Addition of dietary fat to cholesterol in the diets of LDL receptor knockout mice: effects on plasma insulin, lipoproteins, and atherosclerosis [S]

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Abstract **The factors underlying cardiovascular risk in patients with diabetes have not been clearly elucidated. Efforts to study this in mice have been hindered because the usual atherogenic diets that contain fat and cholesterol also lead to obesity and insulin resistance. We compared plasma glucose, insulin, and atherosclerotic lesion formation in LDL receptor knockout (*Ldlr*$^{-/-}$) mice fed diets with varying fat and cholesterol content that induced similar lipoprotein profiles. *Ldlr*$^{-/-}$ mice fed a high-fat diet developed obesity, mild hyperglycemia, hyperinsulinemia, and hypertriglyceridemia. Quantitative and qualitative assessments of atherosclerosis were unchanged in diabetic *Ldlr*$^{-/-}$ mice fed a high-fat diet compared with lean nondiabetic control mice after 20 weeks of diet. Although one group of mice fed diets for 40 weeks had larger lesions at the aortic root, this was associated with a more atherogenic lipoprotein profile. The presence of a human aldose reductase transgene had no effect on atherosclerosis in fat-fed *Ldlr*$^{-/-}$ mice with mild diabetes. Our data suggest that when lipoprotein profiles are similar, addition of fat to a cholesterol-rich diet does not increase atherosclerotic lesion formation in *Ldlr*$^{-/-}$ mice.**—Wu, L., R. Vikramadithyan, S. Yu, C. Pau, Y. Hu, I. J. Goldberg, and H. M. Dansky. **Addition of dietary fat to cholesterol in the diets of LDL receptor knockout mice: effects on plasma insulin, lipoproteins, and atherosclerosis.** ***J. Lipid Res.*** **2006.** 47: **2215–2222.**

Supplementary key words diabetes • insulin resistance • aldose reductase

Type 2 diabetes is an independent risk factor for the development of atherosclerotic vascular disease and its complications (1, 2). The increase in vascular disease in patients with diabetes is thought to be due to the deleterious effects of metabolic abnormalities, such as hyperglycemia, insulin resistance, dyslipidemia, and advanced glycation end products (3, 4). The association of diabetic vascular disease and metabolic factors stems from epidemiologic studies showing quantitative relationships between serum glucose and cardiovascular disease event rates (5, 6), and cell culture models that demonstrate adverse effects of hyperglycemia on vascular cells (as reviewed in Refs. 3, 7). Despite these observations, the results of clinical intervention trials suggest that the specific metabolic factor(s) that directly influence atherogenesis in vivo have not been clearly elucidated. Specifically, data are not available demonstrating reduced vascular disease with better glucose control in subjects with type 2 diabetes (8, 9) who have hyperglycemia, hyperinsulinemia, insulin resistance, and lipid abnormalities. In contrast, clinical studies highlight the importance of lowering plasma lipids to reduce cardiovascular morbidity and mortality in patients with and without diabetes (10). An alternative hypothesis is that defective insulin action, and not hyperglycemia, is vasculotoxic. There are several studies that support this hypothesis. First, macrophages lacking the insulin receptor have increased expression of scavenger receptors and uptake of modified lipoproteins (11). Second, the induction of modest hyperinsulinemia in normal volunteers leads to impairments in endothelium-dependent vasodilation (12). Moreover, the increased risk of coronary heart disease in patients with the metabolic syndrome suggests that the insulin-resistant state is atherogenic without concomitant elevations in plasma glucose and glycosylated hemoglobin (13, 14). In contrast, a study in rabbits failed to show that hyperinsulinemia due to insulin infusion altered atherosclerosis (15). Thus, it may be that other processes associated with type 2 diabetes, such as obesity, changes in adipokines, or circulating inflammatory molecules, are vasculotoxic.

Abbreviations: ApoE, apolipoprotein E; AR, aldose reductase; FPLC, fast-protein liquid chromatography; HF1, high-fat diet 1; LF1, low-fat diet 1.

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Within the context of the National Institutes of Health-sponsored program to develop mouse models of diabetic macrovascular disease (see AMDDC.org), we created hyperlipidemic, atherosclerosis-prone mice. The main objective was to determine whether ingestion of a diet containing either lard or butterfat altered atherosclerosis when compared with a low-fat diet enriched in cholesterol. Because the goal was to assess whether fat alters atherosclerosis in *Ldlr*$^{-/-}$ mice, exclusive of any effects on plasma lipoproteins, the diets were devised to induce comparable total plasma cholesterol. The fat-fed mice in many ways reflect changes found with the metabolic syndrome. Relatively few studies have evaluated atherosclerosis in murine models of type 2 diabetes (16–18). Most studies have utilized mice with insulin deficiency (19–22). Our current study demonstrates that mild hyperglycemia, obesity, and insulin resistance had little if any effect on atherosclerosis in *Ldlr*$^{-/-}$ mice. In previous studies with streptozotocin diabetes, introduction of a transgene for the glucose-metabolizing enzyme aldose reductase (AR) increased atherosclerosis (23). However, in the setting of the mild dietary hyperglycemia in the present study, this transgene had no effect on atherosclerosis.

MATERIALS AND METHODS

Animals and diets

C57BL/6 *Ldlr*$^{-/-}$ mice for breeding were purchased from the Jackson Laboratory and bred at Columbia University Medical Center. *Ldlr*$^{-/-}$, AR transgenic mice were obtained as described (23). Two separate experiments were performed with two different pairs of low- and high-fat diets (**Table 1**). In the first experiment, 8 week-old male mice were started on low-fat diet 1 (denoted "LF1"; Harlan Teklad cat TD03585) or high-fat diet 1 (denoted "HF1"; Harlan Teklad cat TD03584, see Table 1) and were fed the diets for a period of 20 or 40 weeks. Both LF1 and HF1 contain lard as the fat source, and a diet very similar to HF1 was previously shown to reliably induce diabetes and atherosclerosis in *Ldlr*$^{-/-}$ mice (18, 24). In the second experiment, a different set of low- and high-fat diets were used. Male *Ldlr*$^{-/-}$ mice were fed either a low-fat diet (denoted "LF2"; Research Diets D01061401) or a high-fat diet (denoted "HF2"; Harlan Teklad 88137) for a period of 20 weeks. The HF2 high-fat diet contains milk fat as its fat source and is a diet commonly used to evaluate atherosclerosis in apolipoprotein E (ApoE$^{-/-}$) and *Ldlr*$^{-/-}$ mice. The low-fat diet LF2 has also been used in multiple studies of atherosclerosis in *Ldlr*$^{-/-}$ mice (25, 26). A more detailed analysis of these diets is shown in Table 1. The Institutional Animal Care and Use Committee of Columbia University approved all animal protocols.

TABLE 1. Composition of study diets

Diet type	LF1	HF1	LF2	HF2
Fat (%wt)	4.3	35.2	4.3	21.2
Carbohydrate (%wt)	65.7	36.1	67.2	48.5
Protein (%wt)	20.4	20.4	19.2	17.3
Lard (g/kg)	40	350	—	—
Milk fat (g/kg)	—	—	—	210
Soybean oil (g/kg)	—	—	25	—
Cocoa butter(g/kg)	—	—	20	—
Sucrose (g/kg)	240	150	200	341
Corn starch (g/kg)	335	—	375	150
Cellulose (g/kg)	—	—	50	50
Maltodextrin (g/kg)	100	191	125	—
Casein (g/kg)	230	230	200	195
Cholesterol (%)	0.02	0.03	0.15	0.15

HF1, high-fat 1; HF2, high-fat 2; LF1, low-fat 1; LF2, low-fat 2.

Blood sampling

Blood was collected from the retro-orbital venous plexus after isoflurane anesthesia for biochemical measurements. Plasma was used for measurement of total cholesterol, triglyceride, and free fatty acids using enzymatic assays (Wako; Richmond, VA). Fast performance liquid chromatography was performed as described previously (20). Lipoproteins, [VLDL (d < 1.006 g/ml), IDL plus LDL (d = 1.006–1.063 g/ml), and HDL (d = 1.063–1.21 g/ml)], were separated by sequential density ultracentrifugation of plasma in a TLA100 rotor (Beckmann Coulter). Glucose levels were determined in 6 h-fasted conscious mice from tail blood samples using a clinical glucometer (Glucometer Elite, Bayer Diagnostics). Insulin was measured using ELISA assays from Alpco Diagnostics following the manufacturer's instructions. For glucose tolerance tests, mice were fasted for 6 h and were given an intraperitoneal injection of glucose (1 mg/g body weight) in PBS. Blood glucose and insulin were determined from tail vein as described above at 0, 15, 30, 50, 90 and 120 min after injection.

Tissue collection

Mice were anesthetized with an intraperitoneal injection of ketamine and xylazine. The chest and peritoneal cavity were opened, and the circulatory system was perfused via the left ventricle with PBS. Tissues surrounding the aorta were cleared under a binocular microscope. The heart (containing the aortic root) and the brachiocephalic artery, were either frozen in OCT compound for frozen sectioning (Tissue-Tek; Sakura Finetek USA, Inc.) or processed for paraffin sectioning. Sections were stained with hematoxylin and eosin or with a modified Movats stain as previously described (27). The quantification of aortic root atherosclerosis (n = 5 sections/mouse) was performed as previously described (28). In mice fed the diets for 40 weeks, the remaining aorta was dissected to the iliac bifurcation, opened longitudinally, and fixed with 10% buffered formalin, and the enface lesion area was quantified as previously described (29). In the mice fed the diets for 20 weeks, the percentage of the aorta covered by lesions was very low; therefore, only the aortic root lesion area is included for this time point.

Statistics

Data are represented as mean ± SEM. Significance was assessed by two-tailed Student's *t*-test for parametric data at $P < 0.05$. Statistical tests were performed using Prism software (GraphPad).

RESULTS

We hypothesized that diet-induced obesity and insulin resistance would increase atherosclerosis in hyperlipidemic *Ldlr*$^{-/-}$ mice. In experiment 1, we used a high-fat diet containing lard. After 20 weeks, mice fed HF1 developed a metabolic syndrome-like phenotype characterized by elevated body weight, mild hyperglycemia, and increased plasma insulin (**Table 2**). Glucose tolerance and insulin secretion in response to an intraperitoneal glucose load were also assessed in LF1 and HF1 diet-fed *Ldlr*$^{-/-}$ mice. Compared with LF1 diet-fed mice, HF1-fed

TABLE 2. Effects on weight, glucose, insulin, and lipoproteins in LDLR$^{-/-}$ mice fed low and high fat diets for 20 weeks

	LF1	HF1	LF2	HF2
Time (weeks)	20	20	20	20
Body weight (g)	30 ± 1 (10)	39 ± 2 (10)[b]	29 ± 1 (8)	39 ± 2 (11)[b]
Glucose (mg/dl)	129 ± 7 (10)	169 ± 5 (10)[c]	122 ± 9 (8)	182 ± 9 (11)[c]
Insulin (ng/ml)	1.0 ± 0.1 (10)	2.1 ± 0.1 (10)[d]	1.1 ± 0.2 (6)	1.9 ± 0.2 (5)[a]
Total				
Chol (mg/dl)	505 ± 48 (10)	489 ± 34 (10)	633 ± 2 (8)	701 ± 10 (10)[c]
TG (mg/dl)	155 ± 24 (10)	226 ± 36 (10)	109 ± 8 (8)	250 ± 26 (9)[c]
VLDL				
Chol (mg/dl)	261 ± 59 (10)	154 ± 27 (10)	318 ± 33 (5)	314 ± 126 (6)
TG (mg/dl)	151 ± 27 (10)	216 ± 34 (10)	60 ± 6 (6)	183 ± 49 (6)
(I)LDL				
Chol (mg/dl)	141 ± 9 (10)	173 ± 7 (10)[a]	225 ± 21 (6)	244 ± 52 (6)
TG (mg/dl)	22 ± 3 (10)	19 ± 3 (10)	22 ± 1 (6)	34 ± 2 (6)[b]
HDL				
Chol (mg/dl)	75 ± 5 (10)	88 ± 5 (10)	12 ± 4 (6)	28 ± 2 (6)[b]
TG (mg/dl)	ND	ND	13 ± 0.6 (6)	18 ± 2 (6)

Data are mean ± SE, with the number of mice assayed in parentheses. Chol, cholesterol; ND, not detected; TG, triglyceride.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] $P < 0.0001$.

mice had impaired glucose tolerance and elevated insulin secretion in response to an intraperitoneal glucose load (**Fig. 1A, B**). Total plasma cholesterol was similar in LF1 and HF1 diet-fed *Ldlr*$^{-/-}$ mice (Table 2). Lipoproteins were fractionated by serial ultracentrifugation; VLDL-C decreased, and LDL(I)-C increased in the plasma of HF1 compared with LF1 diet-fed mice (Table 2). Fast-protein liquid chromatography (FPLC) profiles of pool plasma from LF1 and HF1 diet-fed mice corroborated these findings (**Fig. 2A**).

The second group of mice received a semipurified cholesterol-containing diet with (HF2) or without (LF2) butterfat. As with the HF1 diet, HF2 diet-fed mice developed a metabolic syndrome-like phenotype, characterized by elevated body weight, mild hyperglycemia, and hyperinsulinemia (Table 2). Total, VLDL, and LDL(I) cholesterol were quite similar in the two groups, but HDL-C was higher in HF2 than LF2 diet-fed mice (Table 2, and Fig. 2B). Total triglycerides and triglycerides in VLDL and LDL(I) were also higher in HF2 diet-fed mice (Table 2). When lipoprotein profiles of the two sets of low- and high-fat diets were compared, total cholesterol levels were higher in LF2/HF2 diet-fed mice (Table 2), and the FPLC cholesterol profiles were quite different (Fig. 2A vs. Fig. 2B). Specifically, the butterfat led to a greater proportion of cholesterol in the VLDL peak and to lower HDL cholesterol.

Atherosclerotic lesion area was assessed by measurement of mean aortic root cross-sectional area and the percentage of the aortic surface occupied by lesions in LF1 and HF1 diet-fed mice. At the 20 week time point, *Ldlr*$^{-/-}$ mice fed LF1 and HF1 diets had identical mean aortic root lesion area (**Fig. 3A**). In mice fed the LF2 or HF2 diets,

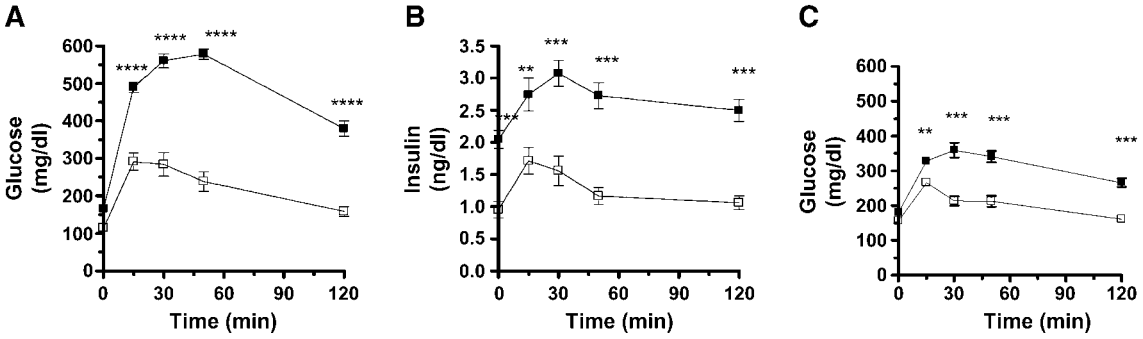

Fig. 1. Intraperitoneal glucose tolerance tests (IPGTTs) in low-fat diet 1- (LF1; open squares) and high-fat diet 1- (HF1; closed squares) fed male *Ldlr*$^{-/-}$ mice. A: Plasma glucose after IPGTT in *Ldlr*$^{-/-}$ mice fed the diets for 20 weeks. Plasma glucose increased significantly in HF1 diet compared with LF1 diet ($F = 304$, $P < 0.0001$ for effect of diet). Area under the curve (AUC) was 57,733 and 26,512 for HF1 and LF1 diet, respectively. B: Plasma insulin after IPGTT in mice fed the LF1 and HF1 diets for 20 weeks. Insulin levels from HF1 diet-fed mice were higher than levels in LF1 diet-fed mice ($F = 132$, $P < 0.0001$). C: IPGTTs in male *Ldlr*$^{-/-}$ mice fed the LF1 and HF1 diet for 40 weeks. Plasma glucose increased significantly in HF1 diet compared with LF1 diet ($F = 120$, $P < 0.0001$ for effect of diet). AUC was 37,242 and 24,123 for HF1 and LF1 diet, respectively. Data are expressed as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

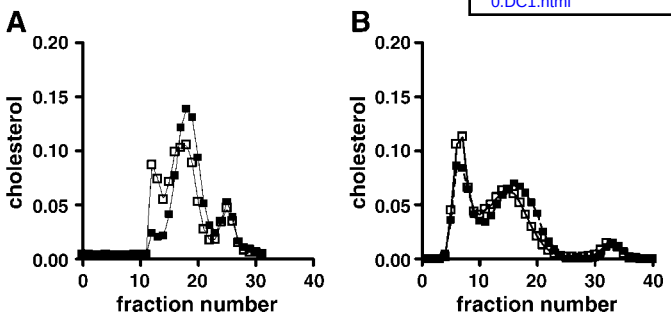

Fig. 2. Fast performance liquid chromatography cholesterol curves obtained from pooled plasma specimens from male *Ldlr*$^{-/-}$ mice fed the LF1 (open squares) and HF1(closed squares) for 20 weeks (A) or LF2 (open squares) and HF2 (closed squares) for 20 weeks (B). Data are expressed as the fraction of total cholesterol mass recovered. Note: Chromatography machine settings were identical for pooled plasma samples from mice fed the low-fat and high-fat diet within each experiment. The fraction numbers corresponding to lipoprotein fractions in A versus B are not comparable due to differences in machine collector settings between the two experiments.

TABLE 3. Effects on weight, glucose, insulin, and lipoproteins in LDLR$^{-/-}$ mice fed low and high fat diets for 40 weeks

	LF1	HF1
Time (weeks)	40	40
Body weight (g)	28 ± 1 (7)	50 ± 2 (8)[d]
Glucose (mg/dl)	158 ± 6 (7)	167 ± 6 (8)
Insulin (ng/ml)	0.42 ± 0.08 (7)	2.9 ± 0.68 (8)[a]
Total		
Chol (mg/dl)	878 ± 98 (7)	933 ± 73 (8)
TG (mg/dl)	119 ± 29 (7)	249 ± 41 (8)[a]
VLDL		
Chol (mg/dl)	545 ± 74 (7)	309 ± 43 (8)[a]
TG (mg/dl)	100 ± 28 (7)	216 ± 39 (8)[a]
(I)LDL		
Chol (mg/dl)	255 ± 29 (7)	512 ± 43 (8)[c]
TG (mg/dl)	37 ± 8 (7)	47 ± 18 (8)
HDL		
Chol (mg/dl)	78 ± 6 (7)	113 ± 7 (8)[b]
TG (mg/dl)	10 ± 2 (7)	27 ± 9 (8)

Data are mean ± SE, with the number of mice assayed in parentheses. Total cholesterol values in this table were calculated from the sum of the individual lipoproteins' fractions.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] $P < 0.0001$.

there was also no significant difference in aortic root lesion area (Fig. 3B). The majority of lesions in the LF1 and HF1 diet-fed mice were fatty streak lesions (see supplementary Fig. I), whereas fibrous caps and necrotic cores were present in the majority of lesions in LF2 and HF2 diet-fed mice (see supplementary Fig. II). No overall qualitative differences in aortic lesion morphology, macrophage foam cell area, or connective tissue were noted in Movats pentachrome-stained sections in the two groups of mice (LF1 vs. HF1 and LF2 vs. HF2; see supplementary Figs. III and IV).

To determine whether diabetes or the altered lipid profile required a longer feeding period to produce more-advanced lesions with the HF1 diet, we evaluated metabolic parameters and atherosclerosis after 40 weeks. HF1 diet-fed *Ldlr*$^{-/-}$ mice had a further increase in body weight and persistent hyperinsulinemia (**Table 3**). However, after 40 weeks, no statistical difference in plasma glucose was noted among the two diet groups (Table 3). Thus, mice fed the high-fat diet appeared to compensate for the continued insulin resistance, possibly through increases in insulin levels. Despite the reduction in fasting glucose, mice fed the HF1 diet remained glucose intolerant (Fig. 1C). There were no differences in total plasma cholesterol (Table 3) in the 40 week LF1 and HF1 diet-fed mice. The HF1 group showed an even greater increase in LDL-C and decrease in VLDL-C than found at the 20 week time point (Table 3). There was also a statistically significant increase in HDL-C, and total and VLDL triglycerides in the 40 week HF1 diet-fed mice (Table 3). Aortic root lesion area was 65% greater (1.6-fold) in the HF1 diet-fed mice at the 40 week time point (**Fig. 4**), but enface lesion area (4.9 ± 2.9% vs. 4.8 ± 1.1%, n = 7–8, P = ns in LF1 vs. HF1, respectively) and brachiocephalic artery cross-sectional lesion area (0.25 ± 0.02 vs. 0.29 ± 0.04 mm^2/section, n = 7–8, P = ns) were identical in the 40 week LF1 and HF1 diet-fed mice. There were no significant correlations among metabolic parameters (cholesterol, glucose, insulin, LDL, VLDL) and lesion area in *Ldlr*$^{-/-}$ mice fed LF1 or HF1 diets for 20 or 40 weeks.

AR may sensitize the arterial wall to the toxic effects of hyperglycemia through the production of mediators that affect the production of reactive oxygen species (7, 23). To determine whether greater AR expression would increase atherosclerosis in the high-fat-diet-induced obese and diabetic *Ldlr*$^{-/-}$ mouse, human (h) AR transgenic mice were bred with *Ldlr*$^{-/-}$ mice. *Ldlr*$^{-/-}$ and hAR transgenic *Ldlr*$^{-/-}$ male mice were fed LF2 and HF2 diets

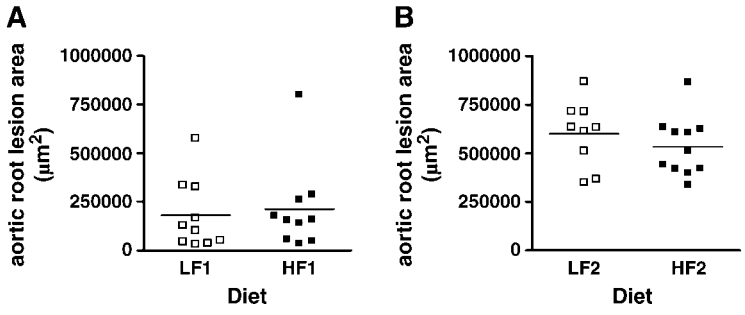

Fig. 3. Atherosclerosis lesion measurements at the aortic root in male *Ldlr*$^{-/-}$ mice fed low-fat (open squares) or high-fat (closed squares) diets for 20 weeks. There were no significant differences in aortic root lesion area in mice fed the LF1 and HF1 diets (A), nor the LF2 and HF2 diets (B).

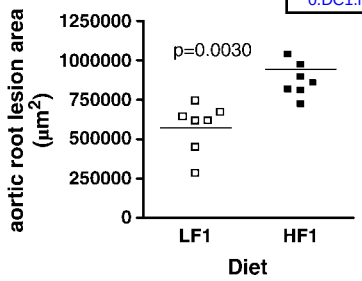

Fig. 4. Atherosclerosis lesion measurements at the aortic root in male $Ldlr^{-/-}$ mice fed low-fat (LF1; open squares) or high-fat (HF1; closed squares) diets for 40 weeks.

for 20 weeks. The transgene had no effect on lipoprotein levels or glucose parameters (data not shown). Aortic root atherosclerosis was not altered by expression of hAR (**Fig. 5**).

DISCUSSION

Using two different diets, high-fat diet feeding of $Ldlr^{-/-}$ mice induced obesity and hyperinsulinemia. These mice also had mild hyperglycemia and, in many ways, were metabolically comparable to humans with metabolic syndrome and impaired glucose tolerance, or type 2 diabetes. Our prediction was that insulin resistance would increase atherosclerotic lesion size in $Ldlr^{-/-}$ mice when compared with low-fat-diet-fed $Ldlr^{-/-}$ mice with similar lipid profiles. Our prediction was wrong! Despite the presence of obesity, mild diabetes, and hyperinsulinemia, atherosclerotic lesion size, the extent of the aortic surface covered by lesions, and the gross appearance of atherosclerotic lesions did not differ in low- and high-fat-fed $Ldlr^{-/-}$ mice fed the diets for 20 weeks. When the period of diet feeding was extended in the lard-containing HF1 diet-fed mice to produce more-advanced lesions, lipoprotein distribution differences were more prominent. With the higher LDL and lower VLDL cholesterol in the HF1 mice, increased aortic root lesion size but not a change in en face area was observed.

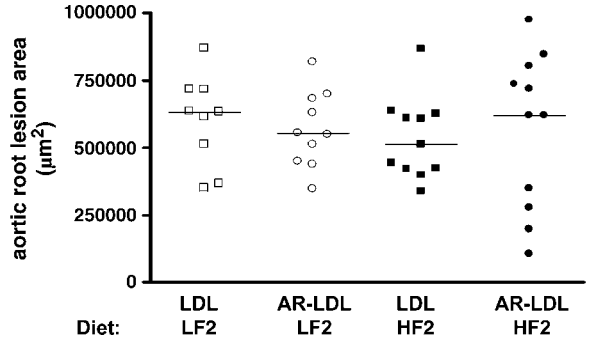

Fig. 5. Atherosclerosis lesion measurements at the aortic root in male $Ldlr^{-/-}$ mice fed low-fat (LF2; open symbols) or high-fat (HF2; closed symbols) diets for 20 weeks. The presence of an aldose reductase transgene (AR-LDL; circles) did not alter aortic root lesion area.

Why did the addition of saturated fat and the presence of metabolic disarray (obesity, mild hyperglycemia, and hyperinsulinemia) have little or no effect on atherosclerosis? A previous study by our group demonstrated that high-fat-induced obesity/diabetes alters endothelial function in mice. HF1-fed mice had impaired endothelium-dependent vasodilation and enhanced reactive oxygen species generation in the aorta (30). Therefore, metabolic changes with the HF1 diet affect the arterial endothelium in mice, even though they do not alter atherosclerosis. It is possible that much higher levels of plasma glucose are needed to affect plaque progression in the mouse. In addition, it may be that cholesterol alone and the lipoprotein changes that it induces were the major factor driving lesion size; the lipid effects obscured any toxic actions of hyperglycemia and hyperinsulinemia.

Both high-fat diets caused mild hyperglycemia; however, this was lost after 40 weeks of the lard diet, HF1. Others have studied the effects of diet on the development of obesity and diabetes in mice. Unlike the relatively atherosclerosis-resistant FVB strain (31, 32), C57BL6 mice do not develop marked diabetes with diets and genetic modification (33). For example, the degree of hyperglycemia in leptin-deficient (ob/ob), leptin receptor-mutant (db/db), and lipodystrophic mice on the C57BL/6 background is relatively mild compared with other genetic backgrounds. Similarly, diet-induced hyperglycemia is less marked on the C57BL/6 background (33–35). The reasons for this are thought to be better islet cell proliferation in response to ambient hyperglycemia (36). It is possible that islet cell proliferation was responsible for the high insulin and normalization of the plasma glucose in the mice fed the HF1 diet for 40 weeks.

Fat feeding is known to cause insulin resistance (37, 38). Insulin clamp studies have previously demonstrated that high-fat diets induce insulin resistance in skeletal muscle, adipose tissue, and liver of mice (39). Although we did not directly study insulin actions by performing insulin clamp studies in the current study, the finding of fasting hyperglycemia despite elevated insulin levels is almost certainly due to insulin resistance. Nevertheless, the fat-fed mice might not have had insulin resistance in vascular cells. In a previous study, we demonstrated that despite the circulating hyperinsulinemia and insulin resistance, insulin signaling was intact in the arterial wall of obese C57BL/6 mice fed the high-fat diet (30). Thus, the lack of demonstrable arterial insulin resistance and changes in atherosclerosis leaves open the question of whether insulin resistance affects atherosclerosis through direct effects on the arterial wall. In vitro studies suggest that loss of macrophage insulin receptors leads to gene changes associated with enhanced atherogenicity (11). Insulin-sensitizing medications, such as thiazolidinediones that are ligands for peroxisomal proliferator receptor γ, reduce atherosclerosis in male $Ldlr^{-/-}$ mice fed the high-fat diet without alterations in plasma lipids (40, 41), and may have direct effects on arterial wall cells (42, 43) and macrophage

biology (40). These agents appear to lead to modest reductions in cardiovascular event rates in patients with type 2 diabetes (9).

Studies of the effects of diabetes on atherosclerosis in mice have produced inconsistent results. Some of these studies have noted a marked increase in lesions in $Ldlr^{-/-}$ and apoE$^{-/-}$ mice with streptozotocin-induced diabetes (20, 21). These studies are all confounded by the development of greater hyperlipidemia in the mice, a condition expected to increase lesion area. Most recently, viral destruction of the pancreas in $Ldlr^{-/-}$ mice led to early lesions, an effect that was not noted when the mice were placed on cholesterol-containing diets (44). Increased atherosclerosis in type 1 diabetic $Ldlr^{-/-}$ mice without altered plasma cholesterol has been reported in some (23, 44) but not all (22) studies. Like our study, a report from Reaven's laboratory found that high-fat diets in $Ldlr^{-/-}$ mice failed to increase atherosclerosis with this mild diabetes (16). In addition, streptozotocin treatment did not alter atherosclerosis in apoB transgenic mice (20). On the basis of the above-mentioned experimental studies, it appears that the relationships between glucose and atherosclerosis in the mouse models are complex and may depend upon interactions between glucose and dietary interventions.

Hyperlipidemia may be a much more potent risk factor than hyperglycemia in the mouse. Marked elevations in plasma cholesterol appear to be a major determinant of atherosclerosis in Western diet-fed $Ldlr^{-/-}$ mice compared with $Ldlr^{-/-}$ mice fed a "diabetogenic" lard-containing diet (17). Although total cholesterol levels were comparable in low-fat, fructose diet-fed $Ldlr^{-/-}$ mice compared with Western diet-fed $Ldlr^{-/-}$ mice, atherosclerosis was significantly reduced in Western diet-fed mice with mild hyperglycemia and insulin resistance (16). The reason for this decrease was not investigated. In the present study, the degree of hyperglycemia was in fact quite mild, and plasma lipids were quite high in $Ldlr^{-/-}$ mice fed the high-fat diet compared with what is observed in human diabetic subjects. In fact, increased plasma lipids were associated with increased atherosclerosis. When the two diet studies are compared, a higher total cholesterol and lower HDL cholesterol in the LF2/HF2 diet-fed $Ldlr^{-/-}$ mice was associated with larger lesion size and progression. The potential atherogenic effects of altered glucose metabolism may have been overridden by the high-fat-diet-induced effects on plasma lipids. The greater HDL-C observed in HF2 versus LF2 diet-fed mice could have counteracted a diabetes-induced increase in atherosclerosis.

We should note that our studies in 40 week-fed mice suggested that the high-fat diet might have altered lesion size. This result is not clear, in that these animals only showed changes in one region, the root but not enface or brachiocephalic, and had atherogenic alterations in their lipoprotein profiles. The HF1-fed mice had a shift to smaller apoB-containing particles (VLDL decreased and LDL increased with no change in total cholesterol); this might have been responsible for the larger lesions at the aortic root. A previous study demonstrated that such a shift in particle size increased atherosclerosis in hyperlipidemic mutant mice (45).

There may be diabetes-associated atherogenic factors that are absent in mice. One such possibility is that AR is expressed at very low levels in mice compared with humans (46, 47). AR catalyzes the reaction of glucose to sorbitol and produces reactive oxygen species in cultured cells (7). The presence of an hAR transgene increased atherosclerosis in hyperglycemic streptozotocin-treated (insulin-deficient) diabetic $Ldlr^{-/-}$ and $Ldlr^{+/-}$ mice (23). In the current study, the hAR transgene had no effect on atherosclerosis, possibly because of the mild hyperglycemia observed in the HF2 diet-fed $Ldlr^{-/-}$ mice.

In summary, we were unable to document a clear atherogenic effect of fat addition to a cholesterol-rich diet in $Ldlr^{-/-}$ mice when lipoprotein profiles were similar. This was despite mild hyperglycemia, increased fasting insulin, and greater obesity in the fat-fed mice. The search continues to find suitable animal models of diabetic vascular disease in which to conduct mechanistic studies. These are needed because the relationship between diabetes and atherosclerosis in humans is also unclear. Clinical studies have clearly demonstrated that treatment of dyslipidemia with statins reduces cardiovascular end points in patients with diabetes (10). Although blood glucose reduction has not been clearly shown to reduce cardiovascular end points in type 2 diabetic subjects (8), tight control of blood glucose has become the standard of care because control of hyperglycemia profoundly affects the incidence and severity of diabetic retinopathy and nephropathy (48). The follow-up of the diabetes control and complications trial did show a decrease in carotid intima media thickness (49) and a reduction in cardiovascular events (50) in patients with type 1 diabetes who were previously randomized to aggressive glucose control. Whether high plasma glucose levels, altered insulin actions, or development of some downstream toxic products harm arteries in humans is unclear. In type 2 diabetes, vasculotoxic glucose/insulin processes may be more difficult to show in the setting of other atherogenic processes that are part of the metabolic syndrome. Uncovering the specific effects of diabetes and insulin resistance using animal models and human investigations holds promise for reducing the diabetes-specific increases in vascular disease.

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