Chronic Hyperglycemia Increases Arterial Low-Density Lipoprotein Metabolism and Atherosclerosis in Cynomolgus Monkeys

Kenneth N. Litwak, William T. Cefalu, and Janice D. Wagner

Diabetes mellitus confers a threefold to fivefold increased risk of mortality from vascular disease. The primary cause of this increased incidence of vascular disease is atherosclerosis, but the mechanisms accounting for the increase are unclear. Chronic hyperglycemia is a common feature of all forms of diabetes mellitus and may contribute greatly to the increased incidence of atherosclerosis, via promotion of both lipoprotein and tissue glycation, which may have atherogenic effects. The present study investigated the effect of chronic hyperglycemia on measures of low-density lipoprotein (LDL) metabolism and atherosclerosis in streptozotocin-induced diabetic (STZ-DM) and control cynomolgus monkeys after 6 months of study. Consistent with a chronic hyperglycemic state, diabetic monkeys had significant increases in glycated hemoglobin (GHb) and glycated plasma LDL concentrations, but had minimal changes in total plasma cholesterol (TPC) or triglyceride (TG) concentrations during the study. Forty-eight hours before necropsy, control and in vitro–glycated LDL were differentially radiolabeled and coinjected into diabetic and control monkeys. There was a significant increase in arterial LDL accumulation in femoral arteries from diabetic monkeys compared with controls, with similar trends in other arterial sites. The effect of LDL glycation on arterial LDL accumulation was minimal in both groups. Arterial segments from diabetic monkeys also had greater amounts of arterial cholesterol content compared with controls. Histomorphometric analyses showed that diabetic monkeys had significantly greater intimal areas in the femoral artery and abdominal aorta compared with controls. Diabetic monkeys also had reduced arterial remodeling, or compensation, in the femoral artery and abdominal aorta. However, there was no difference in advanced glycation end products (AGE) in arterial collagen between groups. In conclusion, experimentally induced diabetes mellitus increases arterial LDL accumulation and atherosclerosis extent in cynomolgus monkeys before changes in AGE formation. The increased atherogenesis may be due to changes in lipoproteins or direct effects of hyperglycemia on the artery wall.

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IN HUMANS, diabetes mellitus confers a threefold to fivefold increased risk of mortality from vascular disease.[1-4] Cerebrovascular disease, coronary heart disease, and peripheral vascular disease are all more common and occur at an earlier age in diabetic subjects compared with the nondiabetic population.[1] The primary cause of this increased incidence of vascular disease is atherosclerosis[1,3,5]; however, the mechanisms accounting for the increase are unclear. Epidemiologic evidence suggests that only 25% of the excess coronary heart disease can be accounted for by traditional risk factors such as hypercholesterolemia, hypertriglyceridemia, hypertension, and obesity.[5] Furthermore, subjects with relatively normal plasma lipid and lipoprotein concentrations still have increased atherosclerosis.[3] This suggests that the increased vascular disease may involve factors unique to diabetic subjects.

Chronic hyperglycemia is a common feature of all forms of diabetes mellitus and has been linked to the increased incidence of atherosclerosis.[6] Chronic hyperglycemia results in increased levels of nonenzymatic glycation of both circulating and tissue proteins, which may affect their metabolic behavior.[7,8] Furthermore, over time, a complex series of dehydration and oxidation reactions can occur, resulting in the formation of irreversible advanced glycation end products (AGE). AGE form in both diabetic and nondiabetic subjects and accumulate with age; however, with diabetes mellitus, the amount of AGE formation is greater.[9] Numerous potentially atherogenic effects of AGE relevant to the vasculature have been described, including alterations of matrix ligands and cellular receptors, increased levels of cytokines, and increased oxidant stress.[9-11]

In addition to the postulated proatherogenic effects of tissue AGE formation, glycated plasma lipoproteins may also be atherogenic. Previous reports have demonstrated decreased uptake of glycated low-density lipoprotein (gLDL) by endothelial cells,[7,12,13] as well as decreased plasma LDL fractional catabolic rate (FCR) in nondiabetic rabbits[14] and guinea pigs[8] compared with control LDL (cLDL), suggesting decreased recognition of gLDL by the classic LDL receptor. However, there is increased uptake of gLDL by macrophages, suggesting a mechanism for formation of foam cells.[12] Other reports examining the effect of diabetes mellitus on LDL metabolism in diabetic rabbits[8,14,15] and diabetic humans[16] have demonstrated either no difference or increased plasma LDL FCR for diabetic LDL compared with nondiabetic LDL. The present study examined the effects of chemically induced diabetes mellitus on atherosclerosis progression in cynomolgus monkeys. The effects of chronic hyperglycemia on LDL glycation and arterial collagen AGE content, as well as the effect of hyperglycemia on LDL metabolism were also examined.

MATERIALS AND METHODS

Animal Study

Sixteen adult male cynomolgus monkeys (*Macaca fascicularis*) were used in this study. The monkeys were part of an ongoing investigation of the effects of dietary cholesterol on the development of atherosclerosis and had varying dietary histories. For the current study, all monkeys were fed a moderately atherogenic diet (0.28 mg cholesterol/kcal, 45% of calories from fat) for 2 months before baseline assessments and

From the Departments of Comparative Medicine and Internal Medicine, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC.

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Address reprint requests to Janice D. Wagner, DVM, PhD, Department of Comparative Medicine, Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1040.

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throughout the 6-month trial period. The monkeys were randomized into the two groups (control and streptozotocin-induced diabetes mellitus [STZ-DM]) based on lifetime dietary cholesterol consumption (mg dietary cholesterol/d × days of diet consumption), as well as total plasma cholesterol (TPC) concentrations to account for pretrial variation in exposure and response to the atherogenic diets. In addition, because of the varying exposure to atherogenic diets, pretreatment atherosclerosis extent was assessed via a biopsy of the right common iliac artery approximately 1 month before day 0.[17] Due to the small size of the arterial biopsy, only cholesterol content was measured on the segment. One monkey from the control group died during the preexperimental period of causes unrelated to the experiment. Thus, data are reported for 15 animals.

Eight monkeys were entered into the study at one time (four STZ-DM and four controls) and the remaining seven (four STZ-DM and three controls) 6 months later. On day 0, diabetes mellitus was induced in STZ-DM monkeys with STZ (Zanosar, 30 mg/kg intravenously; Upjohn, Kalamazoo, MI). All procedures involving animals were conducted in compliance with state and federal laws, standards of the Department of Health and Human Services, and guidelines established by the Institutional Animal Care and Use Committee. Surgical procedures (iliac artery biopsy and vascular catheterization) were performed while animals were anesthetized with ketamine hydrochloride (15 mg/kg intramuscularly) and butorphanol tartrate (0.05 mg/kg intramuscularly), whereas minor procedures (blood sampling) were performed while the animals were sedated with ketamine hydrochloride (10 mg/kg intramuscularly).

Clinical Chemistry Measurements

Blood samples were collected into tubes containing EDTA (1 mg/mL final concentration) after an 18-hour fast and before administration of morning insulin dose (in insulin-dependent STZ-DM monkeys). Plasma was separated at 4°C by low-speed centrifugation. Detailed measurements of glycemic control and glucose tolerance have been discussed in a previous report.[18] Glycated hemoglobin (GHb) concentrations were determined by automated-affinity high-performance liquid chromatography (model CLC-330; Primus, Kansas City, MO), as described previously.[19] TPC, triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) concentrations were determined at baseline and after 2 and 6 months of the experimental period.[20-22] Very–low plus low-density lipoprotein cholesterol (VLDL + LDL-C) was determined by subtracting HDL-C from TPC. Treatment values are reported as the mean of both samples. Plasma lipid and lipoprotein measurements are in full standardization with the Centers for Disease Control–National Heart, Lung and Blood Institute Lipid Standardization Program.

LDL ($1.019 < d < 1.063$) were isolated from study monkeys at baseline and 3 months into the trial period, using density-gradient ultracentrifugation.[23] LDL protein content was determined by the method of Lowry et al.[24] LDL glycation was determined by measuring the fructosamine content of the LDL, as previously described,[25] and is reported as millimoles of fructosamine per milligram LDL protein. LDL size was determined by nuclear magnetic spectroscopic analysis of plasma taken concurrently with baseline and 2-month samples.[26]

LDL Metabolism Studies

LDL metabolism studies were performed in monkeys 6 months after STZ treatment and 48 hours before necropsy. For LDL metabolic studies, LDL were isolated from a pooled sample of nondiabetic adult male cynomolgus monkeys consuming the same atherogenic diet as the study monkeys. The same monkeys were used to obtain LDL for injection for all 15 monkeys. Pooled blood (≈200 mL for each trial) was collected after an overnight fast in the presence of Na_2EDTA, aprotinin, and PPACK (D-phenylalanyl-*L*-prolylarginine chloromethyl-ketone) at final concentrations of 1 mg/mL, 25 KIUs/mL, and 1 μmol/L, respectively, to limit proteolysis and oxidation.[23] TPC and TG concentrations for the pooled samples were 8.9 mmol/L and 0.45 mmol/L for the first trial and 9.6 mmol/L and 0.49 mmol/L for the second trial, respectively. The serine protease inhibitor phenyl methyl sulfonyl fluoride and the antioxidant butylated hydroxytoluene (BHT) were added to isolated plasma at a final concentration of 0.5 mmol/L and 20 μmol/L, respectively, to inhibit proteolysis and oxidation.[23]

LDLs were isolated by density gradient ultracentrifugation followed by exhaustive dialysis against buffer (0.9% NaCl, 2 mmol/L EDTA, pH 7.4). LDL were sterilized by filtration (0.45-μm Millipore filter, Beverly, MA) and split into two equal aliquots. One aliquot (gLDL) was incubated in a water bath at 37°C with 200 mmol D-glucose in phosphate-buffered saline (PBS; pH 7.4) for 72 hours in 2-mL polypropylene vials with the caps slightly loosened (ie, under aerobic conditions). The second aliquot (cLDL) was incubated for 72 hours without glucose and in the presence of 1 mg/mL EDTA and 20 μmol/L BHT in tightly capped polypropylene vials to inhibit oxidation. After 72 hours, the LDL were removed from the water bath. cLDL were stored at 4°C under argon until radioisotope labeling. gLDL were dialyzed for 8 hours against PBS buffer to remove free glucose and then stored at 4°C under argon.

Glycation of the LDL aliquots was determined as described earlier. In vitro glycation of LDL resulted in an approximately threefold increase in the level of LDL glycation (cLDL, 32.1 mmol/mg; gLDL, 106.1 mmol/mg). LDL aliquots were coupled to either ^{131}I-tyramine cellobiose (TC) or to ^{125}I-TC, as previously described.[23,27] To account for possible differences in LDL accumulation due to the radioactive isotope, for the first eight monkeys, gLDL were coupled to ^{125}I-TC and cLDL to ^{131}I-TC, while for the second seven monkeys, gLDL were coupled to ^{131}I-TC and cLDL to ^{125}I-TC. Specific activities of ^{125}I-TC-LDL were 280 cpm/ng and 710 cpm/ng for the first and second set, respectively, and for ^{131}I-TC-LDL, 550 cpm/ng and 440 cpm/ng for the first and second set. Trichloroacetic acid (10% final concentration)-soluble radioactivities for the ^{125}I-TC-LDL were 5.7% for both trials and for ^{131}I-TC-LDL were 2.5% and 5.8% for the first and second trials, respectively. Radioactivities extractable in chloroform-methanol[28] for ^{125}I-TC-LDL were 5.5% and 4.2% and for ^{131}I-TC-LDL were 4.9% and 5.0%, for the first and second trials, respectively. LDL aliquots were used within 1 week of labeling. Just before injection, the two aliquots of LDL were sterilized by filtration (0.45-μm Millipore filter), combined, and coinjected into each monkey. Thus, each monkey served as its own control when comparing gLDL with cLDL, as well as allowing for comparisons of LDL metabolism between groups.

Before injection of labeled LDL, indwelling catheters were inserted into the left femoral artery and vein. Animals were fitted with a nylon mesh jacket and attached to a flexible metal tether (Alice King Chatham Medical Arts, Los Angeles, CA) in their cage to ease collection of multiple blood samples. Radiolabeled LDL (first trial, 2.3×10^8 cpm ^{125}I and 4.8×10^8 cpm ^{131}I; second trial, 2.0×10^9 cpm ^{125}I and 9.8×10^8 cpm ^{131}I) were injected into each animal through the venous catheter 48 hours before necropsy. Subsequent blood samples were collected from the arterial catheter into tubes containing EDTA (0.1% final concentration) at 4, 8, 15, 20, 40, and 60 minutes and 2, 4, 6, 20, 24, 28, and 48 hours after injection to determine the plasma decay of labeled LDL. The plasma FCR of LDL was calculated from exponents and coefficients determined by the biexponential equation fitted to data for the decrease of protein-bound radioactivity in the plasma.[23]

Animals underwent necropsy immediately after the 48-hour blood sample was collected. Animals were sedated with ketamine hydrochloride, then deeply anesthetized with sodium pentobarbitol (80 mg/kg intravenously) and perfused with lactated Ringer's solution containing 50 mmol EDTA and 2.4 μmol BHT through a catheter introduced into the left ventricle. An incision was made in the caudal vena cava, through which blood containing perfusate was removed until the perfusate became colorless. The heart was removed, the major cardiac vessels

clamped, and then perfusion-fixed with modified Karnovsky's fixative at 100 mm Hg for 30 minutes. Arterial segments (thoracic aorta, abdominal aorta, right common carotid and carotid bifurcation, left common iliac, and left femoral arteries) and tissues (liver, spleen, and adrenal gland) were fixed in modified Karnovsky's fixative for 24 hours to preserve radiolabeled TC trapped in lysosomes and undegraded TC-LDL not yet taken up by the cells or degraded by the tissues.[23] As such, radioactivity measured in the tissues represents the combined products of degraded and undegraded LDL (ie, LDL accumulation). ^{125}I and ^{131}I radioactivities in tissues and plasma were determined in a well-type gamma counter (model 5500B; Packared Instruments, Meridian, CT). Arterial ^{125}I and ^{131}I radioactivity (cpm/g) was normalized by the area under the curve of protein-bound ^{125}I and ^{131}I radioactivity in plasma during the metabolic experiment ([cpm/μL] × h) to express the arterial radioactivity in a form (μL/g/h) independent of the plasma LDL concentration and amount of labeled LDL injected.[23] Radioactivities of the samples were corrected for overlap of energy spectra of the two isotopes, for background radiation, and for isotopic decay. Samples were counted for 2 hours, giving a 2σ counting error of less than 1.0% for ^{125}I and less than 3.0% for ^{131}I. Background was counted until a minimum of 10,000 counts accumulated, resulting in a 2σ counting error of less than 2.0%.

Arterial Cholesterol Content and Measurement of Atherosclerosis

Lipid extracts of arterial tissue were prepared from the baseline iliac artery biopsy, the entire arterial segment used to measure LDL accumulation for the thoracic and abdominal aorta; iliac, carotid, and carotid bifurcation; and a segment of the femoral artery adjacent to that used for the metabolic experiment, using the method of Folch et al.[28] Total and free cholesterol concentrations were determined enzymatically for the thoracic and abdominal aorta, and iliac, carotid, and carotid bifurcation, as described previously.[29] Esterified cholesterol (CE) content was determined as the difference between measured total and free cholesterol. As the femoral artery and baseline iliac biopsy were too small to accurately measure cholesterol content enzymatically, the cholesterol content was measured using gas chromatograph analysis of the lipid extract from these arteries.

Arterial samples for histologic analysis were taken from segments of the proximal abdominal aorta and femoral artery, which had been frozen in liquid nitrogen and stored at −70°C, and the left main and left circumflex coronary artery, which had been stored in 10% buffered neutral formaldehyde. After fixation in 10% buffered neutral formaldehyde, segments were embedded in paraffin and stained with Verhoeff-van Gieson's stain. The extent of atherosclerosis was determined by digitizing the area between the internal elastic lamina and luminal surface, using methods previously described,[23,30] and expressed as the mean intimal area (in millimeters squared). Data reported for the coronary arteries represent the mean of the left main and left circumflex coronary artery.

Arterial AGE Content

Arterial AGE content was determined by quantifying total fluorescence in arterial collagen. Sections of the thoracic aorta and carotid artery were taken from each monkey, quick-frozen in liquid nitrogen, and stored at −70°C. As described previously,[31] lipids and soluble proteins were extracted sequentially with 1 mol/L NaCl, chloroform: methanol (2:1), and 0.5N acetic acid. Collagen was then digested with 1% pepsin (wt/wt) in 0.25 mol/L acetic acid and the hydroxyproline content was measured by the procedure of Stegemann and Stalder,[32] as modified by Maekawa et al.[33] Fluorescence was measured in the collagen digest at excitation and emission wavelengths of 325 and 375 nm, respectively. Total fluorescence was normalized for hydroxyproline content and reported as units per milligram hydroxyproline.

Statistical Analyses

Treatment groups were compared statistically by using repeated-measures ANOVA and ANCOVA to determine treatment differences across multiple arterial sites within each animal, as well as multiple measurements over time in each animal. Pretreatment iliac artery CE content was used as a covariate for arterial LDL accumulation and arterial cholesterol content, since this measure of preexisting atherosclerosis was a significant predictor of arterial LDL accumulation and cholesterol content in multiple arterial sites. To reduce skewness and stabilize variability between groups, square-root transformations of intimal areas were used in analyses. Multiple regression analysis was performed to determine the relationship between intimal area and lumen area and to test for group differences. Pearson's correlation coefficients were used to assess the relationships among variables. Analyses were performed using the BMDP statistical package (BMDP Statistical Software, Los Angeles, CA). All values are reported as the mean ± SEM and are based on original units. P values less than .05 were considered significant.

RESULTS

Within 3 weeks of STZ injection, there was a significant increase in GHb (baseline, 4.6% ± 0.3%; 3 weeks, 7.4% ± 0.8%; $P < .01$) in STZ-DM monkeys, which then remained stable for the remainder of the trial (GHb at 6 months, 8.3% ± 0.9%), whereas there were no significant changes in control monkeys (baseline GHb, 4.5% ± 0.3%; 6 months, 3.7% ± 0.3%). Group means for plasma lipid and lipoprotein concentrations in the baseline and treatment period are shown in Table 1. One STZ-DM monkey was not adequately controlled with exogenous insulin at the time of the 2-month treatment measurements (TPC, 25 mmol/L; TG, 7.8 mmol/L) and was not included in the lipid measurements for the STZ-DM monkeys. There were no significant changes with time or group differences in plasma lipid and lipoprotein concentrations. However, there was a significant decrease in average LDL size from

Table 1. Changes in Plasma Lipids and Lipoproteins Over 6 Months in Control and STZ-DM Monkeys (mean ± SEM)

Variable	Control (n = 7)	STZ-DM (n = 7)
Total plasma cholesterol (mmol/L)		
Baseline	9.3 ± 1.5	11.6 ± 1.0
Treatment	8.9 ± 1.2	11.1 ± 1.0
TG (mmol/L)		
Baseline	0.30 ± 0.05	0.19 ± 0.03
Treatment	0.30 ± 0.05	0.30 ± 0.07
HDL-C		
Baseline	1.0 ± 0.2	0.8 ± 0.2
Treatment	1.1 ± 0.1	0.9 ± 0.1
VLDL + LDL-C		
Baseline	8.3 ± 1.6	10.8 ± 1.1
Treatment	7.8 ± 1.2	10.2 ± 1.0
LDL size (nm)		
Baseline	346 ± 16	369 ± 8
Treatment	347 ± 15	360 ± 8*
LDL glycation (mmol/L/mg)		
Baseline	20.9 ± 1.6	23.2 ± 1.8
Treatment	23.0 ± 3.8	36.1 ± 1.8†‡

*$P < .05$, time difference.
†$P < .01$, group difference.
‡$P < .01$, time difference.

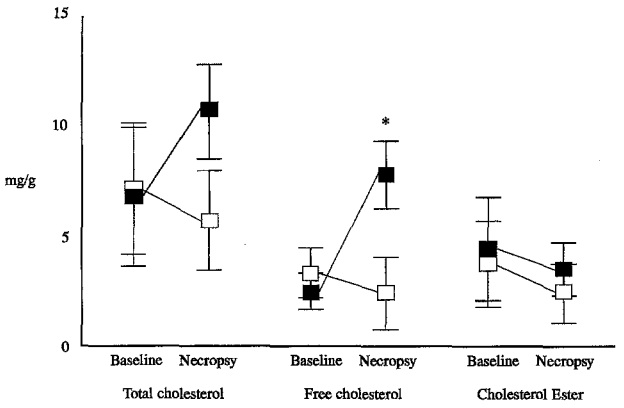

Fig 1. Changes in iliac artery cholesterol content after 6 months in control (□) and diabetic (STZ-DM; ■) monkeys. *$P < .05$, time difference.

baseline in STZ-DM monkeys. LDL glycation increased significantly over time in the STZ-DM monkeys and was significantly higher than the control monkeys.

Arterial Cholesterol Content and Measurement of Atherosclerosis

At baseline, there were no group differences in total or free cholesterol or CE content of the iliac artery. However, in STZ-DM monkeys, after 6 months, the iliac arteries had 58% more total arterial cholesterol ($P = .16$) and 217% more free arterial cholesterol ($P < .05$) compared with baseline, whereas in the control monkeys, there was a slight decrease in arterial cholesterol content (Fig 1). In addition to the iliac artery cholesterol content, across all arterial sites, STZ-DM monkeys had significantly greater arterial cholesterol content compared with control monkeys (Table 2; $P < .05$, repeated-measures ANCOVA). There was also a significant regional difference in the amount of total arterial cholesterol present ($P < .05$, repeated-measures ANCOVA), with the carotid bifurcation having the most cholesterol and the femoral artery having the least amount of cholesterol in both groups.

Atherosclerosis extent, as measured by intimal area, was also increased in STZ-DM monkeys. Intimal areas were significantly greater in the femoral artery and abdominal aorta of STZ-DM monkeys compared with control monkeys, with similar trends in the coronary artery (Table 3). In the femoral artery, intimal lesions (fatty streak or plaque) were present in seven of eight STZ-DM monkeys, but only 2 of 7 control monkeys. In the femoral artery, four of eight STZ-DM monkeys had atherosclerotic plaques, defined as mean intimal thickness equal to or greater than half the medial thickness,[30] whereas none of the control monkeys had plaques. In the abdominal aorta and coronary artery, the number of monkeys with atherosclerotic plaque were similar, although the extent was greater in STZ-DM monkeys (abdominal aorta: control, five of seven monkeys, STZ-DM, eight of eight; coronary artery: control, four of seven, STZ-DM, five of eight). In addition, in the abdominal aorta (Fig 2) from STZ-DM monkeys, there was a lack of remodeling, or compensation, with increasing atherosclerosis (eg, increasing lumen area in response to increasing plaque area[30]) compared with control monkeys ($P < .05$). There was a similar response demonstrated in the femoral artery from STZ-DM monkeys; however, as only two control monkeys had intimal thickening, it was not possible to determine group differences.

Table 3. Atherosclerosis Measurements in Control and STZ-DM Monkeys After 6 Months of Treatment

Artery	Intimal Area (mm^2)	
	Control	STZ-DM
Femoral artery	0.01 ± 0.10	0.30 ± 0.10*
Abdominal aorta	0.97 ± 0.20	1.58 ± 0.18*
Coronary artery	0.24 ± 0.16	0.45 ± 0.15

*$P < .05$.

LDL Metabolism

Arterial LDL accumulation was analyzed for each arterial site separately, as well as across all sites. In vitro glycation of LDL was associated with only a 0% to 5% increase in arterial LDL accumulation ($P = .88$, repeated-measures ANCOVA). Since there was no overall treatment difference due to in vitro glycation of LDL, data shown represent the adjusted mean for gLDL and cLDL (Fig 3). There was an 85% increase in femoral artery LDL accumulation in STZ-DM monkeys compared with control monkeys ($P < .05$, ANCOVA). In all other arterial sites from STZ-DM monkeys, there was a 25% to 30% increase in LDL accumulation, although this was not significant. When analyzed across all arterial sites, there was a tendency for increased LDL accumulation in STZ-DM monkeys ($P = .21$, ANCOVA). Similar to regional differences in arterial cholesterol content, greater amounts of LDL accumulation were found in the carotid bifurcation and carotid arteries, with lesser amounts found in the aorta and femoral arteries ($P < .01$).

Table 2. Arterial Cholesterol Content in Control and STZ-DM Monkeys After 6 Months of Treatment (mg/g)*

	Total Cholesterol†		Free Cholesterol		Cholesterol Ester	
	Control	STZ-DM	Control	STZ-DM	Control	STZ-DM
Femoral	2.6 ± 0.9	3.2 ± 0.9	1.4 ± 0.3	1.7 ± 0.3	1.1 ± 0.6	1.6 ± 0.5
Iliac	5.6 ± 2.2	10.4 ± 2.1	2.4 ± 1.6	7.6 ± 1.5*	2.4 ± 1.3	3.4 ± 1.2
Abdominal aorta	6.1 ± 2.0	10.2 ± 1.8	1.9 ± 0.7	3.5 ± 0.7	4.2 ± 1.3	6.6 ± 1.2
Thoracic aorta	6.8 ± 2.6	12.7 ± 2.4*	3.1 ± 0.9	3.8 ± 0.9	3.9 ± 1.8	8.8 ± 1.6
Carotid	6.6 ± 2.1	8.7 ± 2.0	2.5 ± 0.6	2.9 ± 0.6	4.1 ± 1.5	5.9 ± 1.6
Carotid bifurcation	12.1 ± 3.2	15.6 ± 2.7	4.7 ± 1.2	5.5 ± 1.0	7.4 ± 2.0	10.1 ± 2.0

NOTE. Values adjusted for baseline iliac artery total cholesterol, free cholesterol, or cholesterol ester content.

*$P < .05$, group difference.

†$P < .05$, overall group difference as analyzed by repeated-measures ANOVA.

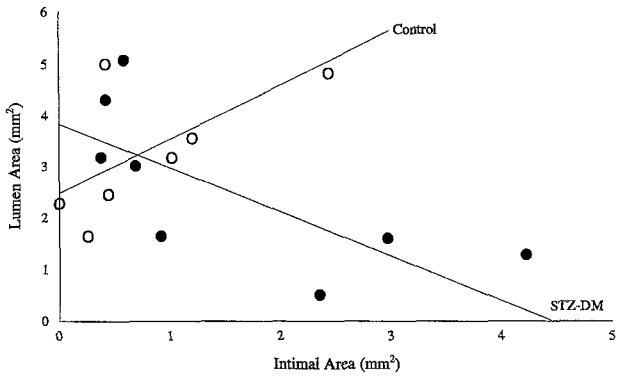

Fig 2. Lumen area–intimal area relationship in the abdominal aorta from control (○; Y = 2.40 + 0.98X, $P = .12$) and diabetic (STZ-DM: ●; Y = 3.78 − 0.79X, $P = .04$) monkeys, $P < .05$.

To determine if diabetes resulted in increased arterial LDL accumulation independent of atherosclerosis extent, we covaried each arterial site by its respective arterial cholesterol content determined after necropsy. In the femoral artery from STZ-DM monkeys, there still was a 67% increase in LDL accumulation ($P > .05$, ANCOVA), whereas in other arterial sites, the increased LDL accumulation was associated with increased cholesterol content. There were significant correlations between amount of LDL accumulation and arterial CE content in arterial segments ($r = .65$ to $.92$; $P < .01$), with a similar trend in the femoral artery ($r = .47$; $P < .1$). There were also significant correlations between LDL accumulation and intimal area in the abdominal aorta ($r = .69$, $P < .01$) and in the femoral artery ($r = .64$, $P < .01$). LDL size was correlated with LDL accumulation in all animals in the iliac artery ($r = .54$, $P < .05$) and the carotid bifurcation ($r = .53$, $P < .05$), with similar trends in the carotid artery ($r = .49$) and abdominal aorta ($r = .42$).

Effects of chronic hyperglycemia and LDL glycation on measures of LDL accumulation in the organs and plasma LDL-FCR are presented in Table 4. There was no difference in plasma LDL FCR between gLDL and cLDL in either group. However, STZ-DM monkeys had significantly decreased plasma LDL FCR regardless of LDL type. Consistent with decreased FCR, in STZ-DM monkeys, accumulation of LDL was also significantly decreased in the liver and adrenal gland, with a similar trend in the spleen. In addition, the rate of hepatic gLDL accumulation was significantly lower compared with cLDL

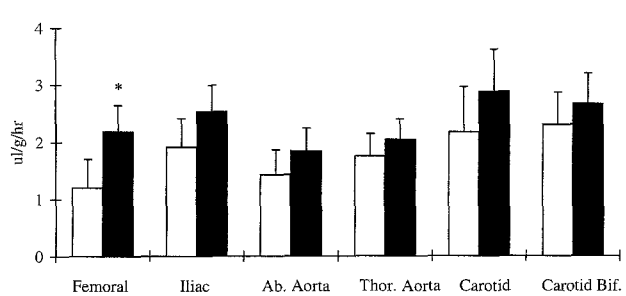

Fig 3. Comparisons of arterial LDL accumulation in multiple arterial sites in control (□) and STZ-DM (■) monkeys. Means (adjusted for LDL glycation) ± SEM. *$P < .05$, group difference; repeated-measures ANCOVA, $P = .21$.

($P < .01$), whereas gLDL accumulated at a greater rate in the adrenal gland compared with cLDL ($P < .01$). There were significant negative correlations between plasma VLDL + LDL-C concentrations and hepatic LDL accumulation (gLDL, −.68; cLDL, −.58; $P < .05$) and LDL accumulation in the adrenal gland (gLDL, −.82; cLDL, −.83; $P < .01$).

Arterial AGE Content

There were no differences in arterial collagen AGE content in the carotid artery (control, 29.2 ± 3.5; STZ-DM, 29.7 ± 3.2 U/mg hydroxyproline; $P = .93$), or in the thoracic aorta (control, 36.9 ± 5.1; STZ-DM, 44.3 ± 4.7 U/mg hydroxyproline; $P = .31$).

DISCUSSION

This study was designed to determine if diabetic monkeys had increased atherosclerosis and to examine mechanisms whereby the chronic hyperglycemia of diabetes mellitus may contribute to the development of atherosclerosis. Similar to diabetic humans[5] and previous reports in other species of experimentally induced diabetic monkeys,[34,35] STZ-DM monkeys had increases in multiple measures of atherogenesis, despite only moderate changes in plasma lipid and lipoprotein concentrations. After 6 months of hyperglycemia, STZ-DM monkeys had a 58% increase in total cholesterol content in the iliac artery, whereas control monkeys had a slight decrease (Fig 1). STZ-DM monkeys also had more arterial cholesterol in all arterial sites assessed compared with control monkeys ($P < .05$, repeated measures ANCOVA; Table 2). Consistent with increased arterial cholesterol content, STZ-DM monkeys had significantly increased intimal areas in both the femoral artery and abdominal aorta, with a similar trend in the coronary artery (Table 3). Moreover, the femoral artery and abdominal aorta (Fig 2) from STZ-DM monkeys did not remodel, or compensate, in response to increasing atherosclerosis, in contrast to control monkeys ($P < .05$ in the abdominal aorta) and a previous report in nondiabetic humans and nondiabetic cynomolgus monkeys.[30] Decreased arterial remodeling or compensation may be a major determinant of future clinical sequelae.[30] Even more noteworthy was the marked atherosclerosis in femoral arteries in STZ-DM monkeys (seven of eight monkeys with measurable intimal lesions), whereas control monkeys had virtually no atherosclerosis present (only two of seven with measurable lesions). The striking increase in femoral LDL accumulation, atherosclerosis, and lack of arterial remodeling in the STZ-DM monkeys is consistent with increased vascular disease at this site in diabetic patients.[5]

Increased progression of atherosclerosis in STZ-DM monkeys may be due to increased LDL uptake and accumulation. In support of this, the rate of LDL accumulation was 85% greater in the femoral artery ($P < .05$) in STZ-DM monkeys, with a 20% to 30% increase in the rates of arterial LDL accumulation in other arterial segments compared with control monkeys. However, as there are also differences in atherosclerosis extent in this study, there may be increased LDL accumulation due to increased lesion.[36] Thus, in this study, it is difficult to determine whether the increased LDL accumulation resulted in increased atherosclerosis or whether the increased atherosclerosis resulted in increased LDL accumulation. However, there was 67%

Table 4. Effects of Glycation on Tyramine Cellobiose Accumulation in Tissue Samples and Individual Organs in STZ-DM Monkeys After 6 Months of Treatment

	Control		STZ-DM	
Group/ LDL Type	Control	Glycated	Control	Glycated
FCR (pools/h)*	0.033 ± 0.004	0.035 ± 0.003	0.026 ± 0.003	0.025 ± 0.002
Accumulation of tyramine cellobiose (μL/g wet wt/h)				
Liver*†	34.6 ± 8.4	32.7 ± 8.3	20.1 ± 1.9	16.9 ± 1.5
Adrenal gland*†	92.6 ± 27.3	124.0 ± 29.1	55.6 ± 15.0	70.5 ± 13.8
Spleen	20.3 ± 2.4	19.1 ± 2.0	18.7 ± 2.5	17.9 ± 1.4
Whole-organ accumulation of tyramine cellobiose (μL/organ/h)				
Liver*†	4,210 ± 558	3,943 ± 522	2,448 ± 349	2,043 ± 298
Adrenal gland†	48 ± 19	63 ± 21	29 ± 10	35 ± 10
Spleen	221 ± 34	208 ± 32	133 ± 9	140 ± 16

*$P < .05$, control *v* STZ-DM.
†$P < .01$, gLDL *v* cLDL.

greater LDL accumulation in the femoral artery even after correcting for necropsy arterial cholesterol content, suggesting an independent effect of diabetes mellitus on arterial LDL metabolism at this site. Regardless, in vitro glycation of LDL was only associated with a small increase in arterial accumulation (0% to 5% greater than cLDL). While this is consistent with a previous report in which there was no difference in aortic LDL uptake due to mild in vitro LDL glycation in normal or cholesterol-fed rabbits, this does not imply that in vivo–glycated LDL is not atherogenic.[37] For example, an additional report from this study found that in vivo–glycated LDL was associated with increased proteoglycan binding,[38] consistent with other reports which suggest that gLDL is atherogenic.[7,12] Moreover, this study only reflects accumulation over a 48-hour period, which might underestimate the effect over a longer period of time (ie, months or years). However, the 0% to 5% increased LDL accumulation due to in vitro glycation versus the 20% to 85% increase in total LDL accumulation in STZ-DM monkeys suggests differences in either the artery itself, or other differences in LDL from diabetic subjects, such as size, composition, or oxidative potential, which may affect arterial LDL accumulation. Also, there may be differences between in vivo glycation and in vitro glycation that would effect the results.

Changes to the artery itself, such as increased arterial AGE may also promote atherogenesis by leading to increased retention of LDL particles within the matrix.[9] This could promote plaque formation by preventing diffusion of LDL out of the intima and possibly by restricting the removal of LDL particles by macrophages resulting in increased susceptibility to oxidative damage or formation of AGE directly on the LDL particle.[9] In this study, there was no increase in arterial collagen AGE as determined by fluorescence. One reason why AGE formation may not have been increased is macrophage degradation of AGE. Decreased collagen-linked fluorescence (ie, decreased collagen-AGE) has been reported in areas of human aorta covered with superficial plaque, suggesting that macrophage accumulation in the atherosclerotic plaque may actually lead to enhanced degradation of AGE modified proteins.[39] As the plaque becomes more complex and fibrotic, the amount of AGE would increase in diabetic subjects reflecting the differences in glycemia.[39] Second, the 6-month trial may have been too short in duration as collagen has a long half-life. With longer treatment, we speculate that there would likely be an increase in arterial collagen AGE and further exacerbation of atherosclerosis.

Differences in LDL composition in diabetic subjects may also enhance atherogenesis. Consistent with this, there was a significant decrease in LDL size and a significant increase in LDL glycation in the STZ-DM monkeys over time. These changes in LDL particles may result in increased susceptibility to oxidation and formation of AGE.[40] Decreased LDL size also suggests TG enrichment of the LDL particles, which has been linked to increased risk of atherosclerosis in humans.[41] However, in nonhuman primates, small LDL have been associated with decreased atherosclerosis,[23] and in the present study, there was a positive correlation between LDL size and arterial LDL accumulation in multiple artery segments. However, other than increased glycation, there were no mass or percentage differences in LDL composition from control and STZ-DM for protein, phospholipid, or free cholesterol and CE.[38] Thus, further studies of LDL composition and atherogenesis in diabetic subjects are required.

As in a previous report in diabetic humans,[16] plasma LDL-FCR was significantly decreased in STZ-DM monkeys compared with control monkeys, most likely due to decreased tissue removal, as suggested by the decreased hepatic and adrenal LDL accumulation (Table 4). Delayed LDL removal from the circulation may predispose the particles to further glycation and oxidation, possibly making them more atherogenic. Also, the slightly higher lipid concentrations in STZ-DM monkeys, may have resulted in downregulation of classic LDL receptors, thus decreasing receptor-mediated accumulation. In support of this, there was a significant negative correlation between plasma VLDL + LDL-C concentrations and LDL accumulation in both the liver and adrenal gland, which contain a preponderance of classic LDL receptors. The lower rate of LDL accumulation in the adrenal glands in STZ-DM monkeys may also suggest decreased adrenal function, although data on adrenal function in

diabetic subjects are contradictory, reporting both increased[42] and decreased function.[43]

Glycation of LDL was not associated with any changes in plasma LDL FCR (Table 4). This is in contradiction to previous reports, which showed altered LDL FCR secondary to in vitro glycation, and suggest that gLDL may be atherogenic.[7,8,12-14] There are several possible explanations for this discrepency. In some previous reports, LDL was glycated in the presence of sodium cyanoborohydride, producing glucitollysine, which differs from the predominant in vivo form and the form in the current study. Also, in our study, LDL was glycated under aerobic conditions, predisposing it to oxidation, which may have increased plasma clearance.[44] However, analysis of the LDL decay rates showed that 99% of the cLDL and 98% of the gLDL remained in circulation 5 minutes after injection, suggesting that the gLDL was not altered so radically as to result in altered clearance.

The results reported herein represent the first study of effects of chronic hyperglycemia on the development of atherosclerosis in the cynomolgus monkey, a well-described model of atherosclerosis and aging.[45] Experimentally induced diabetes mellitus increased arterial LDL accumulation and atherosclerosis (extent and cholesterol content) and reduced arterial remodeling. There was little increase in arterial LDL accumulation due to in vitro glycation and no increase in arterial AGE. Thus, the mechanisms of increased atherosclerosis in diabetic subjects may be related to direct effects of chronic hyperglycemia on the artery wall. Further, the increases in arterial LDL metabolism and atherosclerosis during this 6-month study suggest that changes in atherogenesis precede collagen AGE formation.

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