ASBMB JOURNAL OF LIPID RESEARCH

ApoC-III deficiency prevents hyperlipidemia induced by apoE overexpression

Gery Gerritsen,* Patrick C. N. Rensen,†,§ Kyriakos E. Kypreos,*, Vassilis I. Zannis,** Louis M. Havekes,†,§,†† and Ko Willems van Dijk[1],*,§**

Departments of Human Genetics,* General Internal Medicine,§ and Cardiology,†† Leiden University Medical Center, Leiden, The Netherlands; Netherlands Organization for Applied Scientific Research Quality of Life,† Gaubius Laboratory, Leiden, The Netherlands; and Boston University School of Medicine,** Boston, MA

Abstract **Adenovirus-mediated overexpression of human apolipoprotein E (apoE) induces hyperlipidemia by stimulating the VLDL-triglyceride (TG) production rate and inhibiting the LPL-mediated VLDL-TG hydrolysis rate. Because apoC-III is a strong inhibitor of TG hydrolysis, we questioned whether *Apoc3* deficiency might prevent the hyperlipidemia induced by apoE overexpression in vivo. Injection of 2×10^9 plaque-forming units of AdAPOE4 caused severe combined hyperlipidemia in *Apoe*$^{-/-}$ mice [TG from 0.7 ± 0.2 to 57.2 ± 6.7 mM; total cholesterol (TC) from 17.4 ± 3.7 to 29.0 ± 4.1 mM] that was confined to VLDL/intermediate density lipoprotein-sized lipoproteins. In contrast, *Apoc3* deficiency resulted in a gene dose-dependent reduction of the apoE4-associated hyperlipidemia (TG from 57.2 ± 6.7 mM to 21.2 ± 18.5 and 1.5 ± 1.4 mM; TC from 29.0 ± 4.1 to 16.4 ± 9.8 and 2.3 ± 1.8 mM in *Apoe*$^{-/-}$, *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$, and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice, respectively). In both *Apoe*$^{-/-}$ mice and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice, injection of increasing doses of AdAPOE4 resulted in up to a 10-fold increased VLDL-TG production rate. However, *Apoc3* deficiency resulted in a significant increase in the uptake of TG-derived fatty acids from VLDL-like emulsion particles by white adipose tissue, indicating enhanced LPL activity. In vitro experiments showed that apoC-III is a more specific inhibitor of LPL activity than is apoE. Thus, *Apoc3* deficiency can prevent apoE-induced hyperlipidemia associated with a 10-fold increased hepatic VLDL-TG production rate, most likely by alleviating the apoE-induced inhibition of VLDL-TG hydrolysis.**—Gerritsen, G., P. C. N. Rensen, K. E. Kypreos, V. I. Zannis, L. M. Havekes, and K. Willems van Dijk. **ApoC-III deficiency prevents hyperlipidemia induced by apoE overexpression.** ***J. Lipid Res.*** **2005.** 46: **1466–1473.**

Supplementary key words lipoprotein lipase-mediated triglyceride hydrolysis • adenovirus-mediated gene transfer • mice • very low density lipoprotein • apolipoprotein E • apolipoprotein C-III

The level of circulating plasma VLDL and VLDL remnants is determined by the hepatic production rate of VLDL, the conversion rate of VLDL to VLDL remnants, and their clearance rate. The VLDL-triglyceride (TG) hydrolysis rate is a crucial step in the formation of VLDL remnants that can be efficiently cleared from the plasma by the liver. Both apolipoprotein E (apoE) and apoC-III have long been recognized as modulators of plasma VLDL-TG levels. Plasma levels of apoC-III and apoE are positively correlated with plasma TG levels in human studies (1–5), and apoC-III has been shown to inhibit LPL-mediated TG hydrolysis in vitro (6, 7). Likewise, apoE has been shown to inhibit LPL-mediated TG hydrolysis in vitro and in vivo (8–11). In addition, apoE increases the production rate of VLDL-TG in a gene dose-dependent manner (12–14). However, apoE also functions as a ligand mediating the receptor-mediated uptake of VLDL remnants.

We have previously reported that high levels of apoE expression obtained via adenovirus-mediated transfer of APOE result in hyperlipidemia, characterized by increased plasma cholesterol and TG levels. This was associated with an increased VLDL-TG production rate and a reduced LPL-mediated VLDL-TG hydrolysis rate (13). Apparently, the increased production of VLDLs and their hampered conversion to remnants is not compensated for by an increased apoE-mediated hepatic clearance. We and others have also previously demonstrated that *Apoc3* deficiency prevents the postprandial hypertriglyceridemia induced by an intragastric olive oil load (15, 16), indicative of an enhanced lipolysis of chylomicrons. In the current study, we hypothesize that *Apoc3* deficiency may also prevent apoE-induced combined hyperlipidemia.

To investigate the role of apoC-III in APOE-induced hyperlipidemia, low and high doses of AdAPOE4 were injected into *Apoe*-deficient mice in the presence and absence

Manuscript received 7 December 2004 and in revised form 1 April 2005.

Published, JLR Papers in Press, May 1, 2005.
DOI 10.1194/jlr.M400479-JLR200

Abbreviations: apoE, apolipoprotein E; FPLC, fast-protein liquid chromatography; pfu, plaque-forming units; TC, total cholesterol; TG, triglyceride; TO, triolein; WAT, white adipose tissue.

[1] To whom correspondence should be addressed.
e-mail: kowvd@lumc.nl

Copyright © 2005 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at http://www.jlr.org

Downloaded from www.jlr.org by guest, on July 24, 2017

of endogenous *Apoc3*. The APOE4 isoform was selected because low expression levels rescue the apoE knockout ($Apoe^{-/-}$) phenotype, but high expression levels seem to induce a more pronounced hyperlipidemia compared with the APOE3 isoform (17, 18). We here report that *Apoc3* deficiency gene dose-dependently prevents the hyperlipidemia induced by apoE4 overexpression. Because *Apoc3* deficiency did not affect the VLDL-TG production rate and did result in an enhanced uptake of TG-derived FFA by adipose tissue, we conclude that *Apoc3* deficiency alleviates the block in the formation of lipoprotein remnants from apoE-rich and TG-rich VLDLs.

EXPERIMENTAL PROCEDURES

Mouse studies

$Apoe^{-/-}$ mice that had been generated previously (19) were intercrossed with $Apoc3^{-/-}$ mice (Jackson Laboratories, Bar Harbor, ME) to generate $Apoe^{-/-}.Apoc3^{+/-}$ and $Apoe^{-/-}.Apoc3^{-/-}$ mice. The mice were fed a regular mouse diet (SRM-A; Hope Farms, Woerden, The Netherlands) and given free access to food and water. At least 5 days before adenovirus transfection, mice were transferred to filter-top cages in designated rooms. All animal experimentation protocols were approved by the Committee on Animal Experimentation of the Leiden University Medical Center.

Adenoviral transfection

The recombinant adenoviral vector, expressing the human APOE4 gene and the green fluorescent protein gene under the control of a cytomegalovirus promoter (AdAPOE4), was generated as described (17). A LacZ-expressing recombinant adenovirus (AdlacZ) was used for control virus treatment. The recombinant adenoviruses were propagated in the human embryonic retina cell line 911 and/or the human embryonic kidney cell line 293 as described (20, 21). The viruses were purified via ultracentrifugation in a CsCl gradient, followed by dialysis and titration.

For in vivo administration, 0.5×10^9 to 2×10^9 plaque-forming units (pfu) adenovirus, adjusted to 200 μl with PBS, was injected into the tail veins of female mice. To achieve a linear dose response of AdAPOE4 virus by saturating the uptake of virus particles by Kupffer cells (22), mice were preinjected with 0.5×10^9 pfu AdlacZ at 3 h before injection of the virus of interest.

Plasma lipid and lipoprotein analysis

Two days before and 5 days after adenovirus injection, blood samples of ~50 μl were drawn from the tail veins of 4 h fasted mice. Plasma TG and total cholesterol (TC) levels were measured enzymatically using commercially available kits (Sigma). Lipoprotein fractions were separated using fast-protein liquid chromatography (FPLC). Hereto, a plasma pool obtained from each group of mice before adenovirus injection and 5 days after adenovirus injection was diluted 5–10 times using PBS. A volume of 50 μl was injected onto a Superose 6 column (3.2 × 30 mm; Äkta system; Pharmacia, Uppsala, Sweden). Elution fractions of 50 μl were collected and assayed for TG and TC levels, as described above. Human apoE levels were determined by sandwich ELISA as described previously (23).

Characterization of VLDL

At 5 days after injection of AdlacZ (2×10^9 pfu) or AdAPOE4 (1×10^9 or 2×10^9 pfu), $Apoe^{-/-}$ and $Apoe^{-/-}.Apoc3^{-/-}$ mice were fasted for 4 h and serum was isolated. Sera from each group of mice were pooled, and VLDL ($d < 1.006$ g/ml) was isolated by density gradient ultracentrifugation. The VLDL was enzymatically analyzed for free cholesterol, TC, TG, and phospholipid content using commercially available kits (236691 and 310328, Boehringer-Mannheim; 337-B, Sigma Chemicals; and 99054009, Wako Chemicals). The protein content was determined by the method of Lowry et al. (24).

Hepatic VLDL-TG secretion

The hepatic VLDL-TG secretion rate in $Apoe^{-/-}$ and $Apoe^{-/-}.Apoc3^{-/-}$ mice was measured 5 days after infection with AdlacZ (2×10^9 pfu) or AdAPOE4 (0.5×10^9 and 1×10^9 pfu). Four hour fasted mice were anesthetized with a mixture of vetranquil-dormicum-fentanyl (6.25:6.25:0.3125 mg/kg mouse) and intravenously injected with 500 mg/kg Triton WR 1339 (Sigma) as described (10, 25). Blood samples were drawn via the tail vein at 1, 10, 20, 30, 60, 90, and 120 min after Triton injection, and plasma TG concentrations were measured as described above. The hepatic VLDL-TG secretion rate was determined by the increase in plasma TG concentration after subtraction of the TG concentration in the plasma samples at 1 min after Triton injection.

Preparation of VLDL-like emulsion particles

VLDL-like TG-rich emulsion particles were prepared according to the sonication and ultracentrifugation procedure of Redgrave and Maranhao (26). Hereto, 100 mg of total lipid at a weight ratio of triolein (TO; Fluka)-egg yolk phosphatidylcholine (Lipoid E PC 98%; Lipoid, Ludwigshafen, Germany)-lysophosphatidylcholine (Sigma)-cholesteryl oleate (Sigma)-cholesterol (Sigma) of 70:22.7:2.3:3.0:2.0, supplemented with 200 μCi of glycerol tri[9,10(n)-^{3}H]oleate ([^{3}H]TO; Amersham Biosciences), was sonicated using a Soniprep 150 (MSE Scientific Instruments) at 10 μm output as described (27). An emulsion fraction containing 80 nm emulsion particles was obtained by consecutive density gradient ultracentrifugation steps exactly as described (28). The TG content of the emulsions was determined as described above.

Tissue distribution of VLDL-like emulsion-derived TGs in vivo

Fed $Apoe^{-/-}$ and $Apoe^{-/-}.Apoc3^{-/-}$ mice were anesthetized with a mixture of vetranquil-dormicum-fentanyl (6.25:6.25:0.3125 mg/kg mouse), and their abdomens were opened. [^{3}H]TO-labeled VLDL-like emulsion particles (200 μl) were administered via the inferior vena cava at a dose (1.0 mg of TG) that exceeded the endogenous plasma TG content in both experimental groups. At 10 min after injection of the emulsion particles, the liver, heart, spleen, and aliquots of hindlimb muscle, gonadal white adipose tissue (WAT), perirenal WAT, and intestinal WAT were isolated, dissolved in Soluene (Perkin-Elmer) at 60°C, and counted in 10 ml of Ultima Gold. The ^{3}H activity in the tissues was corrected for wet organ weight.

In vitro LPL activity assay

The effect of apoC-III and apoE on LPL activity was determined essentially as described (9). First, [^{3}H]TO-labeled emulsion particles (0.5 mg/ml TG) were incubated with purified human apoC-III (Academy Biomedical Co., Houston, TX) or recombinant human apoE in 75 μl of PBS (30 min at 37°C). Subsequently, 0.1 M Tris-HCl, pH 8.5, with or without 5% (v/v) heat-inactivated human serum as a source of apoC-II, was added to a total volume of 200 μl. At time zero, LPL (purified bovine milk LPL; 3,300 U/mg; final concentration, 3.5 U/ml; Sigma) was added in 200 μl of 120 mg/ml free fatty acid-free BSA (Sigma) as [^{3}H]oleate acceptor. After 30 min, the [9,10-^{3}H]oleate that was

Downloaded from www.jlr.org by guest, on July 24, 2017

generated during lipolysis was extracted. Hereto, 50 μl samples were added to 1.5 ml of $CH_3OH/CHCl_3$/heptane/oleic acid (1,410:1,250:1,000:1, v/v/v/v) and 0.5 ml of 0.2 N NaOH to terminate lipolysis. ^3H radioactivity in 0.5 ml of the aqueous phase obtained after vigorous mixing and centrifugation (10 min at 1,000 *g*) was counted in 5 ml of Ultima Gold (Perkin-Elmer Life Sciences). Recovery of [9,10-^3H]oleate in the aqueous phase after organic extraction was corrected for a 78.0 ± 0.9% recovery of [1-^{14}C]oleate internal standard.

Statistical analysis

Data were analyzed using the nonparametric Mann-Whitney test. $P < 0.05$ was regarded as statistically significant.

RESULTS

Plasma lipid and lipoprotein levels after injection of AdAPOE4

To determine the role of apoC-III in apoE-induced hyperlipidemia, *Apoe*$^{-/-}$, *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$, and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice were injected with increasing doses of AdAPOE4. Plasma TG and TC levels were measured 5 days after adenovirus injection (**Fig. 1**). In *Apoe*$^{-/-}$ mice, a moderate dose of AdAPOE4 (1×10^9 pfu) resulted in plasma apoE levels of 10–40 mg/dl and significantly reduced plasma TC levels and moderately increased plasma TG levels, whereas a high dose (2×10^9 pfu AdAPOE4) resulted in plasma apoE levels of 40–60 mg/dl and a substantial increase of both plasma TG and TC levels. In contrast, in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice, injection of both the moderate and high doses of AdAPOE4 resulted in near wild-type plasma TG and TC levels and plasma apoE levels of 3–10 mg/dl for both doses. Thus, the absence of apoC-III prevents the apoE-induced hyperlipidemia. This effect of *Apoc3* deficiency is *Apoc3* dose-dependent, because injection of the high dose of AdAPOE4 in *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$ mice resulted in plasma apoE levels of 10–25 mg/dl, moderate hypertriglyceridemia, and unchanged plasma cholesterol levels.

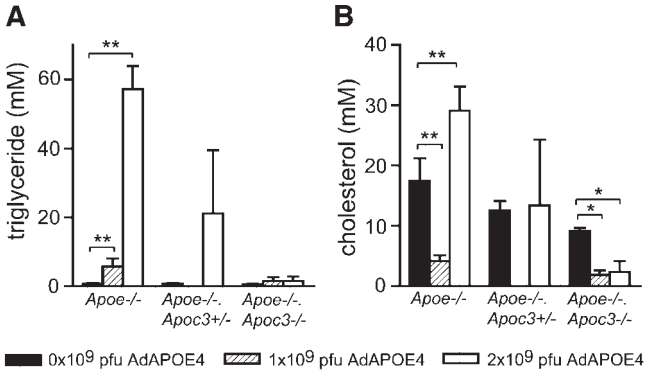

Fig. 1. Plasma triglyceride (TG) and cholesterol levels of *Apoe*$^{-/-}$, *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$, and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice injected with AdAPOE4. Five *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice were injected with 1×10^9 (hatched bars) or 2×10^9 plaque-forming units (pfu; open bars) of AdAPOE4. *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$ mice were injected with a dose of 2×10^9 pfu (open bars) of AdAPOE4 only. As a control virus treatment, five *Apoe*$^{-/-}$, *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$, and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice were injected with 2×10^9 pfu AdlacZ (indicated as 0×10^9 pfu AdAPOE4; closed bars). At day 5 after virus injection, fasted plasma samples were measured for TG (left) and cholesterol (right) concentrations. Values are represented as means ± SD. * $P < 0.05$; ** $P < 0.005$.

The effect of a high dose of 2×10^9 pfu of AdAPOE4 on the distribution of lipids over the lipoprotein fractions was measured after lipoprotein separation via FPLC (**Fig. 2**). The APOE4-induced change in plasma TG and TC was predominantly confined to the VLDL fractions in all types of mice, measured as area under the curve in FPLC lipoprotein profiles. Five days after expression of a high dose of 2×10^9 pfu of AdAPOE4, *Apoe*$^{-/-}$ mice showed an increase in VLDL-TG from 0.8 to 61.2 mM. The VLDL-TC levels also increased from 13.3 to 28.4 mM. *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice have low VLDL-TG levels, although they increased from 0.2 to 0.8 mM. The VLDL-TC levels decreased from 8.5 to 0.6 mM. *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$ mice showed an intermediate response in VLDL lipid levels after expression of AdAPOE4. The VLDL-TG levels increased from 0.14 to 21.6 mM and the VLDL-TC levels decreased from 14.8 to 11.4 mM.

VLDL composition

Sera from 4 h fasted *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice, at day 5 after injection of 2×10^9 pfu of AdlacZ, 1×10^9 pfu of AdAPOE4, or 2×10^9 pfu of AdAPOE4, were pooled, and VLDL was isolated via ultracentrifugation. The lipid composition and protein content of the VLDL were determined (**Table 1**). Strikingly, the TG level of VLDL in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice was unchanged after identical doses of AdAPOE4. At a high dose of 2×10^9 pfu of AdAPOE4, the VLDL in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice contained less CE in the particle and more PL on the surface of the VLDL than in Apoe$^{-/-}$ mice after equal treatment. This indicates that this VLDL particle is decreased in size, although this is a small effect. The total VLDL protein content in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice after 2×10^9 pfu of AdAPOE4 was 6-fold lower than that in *Apoe*$^{-/-}$ mice, indicating that *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice accumulate less VLDL in their blood circulation.

Hepatic VLDL-TG secretion after injection of AdAPOE4

To evaluate whether the absence of apoC-III prevents apoE-induced hyperlipidemia by decreasing the VLDL production rate, we determined the hepatic VLDL-TG secretion rate. To this end, Triton WR 1339 was injected intravenously to block peripheral TG clearance, and the VLDL-TG secretion rate was calculated from the increase in plasma TG levels. In both *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice injected with control LacZ virus, the hepatic VLDL-TG secretion was low, as reported previously for *Apoe*$^{-/-}$ mice (9). After AdAPOE4 injection, the VLDL-TG secretion rate increased dose-dependently, as indicated by a more pronounced increase in plasma TG after Triton treatment. A dose of 0.5×10^9 pfu of AdAPOE4 increased the VLDL-TG production rate to that observed in wild-type mice (13), whereas a dose of 1×10^9 pfu increased the VLDL-TG secretion rate ~10-fold (**Fig. 3**). Because in both *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice the VLDL-TG se-

Downloaded from www.jlr.org by guest, on July 24, 2017

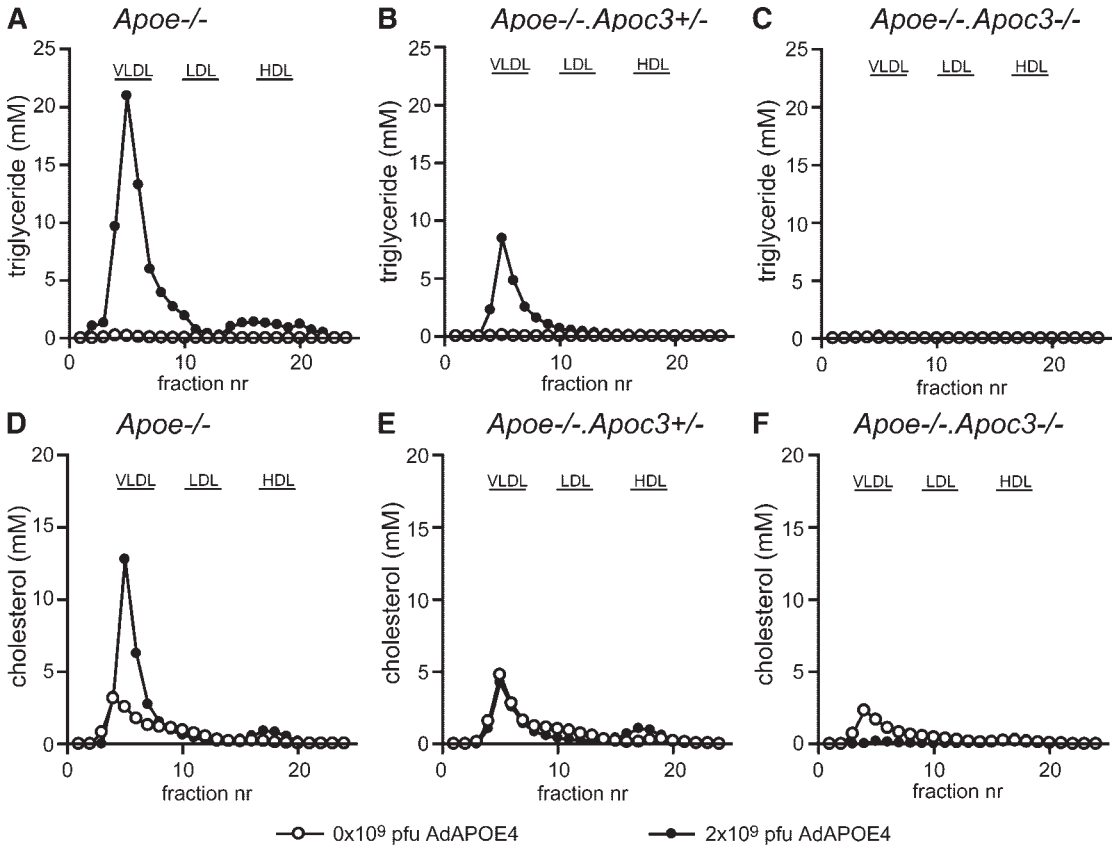

Fig. 2. Lipoprotein profiles of *Apoe*$^{-/-}$, *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$, and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice injected with AdAPOE4. Plasma pools of 4 h fasted *Apoe*$^{-/-}$ (A, D), *Apoe*$^{-/-}$.*Apoc3*$^{+/-}$ (B, E), and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ (C, F) mice, obtained before (open circles) and 5 days after injection of 2 × 10^9 pfu of AdAPOE4 (closed circles), were subjected to fast-protein liquid chromatography. The separate lipoprotein fractions were assayed for TG (A–C) and cholesterol (D–F) levels.

cretion rate was increased significantly and to a similar extent by high levels of apoE expression, we concluded that *Apoc3* deficiency does not affect the VLDL production rate.

Tissue distribution of VLDL-like emulsion-derived TGs in vivo

To investigate whether *Apoc3* deficiency leads to an enhanced rate of LPL-mediated VLDL-TG hydrolysis in vivo, *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice were given bolus injections of [^{3}H]TO-labeled emulsion particles. To achieve a high availability of [^{3}H]TO-labeled particles to peripheral LPL, the mice were given a high dose of 1 mg of TG that exceeded the endogenous plasma TG content of both groups of mice. The tissue-specific uptake of [^{3}H]oleate was determined at 10 min after bolus injection. No strain-specific differences were observed with respect to the uptake of [^{3}H]TO-derived activity by liver, heart, spleen, and skeletal muscle. In contrast, the uptake of ^{3}H activity by WAT was significantly higher in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ versus *Apoe*$^{-/-}$ mice, reaching statistical significance for gonadal WAT (3.2-fold; $P < 0.05$) and intestinal WAT (2.4-fold; $P < 0.05$) (**Fig. 4**). These data indicate that *Apoc3* deficiency results in more efficient peripheral LPL-mediated TG hydrolysis.

LPL activity in the presence of apoC-III or apoE

Both apoE and apoC-III have been shown to inhibit LPL-mediated TG hydrolysis. To determine the inhibitory potency of apoC-III versus apoE, the activity of LPL-medi-

TABLE 1. VLDL composition in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice after adenovirus treatment

Apoe$^{-/-}$	TG	CE	FC	PL	Protein	*Apoe*$^{-/-}$.*Apoc3*$^{-/-}$	TG	CE	FC	PL	Protein
AdlacZ (2 × 10^9 pfu)	12.7	40.6	25.9	20.8	0.06	AdlacZ (2 × 10^9 pfu)	6.1	54.4	11.9	27.6	0.05
AdAPOE4 (1 × 10^9 pfu)	64.1	8.8	6.9	20.3	0.04	AdAPOE4 (1 × 10^9 pfu)	67.6	7.1	6.5	18.8	0.02
AdAPOE4 (2 × 10^9 pfu)	64.7	17.5	7.0	10.8	1.57	AdAPOE4 (2 × 10^9 pfu)	66.4	11.3	4.0	18.3	0.26

pfu, plaque-forming units. The VLDL triglyceride (TG), cholesteryl ester (CE), free cholesterol (FC), and phospholipid (PL) contents were measured as percentages of total weight per volume. VLDL protein was measured in mg/ml.

Downloaded from www.jlr.org by guest, on July 24, 2017

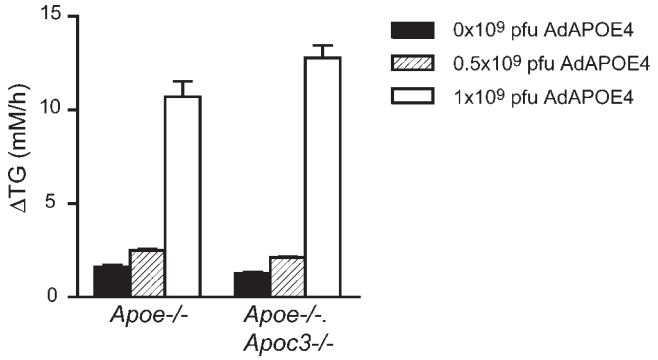

Fig. 3. Hepatic VLDL-TG production rate in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$. *Apoc3*$^{-/-}$ mice injected with AdAPOE4. *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$. *Apoc3*$^{-/-}$ mice were injected with AdlacZ (closed bars; indicated as 0×10^9 pfu AdAPOE4; n = 4), 0.5×10^9 pfu of AdAPOE4 (hatched bars; n = 4), or 1×10^9 pfu of AdAPOE4 (open bars; n = 2). At day 5 after virus injection, 4 h fasted mice were injected with Triton WR 1339. The VLDL-TG production rate was determined as the increase in plasma TG concentration during 2 h after Triton injection. Values are represented as change in TG (ΔTG) in mM/h ± SD.

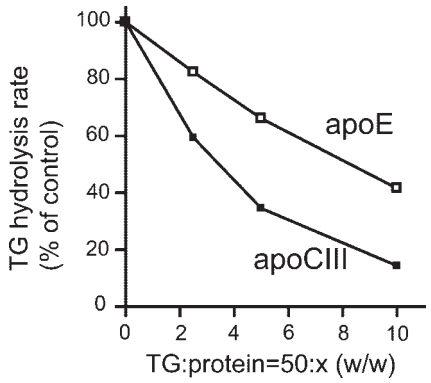

Fig. 5. Effect of apolipoprotein C-III (apoC-III) and apoE on LPL-mediated TG hydrolysis in vitro. Glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles were incubated (30 min at 37°C) with apoC-III (closed squares) or apoE (open squares) at TG/protein weight ratios of 50:0, 2.5, 5, and 10. At time zero, LPL was added and [^{3}H]oleate was extracted as described. The reaction velocities in the presence of apoC-III and apoE were calculated as percentages of [^{3}H]oleate release per minute and are expressed relative to control incubations (100%).

ated TG hydrolysis in the presence of varying amounts of apoC-III or apoE was determined in vitro. Preincubation of VLDL-like emulsion particles with apoC-III resulted in a dose-dependent inhibition of the release of [^{3}H]oleate from the emulsion-incorporated glycerol tri[^{3}H]oleate, reaching 86% inhibition at a TG/apoC-III ratio of 50:10 (w/w) (**Fig. 5**). In agreement with previous observations (9), apoE also dose-dependently inhibited LPL activity. However, the inhibitory efficacy of apoC-III was higher than that of apoE.

DISCUSSION

We and others have shown previously that high levels of adenovirus-mediated expression of apoE result in hyperlipidemia. This effect is attributable to a dramatic increase of the VLDL-TG production rate (12, 13, 18, 29) and inhibition of LPL-mediated VLDL-TG hydrolysis, caused by enrichment of VLDL with apoE (8, 9). Our current data show that deletion of *Apoc3* dose-dependently reduces the hyperlipidemia induced by high doses of AdAPOE4 (Fig. 1). Neither the VLDL composition (Table 1) nor the apoE-induced increase in the VLDL-TG production rate was affected by the absence of apoC-III (Fig. 3). However, the absence of apoC-III clearly increases the rate of peripheral TG hydrolysis, as indicated by an enhanced uptake of fatty acids from [^{3}H]TO by WAT (Fig. 4). These data indicate that the apoE-induced inhibition of VLDL-TG hydrolysis can be alleviated by the elimination of endogenous apoC-III. To determine whether apoC-III is a more potent inhibitor of VLDL-TG hydrolysis than apoE, in vitro lipolysis experiments were performed (Fig. 5). Indeed apoC-III seems a more potent inhibitor of VLDL-TG hydrolysis than does apoE. Thus, we conclude that the absence of apoE-induced hyperlipidemia in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice is explained by the alleviation of the apoE-induced inhibition of VLDL-TG lipolysis.

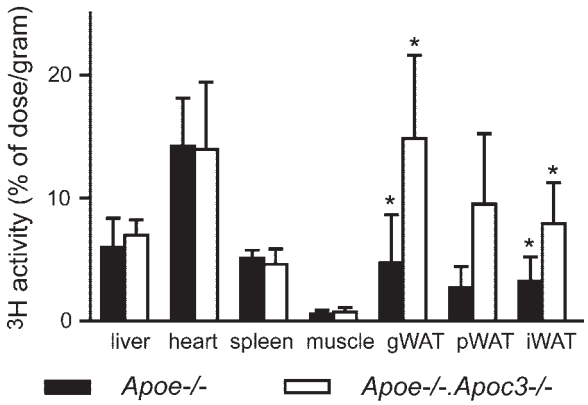

Fig. 4. Tissue distribution of TG-derived fatty acids from VLDL-like emulsion particles. Five *Apoe*$^{-/-}$ (closed bars) and *Apoe*$^{-/-}$. *Apoc3*$^{-/-}$ (open bars) mice were intravenously injected with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles. At 10 min after bolus injection, the indicated organs were isolated and dissolved, and the uptake of [^{3}H]oleate was determined as percentage of the injected dose per gram wet tissue weight. Values are represented as means ± SD. * $P < 0.05$. gWAT, pWAT, and iWAT denote gonadal, perirenal, and intestinal white adipose tissue, respectively.

The effect of apoC-III on VLDL production is somewhat controversial. Overexpression of APOC3 in transgenic mice has been associated with both an increased VLDL-TG secretion rate (30) and, in a different transgenic line, with no change in the VLDL-TG secretion rate (31). Hirano et al. (32) have reported an increased VLDL-TG secretion rate in *Apoc3*-deficient mice. However, we have found that endogenous *Apoc3* deficiency does not affect the VLDL-TG production rate in the absence or presence of endogenous *Apoe* (15). Moreover, the apoE-induced increase in VLDL-TG secretion is also not affected by the presence or absence of apoC-III (Fig. 3). Thus, although the discrepancy with respect to this effect of apoC-III on hepatic VLDL-TG production between various re-

Downloaded from www.jlr.org by guest, on July 24, 2017

search groups remains to be resolved, we have consistently and repeatedly found no effect of *Apoc3* deficiency on VLDL-TG production rates.

The VLDL particles circulating in the plasma of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice have a very similar TG content after treatment with AdAPOE4 (Table 1). Also, there was no effect of *Apoc3* deficiency on the composition of nascent VLDL isolated after Triton injection in mice treated with 5×10^8 or 1×10^9 pfu of AdAPOE4 (data not shown). Importantly, the VLDL protein content after injection of 2×10^9 pfu of AdAPOE4 is 6-fold higher in *Apoe*$^{-/-}$ mice compared with *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice. This indicates that *Apoc3* deficiency prevents the accumulation of APOE-rich VLDL particles in the circulation (Table 1) (13).

It has been demonstrated that apoE dose-dependently inhibits LPL-mediated TG hydrolysis in vitro (8, 9, 13). In the *Apoe*$^{-/-}$ mice injected with a high dose of AdAPOE4, it is more than likely that circulating apoE-rich particles are poor LPL substrates and thereby remain very TG-rich. This is also supported by the positive correlation between plasma apoE levels and plasma TG levels in both transgenic mice and humans (10, 33). The mechanism underlying the inhibition of LPL by excess apoE has been proposed to be the displacement of apoC-II from the particle, which is an essential cofactor of LPL (10). However, our current data indicate that apoC-II may not be the rate-limiting factor. It seems likely that nascent VLDL produced in apoE-overexpressing *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice is equally rich in apoE; thus, the exclusion pressure for apoC-II will be equal on particles from both mouse lines. Nevertheless, the hypolipidemic phenotype of *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice injected with a high dose of AdAPOE4 is illustrative of an efficient TG hydrolysis and clearance of subsequently formed remnants. Thus, in the absence of apoC-III, the apoC-II particle level does not seem to be limiting for efficient LPL-mediated TG hydrolysis.

The mechanism responsible for LPL inhibition by apoC-III is similarly not known. It has been proposed that apoC-III acts as a direct noncompetitive inhibitor of LPL (34). However, attempts to identify the domain in the apoC-III protein responsible for direct LPL inhibition have yielded conflicting results (6, 7). Whether apoC-III affects LPL activity via direct protein-protein interaction (with apoC-II and/or LPL) or indirectly by, for example, inhibiting the binding of TG-rich particles to endothelial surfaces where LPL is active remains to be determined. If the interaction of apoC-III with LPL is nonspecific or based on steric hindrance, then the size of the protein and the occupation of the VLDL particle surface would determine the inhibitory effect on LPL. We incubated VLDL-like emulsion particles with equal amounts of apoC-III and apoE, based on the TG-to-apolipoprotein weight-weight ratio (Fig. 5). At equal weight-weight ratio, and thus presumably equal apolipoprotein occupation of the particle surface, apoC-III inhibits LPL-mediated TG hydrolysis to a greater extent than apoE. This indicates that apoC-III is indeed a specific and efficient inhibitor of LPL in vitro. Interestingly, the absence of an effect on plasma TG levels even after significant overexpression of apoE in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice (Fig. 1) could also indicate that apoC-III and excess apoE act in a supra-additive manner to inhibit LPL-mediated TG hydrolysis in vivo.

The mechanism underlying the enhanced VLDL-TG clearance by *Apoc3* deficiency in vivo was investigated by injecting the mice with radiolabeled TG-rich emulsion particles. Previously, it has been shown that these emulsion particles rapidly acquire apolipoproteins in the blood circulation. Also, these TG-rich emulsion particles are processed by LPL and cleared by hepatic lipoprotein receptors similar to nascent chylomicron and VLDL particles (9, 27). The size of the emulsion particles was similar to that reported for nascent VLDL particles (14). In a previous study, we specifically focused on the plasma kinetics of VLDL-TG in *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice (15). Mice were given an intravenous bolus injection with glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles that contained 150 μg of TG. This resulted in a significantly faster decay of label from plasma because of the *Apoc3* deficiency. In the present study, we focused on the effect of *Apoc3* deficiency on local LPL activity in the periphery. To achieve an optimal availability of LPL substrate, mice were given a bolus injection of glycerol tri[^{3}H]oleate-labeled VLDL-like emulsion particles that contained a total of 1 mg of TG (Fig. 4). Because of the excess of TG that was administered, the plasma levels of the label remained very high in both *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ and *Apoe*$^{-/-}$ mice (data not shown). However, the increased [^{3}H]oleate uptake by WAT clearly indicated a higher local LPL activity in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice. Thus, *Apoc3* deficiency results in both an enhanced clearance of VLDL-TG from the plasma and subsequent uptake by adipose tissue.

ApoE- and TG-rich VLDL particles are poor substrates for receptor-mediated hepatic clearance. After processing by LPL, the VLDL particles will become smaller, thereby increasing the affinity of apoE for hepatic clearance receptors (28). We have shown that *Apoc3* deficiency stimulates LPL-mediated TG hydrolysis, thereby stimulating remnant formation. This might lead to a faster hepatic clearance of these particles, accompanied by a higher rate of apoE-mediated remnant clearance. This was confirmed by the lower circulating apoE levels in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice compared with *Apoe*$^{-/-}$ mice after equal treatment with AdAPOE4. Also, the VLDL-cholesterol levels were low in *Apoe*$^{-/-}$.*Apoc3*$^{-/-}$ mice after AdAPOE4 treatment, without increasing the LDL-cholesterol levels, indicating enhanced clearance of VLDL remnants. However, from our current analyses, we cannot exclude the possibility that apoC-III influences the hepatic clearance of VLDL remnants directly (35).

Numerous studies have reported associations between plasma apoC-III levels, plasma TG levels, and premature cardiovascular disease, both in healthy and patient populations (1, 36–39). Our current results demonstrate that *Apoc3* deficiency has the potential to alleviate the hyperlipidemia associated with a 10-fold increased VLDL-TG production rate. Because peripheral uptake of fatty acids is

ASBMB JOURNAL OF LIPID RESEARCH

Downloaded from www.jlr.org by guest, on July 24, 2017

increased by *Apoc3* deficiency, we conclude that the processing of VLDL into remnant particles is stimulated, which leads to a faster remnant particle clearance by the liver. Therefore, these data confirm the notion from human association studies that apoC-III is a potentially powerful molecule in modulating plasma TG levels and thus in modulating the predisposition to cardiovascular disease.

This research was conducted in the framework of the Leiden Center for Cardiovascular Research Leiden University Medical Center-Netherlands Organization for Applied Scientific Research and was supported by the Netherlands Heart Foundation (Project 2000.099), by the Leiden University Medical Center (Gisela Thier Fellowship to P.C.N.R.), and by the Netherlands Organization for Scientific Research (VIDI Grant 917.36.351 to P.C.N.R. and Netherlands Organization for Scientific Research Program Grant 903-39-291 to L.M.H.). The authors thank A. van der Zee for expert technical assistance.

REFERENCES

1. Talmud, P. J., E. Hawe, S. Martin, M. Olivier, G. J. Miller, E. M. Rubin, L. A. Pennacchio, and S. E. Humphries. 2002. Relative contribution of variation within the APOC3/A4/A5 gene cluster in determining plasma triglycerides. *Hum. Mol. Genet.* **11:** 3039–3046.
2. Waterworth, D. M., P. J. Talmud, S. R. Bujac, R. M. Fisher, G. J. Miller, and S. E. Humphries. 2000. Contribution of apolipoprotein C-III gene variants to determination of triglyceride levels and interaction with smoking in middle-aged men. *Arterioscler. Thromb. Vasc. Biol.* **20:** 2663–2669.
3. Busch, C. P., and R. A. Hegele. 2000. Variation of candidate genes in triglyceride metabolism. *J. Cardiovasc. Risk.* **7:** 309–315.
4. Ginsberg, H. N., N. A. Le, I. J. Goldberg, J. C. Gibson, A. Rubinstein, P. Wang-Iverson, R. Norum, and W. V. Brown. 1986. Apolipoprotein B metabolism in subjects with deficiency of apolipoproteins CIII and AI. Evidence that apolipoprotein CIII inhibits catabolism of triglyceride-rich lipoproteins by lipoprotein lipase in vivo. *J. Clin. Invest.* **78:** 1287–1295.
5. Ganesan, D., H. B. Bass, W. J. McConathy, and P. Alaupovic. 1976. Is decreased activity of C-II activated lipoprotein lipase in type III hyperlipoproteinemia (broad-beta-disease) a cause or an effect of increased apolipoprotein E levels? *Metabolism.* **25:** 1189–1195.
6. Liu, H., P. J. Talmud, L. Lins, R. Brasseur, G. Olivecrona, F. Peelman, J. Vandekerckhove, M. Rosseneu, and C. Labeur. 2000. Characterization of recombinant wild type and site-directed mutations of apolipoprotein C-III: lipid binding, displacement of apoE, and inhibition of lipoprotein lipase. *Biochemistry.* **39:** 9201–9212.
7. McConathy, W. J., J. C. Gesquiere, H. Bass, A. Tartar, J. C. Fruchart, and C. S. Wang. 1992. Inhibition of lipoprotein lipase activity by synthetic peptides of apolipoprotein C-III. *J. Lipid Res.* **33:** 995–1003.
8. Jong, M. C., V. E. Dahlmans, M. H. Hofker, and L. M. Havekes. 1997. Nascent very-low-density lipoprotein triacylglycerol hydrolysis by lipoprotein lipase is inhibited by apolipoprotein E in a dose-dependent manner. *Biochem. J.* **328:** 745–750.
9. Rensen, P. C., and T. J. van Berkel. 1996. Apolipoprotein E effectively inhibits lipoprotein lipase-mediated lipolysis of chylomicron-like triglyceride-rich lipid emulsions in vitro and in vivo. *J. Biol. Chem.* **271:** 14791–14799.
10. Huang, Y., X. Q. Liu, S. C. Rall, Jr., J. M. Taylor, A. von Eckardstein, G. Assmann, and R. W. Mahley. 1998. Overexpression and accumulation of apolipoprotein E as a cause of hypertriglyceridemia. *J. Biol. Chem.* **273:** 26388–26393.
11. McConathy, W. J., and C. S. Wang. 1989. Inhibition of lipoprotein lipase by the receptor-binding domain of apolipoprotein E. *FEBS Lett.* **251:** 250–252.
12. Mensenkamp, A. R., M. C. Jong, H. van Goor, M. J. van Luyn, V. Bloks, R. Havinga, P. J. Voshol, M. H. Hofker, K. W. van Dijk, L. M. Havekes, et al. 1999. Apolipoprotein E participates in the regulation of very low density lipoprotein-triglyceride secretion by the liver. *J. Biol. Chem.* **274:** 35711–35718.
13. van Dijk, K. W., B. J. van Vlijmen, H. B. van't Hof, A. van der Zee, S. Santamarina-Fojo, T. J. van Berkel, L. M. Havekes, and M. H. Hofker. 1999. In LDL receptor-deficient mice, catabolism of remnant lipoproteins requires a high level of apoE but is inhibited by excess apoE. *J. Lipid Res.* **40:** 336–344.
14. Kuipers, F., M. C. Jong, Y. Lin, M. Eck, R. Havinga, V. Bloks, H. J. Verkade, M. H. Hofker, H. Moshage, T. J. Berkel, et al. 1997. Impaired secretion of very low density lipoprotein-triglycerides by apolipoprotein E-deficient mouse hepatocytes. *J. Clin. Invest.* **100:** 2915–2922.
15. Jong, M. C., P. C. Rensen, V. E. Dahlmans, H. van der Boom, T. J. van Berkel, and L. M. Havekes. 2001. Apolipoprotein C-III deficiency accelerates triglyceride hydrolysis by lipoprotein lipase in wild-type and apoE knockout mice. *J. Lipid Res.* **42:** 1578–1585.
16. Maeda, N., H. Li, D. Lee, P. Oliver, S. H. Quarfordt, and J. Osada. 1994. Targeted disruption of the apolipoprotein C-III gene in mice results in hypotriglyceridemia and protection from postprandial hypertriglyceridemia. *J. Biol. Chem.* **269:** 23610–23616.
17. Kypreos, K. E., K. W. van Dijk, A. van der Zee, L. M. Havekes, and V. I. Zannis. 2001. Domains of apolipoprotein E contributing to triglyceride and cholesterol homeostasis in vivo. Carboxyl-terminal region 203–299 promotes hepatic very low density lipoprotein-triglyceride secretion. *J. Biol. Chem.* **276:** 19778–19786.
18. Tsukamoto, K., C. Maugeais, J. M. Glick, and D. J. Rader. 2000. Markedly increased secretion of VLDL triglycerides induced by gene transfer of apolipoprotein E isoforms in apoE-deficient mice. *J. Lipid Res.* **41:** 253–259.
19. van Ree, J. H., W. J. van den Broek, V. E. Dahlmans, P. H. Groot, M. Vidgeon-Hart, R. R. Frants, B. Wieringa, L. M. Havekes, and M. H. Hofker. 1994. Diet-induced hypercholesterolemia and atherosclerosis in heterozygous apolipoprotein E-deficient mice. *Atherosclerosis.* **111:** 25–37.
20. Fallaux, F. J., O. Kranenburg, S. J. Cramer, A. Houweling, H. Van Ormondt, R. C. Hoeben, and A. J. Van Der Eb. 1996. Characterization of 911: a new helper cell line for the titration and propagation of early region 1-deleted adenoviral vectors. *Hum. Gene Ther.* **7:** 215–222.
21. McGrory, W. J., D. S. Bautista, and F. L. Graham. 1988. A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology.* **163:** 614–617.
22. Tao, N., G. P. Gao, M. Parr, J. Johnston, T. Baradet, J. M. Wilson, J. Barsoum, and S. E. Fawell. 2001. Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol. Ther.* **3:** 28–35.
23. van Vlijmen, B. J., H. B. van't Hof, M. J. Mol, H. van der Boom, A. van der Zee, R. R. Frants, M. H. Hofker, and L. M. Havekes. 1996. Modulation of very low density lipoprotein production and clearance contributes to age- and gender-dependent hyperlipoproteinemia in apolipoprotein E3-Leiden transgenic mice. *J. Clin. Invest.* **97:** 1184–1192.
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
25. Sullivan, P. M., H. Mezdour, Y. Aratani, C. Knouff, J. Najib, R. L. Reddick, S. H. Quarfordt, and N. Maeda. 1997. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. *J. Biol. Chem.* **272:** 17972–17980.
26. Redgrave, T. G., and R. C. Maranhao. 1985. Metabolism of protein-free lipid emulsion models of chylomicrons in rats. *Biochim. Biophys. Acta.* **835:** 104–112.
27. Rensen, P. C., M. C. van Dijk, E. C. Havenaar, M. K. Bijsterbosch, J. K. Kruijt, and T. J. van Berkel. 1995. Selective liver targeting of antivirals by recombinant chylomicrons—a new therapeutic approach to hepatitis B. *Nat. Med.* **1:** 221–225.
28. Rensen, P. C., N. Herijgers, M. H. Netscher, S. C. Meskers, M. van Eck, and T. J. van Berkel. 1997. Particle size determines the specificity of apolipoprotein E-containing triglyceride-rich emulsions for the LDL receptor versus hepatic remnant receptor in vivo. *J. Lipid Res.* **38:** 1070–1084.
29. Teusink, B., A. R. Mensenkamp, H. van der Boom, F. Kuipers, K. W. van Dijk, and L. M. Havekes. 2001. Stimulation of the in vivo production of very low density lipoproteins by apolipoprotein E is

Downloaded from www.jlr.org by guest, on July 24, 2017

independent of the presence of the low density lipoprotein receptor. *J. Biol. Chem.* **276:** 40693–40697.
30. de Silva, H. V., S. J. Lauer, J. Wang, W. S. Simonet, K. H. Weisgraber, R. W. Mahley, and J. M. Taylor. 1994. Overexpression of human apolipoprotein C-III in transgenic mice results in an accumulation of apolipoprotein B48 remnants that is corrected by excess apolipoprotein E. *J. Biol. Chem.* **269:** 2324–2335.
31. Ebara, T., R. Ramakrishnan, G. Steiner, and N. S. Shachter. 1997. Chylomicronemia due to apolipoprotein CIII overexpression in apolipoprotein E-null mice. Apolipoprotein CIII-induced hypertriglyceridemia is not mediated by effects on apolipoprotein E. *J. Clin. Invest.* **99:** 2672–2681.
32. Hirano, T., T. Takahashi, S. Saito, H. Tajima, T. Ebara, and M. Adachi. 2001. Apoprotein C-III deficiency markedly stimulates triglyceride secretion in vivo: comparison with apoprotein E. *Am. J. Physiol. Endocrinol. Metab.* **281:** E665–E669.
33. Wang, C. S., W. J. McConathy, H. U. Kloer, and P. Alaupovic. 1985. Modulation of lipoprotein lipase activity by apolipoproteins. Effect of apolipoprotein C-III. *J. Clin. Invest.* **75:** 384–390.
34. Lambert, D. A., A. L. Catapano, L. C. Smith, J. T. Sparrow, and A. M. Gotto, Jr. 1996. Effect of the apolipoprotein C-II/C-III1 ratio on the capacity of purified milk lipoprotein lipase to hydrolyse triglycerides in monolayer vesicles. *Atherosclerosis.* **127:** 205–212.
35. Windler, E., and R. J. Havel. 1985. Inhibitory effects of C apolipoproteins from rats and humans on the uptake of triglyceride-rich lipoproteins and their remnants by the perfused rat liver. *J. Lipid Res.* **26:** 556–565.
36. Olivieri, O., A. Bassi, C. Stranieri, E. Trabetti, N. Martinelli, F. Pizzolo, D. Girelli, S. Friso, P. F. Pignatti, and R. Corrocher. 2003. Apolipoprotein C-III, metabolic syndrome, and risk of coronary artery disease. *J. Lipid Res.* **44:** 2374–2381.
37. Lee, S. J., L. A. Moye, H. Campos, G. H. Williams, and F. M. Sacks. 2003. Hypertriglyceridemia but not diabetes status is associated with VLDL containing apolipoprotein CIII in patients with coronary heart disease. *Atherosclerosis.* **167:** 293–302.
38. Gerber, Y., U. Goldbourt, S. Segev, and D. Harats. 2003. Indices related to apo CII and CIII serum concentrations and coronary heart disease: a case-control study. *Prev. Med.* **37:** 18–22.
39. Onat, A., G. Hergenc, V. Sansoy, M. Fobker, K. Ceyhan, S. Toprak, and G. Assmann. 2003. Apolipoprotein C-III, a strong discriminant of coronary risk in men and a determinant of the metabolic syndrome in both genders. *Atherosclerosis.* **168:** 81–89.

Downloaded from www.jlr.org by guest, on July 24, 2017