ApoA-II maintains HDL levels in part by inhibition of hepatic lipase: studies in apoA-II and hepatic lipase double knockout mice

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Abstract High density lipoprotein (HDL) cholesterol levels are inversely related to the risk of developing coronary heart disease. Apolipoprotein (apo) A-II is the second most abundant HDL apolipoprotein and apoA-II knockout mice show a 70% reduction in HDL cholesterol levels. There is also evidence, using human apoA-II transgenic mice, that apoA-II can prevent hepatic lipase-mediated HDL triglyceride hydrolysis and reduction in HDL size. These observations suggest the hypothesis that apoA-II maintains HDL levels, at least in part, by inhibiting hepatic lipase. To evaluate this, apoA-II knockout mice were crossbred with hepatic lipase knockout mice. Compared to apoA-II-deficient mice, in double knockout mice there were increased HDL cholesterol levels (57% in males and 60% in females), increased HDL size, and decreased HDL cholesteryl ester fractional catabolic rate. In vitro incubation studies of plasma from apoA-II knockout mice, which contains largely apoA-I HDL particles, showed active lipolysis of HDL triglyceride, whereas similar studies of plasma from apoA-I knockout mice, which contains largely apoA-II particles, did not. In summary, these results strongly suggest that apoA-II is a physiological inhibitor of hepatic lipase and that this is at least part of the mechanism whereby apoA-II maintains HDL cholesterol levels.—Weng, W., N. A. Brandenburg, S. Zhong, J. Halkias, L. Wu, X-c. Jiang, A. Tall, and J. L. Breslow. **ApoA-II maintains HDL levels in part by inhibition of hepatic lipase: studies in apoA-II and hepatic lipase double knockout mice.** *J. Lipid Res.* **1999.** 40: **1064–1070.**

Supplementary key words apoA-II • apoA-I • hepatic lipase • HDL cholesterol

High density lipoprotein (HDL) cholesterol levels are inversely correlated with coronary heart disease susceptibility within populations (1–4). HDL is a macromolecular complex consisting of approximately 50% lipid, mainly phospholipid and cholesteryl ester, and 50% protein, of which apoA-I accounts for 70% and apoA-II 20% of protein mass (5). Mature HDL particles are formed in plasma. Nascent HDL consists of protein phospholipid discs that are derived from liver and intestine (5–7). These combine with free cholesterol from cell membranes and other lipoproteins. The free cholesterol is esterified by lecithin:cholesterol acyltransferase (LCAT). The more hydrophobic cholesteryl ester changes the shape of the particle to spherical and resides in the core with protein, phospholipid, and free cholesterol on the HDL surface. Triglyceride from very low density (VLDL) and intermediate density lipoproteins (IDL) can exchange with HDL cholesteryl ester via cholesteryl ester transfer protein (CETP). Lipoprotein lipase (LPL)-mediated lipolysis of triglyceride-rich lipoproteins can provide surface components that can transfer to HDL, enlarging the particles, and hepatic lipase (HL) can act as an HDL triglyceride lipase and phospholipase, diminishing HDL size. Various metabolic studies in humans and mice have shown a strong inverse correlation between HDL size and fractional catabolic rate (8, 9).

In previous studies of apoA-II function using transgenic mice, Zhong et al. (10) showed that expression of a human apoA-II transgene did not prevent the reduction of HDL cholesterol mediated by CETP, but did result in enhanced CETP-mediated accumulation of triglyceride in HDL. The presence of the human apoA-II transgene also inhibited the normal CETP-mediated reduction in HDL size, and HDL containing human apoA-II inhibited HL in an emulsion-based assay and resisted the endogenous HL activity in mouse plasma. This was interpreted to mean that apoA-II does not inhibit CETP-mediated cholesteryl ester–triglyceride exchange, but does inhibit the hydrolysis of HDL triglyceride and phospholipid by HL. ApoA-II knockout mice were created and found to have a marked

Abbreviations: HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; CETP, cholesteryl ester transfer protein; LPL, lipoprotein lipase; HL, hepatic lipase; FCR, fractional catabolic rate; TR, transport rate; CE, cholesteryl ester.

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reduction of HDL cholesterol levels and size (9). This suggested that in the absence of apoA-II there may be unopposed and therefore overactive HL activity which, by diminishing the HDL phospholipid pool, could decrease HDL by affecting synthesis or clearance of HDL particles.

In the current report we further explored the hypothesis that apoA-II maintains HDL levels by inhibiting HL by studying apoA-II and HL double knockout mice. We reasoned that if apoA-II deficiency lowered HDL levels by an HL-dependent mechanism, then HDL levels should be restored in mice deficient in both apoA-II and HL. Compared to apoA-II-deficient mice, we found considerable restoration of HDL levels, increased HDL size, and decreased HDL fractional catabolic rate in double knockout mice, supporting our hypothesis.

METHODS

Generation of apoA-II and HL double knockout mice

ApoA-II knockout mice were generated at Rockefeller University (9) and HL knockout mice were generated by Homanics et al. (11). Heterozygous apoA-II knockout mice (50% C57Bl/6J and 50% 129/Ola) were bred to produce wild-type controls and homozygous apoA-II knockout mice. Homozygous apoA-II knockout mice and homozygous HL knockout mice (on the C57Bl/6J background, Jackson Laboratory) were crossbred. Doubly heterozygous knockout mice were backcrossed to homozygous apoA-II knockout mice to generate heterozygous HL knockout mice on the background of homozygosity for the apoA-II knockout. These mice were then intercrossed to generate apoA-II knockout and apoA-II, HL double knockouts, which were then directly compared. Comparisons were done on littermate mice that were between 4 and 5 months of age at the time of study.

DNA was isolated from the tail tips and PCR-based assays were used to identify the wild-type and mutant alleles in the colony. The PCR assay used to identify apoA-II knockout alleles was described in our previous paper (9). The PCR assay used to identify the wild-type HL allele used primer pairs HL7us, located at the beginning of the HL gene 4th exon with a sequence of 5′-GAATCTGCGAAGTTTTCTCGGAG-3′, and HL6ls, located at the end of the 4th exon with a sequence of 5′-CCTGTGATTCTTCCAATCTTGTTC-3′ and produced a band of 0.12 kb. The PCR used to identify the mutant allele used primer HL7us, as above, and primer 3094, located in the end portion of the neo gene cassette with a sequence of 5′-GACTGTGGCCGGCTGGGTGTGGCGGACCGC-3′. With these primers the mutant allele gave a PCR fragment of 0.3 kb.

Animal maintenance

Animals were housed at the Rockefeller University Laboratory Animal Research Center (LARC). The animal room was maintained on 12 h (7 am to 7 pm) light/dark cycles. Animals were fed a low fat–low cholesterol diet (chow diet) (no. 5001; Ralston Purina. The chow diet contained (wt/wt) 4.5% fat, 59.8% carbohydrate, 23.4% protein, 5.0% fiber, 7.3% minerals, and 0.02% cholesterol. In this diet fat provided 9% of calories, which were divided equally among saturated, monounsaturated, and polyunsaturated fats.

Plasma lipid and lipoprotein analysis

Blood was drawn from the retroorbital venous plexus into EDTA tubes. Fasting specimens were used and for these food was removed from the cages at 9 am and the animals were bled between 4 and 5 pm. Cholesterol and triglyceride levels were determined enzymatically using Boehringer Mannheim reagents. The distribution of cholesterol among the lipoprotein size classes was analyzed by fast protein liquid chromatography (FPLC). For this analysis, 500 μl of pooled mouse plasma was first filtered through a 0.45-μm filter and then passed through two Superose 6 columns connected in series (Pharmacia). Forty-five fractions of 0.5 ml each were collected and assayed as above for cholesterol. Mouse plasma was also subjected to sequential preparative ultracentrifugation to determine lipoprotein cholesterol concentrations as previously described.

Lipoprotein size analysis

HDL particle size distribution was determined by 4–20% nondenaturing gradient gel electrophoresis (12, 13). The HDL isolated by preparative ultracentrifugation was electrophoresed for 20 min at 70 V and then overnight at 150 V. The gel was then stained with 0.05% Coomassie blue and destained with 9% acetic acid and 20% methanol. HDL particle size was judged in comparison to the size of known protein standards (high molecular weight calibration kit; Pharmacia). Human HDL subpopulations and particle diameters determined by this technique are as follows: HDL2b, 12.9–9.8 nm; HDL2a, 9.8–8.8 nm; HDL3a, 8.8–8.2 nm; HDL3b, 8.2–7.8 nm; and HDL3c, 7.8–7.2 nm. To avoid the manipulation of ultracentrifugation, we also developed a method to run plasma on a native polyacrylamide gel as above and detect HDL by immunoblotting. To do this the polyacrylamide gel was blotted onto PVDF membranes (DuPont), which were then probed with goat anti-mouse apoA-I antibody at a dilution of 1:3000 (plasma:PBS). The location of apoA-I on the blot was detected by staining with peroxidase-labeled rabbit anti-goat IgG antibodies followed by a chemiluminescence reaction (ECL kit, Amersham).

HDL turnover study

The cholesteryl ester in HDL was radiolabeled as [^{3}H]cholesteryl oleoyl ether as follows: 10 μCi (1 Ci = 37 GBq) of [^{3}H]cholesteryl oleoyl ether was dried under nitrogen gas for 20 sec. Subsequently, 1 ml of 0.9% NaCl and 1.5 μl of 10–20% intralipid (KabiVitrum, Stockholm) were added. After sonication, 500 μl of plasma from control mice was added with 2.0 μg of purified human CETP. The mixture was incubated for 8 h at 37°C, and radiolabeled HDL was then isolated by preparative ultracentrifugation. HDL turnover was done by injecting 100,000–200,000 dpm of ^{3}H-labeled HDL. Serial blood samples were obtained from the retroorbital plexus at 10 and 90 min and 3, 8, and 24 h. HDL cholesteryl ester fractional catabolic rates were calculated by the Matthew's two pool model (12).

Hepatic lipase activity determinations

Heparin (100 U/kg body wt; Elkins-Sinn, Inc., Cherry Hill, NJ) was injected into the leg vein of apoA-I and apoA-II knockout mice and wild-type littermate controls under isoflurane anesthesia. Post-heparin plasma was collected after 5 min. Hepatic lipase activity in post-heparin plasma (10 μl) was measured using a gum arabic-stabilized emulsion containing 0.88 m NaCl to inactivate lipoprotein lipase. To assess the effects of HDL isolated from mice of different genotypes on hepatic lipase activity, 0–50 μg of dialyzed mouse HDL was added to 300 μl of a gum arabic-stabilized emulsion, pH 8.8, in 1.25 m NaCl prepared by the method of Baginsky and Brown (13). The assay was begun by addition of 10 μl of mouse post-heparin plasma (from wild-type mice) and allowed to continue for 1 h at 27°C. The reaction was terminated by addition of 3.5 ml of a solution of methanol–chloroform–heptane–oleic acid 1,410:1,250:1,000:1,000 (vol:vol) and 1 ml of 0.05 m potassium borate buffer, pH 10. After centrifugation, 1 ml of the

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aqueous phase was removed, 3.5 ml of Hydrofluor (National Diagnostics, Manville, NJ) was added, and the amount of liberated free fatty acids was determined by liquid scintillation spectroscopy in a beta counter (LS 1000; Beckman Instruments). All assays were performed in triplicate and the experiment was performed twice using different pooled preparations of HDL from mice of each genotype.

Plasma HDL cholesterol and triglyceride determinations after in vitro incubation with rCETP and diethyl *p*-nitrophenylphosphate (E600)

Plasma was obtained from apoA-I and apoA-II knockout, and wild-type littermate controls. An aliquot (200 μl) of such pooled plasma was immediately stored on ice. At the same time, two other aliquots (200 μl each) were incubated with purified human CETP (20 μg/ml), with or without E600 (500 μm) for 5 h. Plasma HDL was subsequently isolated by ultracentrifugation between densities 1.055 and 1.21 g/ml and cholesterol and triglyceride were determined.

Statistical analysis

Results are given as mean ± SD unless otherwise indicated. The Student's *t*-test was used to compare differences between groups. Statistical significance was defined as $P < 0.05$.

RESULTS

ApoA-II and HL knockout mice and the double knockout mice all appeared healthy without any gross phenotypic abnormality, and gained weight normally (data not shown). As shown in **Table 1**, compared to wild-type mice the apoA-II knockout mice had reduced total, HDL, and non-HDL cholesterol levels, as previously shown (9). Compared to the apoA-II knockout mice, in the apoA-II, HL double knockout mice HDL cholesterol levels increased 57% and 60% in males and females, respectively.

TABLE 1. Plasma cholesterol level under fasting conditions from apoA-II knockout and double knockout mice, and wild-type mice on chow

Mouse	n	TC	HDL	LDL	VLDL
			mg/dl		
Male					
AII2HL2	5	93 ± 9[b]	57 ± 5[b]	14 ± 2[b]	16 ± 3[b]
AII0HL2	10	35 ± 9[d]	21 ± 7[d]	6 ± 1[c]	3 ± 1[c]
AII0HL0	10	52 ± 9	33 ± 5	8 ± 3	6 ± 3
Female					
AII2HL2	4	62 ± 11	38 ± 8	11 ± 4	8 ± 2[a]
AII0HL2	9	39 ± 12	20 ± 3[d]	8 ± 3	4 ± 2
AII0HL0	10	50 ± 10	32 ± 6	11 ± 3	4 ± 2

Food was removed at 9 am with the animal having ad lib access to water, and blood was drawn from the retroorbital venous plexus at 5 pm the same day. The values of HDL, LDL, and VLDL were determined after micro-ultracentrifugation (Beckman). Lipid values are shown as mean ± SD. AII0HL2, AII0HL0, and AII2HL2 represent apoA-II knockout, double knockout, and wild-type control mice, respectively.

[a] $P < 0.01$ as double knockouts compared with control mice.

[b] $P < 0.001$ as double knockouts compared with control mice.

[c] $P < 0.05$ as double knockouts compared with apoA-II knockout mice.

[d] $P < 0.001$ as double knockouts compared with apoA-II knockout mice.

The percent restoration of wild-type HDL cholesterol levels was greater in females than males, due to higher HDL cholesterol levels in wild-type male compared to female mice. These results using preparative ultracentrifugation were confirmed by FPLC analysis as shown in **Fig. 1**, **A** and **B**.

The size distribution of lipoprotein particles in mice with the various genotypes was next examined by two techniques. In the first, HDL was isolated by subsequential preparative ultracentrifugation and subjected to non-denaturing gradient gel electrophoresis. As shown in **Fig. 2A**, the mean particle diameter of HDL in wild-type mice was 9.7 nm, in apoA-II knockouts it was 8.9 nm, and in the apoA-II HL double knockouts it was 9.5 nm. Thus the lack of apoA-II results in smaller HDL, and this is restored when HL is also deficient. HDL size was also determined by non-denaturing PAGE using immunoblotting of whole plasma. This avoids possible artifacts that might be introduced by ultracentrifugation, which can spin off some HDL components. The results shown in Fig. 2B were quite similar to those using the first technique. HDL size was reduced in the apoA-II knockout mice and size largely returned to normal in the double knockout mice.

The metabolic mechanism underlying the changes observed in HDL cholesterol levels was also studied. As shown in **Table 2**, compared with the apoA-II knockout mice, in the apoA-II HL double knockout mice there was a 67% decrease in HDL cholesteryl ester fractional catabolic rate with no change in transport rate. The increased HDL cholesteryl ester fractional catabolic rate in the apoA-II knockout mice was decreased to nearly normal in the double knockout mice. Thus the partial restoration of HDL cholesterol levels in the double knockout mice is entirely due to changes in the fractional catabolic rate.

The data thus far are compatible with the hypothesis that the lack of apoA-II results in increased functional HL activity and that HL by its ability to hydrolyze HDL triglyceride and phospholipid diminishes HDL size, causing increased HDL fractional catabolic rate. To measure the effect of apoA-II deficiency on functional HL activity, plasma samples from wild-type, apoA-I, and apoA-II-deficient mice were incubated with rCETP with and without a lipase inhibitor (E600), HDL was isolated, and HDL triglycerides and cholesterol were measured. In all cases incubation resulted in increased HDL triglycerides; however, as shown in **Fig. 3**, in the presence of apoA-II (apoA-I knockout mice) the HL inhibitor did not further increase HDL triglycerides, whereas in the presence of apoA-I (apoA-II knockout mice) the HL inhibitor substantially further increased HDL triglyceride levels. The HL inhibitor had no effect on HDL cholesterol levels as expected (not shown). This experiment provides further evidence that apoA-II can serve as a functional inhibitor of HL activity, comparable to exogenously added E600.

DISCUSSION

In the current study, apoA-II and HL double knockout mice were generated to explore the mechanism whereby

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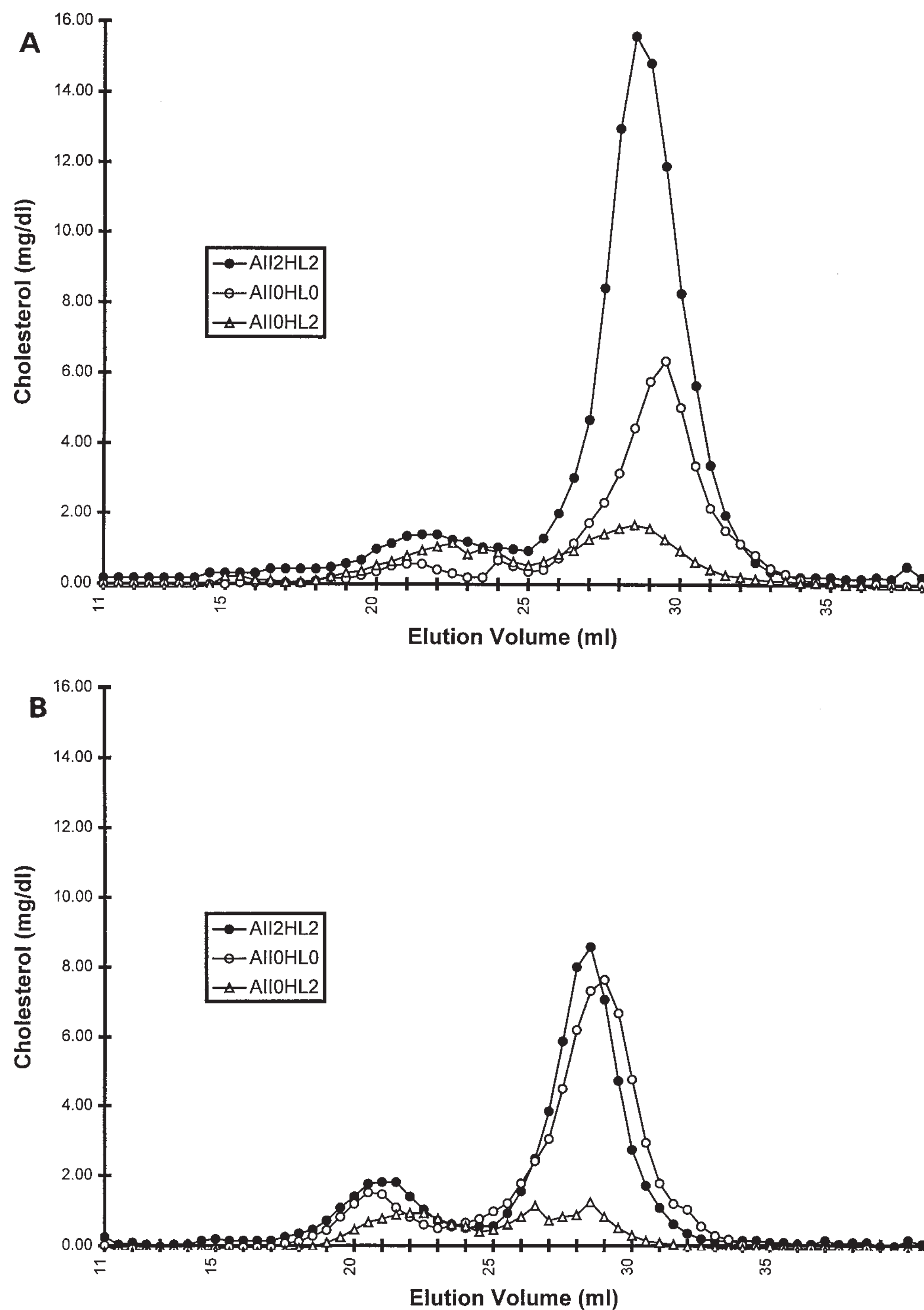

Fig. 1. FPLC fractionation of plasma from apoA-II knockout, double knockout, and wild-type mice on chow diet. Two hundred μl of fasted, pooled, and filtered mouse plasma was applied to two Superose 6 columns (Phamacia) connected in series. The lipoproteins were eluted at a constant flow rate of 0.3 ml/min with 1 mm sodium EDTA and 0.15 m NaCl. Fractions of 0.5 ml were collected and cholesterol concentrations were measured enzymatically. Cholesterol values are shown as mg/dl in each fraction; A, males; B, females.

apoA-II regulates HDL cholesterol levels. Based on published studies with HL knockout mice (11), if there were no interaction between apoA-II and hepatic lipase, double knockouts would have had only a 10–15% increase in HDL cholesterol compared to apoA-II knockout mice rather than the 60% increase observed. Compared with apoA-II deficient mice, mice deficient in both apoA-II and HL had increased HDL size and decreased HDL cholesteryl ester fractional catabolic rate restoring both to near normal levels. In vitro biochemical assays also demonstrated functional inhibition of HL HDL triglyceride hydrolysis in apoA-II but not apoA-I containing HDL. These studies strongly suggest that apoA-II is a physiological inhibitor of HL and that apoA-II maintains HDL cholesterol

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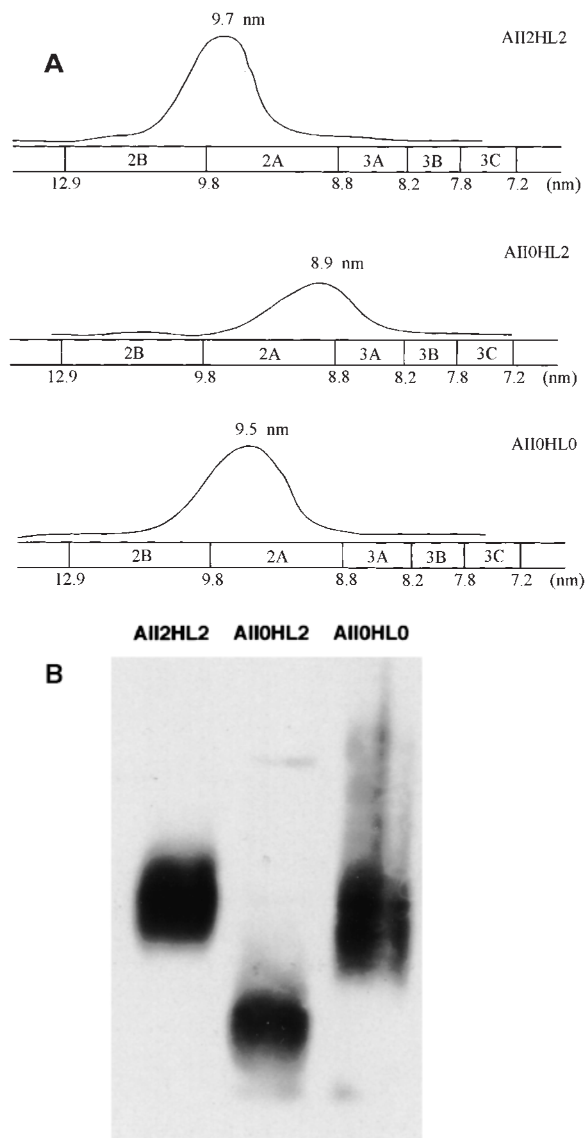

Fig. 2. The distribution of HDL particle size diameters. AII0HL2, AII0HL0, and AII2HL2 represent apoA-II knockout, double knockout, and wild-type control mice, respectively. A: HDL was isolated by sequential ultracentrifugation and HDL from an equal amount of plasma was subjected to non-denaturing gradient gel electrophoresis (4–16% gradient). The gel was then stained and scanned by LKB Laser Densitomerlittermates. B: equal amounts of plasma were subjected to non-denaturing gradient gel electrophoresis (4–16% gradient). The gel was transferred and apoA-I was identified by chemiluminescence reaction.

levels at least in part by inhibition of HL activity. Our studies do not elucidate whether this reflects a direct interaction of apoA-II with HL, or whether apoA-II changes HDL structure and thereby indirectly influences HL activity. Another caveat in extending our studies to humans is that human HL is more than 90% in liver, whereas mouse HL is 80% in plasma. Thus there may be important species differences in apoA-II–HL interaction.

In a previous study, we showed that apoA-II knockout mice had severely diminished HDL cholesterol levels, demonstrating that mouse apoA-II plays an important role

TABLE 2. HDL cholesterol metabolism in apoA-II knockout, double knockout, and wild-type nonfasted mice, respectively

Mouse (Male)	n	HDL-C	HDL CE FCR	HDL CE TR
		mg/dl	*pools/h*	*U*
AII2HL2	6	42.6 ± 3.8	0.18 ± 0.01[a]	7.49 ± 0.45[b]
AII0HL2	5	18.9 ± 3.1	0.30 ± 0.01[c]	5.64 ± 0.18
AII0HL0	4	25.1 ± 3.6	0.22 ± 0.02	5.46 ± 0.53[b]

HDL isolated from controls was labeled with ^{3}H and injected into each genotype mouse; HDL CE FCR, HDL cholesteryl ester fractional catabolic rate; HDL CE TR, HDL cholesteryl ester transport rate.
[a] $P < 0.01$ as double knockouts compared with control mice.
[b] $P < 0.001$ as double knockouts compared with control mice.
[c] $P < 0.001$ as double knockouts compared with apoA-II knockout mice.

in maintaining the plasma HDL pool (9). The exact mechanism underlying this observation was uncertain. One possibility was that apoA-II acted solely as a major structural protein of HDL. However, another possibility was that apoA-II acted as a physiological inhibitor of HL, and that increased functional HL activity decreased HDL cholesterol levels in the apoA-II-deficient mice. The latter possibility gains favor from the current double knockout studies, from various studies in vivo implicating HL as a major regulator of HDL cholesterol levels, and from some in vitro studies demonstrating apoA-II interactions with HL.

In clinical studies it has repeatedly been shown that there is an inverse relationship between heparin-releasable hepatic lipase activity and HDL cholesterol levels (14–17). A sib pair analysis indicates a role for the HL gene in regulation of HDL cholesterol levels and there is an association of an HL gene promoter polymorphism and HDL cholesterol levels (18). In transgenic mice (19) and rabbits (20) the human HL transgene lowered HDL cholesterol lev-

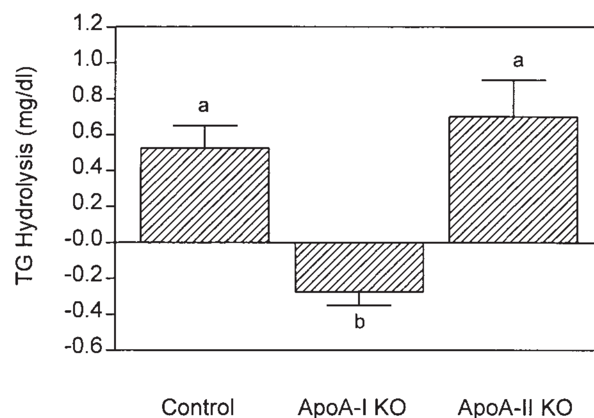

Fig. 3. The difference in plasma HDL triglyceride contents before and after incubation with rCETP and diethyl *p*-nitrophenylphosphate. Two hundred μl pooled plasma was incubated with rCETP (20 μg/ml plasma) and E600 (500 μm). HDL with and without the rCETP and E6000 incubation was then isolated by ultracentrifugation between densities 1.055 and 1.21 g/ml. Triglyceride was determined by enzymatic reaction (Wako Bioproducts). The difference in plasma HDL triglyceride contents before and after the rCETP and E6000 incubation is shown; a and b are significantly different from each other at $P < 0.01$.

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els, and HL knockout mice (11) and HL-deficient humans (21–23) have mildly increased HDL cholesterol levels.

ApoA-II HL interactions have been demonstrated in many in vitro studies. Using a surface monolayer model, Thuren et al. (24) showed that apoA-II inhibits both the penetration of HL into a phospholipid monolayer and the triglyceride hydrolase activity of HL within the monolayer (24). Hime, Barter, and Rye (25) recently showed, using recombinant HDL particles with either apoA-I or apoA-II, that HL has a higher affinity but a much lower maximum rate of hydrolysis for HDL phospholipids and triglyceride for recombinant apoA-II HDL compared to recombinant apoA-I HDL. In another study, Jahn et al. (26) showed that isolated apoA-II could act either as a stimulator or inhibitor of HL depending on assay condition. A number of other in vitro studies have suggested that apoA-II might stimulate the hydrolysis of HDL triglyceride (27–29). Thus it is clear that apoA-II and HL can interact but the role of apoA-II as a physiological inhibitor was affirmed using human apoA-II transgenic mice. In this study in vivo apoA-II prevented HL-mediated HDL triglyceride hydrolysis and reduction in HDL size. In vitro HDL containing human apoA-II inhibited hepatic lipase activity in an emulsion-based assay and resisted the endogenous hepatic lipase activity in mouse plasma (10). In a similar type of assay in the current paper, we showed strong inhibition of HL triglyceride lipase activity in apoA-II but not in apoA-I-containing HDL. These results also support the hypothesis that apoA-II maintains the plasma HDL pool by inhibiting HL.

In this study wild-type female mice were found to have lower HDL cholesterol levels than males. However, in the apoA-II knockout and the apoA-II HL double knockout mice, the male–female difference disappears. This implies that the difference in HDL cholesterol levels between wild-type female and male mice is due to apoA-II or the apoA-II–HL interaction. Sex hormone effects on HL levels and activity have been well documented with androgens increasing HL (30, 31) and estrogens, in some species, decreasing HL (32, 33). There are also some sex hormone effects on apoA-II expression (34, 35).

HDL cholesterol levels were almost completely restored in the double knockout mice. The failure to completely restore levels may have been due to effects of the apoA-II knockout on apoA-I levels. A previous study showed that apoA-II knockout mice had a 45% reduction in apoA-I transport rate (9). Although we did not do apoA-I turnovers in the current experiments, it is probable that this also occurs in the double knockout mice and accounts for the failure to fully restore HDL cholesterol levels to normal.

In summary, utilizing induced mutant mice, we have shown that the severe reduction in HDL cholesterol levels in the apoA-II knockout mouse can be restored almost fully in female and partially in male mice by HL deficiency. These results suggest that the interaction between apoA-II and HL is important to maintaining the plasma HDL pool. It also suggests that postheparin HL activity may differ from functional HL activity, and that the latter may be regulated by the concentration of apoA-II in plasma or in specific HDL particles.

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