

# Cholesteryl ester transfer protein expression attenuates atherosclerosis in ovariectomized mice

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**Abstract** Reduced estrogen levels result in loss of protection from coronary heart disease in postmenopausal women. Enhanced and diminished atherosclerosis have been associated with plasma levels of cholesteryl ester transfer protein (CETP); however, little is known about the role of CETP-ovarian hormone interactions in atherogenesis. We assessed the severity of diet-induced atherosclerosis in ovariectomized (OV) CETP transgenic mice crossbred with LDL receptor knockout mice. Compared with OV CETP expressing (+), OV CETP non-expressing (-) mice had higher plasma levels of total, VLDL-, LDL-, and HDL-cholesterol, as well as higher antibodies titers against oxidized LDL. The mean aortic lesion area was 2-fold larger in OV CETP<sup>-</sup> than in OV CETP<sup>+</sup> mice ( $147 \pm 90$  vs.  $73 \pm 42 \times 10^3 \mu\text{m}^2$ , respectively). Estrogen therapy in OV mice blunted the CETP dependent differences in plasma lipoproteins, oxLDL antibodies, and atherosclerosis severity. Macrophages from OV CETP<sup>+</sup> mice took up less labeled cholesteryl ether (CEt) from acetyl-LDL than macrophages from OV CETP<sup>-</sup> mice. Estrogen replacement induced a further reduction in CEt uptake and an elevation in HDL mediated cholesterol efflux from pre-loaded OV CETP<sup>+</sup> as compared with OV CETP<sup>-</sup> macrophages. **These findings support the proposed anti-atherogenic role of CETP in specific metabolic settings.**—Cazita, P. M., J. A. Berti, C. Aoki, M. Gidlund, L. M. Harada, V. S. Nunes, E. C. R. Quintão, and H. C. F. Oliveira. **Cholesteryl ester transfer protein expression attenuates atherosclerosis in ovariectomized mice.** *J. Lipid Res.* 2003. 44: 33–40.

**Supplementary key words** LDL receptor knockout mice • CETP transgenic mice • lecithin cholesterol acyl transferase • oxidized LDL • phospholipid transfer protein • estrogen

Remodelling of plasma lipoproteins through the transfer of neutral lipids such as cholesteryl ester (CE) and triacylglycerols (TAG) is the best characterized function of

the cholesteryl ester transfer protein (CETP) (1). Epidemiological and experimental evidences have shown that CETP may play an important role in the development of atherosclerosis (2); however, the precise effects of CETP on atherogenesis are controversial. In humans, increased incidence of coronary heart disease has been associated with both CETP deficiency (3) and augmentation (4). In several animal models of atherosclerosis, the effects of CETP on vascular health are clearly dependent upon the metabolic context (5–11).

Various researchers are attempting to target CETP as a form of therapy (9–13), but these approaches will be useless unless the circumstances where CETP acts as pro- or anti-atherogenic are properly clarified.

Deficiency in endogenous estrogen accounts for the loss of protection against coronary heart disease after menopause or following bilateral ovariectomy (14). Estrogen deficiency per se does not alter plasma CETP activity as shown in castrated CETP transgenic mice (15). Also, estrogen therapy has no impact on the plasma CETP activity in humans (16) as well as in apolipoprotein B/CETP double transgenic mouse model (17).

The present study aimed at investigating whether CETP expression would alter the development of atherosclerosis in a moderately hypercholesterolemic mouse model lacking ovarian hormones. For this purpose, mice expressing the human CETP gene were crossbred with LDL receptor (LDLR) knockout mice. On a high fat diet, the LDLR knockout mice develop extensive aorta atherosclerosis in a pattern similar to humans (18, 19). We have shown here that the expression of the CETP gene significantly reduced the development of atherosclerotic lesions in ovariectomized hypercholesterolemic mice. Furthermore, this antiatherogenic effect of CETP was blunted by the estrogen replacement therapy.

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### Animal procedure

The animal protocols were approved by the University of São Paulo Medical School Ethics Committee. Hemizygous human CETP transgenic mice (line 5203, C57BL6/J background) (20) expressing a human CETP minigene under the control of natural flanking sequences were derived from Dr. Alan R. Tall's colony (Columbia University, New York, NY) and crossbred with LDLR knockout mice purchased from Jackson Laboratory (Bar Harbor, ME). The pups' tail tips were utilized for screening for the presence of the CETP gene promoter by polymerase chain reaction (PCR) DNA amplification of the -538 to -222 CETP promoter region (GeneBank U71187). Tail blood was also drawn for determining plasma CETP activity (21). Female littermates, 8–12 weeks of age, heterozygous for the LDLR null allele expressing CETP (+) or not (-) were bilaterally ovariectomized (OV) or sham-operated (Sham). All mice were anesthetized for surgery using ketamine (50 mg/kg, ip, Ketalar, Parke-Davis, São Paulo, Brazil) and xylazine (16 mg/kg, ip, Rompum, Bayer S.A., São Paulo, Brazil).

The success of the ovariectomy was checked by analyzing vaginal smear during 5 consecutive days after the surgery. OV mice presented only the diestrus pattern while in Sham mice the four stages of the estrous cycle (proestrus, estrus, metestrus, and diestrus) were clearly verified (22). Five days after surgery, all animals were placed on an atherogenic high fat and high cholesterol (HFHC) diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid (Cat. # 611208, Dyets, Inc. Bethlehem, PA) for 19 weeks. It has been previously demonstrated that LDLR deficient mice exhibited similar distribution pattern and histological features of the atherosclerotic lesions when fed cholate-free or cholate-containing high fat and high cholesterol diets (23). Blood samples drawn from mice fasted for 6 h on the chow diet (5 days after surgery), corresponding to a baseline period and after 19 weeks on the HFHC diet, were collected into pre-cooled tubes containing 1 mM EDTA and centrifuged at 2,500 *g* at 4°C for 10 min. Aliquots of plasma were stored at -70°C until analysis. In order to compare OV and estrogen treated OV mice, a second experiment was performed. Estrogen replacement was done utilizing 60-day release pellets that released 6 µg/day of 17-β-estradiol (E2) or placebo (Innovative Research, Toledo, OH) subcutaneously implanted in the middle of the HFHC diet period. At the end of this experiment, the uterus weight was monitored exactly as described by Marsh et al. (24). Uterus from estrogen deficient mice were consistently smaller (<0.15 g) than those treated with estrogen (>0.15 g) (*P* < 0.01). Based on the uterus weight criterion, two mice were excluded from OV CETP<sup>+</sup> placebo and one mouse in each of the other three groups: OV CETP<sup>-</sup> placebo, OV CETP<sup>+</sup> E2, and OV CETP<sup>-</sup> E2. Mice body weight (g ± SD) at the end of the studies was slightly but significantly higher in OV than in Sham groups (*P* < 0.05): 22.7 ± 1.2 (CETP<sup>+</sup> Sham) versus 24.4 ± 1.3 (CETP<sup>+</sup> OV) and 24.1 ± 2.0 (CETP<sup>-</sup> Sham) versus 25.3 ± 1.2 (CETP<sup>-</sup> OV). In estrogen treated mice final weights were: OV CETP<sup>+</sup> (23.9 ± 1.1) versus OV CETP<sup>-</sup> (24.6 ± 0.7); OV CETP<sup>+</sup> E2 (22.7 ± 1.1) versus OV CETP<sup>-</sup> E2 (22.8 ± 1.7).

### 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 carried out according to Paigen et al. (25).

The lipid-stained lesions were quantified as described by Rubin et al. (26) using Image Pro Plus software (version 3.0) for image analysis (Media Cybernetics, Silver Spring, MD). The slides were read by an 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 distant from each other in a total aorta length of 480 µm. Because several other studies revealed a predilection for development of lesions in the aortic root, the segment that was chosen for analysis extended from beyond the aortic sinus up to the point where the aorta first becomes rounded (26).

### Lipid transfer proteins and cholesterol esterification assays

CETP activity, which reflects the plasma CETP concentration (27), was measured using exogenous substrates as previously described (21). Lecithin cholesterol acyl transferase (LCAT) mediated cholesterol esterification reaction was measured using endogenous substrates (28). Plasma phospholipid transfer protein (PLTP) activity was measured by the method of Albers et al. (29) that utilize exogenous substrates.

### Analysis of plasma lipoproteins and lipids

Lipoproteins from the pooled plasma of mice were separated by fast protein liquid chromatography (FPLC) using a HR10/30 Superose 6 column (Amersham-Pharmacia Biotech., Uppsala, Sweden) as described (30). Total cholesterol (Chod-Pap, Merck S.A., São Paulo, Brazil) and triacylglycerols (Enz-Color, Biodiagnostica, Paraná, Brazil) were determined by enzymatic methods according to the manufacturer's instructions.

### Detection of antibodies to oxidized LDL

Antibodies against holo-oxidized LDL (oxLDL) or antibodies anti-apoB epitope derived from oxLDL (apoB-D) were measured in mouse plasma by ELISA (31). ApoB-D is a 22 amino acid peptide from a region of apoB-100 not accessible to trypsin (32). Polystyrene microtiter plates (Costar, Cambridge, MA) were coated with 1 µg/ml of human oxLDL (20 mM Cu<sup>2+</sup>, 24 h) or 0.1 µg/ml of apoB-D in carbonate/bicarbonate buffer (20 µl/well), pH 9.4, and kept overnight at 4°C. The plates were blocked with a 5% solution of fat-free milk (Molico/Nestlé, SP, Brazil), and then incubated for 2 h at room temperature followed by washing four times with PBS (100 µl). Plasma samples (20 µl) were added and the plates were incubated overnight at 4°C followed by washing with 1% Tween 20 in PBS. A peroxidase-conjugated rabbit anti-mouse IgG antibody (20 µl; 1:1,500) was added, and after 1 h at room temperature, the plates were washed. Finally, 75 µl of substrate solution (250 mg of tetramethylbenzidine diluted in 50 ml of DMSO, 10 µl of 30% H<sub>2</sub>O<sub>2</sub>, 12 ml of citrate buffer, pH 5.5) were added and, after incubation at room temperature for 15 min, the reaction was stopped by adding 25 µl of 2.0 M sulfuric acid. The optical density (OD) was then measured in a microplate reader (Titertek Multiskan MCC/340P, model 2.20, Labsystems, Finland) at 450 nm. Results were expressed in relation to total plasma IgG concentration. For detection of total IgG levels, the plates were not treated with oxLDL but instead they were incubated with the individual plasma samples diluted 20,000× in carbonate buffer. The procedure then followed exactly as described above.

### Cell culture studies

Peritoneal macrophages were harvested from OV and estrogen treated OV CETP<sup>+</sup> and CETP<sup>-</sup> mice in PBS (0.8% NaCl, 0.06% Na<sub>2</sub>HPO<sub>4</sub>, 0.02% KCl, and 0.04% KH<sub>2</sub>PO<sub>4</sub>), pH 7.4. Pelleted cells obtained after centrifugation at 500 *g*, 4°C for 3 min were resuspended at a final concentration of 3 × 10<sup>6</sup> cells/ml in RPMI 1640 medium containing 20% (v/v) fetal calf serum (FCS), penicillin

(100 U/ml), streptomycin (100 U/ml), and fungizone (2.5 µg/ml). An aliquot of 0.5 ml was transferred into 24-well tissue culture plates and incubated in a humidified incubator with 5% CO<sub>2</sub> atmosphere at 37°C. To remove non-adherent cells, after 2 h incubation, each plate was washed twice with RPMI 1640 medium without FCS and used for subsequent experiments. Control incubations in wells without cells were performed, and their values subtracted from the experimental values.

**HDL mediated cellular cholesterol efflux.** Adhered macrophages were loaded with CE according to the method described by Brown et al. (33). Briefly, macrophages were incubated in RPMI 1640 medium containing 2 mg/ml fatty acid-free BSA in the presence of [<sup>14</sup>C]cholesteryl oleate-labeled acetylated LDL ([<sup>14</sup>C]CE-acLDL, 50 µg of protein/ml) for 24 h, and washed once with DMEM (Dulbecco's Minimum Essential Medium) containing antibiotics. [<sup>14</sup>C]CE-acLDL loaded macrophages were incubated for 6 h with DMEM containing 2 mg/ml BSA in the presence of human HDL (100 µg protein/ml) as cellular cholesterol acceptor and the medium was collected for radioactivity counting (Ultima Gold Packard, Meriden, CT) in a β scintillation counter (LS6000-TA8, Beckman Instruments, Palo Alto, CA). Cells were washed with PBS and dissolved in 0.2 N NaOH for the measurement of the radioactivity that remained in the cells and protein content. Efflux was defined as the amount of radioactivity in the medium expressed as a percentage of that in the medium plus cells. Blank values were obtained by the incubation of labeled cells in medium containing only 2 mg/ml BSA and no lipoprotein.

**acLDL cholesteryl ether uptake.** Adhered macrophages were incubated in RPMI 1640 medium containing 10% (v/v) lipoprotein deficient serum (3.5 mg protein/ml of medium) in the presence of acLDL labeled with [<sup>3</sup>H]cholesteryl oleoyl ether ([<sup>3</sup>H]CET-acLDL), 50 µg of protein/ml, for 6 h at 37°C in a humidified incubator with 5% CO<sub>2</sub> atmosphere. At the end of the incubation, cells were washed with PBS and solubilized in 0.2 N NaOH for the measurement of the cell-associated radioactivity and protein content. Cellular CET uptake was defined as the amount of radioactivity in the cells expressed as a percentage of that offered to the cells per mg of cellular protein.

## RESULTS

In this study, we compared the plasma lipoprotein profiles, lipid transfer protein activities, oxLDL antibodies titers, and the extent of atherosclerotic lesions in control Sham, ovariectomized (OV), and estrogen treated OV mice that expressed the human CETP transgene or not.

The baseline (chow diet) plasma lipid and lipoprotein profile determined 5 days after the surgery are shown on **Table 1**. Total cholesterol (TC) and triacylglycerol (TAG) concentrations were similar among all experimental groups. As expected, the HDL-cholesterol (HDL-C) concentrations were higher and LDL-C lower in CETP<sup>-</sup> compared with CETP<sup>+</sup> mice in both Sham and ovariectomized groups.

After 19 weeks on the atherogenic diet (**Table 2**), the TC concentrations rose in all groups. The TC and absolute cholesterol distributions in plasma lipoproteins were not different in Sham CETP<sup>+</sup> and Sham CETP<sup>-</sup> mice, whereas TC, LDL-, HDL-, and non-HDL-C concentrations were significantly lower in OV CETP<sup>+</sup> than in OV CETP<sup>-</sup> mice; however, the TC/HDL-C and non-HDL-C/HDL-C ratios, 3.0 and

TABLE 1. Plasma lipid and lipoprotein concentrations from CETP<sup>+</sup> and CETP<sup>-</sup> mice 5 days after Sham and ovariectomy on a chow diet

	Sham		Ovariectomized	
	CETP <sup>+</sup>	CETP <sup>-</sup>	CETP <sup>+</sup>	CETP <sup>-</sup>
	mg/dl			
TC	110 ± 10	109 ± 10	106 ± 14	103 ± 16
VLDL-C	17 ± 4	16 ± 3	17 ± 3	15 ± 3
LDL-C	45 ± 1 <sup>a</sup>	36 ± 1	43 ± 1 <sup>a</sup>	34 ± 1
HDL-C	48 ± 3 <sup>b</sup>	57 ± 4	46 ± 3 <sup>b</sup>	54 ± 4
Non-HDL-C	62 ± 2 <sup>b</sup>	52 ± 3	60 ± 3 <sup>b</sup>	49 ± 4
TAG	61 ± 14	59 ± 14	66 ± 15	56 ± 16

Mean ± SD (n = 8–12). TC, total cholesterol; TAG, triacylglycerol. The cholesterol distribution in the plasma lipoproteins was calculated as the area under the peaks of the FPLC profiles of pooled plasma samples (n = 3). Statistical comparisons by one way ANOVA followed by Student-Newman-Keuls test.

<sup>a</sup>P < 0.001 (Sham CETP<sup>+</sup> vs. Sham CETP<sup>-</sup>; Ov CETP<sup>+</sup> vs. OVCETP<sup>-</sup>).

<sup>b</sup>P < 0.05 (Sham CETP<sup>+</sup> vs. Sham CETP<sup>-</sup>; Ov CETP<sup>+</sup> vs. OVCETP<sup>-</sup>).

2.0 respectively, were similar in all four groups. Plasma triacylglycerol (TAG) concentrations were not altered.

The plasma activities of CETP, PLTP, and LCAT are also shown on Table 2. Neither CETP nor PLTP activities changed after ovariectomy. Thus, PLTP activity is not influenced by ovariectomy or by the expression of the CETP gene. In agreement with a previous report (34), the LCAT-dependent cholesterol esterification rate was higher in CETP<sup>+</sup> than in CETP<sup>-</sup> mice in both Sham and OV mice.

TABLE 2. Plasma lipid and lipoprotein concentrations, lipid transfer protein activities, oxLDL, and apoB-D antibodies titers in Sham and ovariectomized CETP<sup>+</sup> and CETP<sup>-</sup> mice fed a high fat and high cholesterol diet for 19 weeks

	Sham		Ovariectomized	
	CETP <sup>+</sup>	CETP <sup>-</sup>	CETP <sup>+</sup>	CETP <sup>-</sup>
	mg/dl			
Lipids				
TC	194 ± 15	200 ± 37	227 ± 40 <sup>a</sup>	266 ± 54 <sup>b</sup>
VLDL-C	56 ± 4	60 ± 0.2	60 ± 3	72 ± 4 <sup>b</sup>
LDL-C	73 ± 0.3	74 ± 3	90 ± 3 <sup>a</sup>	100 ± 3 <sup>b</sup>
HDL-C	65 ± 4	66 ± 3	77 ± 3 <sup>a</sup>	94 ± 2 <sup>b</sup>
Non-HDL-C	129 ± 4	134 ± 3	150 ± 3 <sup>a</sup>	172 ± 2 <sup>b</sup>
TAG	67 ± 14	64 ± 14	55 ± 25	44 ± 25
	%			
Protein activities				
CETP	48 ± 16	—	55 ± 9	—
PLTP	21 ± 5.3	20 ± 1.3	19 ± 4.4	20 ± 1.3
LCAT	5 ± 2 <sup>c</sup>	3 ± 1	14 ± 3 <sup>d</sup>	9 ± 3 <sup>e</sup>
Antibodies				
oxLDL	1.9 ± 0.8	1.7 ± 1.3	1.8 ± 1.1	2.7 ± 1.3 <sup>f</sup>
ApoB-D	1.9 ± 1.0	1.6 ± 0.9	1.2 ± 0.6	2.5 ± 1.0 <sup>b</sup>

Mean ± SD (n = 8–12). The cholesterol distribution in the plasma lipoproteins was calculated as the area under the peaks of the FPLC profiles of pooled plasma samples (n = 3). CETP as a percentage of CE transfer over 2 h, PLTP as percentage of PL transfer over 1 h, and LCAT as percentage of CE formation over 30 min. Antibodies as optical density at 450 nm corrected by total IgG. Statistical comparisons by one way ANOVA followed by Student-Newman-Keuls test.

<sup>a</sup>P < 0.001 (OV CETP<sup>+</sup> vs. Sham CETP<sup>+</sup>, Sham CETP<sup>-</sup>).

<sup>b</sup>P < 0.01 (OV CETP<sup>-</sup> vs. OV CETP<sup>+</sup>, Sham CETP<sup>+</sup>, Sham CETP<sup>-</sup>).

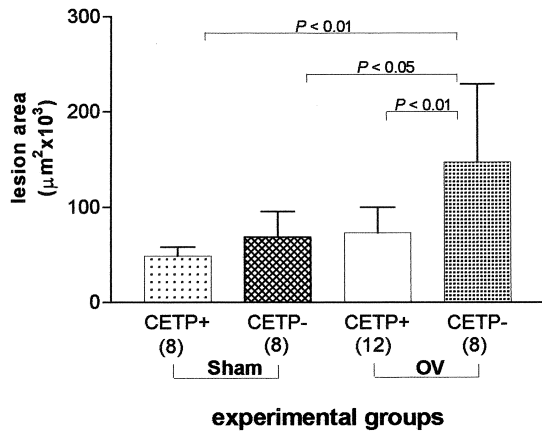
<sup>c</sup>P < 0.01 (Sham CETP<sup>+</sup> vs. Sham CETP<sup>-</sup>).

<sup>d</sup>P < 0.01 (OV CETP<sup>+</sup> vs. OV CETP<sup>-</sup>, Sham CETP<sup>+</sup>, Sham CETP<sup>-</sup>).

<sup>e</sup>P < 0.001 (OV CETP<sup>-</sup> vs. Sham CETP<sup>+</sup>, Sham CETP<sup>-</sup>).

<sup>f</sup>P = 0.06 (OV CETP<sup>-</sup> vs. OV CETP<sup>+</sup>).





**Fig. 1.** Area of aortic lesions in Sham CETP<sup>+</sup>, Sham CETP<sup>-</sup>, OV CETP<sup>+</sup>, and OV CETP<sup>-</sup> mice on a high fat and high cholesterol diet for 19 weeks. The columns represent the mean  $\pm$  SD of the number of mice indicated. Comparison of groups was determined by one-way ANOVA followed by the Student-Newman-Keuls test.  $P < 0.01$  for OV CETP<sup>-</sup> versus OV CETP<sup>+</sup>;  $P < 0.05$  for OV CETP<sup>-</sup> versus Sham CETP<sup>+</sup>; and  $P < 0.01$  for OV CETP<sup>-</sup> versus Sham CETP<sup>+</sup>.

Comparison of the two ovariectomized groups with their respective Sham groups showed that ovariectomy markedly increased the plasma cholesterol esterification rate. This result is compatible with an increased cholesterol esterification rate shown in postmenopausal women (35).

Since estrogens protect LDL particles against oxidation (36), the levels of oxLDL were measured indirectly by determining the antibody titers against the whole oxLDL particle and against a specific apoB epitope derived from oxLDL (apoB-D). Table 2 shows that these antibody titers were elevated in the plasma of OV CETP<sup>-</sup> as compared with OV CETP<sup>+</sup> mice. Anti-oxLDL was borderline higher ( $P = 0.06$ ) while anti-apoB-D antibody was significantly higher ( $P < 0.01$ ) in OV CETP<sup>-</sup> mice. Thus, CETP expression reduced the plasma levels of antibodies against oxLDL in ovariectomized mice.

Morphometric analysis of the lipid-stained areas in the aortic root (Fig. 1) showed that ovariectomy resulted in more extensive lesion area in the absence of CETP, with no differences among the other three groups (Sham CETP<sup>-</sup>, Sham CETP<sup>+</sup>, and OV CETP<sup>+</sup>). Thus, CETP expression reduced atherosclerosis formation in ovariectomized hypercholesterolemic mice.

In order to investigate whether the beneficial role of CETP was restricted to the hormone deficiency, new experiments were performed where HFHC diet fed OV mice were treated with 17 $\beta$ -estradiol. Confirming the data on Table 2 and Table 3 shows OV CETP<sup>-</sup> mice have higher lipoprotein cholesterol and higher titers of both antibodies against oxidized forms of LDL and lower LCAT activity than OV CETP<sup>+</sup> mice. Estrogen replacement abolished the differential responses between CETP<sup>+</sup> and CETP<sup>-</sup> mice of total and lipoprotein cholesterol concentrations, as well as markedly decreased both antibodies titers against oxLDL in both CETP<sup>+</sup> and CETP<sup>-</sup> mice. Accordingly, estrogen therapy blunted the genotype specific dif-

**TABLE 3.** Plasma lipid and lipoprotein concentrations, lipid transfer proteins activities, oxLDL and apoB-D antibodies titers in CETP<sup>+</sup> and CETP<sup>-</sup> mice, ovariectomized with and without estrogen replacement (E<sub>2</sub>) and fed a high fat and high cholesterol diet for 19 weeks

	Ovariectomized		Ovariectomized + E <sub>2</sub>	
	CETP <sup>+</sup>	CETP <sup>-</sup>	CETP <sup>+</sup>	CETP <sup>-</sup>
	<i>mg/dl</i>			
<b>Lipids</b>				
TC	211 $\pm$ 58	274 $\pm$ 58	209 $\pm$ 72	235 $\pm$ 90
VLDL-C	69 $\pm$ 7	78 $\pm$ 32	79 $\pm$ 4	77 $\pm$ 10
LDL-C	82 $\pm$ 4	107 $\pm$ 16 <sup>a</sup>	75 $\pm$ 3	91 $\pm$ 6
HDL-C	61 $\pm$ 3	90 $\pm$ 17 <sup>a</sup>	55 $\pm$ 2	68 $\pm$ 5
Non-HDL-C	151 $\pm$ 3	184 $\pm$ 17 <sup>a</sup>	154 $\pm$ 2	167 $\pm$ 5
TAG	52 $\pm$ 28	46 $\pm$ 21	56 $\pm$ 30	50 $\pm$ 15
	<i>%</i>			
<b>Protein activities</b>				
CETP	40 $\pm$ 3.9	—	47 $\pm$ 9.4 <sup>b</sup>	—
PLTP	18 $\pm$ 2.7	18 $\pm$ 3.1	16 $\pm$ 2.9	16 $\pm$ 3.9
LCAT	8 $\pm$ 1.8 <sup>c</sup>	6 $\pm$ 1.6	11 $\pm$ 3.9 <sup>c</sup>	4 $\pm$ 1.0
<b>Antibodies</b>				
oxLDL	1.4 $\pm$ 1.8	4.0 $\pm$ 2.0 <sup>d</sup>	0.94 $\pm$ 0.26	0.88 $\pm$ 0.35
ApoB-D	1.8 $\pm$ 1.5	2.4 $\pm$ 0.9 <sup>d</sup>	0.65 $\pm$ 0.17	0.51 $\pm$ 0.14

Mean  $\pm$  SD (n = 5–8). The cholesterol distribution in the plasma lipoproteins was calculated as the area under the peaks of the FPLC profiles of pooled plasma samples (n = 3). CETP as a percentage of CE transfer over 2 h, PLTP as a percentage of PL transfer over 1 h, and LCAT as a percentage of CE formation over 30 min. Antibodies as optical density at 450 nm corrected by total IgG. Statistical comparisons by one way ANOVA followed by Student-Newman-Keuls test.

<sup>a</sup>  $P < 0.05$  (OV CETP<sup>-</sup> vs. OV CETP<sup>+</sup> and OVE<sub>2</sub> CETP<sup>+</sup>).

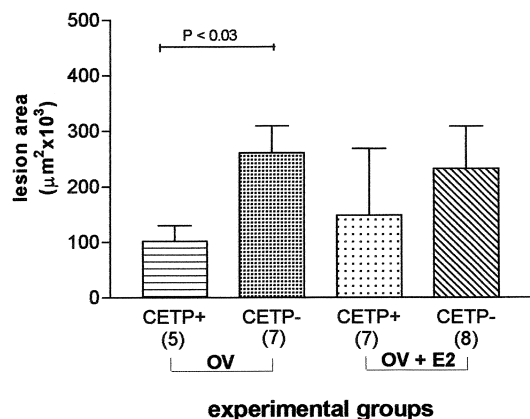
<sup>b</sup>  $P < 0.05$  (OVE<sub>2</sub> CETP<sup>+</sup> vs. OV CETP<sup>+</sup>).

<sup>c</sup>  $P < 0.05$  (OV CETP<sup>+</sup> vs. OV CETP<sup>-</sup>) and (OVE<sub>2</sub> CETP<sup>+</sup> vs. OVE<sub>2</sub> CETP<sup>-</sup>).

<sup>d</sup>  $P < 0.01$  (OV CETP<sup>-</sup> vs. OV CETP<sup>+</sup>, OVE<sub>2</sub> CETP<sup>+</sup> and OVE<sub>2</sub> CETP<sup>-</sup>).

ferences in severity of aorta atherosclerotic lesion between CETP<sup>+</sup> and CETP<sup>-</sup> mice (Fig. 2). Although the average lesion areas were not changed by the E<sub>2</sub> treatment, the hormone replacement therapy was specially beneficial for CETP<sup>-</sup> mice as shown by the analysis of frequency distribution based on two levels of lesion size, small (below median) and large (above median), displayed in Fig. 3. When ovaries were removed from CETP<sup>+</sup> mice, little change was observed in the mice distribution according to their lesion size; however, ovaries removal from CETP<sup>-</sup> mice led to a marked shift in the proportion of mice toward lesions with larger areas, from 50% to 87.5% (Fig. 3A). On the other hand, estrogen replacement of OV mice had little effect on the CETP<sup>+</sup> mice distribution and markedly improved the CETP<sup>-</sup> mice distribution by increasing the number of mice with smaller size lesions from 14% to 50% (Fig. 3B).

To gain insight on the mechanisms of atheroprotection played by CETP, cell cholesterol uptake and efflux were measured in peritoneal macrophages from OV and estrogen treated OV CETP<sup>+</sup> and CETP<sup>-</sup> mice. As shown in Table 4, macrophages from OV CETP<sup>+</sup> mice displayed lower uptake of labeled cholesteryl ether (CET) from acetyl-LDL than macrophages from OV CETP<sup>-</sup> mice. In addition, estrogen replacement induced a further reduction in CET uptake and an elevation in HDL mediated cholesterol efflux from pre-loaded OV CETP<sup>+</sup> as compared with OV CETP<sup>-</sup> macrophages.



**Fig. 2.** Area of aortic lesions in OV CETP<sup>+</sup> and OV CETP<sup>-</sup> mice on a high fat and high cholesterol diet for 19 weeks, without and with 17- $\beta$ -estradiol (E2) replacement during the last 8 weeks. The columns represent the mean  $\pm$  SD of the number of mice indicated.  $P < 0.03$  for OV CETP<sup>-</sup> versus OV CETP<sup>+</sup> by Mann-Whitney test.

## DISCUSSION

The precise role of CETP in atherogenesis has been controversial because of divergent effects of CETP on the incidence or development of atherosclerotic disease in humans and animals. Studies in CETP transgenic mice have shown that CETP expression in the wild-type background is atherogenic in males (5) but not in females (8) or in animal heterozygotes for LDLR deficiency (present data, Sham groups). In contrast, CETP expression is

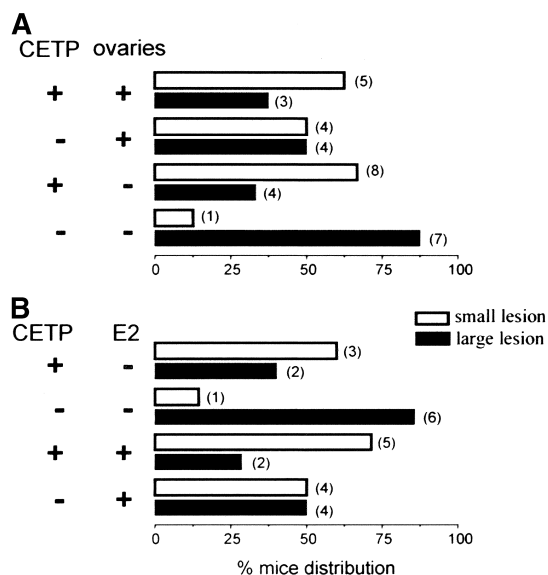
atheroprotective in hypertriglyceridemia (7) and also when there is overexpression of LCAT (8); however, when cholesterol fed rabbits are used as the experimental model, the inhibition of CETP activity by antisense oligonucleotides (9), chemical inhibitors (10), or vaccination against CETP (11) decreases the atherosclerotic areas by 25%, 67%, and 40%, respectively, compared with control animals.

The data presented here showed that CETP expression in moderate hypercholesterolemia and deficiency in ovarian hormones leads to a 50% reduction in atherosclerosis. Estrogen replacement therapy in OV mice blunted the CETP dependent differences in atherosclerosis severity; however, estrogen treatment did not rescue OV mice from developing aortic lipid deposits, most likely because it was administered only during the last 8 weeks of the atherogenic diet period. Nonetheless, the beneficial effect of estrogen treatment was clearly demonstrated by three effects: 1) inhibition of the rise in lipoprotein cholesterol level in CETP<sup>-</sup> mice, 2) decrease of antibodies to oxLDL in both CETP<sup>+</sup> and CETP<sup>-</sup> mice, and 3) marked increase in the proportion of CETP<sup>-</sup> mice presenting smaller lesion size from 13% to 50%. These observations lead to the conclusion that, concerning the parameters evaluated, CETP expression can compensate for the lack of estrogen, and on the other hand, estrogen replacement can partly compensate for the lack of CETP; however, when CETP and estrogen were present simultaneously, 70% of the mice exhibited lesions of smaller size, while when both CETP and estrogen were absent, 80% of the mice presented lesions of larger size.

The atheroprotective role of CETP may have been exerted by CETP per se or through its combined secondary actions on LCAT reaction rate, cholesterol, and oxLDL levels. The effect of CETP in increasing the LCAT mediated cholesterol esterification rate was independent of the presence of ovarian hormones (Table 2, 3) whereas the protective effect of CETP against atherosclerosis was seen only in the estrogen deficient group (OV) (Figs. 1, 2). Furthermore, endogenous LCAT activity did not correlate with the size of the arterial lipid deposits in any combination of the data. Thus, although LCAT may have an anti-atherogenic role, the protection in OV CETP<sup>+</sup> mice cannot be ascribed to the rate of the LCAT reaction.

The CETP mediated decrease in plasma total cholesterol concentrations and in the quantity of cholesterol in each lipoprotein fraction was observed only when mice were ovariectomized and was abolished when OV mice received estrogen treatment. Combined analysis of the four experimental groups shown in Fig. 1 revealed a strong positive correlation between the TC levels and the atherosclerotic lesion areas ( $r = 0.7964$ ,  $P = 0.0001$ ,  $n = 36$ ). Within each group, where the cholesterolemia range is narrow, this statistical correlation was not observed. Thus, the extent of the arterial lesions could be explained, at least in part, by the higher plasma TC concentration in OV CETP<sup>-</sup> mice.

How CETP expression impeded the ovariectomy-induced rise in non-HDL-C is not known. The expression



**Fig. 3.** Percent frequency distribution of mice according to two levels of atherosclerotic lesion size: small (below median) and large (above median). Number of mice given in parentheses. A: (Data from Fig. 1) CETP<sup>+</sup> and CETP<sup>-</sup> with or without ovaries. Median =  $58.5 \times 10^3 \mu\text{m}^2$ , range = 15 to 284,  $n = 36$ . B: (Data from Fig. 2) ovariectomized CETP<sup>+</sup> and CETP<sup>-</sup> mice with or without 17- $\beta$ -estradiol (E2) replacement. Median =  $157.6 \times 10^3 \mu\text{m}^2$ , range = 18 to 524,  $n = 27$ .

TABLE 4. Peritoneal macrophage uptake of [<sup>3</sup>H]CEt from acetyl-LDL and efflux of cholesterol from macrophages pre-loaded with [<sup>14</sup>C]CE

Animal treatments	CETP Genotype	Cell Uptake	Efflux to HDL
		% [ <sup>3</sup> H]CEt/mg protein	% [ <sup>14</sup> C]CE/mg protein
Ovariectomy	+	49 ± 8 <sup>a</sup>	41 ± 8 <sup>c</sup>
	-	63 ± 9 <sup>a</sup>	44 ± 8 <sup>d</sup>
Ovariectomy + Estrogen	+	34 ± 3 <sup>a,b</sup>	51 ± 4 <sup>c,e</sup>
	-	56 ± 10 <sup>b</sup>	31 ± 5 <sup>d,e</sup>

Mean ± SD. Eight replicates of cell pools from five animals in each condition.

Conditions with the same superscripts are statistically different:  $P < 0.01$  by one way ANOVA.

of LDLRs is certainly not upregulated in CETP<sup>+</sup> mice. On the contrary, CETP expression per se downregulates LDLRs in a dose-dependent manner (37). In addition, high cholesterol diet also downregulates LDLRs (38) in an animal already deficient in these receptors. Castration of female C57Bl6 mice, by itself, does not change LDLRs and apoB mRNA abundance (39). On the other hand, estrogen increases hepatic HMG-CoA reductase activity by stabilizing its mRNA level (40) while CETP expression reduces the hepatic HMG-CoA reductase mRNA (37). Thus, estrogen deficiency and CETP expression together may have additive effects on reducing HMG-CoA reductase. This could lead to a lower hepatic cholesterol synthesis, lower VLDL-C secretion, and lower plasma LDL-C generation in OV CETP<sup>+</sup> as compared with OV CETP<sup>-</sup> mice, the exact phenotype that was observed in the present study.

The higher plasma concentration of HDL-C in OV CETP<sup>-</sup> compared with OV CETP<sup>+</sup> mice did not protect them against atherosclerosis formation, as also shown in some studies in humans (3, 41) and mice (8, 42). The protective effect of HDL particles may be related as much to their kinetics as to their plasma concentration (8, 12). The remodelling of HDL by CETP was shown to facilitate uptake of HDL-cholesteryl ester by mouse liver and by SRBI overexpressing cells (43). Thus, the reduction in HDL-C levels observed in OV CETP<sup>+</sup> as compared with OV CETP<sup>-</sup> mice could be ascribed to increased HDL-cholesteryl ester selective uptake, especially in estrogen deficiency that upregulates SRBI in the liver (44, 45, 46).

Protection against lipoprotein oxidation is a well-known anti-atherogenic action of estrogen (36, 47) and is clearly demonstrated by the marked reduction in the levels of antibodies against oxidized forms of LDL in both groups of estrogen treated OV mice (Table 3). In the absence of estrogen, the areas of the atherosclerotic lesions correlated positively with the levels of antibodies against oxLDL ( $r = 0.41$ ,  $P = 0.01$ ,  $n = 32$ , all OV groups) as well as against apoB-D ( $r = 0.32$ ,  $P = 0.04$ ,  $n = 32$ , all OV groups). Thus, the greater extent of atherosclerosis in OV CETP<sup>-</sup> mice is also dependent on their higher titers of oxLDL antibodies. High serum titers of autoantibodies to malondialdehyde epitopes of oxLDL have previously been demonstrated in apoE knock out mice (48). Moreover, circulating antibodies to oxLDL correlated positively with the oxLDL content in the atherosclerotic lesions of LDLR deficient mice (49). We have disclosed in this study a new role for

CETP specifically related to the estrogen deficient state, i.e., CETP may function as a back up mechanism to reduce circulating levels of oxLDL as shown by the lower levels of anti-oxLDL antibodies in OV CETP<sup>+</sup> mice. This role of CETP seems to be even more relevant considering that CETP deficient patients have higher levels of oxLDL (50). In this regard, we have recently shown that CETP transfers esterified cholesterol from oxLDL to HDL more efficiently than from native LDL (51). In doing so, CETP may facilitate the HDL removal of oxidized lipids and diminish the levels of oxLDL in plasma.

Recently, it was reported that CETP is expressed in foam cells (52) and in smooth muscle cells (53) in human atherosclerotic lesions, thus suggesting that CETP may have a direct local involvement in atherogenesis. Additional insight on the interplay of the CETP expression and ovarian hormones on the development of arterial fat deposits was obtained by investigating macrophages' capacity to take up modified LDL cholesteryl ester and to efflux their cholesterol content (Table 4). This experiment clearly showed that, in the deficiency of estrogen, the expression of the CETP impaired the macrophage uptake of acLDL cholesteryl ester. Moreover, after in vivo replacement of estrogen, further inhibition of cholesteryl ester uptake and facilitation of cell cholesterol efflux to HDL was observed only in the CETP expressing macrophages. Previous works had already shown that addition of CETP to the culture media of smooth muscle cell (54) or foam cell (55) stimulated cholesterol efflux rate. Therefore, CETP seems to have anti-atherogenic effects on the foam cell formation, either directly or in an estrogen synergistic manner.

In summary, we have provided evidence supporting the proposed anti-atherogenic role of CETP in a specific metabolic setting, showing that experimental atherosclerosis is more severe when the estrogen levels are suppressed and CETP is absent. Estrogen replacement therapy abolishes genotype specific differences in atherosclerosis mainly because it shifts the proportion of mice with large lesions toward to small lesion size. There have been major disagreements about whether inhibitors of CETP would be anti- or pro-atherogenic in humans (12, 13). Considering the limitations of the mouse model to human physiology, additional data from specific post-menopause or oophorectomy conditions are needed before devising strategies such as the inhibition of CETP to reduce the risk of premature atherosclerosis in humans. ■■



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