Aspects involved in the (patho)physiology of the metabolic syndrome

Duivenvoorden, I.

Chapter 2

Apolipoprotein C3-deficiency results in diet-induced obesity and aggravated insulin resistance in mice

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Abstract

Our aim was to study whether the absence of apoC3, a strong inhibitor of lipoprotein lipase (LPL), accelerates the development of obesity and consequently insulin resistance.

Apoc3$^{-/-}$ mice and wild-type littermates were fed a high-fat (46 energy %) diet for 20 weeks. After 20 weeks of high-fat feeding, *apoc3*$^{-/-}$ mice showed decreased plasma triglyceride (TG) levels (0.11 ± 0.02 vs. 0.29 ± 0.04 mmol/l; $P < 0.05$), and were more obese (42.8 ± 3.2 vs. 35.2 ± 3.3 g; $P < 0.05$) compared with wild-type littermates. This increase in body weight was entirely explained by increased body lipid mass (16.2 ± 5.9 vs. 10.0 ± 1.8 g; $P < 0.05$). LPL-dependent uptake of TG-derived fatty acids by adipose tissue was significantly higher in *apoc3*$^{-/-}$ mice. LPL-independent uptake of albumin-bound fatty acids did not differ. Interestingly, whole-body insulin sensitivity using hyperinsulinemic-euglycemic clamps was decreased by 43% and suppression of endogenous glucose production was decreased by 25% in *apoc3*$^{-/-}$ mice compared with control mice. Absence of apoC3, the natural LPL inhibitor, enhances fatty acid uptake from plasma TG in adipose tissue, which leads to higher susceptibility to diet-induced obesity followed by more severe development of insulin resistance. Therefore, apoC3 is a potential target for treatment of obesity and insulin resistance.

Introduction

Lipoprotein lipase (LPL) hydrolyzes plasma triglycerides (TG) contained in circulating very low density lipoprotein (VLDL) particles and chylomicrons. Subsequently, these TG-derived fatty acids (FA) are taken up by the underlying tissues[1,2]. LPL activity is an important determinant of the rate of FA storage into white adipose tissue (WAT) and other tissues. For instance, overexpression of LPL in muscle leads to enhanced TG storage in muscle[3-5], whereas adipose tissue-specific LPL deficiency prevents excessive adipose tissue TG-storage in leptin-deficient mice[6]. The latter observation indicates a link between adipose tissue-specific LPL activity and obesity. Inhibition of LPL activity therefore may be an effective strategy for prevention of obesity. This concept is further confirmed by mouse models such as VLDL-receptor knockout and human apolipoprotein (apo) C1 overexpressing mice. These mice show decreased *in vivo* VLDL-TG lipolysis and, as a consequence, are protected from diet- and genetically-induced obesity[7-9], as well as insulin resistance.

These data suggest that overall reduction of the LPL activity can protect against obesity. It is unclear, however, whether the effect of LPL modulation acts in both directions, *i.e.*, whether *activation* of LPL can also lead to *enhanced* susceptibility to diet-induced obesity followed by aggravated development of insulin resistance. Adipose tissue-specific overexpression of LPL seemed to result in a relatively mild (20%) increase in fat pad weight[10]. This mild effect could be related to the fact that natural inhibitors of LPL, *e.g.*, apoC3, are still present to regulate *in vivo* adipose tissue LPL activity. Alternatively, it

might relate to the LPL activity ratio between adipose tissue and muscle tissue as discussed by Preiss-Landl et al.[11].

To elucidate the effect of deletion of the main endogenous LPL inhibitor apoC3 on diet-induced obesity and insulin resistance *in vivo*, we have used apoC3 knockout mice[12]. ApoC3 is mainly produced by the liver and is a well-known inhibitor of LPL activity[13]. *Apoc3*$^{-/-}$ mice have greatly enhanced *in vivo* VLDL-TG clearance, as caused by the absence of the endogenous block on LPL activity[14], which is reflected by a total absence of a postprandial TG response after a fat load[12,14]. The present study indeed showed that *apoc3*$^{-/-}$ mice are more sensitive to diet-induced obesity followed by a more aggravated development of insulin resistance compared with their control littermates. ApoC3, therefore, may be a potential therapeutic target for the treatment of obesity and insulin resistance.

Materials and methods

Animals and diet

Male and female *apoc3*$^{-/-}$ mice and their wild-type (WT) littermates (C57Bl/6 background) were originally obtained from The Jackson Laboratories (Bar Harbor, ME, USA) and further bred in our institution. The 4 to 5 month old animals (n=15) were individually housed, allowed free access to food and water, and were kept on a 12 h light cycle (lights on at 7.00 A.M.), under standard conditions. After a standard rat-mouse chow diet (Standard Diet Services, Essex, UK), the mice were given a high-fat corn oil diet (Hope Farms, Woerden, the Netherlands) until the end of the experimental period. This diet contained 24% corn oil, 24% casein, 20% cerelose, 18% corn starch and 6% cellulose by weight, resulting in 46.2% of calories derived from corn oil. Body weight and food intake were followed through the duration of the experiment. Food intake was assessed by determining the difference in food weight during a 7-day period to ensure reliable measurements. Food intake was assessed as food weight (g) per mouse per day. From these data, the “feed efficiency” was calculated as total body weight gained per week divided by the total amount of food consumed per week. All experiments were approved by the animal care committee of TNO Quality of Life (Leiden, the Netherlands).

Plasma parameters

Plasma levels of cholesterol, free fatty acids (FFA), TG (without free glycerol), glucose, keton bodies (β-hydroxybutyrate), insulin, and leptin were determined after an overnight fast in *apoc3*$^{-/-}$ and WT littermates after 0 and 20 weeks of high-fat diet feeding. Blood samples were taken from the tail vein in paraoxon-coated capillaries to prevent lipolysis[15]. The plasma was collected via centrifugation, and plasma cholesterol, TG, glucose, keton body, and FFA levels were determined using standard commercial kits, according to the manufacturer’s instructions (Roche Diagnostics, Mannheim, Germany; Triglyceride GPO-

Trinder, glucose Trinder 500 and β-hydroxybutyrate, Sigma Diagnostics, St. Louis, MO, USA, and NEFA-C, Wako chemicals, Neuss, Germany, respectively). Plasma insulin and leptin levels were measured by radioimmunoassay (RIAs), using rat insulin standards, that show 100% cross-reaction with mouse and human insulin, or mouse leptin standards (sensitive rat insulin RIA kit, mouse leptin RIA kit, Linco Research, St. Charles, MO, USA).

Body mass composition analysis

Mouse carcasses (wet weight) were dehydrated at 65°C until a constant mass was achieved (dry weight). The bodies were hydrolyzed in 100 ml ethanolic potassium hydroxide (3 M in 65% ethanol) for determination of body lipid, using enzymatic measurement of glycerol (Sigma Diagnostics), and body protein content by the Lowry assay[16]. Total water content was calculated as wet weight minus dry weight, and lean body mass (LBM) was calculated as wet weight minus total lipid weight.

WAT histology

Pieces of WAT from reproductive fat pads were fixed in formalin and embedded in paraffin. Sections of 3 µm were cut and stained with hematoxylin-phloxine-saffron. Adipocyte size was quantified using Leica Qwin v1.0 (Leica Micro systems, Wetzlar, Germany).

Tissue-specific FFA uptake from plasma TG

To exclude obesity-induced differences in adipose tissue FA uptake, we used body weight-matched *apoc3*$^{-/-}$ and WT mice in the fed state, which had been treated with the high-fat diet for 2 weeks. The mice were sedated by i.p. injection of hypnorm (0.5 ml/kg; Janssen Pharmaceutical, Tilburg, The Netherlands) and midazolam (12.5 mg/kg; Roche Netherlands, Mijdrecht, The Netherlands) and equipped with a catheter for tail-vein i.v. infusion. Large (150 nm) glycerol [^{3}H]triolein-labeled chylomicron-like particles, prepared as described by Rensen et al.[17], were mixed with a trace amount of [^{14}C]oleic acid complexed to bovine serum albumin (BSA) (both isotopes obtained from Amersham, Little Chalfont, UK) and continuously infused for 2 h into the animals as described earlier[18]. Blood samples were taken using chilled paraoxon-coated capillaries by tailbleeding at 1.5 and 2 h of infusion to quantify steady-state conditions. Subsequently, mice were sacrificed and organs were quickly harvested and snap-frozen in liquid nitrogen. Analyses and calculations were performed as described by Teusink et al.[18].

Total plasma and tissue LPL level

For determining the total LPL activity level, body weight-matched *apoc3*$^{-/-}$ and WT mice, treated with the high-fat diet for 1 week, were fasted for 4 h and received an i.p. injection of heparin (1 U/g body weight; Leo Pharmaceutical Products, Weesp, The Netherlands). Blood samples were taken after 20 min. Plasmas of these samples were snap-frozen and stored at -80°C until analysis. One week later, the animals in the fed state were sacrificed, and liver, heart, skeletal muscle (quadriceps), and WAT samples were collected. The

organ samples were cut into small pieces and put in 1 ml 2% BSA-containing DMEM medium. Heparin (2 U) was added, and samples were shaken at 37°C for 60 minutes. After centrifugation (10 min at 13,000 rpm), the supernatants were taken and snap-frozen until analysis. Total LPL activity of all samples was determined as modified from Zechner[19]. In short, the lipolytic activity of plasma or tissue supernatant was assessed by determination of [^{3}H]oleate production upon incubation of plasma or tissue supernatant with a mix containing an excess of both [^{3}H]triolein, heat-inactivated human plasma as source of the LPL coactivator apoC2, and FA-free BSA as FFA acceptor. Hepatic lipase (HL) and LPL activities were distinguished in the presence of 1 M NaCl, which specifically blocks LPL.

Modulated plasma LPL activity

To allow for studying the effect of apoC3-deficiency on the modulated LPL activity in plasma, post-heparin mouse plasma (as a source of LPL, apoC2, and apoC3) was incubated with a mix of [^{3}H]triolein-labeled 75 nm-sized VLDL mimicking protein-free emulsion particles[20] (0.25 mg TG/ml) and excess FA-free BSA (60 mg/ml). HL and LPL activities were distinguished as described above, and the LPL activity was calculated as the amount of FFA released per min per ml.

Hyperinsulinemic-euglycemic clamp

After 20 weeks of high-fat feeding and an overnight fast, the animals were anesthetized, as described earlier, and basal rates of glucose turnover were determined followed by a hyperinsulinemic-euglycemic phase (plasma glucose at ~ 7.5 mmol/l) as described previously[5,21]. After the final blood sample, mice were sacrificed, and liver, cardiac muscle, skeletal muscle (quadriceps), and adipose tissue samples were immediately frozen in liquid nitrogen and stored at -20°C for subsequent analysis. Carcasses were stored at -20°C until body mass composition was analyzed. Whole-body insulin-mediated glucose uptake and insulin-mediated suppression of endogenous glucose production were calculated on the basis of LBM. The whole-body insulin sensitivity index was expressed as the ratio between insulin-induced whole-body glucose disposal and hyperinsulinemic plasma insulin concentration. The endogenous glucose production insulin sensitivity index was expressed as ratio between insulin-mediated inhibition of endogenous glucose production and hyperinsulinemic plasma insulin concentration. Insulin clearance (ml/min) was calculated from steady-state insulin concentrations and insulin infusion rates:

$$\frac{\text{Insulin infusion (ng/min)}}{\text{Insulin concentration [Hyperinsulinemic] - [Basal] (ng/ml)}}$$

Tissue lipid levels

Tissues were homogenized in phosphate buffered saline (PBS) (~ 10% wet weight/vol), and samples were taken to measure protein content by the Lowry assay[16]. Lipid content was determined by extracting lipids using the Bligh and Dyer method[22] and by separating the lipids using high-performance thin-layer chromatography (HPTLC) on silica gel plates as described before[23], followed by TINA2.09 software analysis[24] (Raytest Isotopen meβgeräte, Straubenhardt, Germany).

Hepatic VLDL-TG Production

To determine the effect of apoC3-deficiency on the hepatic VLDL-TG production rate, animals that were fed the high-fat diet for 2 weeks were fasted for 4 h and anesthetized, followed by an i.v. injection of 10% Triton WR1339 (500 mg/kg body weight; Tyloxapol, Sigma Chemicals, Steinheim, Germany) to inhibit lipolysis and hepatic uptake of VLDL-TG. Blood samples were drawn at 0, 15, 30, 60, and 90 min after Triton injection and TG concentrations were determined in the plasma as described above.

Statistical analysis

The Mann-Whitney U test was used to determine differences between *apoc3*$^{-/-}$ and WT mice. The criterion for significance was set at $P < 0.05$. All data are presented as mean ± SD. Statistical analyses were performed using SPSS11.0 (SPSS, Chicago, IL, USA).

Results

High-fat feeding increased body weight in apoc3$^{-/-}$ mice as a result of an increase in body fat content

Male *apoc3*$^{-/-}$ and WT littermate mice were put on the high-fat diet for a period of 20 weeks. Already after 2 weeks, *apoc3*$^{-/-}$ mice showed a significant increase in body weight on high-fat diet compared with littermate controls, leading to a 22% higher body weight in *apoc3*$^{-/-}$ mice compared with WT mice at the end of the experiment (week 20), as shown in **Figure 1A**. A significant increase in body weight was also observed in female *apoc3*$^{-/-}$ mice compared with WT littermates, although less extreme (data not shown).

Food intake of male *apoc3*$^{-/-}$ and WT mice was comparable during the first 11 weeks. After 11 weeks until the end of the experiment the food intake of *apoc3*$^{-/-}$ mice was increased 5-15% compared with that of WT littermates (**Figure 1B**). The calculated feed efficiency (**Figure 1C**) was significantly increased in the *apoc3*$^{-/-}$ mice compared with WT littermates. The greatest difference in feed efficiency between the genotypes was seen at week 4 of high-fat feeding (0.048 ± 0.009 vs. 0.019 ± 0.007 g weight gain/g food consumed, for *apoc3*$^{-/-}$ and WT mice, $P < 0.05$). This difference between the groups gradually decreased towards the end of the experiment, although *apoc3*$^{-/-}$ mice still had a higher feed efficiency at 20 weeks of high-fat feeding compared with WT littermates (0.031 ± 0.006 vs. 0.019 ± 0.007 g weight gain/g food consumed, respectively, $P < 0.05$).

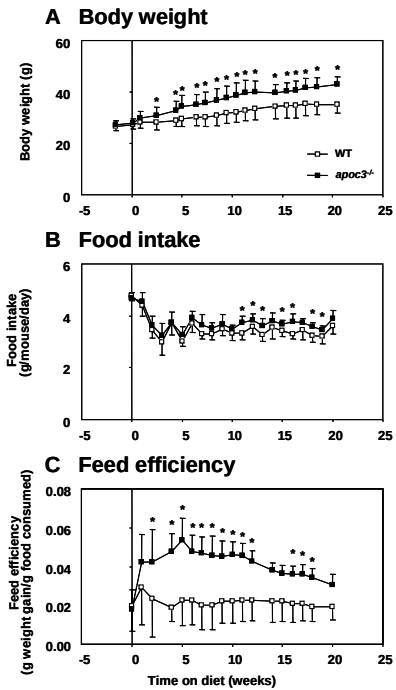

Figure 1. Growth (A), food intake (B) and feed efficiency (C) curves of *apoc3*$^{-/-}$ (closed squares) and WT (open squares) mice during a 20-week period of high-fat feeding

Body weight and food intake were measured periodically over the course of the experiment. Feed efficiency was calculated as the total weight gain divided by the total amount of food consumed during the experiment. Values represent the mean ± SD of 10 *apoc3*$^{-/-}$ and 13 WT mice. *P < 0.05, using nonparametric Mann-Whitney U tests

To investigate alterations in body composition, we analyzed mouse carcasses after 20 weeks of high-fat diet. Body weight, LBM, and the proportion of water, protein and lipid of *apoc3*$^{-/-}$ mice and WT littermates are shown in **Table 1**. Although body weight was ~ 7 g higher in *apoc3*$^{-/-}$ mice compared with WT mice, the LBM was comparable for both groups of mice. The absolute amount of body lipid in *apoc3*$^{-/-}$ mice was ~ 6 g higher compared with WT mice. No differences were found in protein content and amount of body water between *apoc3*$^{-/-}$ mice and WT littermates. Analysis of adipocyte size in the reproductive fat pads revealed that after 20 weeks of high-fat feeding, adipocytes of *apoc3*$^{-/-}$ mice and of WT mice are comparable in size (**Table 1**).

Table 1. Body mass composition and adipocyte size of *apoc3*$^{-/-}$ and WT mice after 20 weeks of high-fat feeding and an overnight fast

Genotype	Body Weight (g)	LBM (g)	Protein (g)	Water (g)	Lipid (g)	Adipocyte size (µm²)
WT	32.4 ± 5.5	23.9 ± 4.5	3.4 ± 0.4	17.9 ± 0.9	10.0 ± 1.8	6035 ± 761
apoc3$^{-/-}$	39.5 ± 3.4*	23.3 ± 3.3	3.9 ± 0.5	18.3 ± 0.8	16.2 ± 5.9*	5771 ± 413

Values represent the mean ± SD of 6 *apoc3*$^{-/-}$ and 10 WT mice. Lean body mass (LBM) and body total protein, water, and lipid content were determined as described in the materials and methods section. Reproductive fat pads were used for freeze sectioning, and subsequent staining and adipocyte size was measured as described.*P < 0.05, using nonparametric Mann-Whitney U tests

Apoc3$^{-/-}$ mice showed increased plasma TG-derived FA uptake by adipose tissue

To show that indeed the increased adipose tissue mass was due to increased LPL-dependent TG-derived FA uptake, we determined the tissue-specific uptake of FA derived from either plasma TG or albumin in several tissues of non-fasted, body weight-matched *apoc3*$^{-/-}$ and WT mice that were fed the high-fat diet for 2 weeks (**Figure 2**). The small difference in body weight after only 2 weeks of high-fat diet feeding ensured the availability of body weight-matched *apoc3*$^{-/-}$ and WT mice. We observed no differences in uptake of albumin-bound FA in liver, heart, muscle, and adipose tissue between *apoc3*$^{-/-}$ and WT littermates. Interestingly, TG-derived FA uptake was significantly increased in visceral, subcutaneous and reproductive fat pads from *apoc3*$^{-/-}$ mice compared with WT mice. No differences were found in TG-derived FA uptake in liver, heart, and skeletal muscle in *apoc3*$^{-/-}$ mice compared with littermates.

Apoc3$^{-/-}$ mice showed increased modulated plasma LPL activity

Because LPL-mediated TG-derived FA clearance was increased in WAT of *apoc3*$^{-/-}$ mice, we determined the total plasma and tissue LPL activity in body weight-matched *apoc3*$^{-/-}$

mice and littermate controls that were fed the high-fat diet for 1 week (plasma LPL) and 2 weeks (tissue LPL).

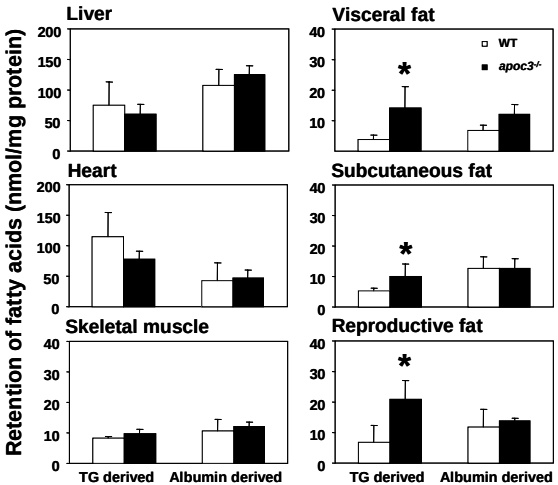

Figure 2. Retention of [^{3}H]TG and [^{14}C]FA label in tissues of *apoc3*$^{-/-}$ and WT mice
After 2 weeks on a high-fat diet, body weight-matched, fed, male *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice were infused for 2h with a solution containing [^{3}H]TG chylomicron-sized particles and albumin-bound [^{14}C]FA. Label content in lipids was measured and corrected for specific activities in plasma of [^{3}H]TG and [^{14}C]FA, respectively, in liver, heart, skeletal muscle tissue, and visceral, subcutaneous, and reproductive fat pads. Values represent the mean ± SD of n = 4 mice per group. *P < 0.05, using nonparametric Mann-Whitney U tests

Total post-heparin plasma LPL activity was similar in *apoc3*$^{-/-}$ and WT mice (7.0 ± 5.6 vs. 5.4 ± 3.2 µmol FFA/h/ml, respectively), demonstrating that the absence of apoC3 does not affect LPL expression. Likewise, tissue-specific LPL activity measured in liver, heart, skeletal muscle, and visceral, subcutaneous, and reproductive fat pads was not different between *apoc3*$^{-/-}$ and control mice (**Figure 3**). We next studied the LPL activity in post-heparin plasma in absence of excess heat-inactivated human plasma, and in presence of limited amounts of VLDL-like emulsion particles rather than excess solubilized TG.

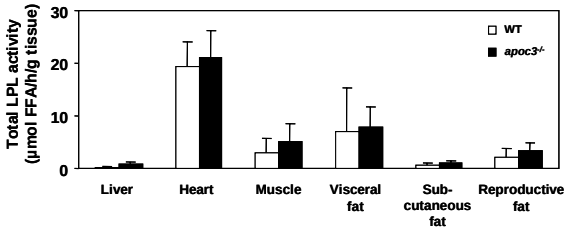

Figure 3. Tissue LPL activity levels in *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice
Fed body weight-matched *apoc3*$^{-/-}$ and WT mice were sacrificed, and liver, heart, skeletal muscle tissue, and visceral, subcutaneous, and reproductive fat pad samples were added to DMEM medium containing 2 U heparin. Supernatant was assayed with [^{3}H]triolein containing substrate mixture in absence or presence of 1 M NaCl to determine LPL activity based on generation of [^{3}H]oleate. Values represent the mean ± SD for n= 4 per group. *P < 0.05, using nonparametric Mann-Whitney U tests

Under these conditions, the LPL activity as modulated by endogenous mouse plasma factors (*e.g.*, apoC3) can be studied. Indeed, *apoc3*$^{-/-}$ mouse plasma showed 78% increased TG hydrolase activity compared with WT littermates judging from [^{3}H]oleate production (1.33 ± 0.20 vs. 0.75 ± 0.17 nmol oleate/ml/min, respectively, P < 0.05).

Collectively, these data clearly show that apoC3 modulates LPL activity by interfering with the interaction between LPL and TG-rich lipoproteins (*i.e.*, VLDL and chylomicrons), rather than by affecting total LPL levels.

Apoc3$^{-/-}$ mice had increased plasma glucose levels and strongly decreased whole-body insulin sensitivity

High-fat feeding induced increased total plasma cholesterol in both groups (**Table 2**). In *apoc3*$^{-/-}$ mice, plasma glucose, keton body, and leptin were also increased after 20 weeks high-fat compared with t=0 (chow diet). Plasma total cholesterol and FFA were comparable between the mice that were on chow and on the high-fat diet (**Table 2**). After 20 weeks of high-fat feeding plasma keton body and leptin levels were increased in *apoc3*$^{-/-}$ mice compared with littermates. Plasma TG levels were significantly lower in *apoc3*$^{-/-}$ mice compared with WT mice as reported earlier[12]. No significant differences were found with respect to plasma glucose and insulin levels in *apoc3*$^{-/-}$ versus WT animals before high-fat feeding (**Table 2**). At the end of the 20-week period of high-fat feeding, *apoc3*$^{-/-}$ mice showed significantly higher plasma glucose and slightly but not significantly increased plasma insulin levels compared with control littermates (**Table 2**).

Table 2. Plasma parameters determined in overnight-fasted *apoc3*$^{-/-}$ and WT mice at 0 and 20 weeks of high-fat diet exposure

	t=0 weeks		t=20 weeks	
	WT	*apoc3*$^{-/-}$	WT	*apoc3*$^{-/-}$
Total Cholesterol (mmol/l)	2.18 ± 0.87	2.09 ± 0.28	3.73 ± 1.09‡	3.36 ± 0.63‡
Triglycerides (mmol/l)	0.28 ± 0.09	0.15 ± 0.03*	0.29 ± 0.04	0.11 ± 0.02‡*
Free fatty acids (mmol/l)	1.08 ± 0.23	1.10 ± 0.25	1.07 ± 0.14	0.92 ± 0.15
Glucose (mmol/l)	4.65 ± 0.67	4.74 ± 0.46	5.69 ± 0.92	7.25 ± 1.21‡*
Keton body (mmol/l)	1.10 ± 0.31	1.21 ± 0.33	1.29 ± 0.19	1.75 ± 0.23‡*
Insulin (pmol/l)	80 ± 59	38 ± 20	161 ± 60	317 ± 155
Leptin (ng/ml)	4.68 ± 2.89	3.54 ± 2.41	6.41 ± 1.55	15.99 ± 5.11‡*

Values represent the mean ± SD of 7 *apoc3*$^{-/-}$ and 10 WT mice. Total cholesterol, triglycerides, free fatty acids, glucose, keton body, insulin, and leptin levels were determined in plasma obtained from the mice via tail-tip incision. *P < 0.05 vs. WT, ‡P < 0.05 vs. t=0, using nonparametric Mann-Whitney U tests

Insulin sensitivity was tested using hyperinsulinemic-euglycemic clamp analyses (**Table 3**). Under fasted conditions, plasma glucose levels were higher in *apoc3*$^{-/-}$ mice compared with WT littermates. During hyperinsulinemic conditions, no significant differences were observed in plasma glucose. Insulin levels were increased in *apoc3*$^{-/-}$ mice as compared with WT mice (**Table 3**). This increase in plasma insulin levels was explained by decreased insulin clearance in *apoc3*$^{-/-}$ mice (**Table 3**).

Table 3. Clamp conditions in *apoc3*$^{-/-}$ and WT mice at 20 weeks of high-fat feeding

Genotype	LBM (g)	Basal conditions		Hyperinsulinemic conditions		
		Glucose (mmol/l)	Insulin (pmol/l)	Glucose (mmol/l)	Insulin (pmol/l)	Insulin clearance (ml/min)
WT	23.9 ± 4.5	5.7 ± 0.9	161 ± 60	7.1 ± 1.2	234 ± 75	480 ± 133
apoc3$^{-/-}$	23.3 ± 3.3	7.3 ± 1.2*	317 ± 155	7.8 ± 0.8	524 ± 149*	110 ± 48*

Values represent the mean ± SD of n = 4 per group. Lean body mass (LBM) and concentrations of plasma glucose and insulin measured before (basal) and during the hyperinsulinemic-euglycemic clamp in *apoc3*$^{-/-}$ and WT mice. Insulin clearance was calculated using steady-state insulin concentrations and insulin infusion rates. Animals were fed high-fat diet for 20 weeks and were fasted overnight before the clamp experiment. *P < 0.05, using nonparametric Mann-Whitney U tests

The clamp results revealed that insulin-mediated whole-body glucose uptake was significantly lower in *apoc3*$^{-/-}$ mice compared with littermate controls (15 ± 5 vs. 35 ± 9%, respectively, P < 0.05; **Figure 4A**). Moreover, the endogenous glucose production was only slightly suppressed under hyperinsulinemic conditions in *apoc3*$^{-/-}$ mice as compared with WT mice (7 ± 1 vs. 27 ± 10% respectively, P < 0.05; **Figure 4B**).

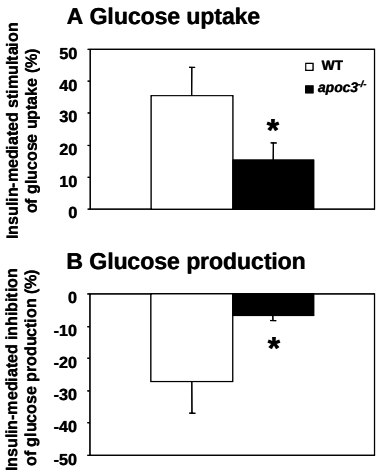

Figure 4. Hyperinsulinemic-euglycemic clamp results of *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice after 20 weeks of high-fat feeding

Whole-body insulin-mediated glucose uptake and insulin-mediated suppression of endogenous glucose production were calculated on the basis of lean body mass. The insulin-mediated stimulation of whole-body glucose uptake (A) was expressed as the ratio between insulin-induced whole-body glucose disposal and hyperinsulinemic plasma insulin concentration. Insulin-mediated inhibition of endogenous glucose production (B) was expressed as ratio between insulin-mediated inhibition of endogenous glucose production and hyperinsulinemic plasma insulin concentration. Values represent the mean ± SD for n= 4 per group.
*P < 0.05, using nonparametric Mann-Whitney U tests

Because *apoc3*$^{-/-}$ mice were insulin resistant compared with WT littermates, we analyzed liver and muscle lipid content. In *apoc3*$^{-/-}$ mice, hepatic lipid content was significantly higher compared with that in WT littermates (**Figure 5A**). Skeletal and cardiac muscle TG content did not significantly differ between both mouse groups.

Apoc3$^{-/-}$ mice showed unaltered VLDL-TG production rates

Since increased liver TG levels were observed in *apoc3*$^{-/-}$ mice, the ability of the liver to secrete VLDL-TG was investigated. Mice that were fed the high-fat diet for 2 weeks were

fasted for 4 h and received an injection of Triton WR1339. The accumulation of endogenous VLDL-TG in plasma was determined over time (**Figure 5B**). The VLDL-TG production rate as determined from the slope of the curve was unchanged in *apoc3*$^{-/-}$ mice compared with WT littermates (3.89 ± 0.54 vs. 3.73 ± 0.58 mmol TG/h, respectively). Therefore, the hepatic TG accumulation as observed in *apoc3*$^{-/-}$ mice is not caused by reduced VLDL secretion.

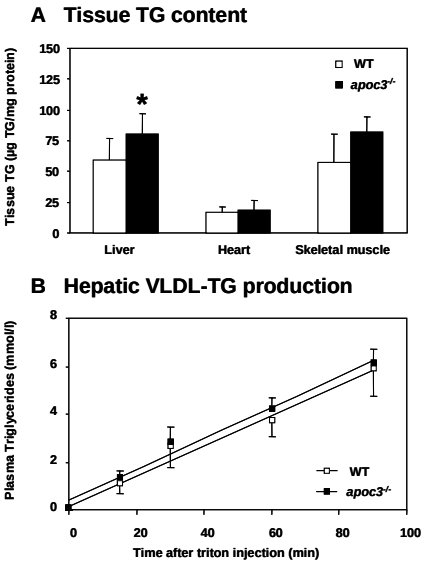

Figure 5. Tissue TG levels (A) and hepatic VLDL-TG production (B)
Hepatic, cardiac, and skeletal muscle TG levels were determined in *apoc3*$^{-/-}$ (closed bars) and WT (open bars) mice after 20 weeks of high-fat feeding. Lipids were extracted from tissue homogenates by the Bligh and Dyer method and subsequently separated on thin-layer silica gel plates (A). Hepatic VLDL-TG production was assessed by Triton WR1339 injection and plasma TG analyses in *apoc3*$^{-/-}$ (closed squares) and WT (open squares) mice after 2 weeks of high-fat feeding and a 4 h fast (B). Values represent the mean ± SD for n=5 per group. *P < 0.05, using nonparametric Mann-Whitney U tests

Discussion

LPL plays an important role in the delivery of FA into peripheral tissues. Several mouse studies indicate that decreased LPL activity in adipose tissue decreases the propensity to develop obesity[6-9,25]. However, it is unclear whether the opposite is true as well, *i.e.*, whether activation of LPL leads to an enhanced susceptibility to diet-induced obesity and associated insulin resistance. Adipose tissue-specific LPL overexpression leads to only a slight increase in fat pad weight[10]. However, this relatively minor effect can result from an altered muscle versus adipose tissue LPL activity ratio and/or the presence of natural regulators of *in vivo* LPL activity[11]. ApoC3 is a known inhibitor of LPL activity, and disruption of the gene in mice increases TG clearance[12,14].

In this study, we investigated the possible effects of enhanced whole-body LPL activity (resulting from deletion of the LPL inhibitor apoC3) on development of obesity and insulin resistance making use of the apoC3 knockout mouse (*apoc3*$^{-/-}$). The data show that *apoc3*$^{-/-}$ mice developed a higher adipose tissue mass during high-fat feeding compared with WT littermates. The higher adipose tissue mass in these mice was caused by a higher uptake of FA derived from plasma TG, leading to insulin resistance of whole-body glucose uptake and production. These data indicate that increased LPL activity, resulting from the absence of apoC3-dependent attenuation of the LPL activity, indeed profoundly increases the propensity to develop obesity and insulin resistance on a high-fat diet.

Apoc3$^{-/-}$ and WT littermates were fed a high-fat, high-caloric diet. Within 2 weeks of high-fat feeding, male *apoc3*$^{-/-}$ mice developed a significantly higher body weight. Body composition analysis, after 20 weeks of high-fat feeding, revealed that the increase in body weight in *apoc3*$^{-/-}$ mice, compared with WT littermates was completely explained by the increase in body fat mass. LBM, body protein, and body water were not different between the two mouse groups (**Table 1**). Up to a period of 11 weeks, no difference in daily food intake was observed between *apoc3*$^{-/-}$ and WT mice, although body weight already differed after a relative short period of two weeks. This suggests that the increase in body weight and fat mass is primarily due to the enhanced LPL activity and not due to a higher food intake. As a consequence, feed efficiency, as expressed as total weight gained per week divided by the total amount of food consumed per week, was clearly higher in *apoc3*$^{-/-}$ mice (**Figure 1C**). The unchanged adipocyte cell size found in *apoc3*$^{-/-}$ mice after 20 weeks of a high-fat diet suggests that more adipocytes should be present (**Table 1**). One might in fact expect the opposite, and we can only speculate on the effects of increased FA flux to adipose tissue and adipocyte differentiation. One clue seems to come from studies using peroxisome proliferator-activated receptor (PPAR) γ agonists. Treatment with PPARγ agonists leads to increased adipose tissue FA flux and accumulation, interestingly with increased adipose tissue LPL expression[26]. Consequently, more adipocytes occur with similar or even smaller size[27].

The observation that apoC3-deficiency has no effect on the uptake of albumin-bound FA by the liver, heart, muscle or adipose tissue (**Figure 2**) indicates that the effect of apoC3 on FA uptake in the respective tissues occurs solely via the modulation of LPL activity. We showed that TG-derived FA uptake in the various tissues is dependent on LPL activity in those tissues[18]. In the fasted state, skeletal muscle is relatively enriched in LPL, whereas adipose tissue is relatively rich in LPL in the fed state[2,6,28-31]. Thus, in the fasted state relatively more TG-derived FA is transported to the muscle, whereas in the fed state, transport of TG-derived FA to the adipose tissue is more pronounced. In the current study, we show that apoC3-deficiency does lead to increased uptake of TG-derived FA by adipose tissue. This implicates, therefore, that the effect of apoC3 on LPL-mediated FA uptake is relevant only in the (high-fat) fed condition. Despite that LPL activity is the highest in the heart (**Figure 3**), we did not observe an effect of apoC3-deficiency on TG-derived FFA uptake by this organ (**Figure 2**). These data are in agreement with our previous observations that either reduced expression of LPL, as seen in VLDLreceptor-deficient mice[32], or increased LPL activity by overexpression of the LPL activator apoA5[33] did not modulate the uptake of TG-derived FFA by the heart. Apparently, the cardiac uptake of TG-derived FFA is irrespective of LPL modulation, as related to its continuous need of vast amounts of fuel.

Apolipoproteins can alter LPL activity by interfering with the (physical) interaction between LPL and TG-rich lipoproteins such as VLDL and chylomicrons, without regulating the LPL protein and/or gene expression in tissues. To exclude an effect of apoC3-deficiency on total LPL levels, we analyzed post-heparin plasma and tissue-specific LPL levels in *apoc3*$^{-/-}$ and WT littermates. In this assay, plasma and tissue samples are diluted into a very large pool of solubilized TG and heat-inactivated human plasma, thereby abolishing the interaction of LPL with potential modulators of LPL activity (*e.g.*, apoC3) contained in the samples. Indeed, no differences were observed in total plasma or tissue LPL activity between *apoc3*$^{-/-}$ and littermate controls (**Figure 3**). However, under conditions in which mouse plasma is added to limited amounts of VLDL-like particles in absence of excess human plasma, the TG hydrolase activity of plasma LPL from *apoc3*$^{-/-}$ mice was indeed 78% higher than that of WT mice. Our data thus clearly shows that the absence of apoC3 from lipoprotein particles is responsible for enhancing LPL activity *in vitro* as evident from increased TG-derived FFA liberation and *in vivo* as evident from increased TG-derived FA flux into adipose tissue of *apoc3*$^{-/-}$ mice. Furthermore, the plasma TG half-life was only 6 ± 3 min in *apoc3*$^{-/-}$ mice vs. 22 ± 6 min for littermate controls. This clearly demonstrates increased LPL-mediated TG clearance in *apoc3*$^{-/-}$ mice, which is in agreement with previous reports[12,14]. Therefore, it would be interesting to investigate whether human apoC3-overexpressing mice could be protected from diet-induced obesity.

Although we cannot exclude that altered adipose tissue lipid handling, *e.g.*, hormone sensitive lipase (HSL) activity, leads to a similar adipose tissue phenotype, the

absence of HSL leads to decreased adipose tissue mass instead of an increase, despite increased adipose tissue LPL activity[34].

Using hyperinsulinemic-euglycemic clamp analyses, we observed that the insulin-mediated stimulation of whole-body glucose uptake was strongly impaired in *apoc3*$^{-/-}$ mice as compared with WT littermates (**Figure 4A**), indicating decreased insulin sensitivity in *apoc3*$^{-/-}$ mice. Insulin sensitivity was found to be negatively correlated with muscle TG content in several studies[3-5]. However, we could not observe a statistically significant increase in muscle TG content in *apoc3*$^{-/-}$ mice when compared with WT littermates (**Figure 5A**), in line with the observation that there was no increase in muscle uptake of albumin-derived FA or TG-derived FA in fed mice. Presumably, apoC3-deficiency leads to insulin resistance as a result of increased adipose tissue mass. For instance, apoC3-deficiency might affect secretion of various endocrine factors by adipose tissue, such as leptin, resistin, and adiponectin. These hormones are known to affect insulin sensitivity and are correlated with adipose tissue mass[35-38]. In this study, we found that *apoc3*$^{-/-}$ mice have increased plasma leptin levels (**Table 2**), in accordance with the increase in adipose tissue mass (**Table 1**). However, it is likely that the hyperleptinemia observed in these mice is the consequence rather than the cause of insulin resistance, as has been observed earlier in humans[35,39-42].

Suppression of the endogenous glucose production (largest contribution by liver) by insulin in *apoc3*$^{-/-}$ mice on a high-fat diet was absent compared with WT littermates (**Figure 4B**). In the liver, TG content is inversely correlated with hepatic insulin sensitivity[4]. Indeed, the decreased hepatic insulin sensitivity in *apoc3*$^{-/-}$ mice coincides with an increased hepatic TG content in these mice (**Figure 5A**). The higher TG levels in livers of *apoc3*$^{-/-}$ mice seem to be unrelated to increased hepatic total FA uptake in these mice (**Figure 2**). Also, the hepatic VLDL-TG production was not affected in *apoc3*$^{-/-}$ mice on high-fat diet (**Figure 5B**), which is similar to our previous observations on chow diet[14]. Alterations in intrahepatic FA metabolism and/or other factors, *e.g.*, adipokines, might in fact be fundamental to the observed mild hepatic steatosis in these high-fat-fed *apoc3*$^{-/-}$ mice.

In conclusion, enhanced LPL activity in mice as a result of absence of the natural inhibitor of LPL, apoC3, leads to enhanced susceptibility to diet-induced obesity. This enhanced obesity seems to be fully explained by the increased TG-derived FA flux towards adipose tissue rather than by differences in food intake. The increased obesity and probably the altered FA partitioning result in severe development of peripheral and hepatic insulin resistance in *apoc3*$^{-/-}$ mice. Absence of hepatic production of apoC3 decreases plasma TG levels by enhancing adipose tissue TG-derived FA uptake. Since TG-rich lipoproteins are atherogenic, the decrease in plasma TG levels might be beneficial in the prevention of cardiovascular diseases. Adversely, the higher *in vivo* LPL activity in turn leads to higher susceptibility to diet-induced obesity and insulin resistance. Intriguingly, our results show that the liver can modulate plasma lipid levels and at the same time fat mass and insulin sensitivity through production of just one protein, apoC3. It warrants

further investigation, whether in humans, apoC3 is a potential therapeutic target for treatment of obesity and insulin resistance, and at the same time for prevention of cardiovascular risk.

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