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ApoAV Reduces Plasma Triglycerides by Inhibiting Very Low Density Lipoprotein-Triglyceride (VLDL-TG) Production and Stimulating Lipoprotein Lipase-mediated VLDL-TG Hydrolysis*

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ApoAV has been discovered recently as a novel modifier of triglyceride (TG) metabolism, but the pathways involved are currently unknown. To gain insight into the function of apoAV, adenovirus-mediated gene transfer of murine *apoa5* to C57Bl/6 mice was employed. The injection of low doses of Ad-*apoa5* (1–5 × 10^8 plaque-forming units/mouse) dose-dependently reduced plasma very low density lipoprotein (VLDL)-TG levels. First, we evaluated whether a reduced hepatic VLDL production contributed to the TG-lowering effect. Ad-*apoa5* treatment dose-dependently diminished (29–37%) the VLDL-TG production rate without affecting VLDL particle production, suggesting that apoAV impairs the lipidation of apoB. Second, Ad-*apoa5* treatment dose-dependently reduced (68–88%) the postprandial hypertriglyceridemia following an intragastric fat load, suggesting that apoAV also stimulates the lipoprotein lipase (LPL)-dependent clearance of TG-rich lipoproteins. Indeed, recombinant apoAV was found to dose-dependently stimulate LPL activity up to 2.3-fold *in vitro*. Accordingly, intravenously injected VLDL-like TG-rich emulsions were cleared at an accelerated rate concomitant with the increased uptake of emulsion TG-derived fatty acids by skeletal muscle and white adipose tissue in Ad-*apoa5*-treated mice. From these data, we conclude that apoAV is a potent stimulator of LPL activity. Thus, apoAV lowers plasma TG by both reducing the hepatic VLDL-TG production rate and by enhancing the lipolytic conversion of TG-rich lipoproteins.

Hypertriglyceridemia is a risk factor for coronary heart disease independent from the well known risk factors such as elevated LDL[1] and reduced HDL cholesterol levels (1). Recently, a novel apolipoprotein, apoAV, has been identified that strongly influences plasma triglyceride (TG) levels (2, 3). The human *APOA5* gene is part of the apolipoprotein gene cluster on chromosome 11q23 that also encompasses *APOA1*, *APOC3*, and *APOA4*. An initial study revealed the association of three single nucleotide polymorphisms within the *APOA5* locus with plasma TG levels and VLDL mass in humans (2). Importantly, these metabolic effects were not associated with a genetic marker in the nearby *APOC3* gene that is also known to affect plasma TG levels (2). Subsequent studies in diverse ethnic groups uncovered additional single nucleotide polymorphisms including apoAV protein variants and further supported a role for common genetic variations in *APOA5* in influencing plasma TG levels (4, 5). Interestingly, in a recent study, minor allele frequencies of 3 of 5 studied single nucleotide polymorphisms were found to be significantly higher in a hypertriglyceridemic population (4).

Mouse models confirmed the TG-modulating effects of apoAV observed in humans. Mice expressing a human *APOA5* transgene showed a 65% decrease in plasma TG levels compared with control mice (2). Conversely, *apoa5* knock-out mice showed a 400% increase in plasma TG concentration (2). Interestingly, the adenovirus-mediated expression of apoAV in mice resulted in a decrease of both plasma TG and total cholesterol (TC) of 70 and 40%, respectively (6).

ApoAV appears to be expressed exclusively in the liver. At present, it is unknown in what form apoAV is secreted by the liver. The circulating mature form has a predicted molecular mass of 39 kDa and is associated mainly with HDL and to a lesser extent with VLDL particles (3). This distribution is somewhat puzzling as apoAV mainly seems to affect VLDL metabolism. Initially, apoAV may be secreted along with VLDL, and as lipolytic conversion of VLDL progresses and excess surface lipids are being transferred to HDL, apoAV may end up in HDL in a similar way. Structure predictions indicate that apoAV is a very hydrophobic, highly α-helical protein (7). At the protein level, apoAV appears most homologous (20–28% amino acid identity) with exchangeable apolipoproteins apoAI, apoAIV, and apoE, prompting the study of lipid efflux and lecithin:cholesterol acyltransferase activation properties of apoAV (8). However, no such activity could be demonstrated in *in vitro* experiments employing recombinant apoAV (8). Little is known regarding the regulation of apoAV levels, but perox-

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[1] The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; LPL, lipoprotein lipase; pfu, plaque-forming unit; PL, phospholipid(s); TC, total cholesterol; TG, triglyceride(s); VLDL, very low density lipoprotein; ER, endoplasmic reticulum.

isome proliferator-activated receptor-α and the farnesoid X receptor have been implicated in the transcriptional regulation of the *APOA5* gene (9, 10).

Animal studies and genetic association studies clearly indicate a strong effect of apoAV on plasma TG levels, implicating a role for apoAV in VLDL metabolism. Based on structural analysis, it has been proposed that, at the intracellular level, apoAV may affect hepatic VLDL production (7). Alternatively, apoAV may stimulate lipolytic conversion of TG-rich lipoproteins. To address the mechanism(s) underlying the hypotriglyceridemic action of apoAV, we employed the adenovirus-mediated gene transfer of murine *apoa5* to C57Bl/6 mice.

Our results show that the expression of apoAV in mice reduces plasma TG and TC levels. ApoAV dose-dependently inhibited the hepatic VLDL-TG production rate without affecting the VLDL-apoB production rate. Moreover, our present studies clearly demonstrate that apoAV accelerates the rate of LPL-mediated TG hydrolysis both *in vitro* and *in vivo*. Thus, we propose that apoAV has a dual effect on VLDL metabolism, diminishing hepatic apoB lipidation and increasing the efficiency of TG disposal from circulating lipoproteins. Interestingly, this hypotriglyceridemic effect of apoAV is associated with a decrease of plasma TC levels. These observations suggest that apoAV is an interesting target in the treatment of hypertriglyceridemia.

EXPERIMENTAL PROCEDURES

Materials—Glycerol tri[9,10(n)-^{3}H]oleate ([^{3}H]triolein) and [1-^{14}C]oleate were from Amersham Biosciences. Trans^{35}S label was from ICN. Egg yolk phosphatidylcholine was a generous gift from Lipoid. Triolein was obtained from Fluka. Hypnorm was from Janssen Pharmaceuticals, and midazolam was from Roche Applied Science. L-α-Lysophosphatidylcholine, cholesterol, cholesteryl oleate, purified bovine milk LPL, essentially free fatty acid-free bovine serum albumin, Triton WR1339 (tyloxapol), and diethyl-*p*-nitrophenylphosphate (paraoxon) were from Sigma. Purified human apoCII and apoCIII were from Labconsult. Olive oil was from Carbonell. Heparin was from Leo Pharmaceutical Products. Solvable™ was from Packard Bioscience. All of the other chemicals were of analytical grade and purchased from various suppliers. For the determination of lipids, commercially available kits (TG and TC (Sigma) and phospholipids (PL) (Wako Chemicals)) were used according to the manufacturer's instructions.

Animals—13–17-week-old male C57Bl/6 mice were taken from the breeding colony at the University of Leiden. Mice were kept in a temperature- and humidity-controlled environment and had free access to standard laboratory chow and water. All of the animals used in the experiments received humane care, and all of the experiments were approved by the animal ethics committees from the University of Leiden and TNO-Prevention and Health.

Adenoviral Expression of ApoAV in C57Bl/6 Mice—The construction of a recombinant replication-deficient adenoviral vector expressing mouse apoAV (Ad-*apoa5*) has been described previously (6). 0–5 $\times$ 10^8 pfu of Ad-*apoa5* (total viral dose adjusted to 5 $\times$ 10^8 with empty vector (Ad-mock)) were injected into the tail vein of the mice (n = 4–6/group) at 3 h after the injection of Ad-LacZ (5 $\times$ 10^8 pfu) to saturate the uptake of viral particles by Kupffer cells (11). Prior to injection and 4 days after injection, mice were fasted for 4 h and a blood sample for lipid determination was collected in paraoxon-coated capillaries by tail bleeding. Mice were bled by retroorbital bleeding, and serum was prepared. For each group, sera were pooled and lipoproteins were fractionated by gel permeation chromatography (Superose 6). Following exsanguination, livers were excised, rinsed briefly in phosphate-buffered saline, and immediately frozen in liquid nitrogen. For determination of hepatic gene expression, mRNA was isolated from liver samples and converted to cDNA. The expression levels of *apoa5* were determined relative to cyclophilin A by real-time PCR (Taqman). Western blot analysis of serum samples was used to estimate the extent of apoAV expression as described previously (6).

Hepatic VLDL-Triglyceride Production—To determine the effect of apoAV expression on the hepatic VLDL-TG production rate, mice were injected via the tail vein with 5 $\times$ 10^8 pfu of Ad-*apoa5* or Ad-LacZ (n = 5/group). After 4 days, a 4-h fasted blood sample was taken (t = 0). Mice were anesthetized with Hypnorm (0.1 mg/kg fentanyl citrate and 25 mg/kg fluanisone) and midazolam (12.5 mg/kg) and injected via the tail vein with Trans^{35}S label (150 μCi/mouse). After 30 min, 12.5 mg of Triton WR1339 (10% solution in saline) was injected to block lipolysis (12). Subsequent blood samples were drawn at 10, 30, 60, and 90 min after injection via tail bleeding. At t = 120 min, mice were exsanguinated and the VLDL fraction of each mouse was isolated quantitatively from 400 μl of serum after density gradient ultracentrifugation (13). VLDL-TG was measured as described above. VLDL-apoB was precipitated selectively by 2-propanol (14) and counted for the incorporated ^{35}S.

Preparation of Recombinant ApoAV—Rat apoAV protein was produced by transformed *Escherichia coli* BL21(DE3) cells as described previously (3). The N-terminally hexahistidine-tagged apoAV protein was purified from guanidinium hydrochloride-solubilized inclusion bodies by metal affinity chromatography (AP Biotech) using an fast protein liquid chromatography system. Renaturation was achieved by on-column refolding of the protein in a linear 6–0 M urea gradient. Elution was performed with a linear 20–500 mM imidazole gradient in the presence of 0.1% Triton X-100. ApoAV-containing fractions were pooled, and buffer was exchanged to phosphate-buffered saline containing 0.1% Triton X-100. Finally, preparations were filter-sterilized (0.22 μm) and stored at −20 °C. The presence of 0.1% Triton X-100 proved to be essential to prevent the precipitation of apoAV during column elution and subsequent storage. Recombinant apoAV was essentially pure as judged from SDS-PAGE analysis. Protein concentrations were determined using the bicinchoninic acid method.

Preparation of VLDL-like Emulsions—VLDL-like TG-rich emulsion particles were prepared as described previously (15). Hereto, 100 mg of total lipid at a weight ratio of triolein:egg yolk phosphatidylcholine: lysophosphatidylcholine:cholesterYl oleate:cholesterol (70:22.7:2.3:3.0: 2.0) supplemented with 50–200 μCi of [^{3}H]triolein was sonicated using a Soniprep 150 (MSE Scientific Instruments) at an output of 10 μm. An emulsion fraction containing 80-nm sized emulsion particles was obtained by consecutive density gradient ultracentrifugation steps exactly as described previously (16). The TG content of emulsions was determined as described above.

In Vitro LPL Activity Assay—The effect of apolipoproteins on LPL activity was determined essentially as described (17). First, [^{3}H]triolein-labeled emulsion particles (200 μg of TG) were incubated (30 min at 37 °C) with the indicated amounts of human apoCII, human apoCIII, and/or rat apoAV in 75 μl of phosphate-buffered saline. Subsequently, 0.1 M Tris·HCl pH 8.5 was added to a total volume of 200 μl. At t = 0, LPL (final concentration 3.5 units/ml) was added in 200 μl of 12% bovine serum albumin (as [^{3}H]oleate acceptor). At t = 15, 30, 60, 90, and 120 min, [9,10-^{3}H]oleate that was generated during lipolysis was extracted. Hereto, 50-μl samples were added to 1.5 ml of CH_3OH:$CHCl_3$: heptane:oleic acid (1410:1250:1000:1 (v/v/v/v)) and to 0.5 ml of 0.2 M NaOH. Following vigorous mixing and centrifugation (10 min at 1000 $\times$ g), ^{3}H radioactivity in 0.5 ml of the aqueous phase was counted in 5 ml of Ultima Gold (Packard Bioscience). The recovery of [^{3}H]oleate in the aqueous phase following organic extraction was corrected for a 78.0 $\pm$ 0.9% recovery of [1-^{14}C]oleate internal standard. Lipolysis rates were calculated by linear regression (regression coefficients were typically greater than 0.995) and expressed as nanomole oleate released per milliliter per min. The inclusion of 1 M NaCl during incubations resulted in negligible lipolytic activity, indicating that the employed LPL preparation was not contaminated with salt-insensitive hepatic lipase.

Postprandial TG Response—To determine the effect of apoAV expression on the postprandial TG response, mice were injected via the tail vein with 1–5 $\times$ 10^8 pfu of Ad-*apoa5* or Ad-mock (n = 5–6/group) as described above. After 5 days, the 4-h fasted blood samples were drawn and the mice received an intragastric load of 200 μl of olive oil. Additional blood samples were drawn after 1, 2, 4, and 6 h, and the resulting sera were assayed for TG as described above.

Clearance of VLDL-like Emulsions in Vivo—To determine the effect of apoAV expression on the kinetics of TG-rich emulsions *in vivo*, mice were injected via the tail vein with 1 $\times$ 10^9 pfu of Ad-*apoa5* or Ad-mock (n = 4/group). At 5 days after adenoviral injection, serum clearance and tissue distribution of VLDL-like emulsion particles was studied as detailed elsewhere (16). The fed mice were anesthetized as described above, and their abdomens were opened. 200 μl of VLDL-like [^{3}H]triolein-labeled emulsion particles was administered via the vena cava inferior at a dose (1.0 mg of TG) that exceeded the endogenous plasma TG content in both experimental groups. Venous blood was sampled at 1, 2, 5, 10, and 15 min following injection, and the respective sera were assayed for ^{3}H radioactivity. After the last sampling, gonadal, perirenal, and intestinal white adipose tissue, heart, hind limb muscle tissue, liver, and spleen were harvested and solubilized in Solvable (overnight at 60 °C) and ^{3}H radioactivity was determined.

Statistical Analysis—The Mann-Whitney nonparametric test for two

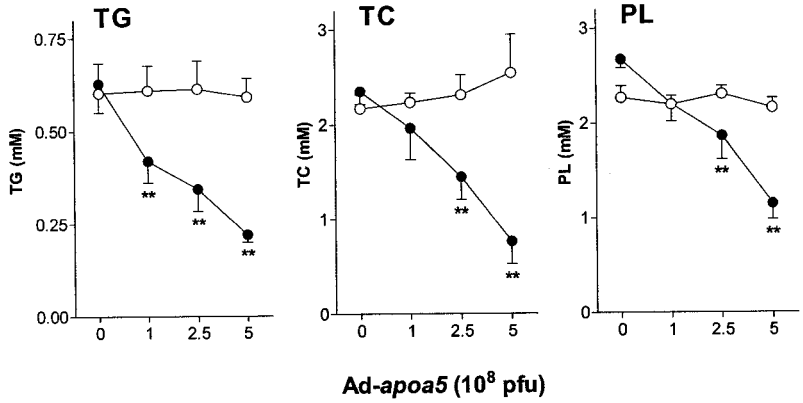

FIG. 1. **Effect of apoAV on plasma lipid levels.** C57Bl/6 mice (n = 5–6/group) were injected consecutively with Ad-LacZ (5×10^8 pfu) and Ad-*apoa5* (0–5×10^8 pfu; total virus dose adjusted to 5×10^8 pfu with Ad-mock). Before injection (○) and at 4 days after injection (●), fasted plasma was collected from the individual mice and assayed for TG (*left panel*), TC (*middle panel*), and choline PL (*right panel*). Values are depicted as means ± S.D. **, $p < 0.01$.

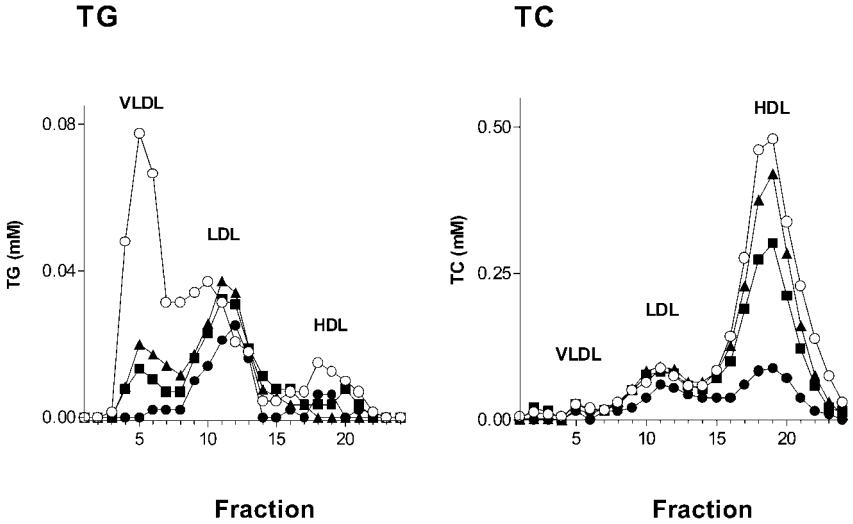

FIG. 2. **Effect of apoAV on distribution of lipids among lipoproteins.** C57Bl/6 mice (n = 5–6/group) were injected consecutively with Ad-LacZ (5×10^8 pfu) and Ad-*apoa5* (0 (○), 1 (▲), 2.5 (■), and 5×10^8 (●) pfu; total virus dose adjusted to 5×10^8 pfu with Ad-mock). After 4 days, the fasted plasma was collected, pooled per group, and subjected to gel permeation chromatography. Fractions were assayed for TG (*left panel*) and TC (*right panel*).

independent samples was used to define differences between the experimental groups. The criterion for significance was set at $p < 0.05$.

RESULTS

Plasma Lipid and Lipoprotein Levels after Adenovirus-mediated Gene Transfer of apoa5 to C57Bl/6 Mice—The injection of low doses of Ad-*apoa5* (1, 2.5, and 5×10^8 pfu) dose-dependently reduced plasma TG, TC, and PL (Fig. 1). At the highest dose, plasma TG was lowered to the same extent (−65%) as in mice transgenic for the human *APOA5* gene (2). In contrast with these transgenic animals, which are normocholesterolemic, we found consistent (6) lowering of plasma TC with 68% at the highest viral dose. However, moderate overexpression by injection of 1×10^8 pfu of Ad-*apoa5* reduced only plasma TG levels significantly (−33%, $p < 0.01$ *versus* control virus), whereas plasma TC and PL were unaffected. Compared with mice receiving control virus, the mice that were injected with 1, 2.5, and 5×10^8 pfu of Ad-*apoa5* had ~10-, 20-, and 30-fold higher hepatic *apoa5* mRNA levels, respectively, at day 4 postinjection (data not shown). When compared with preinjection values, apoAV protein levels in plasma were elevated ~10-, 20-, and 35-fold at day 4 after the injection of 1, 2.5, and 5×10^8 pfu of Ad-*apoa5*, respectively (data not shown).

An analysis of lipoprotein fractions separated by fast protein liquid chromatography revealed that apoAV-mediated reduction of plasma TG was mainly confined to the VLDL fraction resulting in the virtual absence of VLDL-TG at the highest viral dose (Fig. 2, *left panel*). Both the TG content of the intermediate density lipoprotein/LDL fraction and size of the intermediate density lipoprotein/LDL-TG peak fraction decreased in an Ad-*apoa5* dose-dependent manner. In addition, Ad-*apoa5* treatment reduced HDL-TG. The TC-reducing effects of apoAV overexpression were attributed mainly to the reduction of HDL, which is the main carrier of cholesterol in normolipidemic mice (Fig. 2, *right panel*).

Hepatic VLDL Production after Adenovirus-mediated Gene Transfer of apoa5 to C57Bl/6 Mice—To evaluate whether reduced VLDL production contributed to the TG-lowering effect of apoAV, the hepatic VLDL production rate was assessed using Triton WR1339 to block lipolysis and the clearance of VLDL. As compared with control virus, the injection of 1×10^8 or 5×10^8 pfu of Ad-*apoa5* dose-dependently reduced the VLDL-TG secretion rate with 29–37% (5.08 ± 0.54, 3.63 ± 0.20, and 3.22 ± 0.68 mM/h, respectively; $p < 0.01$) without affecting the VLDL-[^{35}S]apoB production rate (Fig. 3, *left* and *right panels*). Apparently, apoAV inhibits the intrahepatic lipidation of apoB without affecting VLDL particle production.

An analysis of VLDL isolated 2 h after Triton WR1339 injection indicated significant reductions of VLDL lipid content. The highest Ad-*apoa5* dose (5×10^8 pfu) mainly affected VLDL-TG (−39%; 6.37 ± 1.39 *versus* 10.4 ± 1.43 mM; mean ± S.D. (Fig. 3, *middle panel*)), although TC (−24%; 1.10 ± 0.15 *versus* 1.45 ± 0.25 mM) and PL (−32%; 1.21 ± 0.25 *versus* 1.79 ± 0.29 mM)) also was reduced ($p < 0.05$ *versus* Ad-LacZ)). An analysis of the lipid composition of VLDL obtained from mice injected with the lowest dose of Ad-*apoa5* (1×10^8 pfu) revealed a significant reduction of the TG content (−26%; 7.74 ± 0.16 *versus* 10.4 ± 1.43 mM), whereas the TC and PL content only tended to be lower (data not shown). The predominant effect of Ad-*apoa5* on VLDL-TG levels is indicative of reduction of VLDL particle size.

Postprandial TG Response after Adenovirus-mediated Gene Transfer of apoa5 to C57Bl/6 Mice—In addition to reducing the

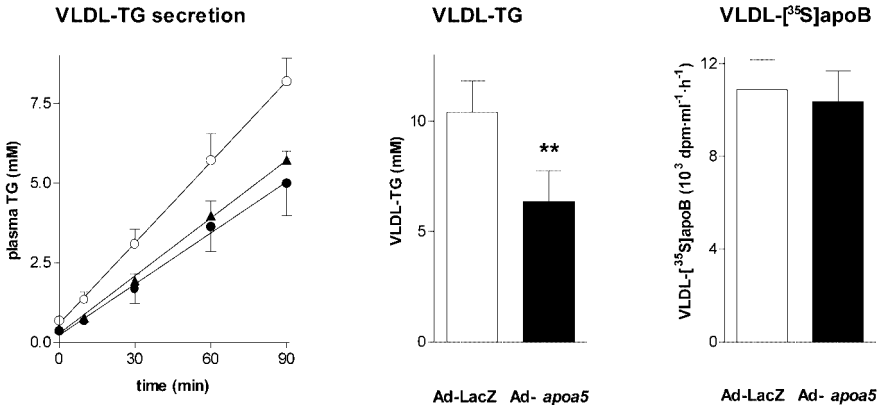

FIG. 3. **Effect of apoAV on hepatic VLDL production.** C57Bl/6 mice (n = 5/group) were treated with Ad-*apoa5* (1×10^8 pfu (▲) or 5×10^8 pfu (●)) or Ad-LacZ (○). After 5 days, the fasted animals received consecutive intravenous injections of Trans^{35}S label and Triton WR1339 to block lipolysis. Plasma samples were drawn within 90 min at the indicated time points and analyzed for TG (*left panel*). Values are depicted as the means ± S.D. Curves were fitted by linear regression. VLDL production rates were significantly different between Ad-LacZ (5.25 ± 0.56 mM/h) and Ad-*apoa5* (3.63 ± 0.20 and 3.22 ± 0.68 mM/h) treated mice ($p < 0.01$). After 120 min, mice were exsanguinated and VLDL was isolated and assayed for triglycerides (*middle panel*) or [^{35}S]apoB (*right panel*). ** $p < 0.01$.

rate of hepatic VLDL-TG secretion, we questioned whether apoAV could also enhance the lipolytic conversion of TG-rich lipoproteins. First, we studied whether apoAV affected postprandial lipemia, anticipating enhanced catabolism of TG-rich lipoproteins if apoAV would stimulate LPL activity. For this purpose, mice were injected with adenovirus and were given an intragastric gavage of olive oil after 5 days to force rapid and extensive chylomicron production. Animals injected with Ad-*apoa5* showed a dose-dependent lowering of the postprandial TG response (68, 73, and 88% reduction compared with control virus at Ad-*apoa5* doses of 1×10^8, 2.5×10^8, and 5×10^8 pfu, respectively) (Fig. 4). These findings indicate that apoAV overexpression results in a rapid clearance of chylomicron-TG. Because apoAV is not expressed by the intestine, a direct effect of apoAV on intestinal lipid absorption is unlikely. Thus, these findings strongly suggest that the diminished postprandial TG response is the result of a stimulating effect of apoAV on LPL-mediated lipolysis.

Effect of Purified apoAV on LPL-mediated TG Hydrolysis in Vitro—To directly evaluate the effect of recombinant rat apoAV on the triacylglycerol hydrolase activity of LPL, we determined the effect of purified apoAV on LPL-mediated TG hydrolysis from [^{3}H]triolein-labeled VLDL-like emulsions *in vitro*. The incubation of 80-nm sized VLDL-like protein-free emulsion particles with LPL in the presence of apoCII resulted in an apoCII dose-dependent increased rate of [^{3}H]oleate generation from emulsion-incorporated glycerol-tri[^{3}H]oleate (Fig. 5*A*) obeying initial linear kinetics up to 120 min. Preincubation of particles with 0.48 nmol of apoAV enhanced the apoCII-stimulated lipolysis rate ~2-fold (Fig. 5, *A* and *B*). In the presence of apoCII, apoAV dose-dependently stimulated LPL activity up to 2.3-fold at the highest dose but it was ineffective in the absence of apoCII (Fig. 5*B*). Preincubation of particles with the well established LPL-inhibitor apoCIII in the presence of a small amount of cofactor (0.11 nmol of apoCII) resulted in a dose-dependent inhibition of the lipolysis rate, reaching 65% inhibition at the highest dose (Fig. 5, *C* and *D*). Importantly, the LPL-inhibitory effect of apoCIII could be overcome fully by a low concentration of apoAV (apoAV:apoCIII = 1:20, mol/mol) (Fig. 5*D*). Taken together, these data suggest that apoAV may act as a stimulatory modifier of apoCII-induced LPL-mediated TG hydrolysis.

Kinetics of VLDL-like Emulsion Clearance after Adenovirus-mediated Gene Transfer of apoa5 to C57Bl/6 Mice—To further evaluate the metabolic consequences of the LPL-activating properties of apoAV *in vivo*, we subsequently determined the tissue distribution of [^{3}H]oleate derived from intravenously injected [^{3}H]triolein-labeled VLDL-like emulsion particles. In line with the concept that apoAV stimulates LPL activity, the

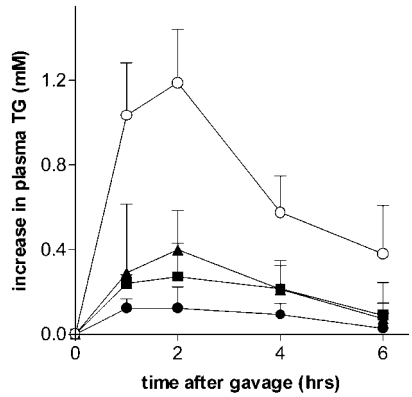

FIG. 4. **Effect of apoAV on the postprandial TG response.** C57Bl/6 mice (n = 5–6/group) were injected consecutively with Ad-LacZ (5×10^8 pfu) and 0×10^8 (○), 1×10^8 (▲), 2.5×10^8 (■), or 5×10^8 (●) pfu of Ad-*apoa5* (total virus dose adjusted to 5×10^8 pfu with Ad-LacZ). After 5 days, animals were fasted and received an intragastric olive oil load. Plasma samples were taken just before the gavage ($t = 0$) and at the indicated time points after the gavage and assayed for TG. TG values are corrected for $t = 0$ and depicted as the means ± S.D.

serum clearance of emulsion-TG was much faster in Ad-*apoa5*-treated mice compared with mice injected with control virus ($t_{1/2}$ = 2.2 ± 0.2 *versus* 7.6 ± 0.2 min, respectively; $p < 0.05$) (Fig. 6, *left panel*). The accelerated TG clearance in Ad-*apoa5*-treated animals was accompanied by a 2.5–3.5-fold increased uptake of ^{3}H radioactivity by gonadal, perirenal, and intestinal white adipose tissue ($p < 0.05$), a 1.8-fold increased uptake by hind limb muscle tissue ($p < 0.05$), and a trend toward an increased uptake by the heart (Fig. 6, *right panel*). The uptake by the liver also was increased (57%, $p < 0.05$). In contrast, the uptake by the spleen, which mainly reflects whole particle uptake because the spleen does not express LPL activity, was unaffected. Collectively, these data indicate the potency of the stimulatory effect of apoAV on LPL-mediated TG-rich lipoprotein clearance *in vivo*.

DISCUSSION

In this study, we have demonstrated that adenovirus-mediated gene transfer of murine *apoa5* results in a dose-dependent reduction of plasma TG in mice (Fig. 1). At least two mechanisms underlie the hypotriglyceridemic effect of apoAV overexpression. First, apoAV diminishes the rate of hepatic VLDL-TG secretion. Second, apoAV increases the efficiency of LPL-mediated TG hydrolysis. We propose that the latter mechanism results in increased conversion of VLDL and chylomicrons in their respective remnants and a subsequently more efficient clearance of remnants by the liver.

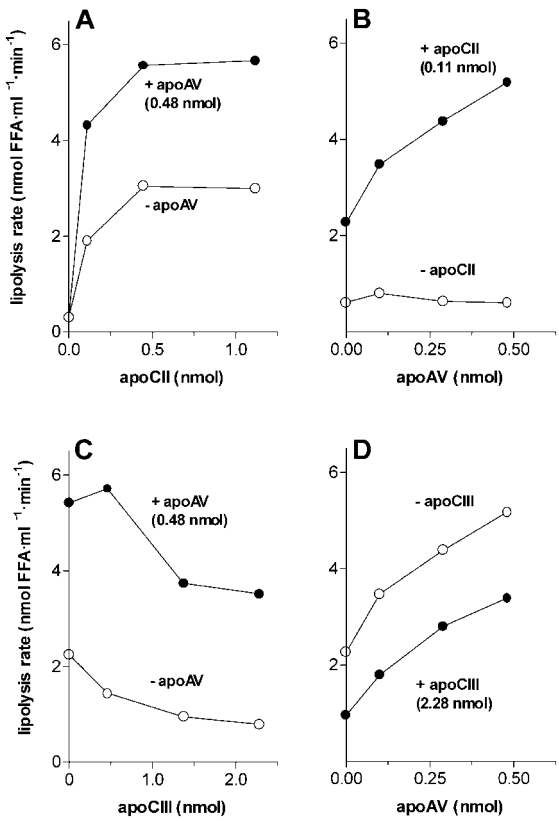

FIG. 5. **Interacting effects of apoAV, apoCII, and apoCIII on LPL-mediated TG hydrolysis *in vitro*.** VLDL-like [^{3}H]triolein-labeled emulsion particles were incubated (30 min at 37 °C) with increasing amounts of apoCII (*A*), apoAV (*B* and *D*) or apoCIII (*C*), and 0.11 nmol of apoCII (*C* and *D*) in the absence (○) or the presence (●) of the indicated amount of apolipoprotein. At $t = 0$, LPL was added and [^{3}H]oleate was extracted at $t = 10$, 30, 60, 90, and 120 min and quantified. Lipolysis rates were calculated by fitting the resulting curves by linear regression. Values are means of duplicate incubations (variation typically < 10%). *FFA* denotes free fatty acid.

Based on structural analysis, it has been proposed recently (7) that apoAV functions in VLDL metabolism by inhibiting hepatic VLDL secretion. In this study, we have provided experimental proof for such a function (Fig. 3). One could argue that the adenoviral overexpression of a secreted apolipoprotein overloads the capacity of the ER/Golgi secretory pathway and thereby interferes with apoB lipid acquisition. Studies with other adenovirally overexpressed apolipoproteins oppose this view. We have reported previously that the adenoviral overexpression of apoE strongly increases the VLDL-TG secretion rate compared with either Ad-GFP (18) or Ad-LacZ (19). In addition, we have observed recently that Ad-*APOC1* does not affect the hepatic VLDL production compared with Ad-LacZ, even at a dose as high as 3.3×10^9 pfu.[2] Hence, the adenoviral overexpression of an apolipoprotein does not alter the rate of VLDL-TG secretion *per se*. Although the underlying mechanism is unknown as yet, the lowering effect of Ad-*apoa5* on VLDL-TG production is thus specific for apoAV.

VLDL assembly is a multicompartemental process proceeding in two major steps (20). First, apoB is lipidated during the translation of its mRNA in the rough ER. Second, this partially lipidated apoB particle fuses with a TG-rich particle preformed in the smooth ER. The latter step takes place in a distal compartment of the secretory pathway and results in the formation of secretion-competent mature VLDL. ApoAV, at least when overexpressed, diminishes the rate of VLDL-TG secretion without affecting the production of VLDL particles. Under the experimental conditions used, the particle levels of major VLDL lipids (*i.e.* TG, TC, and PL) all were found to be lowered, albeit apoAV predominantly affected the VLDL-TG content. As underlipidated apoB protein is subject to rapid degradation before exiting the ER (20), apoAV is more likely to interfere with a step in the second stage of VLDL assembly. For example, apoAV could disturb lipidation of the TG-rich particle in the smooth ER. The hepatic mRNA level for microsomal triglyceride transfer protein, which is essential for this process and for the initial lipidation of nascent apoB, does not change following Ad-*apoa5* treatment (data not shown). The intrahepatic action of apoAV presumably requires the (transient) localization of apoAV to the ER/secretory pathway, which is conceivable for a secreted plasma protein.

In this study, we further demonstrated that apoAV can enhance LPL-mediated TG hydrolysis in a dose-dependent manner *in vitro* (Fig. 5). The molecular mechanism of LPL stimulation remains to be determined, but a role as a cofactor comparable to apoCII seems unlikely because apoAV had no enhancing effect on lipolysis in the absence of apoCII. One could speculate that a potential molecular mechanism of apoAV action is to facilitate the access of LPL to the TG molecules in the core of the lipoprotein particle (*i.e.* enhancing substrate availability). This is in line with the high hydrophobicity of the apoAV protein (7), placing apoAV more toward the core of the lipoprotein particle. The improved presentation of TG to LPL could subsequently lead to a higher efficiency of TG conversion. It is also possible that apoAV interacts directly with LPL and increases the efficiency of TG hydrolysis by enhancing enzymatic activity, for example, by stabilizing the LPL dimer.

Following an intragastric fat load, a diminished postprandial hypertriglyceridemia was observed in mice injected with Ad-*apoa5*, strongly suggesting apoAV-mediated LPL activation to occur *in vivo* as well (Fig. 4). Although intestinal cells do not express apoAV, an indirect effect of apoAV on intestinal lipid absorption involving biliary lipid secretion cannot be excluded. However, bypassing intestinal absorption by intravenous injection of [^{3}H]TG-rich emulsion particles leads to a pronounced shortening of the ^{3}H plasma half-life in Ad-*apoa5*-treated mice, once more indicating the stimulation of LPL-mediated lipolytic processing of TG by apoAV *in vivo* (Fig. 6, *left panel*). Indeed, apoAV expression resulted in an increased accumulation of ^{3}H activity into LPL-expressing tissues representing increased [^{3}H]oleate deposition (Fig. 6, *right panel*). In the case of the liver, increased accumulation of ^{3}H activity may be caused in addition by increased uptake of remnant particles, which often retain some [^{3}H]triolein. From the combined *in vitro* and *in vivo* findings, we thus can conclude that apoAV is a potent stimulator of LPL activity, contributing to the hypotriglyceridemic effect of apoAV. This mechanism can explain fully the markedly diminished postprandial TG response upon apoAV expression, because mice with muscle-specific expression of human LPL, resulting in 2.3-fold increased post-heparin LPL levels (21), also show a >90% diminished TG response upon an intragastric gavage of 200 μl of olive oil.[3]

Although low expression levels of apoAV predominantly affect plasma TG levels (Fig. 1), higher levels of expression also reduced TC levels, mainly by reduction of HDL. Previously, plasma TC levels were reported to be unaffected by the absence (*apoa5*$^{-/-}$ mice) or overexpression (human *APOA5* transgenic

[2] P. C. N. Rensen, C. C. van der Hoogt, and K. Willems van Dijk, unpublished observations.

[3] P. J. Voshol, P. C. N. Rensen, L. M. Havekes, and K. Willems van Dijk, unpublished observations.

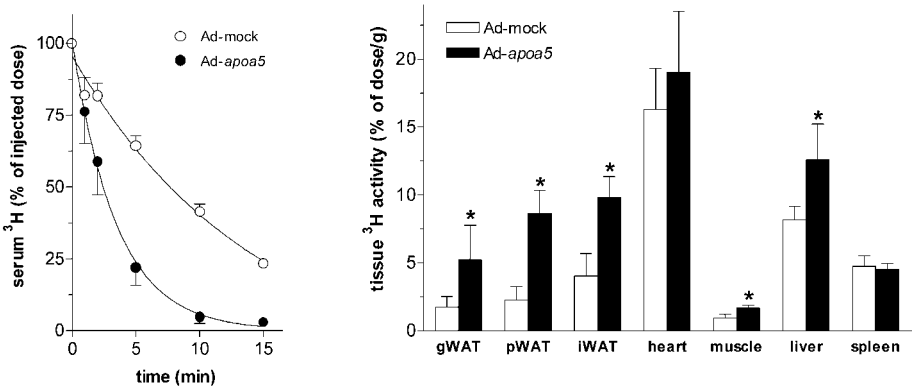

FIG. 6. **Effect of apoAV on the clearance and tissue distribution of VLDL-like emulsion particles.** C57Bl/6 mice (n = 4/group) were treated with Ad-*apoa5* (*black symbols*) or Ad-mock (*white symbols*). After 5 days, fed mice received a large intravenous bolus of [^{3}H]triolein-labeled emulsion particles (1.0 mg of TG) and the serum clearance of ^{3}H label was determined (*left panel*). The curves were fitted according to an exponential model. At 15 min after injection, mice were killed and the tissue distribution of ^{3}H radioactivity was determined (*right panel*). All of the values are depicted as means ± S.D. *Asterisk* denotes a statistically significant difference ($p < 0.05$) between Ad-mock- and Ad-*apoa5*-treated groups of mice. *gWAT*, *pWAT*, and *iWAT* denote gonadal, perirenal, and intestinal white adipose tissue, respectively.

mice) of apoAV (2). Moreover, whereas genetic association studies have revealed consistent association of minor *APOA5* alleles with increased plasma TG levels (2, 4, 5, 21, 22), the influence of *APOA5* polymorphisms on plasma TC levels is less clear. The minor allele of the *APOA5* −1131T→C promoter variant has been associated with a modest decrease of HDL cholesterol in some but not all studies (21, 22) and in one study with a more pronounced increase of VLDL-cholesterol (21). Although apoAV-induced TG reduction thus may be more relevant for human lipid metabolism compared with the TC-reducing effect, the mechanism underlying the effect on HDL is intriguing.

Hepatic mRNA levels of several proteins influencing plasma cholesterol levels (*i.e.* scavenger receptor-BI, ABCA1, LDL receptor, and LDL receptor-related protein) were not affected by Ad-*apoa5* treatment (data not shown). Moreover, preliminary studies indicate that recombinant apoAV does not interfere with apoAI- or HDL-mediated cholesterol efflux.[4] Most likely, however, HDL reduction may relate simply to the LPL-stimulating effect of apoAV. First, systemic LPL activation in C57Bl/6 mice by intravenous injection of heparin (1.0 unit/g body weight) led to a rapid decline of both plasma TG levels (−55% within 10–30 min) and plasma TC levels (−30% after 1 h) as caused by HDL reduction.[5] Hence, systemic LPL activation mimics the hypolipidemic effect of Ad-*apoa5* treatment. Second, enhancing LPL activity in mice by overexpression also generally resulted in the reduction of plasma HDL on chow diet (23–25). Thus, systemic LPL activation in mice results in a reduction of HDL levels, and these observations are consistent with apoAV functioning as an activator of LPL. The mechanism underlying the LPL-mediated decrease in HDL also may involve scavenger receptor-BI mediated clearance. It has been shown that the capacity of HDL to deliver cholesteryl esters via the scavenger receptor-BI pathway increases with decreasing TG content (26). Thus, increased lipolytic conversion of HDL due to the apoAV-stimulated TG-hydrolase activity of LPL may enhance the hepatic clearance of HDL-cholesterol via scavenger receptor-BI. Notwithstanding the above, it is tempting to speculate that apoAV also may have additional yet undiscovered functions. For example, apoAV may influence the activity of hepatic lipase and/or endothelial lipase. Although these lipolytic enzymes do not have major effects on plasma TG metabolism (27, 28), they both act on HDL-phospholipids and are known to influence plasma TC levels.

The potent lipid-lowering effects of apoAV make the *APOA5* gene an obvious target for the treatment of dyslipidemia, especially because it appears to effectively counteract the LPL-inhibiting effect of the main endogenous LPL-modulator apoCIII and to be regulated by nuclear receptors for which ligands exist and are still being developed (9, 10). In primary human hepatocytes, *APOA5* mRNA levels are increased 5-fold following treatment with a peroxisome proliferator-activated receptor-α agonist (10). This is in the same order of magnitude as the increase in *apoa5* expression obtained after the low dose Ad-*apoa5* injection (~10-fold elevation). Thus, the lipid-lowering effects seen upon hepatic *apoa5* overexpression in our studies probably reflect a physiologically relevant mechanism and underscore the importance of apoAV in TG metabolism.

[4] F. G. Schaap and A. K. Groen, unpublished observations.

[5] P. C. N Rensen, J. F. P. Berbée, and L. M. Havekes, unpublished observations.

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ApoAV Reduces Plasma Triglycerides by Inhibiting Very Low Density Lipoprotein-Triglyceride (VLDL-TG) Production and Stimulating Lipoprotein Lipase-mediated VLDL-TG Hydrolysis

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