckman Coulter, Fullerton, CA). HbA_{1c} was quantified after the separation of red blood cell hemolysate on columns packed with a weakly acidic cation exchange resin (Hemoglobin A_{1c} Micro Column Test, BioRad, Hercules, CA). Total cholesterol and triglyceride were measured in whole plasma and isolated lipoproteins using enzymatic, colorimetric assays (Infinity Cholesterol, Infinity Triglyceride, Sigma Chemical, St Louis, MO). High-density lipoprotein-cholesterol (HDL-C) was determined by cholesterol assay after precipitation of apolipoprotein B–containing lipoproteins with sodium phosphotungstate:magnesium chloride as described previously.[13] LDL-C was calculated using the Friedewald equation.[14] Lipoprotein (a) [Lp(a)], uric acid, and fibrinogen were analyzed by automated assays by standardized methods of the Department of Clinical Chemistry at MUSC.

Isolation of G-LDL and N-LDL Subfractions

Blood was collected into 50 mL polypropylene tubes in the presence of a lipoprotein preservative solution comprised of 2.8 mmol/L EDTA, 62 μmol/L chloramphenicol, 50 μg/mL gentamycin sulfate, and 10 mmol/L ε-amino-caproic-acid (final concentrations). Red blood cells were sedimented by centrifugation at 3,500 rpm for 20 minutes. The total LDL fraction was isolated from plasma using a rapid, vertical spin ultracentrifugation technique as previously described.[13] Briefly, plasma was adjusted to 1.21 g/mL with solid potassium bromide (KBr), layered below a salt solution with a density of 1.019 g/mL, and centrifuged in a VTi50 rotor (Beckman, Palo Alto, CA) at 50,000 rpm at 7°C for 150 minutes. LDL, which appears as a yellow band in the middle third of the tube, was needle-aspirated by tube puncture. The isolated LDL fraction was washed and concentrated by centrifugation in a Ti70 rotor (Beckman) (70,000 rpm, 7°C, 12 hours).

LDL from each patient was separated into glycated and nonglycated subfractions using a modification of our previously described affinity chromatography procedure.[6] Briefly, an aliquot (2.75 mg LDL-C) of each total LDL sample was dialyzed against equilibrating buffer (250 mmol/L ammonium acetate, 50 mmol/L magnesium chloride, 5 mmol/L disodium ethylenediaminetetraacetic acid [EDTA], 3 mmol/L sodium azide, pH 8.0 [final concentrations]) and loaded onto a 2-mL column packed with Glycogel II (Pierce, Rockford, IL) equilibrated with the same buffer. The nonbound (relatively nonglycated) LDL fraction, N-LDL, was eluted with 8 mL equilibration buffer. The bound, glycated subfraction of LDL, G-LDL, was then eluted using 4 mL of an elution buffer (200 mmol/L sorbitol, 500 mmol sodium chloride, 5 mmol/L disodium EDTA, 100 mmol/L Tris-HCl, pH 8.0 [final concentrations]). The percentage distribution of N-LDL and G-LDL and the recovery of LDL applied to the column were determined by quantifying the total cholesterol concentration in N-LDL, G-LDL, and unfractionated total LDL.[6] LDL recovery (mean ± SD) from the columns averaged 94% ± 2%. The column fractions were pooled and concentrated by centrifugation in a Ti70 rotor (Beckman) (70,000 rpm, 7°C, 12 hours). The isolated and concentrated N-LDL and G-LDL subfractions from each patient were dialyzed against saline containing 0.3 mmol/L EDTA, pH 8.0, sterilized by filtration, and stored under a nitrogen atmosphere until used. The susceptibility of the subfractions to oxidation was determined within 72 hours of blood collection. Aliquots of LDL subfractions for further characterization were stored frozen at −70°C under nitrogen until analyzed.

Characterization of LDL

The early glycation product fructose-lysine (FL) and the glycoxidation/lipoxidation products (N^{ε}(carboxymethyl)lysine (CML), N^{ε}(carboxyethyl)lysine (CEL), and malondialdehyde-lysine (MDA-lysine) were determined by gas chromatography/mass spectroscopy as described previously.[15] To determine the lipid composition of isolated LDL subfractions, each sample was extracted with chloroform:methanol (2:1, vol/vol) and the free cholesterol, cholesteryl ester, triglyceride, phospholipid, and protein concentrations were determined as described previously.[6] The concentrations of α-tocopherol, γ-tocopherol, α-carotene, β-carotene, and lycopene were determined using high-performance liquid chromatography as previously described.[13] Mean LDL particle size and subclass distribution was determined by NMR spectroscopy.[16,17] The proportion of total LDL within 3 LDL subclasses of different diameter was measured. The diameter (nm) of the largest to smallest LDL subfractions are: L3, 21.3 to 23.0; L2, 19.8 to 21.2; L1, 18.3 to 19.7.[17] There was insufficient G-LDL to enable full characterization of LDL from 2 subjects.

Susceptibility to In Vitro Oxidation

Susceptibility of subfractions to in vitro oxidation by $CuCl_2$ was measured as previously described.[13] Briefly EDTA was removed by passing LDL samples over a Sephadex G25 column (Pharmacia, Piscataway, NJ). N-LDL was diluted with nitrogen purged EDTA-free phosphate-buffered saline (PBS) to the same cholesterol concentration as G-LDL. Each subfraction was diluted to a final concentration of 50 μg/mL cholesterol with oxygen purged EDTA-free PBS and freshly prepared $CuCl_2$ then added to a final concentration of 5 μmol/L. Conjugated diene formation was monitored by determining the absorbance at 234 nm in a Beckman DU650 spectrophotometer with an automatic 6-cell cuvette changer and Peltier temperature controller set at the physiologic temperature of 37°C. Protein oxidation was determined by measuring fluorescence at excitation 360 nm and emission 430 nm of replicate samples in a (Aminco Bowan Series 2, Spectronics, Rochester, NY) fluorimeter. The lag time to oxidation, maximal rate of oxidation, and the extent of oxidation were calculated for each LDL subfraction. The lag time was defined as the interval between initiation (zero time) and the intercept of the tangent of the slope of the absorbance or fluorescence curve during the propagation phase with the time-scale axis expressed in minutes. The maximal rate of oxidation was calculated from the slope of the absorbance or fluorescence curve during the propagation phase and was expressed as units per minute. The extent of oxidation was measured as the difference between the initial and the maximal absorbance or fluorescence.

Statistical Analysis

Results are expressed as the mean ± SD, and statistical significance was assessed using paired *t* and simple regression analyses (SigmaStat, SPSS, Chicago, IL).

RESULTS

Subjects

The clinical and biochemical characteristics of the patients with diabetes are shown in Table 1. Glycemic control as assessed by HbA_{1c} ranged between 4.8% and 9.8%, with a mean ± SD of 6.6% ± 0.4%.

Proportion of G-LDL

The percentage of G-LDL in the total LDL fraction isolated from each of the 15 patients was determined. The (mean ± SD) percentage of LDL-C bound to the column (and regarded as glycated) was 19% ± 2% of the total LDL applied to the columns, with a range of 14% to 25%. As shown by the data presented in Fig 1A, the percentage of LDL-C bound to the column was significantly correlated with HbA_{1c} ($r = 0.71$, $P < .01$). Thus, with increasing HbA_{1c} level, more LDL particles are modified by glycation.

LDL Glycation, Glycoxidation, and Lipoxidation

We determined the extent of glycation of LDL subfractions by quantifying FL, the first stable glycation product, in each subfraction. FL levels in G-LDL averaged 2.7-fold higher compared with N-LDL ($P < .001$) (Fig 1B and Fig 2). However, HbA_{1c} levels were not significantly correlated with FL levels in either G-LDL ($r = 0.30$) or N-LDL ($r = 0.29$) (Fig 1B). Thus, with increasing HbA_{1c} level, there was no significant proportional increase in the extent of glycation of the G-LDL particles.

We also measured levels of the glycoxidation/lipoxidation products CML and CEL in the LDL subfractions from each patient. CML and CEL levels in G-LDL and N-LDL did not differ significantly (Fig 2). However, levels of the lipoxidation product MDA-lysine were significantly higher in N-LDL compared with G-LDL ($P < .05$). Thus, the nonglycated LDL subfraction in type 1 diabetic patients is more oxidized than the glycated subfraction from the same patient.

We further investigated the association between the levels of oxidation/glycoxidation products in the LDL subfractions the level of glycation using continuous analyses. There were significant, inverse correlations between FL and concentrations of CML and MDA-lysine in G-LDL (Fig 3A and C, respectively, $P < .05$). There was also a negative, but not statistically significant, association of CEL with FL in G-LDL (Fig 3B). In contrast, in N-LDL there were positive associations between FL levels and CML, CEL, and MDA (Fig 3A, B, and C, respectively), but only the association with MDA reached statistical significance ($P < .01$).

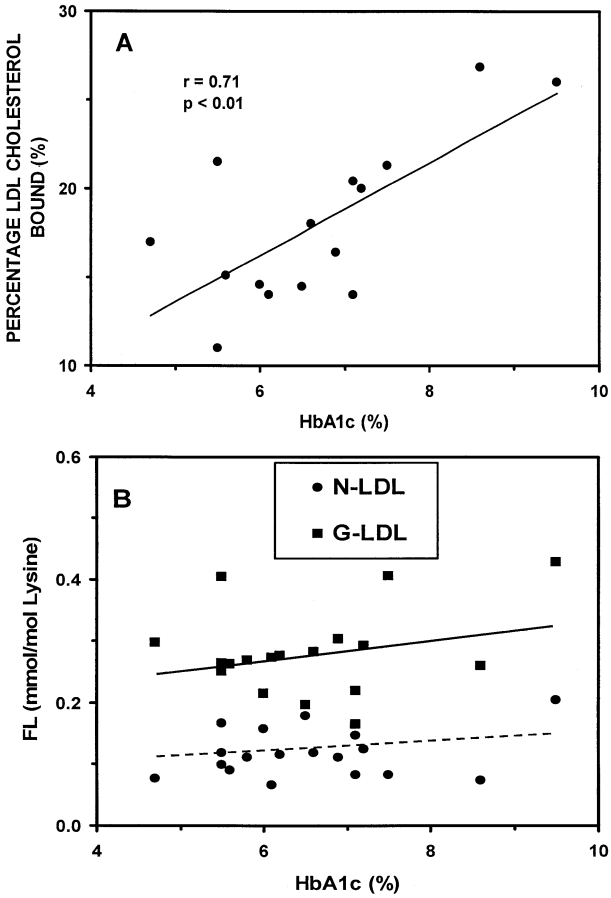

Fig 1. (A) Percentage of LDL-C binding to boronate affinity chromatography column is correlated with the (n = 15) patients' HbA_{1c} levels. (B) The bound LDL represents a more G-LDL subfraction. Associations between FL, the first stable glycation product, in the N-LDL and G-LDL subfractions and the HbA_{1c} level in each patient. There was no statistically significant association in either N-LDL or G-LDL. Thus, with poorer glycemic control (increasing HbA_{1c}), more LDL particles become glycated (G-LDL) but the extent of modification (glycation) in each particle does not increase significantly.

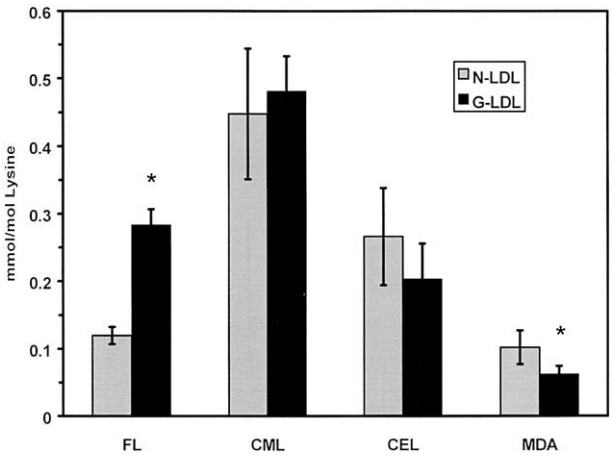

Fig 2. Glycation, glycoxidation, and lipoxidation products in N-LDL and G-LDL from 13 subjects with type 1 diabetes. Data are means ± SD. *$P < .05$. By paired *t* test, FL levels were significantly higher and MDA levels significantly lower in G-LDL than N-LDL. CML and CEL levels of the fractions did not differ significantly. There was insufficient sample for analysis of G-LDL from 2 subjects.

Susceptibility to Oxidation of G-LDL and N-LDL Subfractions

We determined the in vitro susceptibility to copper-induced oxidation of N-LDL and G-LDL. The oxidation of both LDL lipid and protein were assessed. As shown in Fig 4A (upper 3 panels), the lag time to lipid oxidation for N-LDL was significantly shorter than that for G-LDL. For each subject studied, the lipid lag time of the G-LDL fraction was longer than that of N-LDL. Thus, the glycated subfraction of LDL is less susceptible to lipid oxidation than N-LDL isolated from the same patient. There were no significant differences in the rate or extent of lipid oxidation ("delta absorbance") during the reaction. As shown in Fig 4B (lower 3 panels), there were no differences in the lag time, rate, or extent of protein oxidation for N- versus G-LDL.

LDL Composition

To investigate the basis of the difference in susceptibility to lipid oxidation of N-LDL and G-LDL, we determined the chemical composition of N-LDL and G-LDL isolated from each patient. No significant differences in lipid composition or lipid soluble antioxidant content (Table 2) were found.

LDL Size and Subclass Distribution

The mean particle size and subclass distribution of each of the LDL subfractions was determined using NMR spectroscopy. As shown in Table 2, G-LDL was slightly larger than N-LDL, but the difference was not statistically significant. A higher proportion of N-LDL was in the smallest subclass (L1), but the difference in subclass distribution did not reach statistical significance.

DISCUSSION

Despite normal plasma levels in type 1 diabetes, qualitative changes in LDL, such as nonenzymatic glycation and oxidation may potentiate its atherogenicity.[2,3] LDL glycation, which occurs from the onset of diabetes,[4] has been suggested to promote LDL oxidation. A role for oxidized LDL has become central to current theories of atherosclerosis.[2,18,19] Using a combination of ultracentrifugation and boronate affinity chromatography, we have isolated in vivo glycated and relatively nonglycated LDL subfractions from patients with type 1 diabetes. Increased glycation of the subfraction bound by the boronate affinity column was confirmed by higher levels of the early glycation product, FL (Figs 1 and 2). Perhaps surprisingly, the glycated (bound) G-LDL subfraction had similar antioxidant content (Table 2), and, similar or even lower levels of glycoxidation and lipoxidation products than the nonglycated (nonbound) N-LDL subfraction (Fig 2, Table 2). NMR-determined, LDL average particle diameter, and size-based LDL subclass distribution, which may influence oxidizibility, did not differ between the subfractions (Table 2). The G-LDL was less susceptible to in vitro copper oxidation than the N-LDL. These results suggest that glycated LDL is not smaller, is not more oxidized while in the circulation, and is not more susceptible to oxidation when exposed to in vitro oxidative stress. Thus, decreased size and increased oxidizibility may not contribute to the proposed greater atherogenicity of LDL modified by glycation in diabetes.

Previous studies have used native and in vitro G-LDL from nondiabetic subjects or total LDL from nondiabetic and diabetic subjects to assess the susceptibility of LDL to in vitro oxidation.[8,9,13] Stringent precautions must be taken in the preparation of LDL to avoid ex vivo oxidation, particularly if sequential ultracentrifugation, which requires several days and

Table 2. Chemical Composition, Antioxidant Content, Size, and Subfraction Distribution of N-LDL and G-LDL Subfractions Isolated From Type 1 Diabetic Patients

	N-LDL	G-LDL
LDL compositon		
TC/PRO	1.6 ± 0.1	1.6 ± 0.1
FC/PRO	0.4 ± 0.1	0.4 ± 0.1
CE/PRO	2.0 ± 0.1	2.0 ± 0.2
TG/PRO	0.1 ± 0.1	0.2 ± 0.1
PL/PRO	1.0 ± 0.1	0.9 ± 0.1
Antioxidant content (nmol/mg LDL protein)		
α-Tocopherol	20.1 ± 1.6	19.4 ± 2.1
γ-Tocopherol	6.7 ± 0.9	5.4 ± 0.8
α-Carotene	0.09 ± 0.02	0.10 ± 0.03
β-Carotene	0.22 ± 0.06	0.19 ± 0.05
Lycopene	0.14 ± 0.04	0.12 ± 0.02
Size and subclass distribution		
LDL particle size (nm)	20.21 ± 0.19	20.51 ± 0.24
% L3	28	31
% L2	24	36
% L1	48	33

NOTE. LDL composition constituents: TC, total cholesterol; PRO, protein; FC, free (unesterified) cholesterol; CE, cholesteryl ester; TG, triglyceride; PL, phospholipid. LDL particle size and the percentage distribution into large (L3) *v* intermediate (L2) *v* small (L1) sized particles was determined NMR spectroscopy.

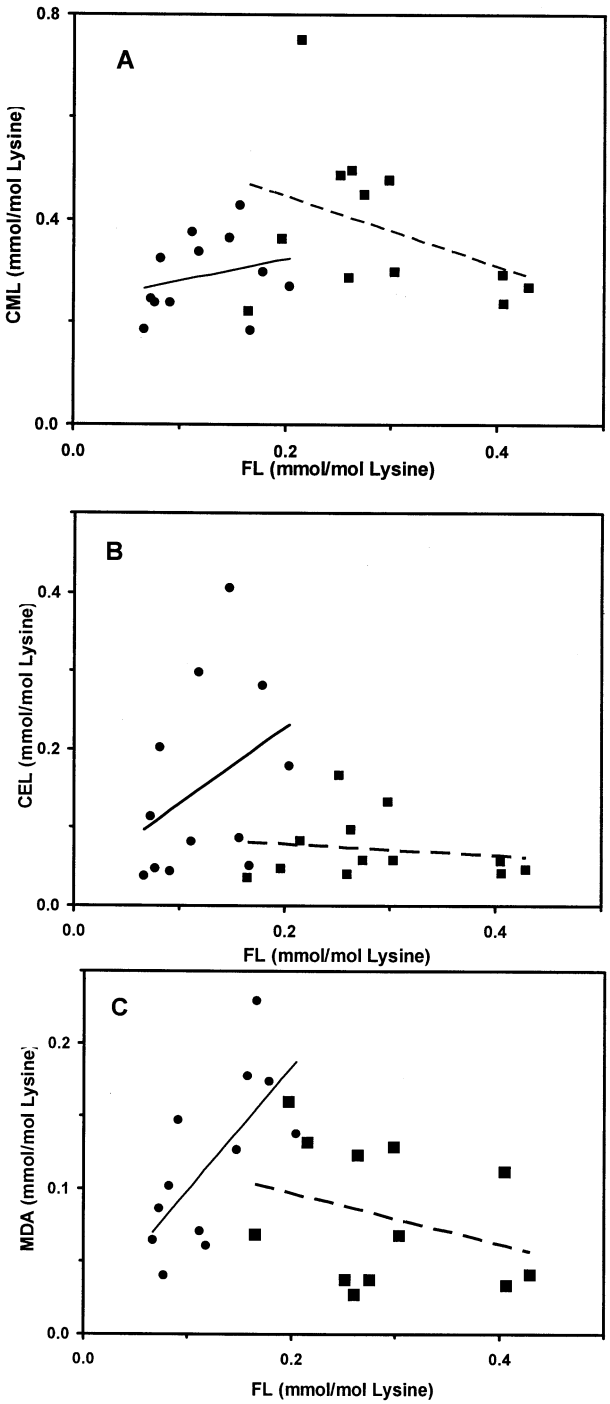

Fig 3. Relationship between FL levels and measures of glycoxidation and lipoxidation in N-LDL (●) G-LDL (■) from 13 patients with type 1 diabetes. There was insufficient sample for analysis of G-LDL from 2 subjects. (A) FL *v* CML; G-LDL, $P < .05$. (B) FL *v* CEL, both were not significant (NS). (C) FL *v* MDA, N-LDL, $P < .01$, G-LDL, $P < .05$).

removal of aqueous phase antioxidant by dialysis, is used. In the present studies, LDL was isolated by rapid vertical spin ultracentrifugation and evaluated soon after preparation. Buffers purged with nitrogen and containing EDTA, low temperatures, and yellow lighting were also used to inhibit in vitro oxidation. EDTA was removed by size exclusion chromatography just prior to in vitro oxidation. We have demonstrated that carefully prepared in vitro G-LDL is not more susceptible to in vitro oxidation than native LDL from nondiabetic subjects (unpublished results). To assess susceptibility of G-LDL to oxidation in diabetes, it is preferable to use in vivo G-LDL from people with diabetes, rather than in vitro G-LDL from nondiabetic subjects. As well as having increased nonenzymatic glycation of apolipoprotein B, in vivo G-LDL may differ in other aspects. We have previously demonstrated that in vivo G-LDL may be relatively triglyceride-enriched in both type 1 diabetic and nondiabetic subjects.[6] In the present study, this was not the case (Table 2) and may relate to better glycemic control as evidenced by a mean HbA_{1c} of 6.6% versus 8.6% in our previous study. Other components, such as fatty acid composition (which we did not measure because of limited sample availability), may differ between glycated and nonglycated fractions.

The proportion of LDL binding to the boronate affinity columns correlated with HbA_{1c} (Fig 1A), with the bound LDL being regarded as glycated LDL, which was confirmed by increased FL content. This is the first report of FL concentrations determined by chemical means in isolated LDL subfractions. Previous studies have demonstrated correlation between HbA_{1c} and the extent of (total) LDL glycation.[4] FL levels within the LDL subfractions did not correlate with HbA_{1c} (Fig 1B), but the trend was in the expected direction. This lack of correlation may have resulted, in part, by the relatively narrow range of FL levels due to selection of a subfraction of LDL and to differences in the half-life of LDL (days) and of HbA_{1c} (months). In addition, there are more than 350 lysine residues in apoprotein B. It is not known if select residues are more susceptible to modification by glycation or if binding to the affinity column is more dependent upon glycation of select residues. Alternatively, FL may not be the sole or major moiety controlling LDL binding to the boronate affinity column. Nonetheless, these results suggest that, at least at the HbA_{1c} concentrations observed in this study, a greater effect of glycation is seen on the number of LDL particles which were glycated rather than on the number of lysine residues which are modified in each particle.

Levels of specific glycoxidation and lipoxidation products, lipid soluble antioxidants, and conjugated diene formation in response to exposure to copper were chosen as measures of oxidative damage. During in vitro oxidation of LDL, an early response is the loss of lipid soluble antioxidants, following which conjugated diene formation ensues.[20] This is paralleled by increases in protein oxidation (measured by fluorescence) and formation of specific oxidation products such as CML, CEL, and MDA.[21] We have previously demonstrated an increase in CML and CEL, without reduction of FL in nondiabetic LDL exposed to copper.[21] The presence of such glycoxidation and lipoxidation products has also been demonstrated in human atherosclerotic lesions.[22,23] Levels of the specific glycoxidation and lipoxidation products (CML, CEL, and MDA) were not significantly higher in G-LDL compared with N-LDL (Fig 2) and, in fact, MDA levels were significantly lower. Within the G-LDL, levels of the glycoxidation and lipoxidation

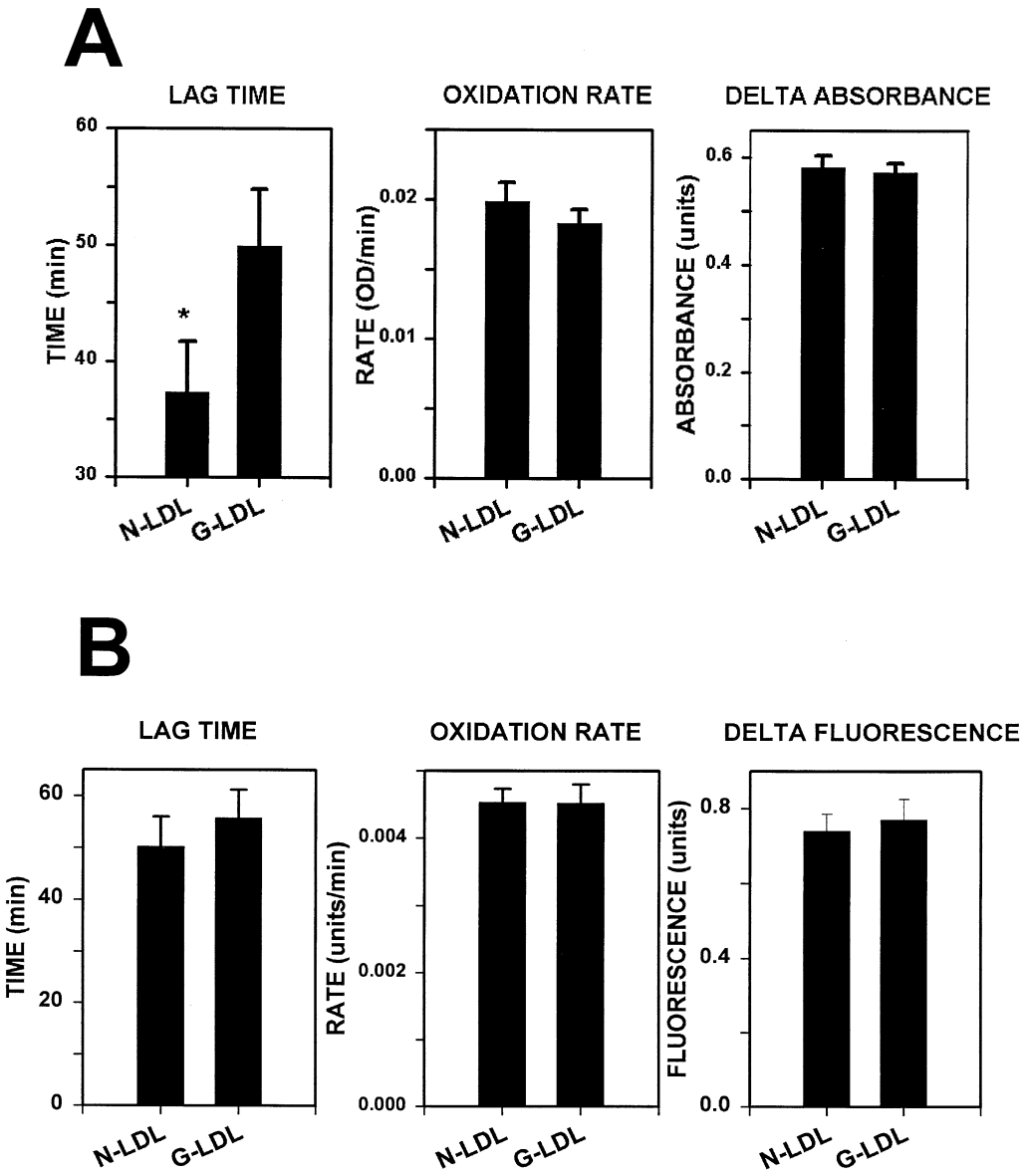

Fig 4. Susceptibility to in vitro oxidation of N-LDL and G-LDL from 15 patients with type 1 diabetes. From left to right, the panels show lag time, the rate of oxidation, and the extent of oxidation. Parameters of lipid oxidation are shown in (A) and parameters of protein oxidation are presented in (B). Apart from the lipid oxidation lag time, there were no statistically significant differences in the copper-induced oxidation of the lipid or protein moieties of N-LDL and G-LDL. Data presented as means ± SD. *$P < .05$.

products were inversely correlated with FL levels (Fig 3). Further, the amounts of glycoxidation and lipoxidation products as a function of the amount of FL in the particle were significantly lower in G-LDL compared with N-LDL (Fig 3). This is further evidence that, at least in the circulation, in vivo glycation of LDL is not associated with an increased amount of oxidative damage. This may reflect the potent antioxidant milieu of plasma, insufficient residence time of LDL within the circulation to enable formation of glycoxidation or lipoxidation products, or the rapid removal of more severely damaged LDL moieties.

Nonenzymatic glycation of LDL slows its rate of catabolism,[10] which would increase its exposure time to lipoprotein lipase and potentially producing a smaller denser LDL, which is thought to be potentially more atherogenic. Contributing mechanisms to enhanced atherogenicity of small LDL include greater binding to vascular wall matrix, greater susceptibility to oxidation,[11,24,25] and potentially, a more ready access to the subendothelial space.[26,27] However, the NMR-determined diameter of G-LDL was not smaller than that of N-LDL. We did not find a significant correlation between LDL size and LDL lipid or protein oxidation (data not shown). These results are in keeping with our previous work in which total LDL size, determined by gradient gel electrophoresis, did not differ be-

tween subjects with type 1 diabetes and nondiabetic control subjects[13] and did not correlate with in vitro lipid oxidation of total LDL.

Although our results demonstrate that in vivo G-LDL is neither more susceptible to oxidation nor smaller than relatively N-LDL, we consider it likely that glycation of LDL may be deleterious. In terms of direct evidence of atherogenic effects of G-LDL, we have previously demonstrated that G-LDL is associated with increased uptake and cholesteryl ester accumulation in macrophages,[6] a hallmark of atherosclerosis. We have also demonstrated that relative to native LDL, in vitro G-LDL increases transforming growth factor-β (TGF-β) mRNA expression in mesangial cells[28] and induces hemeoxygenase-1 mRNA expression, a marker of intracellular oxidative stress, in aortic endothelial cells, mesangial cells, and proximal tubule cells.[29] Ha et al[30] have demonstrated that G-LDL increases mesangial extracellular matrix synthesis. Nonenzymatic glycation of LDL may also adversely alter vascular tone, coagulation, and fibrinolysis.[31] However, in these studies, the extent of in vitro glycation was greater than occurs in vivo in diabetes, and based on the levels of conjugated dienes in LDL, was associated with increased oxidation, so that effects of glycation versus oxidation cannot be directly assessed. LDL glycation is also a marker of glycemic control,[4] although not one that should replace HbA_{1c}. The Diabetes Control and Complications Trial (DCCT) demonstrated that intensive diabetes management, including tighter glycemic control, is associated with reduced risk of microvascular complications, a nonstatistically significant reduction in macrovascular events[32] and in the rate of progression of carotid artery wall thickness.[33] In type 2 diabetes, worse glycemic control is associated with increased risk of macrovascular events, and its reduction is associated with reduced risk.[34] Thus, the effects of G-LDL in mediating vascular complications of diabetes are supported by other evidence and may be attributable to different mechanisms from those addressed in this work.

Lastly, it should be noted that these studies investigated type 1 diabetic patients who were relatively free from diabetic complications and who were in good glycemic control. The impact of diabetes complications and poor glycemic control on LDL subfraction distribution is significant.[35-37] The impact of these factors on LDL subfraction physical and biochemical parameters and metabolism remains to be determined.

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