

# Effects of apoA-V on HDL and VLDL metabolism in APOC3 transgenic mice<sup>1</sup>

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**Abstract** Apolipoprotein A-V (apoA-V) and apoC-III are exchangeable constituents of VLDL and HDL. ApoA-V counteracts the effect of apoC-III on triglyceride (TG) metabolism with poorly defined mechanisms. To better understand the effects of apoA-V on TG and cholesterol metabolism, we delivered apoA-V cDNA into livers of hypertriglyceridemic APOC3 transgenic mice by adenovirus-mediated gene transfer. In response to hepatic apoA-V production, plasma TG levels were reduced significantly as a result of enhanced VLDL catabolism without alternations in VLDL production. This effect was associated with reduced apoC-III content in VLDL. Increased apoA-V production also resulted in decreased apoC-III and increased apoA-I content in HDL. Furthermore, apoA-V-enriched HDL was associated with enhanced LCAT activity and increased cholesterol efflux. This effect, along with apoE enrichment in HDL, contributed to HDL core expansion and  $\alpha$ -HDL formation, accounting for significant increases in both the number and size of HDL particles. As a result, apoA-V-treated APOC3 transgenic mice exhibited decreased VLDL-cholesterol and increased HDL-cholesterol levels. ApoA-V-mediated reduction of apoC-III content in VLDL represents an important mechanism by which apoA-V acts to ameliorate hypertriglyceridemia in adult APOC3 transgenic mice. In addition, increased apoA-V levels accounted for cholesterol redistribution from VLDL to larger HDL particles. **■** These data suggest that in addition to its TG-lowering effect, apoA-V plays a significant role in modulating HDL maturation and cholesterol metabolism.—Qu, S., G. Perdomo, D. Su, F. M. D'Souza, N. S. Shachter, and H. H. Dong. **Effects of apoA-V on HDL and VLDL metabolism in APOC3 transgenic mice.** *J. Lipid Res.* 2007. 48: 1476–1487.

**Supplementary key words** apolipoprotein A-V • apolipoprotein C-III • hypertriglyceridemia • lipoprotein metabolism • very low density lipoprotein • high density lipoprotein

Hypertriglyceridemia is a hallmark of the metabolic syndrome that is closely associated with obesity and type 2 diabetes, and it is characterized by a triad plasma lipid

profile [i.e., increased triglyceride (TG) and LDL levels and decreased HDL levels] (1, 2). Because of its atherogenic potential, hypertriglyceridemia is considered an independent risk factor for coronary artery disease (3, 4). Nevertheless, the pathophysiology of hypertriglyceridemia remains poorly understood.

Recent studies have identified apolipoprotein A-V (apoA-V) and apoC-III as key regulators of plasma TG metabolism. ApoA-V and apoC-III are exchangeable constituents of VLDL and HDL with opposing effects on plasma TG metabolism (5). In humans, genetic variants of apoC-III or apoA-V genes are linked with altered VLDL-TG metabolism (5–14). Furthermore, APOA5 haplotypes are associated with abnormal TG metabolism in hyperlipidemic subjects (15). In animals, transgenic apoC-III production results in hypertriglyceridemia as a result of delayed catabolism of VLDL and chylomicron (16–18). In contrast, transgenic apoA-V expression is associated with hypotriglyceridemia as a result of enhanced clearance of TG-rich particles in mice (6). On the other hand, apoC-III deficiency is associated with significantly lower plasma TG levels (19), whereas apoA-V knockout mice manifest hypertriglyceridemia attributable to decreased lipolysis of TG-rich lipoproteins (6, 20). These data are indicative of an antagonistic interplay between apoC-III and apoA-V in plasma TG metabolism. In support of this notion, genetically engineered mice with cotransgenic expression or double knockout of apoA-V and apoC-III genes exhibit normal plasma TG metabolism (21, 22).

However, the mechanism underlying the apoA-V-mediated TG-lowering effect remains incompletely understood. Although transgenic apoA-V expression prevents the development of hypertriglyceridemia in APOC3 trans-

Abbreviations: apoA-V, apolipoprotein A-V; LDLR, low density lipoprotein receptor; SR-BI, scavenger receptor class B type I; TG, triglyceride.

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genic mice (21), it remains unknown whether augmented apoA-V production can reverse hypertriglyceridemia in adult mice with palpable dyslipidemia. Unlike apoC-III, which is a major constituent of VLDL and an inhibitor of LPL, apoA-V is present mainly in HDL (23, 24). The effect of apoA-V on HDL-cholesterol metabolism remains undetermined. To address these issues, we delivered mouse apoA-V cDNA by adenovirus-mediated gene transfer into livers of hypertriglyceridemic APOC3 transgenic mice. Hepatic apoA-V production ameliorated hypertriglyceridemia in APOC3 transgenic mice, as shown by significantly reduced plasma VLDL-TG levels and improved postprandial TG response. This effect correlated with reduced apoC-III content in VLDL and improved the catabolism of TG-rich particles in apoA-V vector-treated hypertriglyceridemic mice. Furthermore, increased apoA-V levels were shown to enhance LCAT activity and promote cholesterol efflux from cultured macrophage cells. This effect, together with increased apoE and apoA-I content in HDL, contributed to HDL core expansion and  $\alpha$ -HDL formation, resulting in a marked increase in both the number and size of HDL. As a result, apoA-V vector-treated APOC3 transgenic mice displayed decreased VLDL-cholesterol and increased HDL-cholesterol levels without changes in total plasma cholesterol levels. These data validated the concept that increased apoA-V production ameliorated hypertriglyceridemia in adult APOC3 transgenic mice with overt dyslipidemia. In addition, our studies elucidated an important role of apoA-V in HDL-cholesterol homeostasis by modulating the composition of apolipoproteins in HDL.

## MATERIALS AND METHODS

### Adenoviral vectors

Mouse apoA-V cDNA (1.2 kb) was obtained by RT-PCR from C57BL/6J mouse liver RNA using specific primers (forward reaction, 5'-GCAGCCTGAGTTCAGGTTTC-3'; reverse reaction, 5'-CCTAATAGACCATGCTAGCG-3'). ApoA-V cDNA was cloned into plasmid pENTR-1A (Invitrogen, Carlsbad, CA), which was used as an entry vector for the generation of adenovirus Ad-CMV-apoA-V using the ViraPower Adenoviral Expression System (Invitrogen). The control adenovirus Ad-RSV-LacZ expressing the bacterial  $\beta$ -gal gene has been described (25, 26). Adenoviruses were produced in HEK293 cells and purified by CsCl gradient centrifugation, as described (26).

### Animal studies

Transgenic mice expressing human apoC-III in the C57BL/6J background have been described (16). Mice were fed standard rodent chow and water ad libitum in sterile cages with a 12 h light/dark cycle. Under this condition, apoC-III transgenic mice spontaneously developed hypertriglyceridemia with markedly increased plasma TG content (>300 mg/dl at 12 weeks old), compared with gender- and age-matched littermates (plasma TG levels, 60–120 mg/dl). For adenovirus administration, mice were injected via tail vein with 200  $\mu$ l of either apoA-V or control LacZ adenovirus at  $2.5 \times 10^{12}$  viral particles/kg (equivalent to  $1.5 \times 10^{11}$  plaque-forming unit/kg), as described (27). This approach resulted in  $\sim$ 70% transduction of hepatocytes in liver, with little

transduction in extrahepatic organs (26, 28). For blood chemistry measurement, treated mice were fasted overnight (16 h) and aliquots of tail vein blood (100  $\mu$ l) were collected into capillary tubes precoated with potassium-EDTA (Sarstedt, Nümbrecht, Germany). Plasma levels of TG and cholesterol were determined using Thermo Infinity TG and cholesterol reagents (Thermo Electron, Melbourne, Australia). Plasma NEFA levels were determined using the Wako NEFA assay kit (Wako Chemical USA, Richmond, VA). All procedures were approved by the Institutional Animal Care and Usage Committee of Children's Hospital of Pittsburgh (protocol 41-04).

### Fast-protein liquid chromatography fractionation of lipoproteins

Aliquots (250  $\mu$ l) of plasma pooled from a given group of mice were applied to two head-to-tail linked Tricorn high-performance Superose S-6 10/300GL columns using a fast-protein liquid chromatography system (Amersham Biosciences), followed by elution with PBS at a constant flow rate of 0.25 ml/min. Fractions (500  $\mu$ l) were eluted and assayed for TG and cholesterol concentrations using the Thermo Infinity TG and cholesterol reagents (Thermo Electron), as described (29).

### Fat tolerance test

Mice were fasted overnight, followed by oral gavage of olive oil at the dose of 10  $\mu$ l/g body weight. Before and at different times after oral fat administration, aliquots of tail vein blood (25  $\mu$ l) were sampled for the determination of plasma TG levels. Area under the curve of plasma TG profiles during the fat tolerance test was calculated using KaleidaGraph software (Synergy Software, Reading, PA).

### VLDL-TG production assay

Mice were fasted for 5 h, followed by intraperitoneal injection of 500 mg/kg Triton WR1339 (Sigma-Aldrich, St. Louis, MO) to inhibit plasma VLDL-TG hydrolysis and clearance. Aliquots of blood (25  $\mu$ l) were collected at 20, 40, 60, and 80 min after Triton WR1339 injection. Plasma TG levels were determined and plotted as a function of time. The rates of hepatic VLDL-TG production, defined as milligrams of TG produced per minute per kilogram of body weight, were calculated.

### Lipoprotein lipase assay

Mice were injected intravenously with 300 IU heparin/kg body weight, and tail vein blood (20  $\mu$ l) was sampled at 10 min after heparin infusion. Heparinized sera were prepared for the determination of LPL activity using the LPL activity kit (Roar Biochemical, Inc.), as described previously (29).

### Western blot analysis

Aliquots of plasma at a fixed concentration of 15  $\mu$ g of protein per lane were applied to 4–20% polyacrylamide gels. The gels were subjected to Western blot assay using goat anti-apoC-III antibody (1:1,000 dilution; Abcam, Cambridge, MA). This antibody cross-reacts with both human and mouse apoC-III protein. The secondary antibody was rabbit anti-goat IgG conjugated with HRP (1:6,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). ApoC-III proteins were visualized by ECL detection reagents, and the relative intensities of protein bands were quantified by densitometry, as described (29). Likewise, plasma apoA-V levels were determined by Western blot assay using rabbit anti-apoA-V antibody. This antibody was developed in our laboratory by immunizing rabbits with the mouse apoA-V-specific peptide (amino acids 113–128, VGNLEGLRQQLK-

PYT) (Genemed Synthesis, Inc., San Francisco, CA). For the determination of apoB and apoE levels, rabbit anti-apoB (1:1,000 dilution) and anti-apoE (1:1,000 dilution) antibodies were obtained from Abcam.

To determine hepatic protein levels, aliquots (20 mg) of liver tissue were homogenized in 400  $\mu$ l of ice-cold M-PER mammalian protein extraction buffer (Pierce, Rockford, IL) supplemented with 4  $\mu$ l of protease inhibitor cocktail (Pierce). Aliquots (20  $\mu$ g) of liver protein extracts were resolved on 4–20% SDS-polyacrylamide gels, followed by immunoblot analysis using rabbit anti-HMG-CoA reductase (catalog No. 07-457; Upstate Biotechnology, Lake Placid, NY) and goat anti-actin (sc-1615; Santa Cruz Biotechnology).

For the detection of low density lipoprotein receptor (LDLR) and scavenger receptor class B type I (SR-BI), plasma membrane proteins of liver were extracted using the Mem-PER membrane protein extraction kit (Pierce). Aliquots of liver tissue (20 mg) was homogenized in 500  $\mu$ l of TBS (10 mM Tris-HCl, pH 7.0, 10 mM NaCl, and 25 mM EDTA) supplemented with 5  $\mu$ l of protease inhibitor cocktail (Pierce). The samples were centrifuged at 5,000 *g* at 4°C for 5 min. The supernatants were centrifuged at 14,000 rpm at 4°C for 45 min. The pellets containing enriched plasma membrane proteins were subjected to Western blot analysis using rabbit anti-SR-BI (NB400-104; Novus Biologicals, Littleton, CO) and chicken anti-LDLR antibody (NB300-338; Novus Biologicals). As a control, monoclonal mouse antibody against the plasma membrane-specific marker sodium potassium ATPase (ab7671; Abcam) was used. The hepatic abundance of individual proteins was quantified by semiquantitative immunoblot assay using  $\beta$ -actin or sodium potassium ATPase as an internal control, as described (29).

#### Pre- $\beta$ -HDL and $\alpha$ -HDL determination

Native agarose gel electrophoresis was used to determine plasma pre $\beta$ -HDL (also known as lipid-poor HDL) and  $\alpha$ -HDL levels, as described (30). Aliquots of plasma (40  $\mu$ g of protein) were applied to 0.7% agarose gels. After electrophoresis for 2.5 h in 60 mM sodium barbital buffer, pH 8.6 (Sigma-Aldrich), in a 4°C room, proteins were transferred to a nitrocellulose membrane in deionized water by capillary blotting for 16 h. The membrane was probed with rabbit anti-apoA-I antibody (Biodesign, Saco, ME), followed by incubation with anti-rabbit IgG conjugated with HRP (Amersham Biosciences, Piscataway, NJ). ApoA-I proteins were visualized by ECL detection reagents and quantified by densitometry, as described (29).

#### LCAT activity assay

Plasma LCAT activity was assayed using the Calbiochem fluorometric LCAT assay kit (EMD Bioscience, San Diego, CA). This assay is based on the hydrolysis of an artificial LCAT substrate that fluoresces at 470 nm, resulting in a product that fluoresces at 390 nm. Aliquots (2  $\mu$ l) of serum in both control and apoA-V groups were mixed with 1  $\mu$ l of fluorescent LCAT substrate and 200  $\mu$ l of LCAT assay buffer, followed by incubation for 2 h at 37°C. The reaction was stopped by adding 300  $\mu$ l of READ reagent (provided in the kit) to 100  $\mu$ l of the reaction mixture, followed by fluorometry at 390 and 470 nm. LCAT activity is defined as the change in the ratio of 390/470 nm fluorescence emission intensities.

#### Cholesterol efflux assay

Cholesterol efflux was measured as described (31). Mouse macrophage cells (RAW-264.7; American Type Culture Collection, Manassas, VA) were cultured in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin

on six-well plates. Cells grown at  $\sim$ 75% confluence were incubated with [1,2-<sup>3</sup>H]cholesterol (1  $\mu$ Ci/well; specific activity, 40 Ci/mmol; Perkin-Elmer, Boston, MA) in 2 ml of culture medium for 24 h at 37°C. Cells were washed three times with prewarmed PBS and incubated in serum-free medium for 8 h to equilibrate the intracellular cholesterol pool. Afterward, cells were washed three times with PBS and incubated in serum-free DMEM supplemented with 5  $\mu$ l of serum from individual mice in both control and apoA-V groups. Four hours later, the conditioned medium was collected and centrifuged at 13,000 rpm in a microfuge for 5 min to remove potential cell contamination. Cells were washed four times with PBS and collected in 1 ml of 1 N NaOH. After incubation at 60°C for 1 h, the cell lysates were centrifuged at 13,000 rpm for 5 min. The radioactivity of conditioned medium and cell lysates was counted in a scintillation counter (Wallac 1410 Liquid Scintillation Counter; Perkin-Elmer). Cholesterol efflux was calculated as the percentage of radioactivity in conditioned medium out of total radioactivity in both conditioned medium and cells.

#### Statistics

Statistical analyses of data were performed by ANOVA using the JMP statistics software (Cary, NC). Dunnett's test was performed to study the significance of differences between control and apoA-V groups. Data are expressed as means  $\pm$  SEM. *P* < 0.05 was considered statistically significant.

## RESULTS

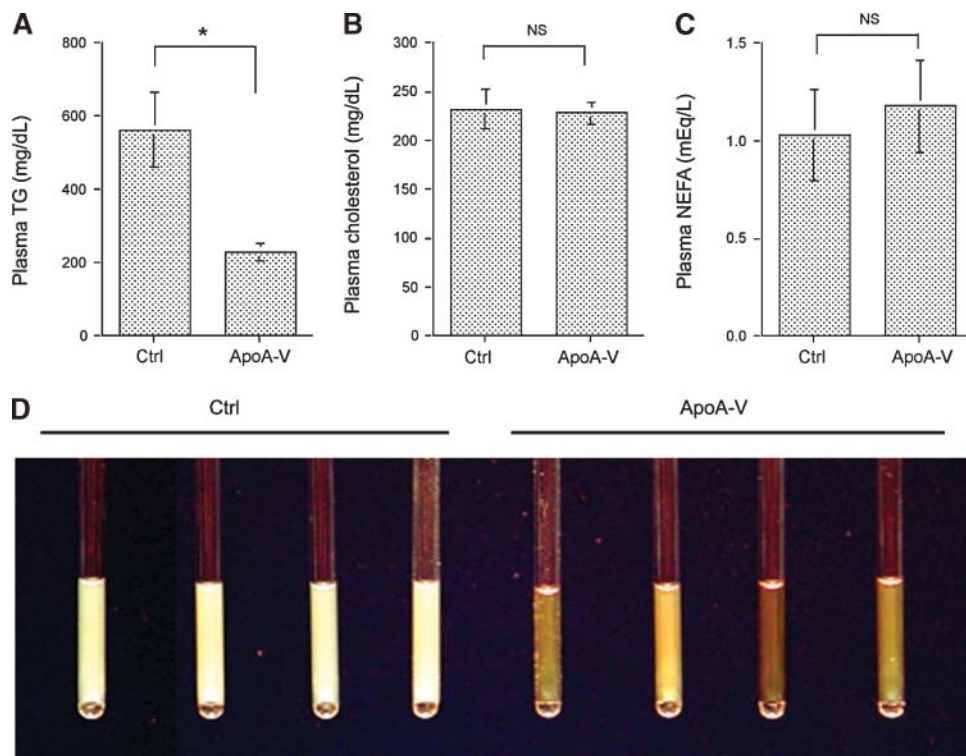
#### Effect of apoA-V on plasma TG metabolism in APOC3 transgenic mice

To investigate whether apoA-V counteracts the effect of apoC-III on TG metabolism, we transferred apoA-V cDNA by adenovirus-mediated gene delivery to APOC3 transgenic mice. This APOC3 transgenic line expresses human apoC-III under the control of its native promoter in the C57BL/6J background (16). When fed regular chow, APOC3 transgenic mice developed hypertriglyceridemia at 4 weeks of age, as reflected in markedly increased plasma TG levels with normal blood glucose (80–140 mg/dl). In this study, male APOC3 transgenic mice (12 weeks old; mean body weight, 27  $\pm$  3.1 g) were stratified by plasma TG levels and randomly assigned to two groups to ensure similar mean nonfasting plasma TG levels (670  $\pm$  43). Mice were treated with either control LacZ or apoA-V vector for 2 weeks.

As shown in **Fig. 1A**, hepatic apoA-V production ameliorated hypertriglyceridemia, as indicated by an  $\sim$ 3-fold reduction in plasma TG levels in APOC3 transgenic mice. In contrast, hypertriglyceridemia persisted in control vector-treated APOC3 transgenic mice. To study the effect of apoA-V on cholesterol and NEFA metabolism, we determined total plasma cholesterol and NEFA levels. No significant differences in plasma cholesterol (**Fig. 1B**) and NEFA (**Fig. 1C**) levels were detected between apoA-V and control vector-treated APOC3 transgenic mice.

The effect of hepatic apoA-V production on plasma TG metabolism was underpinned by the examination of sera extracted from control and apoA-V vector-treated mice. As shown in **Fig. 1D**, sera of control vector-treated APOC3





**Fig. 1.** Lipid profiles. APOC3 transgenic mice treated with apolipoprotein A-V (apoA-V) and control (Ctrl) vectors were euthanized on day 14 after 16 h of fasting. Plasma levels of triglyceride (TG) (A), cholesterol (B), and NEFA (C) were determined. Aliquots of plasma (20  $\mu$ l) of individual mice in capillaries were photographed (D). Data are expressed as means  $\pm$  SEM. \*  $P < 0.05$ .

transgenic mice appeared milky, which is characteristic of severe hypertriglyceridemia. In contrast, sera of apoA-V vector-treated APOC3 transgenic mice were relatively clear, correlating with their significantly reduced plasma TG levels after 2 weeks of hepatic apoA-V production.

In addition, we determined the body weights of individual mice in both control and apoA-V vector-treated APOC3 transgenic groups. No significant differences in body weight between apoA-V ( $28.8 \pm 2.4$  g) and control ( $29.3 \pm 1.5$  g) groups of mice were detected during the course of this study. These results rule out the possibility that the reduction in plasma TG levels in apoA-V vector-treated APOC3 transgenic mice was attributable to body weight changes.

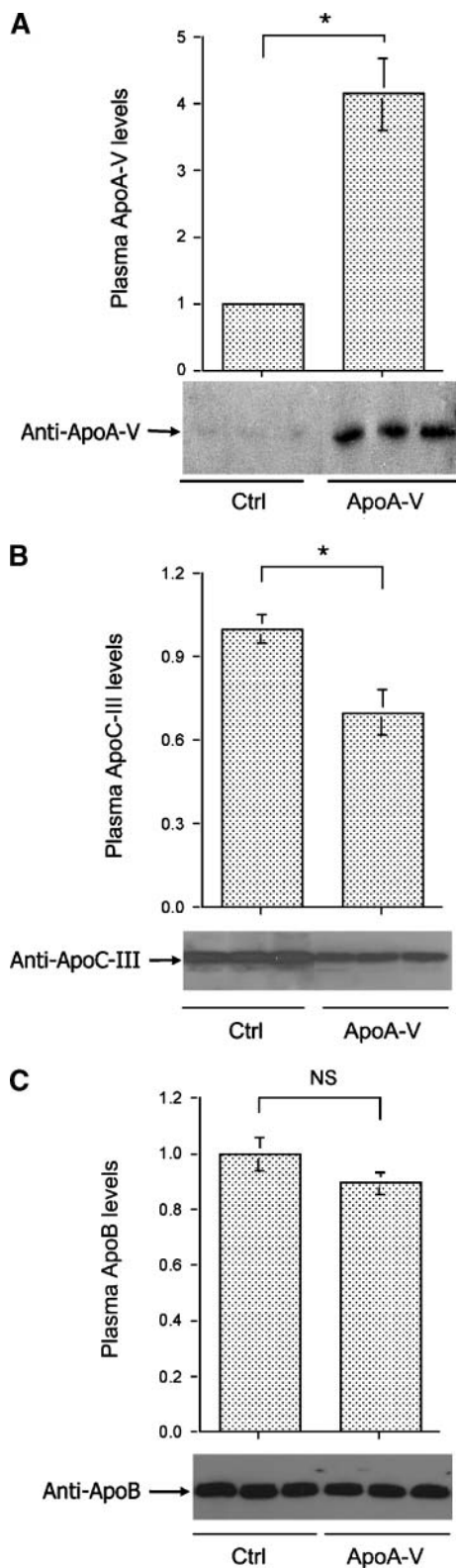
To correlate the reduction in plasma TG levels with hepatic apoA-V production, we determined plasma apoA-V, apoC-III, and apoB protein levels in both control and apoA-V vector-treated APOC3 transgenic mice. As shown in **Fig. 2A**, APOC3 transgenic mice exhibited relatively low basal plasma apoA-V levels, in agreement with previous observations that apoA-V is one of the least abundant apolipoproteins in plasma in mice and humans (5, 22). However, after 2 weeks of hepatic apoA-V production,  $\sim$ 4-fold higher levels of plasma apoA-V protein were detected in apoA-V vector-treated APOC3 transgenic mice (**Fig. 2A**). In addition, we detected a significant reduction in plasma apoC-III levels in apoA-V vector-treated APOC3 transgenic mice (**Fig. 2B**). In contrast, plasma apoB levels

remained unaltered in response to hepatic apoA-V production (**Fig. 2C**).

#### Effect of apoA-V on VLDL-TG production versus catabolism

To characterize the mechanism of apoA-V-mediated amelioration of hypertriglyceridemia in APOC3 transgenic mice, we determined hepatic VLDL-TG production versus catabolism. Mice in both groups at day 8 were fasted for 5 h, followed by intraperitoneal injection of 500 mg/kg Triton WR1339 to inhibit VLDL-TG hydrolysis and clearance. Aliquots of blood (25  $\mu$ l) were collected from tail veins for the determination of plasma TG levels at 20 min intervals. As shown in **Fig. 3A**, plasma TG levels in both control and apoA-V vector-treated APOC3 transgenic mice increased after Triton WR1339 administration. The rates of VLDL-TG production, defined as milligrams of TG per minute per kilogram of body weight, were statistically indistinguishable between apoA-V and control groups (**Fig. 3B**). These results were consistent with the lack of alterations in plasma apoB levels in response to hepatic apoA-V production (**Fig. 2C**).

To determine the effect of apoA-V on VLDL-TG catabolism, we performed oral fat tolerance tests. Mice were fasted overnight, followed by an oral bolus of olive oil. Aliquots of tail vein blood (25  $\mu$ l) were sampled for the determination of plasma TG levels before and at different times after fat administration. As shown in **Fig. 3C**, control



**Fig. 2.** Plasma apolipoprotein levels. Aliquots of plasma (20  $\mu$ g of protein) from euthanized APOC3 transgenic mice in control (Ctrl) and apoA-V groups were resolved on 4–20% SDS-polyacrylamide gels, which were subjected to immunoblot assay. Plasma levels of apoA-V (A), apoC-III (B), and apoB (C) were determined. Data are expressed as means  $\pm$  SEM. \*  $P < 0.05$ .

vector-treated APOC3 transgenic mice were associated with severely impaired fat tolerance. Compared with apoA-V vector-treated APOC3 transgenic mice, significantly higher plasma TG levels were detected at all time points in control vector-treated APOC3 transgenic mice (Fig. 3C). At 6 h after fat administration, plasma TG levels were still maintained at markedly higher levels in control vector-treated APOC3 transgenic mice ( $1,674 \pm 142$  mg/dl vs.  $1,028 \pm 108$  mg/dl in the apoA-V group;  $P < 0.01$ ).

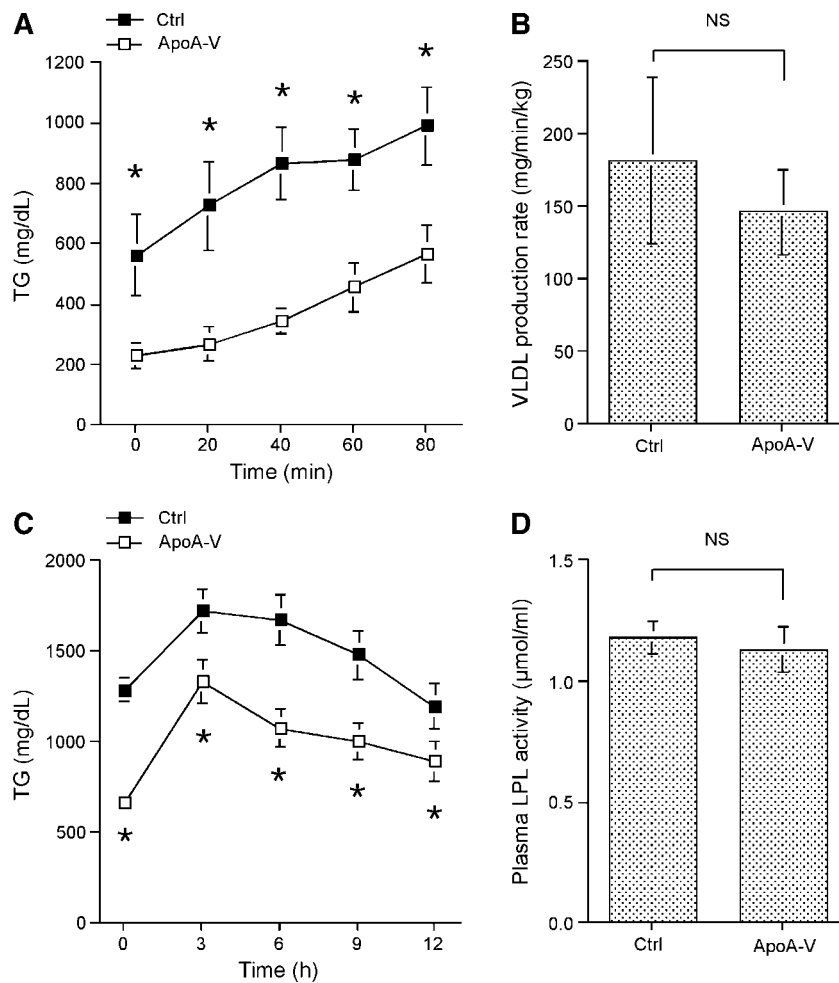
To study the impact of hepatic apoA-V production on plasma LPL activity, we heparinized mice in both groups by intravenous injection of heparin. Aliquots of tail vein blood were collected to measure plasma LPL activity. As shown in Fig. 3D, no significant differences in postheparin plasma LPL activities were detected between control and apoA-V vector-treated APOC3 transgenic mice, suggesting that the observed reduction in plasma TG levels in apoA-V vector-treated mice was not attributable to increased LPL levels in plasma.

#### Impact of apoA-V on lipoprotein and cholesterol metabolism

To study the effect of apoA-V on lipoprotein metabolism, aliquots of plasma (250  $\mu$ l) pooled from individual mice in the control or apoA-V group were subjected to gel filtration column chromatography for the fractionation of lipoprotein particles. ApoA-V vector-treated APOC3 transgenic mice were associated with reduced VLDL-TG levels (Fig. 4A), which were consistent with their significantly decreased plasma TG levels (Fig. 1A). Increased apoA-V production also resulted in decreased VLDL-cholesterol levels, but the fractional concentrations of cholesterol in HDL<sub>1</sub>, HDL<sub>2</sub>, and HDL<sub>3</sub> were markedly increased in apoA-V vector-treated APOC3 transgenic mice (Fig. 4B). Thus, despite the lack of changes in total plasma cholesterol levels (Fig. 1B), increased apoA-V levels were associated with skewed cholesterol distribution from VLDL to large HDL particles (Fig. 4B).

To account for these observations, we performed immunoblot assays to determine the fractional concentrations of apoA-I, a major constituent of HDL particles. As shown in Fig. 4C, significantly higher levels of apoA-I were detected in HDL<sub>1</sub>, HDL<sub>2</sub>, and HDL<sub>3</sub> fractions of apoA-V vector-treated APOC3 transgenic mice. Thus, hepatic apoA-V production resulted in increased apoA-I levels, contributing to increased HDL-cholesterol levels in apoA-V vector-treated APOC3 transgenic mice. These results raise the possibility that apoA-V modulates nascent HDL formation and maturation.

To address this possibility, we used native agarose gel electrophoresis to separate nascent pre $\beta$ -HDL from  $\alpha$ -HDL particles, followed by immune detection and quantification. Pre $\beta$ -HDL levels remained unchanged in response to hepatic apoA-V production (Fig. 5A, B). Instead, we detected a significant increase in  $\alpha$ -HDL levels (Fig. 5A, C), correlating with the relatively higher HDL-cholesterol levels in apoA-V vector-treated APOC3 transgenic mice (Fig. 4B). Together, these results indicate that increased apoA-V production promoted  $\alpha$ -HDL formation, resulting



**Fig. 3.** Hepatic VLDL-TG production versus catabolism. **A:** Hepatic VLDL-TG production. APOC3 transgenic mice treated with apoA-V and control (Ctrl) vectors were fasted at day 9 after vector administration for 5 h, followed by intraperitoneal injection of 500 mg/kg Triton WR1339. Aliquots of blood (25  $\mu$ l) were collected at 20, 40, 60, and 80 min for the determination of plasma TG levels. **B:** The rates of hepatic VLDL-TG production, defined as milligrams of TG produced per minute per kilogram of body weight, were calculated based on the data in panel A. **C:** Postprandial TG response. Treated mice were fasted at day 5 after vector administration for 16 h, followed by an oral bolus of olive oil (10  $\mu$ l/g body weight). Aliquots of tail vein blood (25  $\mu$ l) were sampled at different times for the determination of plasma TG levels. **D:** Postheparin LPL activity. Treated APOC3 transgenic mice at day 7 were injected intravenously with 300 IU heparin/kg body weight, and tail vein blood (20  $\mu$ l) was sampled at 10 min after heparin infusion. Heparinized sera were prepared for the determination of LPL activity. Data are expressed as means  $\pm$  SEM. \*  $P < 0.01$ .

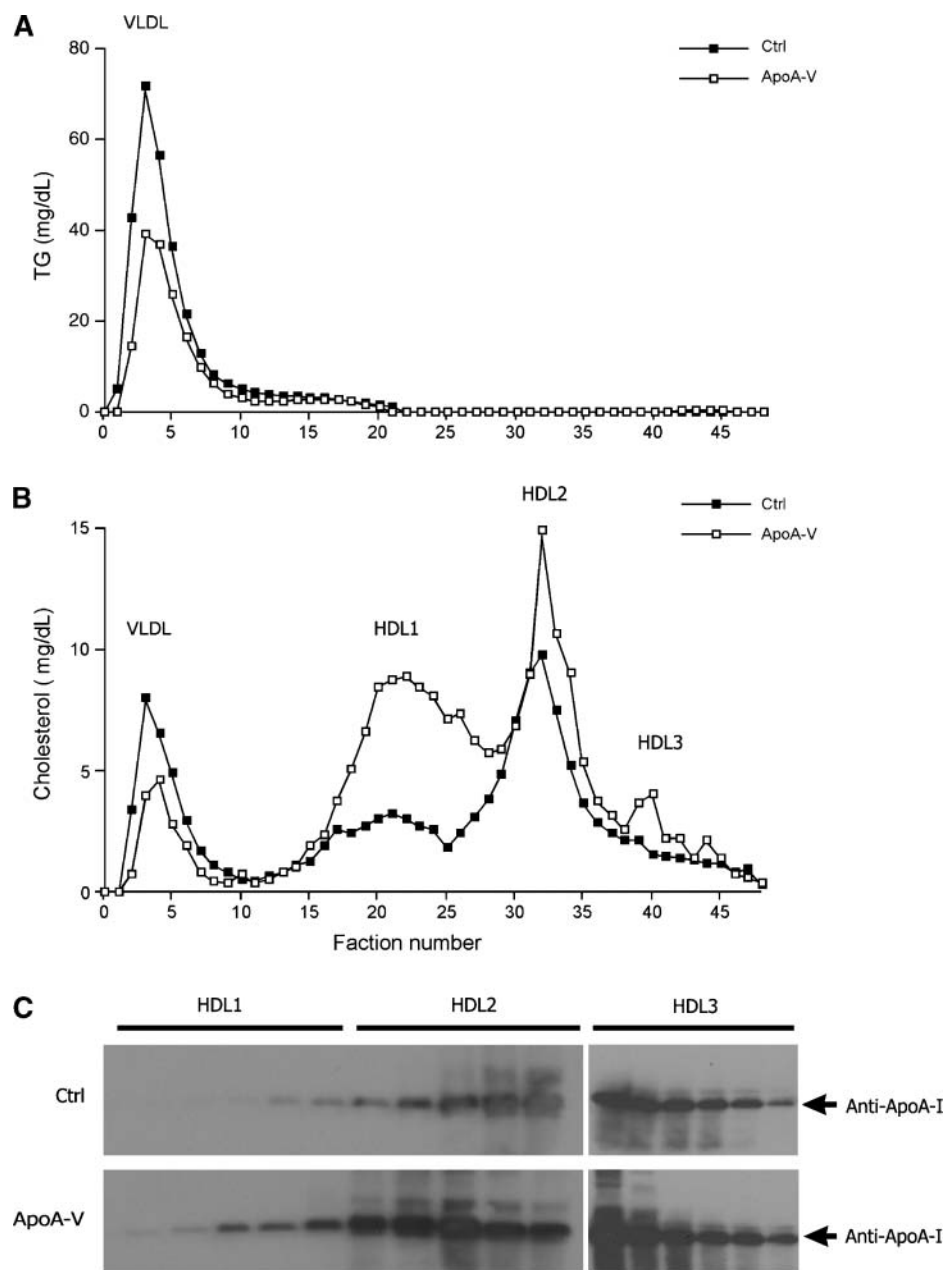
in a significant increase in both the size and number of HDL particles.

#### Mechanism of apoA-V-mediated improvement in VLDL-TG catabolism

To probe the molecular basis underlying apoA-V-mediated improvement in VLDL-TG catabolism, we determined apoC-III and apoA-V content in lipoprotein particles by semiquantitative immunoblot assay. As shown in **Fig. 6A**, hepatic apoA-V production resulted in a significant reduction in apoC-III content in VLDL particles, correlating with the reduction in plasma VLDL-TG levels in apoA-V vector-treated APOC3 transgenic mice. Similarly, we detected a significant reduction in apoC-III content

in HDL<sub>2</sub> particles in response to hepatic apoA-V production (**Fig. 6A**). Interestingly, this observed reduction in apoC-III content in HDL was accompanied by a corresponding increase in apoA-V levels in both HDL<sub>1</sub> and HDL<sub>2</sub> fractions in apoA-V vector-treated APOC3 transgenic mice (**Fig. 6B**). ApoA-V levels in VLDL fractions were undetectable in both control and apoA-V vector-treated APOC3 transgenic mice.

In addition, we detected a significant increase in apoE levels in HDL<sub>1</sub> fractions (**Fig. 6C**), correlating with increased HDL-cholesterol levels in apoA-V vector-treated APOC3 transgenic mice. Together, these results suggest that in addition to its beneficial effect on VLDL-TG catabolism, apoA-V appears to regulate HDL-cholesterol



**Fig. 4.** Plasma lipoprotein profiles. Mice were euthanized after a 16 h fast at 2 weeks after vector administration. Aliquots (250  $\mu$ l) of plasma pooled from each group of mice were fractionated by column gel filtration chromatography. Fractions (500  $\mu$ l) were eluted and assayed for TG (A) and cholesterol (B) levels. In addition, aliquots of HDL<sub>1</sub> and HDL<sub>2</sub> fractions (15  $\mu$ l) were resolved on 4–20% SDS-polyacrylamide gels, which were subjected to immunoblot assay using anti-apoA-I antibody (C). Ctrl, control.

metabolism by modulating the composition of apolipoproteins in HDL particles.

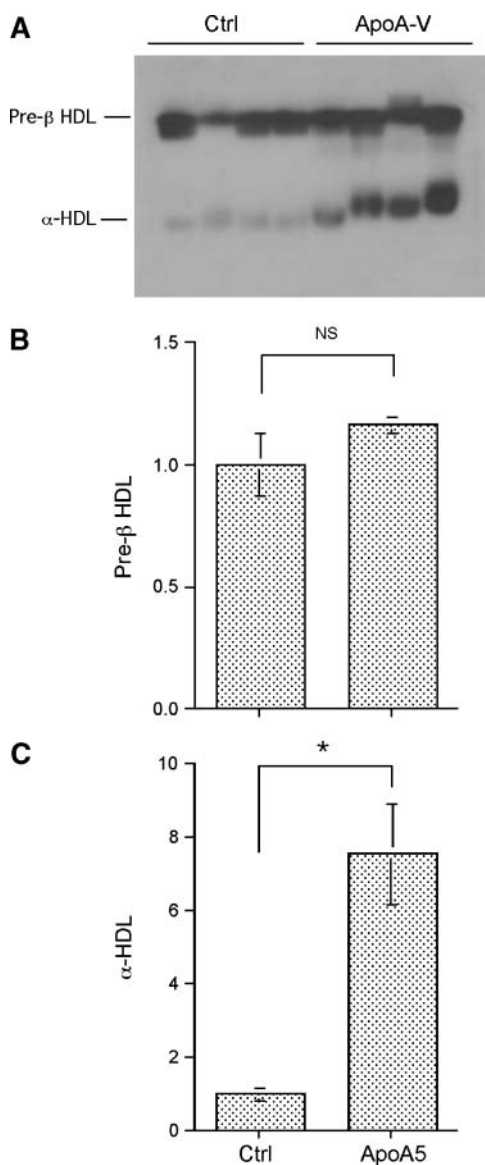
#### Mechanism of apoA-V-mediated increase in HDL particles

To account for the increase in the size and number of HDLs in apoA-V vector-treated APOC3 transgenic mice, we determined the activity of LCAT, a plasma protein that plays a key role in HDL maturation. LCAT catalyzes the conversion of free cholesterol to cholesteryl esters, resulting in the accumulation of cholesteryl esters in the core of HDL. As shown in **Fig. 7A**, plasma LCAT activity was

increased significantly in response to increased apoA-V production. This effect mirrored the enrichment of apoE in HDL and the increases in the size and number of HDL particles in apoA-V vector-treated APOC3 transgenic mice (**Fig. 6**).

In parallel, we determined the effect of apoA-V on cholesterol efflux in macrophages. As shown in **Fig. 7B**, increased apoA-V levels in plasma were associated with increased efflux of [<sup>3</sup>H]cholesterol from macrophages in culture. This effect, along with increased LCAT activity, indicates that apoA-V-enriched HDL particles are





**Fig. 5.** Effect of apoA-V on pre-β-HDL and α-HDL metabolism. Aliquots of plasma (40 μg of protein) from euthanized mice were resolved on 0.7% agarose gels (A). Lipoproteins were transferred to nitrocellulose membrane by capillary blotting, followed by immune detection using rabbit anti-apoA-I antibody. Protein bands corresponding to pre-β-HDL (B) and α-HDL (C) were quantified by densitometry. Data are expressed as means ± SEM. \*  $P < 0.001$ . Ctrl, control.

associated with an enhanced ability to conduct cholesterol efflux from peripheral tissue, contributing to increased plasma HDL-cholesterol levels in apoA-V vector-treated APOC3 transgenic mice.

To gain insight into hepatic HDL uptake and clearance, we determined hepatic expression levels of SR-BI, an acceptor for selective HDL uptake and clearance (32–34). As shown in Fig. 7C, we detected a small, but insignificant, increase in hepatic SR-BI protein level in response to increased apoA-V production. Likewise, similar levels of hepatic LDLR protein were detected in apoA-V and control groups (Fig. 7D). These results indicate that hepatic LDLR

and SR-BI expression levels were not altered significantly in response to increased apoA-V production in liver.

To address whether increased plasma HDL-cholesterol levels were attributable to increased cholesterol synthesis in liver, we determined the expression levels of HMG-CoA reductase, an enzyme that controls the rate-limiting step in de novo cholesterol synthesis (35). As shown in Fig. 7E, no significant differences in hepatic HMG-CoA reductase levels were detected between apoA-V and control groups. These data suggest that increased HDL-cholesterol levels might not be the result of increased hepatic cholesterol synthesis in apoA-V vector-treated APOC3 transgenic mice.

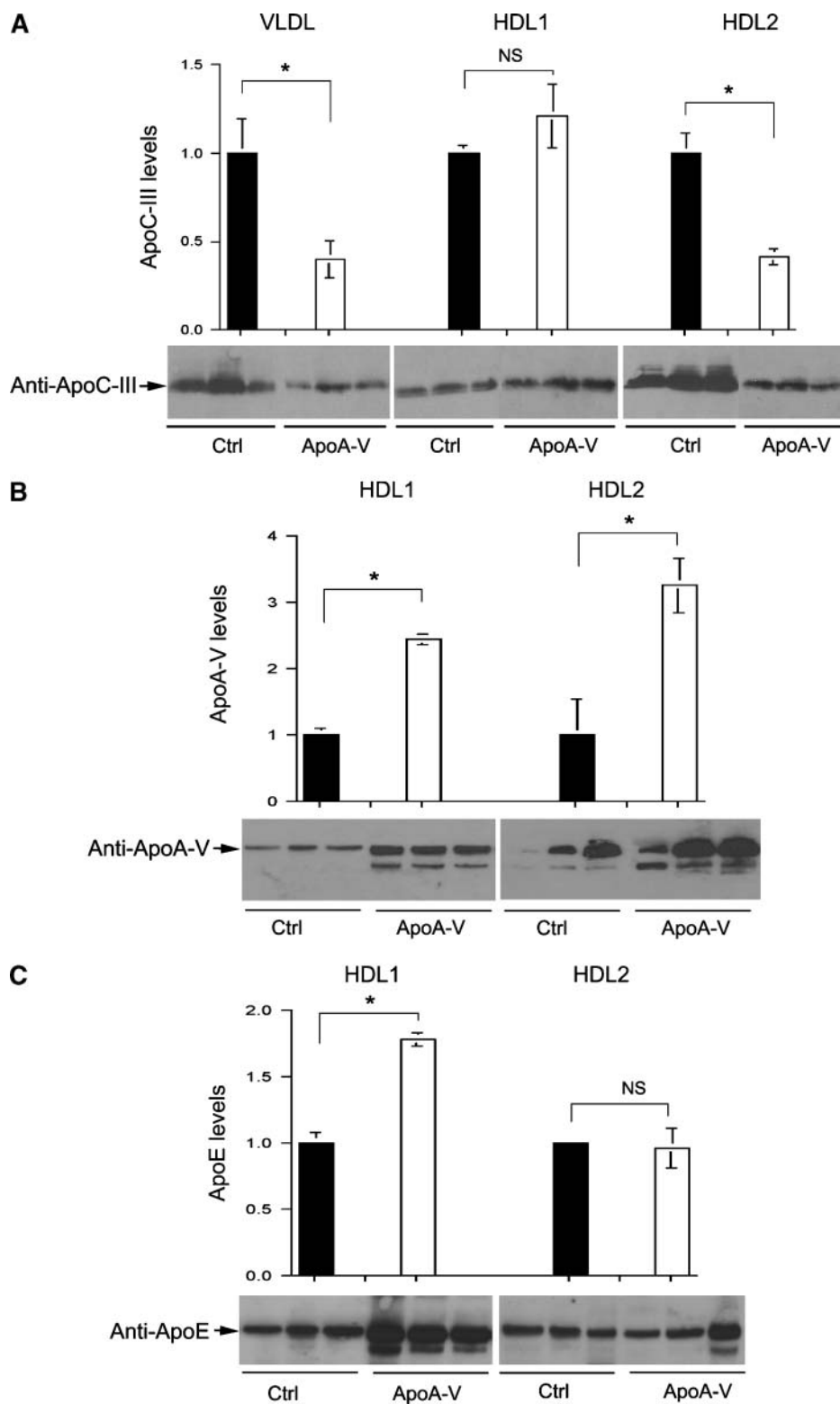
## DISCUSSION

In this study, we determined the effect of apoA-V on plasma TG and cholesterol metabolism in APOC3 transgenic mice. APOC3 transgenic mice fed regular chow develop hypertriglyceridemia as a result of retarded VLDL-TG catabolism (16, 17). Our goal in this study was 2-fold: 1) to address whether apoA-V can counteract apoC-III in improving VLDL-TG metabolism in adult mice with frank hypertriglyceridemia; and 2) to determine whether apoA-V exerts an effect on HDL-cholesterol metabolism.

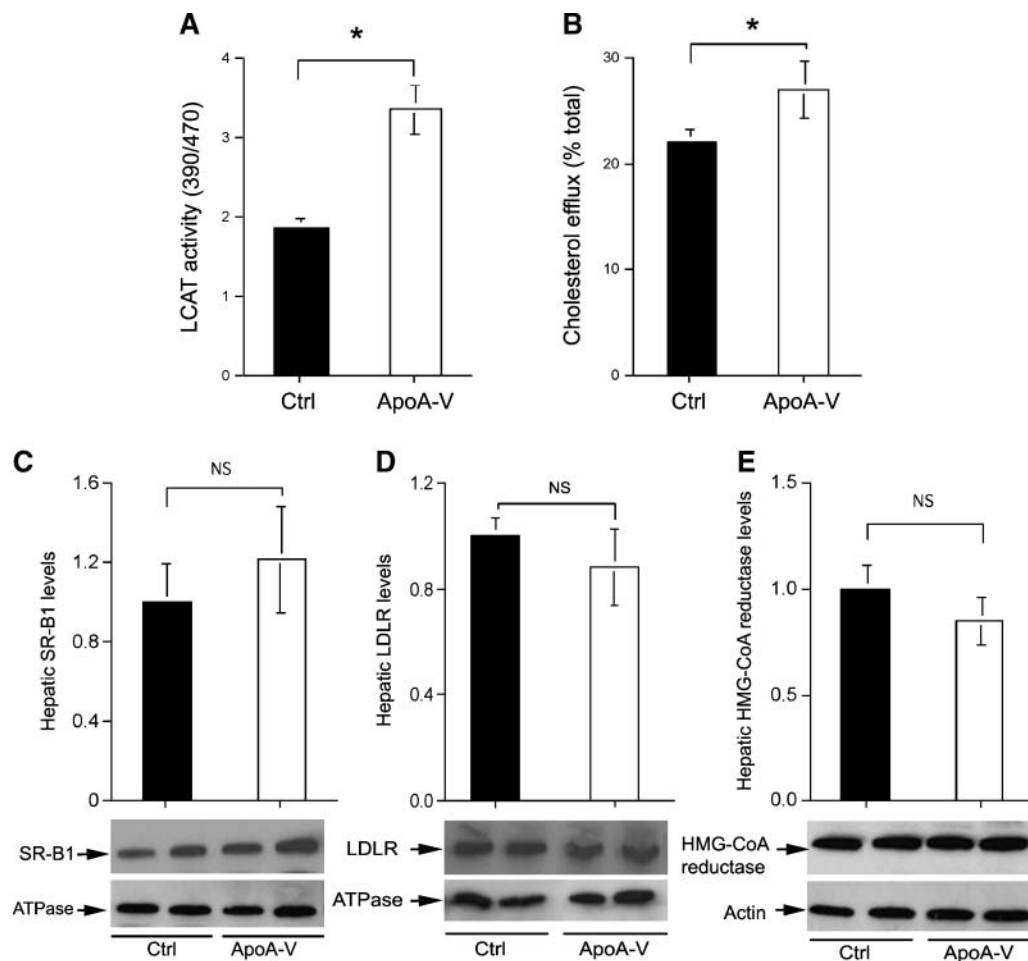
Using an adenovirus-mediated gene expression approach, we achieved a 4-fold increase in plasma apoA-V levels in adult hypertriglyceridemic APOC3 transgenic mice. We show that hepatic apoA-V production resulted in significant reductions in plasma TG levels without alterations in total plasma cholesterol and NEFA levels. APOC3 transgenic mice with increased apoA-V levels exhibited significantly reduced VLDL-TG levels and improved postprandial TG response. This effect correlated with decreased apoC-III and increased apoA-V content in VLDL-TG particles in apoA-V vector-treated APOC3 transgenic mice. These results indicate that increased apoA-V levels modified VLDL apolipoprotein composition by decreasing apoC-III content, which favored VLDL-TG catabolism in apoA-V vector-treated hypertriglyceridemic mice. Using a transgenic approach, Fruchart-Najib et al. (21) showed that transgenic apoA-V expression prevented the development of hypertriglyceridemia in APOC3 transgenic mice. These results, together with our data, support the notion that apoA-V counterbalances the effect of apoC-III on VLDL-TG catabolism, contributing to the amelioration of hypertriglyceridemia in APOC3 transgenic mice.

Despite the improvement in VLDL-TG catabolism, plasma LPL activity remained unchanged in apoA-V vector-treated APOC3 transgenic mice. These results were consistent with previous data that neither apoA-V overexpression nor deficiency is associated with significant alterations in plasma LPL activity (36). How does apoA-V act to improve the clearance of TG-rich particles in the absence of altered LPL activity? To address this fundamental question, Merkel and Heeren (37) postulated that apoA-V augments plasma TG hydrolysis by fostering the interaction of TG-rich lipoproteins with endothelium-bound LPL. In support of this model, apoA-V has been





**Fig. 6.** ApoC-III and apoA-V levels in lipoproteins. Aliquots of peak fractions (15  $\mu$ l) in VLDL, HDL<sub>1</sub>, and HDL<sub>2</sub> fractions were resolved on 4–20% SDS-polyacrylamide gels, followed by semiquantitative immunoblot assay using antibodies against apoC-III (A), apoA-V (B), and apoE (C). ApoA-V and apoE were undetectable in VLDL fractions using the same immunoblot assay. Protein bands were quantified by densitometry. Data are expressed as means  $\pm$  SEM. \*  $P < 0.05$ . Ctrl, control.



**Fig. 7.** A: Plasma LCAT activity. Plasma was prepared from 16 h fasted mice after 2 weeks of hepatic apoA-V production and subjected to LCAT activity assay. The relative LCAT activity was compared between apoA-V and control (Ctrl) groups. B: Cholesterol efflux in macrophages. The ability of apoA-V-enriched HDL to conduct cholesterol efflux was assayed in cultured macrophages using 5  $\mu$ l of plasma from apoA-V and control groups of fasted mice at the end of the 2 week study. C: Hepatic scavenger receptor class B type I (SR-BI) levels. D: Hepatic low density lipoprotein receptor (LDLR) levels. E: Hepatic HMG-CoA reductase levels. Aliquots of 20 mg of liver tissue were homogenized for either the preparation of total liver protein extracts or the enrichment of plasma membrane proteins, which were subjected to semiquantitative Western blot analysis. The relative levels of hepatic proteins SR-BI, HMG-CoA, and LDLR were compared between apoA-V and control groups using actin or the membrane-specific marker sodium potassium ATPase as an internal control. Data are expressed as means  $\pm$  SEM. \*  $P < 0.05$ .

shown to accelerate the hydrolysis of TG-rich lipoproteins in the presence of proteoglycan-bound LPL (36). In the absence of proteoglycans, apoA-V derived from TG-rich lipoproteins and apoA-V transgenic mouse HDL fractions did not affect the LPL-mediated hydrolysis of TG-rich particles (36, 38). This model helps explain the TG-lowering effect of apoA-V, but the underlying molecular basis remains to be elucidated (37, 38).


Although it is accepted that apoA-V-mediated reduction of plasma TG levels is through an enhanced clearance of TG-rich particles, there is a lack of consensus in the literature regarding the inhibitory effect of apoA-V on hepatic VLDL-TG production. We show that increased apoA-V production did not result in significant changes in hepatic VLDL-TG production, correlating with the lack of

alterations in plasma apoB levels in apoA-V vector-treated APOC3 transgenic mice. Consistent with our observations, three independent studies have demonstrated in apoA-V transgenic or knockout mice that apoA-V does not affect hepatic VLDL-TG secretion in mice (20, 21, 36). In contrast, Schaap et al. (39) showed that adenovirus-mediated apoA-V production inhibits hepatic VLDL-TG production in addition to its stimulatory effect on VLDL-TG hydrolysis. Apart from the difference in mouse strains, a potential contributing factor to this discrepancy is the level of hepatic apoA-V production. In our study, we increased plasma apoA-V levels by  $\sim$ 4-fold in hypertriglyceridemic APOC3 transgenic mice, whereas a 10-fold increase of plasma apoA-V levels was achieved in normal C57BL/6J mice in the study by Schaap et al. (39).

In addition to its effect on plasma TG levels, our data revealed a profound effect of apoA-V on HDL metabolism. Increased apoA-V production did not affect pre $\beta$ -HDL levels but promoted  $\alpha$ -HDL formation, resulting in significant increases in both the number and size of HDL particles, as shown by increased HDL<sub>1</sub>- and HDL<sub>2</sub>-cholesterol levels in apoA-V vector-treated APOC3 transgenic mice. This effect correlated with increased apoA-V and decreased apoC-III contents in HDL particles. Furthermore, increased apoA-V levels resulted in increased apoA-I levels in HDL<sub>1</sub> and HDL<sub>2</sub> fractions. In contrast, VLDL-cholesterol levels were markedly reduced in response to hepatic apoA-V production. This reduction in VLDL-cholesterol levels was unlikely to be caused by decreased cholesterol synthesis, as hepatic protein levels of HMG-CoA reductase remained unchanged in apoA-V versus control groups. Thus, despite the lack of changes in total plasma cholesterol levels, increased apoA-V levels contributed to HDL remodeling and expansion with cholesterol, resulting in skewed cholesterol distribution toward large HDLs. It appeared that HDL particles with increased apoA-V content were associated with increased cholesterol-loading capacity.

Consistent with this idea is our observation that larger HDL<sub>1</sub> particles in apoA-V vector-treated APOC3 transgenic mice were enriched with apoE, an apolipoprotein that plays an obligatory role in HDL particle expansion (40, 41). In addition, increased apoA-V levels were associated with enhanced LCAT activity and increased cholesterol efflux from cultured macrophages. A significant increase in LCAT activity, together with augmented efflux of cholesterol from peripheral tissues, contributed to HDL core expansion and enlargement of HDL particles in apoA-V vector-treated APOC3 transgenic mice. In accordance with these observations, increased plasma LCAT activity, resulting from transgenic LCAT expression in both mice and rabbits, is associated with enlarged HDL particles (42, 43). This property of apoA-V in modulating HDL remodeling, along with its ability to improve VLDL-TG catabolism, presages a potential beneficial effect of apoA-V on atherosclerosis. However, because of the infrastructural constraint in our animal facility, we are limited to conducting radioisotope tracer assays to determine the effect of apoA-V on hepatic HDL uptake and clearance in mice. Thus, whether apoA-V can improve reverse cholesterol transport and ameliorate atherosclerotic lesions remains to be determined in animal models with premature atherosclerosis.

In conclusion, our data show that apoA-V at higher levels is able to modulate the apolipoprotein composition of VLDL, resulting in enhanced VLDL-TG catabolism and contributing to the amelioration of hypertriglyceridemia in adult APOC3 transgenic mice. Furthermore, apoA-V was shown to promote  $\alpha$ -HDL formation, accounting at least in part for skewed cholesterol redistribution to large HDL particles. These data underscore the functional importance of apoA-V in plasma VLDL-TG and HDL-cholesterol metabolism. Augmented apoA-V production should be considered a modality for the pharmacological

management of hypertriglyceridemia associated with the metabolic syndrome. 

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