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Effects of diabetes and CETP expression on diet-induced atherosclerosis in LDL receptor-deficient mice

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The role of CETP expression and diabetes in atherogenesis was investigated in mice with heterozygous disruption of the LDL receptor gene (LDLR1). LDLR1 mice with and without CETP expression were treated with streptozotocin (STZ) and maintained on a standard diet for one month before switching to an atherogenic diet for an additional month. STZ-sensitive mice had ~2.5-fold higher glycemia and 7.5- to 8.0-fold higher cholesterolemia. Factorial analysis of variance showed no significant effect of diabetes, CETP or diabetes-CETP interaction on the size of the atherosclerotic lesions. CETP expression in non-diabetic mice resulted in a 50% reduction in the area of the atherosclerotic lesions. Multiple regression analysis showed a positive and independent atherogenic effect of triglyceridemia in LDLR1 mice and of cholesterolemia in diabetic mice. Logistic analysis showed that elevated plasma cholesterol level significantly increased the risk of developing large lesion size (>75th percentile). In conclusion, CETP expression did not alter the lesion formation in response to diabetes, although it may be protective in the euglycemic state; the triglyceride level was an independent risk factor for LDL receptor-deficient mice but not for CETP-expressing mice; and elevated plasma cholesterol levels increased the risk of developing large atherosclerotic lesions, independently of CETP and diabetes.

Key words: Diabetes; CETP expression; atherosclerosis; LDL receptor-deficient mice.

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The incidence of atherosclerotic vascular disease in diabetic patients is 3- to 4-fold higher than in the general population (1–3). Hyperglycemia and hyperlipidemia have been implicated in the development of atherosclerosis in diabetic subjects. While hyperglycemia seems to be associated with microvascular events (4), abnormalities in LDL and HDL are strongly correlated with macrovascular disease (5–7). However, because diabetes frequently clusters hyperglycemia, dyslipidemia, hypertension, and sometimes obesity, it is difficult to isolate the determinants of atherosclerosis in this state (8).

Experimental diabetes produces varying effects on atherosclerosis, depending on the animal model. Atherogenesis is accelerated in diabetic apo E knockout mice (9, 10), increased or unaltered in diabetic LDL receptor knockout mice (11, 12), and decreased in diabetic rabbits (13). Mice lack cholesteryl ester transfer protein (CETP) whereas humans and rabbits express moderate to high levels of this protein (14). CETP mediates the net transfer of cholesteryl ester from HDL to apolipoprotein (apo) B-containing lipoproteins (15). Variations in CETP

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plasma levels and activity may modify the risk of atherosclerotic disease in humans, but conflicting effects have been reported (16). Based on some of these studies, inhibitors of CETP have been considered as potential anti-atherogenic drugs (17–19).

CETP expression in transgenic mice may attenuate or aggravate atherogenesis, a phenomenon clearly dependent on the metabolic context. CETP expression plays a protective role in conditions such as hypertriglyceridemia (20), overexpression of lecithin cholesterol acyl transferase (21), diabetes in excess of apo B and deficiency of lipoprotein lipase (22), and after ovariectomy (23). On the other hand, in the wild type background (24), in severe hypercholesterolemia resulting from deletion of the LDL receptor or apo E genes (25), and in hypertensive rats (26), CETP expression worsens atherosclerosis. Thus, neither diabetes nor CETP expression have a predictable role in the development of atherosclerosis, and require investigation in each specific metabolic setting.

In this work we investigated the effects of CETP expression and streptozotocin -induced diabetes, alone and together, on the development of diet -induced atherosclerosis in mice with a heterozygous disruption of the LDL receptor gene. A partial genetic deficiency in the LDL receptor and a fatty diet are frequent traits in Western populations.

MATERIAL AND METHODS

Animals and treatments

All experimental protocols were approved by the university's Committee for Ethics in Animal Experimentation and followed the "Principles of Laboratory Animal Care" (NIH publication no. 85–23, revised 1985). The mice were housed in a temperature-controlled room on a 12 h light-dark cycle and had free access to food (standard rodent chow; Nuvital CR1, Colombo, Brazil) and water. Hemizygous human CETP transgenic mice (line 5203, C57BL6/J) (27) expressing a human CETP minigene under the control of natural flanking sequences were derived from a colony maintained by Dr. Alan R. Tall (Columbia University, New York, NY) and crossbred with LDL receptor knockout mice (C57Bl6/J background) purchased from the Jackson Laboratory (Bar Harbor, ME). CETP-expressing mice were genotyped by assaying the plasma CETP activity (28). CETP-expressing (CETP/LDLR1, n=16) and non-expressing mice (LDLR1, n=18) received five consecutive daily *ip* injections of streptozotocin (STZ, Calbiochem, Darmstadt, Germany), 50 mg/kg BW in 0.05 M sodium citrate buffer, pH 4.5 (29). Thirty days after STZ treatment, approximately 50% of the mice in each genotype group became hyperglycemic (with at least twice the initial glycemia value) and 50% were resistant to or recovered from the initial hyperglycemia. These euglycemic mice were considered as non-diabetic controls. All mice were maintained on a standard diet for one month after STZ treatment and then switched to an atherogenic, high fat and high cholesterol (HFHC) diet, containing 20% fat, 1.25% cholesterol and 0.5% cholic acid (Cat. # 611208, Dyets Inc., Bethlehem, PA) for an additional month. Body weights were reduced in diabetic mice by the end of the experiment (-20%, $p<0.05$ *vs* basal). At day 60, all mice were anesthetized using ketamine (50 mg/kg, i.p., Ketalar, Parke-Davis, São Paulo, Brazil) and xylazine (10 mg/kg, i.p., Rompum, Bayer S.A., São Paulo, Brazil), blood was drawn from the retro-orbital plexus into heparinized hematocrit tubes, and the hearts were perfused *in situ* and excised.

Lipid, glucose and lipoprotein analyses

Plasma total cholesterol, triglycerides and free fatty acids were determined using enzymatic colorimetric assays (Wako Chemicals, Neuss, Germany). Ten microliters of whole blood precipitated with 5% trichloroacetic acid was used for glucose analysis by the glucose oxidase method (Merck Diagnostic, Chennevières-les-Louvres, France). Plasma lipoproteins were fractionated by fast protein liquid chromatography (FPLC) using an HR10/30 Superose 6 column (Amersham-Pharmacia Biotech, Uppsala, Sweden), equilibrated with Tris-buffered saline, pH 7.2, as previously described (30). Total cholesterol was determined enzymatically in each FPLC fraction.

Cholesteryl ester transfer protein activity assay

The CETP-mediated transfer of cholesteryl ester (CE) was determined as previously described (28). Briefly, a mixture of acceptor lipoproteins (human VLDL and LDL) was incubated with human HDL_3 labeled with [^{14}C]-cholesteryl oleate (CE) (31) and 5 µl of mouse plasma as the source of CETP in a final volume of 100 µl. Blanks were prepared with Tris/saline/EDTA buffer (10 mM/140 mM/1 mM), pH 7.4, and negative controls with plasma from non-transgenic mice. After incubation at 40°C for 1 h, the apo B containing lipoproteins were precipitated with a solution of 1.6% dextran sulfate/1 M $MgCl_2$ (1:1) and the radioactivity was measured in the supernatant using a LS6000 Beckman Beta Counter.

Histological analysis of atherosclerotic lesions

Mice were anesthetized and their hearts were perfused *in situ* with phosphate-buffered saline (PBS)

followed by 10% PBS-buffered formaldehyde, after which they were excised and fixed in 10% formaldehyde for at least 2 days. The hearts were then embedded sequentially in 5%, 10% and 25% gelatin. Processing and staining were done according to Paigen et al. (32). The lipid-stained lesions were quantified as described by Rubin et al. (33) using Image Pro Plus software (version 3.0) for image analysis (Media Cybernetics, Silver Spring, MD). The slides were read by one investigator who was unaware of the treatments. The area of the lesions was expressed as the sum of the lesions in six 10 μm-sections, 80 μm apart, in a total aorta length of 480 μm. Since several studies have shown that lesions develop preferentially in the aortic root, the segment chosen for analysis extended from beyond the aortic sinus up to the point where the aorta first becomes rounded (33).

Statistical analyses

The data were analyzed using one-way and factorial analyses of variance for multiple comparisons and the Mann-Whitney test for two sample comparisons. The Spearman test was used to detect correlations between the variables and atherosclerotic lesion size. A hierarchical, multiple linear regression analysis was used to assess the influence of CETP, diabetes, and plasma lipids and glucose concentrations on the size of the atherosclerotic lesion. These results were expressed as coefficients of determination (R^2), which indicates the percentage of variation in the dependent variable (lesion size) that can be explained by the independent variables. A multiple logistic regression analysis was used to assess the relative risk of developing atherosclerotic lesions larger than the 75th percentile (highest quartile). The level of significance for all tests was 5%; levels between 5 and 10% were considered marginally significant. All statistical calculations were done using the SAS statistical software package (version 8).

RESULTS

The plasma lipid and glucose concentrations in diabetic and non-diabetic LDLR1 and CETP/LDLR1 mice are shown in Table 1. At the end of the study, STZ-sensitive mice showed a ~2.5 fold elevation in glycemia, whereas STZ-resistant mice had no significant change in their basal glycemia levels. Free fatty acids (FFA) and triglycerides (TG) in diabetic mice did not vary during the study and between genotypes. Unexpectedly, TG concentrations were significantly reduced after the HFHC diet in non-diabetic mice in both the LDLR1 and CETP/LDLR1 groups. Thus, diabetes, which is generally accompanied by increases in TG levels (34), masked this TG-reducing diet effect in diabetic mice. A reduction in plasma TG levels by bile salt-containing diet has also been reported by others (29, 35). Plasma total cholesterol levels increased by about 2.5-fold in non-diabetic mice and by 7.5- to 8.0-fold in diabetic mice, indicating that the HFHC diet and diabetes had synergistic effects on plasma total cholesterol levels.

Gel filtration was used to fractionate the plasma lipoproteins of diabetic mice. Since the cholesterolemic response to diabetes was highly variable (205–2213 mg/dL) in these mice, two patterns of lipoprotein profile were found in both genotypic groups. In mice with plasma cholesterol levels >1000 mg/dL (n=6), the VLDL and LDL peaks were not resolved, whereas in mice with cholesterol ranging from 200 to 400 mg/dL (n=6), all three lipoproteins peaks were resolved. The average percent distribution of cholesterol between [VLDL+ LDL] and HDL was, respectively, 70±10% and 30±10% (n=6) in CETP/LDLR1 mice, and 80±6% and 20±6% (n=6) in LDLR1 mice (no significant differences between the corresponding values in each group).

Plasma CETP activity (% CE transfer) in CETP/LDLR1 mice was significantly increased by diabetes, confirming previous work (36), and was further elevated by the HFHC diet: from 32±2 (basal) to 40±3 on day 30 after STZ treatment ($p<0.05$) and 81±3 after STZ+HFHC diet ($p<0.001$). In non-diabetic CETP/LDLR1 mice, CETP activity increased significantly only after the HFHC diet (72±4, $P< 0.05$).

The average areas of atherosclerotic lesions in the aortic roots of the four experimental groups are shown in Fig. 1. Conventional one-way analysis of variance showed that there were no significant differences in the size of the lesions. Within the euglycemic group of mice, CETP expression resulted in a 50% reduction in the area of the atherosclerotic lesion ($p=0.07$, Mann-Whitney test).

Factorial analysis of variance was used to examine the effects of CETP, diabetes and CETP-diabetes interaction on the lesion size, and on cholesterol and triglyceride levels. Only diabetes significantly influenced the plasma cholesterol levels ($p<0.004$). No CETP or CETP-diabetes interaction effects were detected.

Correlation analysis was used to discover the

TABLE 1. *Plasma glucose and lipid concentrations (mg/dL) before and after streptozotocin (STZ) treatment and high fat and high cholesterol (HFHC) diet in LDL receptor-deficient mice with (CETP/LDLR1) and without (LDLR1) expression of CETP*

Groups		*GLUC*	TC	TG	*FFA*
Diabetics					
CETP/LDLR1	Initial	78±4	121±5	98±11	1.4±0.15
	STZ+HFHC diet	188±12[a]	889±266[a]	114±39	1.6±0.16
LDLR1	Initial	85±6	133±9	125±13	1.3±0.07
	STZ+HFHC diet	208±28[a]	822±222[a]	113±24	1.7±0.11[a]
Non-diabetic					
CETP/LDLR1	Initial	71±5	103±4	123±17	1.6±0.17
	STZ+HFHC diet	90±8[b]	251±18[a,b]	70±4[a]	1.3±0.14
LDLR1	Initial	68±4	118±5	142±13	1.6±0.13
	STZ+HFHC diet	84±7[b]	244±25[a,b]	78±4[a]	1.5±0.11

Mean±SE (n=8–10); TC (total cholesterol); TG (triglycerides); FFA (free fatty acids) and GLUC (glucose). Comparisons by the Mann-Whitney test: [a] Within the same genotypic group: STZ+ HFHC diet *vs* Basal: $p<0.05$ (or better); [b] Diabetic *vs* non-diabetic: $p<0.001$. There were no differences between CETP/LDLR1 *vs* LDLR1 in each condition (diabetic and non-diabetic).

determinants of lesion area in these mice. As shown in Table 2, size of lesion area was positively correlated with plasma levels of glucose, TG and total cholesterol levels, when all groups of mice were combined. The same associations were seen when only the LDLR1 group was considered. However, when CETP was present (CETP/LDLR1), all the positive correlations disappeared. As expected, the positive correlation between total cholesterol levels and lesion size was due to the non-HDL-cholesterol fraction (Table 2). This correlation was stronger when non-HDL/HDL ratio was considered as the independent variable.

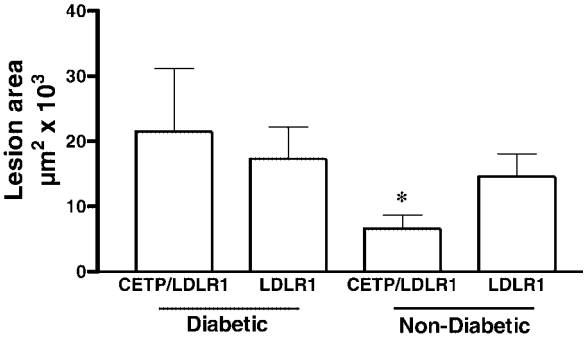

Fig. 1. Area of aortic atherosclerotic lesions in LDL receptor-deficient mice with (CETP/LDLR1) and without (LDLR1) expression of CETP, after 8 weeks of streptozotocin treatment and 4 weeks on a cholate-containing high fat and high cholesterol diet. The columns represent the mean±SE of the number of mice indicated. *p=0.07 between non-diabetic CETP/LDLR1 and LDLR1 mice (Mann-Whitney test).

Since the cholesterol, TG and glucose levels correlate strongly with each other (Table 3), univariate correlation analyses show limited power in determining the relative contribution of each variable to lesion size. Therefore, multiple regression was used to examine the relative influence of cholesterol, TG, glucose and presence of CETP and diabetes (independent covariants) on the size of the atherosclerotic lesions (dependent variable). As shown in Table 4, in LDLR1 mice (n=18), 40% of the variation in lesion size was independently determined by the triglyceridemia (p=0.004); in diabetic mice (n=16), 24% of the variation in lesion size was independently determined by the cholesterolemia (p=0.051); and in all mice (n=34), 10% of the variation in lesion size was independently determined by the glycemia (p=0.085). No independent variables were detected in non-diabetic group (n=18) and in CETP-expressing mice (n=16). The quantitative interpretation of these statistical models is as follows: a) model I predicts that a 1% increase in TG levels would increase the lesion size by 1.4% in LDLR1 mice; b) model II predicts that a 1% increase in plasma cholesterol levels would increase the lesion size by 0.5% in diabetic mice; and c) model III predicts that a 10 mg/dL elevation of glycemia would increase the lesion size by 3.4%.

Logistic regression analysis used to identify the variables (CETP genotype, diabetes, levels of cholesterol, glucose, FFA and TG) that influence the relative risk of developing lesion size

$>75^{th}$ percentile (highest quartile) showed that only cholesterol levels significantly increased the risk of developing such large lesions ($p<0.007$).

DISCUSSION

In this study, the induction of diabetes in LDL receptor-deficient mice significantly affected the plasma cholesterol levels but not the severity of the diet-induced atherosclerosis when compared to non-diabetic mice. These results are supported by previous investigations that reported no effect of diabetes on atherogenesis in wild type C57Bl6 mice (29), in LDL receptor-deficient mice (11), in human apolipoprotein B transgenic mice (37), and in *NOD* (non-obese-diabetic) mice (38). However, in other studies,

TABLE 2. *Univariate correlation coefficients (r) between atherosclerotic lesion area and plasma glucose, triglyceride, total cholesterol and lipoprotein-cholesterol levels in diabetic and control LDL receptor-deficient mice with (CETP/LDLR1) and without (LDLR1) expression of CETP*

		GLUC	TG	TC
Diabetic+non-diabetic	All mice (n=34)	r=0.48 (p=0.004)	r=0.35 (p=0.04)	r=0.60 (p=0.05)
	All LDLR1 (n=18)	r=0.50 (p=0.04)	r=0.71 (p=0.0008)	r=0.56 (p=0.015)
	All CETP/LDLR1 (n=16)	NS	NS	NS
Diabetic	LDLR1 (n=8)	r=0.93 (p=0.0007)	r=0.97 (p=0.0001)	r=0.81 (p=0.01)
	CETP/LDLR1 (n=8)	NS	NS	NS
	All diabetic mice (n=16)	NS	NS	r=0.47 (p=0.05)
		HDL	nHDL	nHDL/HDL
Diabetic	LDLR1 (n=6)	NS	r=0.82 (p=0.04)	r=0.91 (p=0.01)
	CETP/LDLR1 (n=6)	NS	NS	NS
	All diabetic mice (n=12)	NS	r=0.46 (p=0.07)	r=0.58 (p=0.04)

All mice were treated with STZ but approximately 50% were resistant to or recovered from acute hyperglycemia. NS (non-significant). No significant correlation was found when non-diabetic mice were analyzed separately. GLUC (glucose); TG (triglycerides) and TC (total cholesterol).

TABLE 3. *Univariate correlation coefficients (r) between plasma lipid and glucose levels in diabetic and control LDL receptor -deficient mice with (CETP/LDLR1) and without (LDLR1) expression of CETP*

		TG vs *TC*	TC vs *GLUC*	TG vs *GLUC*
Diabetic+non-diabetic	All mice (n=34)	r=0.82 (p<0.0001)	r=0.69 (p<0.0001)	r=0.51 (p=0.002)
	All LDLR1 (n=18)	r=0.91 (p<0.0001)	r=0.87 (p<0.0001)	r=0.82 (p<0.0001)
	All CETP/LDLR1 (n=16)	r=0.80 (p=0.0002)	r=0.48 (p=0.056)	NS
Diabetic	LDLR1 (n=8)	NS	r=0.90 (p=0.002)	r=0.96 (p=0.0001)
	CETP/LDLR1 (n=8)	r=0.81 (p=0.015)	NS	NS
	All diabetic mice (n=16)	r=0.82 (p<0.0001)	r=0.54 (p=0.03)	r=0.47 (p=0.06)

All mice were treated with STZ but approximately 50% were resistant to or recovered from acute hyperglycemia. NS – non significant; TG (triglycerides); TC (total cholesterol) and GLUC (glucose).

TABLE 4. *Influence (coefficient of determination, R^2) of plasma lipids and glucose concentrations on the atherosclerotic lesion areas in diabetic and control LDL receptor-deficient mice with (CETP/LDLR1) and without (LDLR1) expression of CETP*

Multivariate linear regression models	*P*-value	Cumulative R^2
I. LDLR1 only (no CETP expression) (n=18) log[lesion area]=−3.81+1.40 log[triglycerides]	0.004	0.41
II. Diabetics only (n=16) log[lesion area]=−0.51+0.478 log[cholesterol]	0.051	0.24
III. All groups (n=34) log[lesion area]=1.8+0.0039 [glucose]	0.085	0.10

Independent variables: total cholesterol, triglycerides, glucose, presence of CETP and diabetes.

diabetic LDL receptor-deficient mice (12) and apolipoprotein E-deficient mice (9, 10) had more severe atherosclerosis than their non-diabetic controls. Distinct genetic backgrounds, type of dyslipidemia and duration of the studies may account for these contradictory results.

Although diabetes did not alter atherosclerosis in this study, a significant univariate correlation between atherosclerotic lesion size and plasma glucose levels was found when all groups were combined, and was also seen in LDLR1 mice. When all mice were analyzed together, glycemia made a small contribution (10%) and had a marginally significant positive effect (increase) on lesion size (p=0.085). Several mechanisms have been proposed to explain the additive lipid-independent effects of hyperglycemia on atherogenesis. These include oxidative stress and the formation of advanced glycated end products (10, 39).

Plasma cholesterol levels in diabetic mice and plasma TG levels in LDLR1 mice were found to positively and independently influence the size of the lesions in these mice. In addition, elevation of cholesterol concentrations significantly increased the risk of developing large lesions (highest quartile) in all mice.

CETP expression seems to play a beneficial role in non-diabetic mice as shown by the statistical trend (p=0.07) towards decreasing lesion size in these mice, but had no effect on atherosclerosis in STZ-induced diabetic mice. Recently, MacLean et al. (40) reported that CETP expression in obese and diabetic leptin receptor mutant mice (db/db) prevented diet-induced atherosclerosis. Thus, CETP may have a protective role in specific types of diabetes. The results presented here clearly show that CETP expression modified the general determinants of atherosclerosis since there were no significant uni- or multivariate associations of lipids and glucose with lesion size in CETP-expressing mice.

The TG levels were strongly and independently associated with lesion size in CETP non-expressing LDLR1 mice. Epidemiological studies have linked TG-rich and remnant lipoproteins to increased atherosclerosis (41). Why TG is a risk factor for LDLR1 but not for CETP/LDLR1 mice is unknown. Since CETP transfers TG from TG-rich lipoproteins to HDL, and HDL enriched in TG is a better substrate for hepatic lipase, CETP indirectly facilitates the hepatic removal of TG. Collet et al. (42) demonstrated that remodeling of HDL by CETP and hepatic lipase resulted in enhanced uptake of HDL-cholesteryl ester (CE) by cells expressing the scavenger receptor B-I. Thus, CETP could stimulate the hepatic removal of both TG and CE from plasma HDL. These findings could explain why TG and cholesterol levels were not associated with the extent of the atherosclerotic lesion in mice expressing CETP.

In summary, these results showed: 1. CETP expression did not alter lesion formation in STZ-induced diabetes, although it may have been protective in the euglycemic state, 2. triglyceride level was an independent risk factor in LDL receptor-deficient mice but not in mice expressing CETP, 3. elevated plasma cholesterol levels significantly increased the risk of developing large atherosclerotic lesions, independently of CETP expression and diabetes, and 4. hyperglycemia may contribute to the development of atherosclerosis in this mouse model.

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