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Improved Glucose Control Decreases the Interaction of Plasma Low-Density Lipoproteins With Arterial Proteoglycans

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The entrapment and retention of plasma low-density lipoproteins (LDL) by arterial proteoglycans (PG) is a process central to atherogenesis. We postulated therefore that accelerated atherosclerosis of diabetic individuals may result from hyperglycemia-associated modifications in LDL that enhance their interaction with arterial PG. To evaluate the role of clinical treatment on this process, LDL-PG binding was evaluated in uncontrolled type 2 diabetic subjects on monotherapy followed by combination therapy. After a 4-week washout (baseline), subjects were randomized to receive glipizide GITS monotherapy (20 mg/d, n = 12) or metformin monotherapy (2.5 g/d, n = 8) for 6 weeks followed by combination treatment with both agents for 12 weeks. Fasting blood glucose and fructosamine were significantly reduced with monotherapy and further reduced with combination therapy ($P < .01$). With combination therapy, glycated hemoglobin (GHb) levels were significantly reduced from baseline for the group initially treated with glipizide GITS (8.5% *v* 10.5%, $P < .0005$), or initially with metformin (6.7% *v* 9.7%, $P < .0005$). No significant changes in total plasma cholesterol (TPC), LDL, high-density lipoprotein (HDL), or triglycerides (TG) were measured after monotherapy or combination therapy. Plasma LDL was isolated by differential ultracentrifugation. In an in vitro binding assay, LDL from subjects on combination glipizide GITS/metformin demonstrated significantly less binding to arterial PG than LDL obtained after monotherapy with either agent (7.6 ± 0.5 *v* 10.7 ± 0.9 μg LDL cholesterol/μg PG [mean ± SEM], $P < .05$). These data demonstrate that combination treatment with glipizide GITS/metformin will further improve glucose control in type 2 diabetic subjects and may favorably improve diabetes–associated modification of LDL-arterial PG binding.

ALTHOUGH CARDIOVASCULAR disease is the leading cause of morbidity and mortality in type 2 diabetic individuals, mechanisms that link diabetes mellitus and atherosclerosis remain unexplained. Many of the established risk factors for atherosclerosis are more prevalent in the diabetic population, including hypertension, visceral obesity, low plasma high-density lipoproteins (HDL), and increased plasma low-density lipoproteins (LDL).[1] However, both the Multiple Risk Factor Intervention Trial (MRFIT)[2] and the Göteborg[3] study have shown that at every level of known risk factor, the cardiovascular risk is 4 to 6 times higher for diabetic individuals, indicating that other factors unique to the diabetic state contribute to accelerated atherosclerosis. The hallmark of diabetes is hyperglycemia and chronic hyperglycemia may be postulated to contribute greatly to the vascular complications of diabetes.

Atherosclerotic lesions result from the deposition in the arterial wall of lipid that is derived from apolipoprotein B (apoB)-containing lipoproteins.[4,5] Early lipid deposits in the subendothelial extracellular matrix[6,7] are proposed to lead to the recruitment of blood monocytes. These cells are the precursors of lipid-filled foam cells that are the hallmarks of atherosclerosis. Processes that influence the entrapment and retention of LDL may therefore be important targets for therapeutic intervention. Arterial proteoglycans (PG), through their interaction with LDL, are proposed to play a key role in LDL retention in arterial tissue.[8-13] In vitro PG-LDL interaction studies showed that human LDL with high affinity for PG was positively correlated with coronary heart disease[13,14]; ultra-

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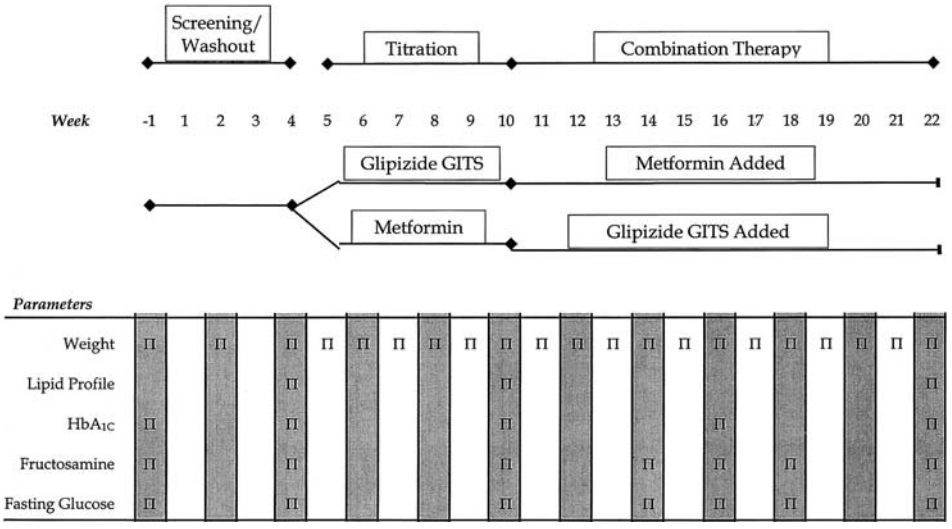

Fig 1. Study design.

structural studies showed that LDL is associated with extracellular matrix PG in rabbit arteries.[12] It is proposed that LDL interaction with arterial PG promotes atherogenesis by delaying the transit of LDL through the tissue, thus increasing LDL exposure to oxidative enzymes. For a detailed discussion of potential consequences of the association of LDL with arterial PG see the review by Hurt-Camejo et al.[15].

We have recently begun to investigate how diabetes-associated abnormalities in LDL particle composition and structure affect interaction with arterial proteoglycans. Using a nonhuman primate model of streptozotocin-induced diabetes, studies demonstrated enhanced PG-binding of LDL from diabetic animals and a role for glycation (nonenzymatic glycosylation) of LDL in the increased interaction.[16] The present report describes studies using LDL from human type 2 diabetic patients before and after therapeutic intervention to reduce glycemia. It demonstrates that as glycemic control is improved, plasma LDL is less reactive with arterial PG.

MATERIALS AND METHODS

Subjects

The effect of improved clinical glycemia on LDL-PG binding was assessed in 20 subjects aged 35 to 69 years with a diagnosis of type 2 diabetes made at least 6 months prior to study entry. Subjects were evaluated after a monotherapy phase with either a sulfonylurea or metformin and after combination of both agents. All subjects had been previously maintained on a stable dose of oral sulfonylureas for a minimum of 2 months and were required to have a glycosylated hemoglobin (HbA_{1C}) greater than 7% at the time of screening. All patients had normal renal and liver function. The study was approved by the Clinical Practices Committee of the Wake Forest University School of Medicine.

Experimental Design

The study was designed as an open-label, randomized, parallel, titration study in patients with type 2 diabetes. The study consisted of a 4-week washout period, a 6-week period of glipizide GITS (Glucotrol XL) or metformin (Glucophage) monotherapy, and a final 12-week period of glipizide GITS and metformin combination therapy. Figure 1 depicts the study design.

Screening (week −1). At this visit, subjects had blood drawn for general chemistries, signed a consent form, and underwent a physical examination and electrocardiogram.

Period 1 (screening/washout). After meeting the criteria for entry into the study, previous oral therapy was stopped and subjects were instructed on home blood glucose monitoring, and continued without any medication for approximately 4 weeks. At the end of the 4-week washout, HbA_{1C} and fructosamine levels were tested. A fasting plasma glucose greater than 200 mg/dL was required for subjects to continue to period 2.

Period 2 (monotherapy titration). Subjects were randomly assigned to 1 of 2 experimental groups. Group 1 received glipizide GITS and group 2 received metformin. Period 2 lasted for a minimum of 6 weeks and during this period subjects were seen weekly in the clinic.

Group 1 started on 5 mg of glipizide GITS every morning. The dosage was increased to 10 mg every morning (to a maximum dose of 20 mg every morning) at intervals of 1 week. Group 2 was started on 850 mg/d of metformin. The dose was increased to 850 mg twice daily (to a maximum dose of 850 mg 3 times daily) at intervals of 2 weeks. Metformin was administered with meals. In the original study design, the dose of either glipizide GITS or metformin was to be increased only until the blood glucose, obtained by fingerstick in the clinic, was well controlled (defined as fasting plasma glucose $\leq$ 140 mg/dL). This was not achieved by monotherapy with either agent and all subjects entering period 3 (combination therapy) were receiving the maximum dose of monotherapy.

Period 3 (combination therapy). A fasting plasma glucose $\geq$140 mg/dL was required for subjects to continue to period 3. Beginning at week 10, glipizide GITS was added to the drug regimen of subjects taking metformin and metformin was added to the drug regimen of subjects taking glipizide GITS. Every effort was made to see each subject every 2 weeks during this period of the study.

Subjects who were inadequately controlled on their final dosage of metformin at the end of period 2 were continued on the same dosage of metformin and given, in addition, 5 mg/d of glipizide GITS. The dosage of glipizide GITS was titrated upward in 5-mg increments at 1-week intervals based on blood glucose levels (to a maximum dosage of 20 mg/d).

Subjects inadequately controlled on their final dosage of glipizide GITS at the end of period 2 were maintained on that dosage and given, in addition, 850 mg/d of metformin. The dose of metformin was titrated

upward at 2-week intervals, based on blood glucose levels, to a maximum daily dosage of 2,550 mg (850 mg administered 3 times daily with meals). The titration schedule was 850 mg/d to 850 mg twice daily to 850 mg 3 times daily of metformin.

If the subject experienced hypoglycemia (defined as blood glucose measured by home blood glucose monitoring < 60 mg/dL with symptoms or blood glucose < 50 mg/dL without symptoms) that was thought to be drug-related, the dose of the added compound was decreased or the added compound was discontinued. At the end of period 3, the study parameters were repeated (see Fig 1).

Glycated Hemoglobin and Fructosamine Measurements

HbA_{1C} was determined by automated affinity high-performance liquid chromatography (HPLC; Primus Corp, Kansas City, MO) as previously described, and fructosamine was measured by an automated nitroblue colorimetric method using Roche reagents in a COBAS-Mira Chemistry Analyzer (Roche Diagnostics, Nutley, NJ).[17-19]

Lipid Measurements and Lipoprotein Preparation

Total plasma cholesterol (TPC), HDL cholesterol, and triglycerides (TG) were measured in the Centers for Disease Control (CDC)-standardized Wake Forest University Baptist Medical Center Lipid Research Laboratory. LDL cholesterol was calculated using the Friedewald formula.[20] Blood samples for LDL isolation were drawn from fasted participants and chilled on wet ice; plasma was isolated within 30 minutes. Plasma was stored at 4°C under argon for less than 1 week, prior to isolation of LDL by differential ultracentrifugation.[21] LDL was stored at 4°C under argon for less than 3 months prior to LDL characterization and PG binding studies.

Proteoglycan Preparation

The proteoglycan preparation used for these studies has been previously described.[21] Briefly, at necropsy of 2 male cynomolgous monkeys fed a monkey chow diet, thoracic aortas were excised, stripped of adventitia, and quick-frozen in liquid nitrogen for storage at -70°C. For PG extraction, tissues were thawed, minced into 2- to 3-cm^2 sections, and incubated for 36 hours at 4°C in 4.0 mol/L GdnHCl, 0.05 mol/L sodium acetate (pH 4.5, 30 mL/g wet tissue) containing protease inhibitors.[22] Following removal of extracted tissues by filtration through Whatman #1 paper (Whatman International, Maidstone, England), the extract was concentrated using CF 25 cones (Amicon, Beverly, MA). The extract was exchange-dialyzed into 7.0 mol/L urea, 0.05 mol/L Tris, 0.15 mol/L NaCl, pH 7.2, and chromatographed on

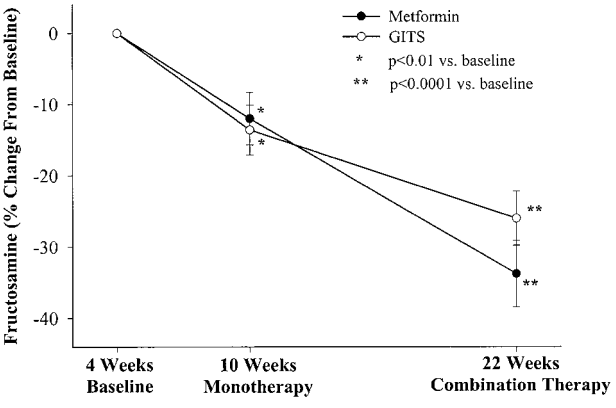

Fig 2. Univariate analysis of fructosamine. The decrease in fructosamine at the end of the monotherapy and combination therapy periods *v* baseline was significant.

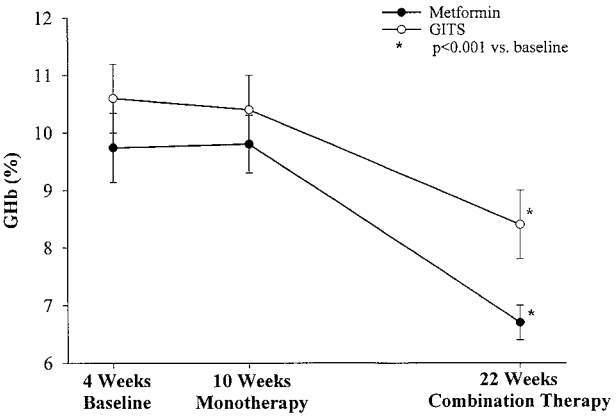

Fig 3. Univariate analysis of HbA_{1C}. Although no difference was observed with monotherapy with either agent, combination therapy significantly improved glycemic control.

Diethylaminoethyl (DEAE) Sephacel (24-mL column; Sigma-Aldrich, St Louis, MO), by washing with 3 bed volumes of loading buffer and eluting with 3 bed volumes of 7.0 mol/L urea, 0.05 mol/L Tris, 1.0 mol/L NaCl, pH 7.2. Dialysis and chromatography was repeated once to improve purification. Recoveries were monitored by dimethylene blue assay[23] and hexuronic acid content of the final preparation was measured by the method of Blumenkrantz and Asboe-Hansen.[24] Agarose electrophoresis of papain-liberated glycosaminoglycan (GAG)[25] demonstrated that the PG preparation was predominantly chondroitin sulfate PG. Aliquots of the PG preparation were stored at -20°C until used in LDL binding assays.

PG-LDL Interactions

Previous publications have extensively described the assay system used for these studies.[21,26-29] Briefly, 120 μg LDL (as cholesterol) was incubated with 1 μg PG (as hexuronic acid) in 1.1 mL of buffer containing 5 mmol/L Tris, 6 mmol/L KCl, I5 mmol/L $CaCl_2$, 1 mmol/L $MgSO_4$, pH 7.2, at room temperature for 30 minutes. Insoluble PG-LDL complexes that formed were separated by centrifugation at 1,000 × *g* for 30 minutes and LDL in the pellet was measured using an enzymatic assay for cholesterol. Data are presented as amount of LDL cholesterol present in insoluble PG complexes.

Data Analysis

Statistically significant differences between measurements taken at baseline, post-monotherapy and post-combination therapy were determined by paired *t* tests. The effect of treatment was estimated and tested for statistical significance by repeated measures analysis of covariance using the baseline level of the outcome measure as the covariant.

RESULTS

The effects of glucose-lowering treatment on measures of glycemic control are shown in Figs 2 and 3. Six weeks of treatment with metformin reduced serum fructosamine from 401± 23 to 352 ± 23 μmol/L, a 12% change from baseline ($P < .01$) (Fig 2). A significant decrease in fructosamine was also observed for the group receiving glipizide GITS monotherapy (470 ± 31 to 405 ± 33 μmol/L, a 14% change from baseline, $P < .005$) (Fig 2). For both groups, further decreases in fructosamine were measured following 12 weeks of combi-

Table 1. Plasma Lipids

	TPC (mg/dL)	TG (mg/dL)	HDLC (mg/dL)	LDLC (mg/dL)
Week 4	187 ± 8	192 ± 21	34 ± 2	115 ± 8
Week 10	180 ± 6	165 ± 31	35 ± 2	116 ± 5
Week 22	177 ± 6	202 ± 40	36 ± 2	108 ± 6

NOTE. Values are means ± SEM (n = 20).

Abbreviations: TPC, total plasma cholesterol; TG, triglycerides; HDLC, high-density lipoprotein cholesterol; LDLC, low-density lipoprotein cholesterol.

nation therapy when a 26% and 34% reduction from baseline fructosamine was achieved by the glipizide GITS and metformin monotherapy groups, respectively. Neither group achieved a decrease in GHb following 6 weeks of monotherapy, whereas 12 weeks of combination therapy resulted in a significant reduction in GHb for both groups with 19% and 30% decrease from baseline for the glipizide GITS and metformin groups, respectively (Fig 3).

Plasma lipids for all patients are shown in Table 1. TPC was not decreased following either monotherapy or combination therapy. LDL, HDL, and TG were also similar following treatments. No group differences in plasma lipids were measured at baseline or following treatment (data not shown).

Although plasma concentrations of LDL were unchanged by treatment, we investigated whether improvement in glycemic control affected properties of plasma LDL important to their interaction with arterial PG. For these studies, isolated LDL was incubated with extracted arterial PG in an in vitro binding assay. Figure 4 shows the mean PG binding of LDL from both groups of patients. No difference in PG binding was measured for LDL following monotherapy for either group. Combination therapy resulted in decreased interaction of LDL for the glipizide GITS monotherapy group (7.5 ± 0.5 *v* 10.7 ± 0.9 μg LDL cholesterol per μg PG, $P < .05$). For the smaller (n = 8) metformin monotherapy group, the decrease in binding following combination therapy (8.8 ± 0.7 *v* 11.6 ± 1.9μg LDL cholesterol per μg PG) did not reach significance.

PG binding of LDL from individual subjects before and after combination therapy is shown in Fig 5. The combined mean of both monotherapy groups was 10.7 ± 0.9 μg LDL cholesterol per μg PG and decreased to 7.6 ± 0.5 μg LDL cholesterol per μg PG following combination therapy. LDL binding to arterial PG was lower following combination therapy in 12 of the 20 patients studied.

DISCUSSION

Improved glucose control was achieved by 6 weeks of treatment with either metformin or glipizide GITS; combination treatment with both agents resulted in further improvement in glycemic control. This is consistent with results of other intervention studies using these agents in type 2 diabetic patients.[30,31] As a result of such studies, combination therapy is considered a standard of care in achieving glycemic control.

The lipid profiles of patients in the present study were not changed as a result of the improvement in glycemic control; this agrees with previous observations that glycemic control may not alter LDL levels in type 2 diabetic subjects. This was, however, an intervention trial of relatively short duration. In addition, although HDL cholesterol was borderline low in these patients, TPC and LDL cholesterol were in the accepted "normal" range and TG were in the "high normal" range. Metformin has been shown previously to improve lipid metabolism in the presence of hypertriglyceridemia but to have little effect in its absence.[32,33]

Although LDL composition was not analyzed in the present study, in isolated LDL from other patients, percent TG was lower following combination therapy (data not shown). Compositional abnormalities in LDL have been shown to be predictors of cardiovascular mortality in patients with type 2 diabetes.[34] Angiographic studies have shown a positive relationship between severity of cardiovascular disease and TG content of LDL.[35] Based on these studies, our data would suggest that improved clinical glycemia may result in the production of LDL that is less atherogenic and thereby reduce the risk of cardiovascular disease in treated individuals.

LDL that were isolated following combination therapy

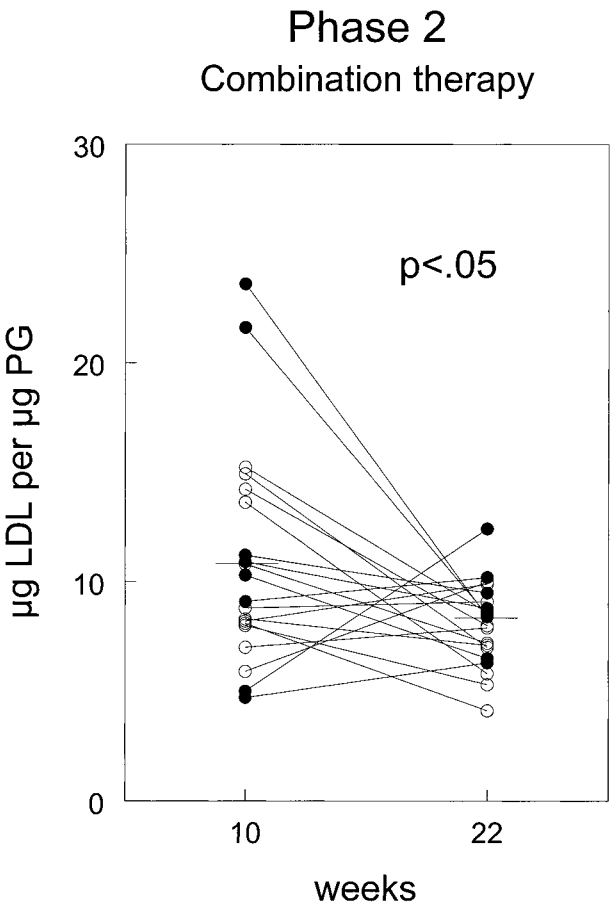

Fig 5. Plot of the changes in formation of PG-LDL complexes during the combination therapy phase of the trial. Interaction of LDL with PG was measured as described in Fig 4 and data are presented as μg of LDL cholesterol bound per μg PG. Values are shown for LDL from individual patients who had received metformin (●) or glipizide GITS (○) in the monotherapy phase of the trial.

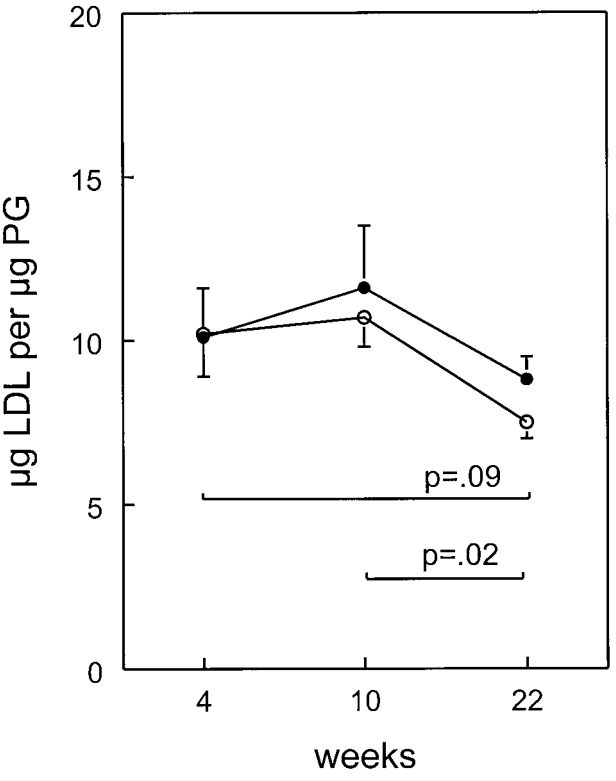

Fig 4. Formation of particulate complexes with PG of LDL from patients who received metformin (●) or glipizide GITS (○) during weeks 4 to 10 and a combination of both agents during weeks 10 to 22. LDL (120 μg as cholesterol) was incubated with PG (1 μg as hexuronate) in 5 mmol/L Tris, 6 mmol/L KCl, 15 mmol/L $CaCl_2$, 1.5 mmol/L $MgSO_4$, pH 7.2, for 30 minutes at room temperature. The formation of particulate complexes was measured as cholesterol in a 1,500 × *g* pellet. Data are presented as μg of LDL cholesterol bound per μg PG and values are group means ± SEM. *P* values are presented for the glipizide GITS monotherapy group.

demonstrated decreased interaction with arterial PG. This may be a result of a decreased TG content since in a previous study of dietary modification of LDL in nonhuman primates, a positive association was observed between the PG-binding potential and TG content of LDL.[21] The association of PG and LDL is mediated by negatively charged sulfate groups of the PG and positively charged amino acids (mainly lysines) of apoB.[15] The charge density of a LDL appears to be a major factor in its PG-binding capacity[36] and it has been shown that different PG-binding sequences of apoB may act cooperatively to enhance the interaction with PG.[37] This suggests that a conformation of apoB that brings positively charged recognition sequences for PG in close proximity to each other will increase the avidity of the particle for PG. We have proposed that this may be one way in which the TG content of the LDL core may influence surface properties of the particles.

Previous studies have shown that glycation may be a major factor mediating the enhanced PG binding of diabetic LDL.[16] In the present study we did not measure glycation of LDL. Based on previous studies in humans[38-41] and in nonhuman primates,[19] the amount of glycation of LDL correlates with glycemic control and parallels GHb levels. With combination therapy, GHb levels were significantly reduced from baseline for both treatment groups. Therefore we would expect a similar reduction in glycated LDL. The lowering of LDL TG content by glipizide GITS/metformin combination therapy also suggests that glycation of LDL may have been lowered by the treatment. This is based on studies showing that glycation of LDL in both type 1 diabetic patients and nondiabetic controls was associated with a TG-enriched subspecies.[42] Since glycation is known to modify positively charged lysines of apoB and may thus reduce the overall charge density of LDL, it is unclear how this modification may lead to enhanced PG binding. Further studies are required to characterize glycation–specific determinants that may affect the interaction of LDL with arterial PG.

Much discussion has focused on the relationship of LDL size to diabetic complications. In diabetic individuals, particle radii were shown to be smaller for all subfractions of LDL compared to nondiabetic controls.[43] Previous studies of nondiabetic human[44,45] and pigeon[46] LDL have shown that particle size may also be an important factor in PG binding. In the present study, no changes in LDL size were detected following glucose reduction therapy. Measurement of LDL size, however, represents the mean of a heterogeneous population of particles and previous studies have reported selective PG binding of specific subpopulations of particles.[44] It may be possible, therefore, by subfractionation studies, to identify hyperglycemia-induced characteristics in LDL that may increase their atherogenicity.

In summary, this report represents one of the first to demonstrate that improved glycemic control may favorably affect qualitative properties of LDL in human subjects, specifically the interaction of human LDL with arterial proteoglycans. The clinical significance of this finding will obviously have to be tested in clinical event trials.

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