

# Apolipoprotein CI Deficiency Markedly Augments Plasma Lipoprotein Changes Mediated by Human Cholesteryl Ester Transfer Protein (CETP) in CETP Transgenic/ApoCI-knocked Out Mice\*

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Transgenic mice expressing human cholesteryl ester transfer protein (HuCETPTg mice) were crossed with apolipoprotein CI-knocked out (apoCI-KO) mice. Although total cholesterol levels tended to be reduced as the result of CETP expression in HuCETPTg heterozygotes compared with C57BL/6 control mice (–13%, not significant), a more pronounced decrease (–28%,  $p < 0.05$ ) was observed when human CETP was expressed in an apoCI-deficient background (HuCETPTg/apoCI-KO mice). Gel permeation chromatography analysis revealed a significant, 6.1-fold rise ( $p < 0.05$ ) in the cholesteryl ester content of very low density lipoproteins in HuCETPTg/apoCI-KO mice compared with control mice, whereas the 2.7-fold increase in HuCETPTg mice did not reach the significance level in these experiments. Approximately 50% decreases in the cholesteryl ester content and cholesteryl ester to triglyceride ratio of high density lipoproteins (HDL) were observed in HuCETPTg/apoCI-KO mice compared with controls ( $p < 0.05$  in both cases), with intermediate –20% changes in HuCETPTg mice. The cholesteryl ester depletion of HDL was accompanied with a significant reduction in their mean apparent diameter ( $8.68 \pm 0.04$  nm in HuCETPTg/apoCI-KO mice versus  $8.83 \pm 0.02$  nm in control mice;  $p < 0.05$ ), again with intermediate values in HuCETPTg mice ( $8.77 \pm 0.04$  nm). *In vitro* purified apoCI was able to inhibit cholesteryl ester exchange when added to either total plasma or reconstituted HDL-free mixtures, and coincidentally, the specific activity of CETP was significantly increased in the apoCI-deficient state ( $173 \pm 75$  pmol/ $\mu$ g/h in HuCETPTg/apoCI-KO mice versus  $72 \pm 19$  pmol/ $\mu$ g/h in HuCETPTg mice,  $p < 0.05$ ). Finally, HDL from apoCI-KO mice were shown to interact more readily with purified CETP than control HDL that differ only by their apoCI content. Overall, the present observations provide direct support for a potent specific inhibition of CETP by plasma apoCI *in vivo*.

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The cholesteryl ester transfer protein (CETP)<sup>1</sup> promotes the exchange of neutral lipid species, *i.e.* cholesteryl esters and triglycerides between plasma lipoproteins (1). *In vivo* studies demonstrated that the CETP-mediated bidirectional transfers of neutral lipids may influence the atherogenicity of the plasma lipoprotein profile, raising a substantial interest in studying the regulation of plasma CETP activity (2–6). The CETP-mediated lipid transfer reaction is a complex process that is influenced by a number of plasma modulators, among them the concentration of active CETP as well as the structure, the lipid composition, and the relative proportions of lipoprotein donors and acceptors (7). In addition, studies from several laboratories support the existence of a protein inhibitor of CETP activity in plasma from distinct vertebrate species, including human. This putative inhibitor might account, at least in part, for the substantial alterations in the CETP-specific activity as observed between plasma samples from distinct subgroups of patients (7).

Although many apolipoproteins, including apoAI, apoAII, apoAIV, apoCs, apoD (8–13), were previously described as putative CETP inhibitors, their inhibitory effects were documented mostly *in vitro* (9, 12, 13), and no clear evidence for physiological relevance has been obtained (14). Briefly, although *in vitro* studies support apoAII as a specific inhibitor of CETP (9, 15), no evidence for a CETP blockade was observed in subsequent studies in transgenic mice expressing both human apoAII and human CETP (16). More recently, apoF was described as a potent CETP inhibitor *in vitro* (13). However, unlike lipid transfer inhibitory activity that is associated with the plasma HDL (17–20) fraction, apoF is almost exclusively localized in LDL (13), and no direct indication of the extent of plasma CETP inhibition by apoF has been yet provided *in vivo*. Several years ago, apoCI was suggested as a possible CETP inhibitor in comparative *in vitro* studies (20, 21). In contrast to other putative inhibitors of CETP activity, we demonstrated that apoCI as a specific CETP inhibitor meets *in vitro* most of the following required criteria. 1) ApoCI inhibitory activity is specifically localized in HDL; 2) it constitutes a potent inhibitor

<sup>1</sup> The abbreviations used are: CETP, cholesteryl ester (CE) transfer protein; apo, apolipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very LDL; KO, knock out; HuCETPTg mouse, transgenic mouse to human CETP; apoCI-KO mouse, apoCI-deficient mouse; HuCETPTg/apoCI-KO mouse, apoCI-deficient mouse expressing human CETP; NBD, nitrobenz-oxadiazol.

of CETP, and unlike other putative apolipoprotein modulators (22, 23), with the exclusion of activating potential; 3) a complete blockade of CETP can be reached with moderate inhibitor doses; 4) apoCI is active not only as a purified protein but also as a component of the HDL protein moiety; 5) substantial increment in CETP activity can be obtained in the presence of anti-apoCI antibodies in incubation media containing purified CETP and lipoprotein substrates; 6) and immunopurified, apoCI-free HDLs are better substrates for CETP than apoCI-containing particles (21).

The assessment of the physiological relevance of CETP inhibition constitutes a key step along the quest of a specific protein inhibitor, and this was addressed in the present study in apoCI-knocked out mice (24). Whereas previous studies in hyperlipidemic, apoCI-KO mice sustained the concept of a role of apoCI in regulating VLDL catabolism (25), the mouse is deficient in CETP activity (26, 27). Consequently, apoCI-deficient mice (24) have been crossed with mice expressing human CETP under the control of its natural flanking regions (28), and the effects of apoCI deficiency on plasma cholesteryl ester transfer activity and plasma lipoprotein parameters were determined.

#### MATERIALS AND METHODS

**Animals**—Four distinct mouse lines were used in the present study, all of them in a homogenous C57BL6 genetic background: wild-type C57BL/6 mice, mice expressing human CETP under the control of natural flanking regions (HuCETPTg) (28), apoCI-knocked out (apoCI-KO) mice (24), and HuCETPTg/apoCI-KO mice obtained by cross-breeding. ApoCI-KO mice were all homozygous for the apoCI-deficient trait. The mice had free access to water and food, and they were placed on a standard chow diet.

**Plasma Samples**—Fresh citrated plasma from fasting normolipidemic subjects was provided by the Centre de Transfusion Sanguine (Hôpital du Bocage, Dijon, France). Fasting blood samples from mice were collected from the retroorbital venous plexus into heparin-containing tubes. Plasma was obtained by low speed centrifugation and stored at  $-80^{\circ}\text{C}$  until analysis.

**Plasma Lipid Analysis**—All assays were performed on a Victor<sup>2</sup> 1420 Multilabel Counter (Wallac). Total cholesterol was measured by the enzymatic method using Cholesterol 100 reagent (ABX Diagnostics), and unesterified cholesterol concentration was determined by the CHOD-PAP method (Sigma). Cholesteryl ester concentration was calculated by the difference between total and free cholesterol. Triglyceride concentration was determined by enzymatic method using Infinity triglyceride reagent (Sigma).

**Immunoassay of CETP Mass Levels in Mouse Plasma**—CETP mass levels in mouse plasma were determined by a specific immunoassay with TP2 anti-CETP monoclonal antibodies. Briefly, plasma samples were diluted (1:9, v/v) in 25 g/liter Tris-buffered saline containing SDS, dithiothreitol, and they were incubated for 15 min at  $80^{\circ}\text{C}$ . Samples were subsequently applied on 8–12% discontinuous polyacrylamide gels in a Mini Protean device (Bio-Rad), and they were transferred to nitrocellulose membranes (Hybond ECL; Amersham Biosciences). The resulting blots were blocked for 1 h in 5% lowfat dried milk in phosphate-buffered saline containing 0.1% Tween, and they were washed in phosphate-buffered saline-Tween. Human CETP was revealed by successive incubations with TP2 anti-CETP antibodies (Heart Institute, Ottawa, Canada) and horseradish peroxidase-coupled second antibodies as previously described (15). Blots were finally developed with an ECL kit (Amersham Biosciences). CETP mass level in each plasma sample was estimated by comparison with a calibration curve that was obtained with serial dilutions of a human plasma standard submitted to electrophoresis together with the samples.

**Fractionation of Plasma Lipoproteins**—Individual plasma samples (200  $\mu\text{l}$ ) were injected on a Superose 6 HR 10/30 column (Amersham Biosciences) that was connected to a fast protein liquid chromatography system (Amersham Biosciences). Lipoproteins were eluted at a constant 0.3 ml/min flow rate with Tris-buffered saline containing 0.074% EDTA and 0.02% sodium azide. Cholesteryl ester and triglyceride concentrations were assayed in individual, 0.3-ml fractions. VLDLs were contained in fractions 5–15, LDLs were contained in fractions 16–29, and HDLs were contained in fractions 30–45.

**Native Polyacrylamide Gradient Gel Electrophoresis**—Total lipopro-

teins were separated by ultracentrifugation as the  $d < 1.21$  g/ml plasma fraction with one 5.5-h, 100,000-rpm spin in a TLA100 rotor in a TLX ultracentrifuge (Beckman). Lipoproteins were then applied to a 15–250 g/liter polyacrylamide gradient gel (Spiragel 1.5–25.0; Spiral, Couteron, France), and electrophoresis was conducted as recommended by the manufacturer. Gels were subsequently submitted to Coomassie staining with Brilliant Blue G (Sigma), and the distribution profiles of HDL were obtained by analysis with a Bio-Rad GS-670 Imaging Densitometer. The mean apparent diameters of HDL were determined by comparison with globular protein standards (high molecular weight kit, Amersham Biosciences) that were submitted to electrophoresis together with the samples (29).

**Purification of Cholesteryl Ester Transfer Protein**—CETP was purified from human plasma by using a sequential chromatography procedure as previously described (30). The CETP preparation was deprived of both lecithin:cholesterol acyltransferase and phospholipid transfer protein activities. The mass concentration of CETP in purified human fractions was determined by using an enzyme-linked immunosorbent assay as previously described (31).

**Purification of Apolipoprotein CI by Chromatofocusing**—Apo CI was purified from delipidated HDL apolipoproteins by using the chromatofocusing method of Tourmier *et al.* (32). This method takes advantage of the high isoelectric point of apoCI as compared with other HDL apolipoprotein components. Purified apoCI, which appeared as a homogeneous band on the polyacrylamide gel, was dialyzed against Tris-buffered saline.

**Measurement of Cholesteryl Ester Transfer Activity**—CETP activity was determined in microplates by a fluorescent method using donor liposomes enriched with nitrobenz-oxadiazol (NBD)-labeled cholesteryl esters (WAK Chemie). For measurement of the activity of isolated CETP, donor liposomes (5  $\mu\text{l}$ ) and partially purified CETP (6.5  $\mu\text{g}$ ) were incubated in the presence of either native LDL acceptors ( $1.019 < d < 1.063$  g/ml plasma fraction; cholesterol, 125 nmol) or native HDL acceptors ( $1.07 < d < 1.21$  g/ml plasma fraction; cholesterol, 125 nmol). For measurement of plasma CETP activity, incubation media contained 5  $\mu\text{l}$  of donor liposomes and 10  $\mu\text{l}$  of total plasma. Final volumes were adjusted to 250  $\mu\text{l}$  with Tris-buffered saline, and incubations were conducted in triplicate for 3 h at  $37^{\circ}\text{C}$  in a Victor<sup>2</sup> 1420 Multilabel Counter (Wallac). The CETP-mediated transfer of NBD-cholesteryl esters from self-quenched donors to acceptor lipoproteins was monitored by the increase in fluorescence intensity (excitation, 465 nm; emission, 535 nm). The amounts of NBD-cholesteryl esters transferred (pmol) were calculated by using a standard curve plotting fluorescence intensity and concentrations of NBD-cholesteryl esters dispersed in isopropanol. Finally, results were expressed as the amount of labeled cholesteryl esters transferred after deduction of blank values (i.e. obtained with control mixtures devoid of active CETP).

**SDS-Polyacrylamide Gel Electrophoresis of HDL Apolipoproteins**—HDL were isolated by ultracentrifugation from control or apoCI-KO plasma as the  $1.07 < d < 1.21$  g/liter fraction with one 7-h, 100,000-rpm spin at the lowest density, and one 10-h, 100,000-rpm spin at the highest density in a 120.2 rotor in a TLX ultracentrifuge (Beckman). Densities were adjusted by the addition of KBr solutions. Isolated HDL (protein, 0.25 g/liter) were incubated for 15 min at  $80^{\circ}\text{C}$  in the presence of SDS (25 g/liter) and dithiothreitol (33 g/liter) in a Tris-buffered saline. Samples were then applied on a SDS-polyacrylamide high density gel (Phastsystem; Amersham Biosciences), and electrophoresis was conducted as recommended by the manufacturer. Protein bands were silver-stained as previously described (33). Apparent molecular weights were determined by comparison with protein standards (Rainbow Markers, Amersham Biosciences) that were submitted to electrophoresis together with the samples.

**Statistical Analyses**—Mann-Whitney U test was used to determine the significance between the data means.

#### RESULTS

Plasma lipid parameters and CETP mass levels in various mouse lines are presented in Table I. In the present studies, the expression of human CETP alone (HuCETPTg mice) did not promote significant alterations in total plasma lipid levels as compared with control mice, although in agreement with previous studies (34), a tendency toward a decrease in total cholesterol levels was observed (Table I). When plasma CETP was expressed in an apoCI-deficient context (HuCETPTg/apoCI-KO mice), decreases in both total cholesterol and cholesteryl ester levels became highly significant when compared with controls

TABLE I  
Plasma lipid and CETP mass concentrations in control, apoCI-KO, HuCETPTg, and HuCETPTg/apoCI-KO mice

Lipid and CETP concentrations were determined in plasmas as described under "Materials and Methods." Values are the mean  $\pm$  S.D. of five distinct animals. ND, not detectable.

Mice	Total cholesterol	Unesterified cholesterol	Cholesteryl esters	Phospholipids	Triglycerides	CETP
			mg/dl			mg/liter
Control	68.8 $\pm$ 21.0	22.0 $\pm$ 4.5	46.9 $\pm$ 18.9	147.6 $\pm$ 33.7	47.6 $\pm$ 23.8	ND
ApoCI-KO	55.9 $\pm$ 9.7	22.6 $\pm$ 6.7	33.3 $\pm$ 13.2	122.0 $\pm$ 21.4	45.5 $\pm$ 8.7	ND
CETPTg	59.8 $\pm$ 6.7	17.7 $\pm$ 4.3	42.1 $\pm$ 10.5	130.4 $\pm$ 9.3	57.6 $\pm$ 29.4	3.6 $\pm$ 0.7
CETPTg/apoCI-KO	49.5 $\pm$ 5.6 <sup>a,b</sup>	23.7 $\pm$ 9.2	25.7 $\pm$ 8.9 <sup>a,b</sup>	109.5 $\pm$ 17.6	42.4 $\pm$ 12.5	2.9 $\pm$ 0.9

<sup>a</sup> Significantly different from CETPTg mice,  $p < 0.05$ .

<sup>b</sup> Significantly different from control mice,  $p < 0.05$ .

(-28% and -45%, respectively;  $p < 0.05$  in both cases) (Table I). The permissive effect of apoCI deficiency was clearly apparent when HuCETPTg/apoCI-KO mice were compared with HuCETPTg mice, with significant -17% and -39% decreases in total cholesterol and cholesteryl ester levels in the former group, respectively ( $p < 0.05$  in both cases). The latter observations could not be explained by differences in CETP expression, with no significant difference in the plasma CETP mass levels between the two groups (2.9  $\pm$  0.9 mg/liter in HuCETPTg/apoCI-KO mice versus 3.6  $\pm$  0.7 mg/liter in HuCETPTg mice; not significant) (Table I).

To bring more insights into the alterations of the plasma lipoproteins in distinct mouse lines, individual plasma samples of control mice, apoCI-KO mice, HuCETPTg mice, and HuCETPTg/apoCI-KO mice were fractionated by gel permeation chromatography, and cholesteryl ester and triglyceride profiles were obtained (Fig. 1; Table II). Although plasma cholesteryl esters were localized mainly in HDL (fractions 30-45) in all the mouse lines studied, HDL cholesteryl ester levels were markedly reduced in HuCETPTg/apoCI-KO mice, with the difference reaching the significance levels when HuCETPTg/apoCI-KO mice were compared with control mice (mean 53% decrease,  $p < 0.05$ ) (Table II). Conversely, the combination of apoCI deficiency and CETP expression produced a marked, 6.1-fold rise in the cholesteryl ester content of VLDL (fractions 5-15) in HuCETPTg/apoCI-KO mice compared with control mice ( $p < 0.05$ ; Table II). A significant rise in the cholesteryl ester content of LDL (fractions 16-29) was observed in mice expressing human CETP compared with control mice (Table II). Overall, as compared with control mice, the redistribution of cholesteryl esters led to a marked 3.3-fold rise in the VLDL + LDL to HDL cholesteryl ester ratio in HuCETPTg/apoCI-KO mice ( $p < 0.05$ ), with only a 1.8-fold rise in HuCETPTg mice (not significant) (Fig. 2). Although no significant alterations in the triglyceride content of individual lipoprotein fractions were observed when comparing the distribution profiles from distinct mouse lines, the cholesteryl ester to triglyceride ratio of HDL was decreased by 57% in HuCETPTg/apoCI-KO mice compared with control mice ( $p < 0.05$ ) (Fig. 3). Alterations in the lipid composition of the plasma lipoproteins from HuCETPTg/apoCI-KO mice were associated with significant changes in their size. Indeed, analysis of total lipoproteins by native polyacrylamide gradient gel electrophoresis revealed a significant reduction in the mean apparent diameter of HDL from HuCETPTg/apoCI-KO mice compared with HDL from both control and apoCI-KO mice (Fig. 4,  $p < 0.05$  in both cases). HDL from HuCETPTg mice were of intermediate size as compared with HDL from either control or HuCETPTg/apoCI-KO mice (Fig. 4).

To determine further whether the above changes in the size and composition of plasma lipoproteins related to alterations in plasma neutral lipid transfer activity, CETP activity was measured in various plasma samples as the rate of transfer of fluorescent cholesteryl esters from exogenous, labeled lipoproteins to endogenous plasma lipoproteins.

As expected from previous interspecies comparisons (26, 27), the mouse is a CETP-deficient animal, and neither control (not shown) nor apoCI-KO mice displayed detectable cholesteryl ester transfer activity (Fig. 5). Plasma from HuCETPTg mice displayed substantial cholesteryl ester transfer activity, with an initial cholesteryl ester transfer rate of 62 pmol/h (Fig. 5, inset). Despite similar CETP mass concentrations in HuCETPTg and HuCETPTg/apoCI-KO mice, CETP activity was markedly increased in the latter animals, with a mean 84% rise in the initial cholesteryl ester transfer rate compared with HuCETPTg mice ( $p < 0.05$ ). As a consequence, the specific activity of human CETP, as calculated as the ratio of cholesteryl ester transfer rate to CETP mass concentration, was remarkably higher in HuCETPTg/apoCI-KO mice than in HuCETPTg mice (173  $\pm$  75 versus 72  $\pm$  19 pmol/ $\mu$ g/h;  $p < 0.05$ ). *In vitro*, the rate of cholesteryl ester transfer in total plasma from HuCETPTg/apoCI-KO mice could be progressively decreased in the presence of increasing concentrations of purified apoCI, with the exclusion of any activating potential (Fig. 6). Finally, and in further support of a direct inhibition of plasma CETP activity by apoCI, the rate of transfer of labeled cholesteryl esters was decreased by purified apoCI when added to reconstituted mixtures made of apolipoprotein-free liposome donors and apoB-containing LDL acceptors (Fig. 7).

All together the above results suggest that plasma apoCI may constitute a potent regulator of CETP activity in total plasma. To give further insights into the biological relevance of lipoprotein-associated apoCI as a specific inhibitor of CETP activity, HDL were isolated by ultracentrifuge from control and apoCI-KO mouse plasma, and their ability to interact with purified human CETP was compared. As shown in Fig. 8, the apolipoprotein composition of HDL from both sources were similar, with the exception of apoCI that was absent from HDL from apoCI-KO mice. As shown in Fig. 9, apoCI deficiency was characterized by a much better ability of isolated HDL to act as a lipoprotein substrate in the lipid transfer assay, with a significant 61% rise in the initial transfer rate measured with apoCI-KO HDL compared with control HDL ( $p < 0.05$ ).

#### DISCUSSION

Recent studies identified a number of pharmacological compounds as potential CETP inhibitors; among them, at least one disulfide derivative (JTT-705) was proven to be efficient in blocking both plasma cholesteryl ester transfer activity and atherosclerosis progression in the rabbit (5). Another relevant strategy consisted of the inhibition of CETP expression in the rabbit by the means of either antisense oligodeoxynucleotides against CETP (3) or anti-CETP immunotherapy (4), again leading in both cases to the prevention of atherosclerosis. In addition to earlier studies in CETP-deficient patients (35), these observations gave rise to an intensive quest for CETP inhibitors that might represent relevant tools for the treatment of dyslipidemia and the prevention of atherosclerosis in future



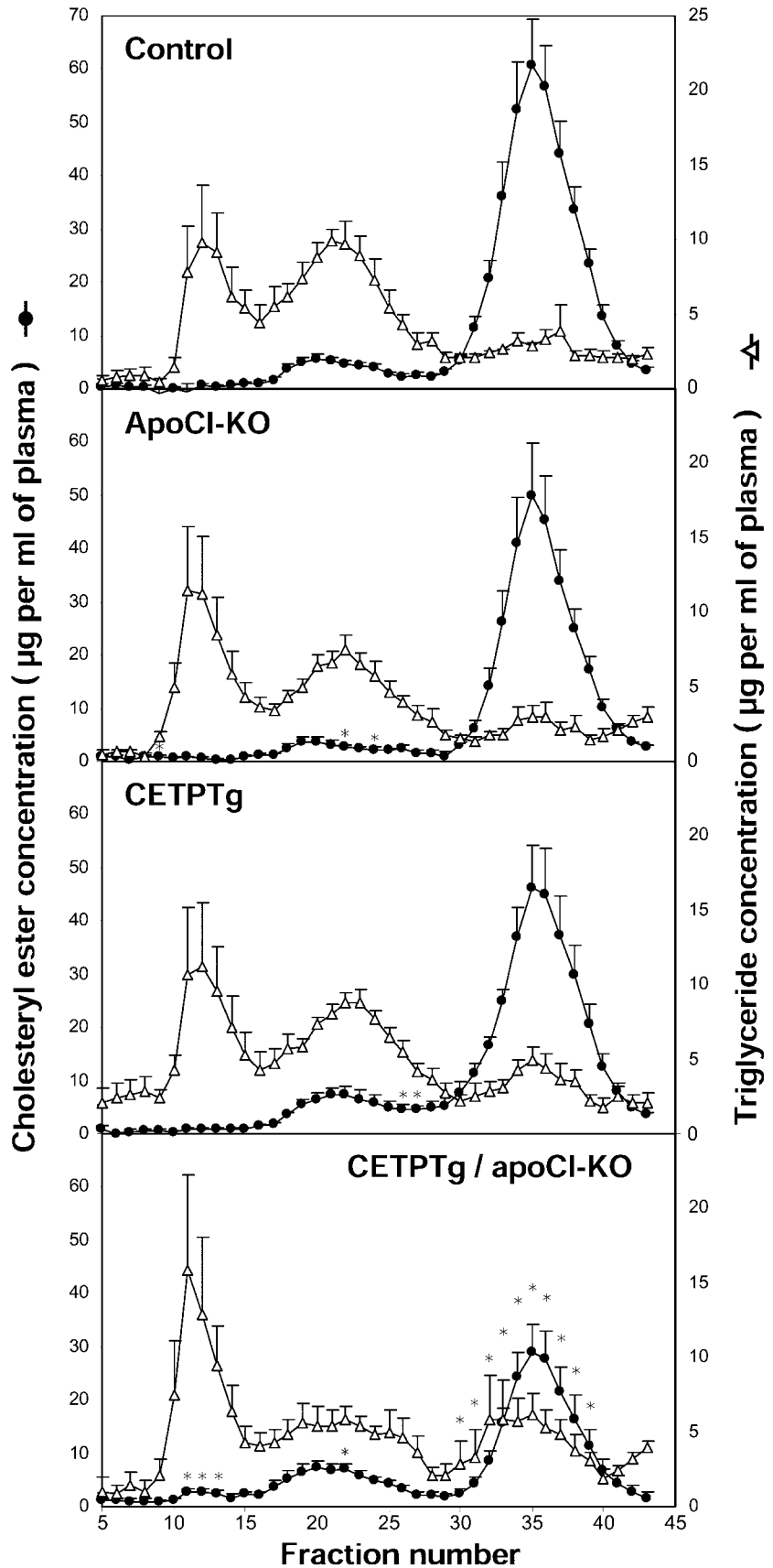


FIG. 1. Gel filtration chromatography of control, apoCI-KO, HuCETPTg, and HuCETPTg/apoCI-KO plasma. Plasma was passed through a Superose 6-HR column on a fast protein liquid chromatograph system, and the cholesteryl ester and triglyceride contents of individual fractions were determined as described under "Materials and Methods." Fractions 5–15, 16–29, and 30–45 contain VLDL, IDL/LDL, and HDL, respectively. Each point is the mean  $\pm$  S.E. for five distinct mice. \*, significantly different from control,  $p < 0.05$ ; Mann-Whitney test.

clinical practice. Besides the quest for new, pharmacological compounds, the presence of a physiological inhibitor of CETP in plasma remains a matter of debate. In our hands, apolipoprotein

CI arose *in vitro* as a relevant candidate, meeting most of the required criteria as a potent regulator of CETP activity (21). However, one important question, *i.e.* the physiological

TABLE II  
Cholesteryl ester and triglyceride contents of VLDL, LDL, and HDL fractions isolated from control, apoCI-KO, HuCETPTg, and HuCETPTg/apoCI-KO mice

Lipid contents were determined as described under "Materials and Methods." Values are expressed in mg of lipid/liter of plasma, and they are mean  $\pm$  S.D. of five distinct animals. TG, triglyceride.

Mice	VLDL-CE	LDL-CE	HDL-CE	VLDL-TG	LDL-TG	HDL-TG
	<i>mg/liter</i>					
Control	3.5 $\pm$ 6.6	48.3 $\pm$ 15.0	377.0 $\pm$ 123.3	45.5 $\pm$ 33.1	86.0 $\pm$ 27.3	35.4 $\pm$ 6.1
ApoCI-KO	11.2 $\pm$ 8.7	31.6 $\pm$ 16.9	287.0 $\pm$ 116.7	51.9 $\pm$ 33.2	65.1 $\pm$ 17.8	30.2 $\pm$ 15.4
CETPTg	9.5 $\pm$ 6.8	71.2 $\pm$ 16.0 <sup>a,b</sup>	308.0 $\pm$ 92.5	68.5 $\pm$ 44.7	83.7 $\pm$ 16.9	42.1 $\pm$ 17.8
CETPTg/apoCI-KO	21.5 $\pm$ 14.4 <sup>a</sup>	63.8 $\pm$ 20.1 <sup>b</sup>	178.2 $\pm$ 77.4 <sup>a</sup>	68.5 $\pm$ 43.5	63.3 $\pm$ 21.1	58.1 $\pm$ 32.9

<sup>a</sup> Significantly different from control mice,  $p < 0.05$ .

<sup>b</sup> Significantly different from apoCI-KO mice,  $p < 0.05$ .

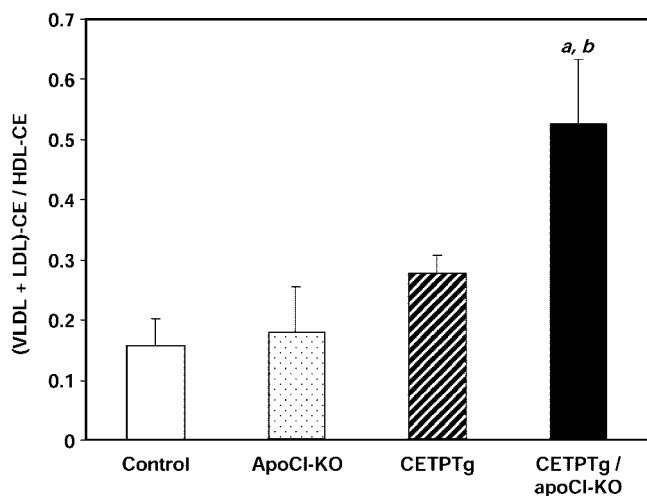


FIG. 2. ApoB-containing lipoprotein to HDL cholesteryl ester ratio in plasma from control, apoCI-KO, HuCETPTg, and HuCETPTg/apoCI-KO mice. Individual lipoprotein fractions were isolated by gel filtration chromatography, and cholesteryl esters were assayed as described in the legend to Fig. 1. Vertical bars are the mean  $\pm$  S.E. for five distinct mice. *a*, significantly different from control,  $p < 0.05$ ; *b*, significantly different from apoCI-KO,  $p < 0.05$ ; Mann-Whitney test.

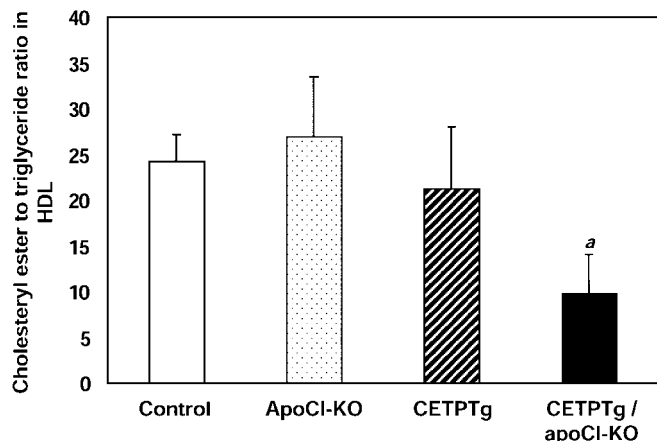


FIG. 3. Cholesteryl ester to triglyceride ratio in HDL from control, apoCI-KO, HuCETPTg, and HuCETPTg/apoCI-KO mice. Individual lipoprotein fractions were isolated by gel filtration chromatography, and cholesteryl esters and triglycerides in HDL were assayed as described in the legend to Fig. 1. Vertical bars are the mean  $\pm$  S.E. of cholesteryl ester to triglyceride molar ratio. *a*, significantly different from control,  $p < 0.05$ ; Mann-Whitney test.

relevance and the consequences of the modulation of plasma CETP activity by apoCI *in vivo* remained unanswered. The present *in vivo* study provides the first, direct evidence in favor

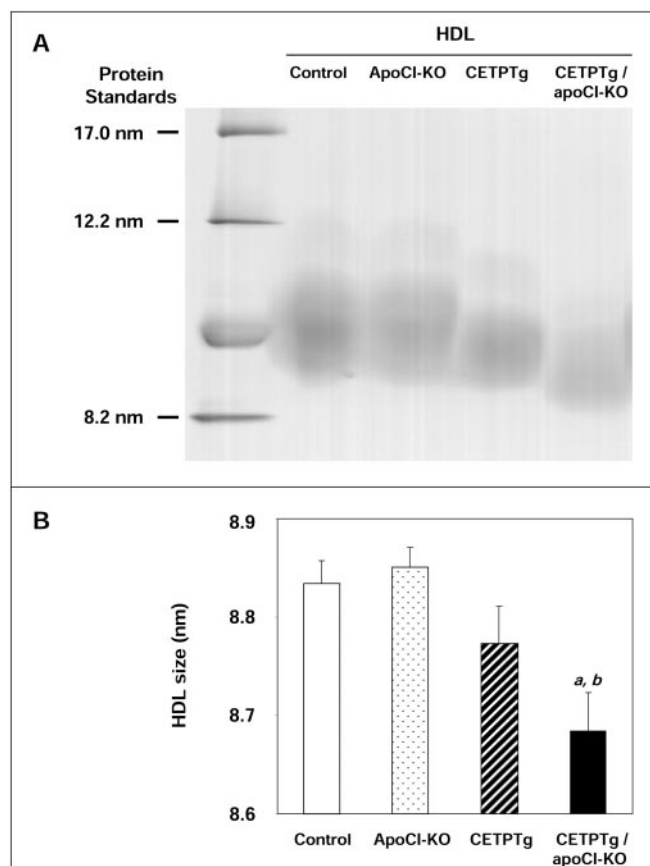


FIG. 4. HDL size distribution in plasma from control, apoCI-KO, HuCETPTg, and HuCETPTg/apoCI-KO mice. Total plasma lipoproteins were submitted to electrophoresis on native 15–250 g/liter polyacrylamide gradient gels that were stained for proteins as described under "Materials and Methods." Coomassie-stained gel in A is representative of 10 distinct experiments that were conducted with plasma samples from 10 distinct mice. HDL profiles were obtained by image analysis, and the mean size was calculated as compared with protein standards (see "Materials and Methods") (Fig. 4B). Vertical bars in Fig. 4B are the mean  $\pm$  S.E. of 10 determinations. *a*, significantly different from control HDL,  $p < 0.05$ ; *b*, significantly different from apoCI-KO HDL,  $p < 0.05$ ; Mann-Whitney test.

of the key role of apoCI as the physiological regulator of plasma cholesteryl ester transfer activity.

Human plasma apolipoprotein CI is a small, exchangeable apolipoprotein with two amphipathic  $\alpha$ -helices that are involved in lipid binding (36). Although the precise physiological function of apoCI remains a matter of debate, transgenic mice overexpressing apoCI show a clear phenotype with severe hyperlipidemia that is due to the inhibition of the hepatic uptake of apoB-containing lipoproteins (37, 38). Recently, apoCI overexpression in mice was also proven to prevent the development of obesity and insulin resistance,

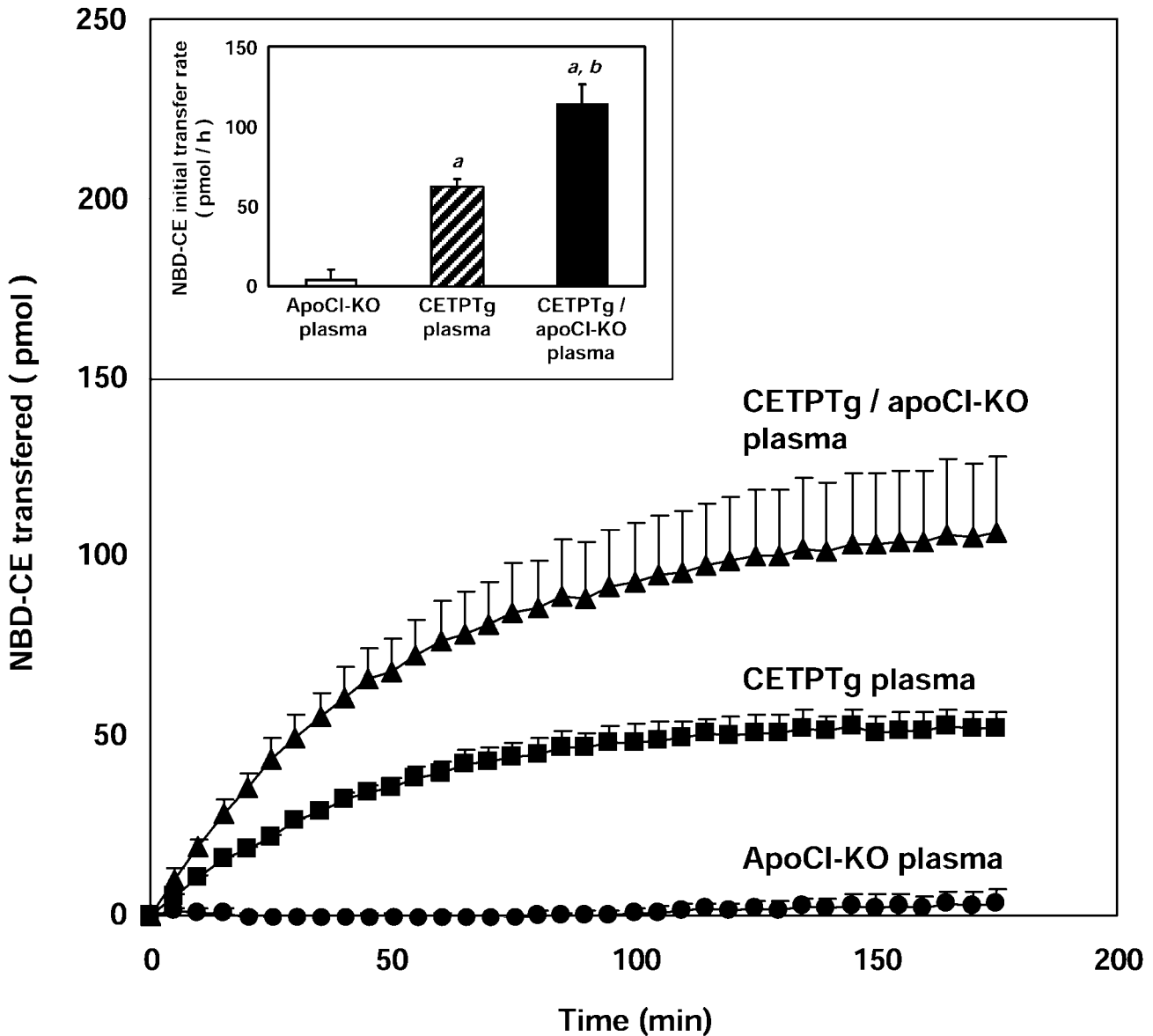
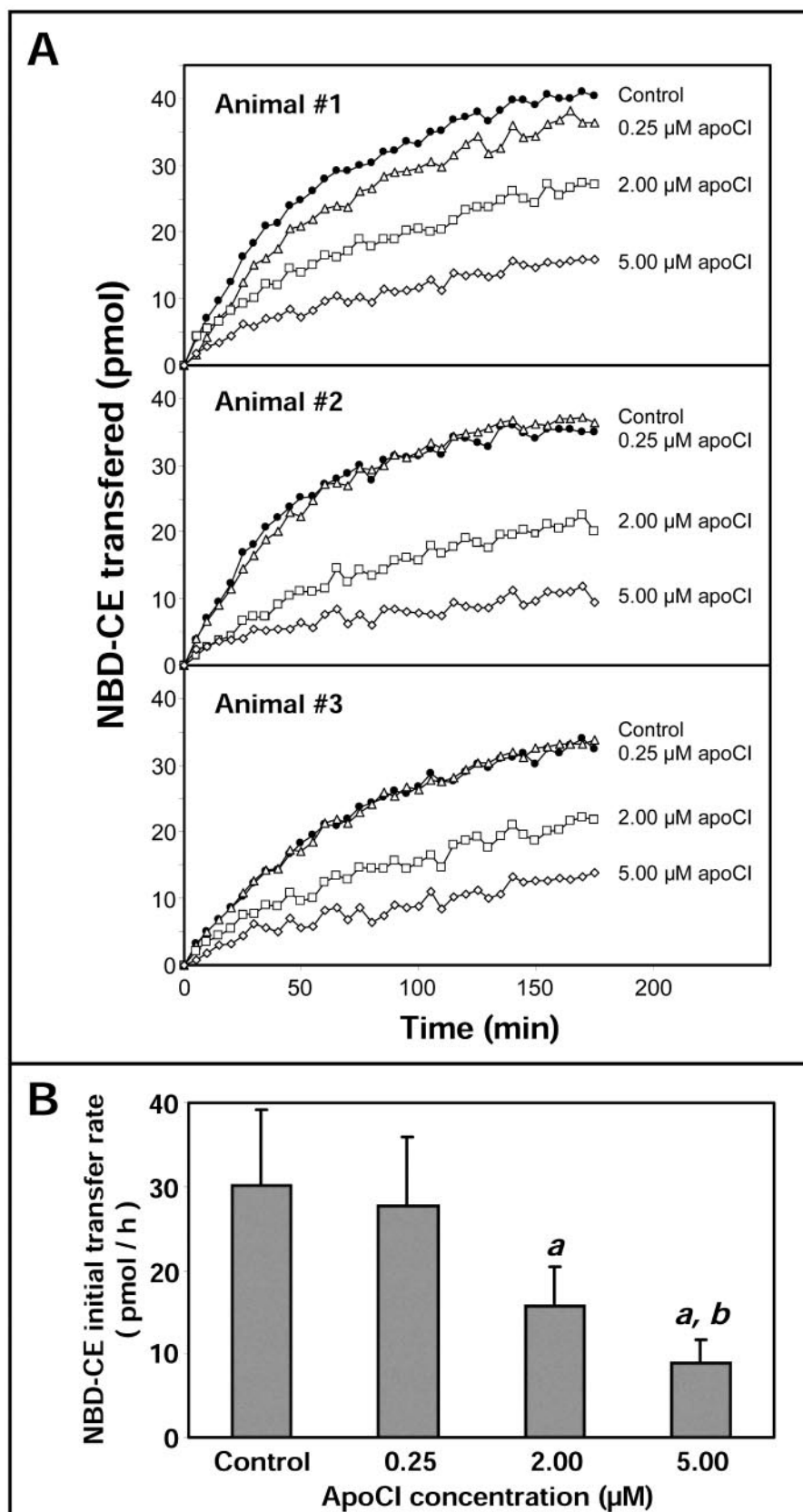


FIG. 5. Time course of cholesteryl ester transfer in plasma from apoCI-KO, HuCETPTg, and HuCETPTg/apoCI-KO mice. Cholesteryl ester transfer activity in individual plasma was determined as the rate of transfer of fluorescent NBD-cholesteryl esters from labeled liposome donors to plasma lipoprotein acceptors along a 3-h incubation period at 37 °C. Transfer rates with plasma from control mice with no detectable cholesteryl ester transfer activity were used as the blank values, and data were expressed as the amount of labeled cholesteryl esters transferred to plasma lipoproteins. The plotted values are the mean  $\pm$  S.D. of three distinct mice. Initial transfer rates (*inset*) were determined from the linear, initial portion of the time-course curves. Vertical bars are the mean  $\pm$  S.D. of three distinct mice. *a*, significantly different from apoCI-KO mice,  $p < 0.05$ ; *b*, significantly different from HuCETPTg mice,  $p < 0.05$ ; Mann-Whitney test.

probably by preventing the uptake of VLDL-derived fatty acids in the periphery (39, 40). However, unlike apoCI transgenic mice, normolipidemic apoCI-knocked out mice fed a standard, lowfat diet display no significant phenotypic alterations (24, 25). This is a peculiar situation since most of *in vivo* studies in genetically engineered mice demonstrated that down-regulation of apolipoprotein gene expression (41–49) is constantly associated with specific disorders of the lipoprotein profile. The lack of a specific phenotype in apoCI-KO mice fed a standard diet suggests either that apoCI has no direct implication in lipoprotein metabolism or, most likely, that the wild-type mouse is not appropriate to the determination of the physiological function of apoCI. In the present study, the effect of apoCI deficiency on lipoprotein metabolism was addressed in transgenic mice expressing the human CETP gene (HuCETPTg mice) under the control of its natural flanking regions (28). As reported earlier (34), the

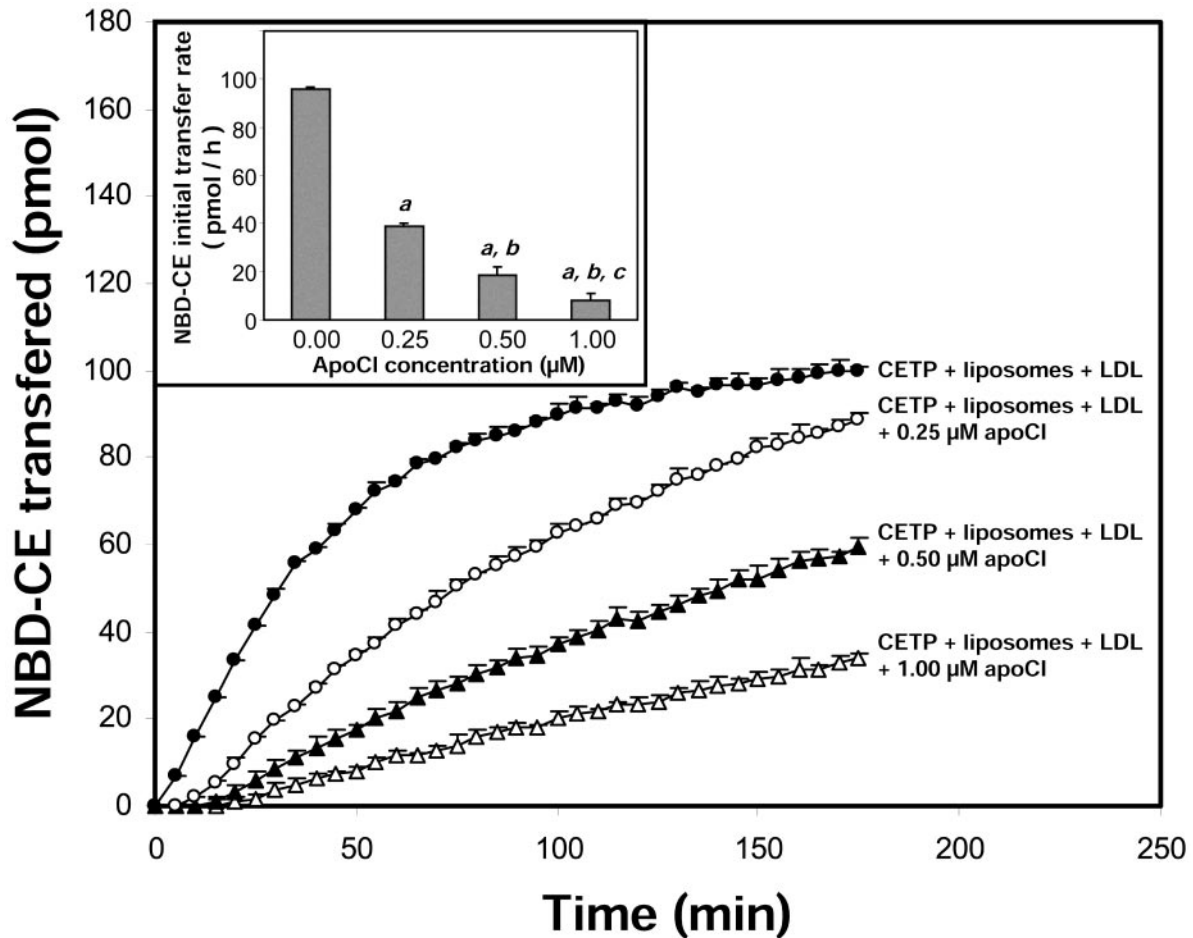
expression of moderate CETP levels in mouse heterozygotes tended to produce a rise in the cholesteryl ester content of apoB-containing lipoproteins but produced a drop in the cholesteryl ester content of HDL. Whereas the lack of a significant effect of apoCI deficiency on plasma lipid parameters was confirmed in the present work, the CETP-mediated redistribution of cholesteryl esters from HDL to apoB-containing lipoproteins was magnified when both CETP expression and apoCI-deficiency were combined, with a nearly 2-fold rise in the VLDL + LDL to HDL cholesteryl ester ratio in HuCETPTg/apoCI-KO mice compared with the HuCETPTg counterparts. The marked reduction in the cholesteryl ester content of HDL from HuCETPTg/apoCI-KO animals accounted for the significant reduction in total plasma cholesterol level in this line. CETP actually proceeds by a hetero-exchange of cholesteryl esters and triglycerides between non-equilibrated pools, *i.e.* triglyceride-rich apoB-containing

**FIG. 6. Concentration-dependent inhibition of cholesteryl ester transfer by purified apoCI in plasma from HuCETPTg/apoCI-KO mice.** Cholesteryl ester transfer activity in plasma from three distinct HuCETPTg/apoCI-KO mice (*Animals 1–3*) was determined as the rate of transfer of fluorescent NBD-cholesteryl esters from labeled liposome donors to plasma lipoprotein acceptors along a 3-h incubation period at 37 °C in the presence of increasing concentrations of purified apoCI. Transfer rates with plasma from apoCI-KO mice with no detectable cholesteryl ester transfer activity were used as the blank values, and data were expressed as the amount of labeled cholesteryl esters transferred to plasma lipoproteins. Plotted values are the means of two distinct assays for each mouse (Fig. 6A). Initial transfer rates (Fig. 6B) were determined from the linear, initial portion of the time-course curves. Vertical bars are the mean  $\pm$  S.D. of three distinct mice. *a*, significantly different from control and 0.25  $\mu$ M apoCI,  $p < 0.05$ ; *b*, significantly different from 2.00  $\mu$ M apoCI,  $p < 0.05$ ; Mann-Whitney test.

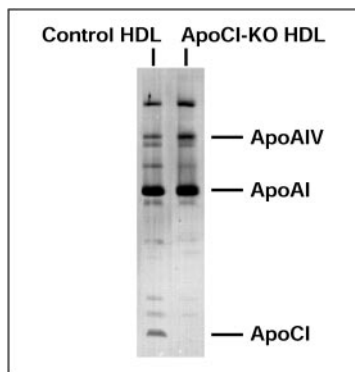


lipoproteins *versus* cholesteryl ester-rich HDL (1, 50). Accordingly, the heteroexchange of neutral lipid species produced a significant, 2-fold drop in the cholesteryl ester to triglyceride ratio in HDL from HuCETPTg/apoCI-KO mice compared with controls, and a weaker tendency was observed with the HuCETPTg line. Unlike cholesteryl esters, triglycerides of

the lipoprotein core are continuously and efficiently hydrolyzed in the plasma compartment through the activity of endothelial lipases. Triglyceride hydrolysis, in conjunction with CETP-mediated neutral lipid exchange is actually a key component of the metabolic process that leads to the emergence of core-depleted, small-sized lipoproteins (51, 52). As a



**FIG. 7. Concentration-dependent inhibition of CETP activity by purified apoCI in reconstituted mixtures containing liposome donors and LDL acceptors.** Cholesteryl ester transfer activity was determined as the rate of transfer of fluorescent NBD-cholesteryl esters from labeled liposome donors to LDL acceptors in the presence of a purified CETP fraction (final protein concentration, 26  $\mu\text{g}/\text{ml}$ ). Incubations were conducted for 3 h at 37  $^{\circ}\text{C}$  in the absence or in the presence of purified apoCI (concentration range, 0–1  $\mu\text{M}$ ) (see “Materials and Methods”). Blank values were obtained with homologous, incubated mixtures to which no purified CETP was added. Initial transfer rates (*inset*) were determined from the linear, initial portion of the time-course curves. Plotted values and *vertical bars* of the *inset* are the mean  $\pm$  S.D. of three determinations. *a*, significantly different from CETP + liposomes + LDL,  $p < 0.05$ ; *b*, significantly different from CETP + liposomes + LDL + 0.25  $\mu\text{M}$  apoCI,  $p < 0.05$ ; *c*, significantly different from CETP + liposomes + LDL + 0.50  $\mu\text{M}$  apoCI,  $p < 0.05$ ; Mann-Whitney test.



**FIG. 8. SDS-polyacrylamide gradient gel electrophoresis of HDL apolipoproteins from control and apoCI-KO mice.** HDL from control and apoCI-KO plasma were isolated by ultracentrifugation, and apolipoproteins were separated by SDS-electrophoresis in a high density polyacrylamide gel (see “Materials and Methods”). Apolipoprotein bands were visualized after silver staining, and molecular weights were calculated by comparison with the relative mobility of protein standards (see “Materials and Methods”).

direct consequence of greater neutral lipid exchanges in HuCETPTg/apoCI-KO mice, we observed an effect on the size distribution of HDL, with a significant reduction in the mean

apparent diameter of HDL from HuCETPTg/apoCI-KO mice compared with control mice, again with an intermediate effect in HuCETPTg mice.

It is worthy to note that the hyperlipidemic response to high fat feeding is exacerbated in apoCI-KO mice (24), and the effect of apoCI deficiency on lipoprotein metabolism *in vivo* was proposed to deal to some extent with the catabolism of apoB-containing lipoproteins (25). Given that the level of circulating apoB-containing lipoproteins tends also to increase in CETP transgenic mice (Ref. 34 and the present study), it may be hypothesized that apoCI deficiency also contributes to the lipid transfer reaction indirectly through its effect on the clearance of cholesteryl ester acceptors. Although the latter hypothesis is rather improbable in apoCI-KO mice fed a standard diet, with no significant lipoprotein alterations in this case (24, 25, present study), it was important to ascertain the real impact of apoCI deficiency on plasma CETP activity in HuCETPTg *versus* HuCETPTg/apoCI-KO mice. The latter point led us to demonstrate that the specific activity of plasma CETP is significantly increased as the result of apoCI withdrawal in HuCETPTg/apoCI-KO mice. Conversely, as observed *in vitro*, the rates of cholesteryl ester transfer in total plasma from HuCETPTg/apoCI-KO mice as well as in reconstituted mixtures could be progressively decreased in the presence of puri-



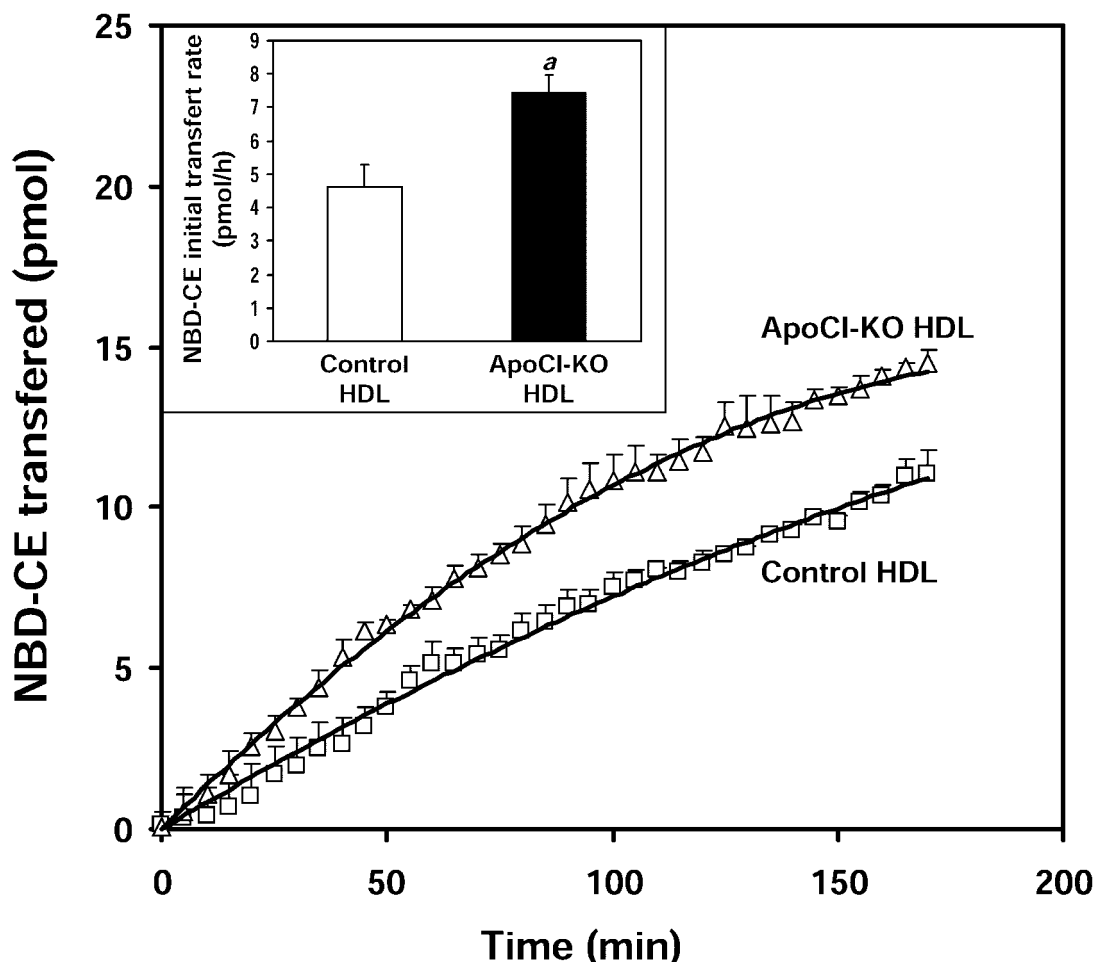


FIG. 9. Comparison of the ability of HDL from control and apoCI-KO mice to interact with CETP. HDL were isolated by sequential ultracentrifugation of pooled plasma from either control ( $n = 8$ ) or apoCI-KO ( $n = 8$ ) mice. Cholesteryl ester transfer activity was determined as the rate of transfer of fluorescent NBD-cholesteryl esters from labeled liposome donors to HDL acceptors in the presence of a purified CETP fraction (final protein concentration, 26  $\mu\text{g}/\text{ml}$ ) along a 3-h incubation period at 37  $^{\circ}\text{C}$  (see "Materials and Methods"). Blank values were obtained with homologous, incubated mixtures to which no purified CETP was added. Initial transfer rates (*inset*) were determined from the linear, initial portion of the time-course curves. Plotted values and vertical bars of the *inset* are the mean  $\pm$  S.D. of three determinations. *a*, significantly different from control HDL,  $p < 0.05$ ; Mann-Whitney test.

fied apoCI. The direct effect of apoCI as a physiological regulator of CETP was further confirmed by the more efficient interaction of CETP with HDL from apoCI-KO mice than with HDL from control mice despite identical size, lipid composition, and apoAI content of the particles from both sources. Interestingly, the ability of plasma HDL to inhibit CETP activity was also shown to disappear as the result of human apoAI overexpression in transgenic mice in previous studies (15). In transgenic mice expressing high levels of human apoAI, the protein moiety of circulating HDL was made mostly of human apoAI at the exclusion of murine HDL apolipoproteins (53). Because apoCI is an HDL apolipoprotein, its removal may then provide a rationale to the previously recognized greater interaction of CETP with HDL from apoAI transgenic animals (15).

The role of apoCI as a physiological inhibitor of CETP activity comes in addition to previous data suggesting that apoCI might be implicated in LCAT activation (54, 55) and in the uptake and clearance of triglyceride-rich lipoproteins and their remnants (56, 57). With regard to the putative role of apoCI as an LCAT cofactor, a non-significant tendency toward a reduction in HDL cholesteryl ester levels was observed in apoCI-KO mice as compared with control mice (Table II). However, it is worthy to note that the physiological relevance of this function, if any, is likely to be out of all proportion to the potent activating potential of apoAI, which has longer been recognized as the

physiological cofactor of LCAT (54, 55). Consistent with this view, no signs of LCAT deficiency (one trait producing marked abnormalities in LCAT-KO mice (58)) was reported in apoCI-KO mice in previous studies. With regard to the role of apoCI in the catabolism of triglyceride-rich lipoproteins, clear evidence appeared only when apoCI-KO mice were fed a high fat diet, but not when they were fed a standard diet (24, 25) as given in the present study. This suggests that the regulation of lipoprotein clearance may not constitute the primary function of apoCI *in vivo*. To our knowledge, the inhibition of CETP by apoCI as described here provides the first evidence for a direct and clear role of apoCI in normolipidemic mice. This new function of apoCI may well have been missed in previous studies of apoCI-KO (24) and apoCI-Tg mouse (59–61), since wild-type mice, unlike humans, do not express detectable levels of CETP in plasma (26, 27).

In conclusion, the present study indicates that the cholesteryl ester transfer activity in total plasma might be largely dependent on the presence of a specific inhibitor, *i.e.* apoCI. Given that some alterations in the plasma cholesteryl ester transfer activity in dyslipidemic patients cannot be explained by abnormalities in the plasma concentration of active CETP (7), the relevance to the human situation of the modulating effect of apoCI deserves further attention.

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**Apolipoprotein CI Deficiency Markedly Augments Plasma Lipoprotein Changes Mediated by Human Cholesteryl Ester Transfer Protein (CETP) in CETP Transgenic/ApoCI-knocked Out Mice**

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