Atherosclerosis is enhanced by testosterone deficiency and attenuated by CETP expression in transgenic mice

A. C. Casquero,* J. A. Berti,* A. G. Salerno,* E. J. B. Bighetti,* P. M. Cazita,† D. F. J. Ketelhuth,§ M. Gidlund,§ and H. C. F. Oliveira[1,*]

Departamento de Fisiologia e Biofísica,* Instituto de Biologia, Universidade Estadual de Campinas, Sao Paulo, Brazil; and Laboratório de Lípides (LIM-10),† Faculdade de Medicina, and Departamento de Imunologia,§ Instituto de Ciências Biomédicas, Universidade de Sao Paulo, Sao Paulo, Brazil

Abstract In this work, we investigated the impact of testosterone deficiency and cholesteryl ester transfer protein (CETP) expression on lipoprotein metabolism and diet-induced atherosclerosis. CETP transgenic mice and nontransgenic (nTg) littermates were studied 4 weeks after bilateral orchidectomy or sham operation. Castrated mice had an increase in the LDL fraction (+36% for CETP and +79% for nTg mice), whereas the HDL fraction was reduced (−30% for CETP and −11% for nTg mice). Castrated mice presented 1.7-fold higher titers of anti-oxidized LDL (Ox-LDL) antibodies than sham-operated controls. Plasma levels of CETP, lipoprotein lipase, and hepatic lipase were not changed by castration. Kinetic studies showed no differences in VLDL secretion rate, VLDL-LDL conversion rate, or number of LDL and HDL receptors. Competition experiments showed lower affinity of LDL from castrated mice for tissue receptors. Diet-induced atherosclerosis studies showed that testosterone deficiency increased by 100%, and CETP expression reduced by 44%, the size of aortic lesion area in castrated mice. In summary, testosterone deficiency increased plasma levels of apolipoprotein B-containing lipoproteins (apoB-LPs) and anti-OxLDL antibodies, decreased LDL receptor affinity, and doubled the size of diet-induced atherosclerotic lesions. The expression of CETP led to a milder increase of apoB-LPs and reduced atherosclerotic lesion size in testosterone-deficient mice.—Casquero, A. C., J. A. Berti, A. G. Salerno, E. J. B. Bighetti, P. M. Cazita, D. F. J. Ketelhuth, M. Gidlund, and H. C. F. Oliveira. **Atherosclerosis is enhanced by testosterone deficiency and attenuated by CETP expression in transgenic mice. *J. Lipid Res.* 2006.** 47: **1526–1534.**

Supplementary key words low density lipoprotein receptor • lipoprotein lipase • lipolysis • plasma lipoprotein kinetics • oxidized low density lipoprotein • aortic atherosclerosis lesion • cholesteryl ester transfer protein

Gender differences in coronary artery disease (CAD) risk observed during the reproductive life period have been attributed to an attenuating effect of estrogen on atherogenesis (1, 2) and/or to a proatherogenic action of androgens (3, 4). However, the unexpected outcomes from the Women's Health Initiative and Heart and Estrogen Replacement Study trials (5, 6) and other controversies about the role of androgens (7) necessitate further investigation of the role of sex steroids, especially in sex-matched individuals. Androgen's effects on lipoprotein metabolism and risk of atherosclerosis are not unequivocal (7, 8). Increased endogenous serum testosterone in men has been associated with a favorable lipid profile (9, 10), and low endogenous levels of testosterone have been associated with an atherogenic lipid profile (11, 12) and CAD (13, 14). However, other studies have not found such relationships (15–18). Aromatization of testosterone into 17β-estradiol seems to be an important determinant of the beneficial effects of androgens observed in men (19, 20) and mice (21, 22). On the other hand, increasing endogenous androgen levels in women (23, 24), androgenic supplementation in men (3), in women (25), and in female animals (26, 27), and nonmedical use of androgenic anabolic steroids (4, 28, 29) are associated with increased risk factors for atherosclerotic diseases.

Cholesteryl ester transfer protein (CETP) transfers cholesteryl ester (CE) from HDL to apolipoprotein B-containing lipoproteins (apoB-LPs) in exchange for triglycerides (TGs), thus remodeling HDL composition in the plasma of several species, such as humans, primates, and rabbits, but it is not present in mice and rats (30). Epidemiological and experimental evidence has shown that CETP may play an important role in the development of atherosclerosis (31). Human subjects with CETP deficiency and high levels of HDL-cholesterol (>60 mg/dl) showed reduced risk of CAD, whereas CETP-deficient subjects whose HDL levels were moderately increased (40–60 mg/dl) presented higher risk of CAD (32). Inhibitors of CETP have now been tested in human subjects and shown to increase the concentration of HDL-cholesterol while decreasing that of LDL-cholesterol and apoB (33, 34). Studies in

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[1] To whom correspondence should be addressed.
e-mail: ho98@unicamp.br

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CETP transgenic (Tg) mice have provided mixed results. The effects of CETP expression in this species can be neutral, proatherogenic, or antiatherogenic, depending upon the metabolic context (35–39).

Plasma CETP levels differ in men and women (40, 41), but sex hormone seems not to affect the CETP plasma levels. Treatment of young women (25) or hypogonadic men (42) with testosterone did not change plasma CETP activity. Similarly, estrogen deficiency (43) or estrogen therapy (44, 45) had no impact on the plasma CETP activity in humans and Tg mice. Although castration did not change CETP plasma levels in female mice, we showed recently that CETP expression reduced atherosclerosis by 50% in ovariectomized Tg mice (36).

In this work, we investigated the effect of testosterone deficiency, in the presence and absence of CETP, on the lipoprotein profile and associated enzyme levels and tested the hypotheses that *i*) androgens are atheroprotective for males, and *ii*) the expression of CETP can modulate the development of atherosclerosis in testosterone-deficient male mice.

MATERIALS AND METHODS

Animal procedure

All animal protocols were approved by the university's Committee for Ethics in Animal Experimentation. 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 Tg mice (line 5203, C57BL6/J background) (46) expressing a human CETP minigene under the control of its natural flanking sequences and nontransgenic (nTg) male littermates, 12–16 weeks of age, were bilaterally orchidectomized or sham-operated. Thirty days after the surgical removal of testis, mice fasted overnight were anesthetized and euthanized by exsanguination through the retro-orbital plexus. To compare the development of diet-induced atherosclerosis, CETP Tg mice were cross-bred with LDL receptor knockout mice purchased from Jackson Laboratory (Bar Harbor, ME) to generate mice heterozygous for the LDL receptor null allele expressing CETP (CETP/R1) or not (R1). One week after castration, all animals were placed on an atherogenic high-fat and high-cholesterol diet containing 15% fat, 1.25% cholesterol, and 0.5% cholic acid (percentage by weight) (catalog number 611208; Dyets, Inc., Bethlehem, PA) for 10 weeks.

Plasma biochemical analyses

Lipoproteins from the pooled or individual plasmas of mice were separated by fast-protein liquid chromatography (FPLC) (Amersham-Pharmacia Biotech, Uppsala, Sweden) as described previously (47). The FPLC HR10/30 Superose 6 column separates three well-defined cholesterol peaks from mouse plasma: fractions 10–15, VLDL ($d < 1.006$), >30–80 nm; fractions 16–26, intermediate and low density lipoproteins (IDL) + LDL ($d = 1.006$–1.063), 16–30 nm; and fractions 27–37, HDL ($d = 1.063$–1.21), 8–16 nm. Total cholesterol and triacylglycerols (Chod-Pap; Roche Diagnostic GmbH, Mannheim, Germany) and plasma free fatty acids (Wako Chemical, Neuss, Germany) were determined by enzymatic/colorimetric methods according to each manufacturer's instructions. Plasma glucose concentrations were determined by the glucose oxidase method using the Merck Diagnostic Biotrol® kit (Chennevires-les-Louvres, France). Insulin concentrations were determined by radioimmunoassay using rat insulin as a standard.

Intravascular lipase activities

Total lipase activity was determined according to Ehnholm and Kuusi (48). Overnight-fasted mouse plasmas, obtained before (basal) and 10 min after subcutaneous injection of heparin (100 U/kg body weight), were incubated with a [^{3}H]triolein/gum arabic substrate {[9,10-^{3}H(N)]triolein; New England Nuclear, Boston, MA} for 1 h. HL activity was determined in tubes in which the LPL was inhibited by 2 M NaCl. The hydrolyzed labeled free fatty acids were extracted with methanol-chloroform-heptane (1.4:1.25:1) and dried under N_2, and radioactivity was determined in a LS6000 Beckman Beta Counter. LPL activity was calculated as the difference between the total lipase and the hepatic lipase activities.

Endogenous lipolysis rate

Basal blood samples (100 μl; source of endogenous substrates) were obtained by the tail tip of 6 h-fasted mice. Next, mice received a subcutaneous injection of heparin (100 IU/kg body weight), and after 10 min, a second blood sample (50 μl) was obtained to be used as the source of lipases. Five microliters of postheparin plasma or saline (blank) was added to 50 μl of preheparin plasma, and the mixtures were incubated at 37°C. Free fatty acids were determined sequentially in time, every 15 min until 60 min, using the Wako Chemical enzymatic/colorimetric kit according to the manufacturer's instructions. Lipolysis rate was calculated as the slope of the linear regression of the released FFA versus time curve.

CETP activity (exogenous assay)

CETP activity, which reflects the plasma CETP concentration, was measured using exogenous substrates as described previously (49). Briefly, a mixture of human VLDL and LDL (100 μg of proteins) was incubated with 10,000 dpm of human HDL_3 labeled with [^{14}C]CE (50) 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 and negative controls with nTg mouse plasma. The incubations were carried out at 40°C for 2 h. The apoB-LPs were precipitated, and the remaining radioactivity was measured in the supernatant in a LS6000 Beckman Beta Counter.

Endogenous CETP-mediated CE transfer

One hundred microliters of plasma from overnight-fasted mice was fractionated by FPLC to obtain the VLDL + LDL fractions. In parallel, 100 μl of plasma was precipitated with 10 μl of 1.6% dextran sulfate-1 M $MgCl_2$ (1:1) solution, and the supernatant (HDL fraction) was recovered. Trace amounts of human HDL labeled with [^{3}H]CE (10^5 dpm) were incubated with the mouse HDL fractions at 4°C overnight. Next, the VLDL + LDL fractions were mixed with the HDL fractions (preequilibrated with [^{3}H]HDL) and incubated at 37°C for 30 min. The transfer reaction was stopped by ice-cooling the tubes. Fifty micrograms of human LDL protein was added in each tube as precipitating carrier, followed by one-tenth volume of the precipitating cocktail. Samples stood on ice for 15 min, after which they were centrifuged at 12,000 rpm for 15 min at 4°C. The supernatants were recovered, and radioactivity remaining in the HDL was determined in a LS6000 Beckman Beta Counter. Blanks used saline and 3.5% BSA instead of the VLDL + LDL fraction.

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In vivo hepatic VLDL secretion rate

After a 12 h fasting period, mice were injected intravenously with Triton WR1339 (500 mg/kg body weight) using a 15% (w/v) Triton solution in 0.9% NaCl (51). Blood samples were drawn at 0, 15, 30, 60, 90, and 120 min after injection and analyzed for cholesterol and TGs. Hepatic VLDL secretion rates were calculated from the slopes of the linear regression curves.

Kinetic studies with labeled LDL and HDL

LDL and HDL were isolated from plasma pools of normolipidemic human donors by sequential ultracentrifugation according to Havel, Eder, and Bragdon (52) and dialyzed against saline containing 2 mM EDTA. Protein moieties of LDL and HDL were labeled with $Na^{131}I$ (Instituto de Pesquisa Energia Nuclear/Universidade de Sao Paulo, Sao Paulo, Brazil) and $Na^{125}I$ (Amersham Biosciences, Buckinghamshire, UK), respectively, according to MacFarlane as modified by Bilheimer, Eisenberg, and Levy (53). LDL and HDL were also labeled with [^{3}H]cholesteryl oleoyl ether (Amersham Biosciences) as described previously (50). Labeled lipoproteins were filtered with a 0.22 μm Millipore membrane and used fresh. Slightly anesthetized mice received an intravenous or intraperitoneal injection of labeled lipoproteins (1×10^6 dpm). Blood samples (50 μl) were obtained by the tail tip at 0.25, 0.5, 1, 2, 4, and 6 h after intraperitoneal injection or at 2 min and 0.5, 1, 2, 4, and 6 h after intravenous injection. Iodine and cholesterol radioactivities were determined in plasma samples in a Gamma Counter (Beckman Gamma 5500) or with liquid scintillation solution in a Beta Counter (Beckman LS6000 TA), respectively.

LDL competition studies

Pooled plasma (1 ml) from sham-operated mice was incubated with [^{14}C]cholesterol (50 μCi dried on a filter paper disk) at 4°C for 24 h. Plasma was then incubated at 37°C overnight with gentle shaking. [^{14}C]LDL was isolated by density gradient ultracentrifugation in Ultraclear tubes for 24 h at 40,000 rpm in a Beckman SW41ti rotor, as described previously (54). Cold LDL was isolated from pooled plasma samples (3.5 ml) from sham-operated and castrated mice. A human plasma sample (3.5 ml) was submitted to ultracentrifugation together with mouse samples as a visual reference for the density gradient lipoprotein isolation. Labeled and cold LDL fractions were thoroughly dialyzed against saline containing 1 mM EDTA. The in vivo competition assay consisted of mixing labeled LDL (5×10^5 dpm, 1 μg) from sham-operated mice with cold LDL (10 μg of cholesterol) from sham-operated or castrated mice and injecting the mixture into the femoral vein of intact mice. Blood samples (50 μl) were obtained at 2 min and 0.5, 1, 2, 3, and 6 h postinjection for measurements of radioactivity in plasma. Comparisons of the areas under the curves and fractional catabolic rates show which LDL, from sham-operated or castrated mice, is able to retard plasma removal of [^{14}C]LDL more efficiently.

Histological analysis of atherosclerotic lesions

Mice were anesthetized, and their hearts were perfused in situ with PBS followed by 10% PBS-buffered formaldehyde, excised, and fixed in 10% formaldehyde for at least 2 days. The hearts were embedded sequentially in 5, 10, and 25% gelatin. Processing and staining were carried out according to Paigen et al. (55). The lipid-stained lesions were quantified as described by Rubin et al. (56) using Image Pro Plus software (version 3.0) for image analysis (Media Cybernetics, Silver Spring, MD). One investigator who was unaware of the treatments read the slides.

Detection of antibodies to oxidized LDL

Antibodies against holo-oxidized LDL (OxLDL) were determined as described previously (36) using microtiter plates coated with 1 μg/ml human OxLDL (20 mM Cu^{2+}, 24 h), blocked with a 5% solution of fat-free milk, and incubated overnight with mouse plasma samples (50 μl; diluted 1:100). A peroxidase-conjugated secondary goat anti-mouse IgG antibody (50 μl; diluted 1:5,000) was used to detect anti-OxLDL. The optical density was measured at 450 nm (Titertek Multiskan MCC/340P, model 2.20; Labsystems).

Statistical analyses

Data, expressed as means ± SEM, were analyzed with InStat GraphPad software (version 3.0) using Student's *t*-test for comparisons between two groups and one-way ANOVA for multiple comparisons, followed by the Student-Newman-Keuls test. Differences between groups were considered significant at $P \leq 0.05$.

RESULTS

Plasma lipid and lipoprotein concentrations were studied in adult male CETP Tg and nTg littermates 4 weeks after bilateral orchidectomy. Both castrated groups of mice lost weight after this period (−4% in both CETP Tg and nTg; $P < 0.05$ vs. initial body weight), probably as a result of a reduction in lean body mass (57). Plasma total cholesterol, TG, FFA, glucose, and insulin fasting levels were not modified by the testosterone deficiency (data not shown). However, the cholesterol distribution in the plasma lipoprotein fractions was modified significantly in castrated mice, as shown in **Table 1**. Castrated mice had an increase in the LDL + IDL fraction: +36% for CETP Tg mice ($P < 0.02$) and +79% for nTg mice ($P < 0.001$); the HDL fraction was reduced: −30% for CETP Tg mice ($P < 0.002$) and −11% for nTg mice ($P < 0.03$). The ratio of apoB-LP to HDL was markedly increased in castrated mice, 1.7-fold for CETP mice and 2-fold for nTg mice.

Possible mechanisms responsible for these lipoprotein variations induced by deficiency of testosterone were further investigated in CETP Tg mice. A higher hepatic VLDL production rate could explain the increased LDL + IDL levels in castrated mice. However, as shown in **Fig. 1**, the VLDL-TG secretion rate was actually decreased by 23% ($P < 0.02$) in castrated compared with sham-operated CETP Tg mice (185.9 ± 12.5 vs. 144.1 ± 6.0 mg/dl/h, respectively; $P < 0.02$). Hepatic VLDL-cholesterol secretion rate was essentially the same in both groups of mice. This means that nascent VLDL from castrated mice contained less TG and more cholesterol relative to the VLDL from sham-operated mice. This modified VLDL may have been metabolized or removed from plasma of castrated mice slower than the VLDL of sham-operated mice in such a way that the total TG concentrations did not differ in the two groups.

The efficiency of the conversion of VLDL to LDL was studied by determining the maximum activities of LPL and HL and the rate of lipolysis of endogenous substrates (**Table 2**). There were no significant differences in the LPL and HL activities, indicating that testosterone deficiency does not influence the expression of these two

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TABLE 1. Cholesterol distribution in plasma lipoproteins from castrated and sham-operated CETP Tg and nTg mice

	CETP Tg		nTg	
Lipoprotein	Sham-Operated	Castrated	Sham-Operated	Castrated
VLDL	3.5 ± 0.3	2.7 ± 0.6	3.7 ± 0.3[d]	5.3 ± 0.3[d]
LDL + IDL	10.7 ± 1.1[a]	14.6 ± 0.2[a]	9.3 ± 0.1[e]	16.7 ± 1.1[e]
HDL	47.4 ± 1.9[b]	33.1 ± 0.8[b]	86.7 ± 0.9[f]	77.2 ± 1.6[f]
Apolipoprotein B-containing lipoprotein/HDL	0.30 ± 0.03[c]	0.50 ± 0.05[c]	0.13 ± 0.03[g]	0.27 ± 0.03[g]

CETP, cholesteryl ester transfer protein; IDL, intermediate density lipoprotein; nTg, nontransgenic; Tg, transgenic. Values shown are mg/dl (means ± SEM; n = 3–5 per group, number of pooled samples from two mice). The same superscripts are statistically different by Student's *t*-test.

[a] $P = 0.025$.
[b] $P = 0.002$.
[c] $P = 0.0303$.
[d] $P = 0.024$.
[e] $P = 0.001$.
[f] $P = 0.003$.
[g] $P = 0.047$.

enzymes. The assay using mouse plasma as the source of both lipases and endogenous substrates showed similar rates of FFA release in castrated and sham-operated mice (Table 2). Therefore, intravascular lipases and lipolysis rate cannot explain the differences in either LDL + IDL or HDL concentration found in the plasma of testosterone-deficient mice.

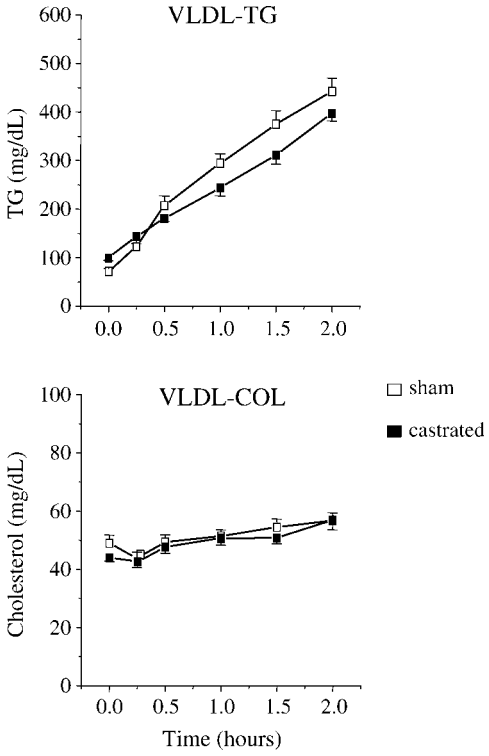

Fig. 1. Hepatic VLDL secretion in castrated and sham-operated cholesteryl ester transfer protein (CETP) transgenic mice measured after Triton WR1339 injection. Values shown are means ± SEM (n = 6–7). Slopes of the triglyceridemia curves are 185.9 ± 12.5 and 144.1 ± 6.0 mg/dl/h for sham-operated and castrated mice, respectively ($P < 0.02$). Slopes of cholesterolemia curves are 6.9 ± 0.2 and 7.3 ± 1.7 mg/dl/h for sham-operated and castrated mice, respectively. COL, cholesterol; TG, triglyceride.

Deficiency of testosterone also did not influence CETP expression, as shown by the unaltered plasma CETP activity (Table 2) measured by an exogenous substrate assay indicative of CETP mass. However, endogenous CETP-mediated CE transfer rate was increased by 20% ($P < 0.05$) in castrated compared with sham-operated CETP Tg mouse plasma (Table 2). This effect may have contributed to the increase in LDL + IDL and the decrease in HDL-cholesterol observed in castrated mice, but it is not necessary to explain these lipoprotein alterations, because they also occurred in castrated nTg mice. It certainly explains the greater decrease in HDL in castrated CETP Tg mice than in castrated nTg mice (30% vs. 11%, respectively).

Potential variations in the number of LDL and HDL tissue receptors were studied by measuring the plasma removal of heterologous lipoproteins from human donors labeled in their protein or CE moiety and injected either intraperitoneally or intravenously in sham-operated and castrated CETP Tg mice (**Table 3**). It can be observed that the amount of labeled lipoprotein remaining in plasma during 6 h, measured as area under the curve, and the fractional catabolic rates of LDL and HDL protein and CE were similar in both castrated and sham-operated CETP Tg mice. These findings suggest that testosterone

TABLE 2. Plasma activities of CETP, LPL, and HL, and lipolysis rate in castrated and sham-operated CETP Tg mice

	CETP Tg	
Variable	Sham-Operated	Castrated
CETP (exogenous assay) (%)	30 ± 2.9	27 ± 3.8
Cholesteryl ester endogenous transfer (%)	44 ± 2.3	53 ± 2.1[a]
LPL (nmol/ml/h)	1,120 ± 117	1,203 ± 174
HL (nmol/ml/h)	1,759 ± 96	1,853 ± 124
Endogenous lipolysis (FFA release) (nmol/l/h)	0.44 ± 0.06	0.35 ± 0.03

Values shown are means ± SEM (n = 5–8 per group).
[a] $P = 0.01$ by Student's *t*-test.

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TABLE 3. Plasma removal of LDL and HDL labeled with ^{125}I, ^{131}I, or [^{3}H]CE in castrated and sham-operated CETP Tg mice

Lipoprotein	Test	Castrated	Sham-Operated
^{125}I-HDL (intraperitoneal)	AUC	372 ± 33	435 ± 21
^{131}I-LDL (intraperitoneal)	AUC	507 ± 19	500 ± 14
[^{3}H]CE-LDL (intraperitoneal)	AUC	517 ± 16	474 ± 52
[^{3}H]CE-LDL (intravenous)	FCR	0.259 ± 0.01	0.287 ± 0.01
	AUC	261 ± 15	260 ± 9
[^{3}H]CE-HDL (intravenous)	FCR	0.220 ± 0.01	0.213 ± 0.01
	AUC	325 ± 10	318 ± 9

AUC, area under the curve; CE, cholesteryl ester; FCR, fractional catabolic rate. Values shown are means ± SEM (n = 4–8 per group). AUC (dpm/h × 10^3) and FCR were calculated from the peak of radioactivity (1 h after intraperitoneal injection or 2 min after intravenous injection) to 6 h after injection. FCR was obtained as the slope of the radioactivity decay curves.

deficiency does not alter the number of LDL and HDL tissue receptors.

Lipoprotein plasma removal rates are affected by *i*) the number of tissue receptors and *ii*) their affinity for these receptors. To check the putative lower affinity for LDL of castrated mice, in vivo competition experiments for plasma removal between LDL from sham-operated or castrated mice and labeled LDL (from sham-operated mice) were performed (**Fig. 2**). The results show that the 10-fold mass excess of LDL from sham-operated mice more efficiently retarded [^{14}C]LDL plasma removal than the same amount of LDL from castrated mice, as indicated by the areas under the radioactivity curves (188.9 ± 18.7 vs. 142.1 ± 7.3 for sham-operated vs. castrated mice, respectively; $P \leqslant 0.01$) and fractional catabolic rates determined from 2 to 60 min (0.020 ± 0.002 vs. 0.027 ± 0.001 for sham-operated vs. castrated mice, respectively; $P \leqslant 0.002$).

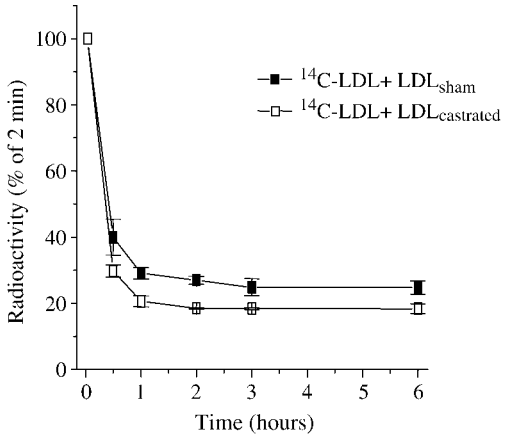

Fig. 2. Competition between LDL from sham-operated (closed symbols) and castrated (open symbols) mice and [^{14}C]cholesterol-LDL for plasma removal. Labeled LDL from sham-operated mice was mixed with cold LDL from sham-operated or castrated mice and injected into the femoral veins of intact mice (for details, see LDL competition studies in Materials and Methods). Values shown are means ± SD (n = 4). LDL from sham-operated mice more efficiently retarded [^{14}C]LDL plasma removal than LDL from castrated mice, as shown by the areas under the radioactivity curves (188.9 ± 18.7 vs. 142.1 ± 7.3, sham-operated vs. castrated mice, respectively; $P < 0.01$) and fractional catabolic rates determined from 2 to 60 min (0.020 ± 0.002 vs. 0.027 ± 0001, sham-operated vs. castrated mice, respectively; $P < 0.002$).

Therefore, this experiment shows that LDL from castrated mice presents lower affinity than control (sham-operated) LDL for tissue receptors.

Because gonadal androgens are the main source of plasma estrogen and the latter has an antioxidant effect (58), we postulated that castrated mice would have increased levels of OxLDL. This was determined indirectly by quantifying the amount of autoantibodies against OxLDL. Indeed, castrated CETP Tg mice presented 1.7-fold higher titers of anti-OxLDL than sham-operated controls (0.20 ± 0.02 vs. 0.12 ± 0.02 absorbance at 450 nm, respectively; $P < 0.015$).

The impact of testosterone deficiency on the development of atherosclerosis and a possible modulating role of CETP expression were tested in CETP Tg mice crossbred with atherosclerosis-susceptible LDL receptor knockout mice. Castrated and sham-operated littermate mice with partial deficiency of LDL receptor, expressing CETP or not (CETP/R1 and R1, respectively), were studied after 10 weeks on a high-fat and high-cholesterol cholate-containing (HFHC) diet. Under these conditions, castration provoked a significant increase in total plasma cholesterol concentration in both genotypic groups: 273 ± 14 versus 222 ± 17 ($P < 0.05$) for R1 and 169 ± 8 versus 144 ± 8 ($P < 0.05$) for CETP/R1 castrated mice compared with their respective sham-operated controls. There were no changes in TG, FFA, and glucose levels after castration in either HFHC diet-fed genotypic group (data not shown). Testosterone deficiency did not influence the expression of hepatic lipase, measured as maximal activity (FFA release in nmol/ml/h) in mice fed this atherogenic diet: 1,915 ± 102 vs. 1,663 ± 75 (n = 5) for R1 groups and 1,478 ± 111 vs. 1,616 ± 61 (n = 16) for CETP/R1 groups. CETP expression (measured as percentage of CE transfer) also was not affected by the testosterone deficiency in HFHC diet-fed sham-operated and castrated CETP/R1 mice: 38 ± 1.9 versus 35 ± 2.1, respectively. Thus, neither the genetic background nor the type of diet changed the lack of response of HL and CETP to testosterone deficiency.

The lipoprotein cholesterol distribution in plasma of mice fed the atherogenic diet is presented in **Fig. 3**. VLDL and LDL + IDL are presented as combined fractions because the FPLC HR30 Superose 6 gel filtration column

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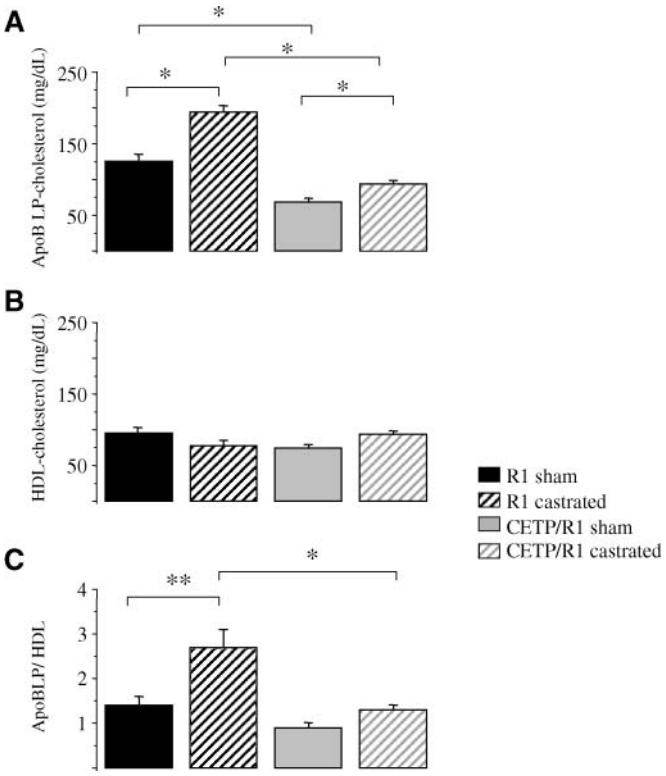

Fig. 3. Cholesterol distribution in plasma lipoprotein fractions from castrated and sham-operated LDL receptor-deficient mice expressing CETP (CETP/R1) or not (R1) after 10 weeks on a high-fat and high-cholesterol diet. A: Apolipoprotein B-containing lipoprotein (apoB-LP)-cholesterol. B: HDL-cholesterol. C: ApoB-LP/HDL ratio. Values shown are means ± SEM (n = 5–9). Statistical comparisons by Student's *t*-test: * $P < 0.001$, ** $P < 0.02$.

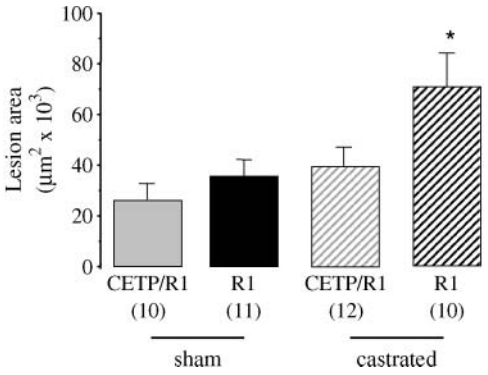

Fig. 4. Areas of aortic lipid-stained lesions in castrated and sham-operated LDL receptor-deficient mice expressing CETP (CETP/R1) or not (R1) after 10 weeks on a high-fat and high-cholesterol diet. Values shown are means ± SEM (n). Statistical comparisons by one-way ANOVA followed by Student-Newman-Keuls test: $P = 0.005$. * Castrated R1 mice differ from the other three groups ($P < 0.05$).

could not resolve these fractions in plasma from mice fed this diet. In agreement with the previous experiments, testosterone deficiency induced significant increases in apoB-LP and apoB-LP/HDL ratio in both R1 and CETP/R1 mice, although the changes were milder in CETP-expressing mice (50% in R1 and 30% in CETP/R1).

Morphometric analysis of lipid-stained lesions in the aortic roots (**Fig. 4**) showed that testosterone deficiency induced an increase of ~100% in atherosclerotic lesion area of R1 ($P < 0.02$) and of ~50% in CETP/R1 ($P = 0.15$) castrated mice compared with their respective sham-operated littermates. CETP expression significantly reduced the lesion area in castrated mice by 44% (CETP/R1 vs. R1; $P < 0.05$).

DISCUSSION

In this work, we investigated some of the mechanisms by which testosterone deficiency, combined or not with CETP expression, altered plasma lipoprotein metabolism and its impact on the development of diet-induced atherosclerosis. One of the major lipoprotein alterations caused by the testosterone deficiency was a marked increase in the apoB-LP concentration. This was observed in four genotypic castrated groups: nTg, CETP Tg, R1, and CETP/R1. Therefore, this effect was independent of the presence of CETP, although the changes in these lipoproteins were always milder in CETP-expressing mice. On a low-fat diet (chow), testosterone deficiency also induced a reduction in HDL-cholesterol levels, which was exacerbated by the CETP activity in Tg mice. The HDL-reducing effect of testosterone deficiency and CETP expression was not seen in high-fat diet-fed mice, probably because this type of diet stimulates hepatic apoA-I synthesis (59), thus increasing HDL levels in all mice consuming this diet. Nonetheless, in both low- and high-fat-fed mice, castration induced significant increases in apoB-LP and apoB-LP/HDL ratio.

LPL and HL play an important role not only in the metabolism of TG-rich lipoproteins but also in the metabolism of HDL (60). Administration of androgenic steroids to humans caused increases in HL activity (3, 61), whereas no effect or increases (62, 63) in LPL activity have been reported. We show here that deficiency of testosterone in mice did not alter the expression of either lipolytic enzyme, which excludes their participation in the changes of LDL and HDL levels observed in castrated mice.

According to our results, the increase in apoB-LP in testosterone-deficient mice could not be explained by changes in the production rate of the precursors (VLDL), or in the lipolysis-mediated VLDL-LDL conversion rate, or in the number of LDL and HDL receptors. However, the hepatic secretion of VLDL particles with lower TG content in castrated mice could generate LDL particles with less affinity for their receptors. These LDLs would then remain longer in the plasma compartment, thus explaining their higher plasma concentrations. Indeed, LDL competition studies for plasma removal (Fig. 2) indicated that LDL from castrated mice presents lower affinity than LDL from sham-operated mice for tissue sites of LDL plasma removal.

Further evidence for other alterations in LDL chemical composition that would affect receptor affinity is the

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higher titer of anti-OxLDL autoantibodies found in the plasma of castrated mice. This means that testosterone deficiency, probably caused by the consequent deficiency of the antioxidant action of estrogen, could trigger LDL oxidation and increase atherogenesis. The critical role of testosterone-derived estrogen in atherogenesis was elegantly demonstrated by Nathan et al. (22) using testosterone replacement with and without aromatase inhibitor in castrated mice. The same authors showed that aromatase-mediated conversion of testosterone in estrogen in endothelial cells attenuated the expression of VCAM-1, an adhesion molecule implicated in atherogenesis (21).

Accordingly, castrated mice developed greater atherosclerotic lesion area than the sham-operated controls. Combining groups by hormone state, the lesion-enhancing effect of testosterone deficiency is confirmed (+90%, n = 21–22 per group, $P < 0.005$), regardless of the genotypes. Therefore, increased atherosclerosis in testosterone deficiency could be ascribed, at least in part, to the higher concentration of LDL and OxLDL and the lower LDL receptor affinity observed in castrated mice.

Castrated CETP-expressing mice (CETP/R1) presented a smaller lesion area (−44%) compared with castrated R1 mice ($P < 0.05$). We reported previously that CETP expression attenuated atherosclerosis in castrated female mice by 50% (36). In this work, we extend this finding showing that CETP also attenuated atherosclerosis in testosterone-deficient male mice. As in the previous work, the beneficial role of CETP can be observed only in the hormone-deficient state. A likely mechanism may be related to the CETP capacity of softening the increase of apoB-LP caused by the hyperlipidemic diet and testosterone deficiency, because apoB-LP levels correlated positively with lesion area ($r = 0.53$, $P < 0.005$). This CETP effect could be related to its capacity to modify LDL's affinity for its specific receptors. Accordingly, Sakai et al. (64) reported that CETP-deficient patients presented LDL particles with decreased affinity for LDL receptors. In another work (65), we showed that CETP transfers CE from OxLDL to HDL more efficiently than from native LDL. As a consequence, CETP may facilitate the plasma removal of oxidized lipids via HDL. This finding is in agreement with the higher levels of OxLDL found in CETP-deficient patients (66). Another potential protective mechanism mediated by CETP is the transfer of esterified 17β-estradiol from HDL particles to LDL (67, 68), conferring protection against oxidation on LDL.

On the other hand, the local events involved in the atheroma formation are also important to explain differences in lesion areas. Previous work showed that the addition of CETP to culture medium of smooth muscle cells (69) or foam cells (70) stimulated the cell cholesterol efflux rate. We reported previously that CETP-expressing macrophages took up less cholesterol from acetylated LDL and released more cell cholesterol to HDL (36). Therefore, CETP may contribute directly to the decreased lipid accumulation in arterial foam cells.

In conclusion, our results show that testosterone deficiency enhances atherosclerosis in male mice, indicating a protective role of androgens in sex-matched individuals. These observations are supported by data from human (13, 14, 19, 20) and mouse (21, 22) studies. Mechanisms involved in the loss of atheroprotection in castrated mice are related to increases in plasma levels of apoB-LPs and anti-OxLDL autoantibodies and the decreased affinity of LDL for its receptors. Although the expression of CETP was not altered by testosterone deficiency, it led to a smaller increase in apoB-LPs and a reduction in the atherosclerotic lesion size of testosterone-deficient mice.

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