

# A role of apolipoprotein D in triglyceride metabolism<sup>S</sup>

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**Abstract** Apolipoproteins (apo) are constituents of lipoproteins crucial for lipid homeostasis. Aberrant expression of apolipoproteins is associated with metabolic abnormalities. Here we characterized apolipoprotein D (apoD) in triglyceride metabolism. Unlike canonical apolipoproteins that are mainly produced in the liver, apoD is an atypical apolipoprotein with broad tissue distribution. We show that circulating apoD is present mainly in HDL and, to a lesser extent, in LDL and VLDL and that its plasma levels were reduced in *db/db* mice with visceral obesity and altered lipid metabolism. Elevated apoD production, derived from adenovirus-mediated gene transfer, resulted in significant reduction in plasma triglyceride levels in mice. This effect was attributable to enhanced LPL activity and improved catabolism of triglyceride-rich particles. In contrast, VLDL triglyceride production remained unchanged in response to elevated apoD production. These findings were recapitulated in high-fat-induced obese mice. Obese mice with elevated apoD production exhibited significantly improved triglyceride profiles, correlating with increased plasma LPL activity and enhanced postprandial fat tolerance. ApoD was shown to promote LPL-mediated hydrolysis of VLDL *in vitro*, correlating with its TG-lowering action *in vivo*. **Apolipoprotein D plays a significant role in lipid metabolism. These data provide important clues to clinical observations that genetic variants of apoD are associated with abnormal lipid metabolism and increased risk of metabolic syndrome.**—Perdomo, G., D. H. Kim, T. Zhang, S. Qu, E. A. Thomas, F. G. S. Toledo, S. Slusher, Y. Fan, D. E. Kelley, and H. H. Dong. **A role of apolipoprotein D in triglyceride metabolism.** *J. Lipid Res.* 2010. 51: 1298–1311.

**Supplementary key words** HDL • VLDL • lipoprotein lipase

ApoD is an atypical apolipoprotein that belongs to the lipocalin superfamily (1). This superfamily comprises a diverse family of lipid-binding proteins, including fatty acid binding proteins (FABP), plasma retinol-binding proteins

(RBP), and apolipoprotein M (ApoM) (1–5). These proteins differ in amino acid sequences, but they share a highly conserved  $\beta$ -barrel structure comprised of an eight-stranded anti-parallel  $\beta$ -sheet (1). Such a tertiary architecture forms a ligand-binding pocket that is responsible for binding and transporting lipids and other small hydrophobic molecules (2, 3). Indeed, apoD has been shown to associate with a number of ligands, including cholesterol (6), steroid hormones (7, 8) and arachidonic acid (9, 10). Unlike other apolipoproteins that are mainly produced in the liver and intestine, apoD is widely expressed in mammalian tissues, including brain, peripheral nerves, liver, intestine, cardiac and skeletal muscle, adipose tissue, spleen, testes, placenta, ovaries, lung, and pancreas (2, 11–13). Plasma apoD is present mainly in HDL and, to a lesser extent, in VLDL (14). This correlates with the ability of apoD to associate with apoA-II, an abundant component of HDL (15). In humans, plasma apoD levels range from 3 to 11  $\mu\text{mol/l}$  (2). The human apoD gene harbors several genetic polymorphisms, including those in the coding, the 3'-untranslated and intronic regions. Linkage analysis studies have found associations between apoD variants and the incidence of obesity and hyperinsulinemia (16) and type 2 diabetes (17). Furthermore, three missense mutations were found to be linked to elevated plasma triglycerides and reduced HDL-cholesterol levels (18).

These data underscore the importance of apoD in metabolism; spurring the hypothesis that apoD dysregulation may contribute to the pathogenesis of dyslipidemia. In this study, we addressed this hypothesis by determining plasma apoD levels in correlation to plasma lipid profiles in obese mice with altered lipid metabolism. Furthermore, we studied the impact of apoD on lipid metabolism in normal and obese mice with elevated apoD production.

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Abbreviations: ALT, alanine aminotransferase; apo, apolipoprotein; AST, aspartate aminotransferase; CETP, cholesterol ester transfer protein; FABP, fatty acid binding protein; MTP, microsomal triglyceride transfer protein; RBP, retinol binding protein; TG, triglyceride.

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<sup>S</sup>The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of one figure and one table.

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## MATERIALS AND METHODS

### Animal studies

CD-1 mice (male, 6 weeks old) were obtained from Charles River Laboratory (Wilmington, MA). Male *db/db* mice and heterozygous *db/+* littermates (6 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME). Male C57BL/6J mice (6 weeks old) obtained from Jackson Laboratory. Mice were fed standard rodent chow and water ad libitum in sterile cages with a 12-h light/dark cycle. To induce obesity, C57BL/6J mice were fed a high-fat diet (fat content, > 60 kcal%, D12492, Research Diets, New Brunswick, NJ) for 14 weeks. For blood chemistry, mice were fasted for 16 h and tail-vein blood samples were collected into capillary tubes precoated with potassium-EDTA (Sarstedt, Nümbrecht, Germany) for the preparation of plasma. Blood glucose levels were measured using Glucometer Elite (Bayer, IN). Plasma insulin levels were measured using ultrasensitive mouse ELISA (Mercodia, Uppsala, Sweden). Plasma triglyceride (TG) and cholesterol levels were determined using TG and cholesterol reagents (Thermo Electron, Melbourne, Australia). The Institutional Animal Care and Usage Committee of Children's Hospital of Pittsburgh approved the protocol for animal studies (#30-07).

### Adenoviral vector construction and delivery

ApoD cDNA was amplified by RT-PCR from CD-1 mouse brain RNA using primers flanking the apoD mRNA for forward reaction (5'-TAAGGCCTCTCCTGCAGC CA-3') and reverse reaction (5'-CTTTACAGGAAGTCCGGGCAG-3'). ApoD cDNA was cloned into adenoviral shuttle plasmid pENTR-1A (Invitrogen, Carlsbad, CA), which was converted into an adenoviral vector using ViraPower™ Adenoviral Expression System (Invitrogen, Carlsbad, CA). The control Adv-null adenovirus has been described (19). Both adenoviruses were propagated in HEK-293 cells and purified by CsCl density centrifugation as described (20). For vector administration, mice were injected through the tail vein with  $5 \times 10^{12}$  viral particles per kg body weight (equivalent to  $1.5 \times 10^{11}$  plaque-forming units/kg), as described (20, 21).

### Fat tolerance test

Mice were fasted overnight, followed by an oral bolus of olive oil (10  $\mu$ l/g body weight). Aliquots of blood from the tail vein were drawn before and after olive oil administration (every 1.5 h) to determine plasma TG.

### VLDL-TG production assay

Mice were fasted for 5 h, followed by intravenous injection of Tyloxapol (Sigma Aldrich, St. Louis, MO) at 500 mg/kg body weight per mouse, to inhibit plasma VLDL clearance. Aliquots of tail-vein blood were taken at different times to determine plasma TG levels.

### Lipoprotein lipase assay

Mice were injected intravenously with 300 IU heparin/kg body weight, and tail-vein blood (20  $\mu$ l) was sampled 10 min after heparin infusion. Heparinized sera were prepared to determine lipoprotein lipase activity using the LPL activity kit (Roar Biochemical Inc., New York, NY), as previously described (22, 23).

### Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) assay

Aliquots (30  $\mu$ l) of blood were collected from the tail vein of mice after a 16-h fast. Fasting plasma levels of AST and ALT were determined using the AST and ALT assay kits (Thermo Electron, Melbourne, Australia), as described (24).

### Hepatic lipid determination

Aliquots of liver tissue (20 mg) were homogenized in 400  $\mu$ l HPLC-grade acetone. After incubation with agitation at room temperature overnight, aliquots (50  $\mu$ l) of acetone-extract lipid suspension were used to determine triglyceride concentrations using the Infinity triglyceride reagent (Thermo Electron). Hepatic lipid content was defined as mg of triglyceride per gram of total liver proteins, as described (21, 24).

### Hepatic lipase assay

Aliquots of liver tissue (20 mg) were homogenized in 200  $\mu$ l PBS supplemented with 2  $\mu$ l Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL). Aliquots of hepatic protein lysates (10  $\mu$ l) were subjected to lipase activity assay (Roar Biochemical Inc., New York, NY).

### Liver histology

Liver tissues from sacrificed mice were placed in Histoprep tissue embedding media and snap frozen for fat staining with Oil red O as described (24).

### In vitro VLDL-TG hydrolysis assay

The effect of apoD on VLDL-TG hydrolysis by proteoglycan-bound LPL was determined using a previously described assay with modifications (25). Heparan sulfate proteoglycan (H4777, Sigma-Aldrich) at a fixed concentration of 1.5  $\mu$ g per well was added to a 96-well microtiter plate. After incubation at 4°C for 18 h, the plate was washed three times with PBS and blocked for 1 h at 37°C with PBS containing 1% (w/v) FFA-free BSA. The proteoglycan-coated plate was incubated with bovine LPL (L-2254, Sigma-Aldrich) at 80 U/well for 3 h at 4°C to couple LPL to proteoglycan. Free, uncoupled LPL was washed off by washing the plate with PBS three times. Aliquots of human VLDL (Biomedical Technologies) at a fixed quantity of 1 mM triglyceride were added to individual wells in the LPL-coated plates, followed by the addition of recombinant human apoD (ab38530, Abcam) at final concentrations ranging from 0 to 1  $\mu$ g/ml. Each condition was run in triplicate. After 10-min incubation at 37°C, the reactions were stopped by the addition of Triton X-100 (final concentration, 1%). Aliquots of the reaction mixture were used for determining FFA concentrations using the FFA kit (Wako Chemicals USA). Likewise, the effect of apoD on the hydrolysis rate of VLDL by free LPL was determined using microtiter plates without proteoglycan precoating. The rate of VLDL hydrolysis, defined by the amount of FFA produced in the reaction per unit time, was compared among different conditions.

### Preparation of native ApoD protein

ApoD protein is prepared from a conditioned medium of HepG2 cells that were pretransduced with apoD vector. HepG2 cells grown in 75 cm flask in 10 ml DMEM supplemented with 10% FBS were transduced with apoD vector or control Adv-null vector at a fixed dose of 200 pfu/cell. Each condition was run in triplicate. After 4-h incubation, DMEM medium was removed. Cells were washed with prewarmed PBS buffer to remove residual DMEM medium, followed by incubation in 10 ml serum-free DMEM medium for 24 h at 37°C in a CO<sub>2</sub> incubator. Conditioned medium (30 ml in total) from apoD and control groups was collected and centrifuged through the Amicon Centricron Plus-20 (molecular mass cut-off at 5,000 Dalton, Millipore Corp., Billerica, MA) at 2,500 *g* for 45 min. Subsequently, both control and apoD samples (1 ml in aliquots) were dialyzed against 1,000 ml of 1  $\times$  PBS buffer for 5 h at 4°C. Aliquots of dialyzed samples (5  $\mu$ g protein) were subjected to electrophoresis

on 4–20% SDS-PAGE under reducing conditions, followed by immunoblot assay using rabbit anti-apoD antibody.

### FPLC fractionation of lipoproteins

Aliquots (250  $\mu$ l) of plasma pooled from control or apoD groups were applied to two head-to-tail-linked Tricorn high-performance Superose S-6 10/300GL columns using an FPLC system (GE Health Care, Piscataway, NJ), 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, as described (21, 22).

### Immunoblot analysis

To determine plasma apolipoprotein levels, aliquots of plasma at a fixed concentration of 20  $\mu$ g protein per lane were resolved on 4–20% polyacrylamide gels under reducing conditions. The gels were subjected to immunoblot assay using rabbit anti-apoD antibody (1:1,000) that was developed in our laboratory by immunizing rabbits with the peptide VKKYLGRWYEIEKIP (corresponding to amino acid residue 18–32 of apoD protein, Genemed Synthesis, San Francisco, CA). As control, polyclonal goat anti-mouse albumin antibody (1:500, Abcam) or polyclonal rabbit anti-human albumin antibody (1:500, Cell Signaling, Danvers, MA) was used in the immunoblot assay. Similarly, plasma levels of apoA-I and apoB100 were determined by immunoblot analysis using rabbit anti-mouse ApoA-I (1:500, Biotest, Saco, ME) and apoB antibodies (1:500, Abcam), respectively. To determine the effect of apoD on LPL protein expression, epididymal fat tissue, hind-limb skeletal muscle, and heart were collected from sacrificed mice and were subjected to immunoblot analysis using rabbit anti-mouse LPL antibody (1:500, Santa Cruz). Likewise, hepatic lipase and microsomal triglyceride transfer protein (MTP) levels were determined using rabbit anti-mouse HL antibody (Santa Cruz) and rabbit anti-mouse MTP antibody as described (21).

### Statistical analysis

Statistical analyses of data were performed by paired Student *t*-test using the statistical module of Microsoft Excel and by ANOVA (ANOVA) using JMP statistics package (SAS Institute Inc., Cary, NC). Data were expressed as mean  $\pm$  SEM. *P* values <0.05 were considered significant.

## RESULTS

### ApoD production in obese *db/db* mice

To investigate the association of apoD with lipid metabolism, we determined plasma apoD levels in *db/db* mice with visceral obesity. Due to the lack of leptin receptor, *db/db* mice are hyperphagic, developing obesity with excessive accumulation of visceral fat, as described (20, 21). When compared with heterozygous control littermates, homozygous *db/db* mice displayed significantly lower plasma apoD levels (Fig. 1A) and higher plasma TG levels (Fig. 1B). Unlike obese human subjects, obese *db/db* mice are associated with increased HDL-cholesterol levels (Fig. 1C). After normalizing to apoA-I content in HDL, a greater reduction in apoD expression levels were detected in obese *db/db* mice (Fig. 1D). Furthermore, plasma apoD levels were inversely correlated with plasma TG levels in *db/db* and control mice (Fig. 1E). Together these data indicate that plasma apoD levels were downregulated in obese *db/db* mice with altered TG metabolism.

### Effect of apoD on lipid metabolism in mice

To determine the effect of apoD on metabolism, we delivered apoD cDNA by adenovirus-mediated gene transfer to the liver of normal CD-1 mice using a null adenovirus as the control, as described (19, 20). This approach resulted in transgene expression mainly in the liver with little transduction of extrahepatic tissues (20, 26). We used a predefined dose of apoD vector ( $1.5 \times 10^{11}$  pfu/kg) to transduce about 70% of hepatocytes in liver (20). This vector dose resulted in 3-fold elevation of plasma apoD levels following a 2-week hepatic apoD production (Fig. 2A). Elevated plasma apoD levels resulted in significantly reduced fasting plasma TG (Fig. 2B) and cholesterol levels in apoD vector-treated mice (Fig. 2C). In contrast, plasma NEFA levels remained unchanged (Fig. 2D). No significant differences in body weight were detected between apoD and control groups during the course of the study.

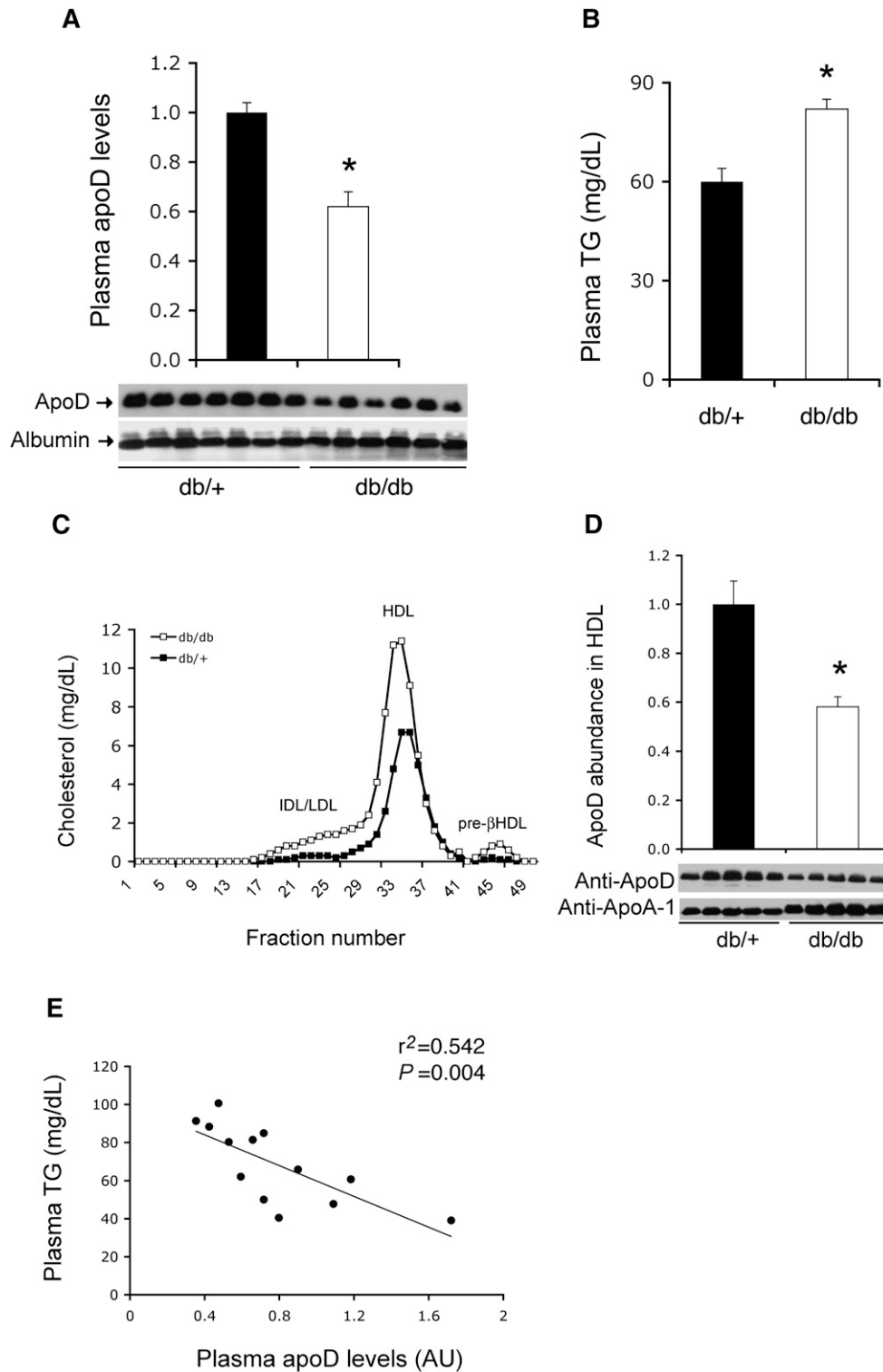
To determine the potential adverse effect of hepatic apoD production on the liver, we determined fasting plasma levels of ALT and AST. No significant differences in these two liver enzymes were detected between apoD and control vector-treated mice, and their fasting plasma ALT and AST levels were within the physiological range (see supplementary Fig. IA, B).

To investigate the potential effect of apoD on hepatic fat metabolism, we sacrificed both control and apoD vector-treated mice 2 weeks post vector administration and determined hepatic lipid content. No significant differences in hepatic lipid content were detected in apoD versus control vector-treated mice (see supplementary Fig. IC). To confirm these findings, liver tissues from both apoD and control groups were cut into sections (10  $\mu$ m thickness) and stained with Oil Red O. Histological examination of liver sections did not reveal significant differences in hepatic lipid content in apoD and control vector-treated mice (Data not shown).

To study the effect of apoD on lipoprotein metabolism, we subjected plasma pooled from individual mice from apoD or control groups to gel filtration column chromatography for the fractionation of lipoprotein particles. Consistent with plasma TG and cholesterol levels, apoD vector-treated mice were associated with markedly reduced VLDL-TG (Fig. 3A) and HDL-cholesterol levels (Fig. 3B). To study apoD distribution in lipoproteins, we subjected the peak fractions of VLDL, LDL, and HDL profiles to immunoblot analysis. ApoD was detected mainly in HDL fractions. In accordance with plasma apoD levels, apoD vector-treated mice displayed significantly higher apoD levels in HDL fractions (Fig. 3C).

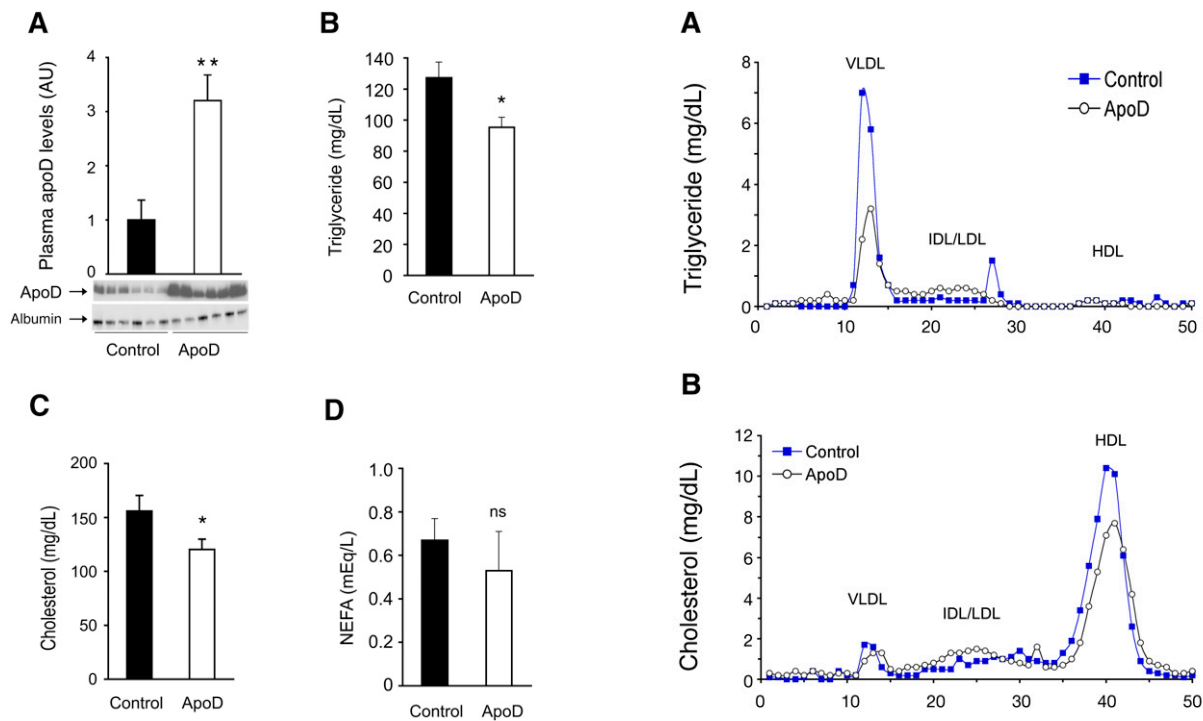
### Impact of apoD on VLDL-TG metabolism

The reduction in plasma triglyceride levels could result from decreased hepatic VLDL-TG production or increased VLDL-TG clearance in plasma or a combination of both. To address the mechanism underlying apoD-mediated reduction in plasma TG levels, we fasted both control and apoD vector-treated mice for 5 h, followed by intraperitoneal injections of Tyloxapol (500 mg/kg body weight), an



**Fig. 1.** ApoD expression in obese mice. Male obese *db/db* mice ( $n = 5$ ) and control littermates ( $n = 7$ ) at 6 months of age were fasted for 16 h. **A:** Aliquots of plasma (20  $\mu$ g of protein) from individual mice in lean and *db/db* groups were subjected to semiquantitative immunoblot assay using anti-apoD and control anti-albumin antibodies. **B:** Fasting plasma triglyceride levels in *db/db* and heterozygous control littermates. **C:** Plasma lipoprotein profiles of *db/db* mice. Aliquots of sera (250  $\mu$ l) pooled from *db/db* ( $n = 6$ ) and sex/age-matched control littermates ( $n = 7$ ) were fractionated by gel filtration column chromatography, followed by the determination of cholesterol concentrations in fractions. **D:** ApoD content in HDL. The peak fractions (34–38) of HDL obtained in panel C were subjected to immunoblot analysis to determine apoD content, using apoA-1 as control. **E:** Correlation between plasma apoD and TG levels. Plasma TG levels were plotted as a function of plasma apoD levels in *db/db* and *db/+* mice. \* $P < 0.05$  vs. control by ANOVA. ApoD, apolipoprotein D; TG, triglyceride.



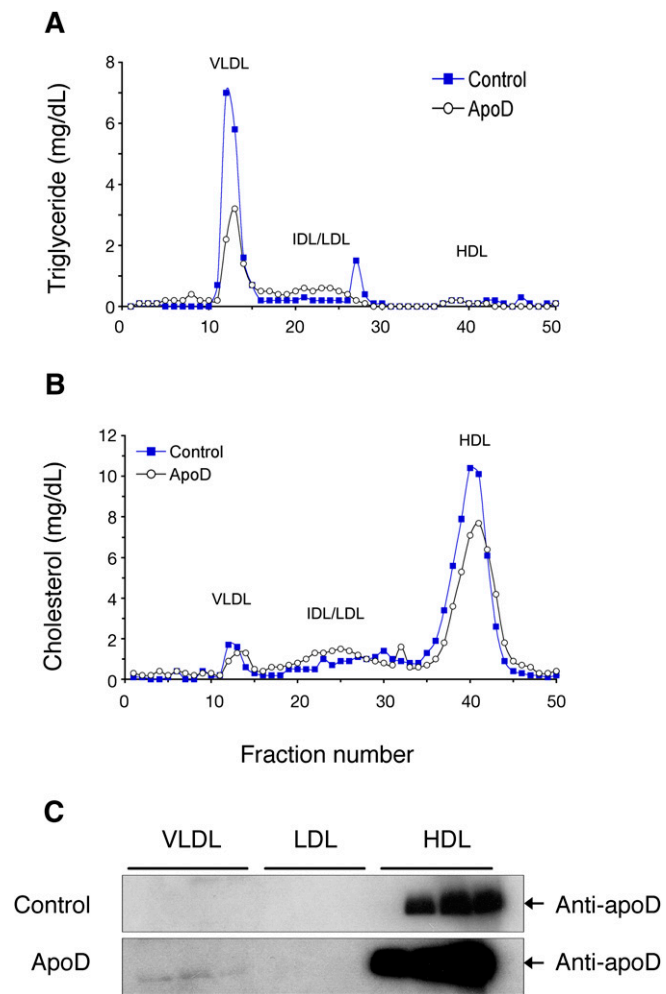


**Fig. 2.** Effect of apoD on lipid metabolism in mice. Male CD-1 mice (10 weeks old) were stratified by body weight and randomly assigned to two groups ( $n = 10$  per group), which were treated with apoD and control vectors. Mice were fasted for 16 h after 2-week of hepatic apoD production to determine fasting plasma levels of apoD (A), triglyceride (B), cholesterol (C), and NEFA (D). \* $P < 0.05$  and \*\* $P < 0.001$  vs. control. ApoD, apolipoprotein D; NS, not significant.

inhibitor of lipoprotein lipase. This approach allows selective inhibition of plasma TG hydrolysis to determine hepatic VLDL-TG production, as previously described (21, 22). As shown in **Fig. 4A**, plasma TG levels were not significantly different between the apoD and control groups at different times for up to 3 h after Tyloxapol administration. Furthermore, the relative rate of VLDL-TG production in apoD group, defined by the slope of TG increase over time, was indistinguishable from the control mice (**Fig. 4B**).

In addition, we determined plasma apoB and MTP levels. MTP is a molecular chaperone that transports lipid to nascent apoB, a rate-limiting step in hepatic VLDL assembly and secretion (27–29). As shown in **Fig. 4C**, plasma apoB100 levels remained unchanged in apoD vector-treated mice. No significant differences in hepatic MTP protein levels between apoD and control groups were detected (**Fig. 4D**). Together these data do not support a significant role of apoD in hepatic VLDL-TG production.

To study the effect of apoD on VLDL-TG catabolism, we studied postprandial fat clearance. Mice were fasted overnight, followed by gavage administration of an oral bolus of olive oil (10  $\mu\text{l/g}$  of body weight). Plasma TG levels were determined before and at 1.5 h intervals after fat administration. When compared with controls, apoD vector-treated mice exhibited significantly improved plasma lipid profiles (**Fig. 4E**).

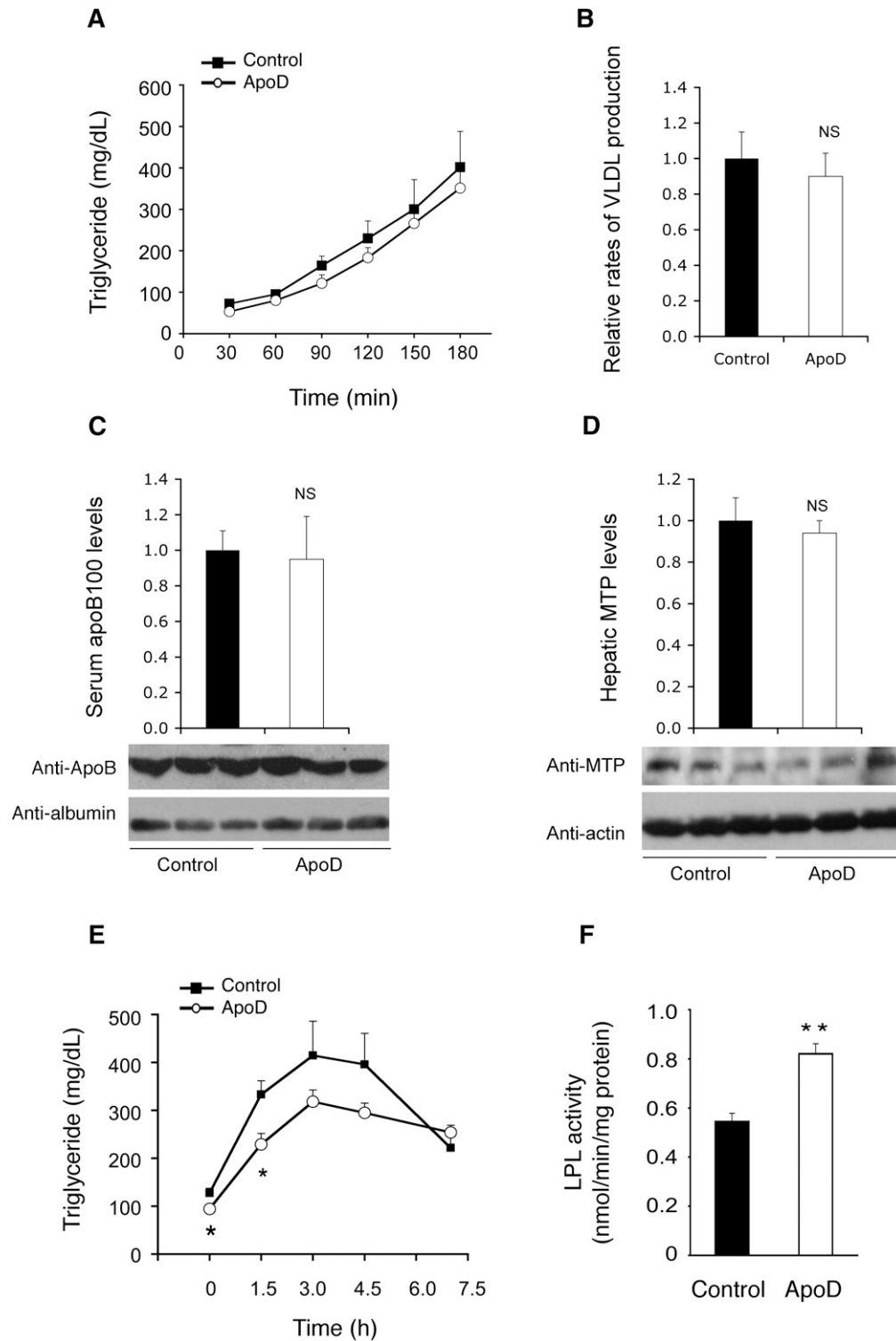


**Fig. 3.** Effect of apoD on lipoprotein metabolism. After two weeks of hepatic apoD production, mice were sacrificed. Aliquots of plasma (250  $\mu\text{l}$ ) pooled from individual mice in apoD ( $n = 10$ ) and control vector-treated mice ( $n = 10$ ) subjected to gel filtration chromatography, followed by the determination of TG (A) and cholesterol (B) concentrations in fractions. The peak fractions of VLDL (11–14), LDL (22–26), and HDL (38–42) in the control (upper panel) and apoD vector-treated (lower panel) groups were analyzed by immunoblot assay using anti-apoD antibody (C). ApoD, apolipoprotein D.

To address the mechanism of apoD-mediated postprandial fat clearance, we determined the plasma activity of LPL, a key enzyme in the hydrolysis and clearance of TG-rich particles. Mice were heparinized by intravenous injections of heparin (300 IU/Kg body weight). Aliquots (20  $\mu\text{l}$ ) of blood were collected 10 min postheparin infusion to determine LPL activity. In keeping with enhanced postprandial TG clearance, significantly higher levels of plasma LPL activity were detected in apoD vector-treated mice (**Fig. 4F**).

### Impact of apoD on LPL and HL expression

To account for the induction of plasma LPL activity in apoD vector-treated mice, we determined postheparin plasma LPL protein levels. As shown in **Fig. 5A**, mice with elevated apoD levels had a small increase in postheparin



**Fig. 4.** Effect of apoD on VLDL-TG production and clearance. **A:** Hepatic VLDL-TG production. Mice in control and apoD groups ( $n = 10$  per group) were fasted for 5 h at day 10 after vector administration, followed by intravenous injection of Tyloxapol to determine plasma TG levels at different times. **B:** VLDL-TG production rates. The relative rate of VLDL-TG production, defined by the slope of TG increase over time, was calculated from data in panel A. **C:** Serum apoB100 levels. Sera obtained from mice at 2 h post-Tyloxapol injection were subject to immunoblot analysis using anti-apoB and anti-albumin antibodies. **D:** Hepatic MTP levels. Aliquots (40 mg) of liver tissues were analyzed by immunoblot assay to determine hepatic MTP levels using actin as an internal control. **E:** Postprandial fat clearance. Mice ( $n = 10$  per group) were fasted overnight at day 11 after vector administration, followed by an oral bolus of olive oil (10  $\mu$ l/g body weight). Aliquots of blood from tail vein were drawn before and after olive oil administration (every 1.5 h) to determine plasma TG levels. **F:** Plasma LPL activity. Mice ( $n = 10$  per group) were intravenously injected with 300 IU heparin/kg body weight at day 12 after vector administration. Tail-vein blood (20  $\mu$ l) was sampled 10 min after heparin infusion to determine postheparin lipoprotein lipase (LPL) activity. \* $P < 0.05$  and \*\* $P < 0.001$  vs. control. ApoD, apolipoprotein D; MTP, microsomal triglyceride transfer protein; TG, triglyceride.

LPL protein levels. To study the impact of apoD on LPL expression in peripheral tissues, we determined LPL protein levels in adipose tissue as well as in cardiac and skeletal muscles. We detected a small, but insignificant, increase in LPL protein levels in adipose tissue (Fig. 5B) and cardiac muscle (Fig. 5C) in apoD vector-treated mice. No differences in LPL expression in skeletal muscle were seen between control and apoD groups (Fig. 5D). In addition, we determined HL protein abundance in the liver, demonstrating that hepatic HL expression remained unchanged in apoD vs. control groups (Fig. 5E). Likewise, no significant difference in HL activity was detected between apoD and control groups (Fig. 5F).

#### Effect of apoD on TG metabolism in obese mice

To corroborate the above findings, we determined the effect of apoD on plasma lipid metabolism in high-fat-induced obese mice. High-fat feeding is associated with hyperlipidemia, a condition characterized by hypercholesterolemia and hypertriglyceridemia. We fed male C57BL/6J mice a high-fat diet for 14 weeks. High-fat feeding resulted in a significant weight gain, accompanied by significantly elevated plasma TG, cholesterol, and NEFA levels (see supplementary Table I). Obese mice were stratified by body weight and randomly assigned to two groups ( $n = 10$  per group), which were treated with apoD and control vectors at a predefined dose of  $1.5 \times 10^{11}$  pfu/kg. Mice were maintained on high fat following vector administration. When compared with control obese mice, apoD vector-treated obese mice exhibited about 2.5-fold elevation in plasma apoD levels (Fig. 6A). In keeping with the observations in CD-1 mice, elevated hepatic apoD production resulted in a significant reduction in plasma TG levels in diet-induced obese C57BL/6J mice (Fig. 6B). While elevated apoD production also reduced plasma cholesterol levels in high-fat-induced obese mice, this reduction did not reach a significant level (Fig. 6C). In contrast, plasma NEFA levels remained unchanged in response to hepatic apoD production (Fig. 6D). In addition, elevated apoD production also resulted in a slight, but significant, reduction in body weight (Fig. 6E), and epididymal fat mass in high-fat-induced obese mice (Fig. 6F).

#### Effect of apoD on hepatic TG production and plasma TG clearance in obese mice

To address the underlying mechanism of apoD-mediated reduction in plasma TG levels, we determined hepatic VLDL-TG production and postprandial TG clearance using the same procedures described above. As shown in Fig. 7A, no significant differences were detected in the relative rate of hepatic VLDL-TG production between control and apoD vector-treated obese mice, similar to the effect observed in nonobese CD-1 mice (Fig. 4). Instead, we detected a significant improvement in plasma TG clearance. This is reflected in significantly improved postprandial TG profiles following an oral bolus of olive oil (10  $\mu$ l/g of body weight) (Fig. 7B) in apoD vector-treated obese mice.

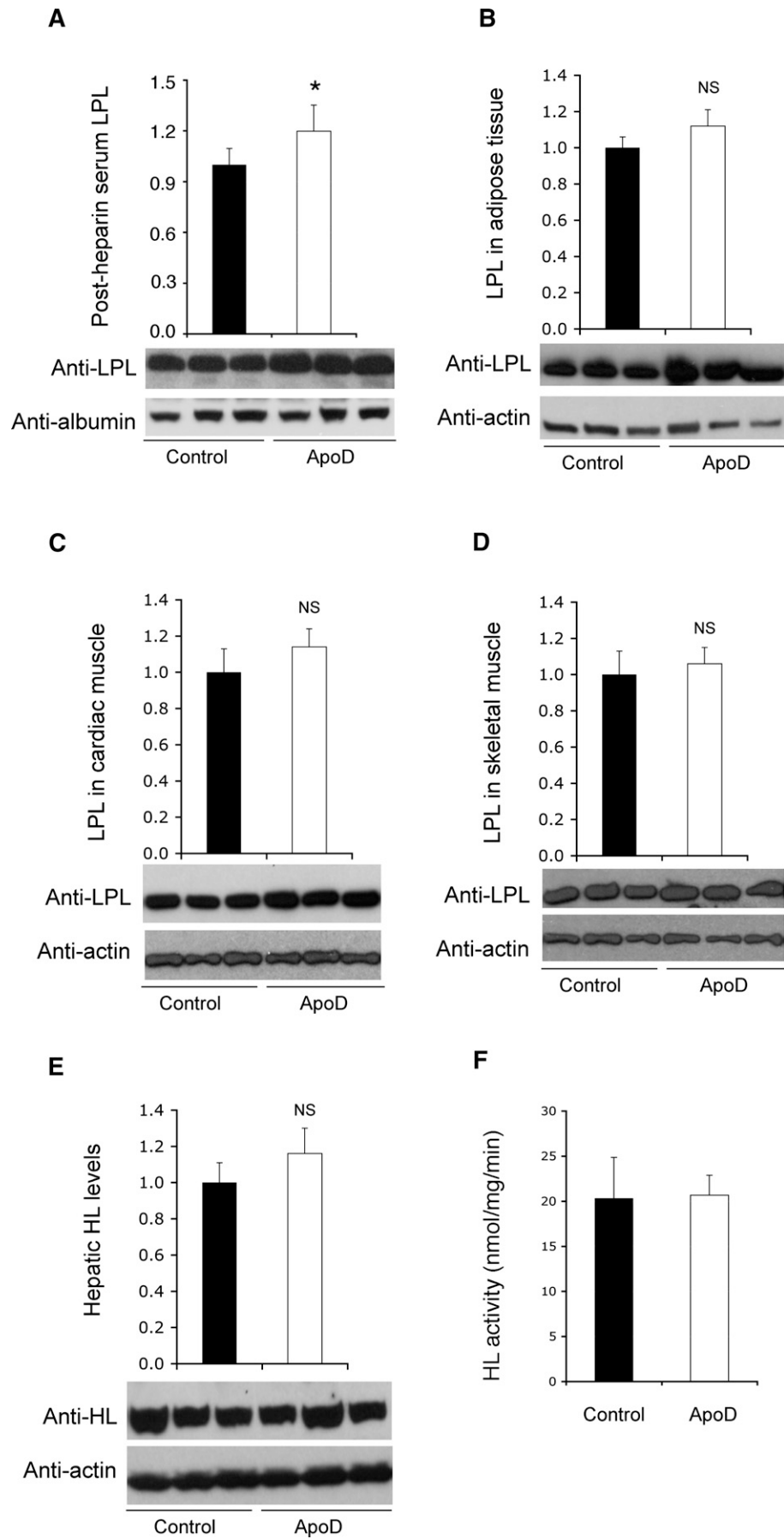
#### Impact of apoD on plasma LPL activity in obese mice

To account for apoD-mediated improvement in TG catabolism, we determined plasma LPL activity. Both control and apoD groups of obese C57BL/6J mice were heparinized by intravenous injections of heparin (300 IU/Kg body weight), followed by the determination of postheparin plasma LPL activity. As shown in Fig. 7C, significantly higher plasma LPL activity levels were detected in apoD vector-treated obese mice, coinciding with enhanced postprandial plasma TG profiles in response to fat tolerance (Fig. 7B). To rule out the possibility that hepatic apoD production exerted a potential adverse effect on liver function, we determined fasting plasma levels of ALT and AST. We did not detect significant alterations in plasma AST (Fig. 7D) and ALT (Fig. 7E) levels in response to hepatic apoD production in diet-induced obese C57BL/6J mice.

#### Effect of recombinant apoD on LPL activity

To address whether apoD is a coactivator of LPL activity, we determined LPL activity in the presence and absence of recombinant human apoD using an *in vitro* LPL activity assay. Addition of purified apoD proteins at escalating concentrations did not result in significant induction of LPL activity (data not shown). To mimic the physiological situation in which LPL is normally attached to endothelium, we determined the effect of apoD on VLDL-TG hydrolysis rates by LPL that was coupled to proteoglycan, a major component of extracellular matrix. No significant effects of apoD on the hydrolysis rates of VLDL-TG by proteoglycan-bound LPL were detected (data now shown).

We reasoned that recombinant human apoD protein may not be active as it consists of four amino acid substitutions (Trp99His, Cys116Ser, Ile118Ser, Leu120Ser) (30). Isolated from bacteria, recombinant apoD lacks posttranslational modifications, such as N-glycosylation, as apoD proteins are N-glycosylated at two sites (Asn45 and Asn78) in mammalian cells (31). To circumvent this limitation, we prepared apoD proteins from HepG2 cells. Cultured HepG2 cells were transduced with Adv-apoD or Adv-null control vectors at a defined dose of 200 pfu/cell. After 24-h incubation, the conditioned medium was enriched and analyzed for apoD production. As shown in Fig. 8A, apoD was detected in the conditioned medium of HepG2 cells that pretransduced with apoD vector. In contrast, apoD was nondetectable in the conditioned medium of HepG2 cells that were pretransduced with control vector. We then applied apoD proteins derived from HepG2 cells to proteoglycan-bound LPL activity assay, in which the substrate was VLDL-TG particles that were isolated from C57BL/6J mice using Superose S-6 gel filtration chromatography. As shown in Fig. 8B, addition of apoD proteins resulted in a dose-dependent induction of FFA production from VLDL-TG in the reaction. To corroborate these findings, we denatured apoD by boiling the apoD protein sample at 100°C for 10 min, followed by assaying its activity. As shown in Fig. 8C, heat-inactivated apoD proteins were no longer able to promote LPL-mediated hydrolysis of VLDL-TG in the reaction.





## DISCUSSION

Our goal in this study was 2-fold: (1) to characterize the role of apoD in triglyceride metabolism, and (2) to determine its functional contribution to dyslipidemia. We show that elevated apoD production resulted in significant reduction in plasma TG levels in mice. This was due to enhanced VLDL-TG hydrolysis and clearance, as hepatic VLDL-TG production remained unchanged in response to elevated apoD production. ApoD is present mainly in HDL and, to a lesser extent, in VLDL and LDL. Plasma apoD levels were downregulated in obese *db/db* mice. These data for the first time revealed an important facet of apoD function in lipid metabolism. Epidemiological studies identified three distinct missense mutations, namely Phe36Val, Tyr108Cys, and Thr158Lys in the apoD gene in African populations. The Phe36Val and Thr158Lys are associated with significantly elevated plasma triglyceride levels and reduced HDL-cholesterol levels, a plasma lipid profile that is characteristic of metabolic syndrome (18, 32). Furthermore, there is evidence that the *TaqI* polymorphism of the apoD gene is associated with increased risk of developing obesity, insulin resistance, and type 2 diabetes in the British Caucasoid population (16), as well as in South Indians and Nauruans (17, 33). Our data suggest that apoD deficiency may be a causative factor for retarding VLDL-TG catabolism and contributing to impaired lipid metabolism in at-risk individuals with increased visceral adiposity. Consistent with this interpretation, we show that elevated apoD production was associated with increased LPL activity, contributing to improved postprandial TG clearance. This observation was reproduced in both normal CD-1 mice and high-fat-induced obese C57BL/6J mice.

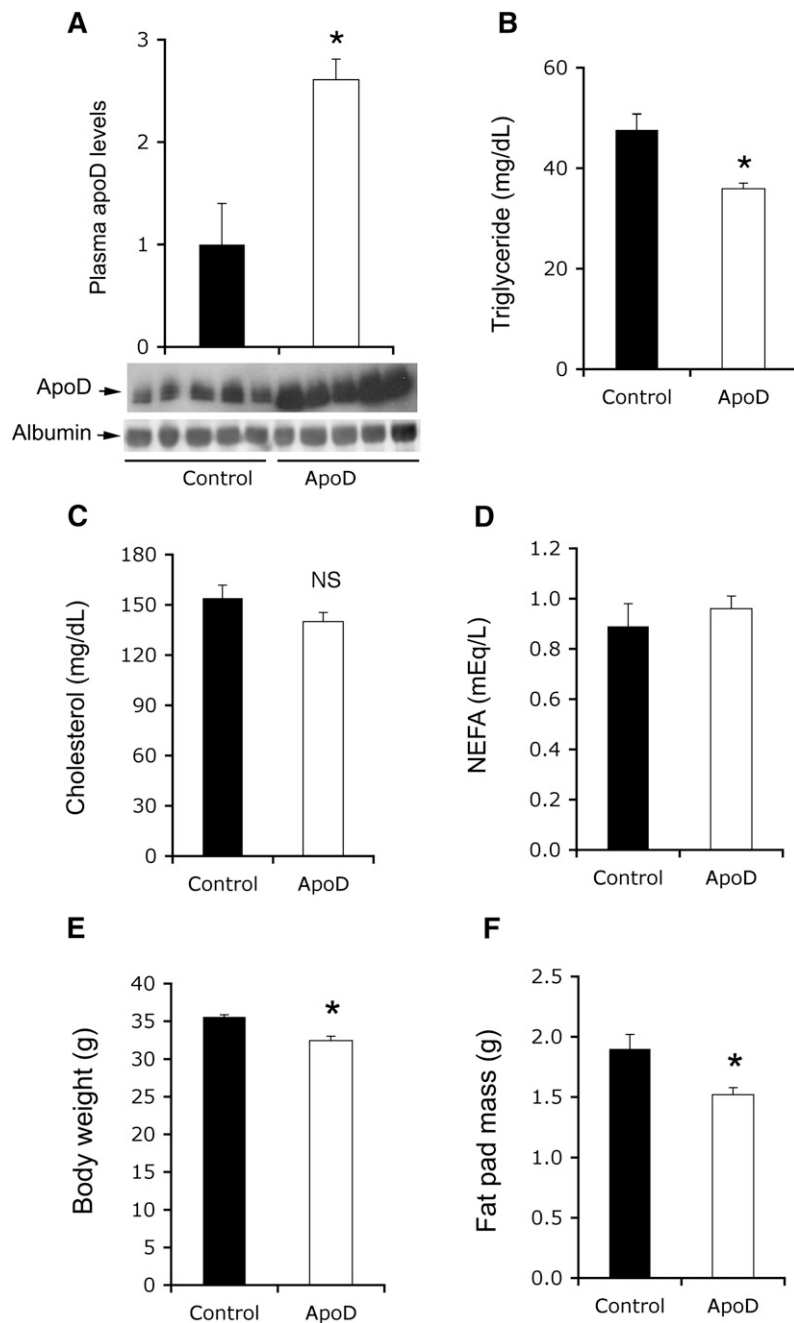
To account for the mechanism by which apoD contributes to enhanced LPL activity, we determined postheparin plasma LPL activity, demonstrating that mice with elevated apoD production displayed significantly higher LPL protein levels and activity. To recapitulate this finding in a cell-free system, we applied recombinant apoD proteins to an *in vitro* LPL activity assay. We show that neither free LPL nor extracellular matrix-bound LPL activities were significantly altered by the addition of recombinant apoD protein into the reaction. The recombinant human apoD protein used in our LPL activity assay is produced from bacteria, following four amino acid substitutions (Trp99His, Cys116Ser, Ile118Ser, Leu120Ser) in the hydrophobic polypeptide chain to enhance its solubility (30). It is plausible that these amino acid substitutions

impact the biological function of apoD as a result of its structural alterations. Alternatively, the recombinant apoD proteins do not behave as its native form because of the lack of secondary modifications, such as N-glycosylation in bacterial cells, because plasma apoD proteins are N-glycosylated at two evolutionally conserved sites (Asn45 and Asn78) in mammalian cells (31). To overcome this limitation, we isolated apoD from conditioned medium of HepG2 cells that were pretransduced with an apoD vector. We show that apoD derived from HepG2 cells was capable of enhancing LPL-mediated hydrolysis of VLDL-TG that was isolated from C57BL/6J mice. These results, together with our data in mice, suggest that the presence of apoD at higher levels render VLDL-TG particles favorable for LPL-mediated hydrolysis. This action of apoD contributed to its TG-lowering effect in normal CD1 and high-fat-induced obese mice. It also helped explain why apoD deficiency was associated with impaired TG metabolism in obese *db/db* mice and human subjects with missense mutations in the apoD gene.

Although categorized as apolipoprotein because of its association with HDL, apoD is distinct from other apolipoproteins in three important aspects: (1) it does not bear significant degrees of homology in the amino acid sequence to other apolipoproteins; (2) it has a broad spectrum of tissue distribution, including its expression in adipose tissue and skeletal muscle, which is in contrast with other apolipoproteins whose expressions are limited primarily to the liver and intestine; and (3) it comprises a highly conserved  $\beta$ -barrel structure that is characteristic of lipocalin superfamily (3, 34). Members of this superfamily include FABPs, apoM, and RBP4. FABPs serve as molecular chaperones for binding and transporting fatty acids for catabolism in cells (35). ApoM is shown to promote cholesterol efflux from peripheral tissues to the liver for excretion and to ameliorate atherosclerotic lesion in atherogenic mice (5). RBP4 is linked to the pathogenesis of insulin resistance in obesity and type 2 diabetes (4). Our studies spotlight the role of apoD, a new member of lipocalin superfamily, in modulating triglyceride metabolism. ApoD dysregulation may be a contributing factor for the development of metabolic abnormalities in obesity.

Aside from its impact on metabolism, altered apoD production is linked to other pathophysiological conditions, such as in women with gross cystic disease (36) and in subjects with Niemann-Pick Type C (NPC) disease, a neurodegenerative disorder characterized by impaired intracellular cholesterol transport (37). ApoD is upregulated in the brain of subjects with chronic schizophrenia and in the

**Fig. 5.** Effect of apoD on LPL and HL expression. A: Postheparin plasma LPL levels. Aliquots of postheparin plasma (20  $\mu$ g protein) from control and apoD groups ( $n = 10$ ) in Fig. 4F were subjected to immunoblot analysis using anti-LPL and anti-albumin antibodies, respectively. After normalizing to albumin, the relative amounts of LPL proteins were compared in control and apoD groups. B: LPL protein levels in adipose tissue. Aliquots (40 mg) of epididymal fat isolated from control and apoD vector-treated mice were subjected to immunoblot analysis using anti-LPL and anti-actin antibodies. C: LPL protein levels in cardiac muscle. Aliquots (40 mg) of cardiac muscle were analyzed by immunoblot assay to determine cardiac LPL protein levels. D: LPL protein levels in skeletal muscle. Aliquots (40 mg) of hind-limb skeletal muscle were analyzed by immunoblot to determine LPL protein levels in skeletal muscle. E: HL protein levels. Aliquots (40 mg) of liver tissue were analyzed by immunoblot to determine HL protein abundance. F: HL activity. Aliquots (20 mg) of liver tissue were homogenized in PBS and hepatic protein lysates (10  $\mu$ l) were subjected to lipase activity assay. HL activity was expressed as the amount of substrates hydrolyzed (nmol) per min per mg of liver protein. \* $P < 0.05$  vs. control. ApoD, apolipoprotein D; NS, not significant.

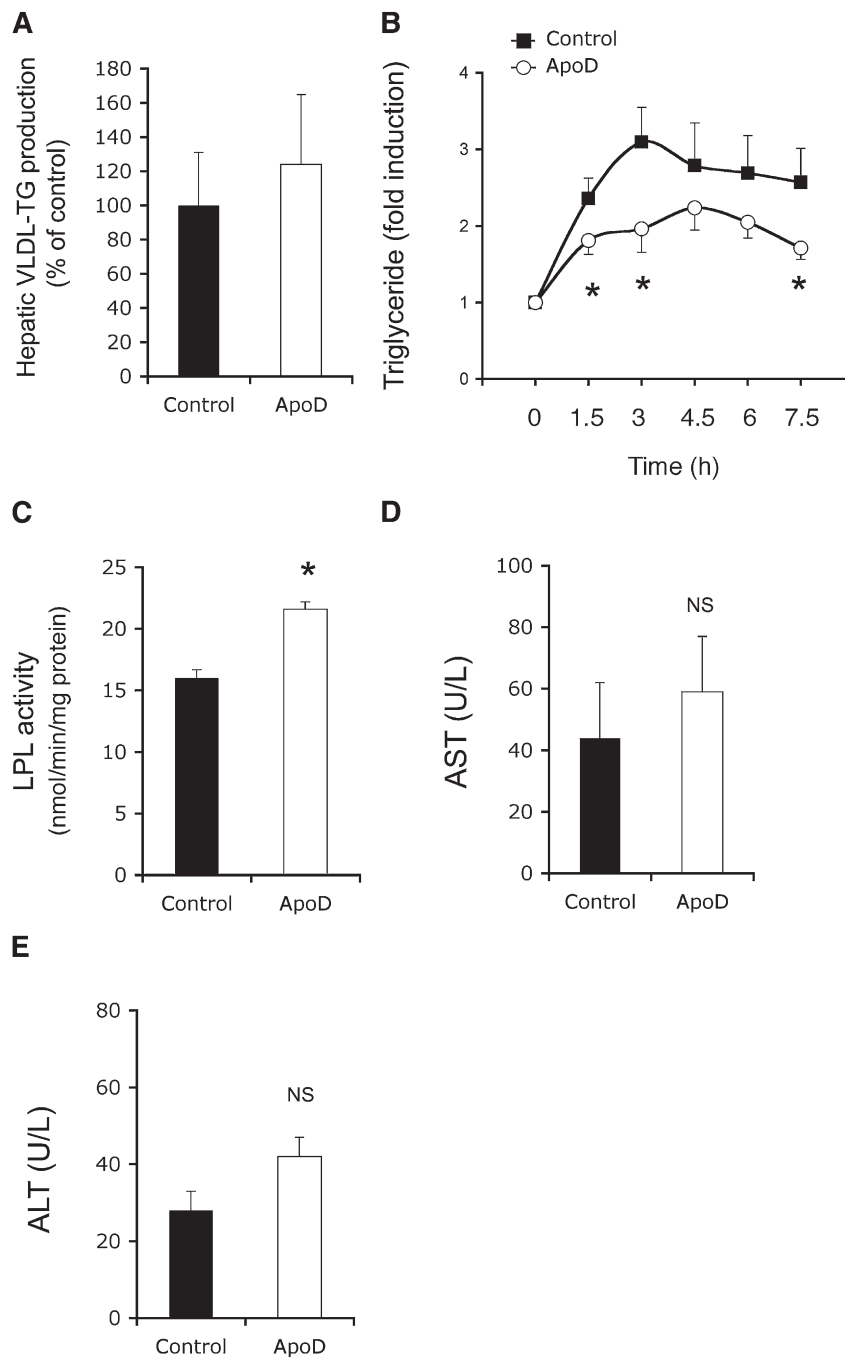


**Fig. 6.** Effect of apoD on lipid metabolism in high-fat-induced obesity. Male C57BL/6J mice were fed a high-fat diet for 14 weeks. High-fat-induced obese mice were stratified by body weight and assigned randomly to two groups ( $n = 10$ ), which were respectively treated with apoD and control vectors. Two weeks after vector administration, mice were fasted for 16 h to determine fasting plasma levels of apoD (A), triglyceride (B), cholesterol (C), NEFA (D), body weight (E), and epididymal fat-pad mass (F). Epididymal fat pads were surgically removed from sacrificed mice to determine fat-pad mass in control and apoD groups ( $n = 10$  per group). \* $P < 0.05$  vs. control by ANOVA. ApoD, apolipoprotein D; NS, not significant.

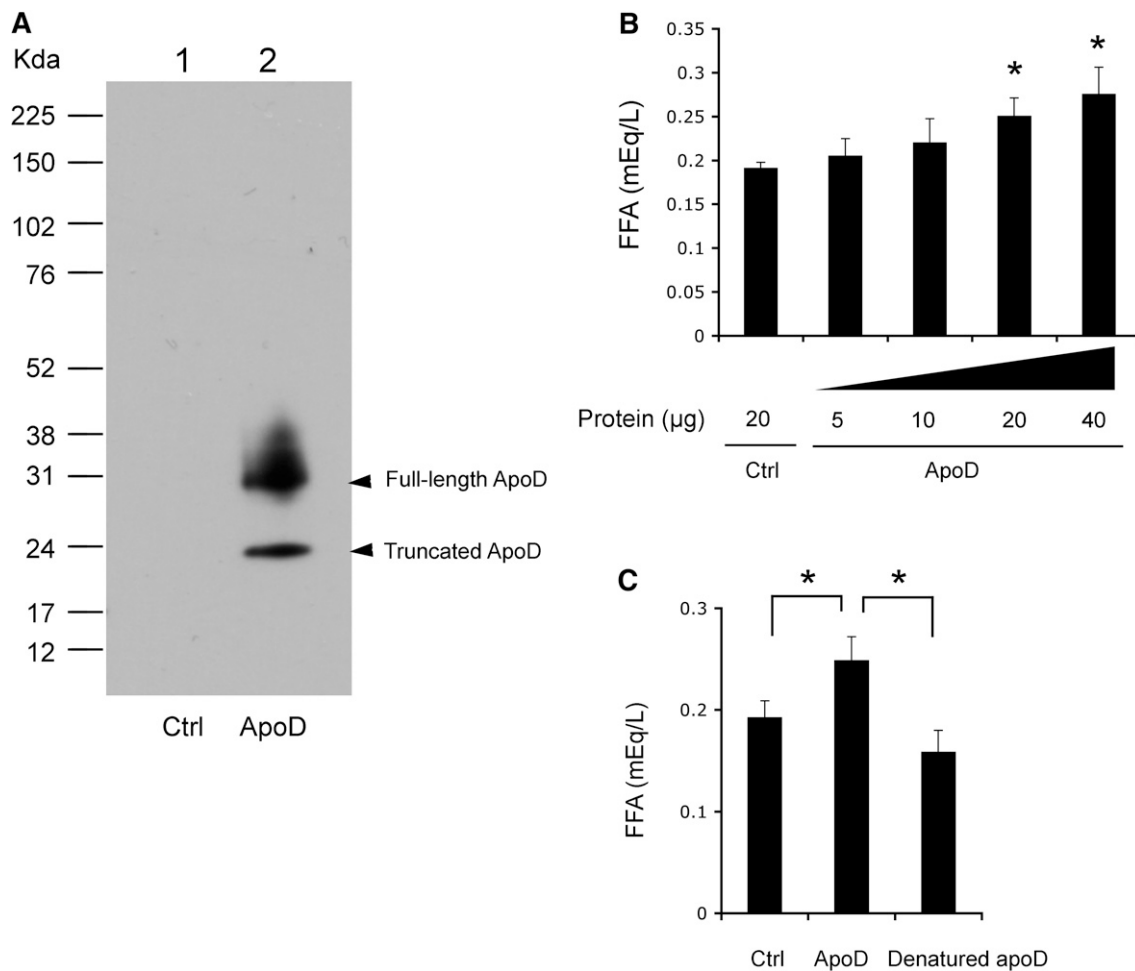
prefrontal cortex of patients with Alzheimer disease (38–40). Treatment with antipsychotic drugs, especially clozapine, results in elevated apoD expression in rodent brains, as well as in human plasma (38, 41, 42). Increased apoD production is seen in the rat brain following traumatic brain injury (43). High plasma apoD levels are also found in patients with failing hearts (44). Elevated apoD production is detected in liver tumors resected from hepatocellular carcinoma (45), as well as in invasive carcinoma of the breast (46, 47). Ganfornina et al. (11) show that apoD overexpression in the brain protects mice from oxidative stress. This effect correlates with the ability of apoD to prevent lipid peroxidation in cells (11). Do Carmo et al. (48) show that apoD confers a neuroprotective effect in the brain of mice. Their studies demonstrate that mice

overexpressing human apoD in neurons, as opposed to normal controls, are more resistant with a 3-fold higher survival rate in response to human coronavirus-induced acute encephalitis. These data are consistent with the idea that apoD is an atypical apolipoprotein with multiple functions, although a cause and effect relationship between aberrant apoD production and pathogenesis of disease under those pathophysiological conditions remains to be determined.

A recent study by Do Carmo et al. (49) reports that transgenic overexpression of human apoD from a neuron-specific promoter is associated with hepatic fat infiltration and insulin resistance in aged mice. This result seems at variance with our data, as we did not detect significant alterations in hepatic lipid content in mice with elevated



**Fig. 7.** Effect of apoD on VLDL-TG production and clearance in obese mice. **A:** Hepatic VLDL-TG production. Obese C57BL/6J mice in control and apoD groups ( $n = 10$  per group) were fasted for 5 h at day 10 after vector administration, followed by intravenous injection of Tyloxapol to determine plasma TG levels at different times. The relative rates of hepatic VLDL-TG production, defined by the slope of plasma TG excursion, were determined. **B:** Postprandial fat clearance. Obese mice treated with apoD and control vectors ( $n = 10$  per group) were fasted overnight at day 11 after vector administration, followed by an oral bolus of olive oil ( $10 \mu\text{l/g}$  body weight). Aliquots of blood from tail vein were taken at 1.5-h intervals following olive oil administration to determine plasma TG levels. **C:** Plasma LPL activity. Obese mice ( $n = 10$  per group) were intravenously injected with 300 IU heparin/kg body weight at day 12 after vector administration. Tail-vein blood ( $20 \mu\text{l}$ ) was sampled 10 min after heparin infusion to determine postheparin LPL activity. After 2 weeks of hepatic apoD production, mice were fasted overnight, followed by the determination of fasting plasma AST (**D**) and ALT (**E**) levels in apoD and control groups ( $n = 10$  per group). \* $P < 0.05$  vs. control. ApoD, apolipoprotein D; NS, not significant; TG, triglyceride.



**Fig. 8.** Effect of apoD on LPL-mediated VLDL-TG hydrolysis in vitro. **A:** Enrichment of apoD from conditioned medium of apoD-expressing HepG2 cells. HepG2 cells were transfected with Adv-apoD or Adv-null control vectors at 200 pfu/cell in triplicate. After 24-h incubation in serum-free medium, conditioned medium was collected for the preparation of apoD proteins as described in "Materials and Methods." Aliquots of protein samples (5-μg) from control (lane 1) and apoD (lane 2) groups were resolved on 4–20% SDS-PAGE, followed by anti-apoD immunoblot assay. Predicted molecular mass of full-length apoD protein, 29 KDa. **B:** ApoD dose-dependent induction of VLDL-TG hydrolysis in vitro. ApoD enriched from conditioned medium of apoD-expressing HepG2 cells were applied to 96-well microplates pre-coated with proteoglycan-bound LPL (purified bovine LPL at 80 U/well). After the addition of murine VLDL-TG particles as substrates (final concentration of TG, 120 μg/ml), the amounts of FFA produced from LPL-mediated hydrolysis of VLDL-TG in the absence and presence of escalating doses of apoD proteins in the reaction during a 15-min incubation were determined. Data were from three independent measurements. **C:** Effect of denatured apoD on LPL-mediated hydrolysis of VLDL. Aliquots of apoD protein sample (20 μg) enriched from conditioned medium of apoD-expressing HepG2 cells were boiled for 10 min for heat inactivation. Its activity to enhance LPL-mediated VLDL hydrolysis was compared with an equivalent amount (20 μg) of control and native apoD protein samples in the in vitro LPL activity assay. Data were from eight independent measurements. \* $P < 0.05$  vs. control. ApoD, apolipoprotein D; TG, triglyceride.


apoD production in the liver. It is noted that in their neuron-specific apoD transgenic mice, apoD is primarily expressed in the brain. Elevated apoD in the central nervous system may interfere with leptin signaling, which in turn impacts whole-body insulin sensitivity and carbohydrate metabolism. There is evidence that apoD and the leptin receptor physically interact with each other in the brain (50). Liu et al. (50) show that apoD and leptin receptor expression are coordinately regulated in the hypothalamus in modulating food intake and energy homeostasis in mice. Dissociation of apoD with the leptin receptor is linked to the development of obesity in leptin receptor-deficient *db/db* mice (50). Therefore, it remains an open question as to how transgenic expression of apoD in the brain results in the development of hepatic steatosis.

We would like to acknowledge an inherent caveat in using rodent models for studies of lipoprotein metabolism. Unlike humans, rodents do not express cholesterol ester transfer protein (CETP), a component that mediates the transfer of cholesteryl ester from HDL to TG-rich lipoproteins (51, 52). As a result, cholesterol is mainly present in HDL in rodents. In particular, the human apoD contains five cysteine residues, four of which are used for intramolecular cross-links, and the fifth unpaired cysteine (Cys-116) is responsible for forming an intermolecular bond with Cys-6 of apoA-II in HDL (1). In contrast, the rodent apoD lacks the fifth cysteine residue (1, 31). The underlying physiology remains unknown. Given the clinical evidence that altered apoD production is associated with metabolic abnormalities, it is of clinical



significance to illustrate the function of human apoD in lipoprotein metabolism.

Although we consistently observed that elevated apoD production was attributable to significantly improved lipid profiles in mice following an oral bolus of fat and that this effect correlated with enhanced plasma LPL activity, it is noteworthy that a reduction in intestinal chylomicron production could also contribute to improved postprandial TG profiles in apoD vector-treated mice. Further investigation is warranted to determine the effect of apoD on chylomicron production in mice. Likewise, we did not detect a significant impact on HL activity in liver, but this finding could not preclude the possibility that apoD affects HL activity in plasma. Studies are needed to selectively determine postheparin HL activity in mice with elevated apoD production.

In conclusion, we focused our studies on apoD in lipid metabolism, demonstrating that apoD contributed to improved VLDL-TG metabolism. This effect can be ascribed to enhanced VLDL-TG hydrolysis and clearance. Obese *db/db* mice with altered triglyceride metabolism exhibited significantly lower plasma apoD levels. These data suggest that apoD plays a significant role in lipid homeostasis and help explain why genetic mutations in the apoD gene predispose at-risk individuals to developing metabolic syndrome. 

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