Endothelial lipase is less effective at influencing HDL metabolism in vivo in mice expressing apoA-II

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Abstract Endothelial lipase (EL) plays an important physiological role in modulating HDL metabolism. Data suggest that plasma contains an inhibitor of EL, and previous studies have suggested that apolipoprotein A-II (apoA-II) inhibits the activity of several enzymes involved in HDL metabolism. Therefore, we hypothesized that apoA-II may reduce the ability of EL to influence HDL metabolism. To test this hypothesis, we determined the effect of EL expression on plasma phospholipase activity and HDL metabolism in human apoA-I and human apoA-I/A-II transgenic mice. Expression of EL in vivo resulted in lower plasma phospholipase activity and significantly less reduction of HDL-cholesterol, phospholipid, and apoA-I levels in apoA-I/A-II double transgenic mice compared with apoA-I single transgenic mice. We conclude that the presence of apoA-II on HDL particles inhibits the ability of EL to influence the metabolism of HDL in vivo.—Broedl, U. C., W. Jin, I. V. Fuki, J. S. Millar, and D. J. Rader. **Endothelial lipase is less effective at influencing HDL metabolism in vivo in mice expressing apoA-II.** *J. Lipid Res.* 2006. **47:** 2191–2197.

Supplementary key words high density lipoprotein metabolism • apolipoprotein A-II • high density lipoprotein containing apolipoprotein A-I but no apolipoprotein A-II • high density lipoprotein containing both apolipoprotein A-I and apolipoprotein A-II • transgenic mice

Endothelial lipase (EL), a member of the triglyceride lipase gene family (1, 2), plays a physiological role in the regulation of HDL metabolism. Hepatic overexpression of EL using adenoviral vectors resulted in markedly reduced high density lipoprotein-cholesterol (HDL-C) levels in mice (1, 3, 4). Transgenic overexpression of EL under the control of the endogenous promoter resulted in modestly reduced HDL-C levels (5). Conversely, inhibition of mouse EL activity in wild-type, apolipoprotein A-I (apoA-I) knockout, and HL knockout mice using a specific antibody resulted in significantly increased HDL-C and phospholipid levels (6). In the EL knockout mouse model, HDL-C and phospholipids were significantly increased (5, 7).

HDL contains two major proteins, apoA-I and apoA-II, that constitute $\sim$70% and $\sim$20% of the total HDL protein mass, respectively. In human plasma, HDL has been classified on the basis of apolipoprotein composition into lipoproteins containing apoA-I but no apoA-II (LpA-I) and lipoproteins containing both apoA-I and apoA-II (LpA-I/A-II) (8). ApoA-II has a higher lipid affinity than apoA-I and is able to displace apoA-I from lipoprotein particles (9, 10). Both apolipoproteins directly affect HDL remodeling catalyzed by the interfacially active proteins found in the plasma. LCAT activity is higher in HDL reconstituted with apoA-I than in HDL reconstituted with apoA-II (11). The lipid transfer activities of both cholesteryl ester transfer protein and phospholipid transfer protein are partially inhibited by apoA-II (12, 13). Conflicting results have been reported regarding the effect of apoA-II on HL: apoA-II has been suggested to be both stimulatory (14) and inhibitory (15, 16) to HL-mediated lipid hydrolysis.

We previously reported that EL activity is inhibited in a dose-dependent manner by heat-inactivated serum in vitro (17). Based on the ability of apoA-II to inhibit other enzymes and transfer proteins involved with HDL metabolism, we hypothesized that apoA-II may be one of the inhibitory factors modulating EL activity. To test this hypothesis, human apoA-I and apoA-I/A-II double transgenic mice were injected with low doses of an adenoviral vector encoding human endothelial lipase (AdhEL). Despite similar levels of EL protein expression, the apoA-I/A-II double transgenic mice had lower plasma phospholipase activity and significantly less reduction of HDL-C, phospholipid, and apoA-I levels than the apoA-I single transgenic mice. Thus, apoA-II expression substantially inhibits the effect of EL on HDL metabolism in vivo.

Abbreviations: AdGFP, adenovirus encoding green fluorescent protein; AdhEL, adenovirus encoding human endothelial lipase; apoA-I, apolipoprotein A-I; DPPC, dipalmitoylphosphatidylcholine; EL, endothelial lipase; HDL-C, high density lipoprotein-cholesterol; HSPG, heparan sulfate proteoglycan; LpA-I, high density lipoprotein containing apolipoprotein A-I but no apolipoprotein A-II; LpA-I/A-II, high density lipoprotein containing both apolipoprotein A-I and apolipoprotein A-II.

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MATERIALS AND METHODS

Animal studies

A recombinant adenovirus encoding human EL was constructed as described previously (1). In brief, the full-length human EL cDNA was subcloned into the shuttle plasmid vector pAdCMVLink1, and the plasmid was linearized with *Nhe*I and cotransfected into HEK 293 cells along with adenoviral DNA digested with *Cla*I. After confirmation of the presence of EL cDNA, the recombinant adenovirus was expanded in HEK 293 cells, purified by cesium chloride ultracentrifugation, and stored in 10% glycerol/PBS at −80°C.

Homozygous human apoA-I and heterozygous human apoA-II transgenic mice were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were fed a chow diet. ApoA-I and apoA-II transgenic mice were interbred, generating either heterozygous apoA-I or heterozygous apoA-I/A-II double transgenic mice. ApoA-I and apoA-I/A-II transgenic mice were injected via the tail vein with 3×10^{10} particles (study 1) or 1×10^{10} particles (study 2) of AdhEL on day 0 of the studies. Blood was drawn from the retro-orbital plexus after 4 h of daytime fasting before and weekly after virus injection for up to 4 weeks.

Electrophoresis and immunoblotting

Twenty microliters of postheparin mouse plasma was incubated overnight at 4°C with heparin-Sepharose CL-6B (Amersham Pharmacia Biotech) in the presence of a protease inhibitor cocktail (Complete; Roche) and subsequently washed three times with sterile PBS. After centrifugation, the pelleted heparin-Sepharose was suspended in SDS loading buffer. Western blot analysis of these samples was performed using the NuPAGE Bis-Tris Electrophoresis System (Invitrogen, Carlsbad, CA) under denaturing and reducing conditions (10% Bis-Tris gel) according to the manufacturer's protocol. Protein was detected with rabbit anti-EL peptide serum (1:3,000) and goat anti-rabbit peroxidase-conjugated antiserum (1:5,000) as the secondary antibody (1). The blot was developed with ECL detection reagents (Amersham Pharmacia Biotech). As a positive control, medium from Cos-7 cells infected with 1×10^{11} particles of AdhEL was used (tissue culture-derived positive control). Medium from Cos-7 cells infected with 1×10^{11} particles of a control virus encoding green fluorescent protein (AdGFP) was used as a negative control (tissue culture-derived negative control).

Lipid and lipoprotein analysis

Plasma total cholesterol, HDL-C, and phospholipid levels were measured enzymatically on a Cobas Fara II (Roche Diagnostic Systems, Inc.) using Sigma reagents (Sigma Chemical Co.). Plasma human apoA-I and human apoA-II levels were quantified using an immunoturbidometric assay (Sigma Chemical Co. and Wako Pure Chemical Industries, respectively) on the Cobas Fara. Pooled plasma samples (250 μl) were subjected to fast-protein liquid chromatography gel filtration (Pharmacia) using two Superose 6 columns in series as described (1). Fractions (0.5 ml) were collected, and cholesterol concentrations were determined using an enzymatic assay (Wako Pure Chemical Industries).

Phospholipase assay

The phospholipase assay was performed as described previously (17). Briefly, the emulsion of cholesteryl oleate (150 mg) and dipalmitoylphosphatidylcholine (DPPC) (8.88 mg of unlabeled DPPC and 17.15 μCi of [1,2-^{14}C]DPPC, 110 mCi/mmol) was prepared by sonication in 2.5 ml of glycerol. The assay tubes contained, in a total volume of 0.3 ml, 0.05 M Tris-HCl, pH 8.0, 0.75% BSA, 4.6 mM cholesteryl oleate, 245 μM DPPC, 0.15 M NaCl, and postheparin mouse plasma. Samples were incubated for 60 min at 37°C. Reactions were stopped, and products were extracted by the method of Belfrage and Vaughan (18) except that lysopalmitoyl-phosphatidylcholine (100 μg/ml) was included as carrier in the organic extraction mix.

HDL binding studies

HDLs (1.063 < d < 1.25) from human apoA-I and apoA-I/A-II transgenic mice were isolated by sequential ultracentrifugation. Apolipoprotein composition of the lipoproteins was confirmed by gel electrophoresis (data not shown). A cell binding assay was used to compare the properties of EL to mediate bridging between the cell surface heparan sulfate proteoglycans (HSPGs) and LpA-I versus LpA-I/A-II as described previously (19, 20). Briefly, Cos-7 cells cultured on 12-well plates were infected with AdhEL or AdGFP as a control. To assess the effects of AdhEL on the surface binding of lipoproteins, cells were incubated with ^{125}I-labeled LpA-I (5 μg/ml) or ^{125}I-labeled LpA-I/A-II (5 μg/ml) for 1 h at 4°C in the absence or presence of heparin (100 μg/ml). At this concentration, heparin specifically blocks interactions of ligands with HSPGs (21) but not lipoprotein binding to the members of the LDL receptor family or other lipoprotein receptors (22). HSPG-dependent binding of lipoproteins was calculated by subtracting the values obtained in the presence of heparin (HSPG-independent) from those obtained in the absence of heparin (total). The data are presented as HSPG-dependent amounts of lipoproteins bound by the cells in the course of 1 h.

Statistical analysis

Graphs are presented as means ± SD. Statistical significance for all comparisons was tested using the unpaired Student's *t*-test and was assigned at $P < 0.05$.

RESULTS

To test the hypothesis that apoA-II inhibits the effect of EL on HDL metabolism in vivo, we injected human apoA-I and human apoA-I/A-II transgenic mice with a relatively low dose of 3×10^{10} particles of AdhEL. Western blot analysis of day 7 postheparin plasma confirmed comparable EL expression between the two groups (**Fig. 1A**). However, expression of EL resulted in significantly lower postheparin phospholipase activity in apoA-I/A-II compared with apoA-I transgenic mice on day 7 after virus injection (Fig. 1B). In addition, expression of EL in apoA-I/A-II double transgenic mice resulted in significantly less reduction of HDL-C (**Fig. 2A**), phospholipid (Fig. 2B), and apoA-I (Fig. 2C) levels over 28 days than in apoA-I single transgenic mice. Furthermore, expression of EL in apoA-I/A-II double transgenic mice resulted in significantly less reduction of apoA-II than of apoA-I levels (Fig. 2D).

In a subsequent experiment, human apoA-I and apoA-I/A-II transgenic mice were injected with an even lower dose of 1×10^{10} particles of AdhEL. Western blot analysis of day 7 postheparin plasma confirmed comparable EL protein levels (**Fig. 3A**). Postheparin phospholipase activity showed a trend toward lower levels in apoA-I/A-II compared with apoA-I transgenic mice ($P = 0.14$) (Fig. 3B). Ex-
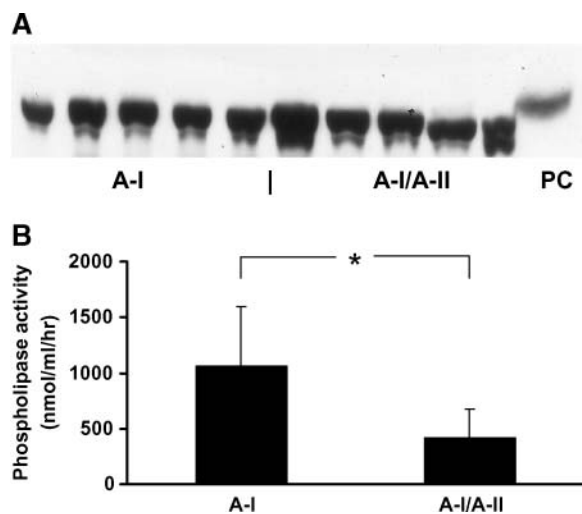
Fig. 1. A: Western blot analysis of day 7 postheparin plasma of apolipoprotein A-I (apoA-I) and apoA-I/A-II transgenic mice injected with 3×10^{10} particles of adenoviral constructs encoding endothelial lipase (EL). PC, tissue culture-derived positive control. B: Phospholipase activity (nmol/ml/h) determined in postheparin plasma at day 7 after injection of apoA-I and apoA-I/A-II transgenic mice with 3×10^{10} particles of adenovirus encoding human endothelial lipase (AdhEL). Data are presented as means ± SD. * $P < 0.05$.

pression of EL in apoA-I/A-II double transgenic mice, however, again resulted in significantly less reduction of HDL-C (**Fig. 4A**), phospholipid (Fig. 4B), and apoA-I (Fig. 4C) levels over 28 days than in apoA-I sin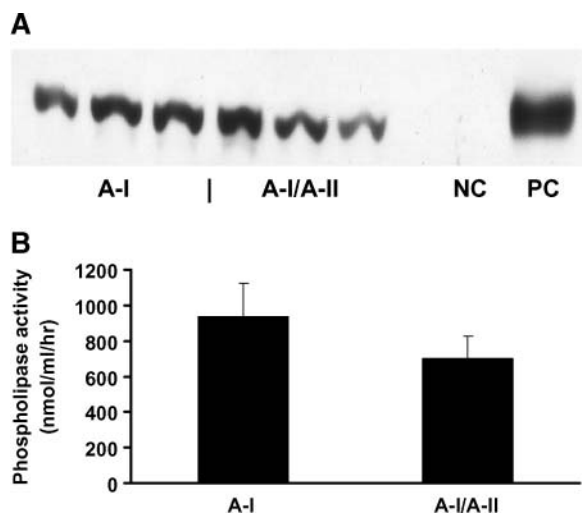gle transgenic mice. Injection of apoA-I/A-II double transgenic mice with 1×10^{10} particles of EL had no effect on apoA-II levels despite significantly reducing apoA-I levels (Fig. 4D). Consistent with these results, analysis of lipoprotein distribution by fast-protein liquid chromatography before and on day 21 after injection showed much less reduction of the HDL cholesterol peak induced by EL expression in apoA-I/A-II double transgenic mice (**Fig. 5A**) than in apoA-I transgenic mice (Fig. 5B).

Because the reduced postheparin phospholipase activity in the apoA-II-expressing mice suggested that apoA-II had an inhibitory effect on endogenous human EL activity, we also wondered whether the plasma from apoA-I/A-II animals (before EL overexpression) inhibits exogenously added EL more than apoA-I-only plasma. Therefore, we expressed EL in vitro and added to the conditioned medium different amounts of plasma from wild-type mice and human apoA-I, apoA-I/A-II, and apoA-II transgenic mice and determined EL phospholipase activity using a synthetic lipid substrate. Unexpectedly, plasma from apoA-I/A-II and apoA-II transgenic mice did not inhibit EL-phospholipase activity to a greater extent than plasma from wild-type or apoA-I transgenic mice (**Table 1**).

To determine whether the ability of EL to "bridge" HDL to cell surface HSPGs, as we have described previously (19, 20), was reduced by apoA-II, we isolated native HDL from apoA-I and apoA-I/A-II transgenic mice and performed a cell binding assay using Cos-7 cells as described previously (19, 20). HDL isolated from apoA-I/A-II double transgenic mice had significantly reduced EL-mediated
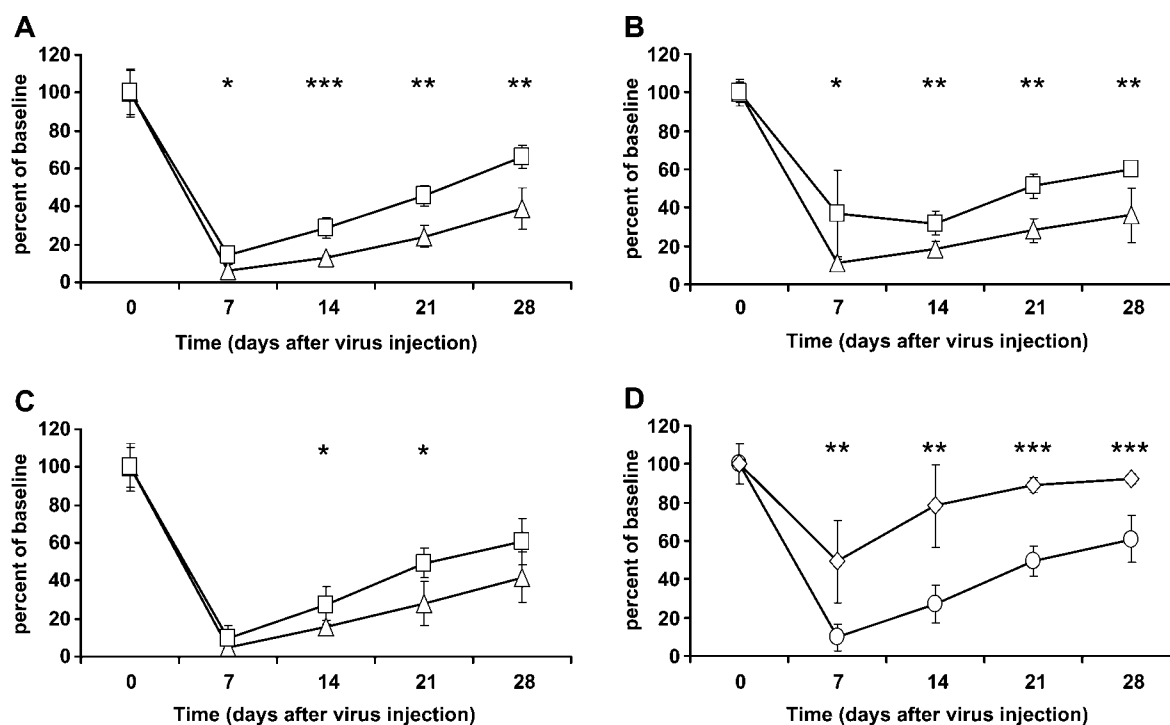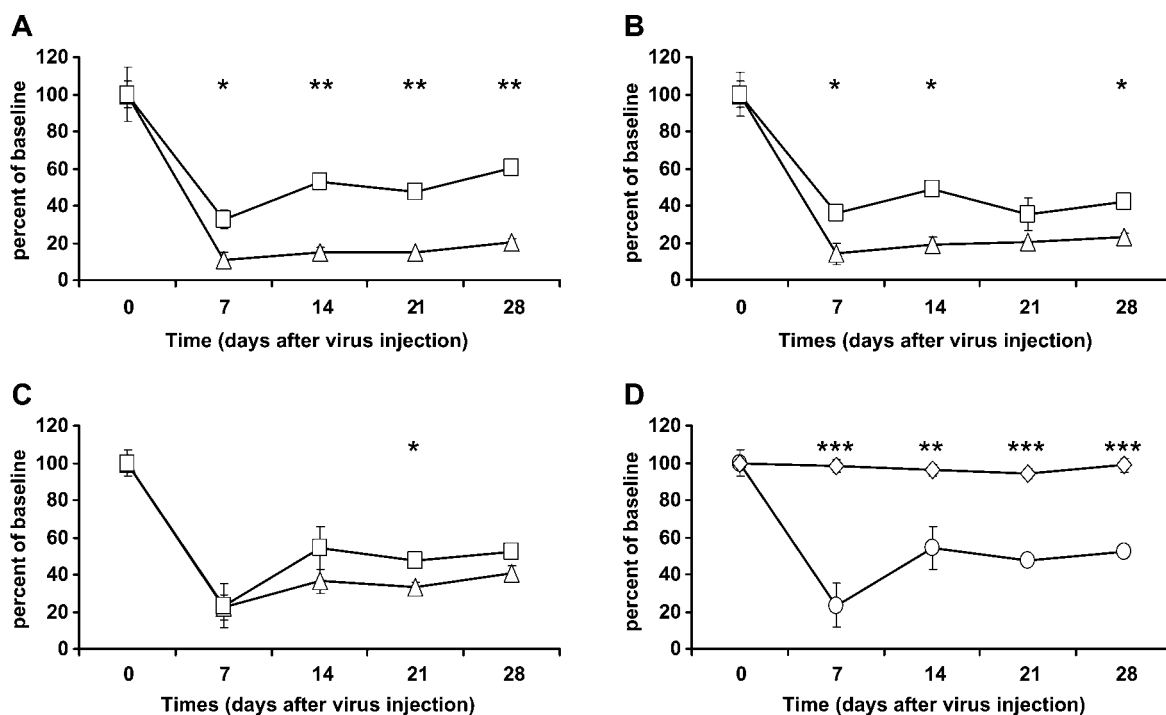

Fig. 2. A–C: Plasma high density lipoprotein-cholesterol (HDL-C) (A), phospholipid (B), and apoA-I (C) levels in apoA-I (triangles; n = 5) and apoA-I/A-II (squares; n = 5) transgenic mice after injection of 3×10^{10} particles of AdhEL. D: ApoA-I (circles) and apoA-II (diamonds) levels in apoA-I/A-II transgenic mice after injection of 3×10^{10} particles of AdhEL. Data are presented as percentage of baseline values ± SD. Baseline HDL-C levels in apoA-I and apoA-I/A-II transgenic mice before virus injection were 176 ± 22 and 149 ± 17 mg/dl, respectively; baseline phospholipid levels were 381 ± 26 and 303 ± 16 mg/dl; baseline apoA-I levels were 340 ± 43 and 250 ± 26 mg/dl. Baseline apoA-II level in apoA-I/A-II transgenic mice was 65 ± 2 mg/dl. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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Fig. 3. A: Western blot analysis of day 7 postheparin plasma of apoA-I and apoA-I/A-II transgenic mice injected with 1×10^{10} particles of adenoviral constructs encoding EL. NC, tissue culture-derived negative control; PC, tissue culture-derived positive control. B: Phospholipase activity (nmol/ml/h) determined in postheparin plasma at day 7 after injection of apoA-I and apoA-I/A-II transgenic mice with 1×10^{10} particles of AdhEL ($P = 0.14$). Data are presented as means ± SD.

binding to cell surface HSPGs than HDL isolated from apoA-I single transgenic mice (**Fig. 6**). Therefore, apoA-II appears to reduce the ability of EL to bridge HDL to cell surface HSPGs.
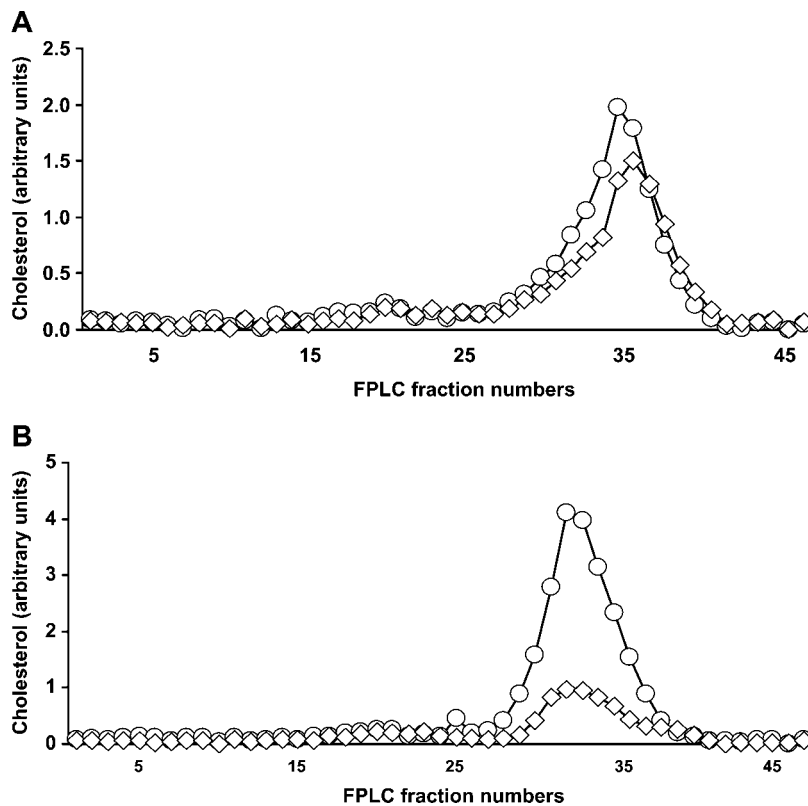
DISCUSSION

Because apoA-II was shown to have inhibitory effects on LCAT (11), cholesteryl ester transfer protein (12), phospholipid transfer protein (13), and HL activity (15, 16), it seems reasonable to speculate that the presence of apoA-II may also moderate the effect of EL on plasma HDL levels by inhibiting the activity of EL. We performed these experiments to test the hypothesis that apoA-II inhibits the effects of EL on HDL metabolism in vivo. Our data indicate that despite comparable levels of EL protein expression, 1) EL expression is less effective at reducing HDL-C and apoA-I levels in apoA-I/A-II double transgenic mice than in apoA-I single transgenic mice; 2) after EL expression, postheparin plasma phospholipase activity is lower in apoA-I/A-II double transgenic mice than in apoA-I single transgenic mice; and 3) HDL from apoA-I/A-II double transgenic mice is reduced in EL-mediated binding to cell surface HSPGs compared with HDL from apoA-I single transgenic mice. Thus, we conclude that apoA-II has the ability to reduce the effects of EL on HDL metabolism in vivo.

Interestingly, expression of EL in apoA-I/A-II transgenic mice had a significantly greater effect on apoA-I than on apoA-II levels. Injection of 1×10^{10} particles of AdhEL did not reduce apoA-II levels at all despite a significant concomitant reduction of HDL-C, phospholipid, and apoA-I levels. It is known that apoA-II has high affinity for lipid and less probability of dissociating from HDL

Fig. 4. A–C: Plasma HDL-C (A), phospholipid (B), and apoA-I (C) levels in apoA-I (triangles; n = 3) and apoA-I/A-II (squares; n = 3) transgenic mice after injection of 1×10^{10} particles of AdhEL. D: ApoA-I (circles) and apoA-II (diamonds) levels in apoA-I/A-II transgenic mice after injection of 1×10^{10} particles of AdhEL. Data are presented as percentage of baseline values ± SD. Baseline HDL-C levels in apoA-I and apoA-I/A-II transgenic mice before virus injection were 223 ± 32 and 96 ± 7 mg/dl, respectively; baseline phospholipid levels were 381 ± 26 and 236 ± 28 mg/dl; baseline apoA-I levels were 255 ± 19 and 135 ± 10 mg/dl. Baseline apoA-II level in apoA-I/A-II transgenic mice was 64 ± 1 mg/dl. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 5. Fast-protein liquid chromatography analysis of the plasma lipoproteins of apoA-I/A-II (A) and apoA-I (B) transgenic mice before (circles) and 21 days after (diamonds) injection of 1×10^{10} particles of AdhEL.

particles than apoA-I (10). Thus, HDL particles bearing apoA-II may be less likely to be hydrolyzed by EL, and/or an HDL particle hydrolyzed by EL may be more likely to shed apoA-I than apoA-II.

Caiazza et al. (23) previously reported the effect of recombinant HDL particles that were comparable in terms of size and lipid composition and contained either apoA-I as the only apolipoprotein (rLpA-I) or apoA-I as well as apoA-II (rLpA-I/A-II) on the kinetic properties of EL in vitro. EL was shown to have a higher affinity for the phospholipids in rLpA-I than in rLpA-I/A-II in vitro, consistent with the results of our bridging study using native lipoproteins from apoA-I and apoA-I/A-II transgenic mice. However, the V_{max} of phospholipid hydrolysis was re-

ported to be greater for rLpA-I/A-II than for rLpA-I (23). This apparent discrepancy between the in vitro results of Caiazza et al. (23) and our in vivo results may relate to the different lipoprotein complexes used for the two studies. In contrast to the recombinant HDL particles used by Caiazza et al. (23), HDLs from apoA-I and apoA-I/A-II transgenic mice differ in both size and lipid composition (24). Importantly, HDLs from the apoA-I and apoA-I/A-II double transgenic mice on a chow diet are known to have a size distribution similar to the LpA-I and LpA-I/A-II population separated from human plasma. LpA-I from apoA-I transgenic mice was demonstrated to be larger and to con-

TABLE 1. Phospholipase activity in the medium of adenovirus encoding human endothelial lipase-infected Cos-7 cells after the addition of 1, 3, or 5 μl of plasma from wild-type mice and apoA-I, apoA-I/A-II, and apoA-II transgenic mice

	Phospholipase Activity			
Plasma Dosage	Wild Type	ApoA-I	ApoA-I/A-II	ApoA-II
	nmol/ml/h			
1 μl	900	940	1,033	961
3 μl	409	207	331	272
5 μl	4	74	83	75

ApoA-I, apolipoprotein A-I. The phospholipase activity assay was performed in duplicate. Data are presented as averages.
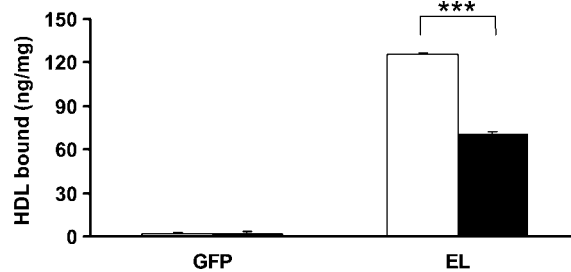
Fig. 6. EL-dependent binding of HDL containing apoA-I but no apoA-II (white bars) and HDL containing both apoA-I and apoA-II (black bars) by Cos-7 cells infected with adenoviral constructs encoding green fluorescent protein (GFP; control) or EL in the course of 1 h. Data are presented as means ± SD. *** $P < 0.001$.

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tain relatively more phospholipids compared with LpA-I/A-II from double transgenic mice (24). In human plasma, apoA-II is found predominantly associated with smaller and less lipid-enriched HDL particles. The more dense HDL fractions, HDL_3, have been shown to contain higher relative amounts of apoA-II than the larger HDL_2 particles (25). We have reported previously that overexpression of EL results in increased phospholipase activity in vivo, thus generating phospholipid-depleted smaller HDL particles that are more rapidly catabolized (3). In this study, we demonstrate that EL prefers larger, phospholipid-rich HDL (LpA-I) particles as a substrate compared with LpA-I/A-II in vivo. It remains to be determined whether in apoA-I/A-II transgenic mice the reduced EL phospholipase activity and altered clearance of cholesterol and phospholipids reflect a direct or an indirect effect of apoA-II (i.e., is a result of different HDL size distribution and lipid composition).

Interestingly, despite the fact that EL-mediated phospholipase activity in apoA-I/A-II transgenic mice was reduced compared with that in apoA-I transgenic mice, addition of plasma from apoA-I/A-II and apoA-II transgenic mice to EL-containing conditioned medium did not inhibit EL-phospholipase activity to a greater extent than plasma from wild-type or apoA-I transgenic mice. We hypothesize that the interaction of produced EL with endogenous HDL in vivo is different from the interaction of EL with a synthetic substrate in vitro. Specifically, apoA-II may influence the ability of EL to hydrolyze an apoA-II-containing HDL particle but not affect the ability of EL to hydrolyze a synthetic substrate. We consider effects on HDL metabolism in vivo a better bioassay of EL activity on its endogenous substrate.

One limitation of using transgenic mice to analyze the in vivo role of the major human HDL-associated apolipoproteins is the presence of murine apoA-I and apoA-II in the plasma of these animals. Transgenic overexpression of human apoA-I, with or without human apoA-II, was shown to be associated with a major decrease ($\sim$90%) in endogenous murine apoA-I and a substantial decrease ($\sim$40%) in the endogenous murine apoA-II plasma concentrations (24, 26). Overexpression of human apoA-II was demonstrated to not significantly alter mouse apoA-I and apoA-II levels (26). The decreased murine apoA-I in the plasma of human apoA-I and apoA-I/A-II transgenic mice simplifies one aspect of the analysis. However, we cannot exclude the possibility that murine apoA-II may modulate the proposed effect of human apoA-II on EL.

In summary, we demonstrate that apoA-II has the ability to reduce the effects of EL on HDL metabolism in vivo. This adds to the list of factors that modulate HDL metabolism that appear to be inhibited by apoA-II.

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