Lipoprotein lipase deficiency and CETP in streptozotocin-treated apoB-expressing mice[S]

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Abstract **Both hyperglycemia and hyperlipidemia have been postulated to increase atherosclerosis in patients with diabetes mellitus. To study the effects of diabetes on lipoprotein profiles and atherosclerosis in a rodent model, we crossed mice that express human apolipoprotein B (HuB), mice that have a heterozygous deletion of lipoprotein lipase (LPL1), and transgenic mice expressing human cholesteryl ester transfer protein (CETP). Lipoprotein profiles due to each genetic modification were assessed while mice were consuming a Western type diet. Fast-protein liquid chromatography analysis of plasma samples showed that HuB/LPL1 mice had increased VLDL triglyceride, and HuB/LPL1/CETP mice had decreased HDL and increased VLDL and IDL/LDL. All strains of mice were made diabetic using streptozotocin (STZ); diabetes did not alter lipid profiles or atherosclerosis in HuB or HuB/LPL1/CETP mice. In contrast, STZ-treated HuB/LPL1 mice were more diabetic, severely hyperlipidemic due to increased cholesterol and triglyceride in VLDL and IDL/LDL, and had more atherosclerosis.**—Kako, Y., M. Massé, L-S. Huang, A. R. Tall, and I. J. Goldberg. **Lipoprotein lipase deficiency and CETP in streptozotocin-treated apoB-expressing mice.** ***J. Lipid Res.*** **2002.** 43: **872–877.**

Supplementary key words diabetes • hypercholesterolemia • lipoproteins • glucose

Although it is well established that humans with type 1 and type 2 diabetes mellitus have more atherosclerotic vascular disease, the reasons for this are uncertain (1, 2). Recent clinical trials have shown that treatments for hypertension and hyperlipidemia reduce the incidence of cardiovascular events (3, 4). In contrast, more vigorous treatment of hyperglycemia caused a significant reduction of microvascular, but not macrovascular disease in diabetic patients (5, 6). The importance of lipid abnormalities in diabetes was also seen in several studies where plasma levels of LDL and HDL cholesterol had a stronger correlation with macrovascular disease than did glycosylated hemoglobin (7, 8). Because lipid abnormalities, hypertension, and diabetes often co-exist, segregating the pathological effects of each has been difficult.

For more than 50 years, investigators have attempted to define the etiology of accelerated atherosclerosis in patients with diabetes. Diabetes induction in rabbits leads to severe hyperlipidemia due to circulating large remnant lipoproteins (9). These particles are too large to enter the artery wall, consequently diabetes decreased atherosclerosis in this model. A number of diabetic-atherosclerosis mouse models that have used severely hypercholesterolemic mice failed to show an independent effect of hyperglycemia on atherogenesis (10–13). In contrast, Park et al. reported more atherosclerosis in streptozotocin (STZ)-induced diabetic apolipoprotein E (apoE) knockout mice and attributed this to complications of hyperglycemia (14). Thus, despite a large number of in vitro observations (15), hyperglycemia does not consistently increase atherosclerosis in vivo.

An ideal mouse model for diabetes and atherosclerosis should have hyperglycemia and a lipoprotein profile that allows for atherogenesis within an experimentally feasible amount of time. The concentration of apoB is ~3.5 mg/dl in mice and 60–120 mg/dl in humans (16, 17). For this reason, genetic alteration is required to produce atherosclerosis in the mice unless they are fed a cholic acid-containing diet (18). Triglyceride (TG) catabolism is faster in mice (19, 20), perhaps due to greater lipoprotein lipase (LPL) activity. Mice lack cholesteryl ester transfer protein (CETP), a key protein required to shuttle core lipids between HDL and the apoB-containing lipoproteins VLDL and LDL.

We attempted to produce mice with a more human-like lipoprotein profile than that found in LDL receptor (LDLR) and apoE knockout mice by over expression of apoB and genetic modulation of LPL and CETP. To specifically assess the effects of hyperglycemia on lipoprotein profiles and atherosclerosis exclusive of obesity and hyperinsulinemia, these dyslipidemic mice were made diabetic using STZ. STZ-treated mice more closely resemble type 1

Abbreviations: CTR, control; HSPG, heparan sulfate proteoglycan; HuB, human apolipoprotein B; LPL1, a heterozygous deletion of lipoprotein lipase; LRP, LDL receptor-related protein; RAGE, receptor for advanced glycosylation endproducts; STZ, streptozotocin; UC, area under the curve; WTD, a Western type diet.

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[S] The online version of this article (available at http://www.jlr.org) contains an additional 2 tables and 1 figure.

than type 2 diabetics because they are insulin deficient, but not insulin resistant. In some of the genotypes we created, diabetes caused hyperlipidemia and accelerated atherosclerosis.

METHODS

Mice housing and diets

Mice were maintained in a temperature-controlled (25°C) facility with a 12 h light/dark cycle and given free access to food and water, except when fasting blood specimens were obtained. Mice were fed either laboratory rodent chow (PMI Nutrition International Inc.) or a Western type diet (WTD, No. 88137, Teklad Premier Laboratory Diets). Rodent chow contained 4.5% (wt/wt) fat; the WTD contained 21% (wt/wt) fat (polyunsaturated/saturated = 0.07), 0.15% (wt/wt) cholesterol, and 19.5% casein. These diets were free of sodium cholate. Body weight was recorded every 4 weeks after 6 h fasting.

Genetically altered mice

Human apoB (16) (HuB), CETP transgenic mice (21), and heterozygous LPL deficient (22) (LPL1) mice were used. Genotyping was done using PCR as described previously (21, 23, 24). HuB, LPL1, and CETP were backcrossed to C57BL/6 for more than five generations. Two separate breeding studies were undertaken to provide sufficient numbers of HuB/LPL1 mice ± diabetes.

Blood sampling

Fasting blood samples were obtained from mice after removal of food for 6 h in the morning. The animals were anesthetized with methoxyflurane, and bled by retroorbital phlebotomy into tubes containing anticoagulant (5 mM EDTA) using heparinized capillary tubes. Glucose was measured using a kit from Sigma (#315), and lipid assays were measured using kits from Boehringer Mannheim Biochemicals. Glucose, cholesterol, and TG were assessed every 4 weeks from 8 weeks of age. The average of the concentrations at 20, 24, 28, and 32 weeks of age was used for some analyses.

Lipoproteins, VLDL ($d < 1.006$ g/ml), IDL/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 TLA 100 rotor (Beckmann Instruments) (23). Insulin was quantified using a rat insulin RIA kit (#SRI-13K, Linco Research Inc.). Free fatty acids were measured using a kit (NEFA C, Wako).

Gel filtration chromatography

Pooled plasma (200 μl) was chromatographed using two Superose 6 columns in series (FPLC; Pharmacia LKB Biotechnology Inc.). Fifty 0.5 ml fractions were collected. VLDL elutes in fractions 5 to 11, IDL and LDL in fractions 11 to 28, and HDL in fractions 28 to 40. Cholesterol and TG levels of FPLC fractions were measured using enzymatic reagents in colorimetric assays modified for a 96-well plate spectrophotometer (SpectraMax 250; Molecular Devices) as described previously (23). The distribution of recovered lipid in each fraction was displayed.

Postheparin lipases

LPL and hepatic TG lipase were measured in the postheparin plasma of mice and humans. Heparinized blood was obtained from mice 5 min after the intravenous injection of 10 units heparin (Elkins-Sinns). Human postheparin plasma was obtained 15 min after the injection of 60 U/kg weight heparin. After the plasma separation at 4°C, samples were stored at −80°C till the assay. LPL activity was measured with a lipid emulsion containing [^{3}H]triolein (25). Human LPL activity was determined using a monoclonal antibody that only inhibits human LPL (25); salt inhibition (1 M NaCl) was used to inhibit mouse LPL.

Diabetes induction

Male mice were divided into two groups at 8 weeks of age; half were treated with 50 mg/kg of STZ (Sigma) using a protocol similar to that described by Kunjathoor et al. (18). Because of the acute release of pancreatic insulin that accompanies STZ treatment, to avoid hypoglycemia, mice were continued on a chow diet for 4 weeks after the treatment. At 12 weeks old mice were switched to WTD for the remainder of the study.

Quantitative atherosclerosis analysis

Mice were killed at 32-weeks-old and atherosclerosis assays were performed on the aortic roots as described previously (23). In brief, hearts were perfused with PBS, fixed in 10% phosphate-buffered formalin, embedded in OCT compound, and sectioned at 10 μm thicknesses in a cryostat. Sections were stained with Oil Red O and hematoxylin, and counter-stained with light green, and lesions of the proximal aorta were measured in 80 μm intervals. The mean lesion area of six sections was calculated and shown as lesion area (μm^2).

Statistical analysis

Statistical analysis for the effects of STZ treatment was done by two tailed Student's *t*-test. Comparisons between three genotypes were performed using one-way ANOVA. When the standard deviations were not equal, analyses were done using nonparametric one-way ANOVA. Correlations between atherosclerotic lesions and plasma glucose, TG, cholesterol, and lipoproteins were analyzed by linear regression.

RESULTS

Effects of LPL deficiency on lipoproteins in HuB mice

HuB mice do not have an appreciable peak of TG in VLDL (23). One reason for this might be that mice have high levels of LPL. To assess this, postheparin lipase activities from normal humans and mice were compared using the same assay conditions. Wild-type mice had >10-fold more LPL activity than humans (82.1 ± 11.8 vs. 6.0 ± 3.8 μmol FFA/h/ml, $P < 0.0001$). Hepatic TG lipase was also significantly increased in the mice, 17.3 ± 5.5 versus 10.0 ± 2.1 μmol FFA/h/ml ($P < 0.05$).

In an effort to produce mice with higher TG levels and a lipoprotein profile more similar to humans, we introduced LPL1 onto the HuB background. In WTD-fed mice, this resulted in an increase in VLDL TG in both males (**Fig. 1**) and females (not shown). However, there was also a reduction in IDL/LDL TG, so that total TG was not affected (**Table 1**). Less LPL is associated with lower HDL in humans; however, the area under the curve (AUC) of HDL-C in HuB/LPL1 mice did not differ significantly from that in HuB mice.

Lipoproteins in HuB/LPL1/CETP mice

To test whether the lack of HDL decrease with LPL deficiency was due to the absence of CETP in mice, HuB were bred with human CETP transgenic mice (21). Although CETP reduced HDL and increased IDL/LDL in HuB mice (26), the lipoprotein profile created was still dissimi-

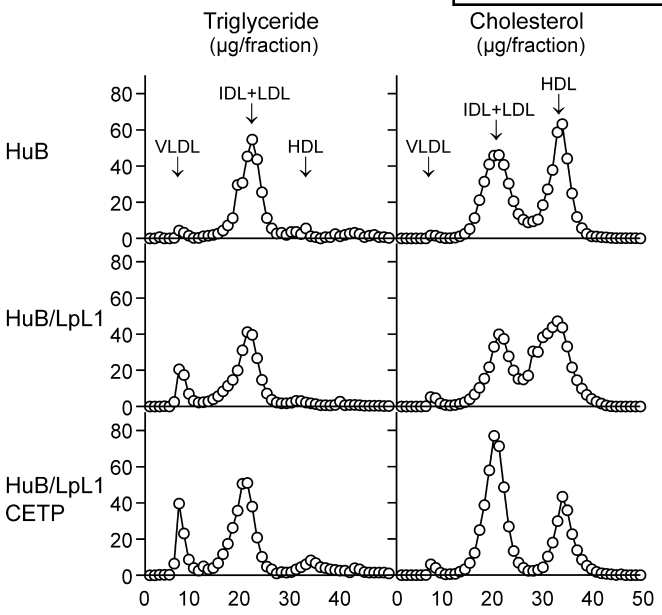

Fig. 1. Fast-protein liquid chromatography profiles of genetically altered human apolipoprotein B (HuB) mice. HuB are human apoB transgenic mice, HuB/heterozygous deletion of lipoprotein lipase (LPL1) are HuB mice with a heterozygous deficiency of lipoprotein lipase, and HuB/LPL1/cholesteryl ester transfer protein (CETP) are HuB/LPL1 mice containing a transgene for human CETP. The distributions of triglyceride and cholesterol in pooled plasmas from three to six 20-week-old male mice fed a Western type diet were determined by gel filtration chromatography as described in Methods.

lar to that found in humans and especially humans with combined hyperlipidemia. To create a partial defect in VLDL catabolism along with the CETP-mediated changes in HDL, HuB/CETP mice were crossed with LPL1 mice. The HuB/LPL1/CETP mice had increased VLDL TG, increased IDL/LDL-C, and decreased HDL-C compared with HuB (Table 1, Fig. 1).

Effects of diabetes on lipoprotein profiles

We next determined whether induction of diabetes with STZ would alter the plasma lipoprotein profiles. Only male mice were studied because the males are more sensitive to STZ treatment. Baseline glucose levels were not significantly different in all three groups prior to the STZ treatment. STZ increased glucose levels to >300 mg/dl and decreased plasma insulin levels; control mice (n = 13) had insulin levels of 2.00 ± 1.02 μg/ml while STZ-treated mice (n = 14) had insulin levels of 0.24 ± 0.18 μg/ml, $P < 0.001$. The glucose levels were monitored every 4 weeks to verify that the diabetes persisted while the mice consumed the WTD. Mice that did not maintain glucose levels >200 mg/dl were excluded from the STZ group (less than 10% of each genotype). Among the three groups of animals, hyperglycemia was most severe in the HuB/LPL1 mice; glucose averaged 434 ± 120 mg/dl in the HuB/LPL1 mice, 302 ± 135 mg/dl in HuB mice, and 328 ± 159 mg/dl in HuB/LPL1/CETP mice at 20 weeks. These mice also had a reduced body weight (Table 1).

Surprisingly, STZ treatment significantly altered plasma lipids only in HuB/LPL1 mice (Table 1, **Fig. 2**). There was a marked increase in TG and cholesterol in VLDL and IDL/LDL. These changes were not observed in mice with normal LPL activity. The presence of the CETP transgene in HuB/LPL1 mice reversed the effects of diabetes on the plasma lipoprotein profile (Table 1, Fig. 2).

Atherosclerosis development

There was no effect of genotype on atherosclerosis in non-STZ treated male mice (**Table 2**). Similarly, the three genotypes of female mice had similar lesion size (n ⩾ 11 of each genotype, data not shown).

STZ-induced diabetes increased atherogenesis, but only in HuB mice with LPL deficiency and no CETP. As reported previously (23), HuB mice with and without STZ treatment had similar amounts of atherosclerosis (Table 2). HuB/LPL1 mice had much more atherosclerosis with STZ treatment (control 3500 ± 3462 μm^2 vs. STZ-treated 50160 ± 68344 μm^2, $P < 0.05$) (Table 2). Remarkably, HuB/LPL1/CETP mice did not have more atherosclerosis, despite STZ treatment (Table 2). Therefore, the CETP transgene reduced atherosclerosis in STZ-treated HuB/LPL1 mice.

Several markers of diabetes were more abnormal in the STZ-treated HuB/LPL1 mice: they did not gain weight during the study, they had the lowest body weight at 32

TABLE 1. Lipid profile of genetically altered HuB mice

Genotype	Group	n	Body Weight	Glucose	n	Triglyceride	Cholesterol	n	HDL-C
			g	*mg/dl*		*mg/dl*	*mg/dl*		*mg/dl*
HuB	CTR	15	34.5 ± 5.8	158 ± 36	14	169 ± 80	328 ± 79	6	138 ± 58
	STZ	11	30.0 ± 2.7[a]	302 ± 135[a]	9	181 ± 105	373 ± 51	4	173 ± 58
HuB/LPL1	CTR	34	34.5 ± 4.8	165 ± 52	34	161 ± 60	339 ± 89	5	137 ± 42
	STZ	22	26.4 ± 2.9[a,b,c]	434 ± 120[a,b,c]	21	484 ± 597[b,d]	533 ± 177[a,b,c]	3	127 ± 44
HuB/LPL1/CETP	CTR	14	35.9 ± 5.7	176 ± 47	14	225 ± 138	309 ± 44	5	70 ± 22
	STZ	15	28.9 ± 2.7[a]	320 ± 159[a]	12	271 ± 146	338 ± 118	5	68 ± 29

The data are means ± SD. The data were obtained at 20 weeks of age from mice fed a Western type diet. One-way ANOVA for triglyceride and cholesterol were done by nonparametric analysis.

[a] $P < 0.01$, versus control.

[b] $P < 0.05$, versus STZ-treated HuB.

[c] $P < 0.05$, STZ-treated HuB/LPL1/CETP.

[d] $P < 0.05$, versus control.

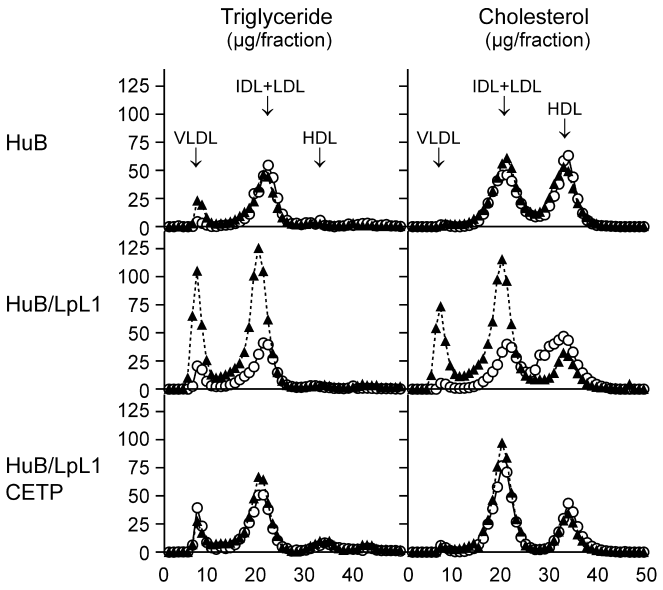

Fig. 2. Effect of streptozotocin (STZ) treatment on lipid distributions. The distribution of triglyceride and cholesterol in the plasma of 20-week-old STZ-treated male mice fed a Western type diet are shown in closed triangles. Profiles from control mice are shown with open circles for comparison. Pooled plasma samples were obtained from three to eight mice.

weeks, and they had the highest free fatty acid levels [1.14 ± 0.30 mM vs. 0.50 ± 0.09 mM (HuB) and 0.60 ± 0.19 mM (HuB/LPL1/CETP), $P < 0.01$]. They also had higher average glucose, TG, and cholesterol levels (Table 2). To determine if the atherogenic response was primarily related to the severity of diabetes or dyslipidemia, data in the HuB/LPL1 mice were analyzed by linear regression. Increased atherosclerosis correlated with averaged plasma cholesterol ($r^2 = 0.319$, $P < 0.05$), but not glucose ($r^2 = 0.034$, $P = 0.857$) or FFA levels ($r^2 = 0.001$, $P = 0.934$). IDL/LDL-C, but not TG or HDL, correlated with lesion size ($r^2 = 0.838$, $P < 0.03$).

DISCUSSION

We created a new model of dyslipidemia in a mouse and used it to explore how STZ-diabetes affects lipoprotein profiles and atherosclerosis. The genetically altered mice contained a transgene for HuB ± LPL1 and ± CETP. The effects of diabetes on these mice were, in most genotypes, rather unremarkable except for the HuB/LPL1 mice. These mice had worse diabetes and developed severe hyperlipidemia due to increased cholesterol and TG in VLDL and IDL/LDL. This hyperlipidemia was associated with more atherosclerosis.

Why did HuB/LPL1 mice develop increased IDL/LDL and cholesterol-rich VLDL? Removal of cholesterol rich VLDL in these mice might be via similar pathways to those required for other remnant lipoproteins. Clearance of chylomicrons and large VLDL involves several steps: initial lipolysis, trapping of the remnants by liver heparan sulfate proteoglycan (HSPG), uptake by either LDLR-related protein (LRP) or the LDLR (27). Marked hyperlipidemia was only found in the LPL1 mice; this suggests that an LPL-mediated process was perturbed. One possibility is that the newly synthesized particles fail to be converted from nascent to remnant particles and remain circulating in the plasma. A second possibility is that reduced LPL leads to a defect in receptor-mediated lipoprotein uptake in the liver; LPL is a ligand for LRP-mediated lipoprotein uptake (28). A third possibility is that the LPL deficiency is unmasking a defect in liver lipoprotein trapping. We have previously reported (29) that liver HSPG and trapping of remnant lipoproteins is defective in STZ-treated mice. A similar situation appears to also exist in apoE deficient mice; these mice develop severe hyperlipidemia with STZ treatment (14). LPL, a strong heparin- and lipoprotein-binding molecule, might normally overcome this defect. Finally, since the diabetes was worse in these mice and they had lower body weight, it is conceivable that they consumed more food and that this exacerbated their hyperlipidemia.

Pancreatic islet cells express LPL and it has been hy-

TABLE 2. Atherosclerosis in control and streptozotocin (STZ)-treated HuB mice

Genotype	Group	n	Atherosclerotic Lesion Area	Body Weight at 32 Weeks	Average of 20 to 32 Weeks of Age		
					Glucose	Triglyceride	Cholesterol
			μm^2	g	*mg/dl*	*mg/dl*	*mg/dl*
HuB	CTR	15	3449 ± 2539	42.8 ± 8.9	196 ± 28	170 ± 79	327 ± 96
	STZ	12	3560 ± 2424	32.4 ± 3.5[d]	293 ± 66[d]	148 ± 52	333 ± 81
HuB/LPL1	CTR	29	3500 ± 3462	42.0 ± 6.9	186 ± 36	187 ± 45	324 ± 58
	STZ	13	50160 ± 68344[a,b,c]	26.6 ± 3.4[d,e,f]	507 ± 72[d,e,g]	612 ± 467[d,e,f]	717 ± 353[d,e,f]
HuB/LPL1/CETP	CTR	11	2047 ± 3653	44.8 ± 7.0	202 ± 37	239 ± 141	314 ± 57
	STZ	13	3058 ± 3304	34.5 ± 5.0[d]	363 ± 137[d]	182 ± 77	325 ± 104

The data are means ± SD. One-way ANOVA was done by nonparametric analysis.
[a] $P < 0.05$, versus control.
[b] $P < 0.05$, versus STZ-treated HuB.
[c] $P < 0.01$, versus STZ-treated HuB/LPL1/CETP.
[d] $P < 0.01$, versus control.
[e] $P < 0.01$, versus STZ-treated HuB.
[f] $P < 0.001$, versus STZ-treated HuB/LPL1/CETP.
[g] $P < 0.05$, versus STZ-treated HuB/LPL1/CETP.

pothesized that in some circumstances LPL promotes fatty acid toxicity (30). For this reason, we initially expected that HuB/LPL1 mice would develop less severe diabetes with STZ. For reasons that are not obvious, the opposite occurred. Islets, like most cells, require glucose or fatty acids for cellular energy. It is possible that STZ-induced insulin deficiency reduced glucose uptake by islets and led to a greater dependence on fatty acids. However, with concomitant LPL deficiency, insufficient local fatty acid availability may have occurred, leading to greater islet cell dysfunction.

CETP led to less dyslipidemia in HuB/LPL1 mice, probably because it transferred cholesterol from LDL and cholesterol-enriched VLDL to particles that had a more favorable route of plasma clearance. One possibility is that the cholesterol in the LDL and VLDL was transferred to larger lipoproteins that were rapidly catabolized by apoE interaction with lipoprotein receptors in the liver. Alternatively, smaller LDL could have been produced that interact better with LDLRs (31). LDL reduction by CETP has been seen in another hypertriglyceridemic model, apoC-III overexpressing mice (32), and was associated with reduced LDL but increased VLDL-C.

The relationship between diabetes and atherosclerosis is complicated by concomitant changes in plasma lipoproteins. In a previous study, we noted that STZ-induced diabetes alone did not increase atherosclerosis in HuB mice (23), and our current study confirmed this. Similar observations were reported in diabetic LDLR knockout mice (10). STZ treatment, however, increased atherosclerosis in mice that had a heterozygous deletion of LPL. Thus, our current study and that of Park et al. (14) illustrate mouse models of diabetes-induced atherosclerosis associated with an increase in cholesterol-enriched lipoproteins. The major issue in both these studies is whether the atherosclerosis increase was due to the hyperglycemia or dyslipidemia. Despite the marked hypercholesterolemia that occurred with STZ treatment in their apoE knockout mice, Park et al. (14) attributed the increased atherosclerosis that they observed to hyperglycemia. This conclusion was supported by studies showing that infusion of a soluble form of the receptor for advanced glycosylation endproducts (RAGE) decreased lesion size. Subsequent work from these investigators has established that soluble RAGE has general anti-inflammatory actions that are not specific for effects of hyperglycemia; soluble RAGE inhibits colitis, decreases amyloid deposition, and suppresses growth of some tumors (33). Thiazolidinediones, a commonly used anti-diabetic medication, also decreased mouse atherosclerosis; however, this appeared to be unrelated to changes in blood glucose (12, 13).

Vessel wall LPL increases atherosclerosis (34) and in non-diabetic mice LPL deficiency is associated with reduced atherosclerosis (35). In our model, however, the extent of atherosclerosis was attributable to the degree of hyperlipidemia that appeared to have negated any beneficial effects of partial macrophage LPL deficiency. Reductions in HDL that accompanied the CETP transgene also did not correlate with an increase in atherosclerosis in either control or STZ-treated animals. It may be that in this Western diet fed model, these reduced HDL of ~70mg/dl were still sufficient to provide maximal protection.

We attempted to use the diabetic HuB/LPL1 mice to evaluate the effects of hyperglycemia and hyperlipidemia on atherosclerosis. Linear regression analysis suggested that the hyperlipidemia was the more potent atherogenic factor; however, because these mice had both more severe hyperlipidemia and hyperglycemia, this analysis may not be conclusive.

In summary, the effects of three different genetic mutations on plasma lipoproteins and their response to STZ-induced diabetes were studied. Neither LPL deficiency nor the CETP transgene altered atherosclerosis in HuB mice. Dyslipidemia was exacerbated by induction of diabetes only in HuB/LPL1 mice. This severe hyperlipidemia in STZ-treated HuB/LPL1 mice was reduced by CETP. Thus, CETP may alleviate hyperlipidemia in situations in which lipolysis is reduced or saturated. Alternatively, CETP may modify the induction of STZ-diabetes in the HuB/LPL1 mouse. Finally, our data suggest that STZ-induced hyperglycemia does not increase atherosclerosis in HuB-expressing mice unless there is an attendant worsening of hypercholesterolemia.

Like most atherosclerosis studies in mice, our animals exhibited some major differences from humans. Almost all studies of atherosclerosis in mice use animals that are severely hyperlipidemic due to genetic alterations, or ingestion of a diet that is enriched in cholic acid as well as fat and cholesterol. Therefore, the effects of hyperglycemia on arteries might be seen in a less flagrant atherogenic model. Alternatively, additional modifications of the mouse may be needed to define the factor(s) that protect it from the toxicity of hyperglycemia.

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