Human Apolipoprotein A-II Determines Plasma Triglycerides by Regulating Lipoprotein Lipase Activity and High-Density Lipoprotein Proteome

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Introduction—Apolipoprotein (apo) A-II is the second most abundant high-density lipoprotein (HDL) apolipoprotein. We assessed the mechanism involved in the altered postprandial triglyceride-rich lipoprotein metabolism of female human apoA-II-transgenic mice (hapoA-II-Tg mice), which results in up to an 11-fold increase in plasma triglyceride concentration. The relationships between apoA-II, HDL composition, and lipoprotein lipase (LPL) activity were also analyzed in a group of normolipidemic women.

Methods and Results—Triglyceride-rich lipoprotein catabolism was decreased in hapoA-II-Tg mice compared to control mice. This suggests that hapoA-II, which was mainly associated with HDL during fasting and postprandially, impairs triglyceride-rich lipoprotein lipolysis. HDL isolated from hapoA-II-Tg mice impaired bovine LPL activity. Two-dimensional gel electrophoresis, mass spectrometry, and immunonephelometry identified a marked deficiency in the HDL content of apoA-I, apoC-III, and apoE in these mice. In normolipidemic women, apoA-II concentration was directly correlated with plasma triglyceride and inversely correlated with the HDL-apoC-II+apoE/apoC-III ratio. HDL-mediated induction of LPL activity was inversely correlated with apoA-II and directly correlated with the HDL-apoC-II+apoE/apoC-III ratio. Purified hapoA-II displaced apoC-II, apoC-III, and apoE from human HDL2. Human HDL3 was, compared to HDL2, enriched in apoA-II but poorer in apoC-II, apoC-III, and apoE.

Conclusion—ApoA-II plays a crucial role in triglyceride catabolism by regulating LPL activity, at least in part, through HDL proteome modulation. (Arterioscler Thromb Vasc Biol. 2010;30:232-238.)

Key Words: VLDL ■ chylomicron ■ transgenic mice ■ lipolysis ■ proteomics

Apolipoprotein (apo) A-II is the second most abundant protein, comprising ~20% of high-density lipoprotein (HDL) total protein mass; however, its function remains unclear.1–4 Evidence for a role of apoA-II in triglyceride (TG) and free fatty acid (FFA) metabolism was provided by reports that showed a positive correlation between apoA-II synthesis and very-low-density lipoprotein apoB in humans, and that showed the apoA-II locus was linked to a locus controlling plasma levels of apoA-II and FFA in mice and humans.4 More recently, several clinical studies reported an association between the −265T>C allele affecting the D element of the apoA-II promoter, altered plasma apoA-II concentration, and postprandial metabolism of large triglyceride-rich lipoproteins (TRL).5–7 Further evidence for a role of apoA-II in TG metabolism in humans has been provided by studies of controls and cases with coronary artery disease, and in families with familial combined hyperlipidemia.8,9 Both studies showed a direct association between apoA-II and plasma TG levels. However, the mechanism whereby apoA-II affects TG metabolism in humans is largely unknown.

A direct effect of apoA-II on TG and FFA metabolism is also supported by studies conducted in genetically modified mice. Mice overexpressing mouse and human apoA-II (hapoA-II) exhibit marked hypertriglyceridemia and increased FFA.1,10,11 Knocking out of apoA-II in mice decreased plasma TRL concentration and increased remnant catabolism.12 However, whether apoA-II variability causes increased TRL synthesis, decreases TRL catabolism, or both, remains controversial.4 Our hapoA-II transgenic mice (hapoA-II-Tg mice) fed an atherogenic, cholate-containing...
diet or cross-bred with apoE knockout (KO) mice presented higher very-low-density lipoprotein production and normal very-low-density lipoprotein TG catabolism and postheparin lipolytic activities when analyzed in fasting conditions.

In contrast, an independently generated line of hapoA-II-Tg mice exhibited marked postprandial hypertriglyceridemia, which was associated with an increase in plasma hapoA-II compared to fasted mice, and impaired lipolysis of TRL enriched with hapoA-II.

To gain more insight into the role of human apoA-II in TG metabolism, we conducted a detailed study of TRL and HDL in our hapoA-II Tg mice and in a group of normolipidemic women. Our data show that accumulation of hapoA-II increases plasma TG through an impaired HDL induction of lipoprotein lipase (LPL) activity that depends, at least in part, on changes induced in the protein composition of discrete HDL subfractions.

Materials and Methods

The hapoA-II-Tg mice were developed as previously described. Twenty-three healthy women were enrolled in the study, which was approved by the Ethics Committee for Clinical Investigation of the Hospital de la Santa Creu i Sant Pau. For further experimental details, see the online data supplement at http://atvb.ahajournals.org.

Results

Mouse Plasma Lipid and Lipoprotein Profile and Functional Distribution of TG After Oral Fat Gavage

The hapoA-II-Tg mice used in this study displayed 2.5-fold higher hapoA-II plasma levels than those of normolipidemic humans. No differences were found in food consumption among mice of different genotypes (4.1 ± 0.3 in control mice vs 3.9 ± 0.3 grams/day in hapoA-II-Tg). Plasma TG levels in female hapoA-II-Tg mice were maximal between 2 hours and 3 hours after the oral fat gavage (OFG), and at that time were 11-fold higher than those in control mice (Figure 1A). Plasma TG in male hapoA-II-Tg mice were 7.5–fold higher 3 hours after the OFG (5.9 ± 1.0 vs 0.8 ± 0.1 mmol/L in controls). Because females were more prone to hypertriglyceridemia, we conducted a series of experiments in our female hapoA-II-Tg mice. Radiolabeled TG mixed with olive oil was individually gavaged, and the radiolabeled TG was determined in plasma and target tissues at the times indicated (Figure 1B, C). Plasma levels of radiolabeled TG in hapoA-II-Tg mice were significantly higher than those of control mice after 3 hours of OFG administration (Figure 1B). At that time, a very high percentage (>99%) of radioactivity present in the lipoprotein-depleted fraction was mainly attributable to FFA, and these were found to be significantly decreased in hapoA-II-Tg mice (1.5%) compared to control mice (8.9%). The hapoA-II-Tg mice showed decreased radiolabeled TG in parametrial White Adipose Tissue (pWAT) 6 hours after OFG (Figure 1C).

Because hypertriglyceridemia is common in patients with inherited HDL deficiency, and because hapoA-II is known to displace mouse apoA-I from the HDL surface, we determined postprandial plasma TG profiles after an OFG in female apoA-I KO and apoA-II KO mice (Figure 1D). Although potential changes in postprandial TG could be observed and were consistent with previous studies, the area under the curve of TG after the OFG in apoA-I KO and apoA-II KO mice did not significantly differ from that of control mice (Figure 1D). This may be because of the limited number of animals studied. However, overall, these results suggested that the increased postprandial TG in hapoA-II-Tg mice was not attributable to their decreased HDL.

Postprandial plasma hapoA-II concentration in the hapoA-II-Tg mice used in the present study (82.6 ± 5.3 mg/dL) did not differ significantly from that found in fasting plasma (75.0 ± 4.2 mg/dL). Fast protein liquid chromatography (FPLC) analyses of postprandial plasma revealed a dramatic increase in TG and cholesterol amount and size of the TRL particles of hapoA-II-Tg mice compared with those of control mice (Figure 2A, B, C).
respective). Decreased HDL cholesterol in hapoA-II-Tg mice was not more pronounced postprandially than in fasting conditions (Figure 2B). The hapoA-II was mainly found in the HDL fraction (77.8% / 110.6 mg/dL), but small amounts could be detected in chylomicrons (3.1% / 0.5 mg/dL; Figure 2C, D). Sixty-eight percent of plasma TG in hapoA-II-Tg mice floated as chylomicrons (Figure 2D). The TG-to-protein ratio confirmed that postprandial plasma transgenic TRL were significantly larger than those of control TRL (online Figure I).

Effect of hapoA-II Expression on TRL Plasma Turnover in Mice

In vivo analyses were performed to evaluate the causes of postprandial hypertriglyceridemia in hapoA-II-Tg mice. Data indicated that TG intestinal and liver secretion in hapoA-II-Tg mice did not significantly differ from those of control mice (Figure 3A, B). In vivo catabolic studies with autologous [3H]-TG-TRL showed that the fractional catabolic rate of hapoA-II-Tg-mice [3H]-TG-TRL was significantly reduced in plasma of hapoA-II-Tg mice compared to that of control mice under postprandial conditions (Figure 3C, D). In contrast, [3H]-TG-TRL fractional catabolic rate did not significantly differ between genotypes under fasting conditions. Changes in TG clearance in hapoA-II-Tg mice under postprandial conditions may reflect the dilution of [3H]-TG-TRL in an expanded TRL pool. For this reason, we also performed cross-infusion experiments consisting of the injection of labeled TRL from transgenic mice into control mice, and labeled TRL from control mice into hapoA-II-Tg mice (see the 2 last bars of Figure 3D). Data indicated that control [3H]-TG-TRL clearance from plasma of hapoA-II-Tg mice was significantly delayed compared to that in control mice, whereas hapoA-II-Tg [3H]-TG-TRL were cleared from plasma of control mice as rapidly as the autologous control [3H]-TG-TRL (Figure 3D). This could indicate that the plasma content, rather than TRL origin, was the main determinant of the changes in TRL catabolism. Although postheparin postpran-

dial or fasting plasma LPL activity toward an artificial triolein-based substrate in hapoA-II-Tg mice did not differ from control mice (Figure 3E), coincubation with HDL of control mice increased bovine LPL activity 60% more than coincubation with apoA-II-Tg mice HDL (Figure 3F). In addition, we conducted experiments to test the ability of HDL from hapoA-II-Tg mice and control mice to modulate in vitro bovine LPL activity toward TRL (data not shown). Coincubation of TRL with HDL from control mice and hapoA-II-Tg mice at ratios similar to those found in vivo in each group of mice (Figures 1 and supplemental Figure I) resulted in 4-fold more enzymatic activity in the former condition compared with the latter. To rule out the possibility that the redistribution of hapoA-II in chylomicrons could affect LPL activity, we incubated control chylomicrons with free apoA-II at 37°C for 3 hours. The rate of FFA formation in apoA-II-enriched chylomicrons, at a hapoA-II concentration of 3 mg/dL (which represents ~4% of the total concentration of proteins in chylomicrons), was similar to that of control chylomicrons (13.8±0.9 vs 11.9±0.7 nmol of FFA/mL per minute).

Effect of hapoA-II on Plasma and HDL-Associated Apolipoproteins in Mice

HDL protein composition was studied by 2-dimensional gel electrophoresis. This analysis showed the existence of 10 significant, differentially expressed spots (Figure 4A) and 2 hapoA-II isoforms in apoA-II-Tg mice HDL. Identification of these spots was performed by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Figure 4A, B). Two spots contained a mixture of paraoxonase 1 and an apoA-I isoform; thus, the respective contributions to their decreased abundance could not be determined. To confirm these changes, we measured several available murine apolipoproteins by immunonephelometry in plasma and in HDL obtained by ultracentrifugation (Figure 4C, D). This method confirmed that the amount of apoC-III was almost negligible in hapoA-II-Tg mice HDL, whereas
plasma apoC-III levels were 3-fold higher in postprandial conditions, thereby indicating a redistribution of this apolipoprotein to TRL (Figure 4C, D). Again, apoA-I was decreased in plasma and in HDL in fasting and postprandial conditions (Figure 4C, D).

**Human Studies**

In a group of healthy normolipemic women (n=23), hapoA-II concentration was found to be directly correlated with plasma TG ($r=0.72$; $P<0.001$; Figure 5A). HDL of these women was incubated in the presence of a triolein-based emulsion
and bovine LPL. ApoA-II was found to be inversely correlated with HDL-mediated induction of LPL activity ($r = -0.45; P < 0.04$; Figure 5B). HDL modulation of LPL activity was also directly correlated with the HDL-apoC-II/apoE/apoC-III, HDL-apoC-II/apoC-III, and apoA-V/apoA-I ratios (see supplemental Table II for detailed correlation coefficients); however, only the first parameter significantly correlated with apoA-II ($r = -0.41; P < 0.05$; Figure 5C).

**Effect of hapoA-II on Human HDL Apolipoprotein Content**

Coincubation of human HDL2 with purified hapoA-II clearly decreased the intensity of the immunoreactive bands, both in denaturing and nondenaturing conditions, corresponding to human apoC-II, apoC-III, and apoE when analyzed by Western blot (Figure 6A). This incubation also generated a novel peak that contained mainly apoA-II and apoA-I (data not shown). ApoA-II was preferentially located in HDL3, and this was concomitant with a strongly decreased content of apoC-II, apoC-III, and apoE in HDL3 compared to that of HDL2 (Figure 6B).

**Discussion**

Evidence favoring a role for apoA-II in TG metabolism stems from experiments in mice and, also, human studies. However, the precise mechanism by which hapoA-II influences TG metabolism remains unclear. In the current study, we show that overexpression of human apoA-II in transgenic mice increased postprandial plasma TG concentration. A similar degree of postprandial hypertriglyceridemia was also found in the double hapoA-IIxCETP-Tg mice having a hapoA-II plasma concentration of 42 mg/dL.
thereby indicating that the effect of hapoA-II on TG metabolism also occurs at more physiologically significant levels. Because liver and intestinal TG secretion was comparable between hapoA-II-Tg mice and control mice, we focused our study on postprandial TRL catabolism. Our data clearly show that the hypertriglyceridemia of hapoA-II-Tg mice was primarily caused by a decrease in TRL clearance. An important question is whether postprandial plasma TG accumulation resulted from the association of hapoA-II with TRL. In a previous report using independently generated hapoA-II-Tg mice, severe postprandial hypertriglyceridemia was associated with a marked increase in plasma hapoA-II concentration in TRL particles, which were found to be poor LPL substrates. In contrast, plasma hapoA-II of our hapoA-II-Tg mice was mainly found associated with HDL during the postprandial state, and we found no nearly complete depletion of the HDL fraction nor a fed-to-fasted switch in hapoA-II concentration. It is possible that the different hapoA-II gene constructions used to generate these 2 hapoA-II-Tg mice could explain their differences in hapoA-II plasma concentration regulation in these 2 different transgenic models. Increased catabolism and decreased production of HDL were the main mechanisms leading to the partial HDL deficiency in our hapoA-II-Tg mice. These changes seemed to be more severe in the other transgenic model, leading to severe hapoA-II accumulation in TRL. Taken together, our data indicate that the amount of hapoA-II present in TRL in our hapoA-II-Tg mice did not impair its catabolism.

ApoA-II variability was also correlated with the concentration of TG in healthy normolipidemic women with hapoA-II levels ranging from 22.5 to 45.4 mg/dL. In agreement with data from our hapoA-II-Tg mice, hapoA-II concentration was found to be directly correlated with plasma TG 3 hours after the fat-loading test ($r=0.59$; $P<0.05$) in 13 subjects who were given a fatty meal with 1 gram of fat per kilogram of body weight, whereas that with HDL cholesterol was only close to significance ($r=-0.51$; $P=0.073$). Importantly, plasma apoA-II levels were unaffected by the fat-loading test (34.8±1.4 vs 35.6±1.5 mg/dL under fasting conditions), and TRL contained negligible amounts of apoA-II. This finding strongly suggests that the marked increase in hapoA-II in TRL that leads to LPL inhibition is not a physiological mechanism in humans. Nevertheless, a direct LPL inhibition by apoA-II may indeed occur in pathological situations, such as hypertriglyceridemia associated with Tangier disease and type V hyperlipidemia in humans, in cases of extreme HDL deficiency in apoA-II-Tg mice, and when apoA-II accumulates in TRL, as in mouse apoA-II-Tg mice. This and other major differences between human and mouse apoA-II, such as the divergent effect on HDL levels, had been attributed to species-specific differences in amino acid sequence, dimer formation, and affinity for lipoprotein surfaces. The reported inhibition of LPL by apoA-II may also be extendable to other lipases, such as hepatic lipase and endothelial lipase.

Data obtained in our hapoA-II-Tg mice suggested that the presence of hapoA-II in HDL might influence HDL apolipoprotein composition or apolipoprotein transfer to TRL, thereby altering TRL postprandial catabolism. To test this hypothesis in detail, LPL assays were performed using mouse HDL from each genotype as the activator apolipoprotein source. Our results clearly indicated that the ability of hapoA-II HDL to modulate LPL activity was lower than that induced by control HDL. We also found that apoA-II levels showed an inverse correlation with the capacity of HDL to induce LPL activity in healthy normolipidemic volunteers in absence of plasma as a source of LPL-activating apolipoproteins, and this parameter was also strongly correlated with the HDL-apoC-II+/apoE/apoC-III ratio. ApoC-II is well-known to be an effective activator of LPL activity, whereas apoC-III acts as an inhibitor. ApoE may also exert enhancing effects on the action of LPL, although apoE is mainly a ligand of different cellular lipoproteins. It is also well-known that at least part of apoC-II, apoC-III, and apoE is acquired by TRL from the HDL reservoir during the postprandial state; thus, the alteration in HDL composition during fasting may strongly affect TRL metabolism and plasma TG levels.

Therefore, it is conceivable that an altered concentration and, therefore, transfer of these HDL apolipoproteins impaired TG catabolism in individuals with high hapoA-II levels. The hapoA-II-Tg mice provided a useful model to gain more insight into this question. Proteomic and immunonephelometric analyses of the HDL of hapoA-II-Tg mice did demonstrate a decreased content in apoA-I and paraoxonase 1, a deficiency previously reported to be caused by displacement of the HDL surface by a high hapoA-II content, and also demonstrated a deficiency in the HDL content of apoC-III and apoE. Thus, it is possible that hapoA-II-Tg mice HDL is a poor acceptor for apoC-III, thereby causing redistribution to TRL during the postprandial state, which impairs LPL activity. Alternatively, apoC-III accumulation in postprandial TRL of hapoA-II-Tg mice could be the consequence, rather than the cause, of the hypertriglyceridemia. ApoC-II, the main cofactor required for LPL activity, was not identified by proteomic analysis and could not be measured by immunonephelometry. Previous reports showed that HDL3 presented a higher apoA-II-to-apoA-I ratio than HDL2 and apoC-II, apoC-III, and apoE were found to be mainly associated with HDL2 whereas apoC-II, apoC-III, and apoE were found to be mainly associated with HDL2. We therefore analyzed whether human HDL subfractions enriched with apoA-II were poorer in apoC-II, apoC-III, and apoE. This was the case, as demonstrated by quantifying these apolipoproteins in pools of HDL2 and HDL3. In an attempt to ascertain whether hapoA-II could affect apoC-II content and whether there was a common mechanism responsible for the rest of the changes found in HDL apolipoproteins, we incubated purified hapoA-II with human HDL2 and found that the increased association of hapoA-II with HDL2 caused a decrease in the content of apoC-II, apoC-III, and apoE. The displacement of these apolipoproteins, especially apoC-II, from HDL and the consequent altered transfer to TRL may thus explain, at least in part, the association found between hapoA-II with TG and with the capacity of HDL to activate LPL, both in mice and humans. These results also demonstrate that hapoA-II is a major regulator of the apoC-II, apoC-III, and apoE distribution in discrete HDL particle subfractions, as previously shown with paraoxonase. This is likely to be attributable to the higher affinity of hapoA-II for the HDL surface compared with these apolipoproteins. Therefore, although
TRL apolipoprotein composition is recognized as modulator TG metabolism, our results indicate that HDL composition could play an active role in this process. Further, based on our observations, we speculate that the relative decrease in HDL2 found in hypertriglyceridemia could play an active role in its pathophysiology by decreasing LPL activity.

In conclusion, our study shows that hapoA-II excess in HDL may contribute to postprandial hypertriglyceridemia by inhibiting TRL lipolysis mediated by LPL attributable, at least in part, to apoC-II, apoC-III, and apoE displacement from the HDL surface. Because the results of this displacement are consistent with the differential distribution of apoA-II and apoC-II, apoC-III, and apoE in HDL2 and HDL3, it is suggested that hapoA-II has a role in determining the proteome of discrete HDL particle subfractions.

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Disclosure

None.

References


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In the article “Human Apolipoprotein A-II Determines Plasma Triglycerides by Regulating Lipoprotein Lipase Activity and High-Density Lipoprotein Proteome” by Julve et al, which appeared in the February 2010 issue of the journal (Arterioscler Thromb Vasc Biol. 2010;30:232–238; DOI: 10.1161/ATVBAHA.109.198226), several errors were introduced during copyediting that the publisher failed to correct in the final, published version:

1. “HDL-to-apoC-II+apoE/apoC-III ratios” should have been “the HDL-apoC-II+apoE/apoC-III ratio” in the following locations:
   a. Page 232, abstract, Methods and Results, lines 6 and 8 (two instances).
   b. Page 236, Figure 5 legend, lines 5 to 6.
   c. Page 237, right column, line 9.


The online version of the article has been corrected.

The publisher sincerely regrets the errors.

DOI: 10.1161/ATV.0b013e3181df5360
In the article, “CD11c Expression in Adipose Tissue and Blood and Its Role in Diet-Induced Obesity” by Wu et al, which appeared in the February 2010 issue of the journal *Arterioscler Thromb Vasc Biol*. 2010;30:232–238; DOI: 10.1161/ATVBAHA.109.198044), the publisher omitted several important corrections from the final published version:

1. Page 187, 5th line of “Animal Models” section: LabDiet is located in Brentwood, Mo, not Brentwood, Miss.
2. Page 187, 1st column, 6th and 7th lines from bottom: the multiplication symbol should not be superscripted, for the final version to read: “. . .(measured in micro-international units per milliliter) × fasting glucose (measured in millimoles per liter)/22.5. . .”
3. Page 189, 11th line of “Effect of CD11c Deficiency on Macrophage Activation Markers and Chemokines in Mouse” section: CD11b⁺/MGL1 should have appeared as CD11b⁺/MGL1–.
4. Page 189, 2nd column, 8th line: “AT of” should have been inserted before “obese WT” for the correct version to read: “. . .also lower in AT of obese CD11c–/– than in AT of obese WT. . .”
5. Page 190, Figure 5 legend, 5th and 6th lines from bottom: “projection” should have been deleted for the final version to read: “. . .macrophage inflammatory protein (MIP). . .”
6. Page 191, 1st column, 2nd paragraph, line 2: CD11c⁺ should have appeared as CD11c–.
7. Page 191, 1st column, 3rd paragraph, line 5: “an” should have been deleted before “HFD” for the final version to read: “. . .resistance induced by HFD.”

The online version has been corrected.

The publisher sincerely regrets the errors.

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Extended Methods

Mice and diets
HapoA-II-Tg (line 11.1) were developed as previously described.1 ApoA-I- (apoA-I-KO) and apoA-II-deficient mice (apoA-II-KO) were kindly provided by Dr. Jesús Osada and Dr. Van der Westhuyzen, respectively (University of Zaragoza, Spain, and University of Kentucky Medical Center). Two-month-old female C57BL/6 (control mice) and hapoA-II-Tg and apoA-I-KO and apoA-II-KO were maintained on a regular chow diet (Rodent Toxicology Diet; B&K Universal, UK) containing 0.02% cholesterol for 3 months. Food and water were available ad libitum. All animal procedures were considered and approved by the Institutional Animal Care Committee of the Institut de Recerca de l’Hospital de la Santa Creu i Sant Pau, Spain.

Plasma lipids and lipoproteins
Plasma total cholesterol, HDL cholesterol, phospholipids, TG and FFA were determined enzymatically using commercial kits adapted to an BM/HITACHI 911 autoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland). 2,3 TG determinations were corrected for the free glycerol present in plasma (Sigma Diagnostics, St. Louis, MO).2 Plasma and HDL levels of murine apoA-I, apoB and apoC-III were measured by immunonephelometric assay using specific mouse polyclonal antibodies.3 HapoA-II, apoC-II, apoC-III, and apoE levels were determined using nephelometric commercial kits (Kamiya, Biomedical Company, Seattle, WA) adapted to an BM/HITACHI 911 autoanalyzer. HapoA-V was determined as described.4 The lipoproteins of 0.2 mL of pooled filtered plasma were fractionated by fast performance liquid chromatography (FPLC) using a Superose 6HR column (Roche Diagnostics) and their content in cholesterol and TG measured as described above.2 Chylomicrons were isolated by ultracentrifugation at 10,000 g at 4°C for 30 min. VLDL, IDL+LDL and HDL were
isolated by sequential ultracentrifugation at 100,000 g for 24 h at densities 1.006, 1.063 and 1.210 g/mL, respectively.\textsuperscript{2–5} The TRL fraction used throughout the study consisted of lipoproteins with a density < 1.006 g/mL which includes VLDL in fasting conditions and chylomicrons and VLDL after postprandial TG response. Lipoprotein protein concentrations were determined by the BCA method (Pierce, Brebières, France).

**Postprandial TG response**

A plasma basal sample was collected by tail bleeding of mice fasted overnight. Each mouse received an oral fat gavage (OFG) consisting of an intragastric administration load of 150 µL of olive oil (Carbonell, Madrid, Spain; 79% of oleic acid). Subsequently, blood samples (75 µL) were taken at the times indicated. To reduce the stress effects of repeated samples, each animal underwent only three postload bleeds, with different animals bled at each time point. Plasma TG were determined enzymatically as described above.\textsuperscript{2,3} For *in vivo* TRL studies and LPL activity assays, larger quantities of TRL and HDL were isolated by sequential ultracentrifugation from pooled plasmas of apoA-II-Tg and control mice fasted overnight and 3h after an OFG.

**Distribution of intragastrically-administered [\textsuperscript{3}H]-TG in mice**

Mice were given an OFG consisting of 20 µCi \textsuperscript{3}H]-labeled triolein (glycerol tri[9, 10(n)-\textsuperscript{3}H]oleate, 21.0Ci/mmol; Amersham Biosciences, Buckinghamshire, UK) in 150 µL of olive oil. Mice were bled by cardiac puncture at the times indicated after the OFG, and target tissues (liver, parametrial white adipose tissue –pWAT–, heart, and leg muscle) were collected after perfusion with 0.9 % NaCl solution. Radiolabeled plasma and tissue TG were separated from FFA using methanol:chloroform:heptane 1.4:1.25:1 (v:v:v) and 0.1 mol/L \textsubscript{3}BO\textsubscript{3}-KCO\textsubscript{3} at pH 10.5.\textsuperscript{6} The radioactivity in the TG and FFA fraction in total plasma and target tissues were determined by scintillation counting.\textsuperscript{6} Lipoprotein fractions were also isolated from pooled plasma at the times indicated after
an OFG and radiolabeled TG and FFA associated with each lipoprotein and non-lipoprotein plasma fraction determined.

**In vivo intestinal TG absorption**

Mice were fasted overnight and received an OFG of 10 µCi glycerol tri[9,10(n)-3H]oleate (Amersham Biosciences) in 150 µL of olive oil directly after intravenous injection of 500 mg/Kg of Triton WR-1339 (Sigma) dissolved in a 15% (v/v) solution of 0.9% NaCl solution to completely block LPL-mediated lipolysis. Blood was collected after intragastric loading at the times indicated. Plasma TG were separated from FFA using methanol/chloroform/heptane mix and radioactivity in the TG fraction determined by scintillation counting.

**Liver TG production**

Hepatic TG production rates in plasma were measured in mice fasted overnight and 3h after an OFG. Anesthetized mice were injected intravenously with 500 mg/Kg of Triton WR-1339 (Sigma) and 10 µCi of [3H]-oleic acid (Amersham Biosciences) complexed to albumin. Blood was collected after Triton injection at the times indicated and the TG radioactivity determined by scintillation counting.

**In vivo clearance of TRL**

The TRL were isolated by ultracentrifugation from plasma of mice after fasting or 3 h after an OFG and were radiolabeled with glycerol tri[9,10(n)-3H] oleate ([3H]-TG-TRL) as previously described. In all cases, 500,000 cpm of the TRL fraction in 100 µL of 0.9% NaCl were injected i.v. into anesthetized mice from each group and serial blood samples were collected for [3H] triglyceride counting. In a preliminary experiment, 90-95% of the label was present in the VLDL plasma fraction as glycerol tri[9,10-(n)-3H]oleate throughout the experiment. Thus, in the following experiments, only remaining total plasma radioactivity was determined. In vivo catabolic studies were
performed with autologous and heterologous [3H]-TG-TRL, respectively. Autologous [3H]-TG-TRL consisted of radiolabeled TRL isolated and injected into mice of the same genotype from which they were previously generated. Heterologous [3H]-TG-TRL consisted of radiolabeled TRL prepared and isolated from plasma of one genotype and injected into the other genotype and their plasma clearance studied. In all mice, the injection of labeled lipid lipoprotein was less than 10% of total mass plasma TG. Fractional catabolic rate (FCR) was calculated using the reciprocal area under the curve.7

**Plasma LPL levels**

LPL activities were measured in hapoA-II-Tg and control mice fasted overnight and 3h after an OFG. In all cases, mice were injected intraperitoneally with 500 U/kg of heparin (Laboratorios Farmaceúticos Rovi, Madrid, Spain). Blood was collected 30 min later and plasma was stored at –80°C until the assays. LPL activity was determined using a glycerol tri[9,10(n)-3H]oleate-based emulsion as previously described.3, 7

**Fluorescence 2-D Gel Electrophoresis and MALDI-TOF Mass Spectrometry**

HDL were isolated as described above. A mixture of 20 µg of protein per gel was diluted two-fold with IEF sample buffer and subjected to 2-D DIGE using GE-Healthcare reagents and equipment (Amersham Biosciences) as previously described.9 Briefly, samples were added to a 7-cm IPG strip (pH 3.5-5.6, non linear IPG DryStrips, Amersham Biosciences) and transferred for the second dimension SDS-PAGE proceed using 4-16% gradient gels (Biorad, Barcelona, Spain). Gels were labeled with Flamingo fluorescent gel stain (Biorad) and the images scanned using a Thyphoon 9400. Image analysis of 2D and quantification of relative protein abundances were performed using SameSpots V. 3.3 software. Protein spots of interest were excised from the gel and subjected to trypsin digestion using autolysis-stabilized trypsin (Promega, Madrid,
Spain). MALDI-TOF analysis of tryptic peptides was performed on an Ultraflex TOF-TOF Instrument (Bruker, Wissembourg, France) as described previously.9

**In vitro HDL-mediated LPL activation assays**

To assess the ability of HDL samples from different mice to serve as LPL activators, a tri[9,10(n)-3H] oleate-based emulsion was prepared as described above without addition of plasma. Mouse HDL at a final concentration of 0.5 g/L was then added and the final volume was adjusted to 200 μL with LPL reaction buffer (50 mM MgCl2, 0.05% fatty acid-free-BSA and 25 mM PIPES, pH 7.5). Mouse HDL samples were obtained by ultracentrifugation as described above. The emulsion was incubated at 37ºC for 60 min to allow activation. A standard source of purified bovine LPL (Sigma Diagnostics) was then added and the assay was allowed to proceed for 60 min at 27ºC.7 All assays were performed in triplicate and LPL activity expressed as TG hydrolysis rate.7

The endogenous TRL fraction isolated by ultracentrifugation from both control and apoA-II-Tg after OFG, coincubated with their own HDL was also used as substrate in lipolysis experiments.3 Enzyme velocity was measured as a function of substrate concentration and Vmax values were found at 0.5mM of triglycerides. Thus, TRL at a final volume of 60 μL were adjusted to 0.5 mM in 2% (w/v) FFA-BSA and 0.1 M Tris-ClH, pH 8.5 and incubated with HDL at different protein concentrations for 60 min at 37ºC. These samples were then incubated with 0.2 U of bovine LPL (Sigma) for 10 min at 37ºC. Reaction was stopped by the addition of 50 mM KH2PO4/0.1% (v/v) Triton X-100. The FFA released were determined enzymatically using commercial kits adapted to a BM/HITACHI 911 autoanalyzer (NEFA C, Wako Chemicals, Neuss, Germany). The rate of FFA formation was expressed as nmols of FFA released per mL per min.3

**Human studies**
Twenty-three healthy apoE3/apoE3 women with no family history of premature cardiovascular disease were enrolled in the study, which was approved by the Ethics Committee for Clinical Investigations, Hospital de la Santa Creu i Sant Pau. All volunteers presented plasma TG less than 2.0 mM (Online Table 1) and none had diabetes, liver or kidney disease or were taking medication known to affect plasma lipids and apolipoproteins. After an 11-h overnight fast, blood samples were collected into EDTA-containing tubes. ApoE genotyping was carried out with a predesigned validated assay (Assays-on-Demand) on an Applied Biosystems Prism 7000 sequence detection system (Applied Biosystems). Plasma was separated from cells by centrifugation and plasma lipids and apolipoproteins determined as described above.\textsuperscript{2-5} 

\textit{In vitro} HDL-mediated LPL activation assays were performed as described above with an artificial \(^{[3]H}\)-TG-based emulsion and HDL isolated by sequential ultracentrifugation.\textsuperscript{2-5} HDL2 (d 1.063 to 1.125 g/mL) and HDL3 (d 1.125 to 1.21 g/mL) were isolated by ultracentrifugation from pooled plasma samples. HDL2 used for hybridization assays with hapoA-II was isolated by ultracentrifugation from pooled human plasma and dialyzed against PBS pH 7.4 as described.\textsuperscript{1} To investigate the apoA-II-mediated displacement of the HDL2 apolipoproteins, a previously reported method was used with minor modifications.\textsuperscript{9} Briefly, working solutions of both lipoproteins and apolipoproteins, and egg lecithin-cholesterol vesicles were prepared as described previously.\textsuperscript{9} Hybridization of HDL-2 with purified hapoA-II (Sigma) at a final concentration of 60 mg/dL was carried out under nitrogen in PBS pH 7.4 at 23°C by mixing the two components and stirring for 1 h, followed by the addition of lecithin-cholesterol vesicles in a molar ratio of vesicle to HDL of 1:3 for 30min to trap free apolipoprotein species from solution.\textsuperscript{9} Apolipoprotein distribution among components were resolved by FPLC and determined by western blot analysis using anti-apoC-II,
apoC-III, apoE, and apoA-II specific goat polyclonal antibodies, respectively. ApoA-I was determined using a nephelometric commercial kit.

**Statistical Methods**

Mann-Whitney U test was used to compare data obtained from hapoA-II-Tg and control mice under both fasting conditions and after an oral fat test. The relationship between apoA-II and other lipid and lipoprotein variables was tested using Pearson’s correlation test. GraphPad Prism 4.0 software (GraphPad, San Diego, CA) was used to perform all statistical analyses. A P value < 0.05 was considered statistically significant.


**Online Table 1.** Mean, SEM, minimum and maximum and lower and upper 95% CI of mean of lipid, lipoprotein and apolipoprotein parameters in healthy apoE3/apoE3 normolipemic women (n=23).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean±SEM</th>
<th>(Minimum-maximum)</th>
<th>Lower 95% CI of mean</th>
<th>Upper 95% CI of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
<td>4.45±0.15</td>
<td>(3.36-5.89)</td>
<td>4.13</td>
<td>4.76</td>
</tr>
<tr>
<td>HDLc</td>
<td>1.61±0.07</td>
<td>(0.94-2.26)</td>
<td>1.46</td>
<td>1.76</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>0.75±0.07</td>
<td>(0.37-1.90)</td>
<td>0.60</td>
<td>0.90</td>
</tr>
<tr>
<td>FFA</td>
<td>0.42±0.04</td>
<td>(0.11-0.82)</td>
<td>0.32</td>
<td>0.51</td>
</tr>
<tr>
<td>ApoA-I</td>
<td>1.52±0.06</td>
<td>(1.06-2.36)</td>
<td>1.40</td>
<td>1.64</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>30.9±1.13</td>
<td>(22.51-45.35)</td>
<td>28.5</td>
<td>33.2</td>
</tr>
<tr>
<td>ApoB</td>
<td>0.71±0.04</td>
<td>(0.42-1.22)</td>
<td>0.63</td>
<td>0.80</td>
</tr>
<tr>
<td>ApoC-II</td>
<td>3.12±0.22</td>
<td>(1.68-6.10)</td>
<td>2.67</td>
<td>3.57</td>
</tr>
<tr>
<td>ApoC-III</td>
<td>19.2±0.90</td>
<td>(6.57-27.67)</td>
<td>17.32</td>
<td>21.06</td>
</tr>
<tr>
<td>ApoE</td>
<td>4.06±0.20</td>
<td>(3.01-6.90)</td>
<td>3.65</td>
<td>4.46</td>
</tr>
<tr>
<td>ApoA-V</td>
<td>264.7±15.0</td>
<td>(152.2-375.0)</td>
<td>233.5</td>
<td>296.0</td>
</tr>
</tbody>
</table>
Online Table 2. Correlations of plasma triglycerides and HDL-mediated LPL coactivation in 23 apoE3/apoE3 normolipidemic women.

<table>
<thead>
<tr>
<th></th>
<th>Triglycerides</th>
<th>HDL-mediated LPL activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>P</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.72</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>-0.19</td>
<td>0.396</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0.28</td>
<td>0.201</td>
</tr>
<tr>
<td>Plasma ApoA-I</td>
<td>0.26</td>
<td>0.224</td>
</tr>
<tr>
<td>Plasma ApoA-II</td>
<td>0.72</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Plasma ApoB</td>
<td>0.65</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Plasma ApoC-II</td>
<td>0.40</td>
<td>0.056</td>
</tr>
<tr>
<td>Plasma ApoC-III</td>
<td>0.60</td>
<td>0.002*</td>
</tr>
<tr>
<td>Plasma ApoC-II/ApoC-III</td>
<td>0.03</td>
<td>0.895</td>
</tr>
<tr>
<td>Plasma ApoE</td>
<td>0.20</td>
<td>0.370</td>
</tr>
<tr>
<td>Plasma ApoA-V</td>
<td>-0.11</td>
<td>0.611</td>
</tr>
<tr>
<td>HDL ApoC-II/ApoA-I</td>
<td>-0.35</td>
<td>0.104</td>
</tr>
<tr>
<td>HDL ApoC-III/ApoA-I</td>
<td>-0.08</td>
<td>0.714</td>
</tr>
<tr>
<td>HDL ApoC-II/C-III</td>
<td>-0.41</td>
<td>0.049*</td>
</tr>
<tr>
<td>HDL ApoE/ApoA-I</td>
<td>-0.45</td>
<td>0.031*</td>
</tr>
<tr>
<td>HDL apoC-II+apoE/apoC-III</td>
<td>-0.44</td>
<td>0.037*</td>
</tr>
<tr>
<td>HDL apoA-V/apoA-I</td>
<td>-0.42</td>
<td>0.0047*</td>
</tr>
</tbody>
</table>
Online figure legend

**Online figure 1. Plasma concentration and relative composition of TRL and HDL in hapoA-II-Tg mice and control mice.** TRL (d<1.006 g/mL) (A and B) and HDL (C and D) were isolated from pooled plasma of postprandial mice by ultracentrifugation. The relative amount (%) of each parameter in the corresponding lipoprotein subfractions and total lipoprotein concentration (g/L) were calculated. Data represent the mean ± SD of up to 4 pools of 8-10 mice per genotype. To permit comparisons of TRL concentration in postprandial plasma between genotypes, relative diameter of the TRL diagram was drawn, considering the plasma concentration control TRL as 1. Otherwise, relative diameter of the HDL diagram was drawn considering the plasma concentration hAapo-II-Tg HDL as 1.
Online Figure 1

A

Control
TRL concentration: 0.8 ± 0.2 g/L

hapoA-II-Tg
TRL concentration: 7.9 ± 3.4 g/L

B

9.0 ± 1.7 %

12.8 ± 0.4 %

1.3 ± 1.1 %

76.9 ± 2.5 %

P<0.001

hapoA-II-Tg
HDL concentration: 1.6 ± 0.5 g/L

C

Control
HDL concentration: 3.5 ± 0.5 g/L

D

0.7 ± 0.2 %

9.0 ± 1.4 %

28.8 ± 2.2 %

61.5 ± 2.1 %

17.5 ± 12.8 %

1.5 ± 0.7 %

5.1 ± 5.8 %

19.5 ± 13.9 %

81.9 ± 12.8 %

P<0.001

hapoA-II-Tg
HDL concentration: 1.6 ± 0.5 g/L

- RELATIVE TG MASS
- RELATIVE TC MASS
- RELATIVE PL MASS
- RELATIVE PROTEIN MASS