Fatty acid metabolism and metabolic inflammation : two important players in the development of insulin resistance

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3 ASPIRIN REDUCES HYPERTRIGLYCERIDEMIA BY LOWERING VLDL-TRIGLYCERIDE PRODUCTION IN MICE FED A HIGH-FAT DIET

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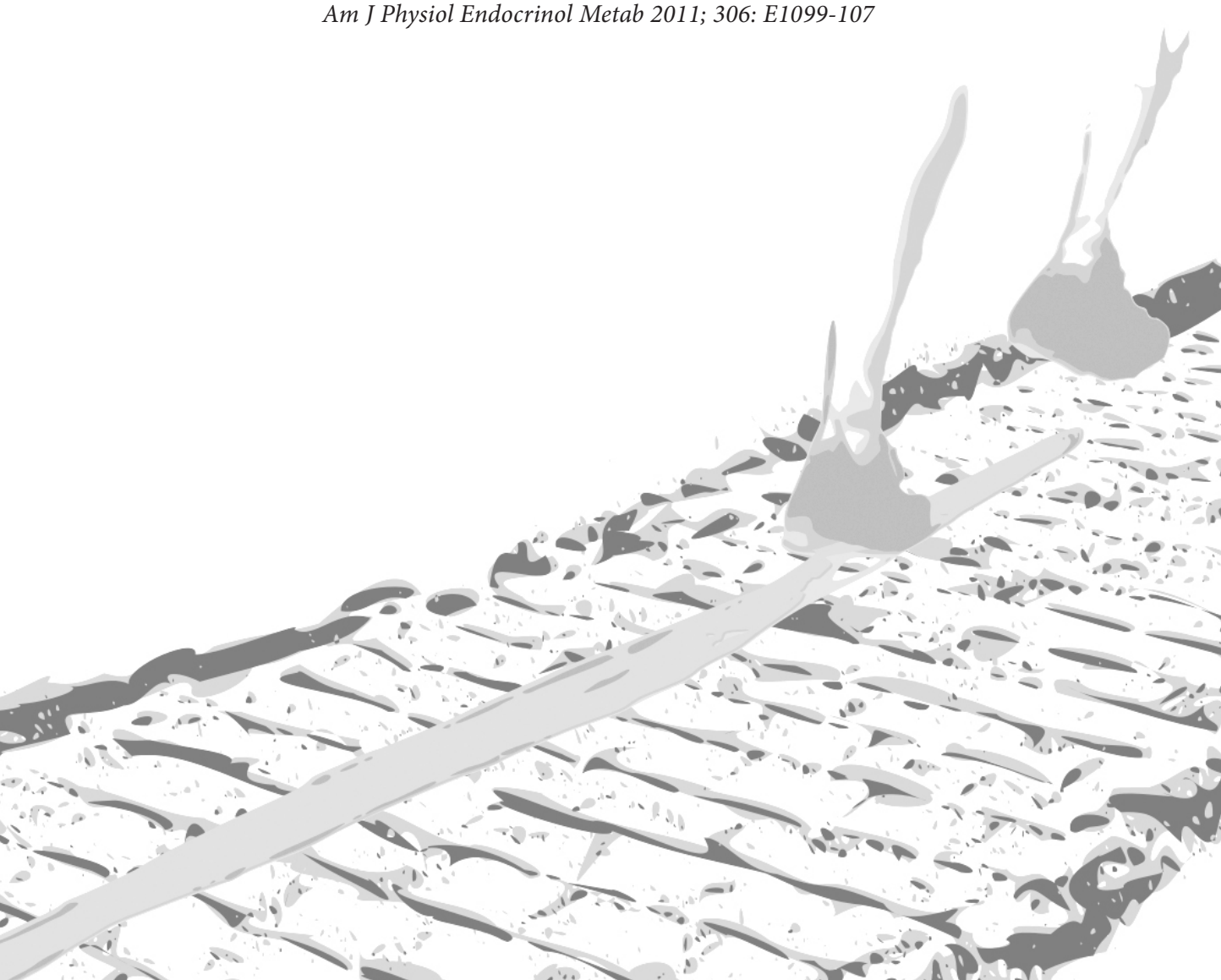

ABSTRACT

Systemic inflammation is strongly involved in the pathophysiology of the metabolic syndrome, a cluster of metabolic risk factors including hypertriglyceridemia. Aspirin treatment lowers inflammation via inhibition of NF-κB activity, but also reduces hypertriglyceridemia in humans. The aim of this study was to investigate the mechanism by which aspirin improves hypertriglyceridemia. Human apolipoprotein CI (apoCI)-expressing mice (*APOC1* mice), an animal model with elevated plasma triglyceride (TG) levels, as well as normolipidemic wild-type (WT) mice were fed a high-fat diet (HFD) and treated with aspirin. Aspirin treatment reduced hepatic NF-κB activity in HFD-fed *APOC1* and WT mice and in addition, aspirin decreased plasma TG levels (-32%; $p<0.05$) in hypertriglyceridemic *APOC1* mice. This TG-lowering effect could not be explained by enhanced VLDL-TG clearance, but aspirin selectively reduced hepatic production of VLDL-TG in both *APOC1* (-28%; $p<0.05$) and WT mice (-33%; $p<0.05$) without affecting VLDL-apoB production. Aspirin did not alter hepatic expression of genes involved in FA oxidation, lipogenesis and VLDL production, but decreased the incorporation of plasma-derived FA by the liver into VLDL-TG (-24%; $p<0.05$), which was independent of hepatic expression of genes involved in FA uptake and transport. We conclude that aspirin improves hypertriglyceridemia by decreasing VLDL-TG production without affecting VLDL particle production. Therefore, the inhibition of inflammatory pathways by aspirin could be an interesting target for the treatment of hypertriglyceridemia.

3.1 INTRODUCTION

The metabolic syndrome is a clustering of metabolic risk factors, including steatosis, insulin resistance and hyperlipidemia, predisposing to the early onset of atherosclerosis and cardiovascular morbidity and mortality. It is well established that the metabolic syndrome is associated with increased systemic inflammation.[1] Moreover, accumulating evidence suggests a strong involvement of systemic inflammation in the pathogenesis of components of the metabolic syndrome.[2] Hypertriglyceridemia, one of the components of the metabolic syndrome and an important risk factor for the development of cardiovascular disease, is strongly associated with increased inflammation.[3] Early studies show that sepsis, infection and inflammation are accompanied by hypertriglyceridemia.[4-6] More recent studies show that administration of LPS induces hypertriglyceridemia.[7,8] In addition, multiple cytokines, such as IL-6 and TNF-α, increase serum triglyceride (TG) levels.[9,10] Inhibition of inflammation might therefore be an attractive therapeutic target in patients with HFD-induced hypertriglyceridemia.

Non-steroidal anti-inflammatory drugs (NSAID) such as aspirin are known to inhibit the enzyme cyclooxygenase (COX). In addition, high doses of aspirin lower activation of inflammatory pathways by inhibition of the NF-κB pathway,[11,12] which plays a crucial role in the inflammation-mediated pathogenesis of the metabolic syndrome.[2] Interestingly, aspirin treatment diminishes hypertriglyceridemia in both obese rodents[13] and patients with type 2 diabetes mellitus.[14] However, the mechanism underlying this TG-lowering effect still has to be elucidated.

We previously found that human apolipoprotein CI (apoCI)-expressing (*APOC1*) mice have increased plasma TG, by a diminished clearance of VLDL particles through apoCI-mediated inhibition of lipoprotein lipase (LPL),[15] which is aggravated by high-fat diet (HFD) feeding (unpublished observation by I.O.C.M. Vroegrijk et al). Therefore, we reasoned that the HFD-fed *APOC1* transgenic mouse is an appropriate model to study the effectiveness of treatments targeting HFD-induced hypertriglyceridemia.

The aim of this study was to investigate the mechanism by which aspirin reverses HFD-induced hypertriglyceridemia. Therefore, we studied the effect of aspirin on VLDL-TG metabolism *in vivo* in HFD-fed hypertriglyceridemic *APOC1* mice as well as in C57Bl/6 wild-type (WT) mice, to extend any findings towards the mouse model that is most widely used for evaluation of treatments for the metabolic syndrome. Our results show that a high dose of aspirin improves hypertriglyceridemia as a consequence of a clear reduction of hepatic VLDL-TG production, mediated by a diminished hepatic incorporation of plasma-derived FA into VLDL-TG.

3.2 MATERIALS AND METHODS

Animals, diet and aspirin treatment

Transgenic *APOC1* mice with hemizygous expression of the human *APOC1* gene were generated as previously described and backcrossed at least 10 times to the C57Bl/6 background. The *APOC1* mouse model develops hypertriglyceridemia mainly due to a diminished clearance of VLDL particles through apoCI-mediated inhibition of lipoprotein lipase (LPL).[16,17] Male *APOC1* mice and WT mice (also on a C57Bl/6 background) were housed under standard conditions with a 12-hour light-dark cycle. At the age of 10–12 weeks, mice received a HFD (45 energy% derived from palm oil; D12451, Research Diet Services, Wijk bij Duurstede, The Netherlands) for a period of 6 weeks. Aspirin treatment (120 mg/kg/day in drinking water; pH 6.4) was given during the last 4 weeks on HFD and mice were subsequently used for experiments after an overnight fast at 9:00 am. Control mice received the same drinking water of pH 6.4 without the addition of aspirin. Mice were allowed free access to food and water. Animal experiments were approved by the institutional ethical committee on animal care and experimentation.

Liver NF-κB activation

Since the most common form of NF-κB is the p50/p65 heterodimer,[18] the activity of both the p50 and p65 subunits in liver tissue was determined using electrophoretic mobility shift assay (EMSA).[19] Shortly, tissues were homogenized in ice-cold Passive Lysis Buffer (Promega, Madison, WI) and centrifuged (14,000 rpm; 20 min; 4°C). Protein content of the supernatant was determined using the BCA protein assay kit (Pierce, Rockford, IL). For the EMSA, the gel shift assay system was purchased from Promega. The probe was end-labeled using T4 polynucleotide kinase and [^{32}P]ATP and purified on a Microspin G-25 column (GE Healthcare, Piscataway, NJ). For each sample, 50 μg protein was incubated with labelled probe and binding buffer (Promega) for 20 min at RT. Specific competition was done by adding unlabeled NF-κB binding probe to the reaction. The mixtures were run on 4.5% polyacrylamide gel electrophoresis in 0.5x Tris/Borate/EDTA (TBE) buffer. The gel was vacuum-dried and exposed to radiographic film.

Plasma parameters

Blood was collected from the tail vein into chilled paraoxon (Sigma, St Louis, MO)-coated capillaries to prevent ongoing lipolysis.[20] Capillaries were placed on ice, centrifuged and plasma was assayed for TG, total cholesterol (TC) and phospholipids (PL) using commercially available enzymatic kits from Roche Molecular Biochemicals (Indianapolis, IN) in 96-wells plates (Greiner Bio-One). Free fatty acids (FFA) were measured using NEFA-C kit from Wako Diagnostics (Instruchemie, Delfzijl, the Netherlands). ß-hydroxybutyrate (ß-HB) was determined using the enzymatic ß-HB Assay kit from BioVision (Mountain View, CA, USA)

Liver lipids

Lipids were extracted from livers according to a modified protocol from Bligh and Dyer.[21] Shortly, a small piece of liver was homogenized in ice-cold methanol. After centrifugation, lipids were extracted by addition of 1800 µL CH_3OH:$CHCl_3$ (3:1 v/v) to 45 µL homogenate. The $CHCl_3$ phase was dried and dissolved in 2% Triton X-100. Hepatic TG and TC concentrations were measured using commercial kits as described earlier. Liver lipids were expressed per mg protein, which was determined using the BCA protein assay kit (Pierce).

Generation of VLDL-like emulsion particles

VLDL-like TG-rich emulsion particles were prepared and characterized as described previously.[22,23] Lipids (100 mg) at a weight ratio of triolein: egg yolk phosphatidylcholine: lysophosphatidylcholine: cholesteryl oleate: cholesterol 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) were sonicated at 10 µm output using a Soniprep 150 (MSE Scientific Instruments, Crawley, UK). Density gradient ultracentrifugation was used to obtain 80 nm-sized emulsion particles, which were used for subsequent experiments. TG content of the emulsions was measured as described above. Emulsions were stored at 4°C under argon and used within 7 days.

In vivo clearance of VLDL-like emulsion particles

To study *in vivo* clearance of the VLDL-like emulsion particles, overnight fasted mice were anesthetized by intraperitoneal injection of acepromazine (6.25 mg/kg Neurotranq, Alfasan International BV, Weesp, The Netherlands), midazolam (6.25 mg/kg Dormicum, Roche Diagnostics, Mijdrecht, The Netherlands), and fentanyl (0.31 mg/kg Janssen Pharmaceuticals, Tilburg, The Netherlands). Mice were injected (t=0) via the tail vein with 200 µL of [^{3}H]TO-labeled emulsion particles at a dose of 100 µg of TG per mouse. Blood samples were taken from the tail vein at 1, 2, 5, 10 and 15 minutes after injection and plasma ^{3}H-activity was counted. Plasma volumes were calculated as 0.04706 x body weight (grams) as determined from ^{125}I-BSA clearance studies as described previously.[24] After taking the last blood sample, the liver, heart, spleen, muscle and white adipose tissue (*i.e.* gonadal, subcutaneous and visceral) were collected. Organs were dissolved overnight at 60°C in Tissue Solubilizer (Amersham Biosciences, Rosendaal, The Netherlands) and ^{3}H-activity was counted. Uptake of [^{3}H]TO-derived radioactivity by the organs was calculated from the ^{3}H activity in each organ divided by plasma-specific activity of [^{3}H]TG and expressed per mg wet tissue weight.

Hepatic VLDL-TG and VLDL-apoB production

To measure VLDL production *in vivo*, mice were fasted overnight as described above. Mice were injected intravenously with Tran^{35}S label (150 µCi/mouse; MP Biomedicals, Eindhoven, The Netherlands) to label newly produced apoB. After 30 minutes, at t=0 min, Triton WR-1339 (Sigma-Aldrich) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Blood samples were drawn before

(t=0) and 15, 30, 60 and 90 min after injection and used for determination of plasma TG concentration as described above. After 120 min, mice were exsanguinated via the retro-orbital plexus. VLDL was isolated from serum after density gradient ultracentrifugation at d<1.006 g/mL by aspiration[25] and counted for incorporated ^{35}S-activity.

Hepatic gene expression analysis

Total RNA was extracted from liver tissues using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The quality of each mRNA sample was examined by lab-on-a-chip technology using Experion Stdsens analysis kit (Biorad, Hercules, CA). 1 μg of total RNA was reverse-transcribed with iScript cDNA synthesis kit (Bio-Rad) and obtained cDNA was purified with Nucleospin Extract II kit (Macherey-Nagel). Real-Time PCR was carried out on the IQ5 PCR machine (Biorad) using the Sensimix SYBR Green RT-PCR mix (Quantace, London, UK). mRNA levels were normalized to mRNA levels of cyclophilin (*Cyclo*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*). Primer sequences are listed in table 1.

Contribution of plasma FA to VLDL-TG production

To measure the contribution of plasma derived FA to the VLDL-TG production *in vivo*, mice were fasted overnight as described above. Mice received a continuous i.v. infusion of

Table 1. Primers used for quantitative real-time PCR analysis.

Gene	Forward primer	Reverse primer
Acox1	TATGGGATCAGCCAGAAAGG	ACAGAGCCAAGGGTCACATC
Apob	GCCCATTGTGGACAAGTTGATC	CCAGGACTTGGAGGTCTTGGA
Cd36	GCAAAGAACAGCAGCAAAATC	CAGTGAAGGCTCAAAGATGG
Cpt1a	GAGACTTCCAACGCATGACA	ATGGGTTGGGGTGATGTAGA
Cyclo	CAAATGCTGGACCAAACACAA	GCCATCCAGCCATTCAGTCT
Dgat1	TCCGTCCAGGGTGGTAGTG	TGAACAAAGAATCTTGCAGACGA
Fasn	TCCTGGGAGGAATGTAAACAGC	CACAAATTCATTCACTGCAGCC
Fabp1	GAGGAGTGCGAACTGGAGAC	GTAGACAATGTCGCCCAATG
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Ppara	ATGCCAGTACTGCCGTTTTC	GGCCTTGACCTTGTTCATGT
Slc27a2	ATCGCCTATGGTATGGGACA	ACTGGCTGGCTGAGAATTTG
Slc27a4	GCTTACTCCACGGCATGACT	GTGGCTGGTTCAGGAGGTAG
Slc27a5	ATGCAGAGCTGATGATGTGG	ATCACTGTTACGCCATGCTG
Srebf1	GGAGCCATGGATTGCACATT	CCTGTCTCACCCCCAGCATA

Acox1, acyl-Coenzyme A oxidase 1, palmitoyl; *Apob*, apolipoprotein B; *Cd36,* fatty acid translocase; *Cpt1a,* carnitine palmitoyltransferase 1a; *Dgat1*, diglyceride acyltransferase 1; *Fabp1*, fatty acid binding protein 1; *Fasn*, fatty acid synthase; *Mttp*, microsomal triglyceride transfer protein; *Ppara*, peroxisome proliferative activated receptor alpha; *Slc27a2*, fatty acid transport protein 2; *Slc27a4*, fatty acid transport protein 4; *Slc27a5*, fatty acid transport protein 5; *Srebf1*, sterol-regulatory element binding protein.

^{3}H-labeled FA ([9,10(n)-^{3}H] palmitic acid in PBS with 2% bovine serum albumin) at a rate of 100μL/h (1.6 μCi/h). After 2 hours of ^{3}H-labeled FA infusion a blood sample was taken (t=0 min), and Triton WR-1339 (Sigma-Aldrich) was injected intravenously (0.5 mg/g body weight, 10% solution in PBS) to block serum VLDL clearance. Additional blood samples were drawn 15, 30, 60 and 90 min after injection and used for determination of ^{3}H activity in the TG fraction. Lipids were extracted by adding 10 μL plasma to 3.25 mL extraction fluid (heptane/methanol/chloroform; 100:128:137 (*v/v/v*)). ^{3}H-TG were subsequently separated from ^{3}H-FA; 1mL potassium carbonate (0.1M K_2CO_3, pH 10.5) was added followed by vortexing and centrifugation (3600 rpm; 15 min), leading to an upper alkaline-methanol-aqueous phase containing saponified ^{3}H-FA and a lower chloroform-organic phase containing ^{3}H-TG.[26] A fraction (0.5 mL) of the total aqueous phase (2.45 mL) was counted for ^{3}H in scintillation fluid. The amount of ^{3}H-TG in each sample was calculated by distracting total ^{3}H-FA activity from total ^{3}H activity.

Statistical analysis

Data are presented as means ± SD. Statistical differences were calculated using the Mann-Whitney test for two independent samples with SPSS 16.0 (SPSS Inc, Chicago, IL). P<0.05 was regarded statistically significant.

3.3 RESULTS

Aspirin reduces hepatic NF-κB activation

To verify that aspirin inhibits hepatic NF-κB activity, the activities of the NF-κB subunits p50 and p65 were measured in livers of *APOC1* and WT mice fed a HFD and treated with or without aspirin using a gel shift assay (Fig. 1). Aspirin indeed reduced the activity of both p50 (-69%; P<0.05) and p65 (-48%; P<0.05) in *APOC1* mice (Fig. 1A) and the activity of p50 in WT mice (-72%; P<0.05), while the reduction in the activity of p65 did not reach statistical significance (P=0.13) (Fig. 1B).

Aspirin lowers plasma triglyceride and cholesterol levels in HFD-fed *APOC1* mice

To examine whether aspirin could reduce hypertriglyceridemia in *APOC1* mice, hyperlipidemic *APOC1* mice were fed a HFD for 6 weeks and treated with or without aspirin and plasma lipids were determined (Fig. 2). Treatment of mice with aspirin reduced plasma TG levels by -32% (3.94 ± 0.15 to 2.67 ± 0.59 mmol/L; P<0.05; Fig. 2A) and plasma TC levels by -33% (4.09 ± 0.52 to 2.76 ± 0.90 mmol/L; P<0.05; Fig. 2B). Aspirin treatment did not affect plasma PL levels (Fig. 2C) and FFA levels (Fig. 2D). The reduction in plasma TG and TC levels was not caused by a reduction in body weight, since aspirin did not affect body weight in *APOC1* mice (control: 30.5 ± 2.1 g; aspirin: 28.9 ± 3.0 g). In WT mice fed a HFD

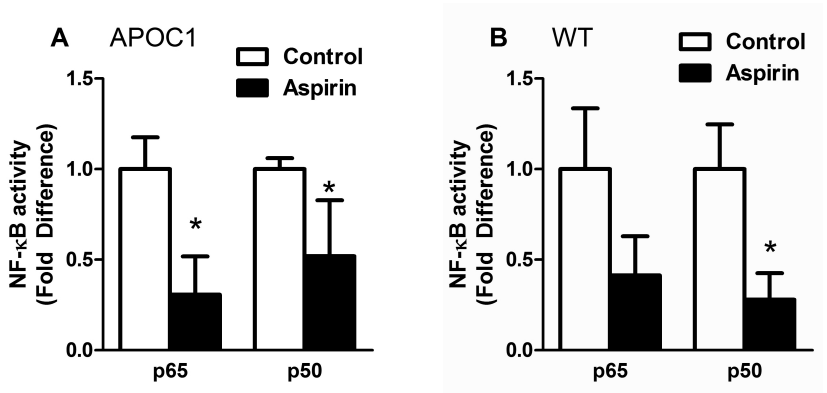

Figure 1. Aspirin reduces hepatic NF-κB activation. *APOC1* and WT mice were fed a HFD for 6 weeks and treated without or with aspirin. Mice were sacrificed after an overnight fast and hepatic NF-κB activity was measured by electrophoretic mobility shift assay in liver tissue of *APOC1 (A)* and WT (B) mice treated without (open bars) or with (closed bars) aspirin. Activities of subunits p50 and p65 were measured. Values are means ± SD (n=3-4). *P<0.05

for 6 weeks, aspirin did not affect plasma TG, TC, PL or FFA levels (Fig. 2E-H). In addition, aspirin did not affect body weight in WT mice (control: 30.3 ± 2.1 g; aspirin: 30.8 ± 1.9 g)

Aspirin attenuates VLDL-like emulsion particle-TG clearance in HFD-fed *APOC1*, but not WT mice

A reduction in fasted plasma TG levels can be explained by an increase in VLDL-TG clearance and/or a decrease in hepatic VLDL-TG production. To determine whether aspirin enhances the clearance of VLDL-TG, the plasma clearance and organ distribution of [^{3}H]-TO-labeled TG-rich VLDL-like emulsion particles was evaluated in aspirin and control treated hypertriglyceridemic *APOC1* mice (Fig. 3). Unexpectedly, aspirin inhibited, rather than enhanced, serum clearance of [^{3}H]TO (t ½ = 15.9 ± 6.6 *vs* 5.6 ± 2.6 min) (Fig. 3A) in *APOC1* mice. This reduction in [^{3}H]TO clearance upon aspirin was reflected by reduced uptake of [^{3}H]TO-derived radioactivity by the liver by -60% (123 ± 1 *vs* 308 ± 75 nmol/g; P<0.05), by skeletal muscle by -66% (11 ± 2 *vs* 31 ± 15 nmol/g; P<0.05) and by white adipose tissue (WAT), which reached statistical significance for gonadal WAT (12 ± 3 *vs* 44 ± 22 nmol/g; P<0.05) (Fig. 3B). Apparently, aspirin reduces rather than enhances TG clearance in *APOC1* mice and can, therefore, not explain the aspirin-induced reduction in VLDL-TG. In WT mice fed a HFD for 6 weeks, aspirin did not affect plasma clearance of [^{3}H]TO (Fig. 3C) or organ specific uptake of [^{3}H]TO-derived radioactivity (Fig. 3D) in WT mice. Apparently, the decreasing effect of aspirin on TG clearance may be specific for *APOC1* mice.

Aspirin lowers VLDL-TG production in HFD-fed *APOC1* and WT mice

Because the decrease in plasma TG levels in *APOC1* mice upon aspirin treatment was not caused by increased TG clearance, we investigated whether the decreased TG levels could be explained by diminished hepatic VLDL-TG production in *APOC1* mice. The rate of hepatic VLDL-TG production was measured by determining plasma TG levels after intravenous

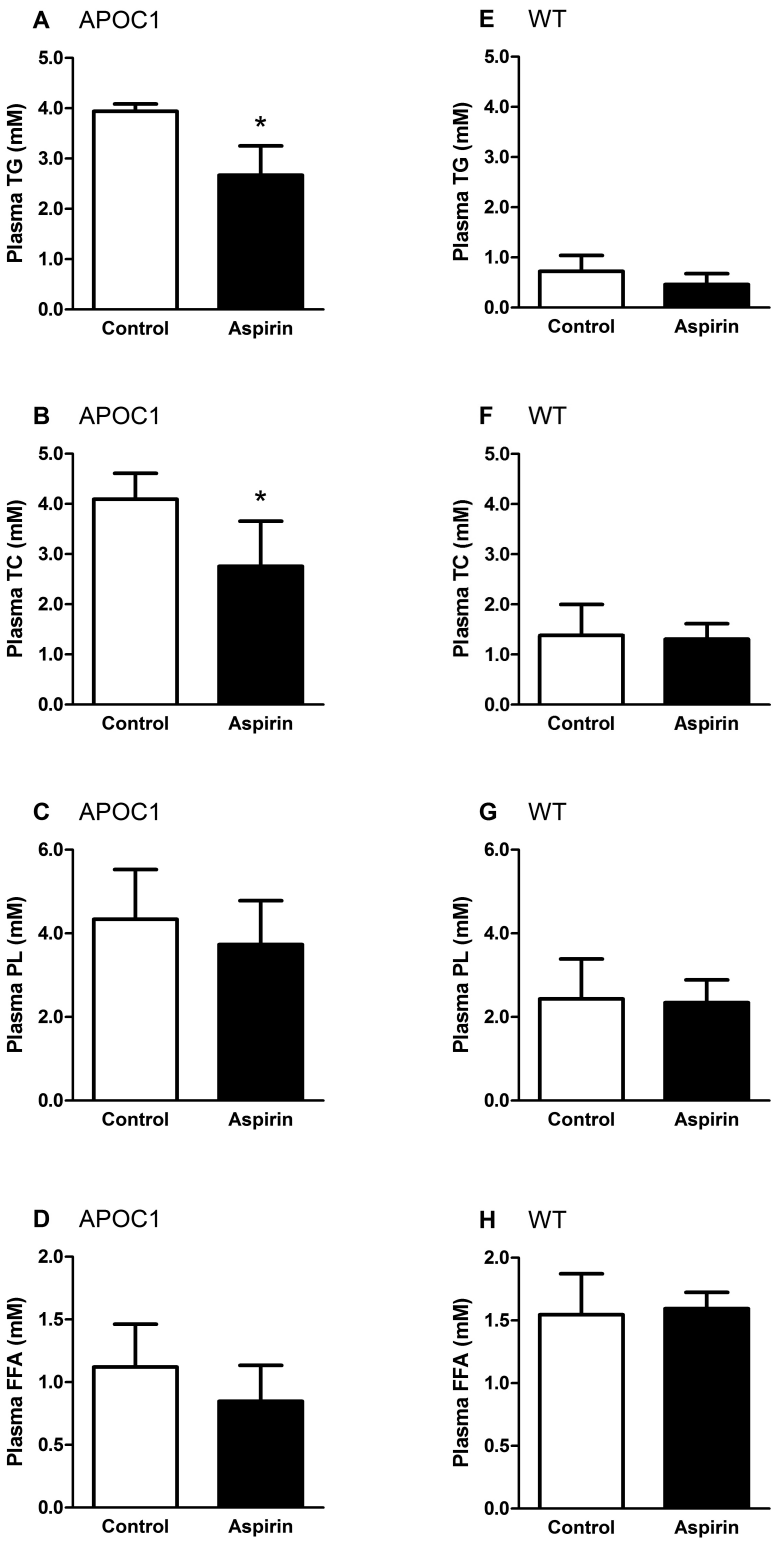

Figure 2. Aspirin lowers plasma triglyceride and cholesterol levels in HFD-fed *APOC1* mice. Plasma triglycerides (TG) (A&E), total cholesterol (TC) (B&F), phospholipids (PL) (C&G) and free fatty acid (FFA) (D&H) levels were measured in plasma of overnight-fasted HFD-fed *APOC1* and WT mice treated without or with aspirin. Values are means ± SD (n=4-5). *P<0.05.

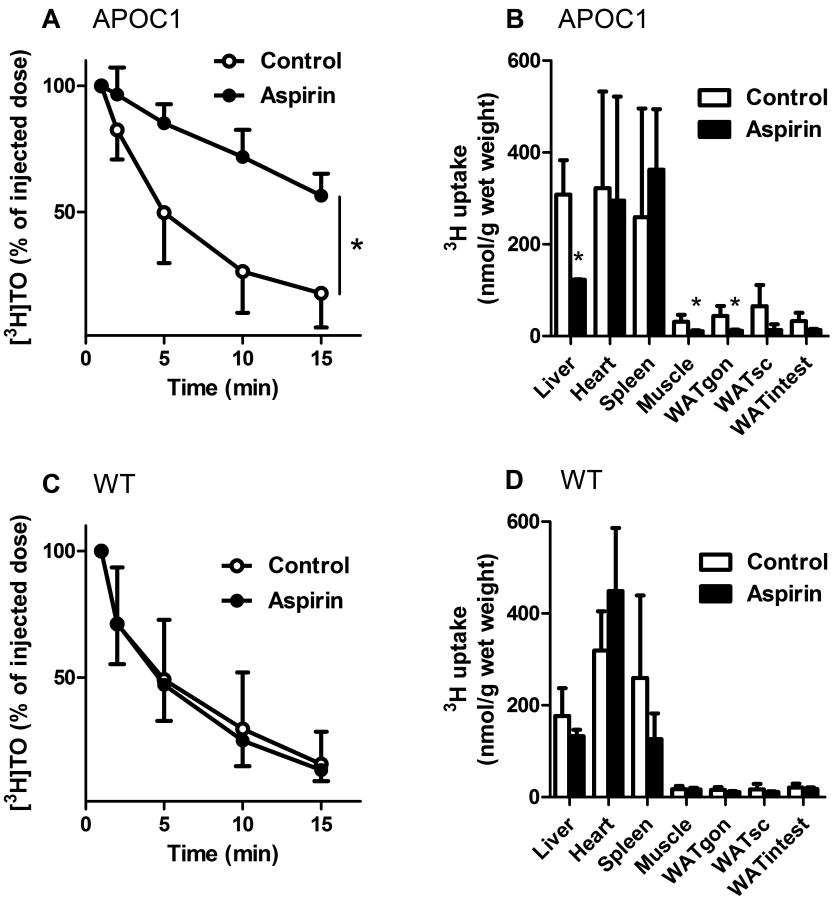

Figure 3. Aspirin attenuates TG clearance of VLDL-like emulsion particles in HFD-fed *APOC1*, but not WT mice. HFD-fed *APOC1* and WT mice that were treated without or with aspirin were fasted overnight and injected with [^{3}H]TO-labeled VLDL-like emulsion particles. Blood was collected at the indicated time points and radioactivity was measured in plasma of *APOC1* (A) and WT (C) mice treated without (open circles) or with (closed circles) aspirin. Uptake of [^{3}H]TO-derived activity by various organs was determined, and total FA uptake was calculated from the specific activity of TG in plasma, and expressed as nmol FA per mg wet tissue weight in *APOC1* (B) and WT (D) mice. Values are means ± SD (n=4). *P<0.05. WAT, white adipose tissue; intest, intestinal; sc, subcutaneous; gon, gonadal.

Triton WR1339 injection (Fig 4). We found a reduction in hepatic VLDL-TG secretion rate in *APOC1* mice treated with aspirin by -28% (3.42 ± 0.53 vs 4.95 ± 1.11 mM/h; P<0.05) (Fig. 4A), whereas aspirin did not affect the rate of VLDL-apoB production (Fig. 4B). Interestingly, similar to our observation in *APOC1* mice, aspirin did reduce the hepatic VLDL-TG secretion rate in HFD-fed WT mice by -33% (2.79 ± 0.47 mM/h vs 4.19 ± 0.48 mM/h; P<0.05; Fig. 4C), whereas VLDL-apoB production rate was also not affected (Fig. 4D). Apparently, aspirin generally reduces the VLDL-TG production in HFD-fed mice, independent of the genotype. Furthermore, since each VLDL particle contains a single apoB molecule, this observation shows that aspirin treatment inhibits hepatic VLDL-TG production, without affecting the rate of VLDL particle production.

Aspirin does not affect liver lipid levels in HFD-fed *APOC1* and WT mice

To determine whether the attenuation in hepatic VLDL-TG production was the result of decreased lipid substrate availability in the liver, the effect of aspirin on hepatic lipid content was measured (Fig. 5). However, aspirin did not affect liver TG levels (Fig. 5A) and TC levels (Fig. 5B) in *APOC1* mice. Also, aspirin did not affect liver TG (Fig. 5C) or TC (Fig. 5D) levels in WT mice.

Aspirin treatment does not affect hepatic expression of genes involved in FA oxidation, lipogenesis or VLDL production

Since changes in hepatic gene expression could underlie the reduction in VLDL-TG production, we determined the effect of aspirin on expression of genes involved in FA

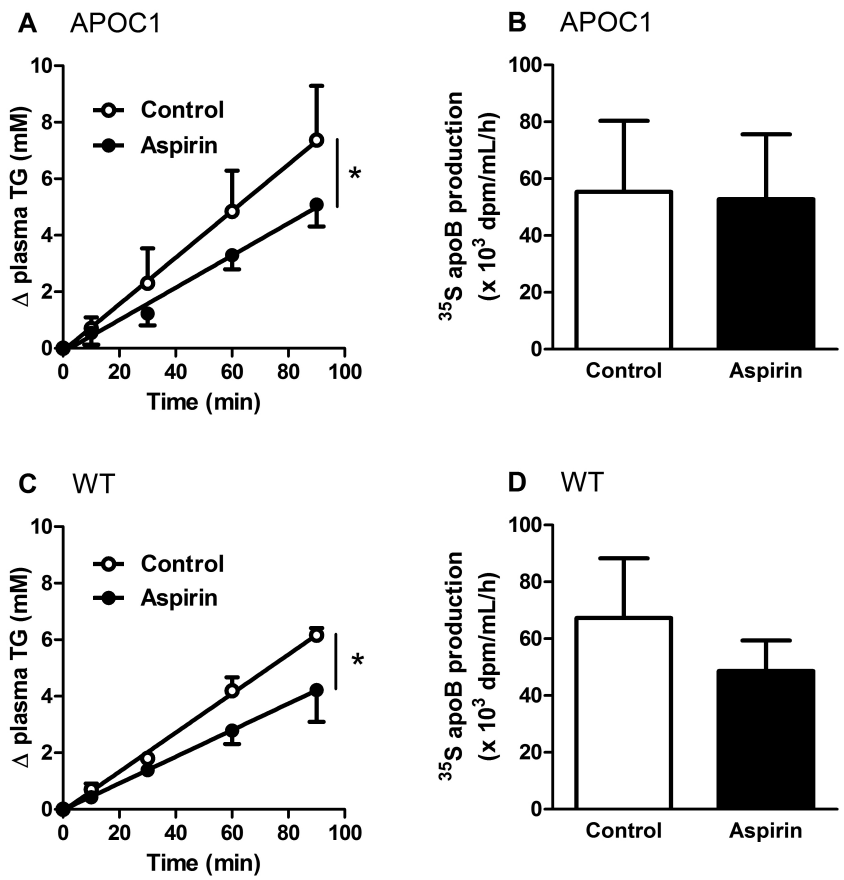

Figure 4. Aspirin decreases VLDL-TG production in HFD-fed *APOC1* and WT mice. *APOC1* and WT mice were fed a HFD and treated without or with aspirin. Overnight fasted mice were injected with Trans^{35}S and TritonWR1339 and blood samples were drawn at the indicated time points. TG concentrations were determined in *APOC1* (A) and WT (C) mice treated without (open circles) or with (closed circles) aspirin and plotted as the increase in plasma TG relative to t=0 (A). After 120 min, VLDL was isolated by ultracentrifugation, ^{35}S-activity was counted and the production rate of newly synthesized VLDL-^{35}S-apoB was determined for *APOC1* (B) and WT (D). Values are means ± SD (n=5). *P<0.05.

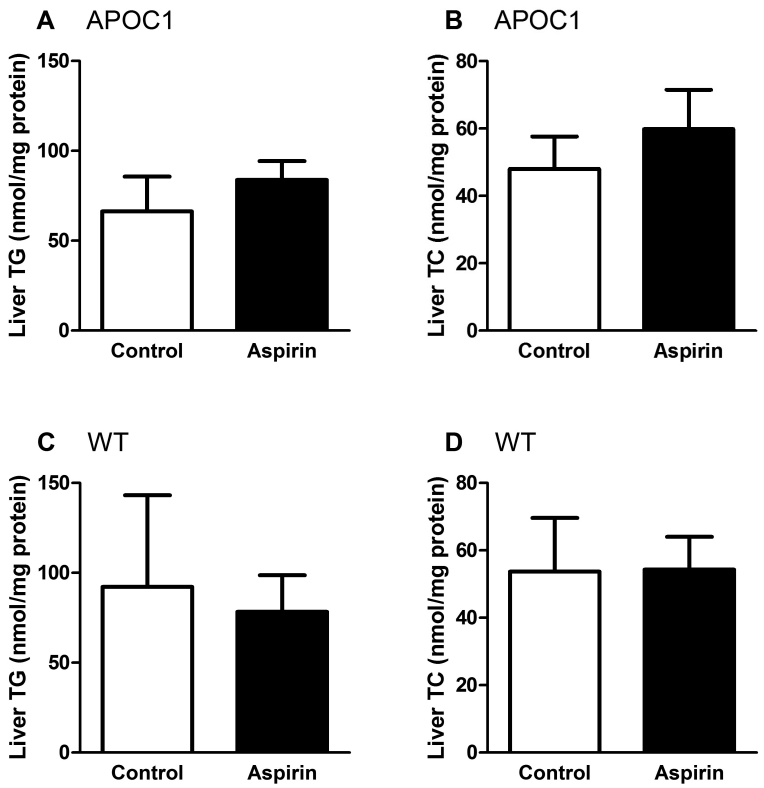

Figure 5. Aspirin does not affect liver lipids in HFD-fed *APOC1* and WT mice. Livers were collected from overnight-fasted HFD-fed *APOC1* and WT mice treated without or with aspirin. Lipids were extracted and triglyceride (TG, A&C) and total cholesterol (TC, B&D) concentrations were measured and expressed per mg protein. Values are means ± SD (n=6).

oxidation, lipogenesis and VLDL production (Table 2). In both *APOC1* and WT mice, aspirin did not affect expression of peroxisome proliferative activated receptor alpha (*Ppara*), a transcription factor that regulates genes involved in FA oxidation and ketogenesis, nor did it affect its target genes acyl-Coenzyme A oxidase 1 (*Acox1*) and carnitine palmitoyltransferase 1a (*Cpt1a*). In line with these results, aspirin did not increase plasma ß-HB levels in WT mice (data not shown), which is a plasma marker for hepatic FA oxidation and ketogenesis. This implies that the reduced VLDL-TG production upon aspirin treatment is not caused by increased hepatic FA oxidation. We additionally determined the effect of aspirin on expression of genes involved in lipogenesis. In both APOC1 and WT mice, aspirin did not affect expression of sterol regulatory element binding protein 1c (*Srebp-1c*), which regulates genes required for de novo lipogenesis, nor did it affect acyl:diacylglycerol transferase 1 (*Dgat1*), which catalyzes the final and only committed step in TG synthesis, or FA synthase (*Fas*), which plays a key role in FA synthesis. These data suggests that aspirin does not affect genetic regulation of *de novo* lipogenesis. In addition, aspirin did not affect hepatic gene expression of microsomal TG transfer protein (*Mttp*) which is involved in the assembly and secretion of VLDL. Furthermore, aspirin does not affect hepatic gene expression of

Table 2. Aspirin generally does not affect hepatic expression of genes involved in FA uptake and transport, FA oxidation, lipogenesis or VLDL secretion.

		APOC1			WT		
Gene	Protein	Control	Aspirin	p-value	Control	Aspirin	p-value
FA uptake and transport							
Fabp1	FABP1	1.00 ± 0.37	0.73 ± 0.35	0.22	1.00 ± 0.41	0.61 ± 0.25	0.14
Slc27a2	FATPa2	1.00 ± 0.45	1.23 ± 0.49	0.46	1.00 ± 0.33	0.74 ± 0.16	0.14
Slc27a4	FATPa4	1.00 ± 0.35	1.74 ± 0.51	0.13	1.00 ± 0.44	1.01 ± 0.47	0.77
Slc27a5	FATPa5	1.00 ± 0.17	1.20 ± 0.39	0.18	1.00 ± 0.40	0.96 ± 0.31	0.62
Cd36	CD36	1.00 ± 0.58	1.75 ± 0.40	0.05	1.00 ± 0.80	0.45 ± 0.22	0.23
FA oxidation							
Ppara	PPARα	1.00 ± 0.29	1.12 ± 0.46	0.62	1.00 ± 0.37	0.72 ± 0.18	0.18
Acox1	ACO	1.00 ± 0.42	1.55 ± 0.55	0.14	1.00 ± 0.36	0.59 ± 0.12	0.09
Cpt1a	CPT1a	1.00 ± 0.55	1.36 ± 0.43	0.22	1.00 ± 0.10	0.96 ± 0.11	0.46
Lipogenesis							
Dgat1	DGAT1	1.00 ± 0.37	1.20 ± 0.11	0.29	1.00 ± 0.42	1.06 ± 0.53	0.85
Fasn	FAS	1.00 ± 0.42	1.05 ± 1.09	0.81	1.00 ± 0.40	0.97 ± 0.27	0.90
Srebf1	SREBP-1c	1.00 ± 0.40	1.22 ± 0.53	0.41	1.00 ± 0.53	0.85 ± 0.68	0.72
VLDL secretion							
Apob	ApoB	1.00 ± 0.46	1.20 ± 0.26	0.73	1.00 ± 0.32	1.59 ± 0.31*	0.03
Mttp	MTP	1.00 ± 0.37	0.87 ± 0.21	0.56	1.00 ± 0.39	1.21 ± 0.12	0.34

Livers were isolated from overnight fasted *APOC1* and WT mice fed a HFD and treated without or with aspirin. mRNA was isolated and mRNA expression of the indicated genes was quantified by RT-PCR. Genes are grouped as genes involved in FA uptake and transport, FA oxidation, lipogenesis and VLDL production. Data are calculated as fold difference as compared to the control group. Values are means ± SD (n=4-5). *P<0.05 compared to control group. *Acox1,* acyl-Coenzyme A oxidase 1, palmitoyl; *Apob*, apolipoprotein B; *Cd36,* fatty acid translocase; *Cpt1a,* carnitine palmitoyltransferase 1a, liver; *Dgat1*, diglyceride acyltransferase 1; *Fabp1*, fatty acid binding protein 1, liver; *Fasn*, fatty acid synthase; *Mttp*, microsomal triglyceride transfer protein; *Ppara*, peroxisome proliferative activated receptor alpha; *Slc27a2*, fatty acid transport protein 2; *Slc27a4*, fatty acid transport protein 4; *Slc27a5*, fatty acid transport protein 5; *Srebpf1*, sterol-regulatory element binding protein.

apoB (*Apob*) in *APOC1* mice, which is in line with the observation that aspirin does not affect VLDL-apoB secretion *in vivo*. However, despite the fact that aspirin did not affect VLDL-apoB secretion in WT mice, gene expression of *Apob* was increased in WT mice.

Aspirin treatment decreases the contribution of plasma-derived FA to the VLDL-TG production

Because the decrease in VLDL-TG production was not caused by a reduced hepatic lipid content or decreased expression of genes involved in *de novo* lipogenesis that could reduce lipid availability for VLDL-TG secretion, we investigated whether the decreased VLDL-TG production could be explained by a diminished contribution of plasma derived FA for VLDL-TG secretion in WT mice (Fig. 6). The contribution of plasma derived FA

was measured by determining plasma ^{3}H-TG levels after continuous ^{3}H-FA infusion and intravenous Triton WR1339 injection. We found that aspirin reduced hepatic ^{3}H-TG secretion rate in WT mice by -24% (3.1 ± 0.4 vs 2.4 ± 0.7 x 10^{3} dpm/h; P<0.05), which suggests that aspirin reduces VLDL-TG production by reducing the incorporation of plasma-derived FA into VLDL-TG. This reduction is not caused by a reduced hepatic expression of genes involved in hepatic FA uptake and transport (Table 2), since aspirin did not affect liver-type FA binding protein (*Fabp1*), FA transport proteins 2, 4 and 5 (*Slc27a2, Slc27a4, Slc27a5*) and even increased expression of FA translocase (*Cd36*) in *APOC1*, but not WT, mice. These data imply that aspirin reduced the VLDL-TG production independent of changes in hepatic expression of genes involved in FA uptake and transport.

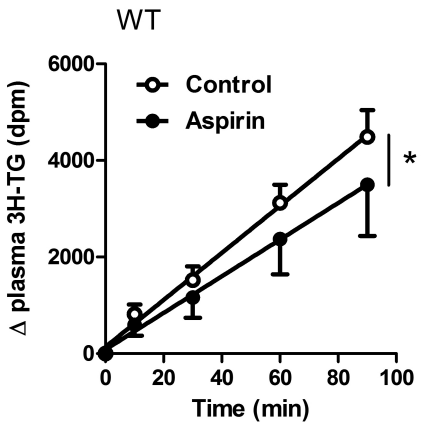

Figure 6. Aspirin reduces the contribution of plasma derived FA to the VLDL-TG production. WT mice were fed a HFD and treated without or with aspirin. Overnight fasted mice received a continuous i.v. infusion of ^{3}H-labeled FA ([9,10(n)-^{3}H] palmitic acid for 2 h, followed by an i.v. injection of TritonWR1339. Blood samples were drawn at the indicated time points and ^{3}H activity in the TG fraction was determined in mice treated without (open circles) or with (closed circles) aspirin and plotted as the increase in plasma ^{3}H-TG relative to t=0. Values are means ± SD (n=7). *P<0.05.

3.4 DISCUSSION

Treatment of obese rodents and patients with type 2 diabetes with high dose aspirin reduces hypertriglyceridemia.[13,14] However, so far, the mechanistic basis for the relation between aspirin intake and reduced plasma TG levels has been poorly understood. In the present study we focused on the effects of aspirin on VLDL-TG metabolism in HFD-induced obese hyperlipidemic *APOC1* mice and additionally evaluated the effects of aspirin on VLDL-TG metabolism in HFD-fed normolipidemic WT mice. Our results document that aspirin treatment improves hypertriglyceridemia by reducing the hepatic production of VLDL-TG as a result of an attenuated hepatic incorporation of plasma derived FA into VLDL-TG, rather than from increased clearance of VLDL-TG from the circulation.

In the present study, aspirin treatment decreased plasma TG and TC levels in HFD fed *APOC1* mice that display hypertriglyceridemia. This improvement in hyperlipidemia is in accordance with earlier studies, showing reduced serum TG concentrations upon aspirin or salicylate treatment in patients with type 2 diabetes mellitus[14] and in diabetic rats.[13]

Our data show that aspirin treatment attenuated the clearance of VLDL-like TG-rich particles in *APOC1* mice. Therefore, the decrease in plasma TG levels by aspirin cannot be explained by increased TG clearance. Earlier studies report that high dose LPS injections reduce the clearance of TG-rich lipoproteins by inhibition of the LPL activity, mediated by cytokines.[6,27] If indeed inflammation inhibits clearance of TG, inhibition of inflammation by aspirin is expected to increase TG-rich lipoprotein clearance, which is in contrast to our observation in *APOC1* mice. It should be noted that aspirin, in addition to inhibition of inflammation via NF-κB, also inhibits prostaglandin synthesis, which has been demonstrated to restore the LPS-induced inhibition of LPL.[28] Moreover, an early report has shown that aspirin treatment inhibits post-heparin LPL activity in humans.[29] It would be interesting to elucidate the mechanism by which aspirin reduces the VLDL-TG clearance, however this is beyond the scope of the current manuscript as it does not explain the reduction in hypertriglyceridemia that we observe. Moreover, the observation may be a specific feature of the *APOC1* transgenic mouse model, since we did not observe such an effect in WT mice.

Aspirin very effectively reduced hepatic secretion of VLDL-TG in *APOC1* mice, explaining the reduction in hypertriglyceridemia upon aspirin treatment. In addition, aspirin equally reduced hepatic secretion of VLDL-TG in WT mice, indicating that the effects of aspirin on the VLDL-TG production do not exclusively occur in hypertriglyceridemic mouse models such as the *APOC1* mouse. To our knowledge, we show for the first time that a decrease in inflammation corresponds with a drop in VLDL-TG production. The reduction of VLDL-TG secretion in our study is not paralleled by a reduction in apoB secretion in both *APOC1* and WT mice, suggesting that aspirin reduces the lipidation of VLDL particles rather than reducing the number of particles that are secreted by the liver. In contrast to our data on apoB secretion, a recent study by Tsai et al[30] observed that suppression of IKK with BMS345541 decreased apoB secretion *in vitro* in primary hamster hepatocytes and HepG2 cells. Even though differences between species might explain these conflicting findings, both these published *in vitro* studies and our present *in vivo* study point towards a link between the IKK/NF-κB pathway and the regulation of VLDL production. Moreover, we have recently shown that *activation* of the hepatic IKK/NF-κB pathway increases VLDL-TG production,[31] supporting the hypothesis that the effects of aspirin on the VLDL-TG production are mediated via a reduction in hepatic NF-κB activity. Nevertheless, activation of hepatic IKK/NF-κB increases hepatic *Fas* expression,[31] while aspirin in the current study did not change hepatic expression of *Fas*, neither did it change expression of other genes involved in TG synthesis, such as *Dgat1* and *Srepb-1c,* suggesting that aspirin more likely lowers VLDL-TG production by other mechanisms than via its effects on hepatic NF-κB activity.

A reduction in hepatic lipid availability by increased lipid oxidation could underlie the mechanism by which aspirin reduces hepatic VLDL-TG production. However, aspirin did not affect expression of genes involved in FA oxidation nor plasma levels of ß-HB, a marker of hepatic FA oxidation and ketogenesis. Similarly, aspirin did not affect expression of genes involved in de novo lipogenesis or VLDL production, suggesting that aspirin does not reduce VLDL-TG production by changing expression of genes involved in hepatic lipid metabolism.

It has been suggested that the decrease in plasma TG concentration that occurs upon aspirin treatment might be secondary to the fall in plasma FFA levels.[32] A reduction in FFA delivery to the liver could result in a reduced availability of FA for the release of VLDL-TG by the liver.[33] Indeed, although aspirin did not change plasma FFA levels, it changed the turnover of FA as reflected by a -24% reduction in the incorporation of plasma derived FA into VLDL-TG, showing that aspirin in fact lowers the availability of plasma derived FA for VLDL-TG production. This reduction of FA incorporation into VLDL-TG upon aspirin treatment was not caused by a reduced hepatic expression of FA transporter proteins, suggesting that aspirin reduces the FA incorporation via another mechanism. It is possible that aspirin reduces posttranscriptional processing of FA transporters independent of mRNA expression, since expression of FA transporters does not always correlate with changes in protein content or the rate of FA transport.[34] Alternatively, aspirin might increase FA uptake and transport via simple diffusion, since FA uptake has been described independent of any FA transporter.[34]

The decrease of FA turnover that we observed could be secondary to an increased insulin sensitivity of adipose tissue, thereby decreasing FA mobilization to plasma. Indeed, high dose salicylates, such as aspirin have shown to increase insulin sensitivity[13] and the reduction in VLDL-TG production in our study is similarly accompanied by an increased insulin sensitivity (unpublished observation). However, the aspirin-induced reduction in FA utilization and subsequent VLDL-TG secretion in our study were determined under fasting conditions, when the role of insulin is marginal. In fasting conditions, the lipolytic activity of adipocytes is stimulated by catecholamines. Interestingly, aspirin has been reported to reduce catecholamine-stimulated lipolysis, which is therefore a more likely explanation for our findings.[35,36] In addition, it has been shown that aspirin reduces release of FA from adipose tissue directly via inhibition of TNF-α induced lipolysis.[37] We therefore propose that the fact that aspirin reduces plasma derived FA utilization by the liver, is likely caused via direct inhibition of intracellular lipolysis in adipose tissue, which reduces plasma FA availability. Adipose tissue lipolysis might be further inhibited in the fed state by an increased sensitivity for insulin.

In conclusion, our data show that aspirin inhibits NF-κB and decreases HFD-induced hypertriglyceridemia by reducing hepatic VLDL-TG secretion rather than by accelerating the tissue distribution of VLDL-TG. The reduction in VLDL-TG is not caused by a decreased steatosis, increased FA oxidation or changes in *de novo* lipogenesis, but by an attenuation of hepatic incorporation of plasma derived FA into VLDL-TG. In scope of our findings, aspirin could potentially be a new therapeutic drug in the treatment of hypertriglyceridemia. However, chronic high-dose aspirin is associated with risk for bleeding. Salsalate on the

other hand is a non-steroidal anti-inflammatory drug with similar structure that is regarded as a safer alternative. High-dose salsalate treatment has recently shown to reduce TG levels in diabetic patients similar to high-dose aspirin treatment,[38] and could therefore potentially be a new drug for the treatment of hypertriglyceridemia.

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REFERENCES

1. Ridker PM, Buring JE, Cook NR, Rifai N. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14 719 initially healthy American women. Circulation 2003;107:391-397.
2. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med 2005;11:183-190.
3. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. J Lipid Res 2004;45:1169-1196.
4. Lequire VS, Hutcherson JD, Hamilton RL, Gray ME. The effects of bacterial endotoxin on lipide metabolism. I. The responses of the serum lipides of rabbits to single and repeated injections of Shear's polysaccharide. J Exp Med 1959;110:293-309.
5. Gallin JI, Kaye D, O'Leary WM. Serum lipids in infection. N Engl J Med 1969;281:1081-1086.
6. Feingold KR, Staprans I, Memon RA, Moser AH, Shigenaga JK, Doerrler W, Dinarello CA, Grunfeld C. Endotoxin rapidly induces changes in lipid metabolism that produce hypertriglyceridemia: low doses stimulate hepatic triglyceride production while high doses inhibit clearance. J Lipid Res 1992;33:1765-1776.
7. Hudgins LC, Parker TS, Levine DM, Gordon BR, Saal SD, Jiang XC, Seidman CE, Tremaroli JD, Lai J, Rubin AL. A single intravenous dose of endotoxin rapidly alters serum lipoproteins and lipid transfer proteins in normal volunteers. J Lipid Res 2003;44:1489-1498.
8. Feingold KR, Hardardottir I, Memon R, Krul EJ, Moser AH, Taylor JM, Grunfeld C. Effect of endotoxin on cholesterol biosynthesis and distribution in serum lipoproteins in Syrian hamsters. J Lipid Res 1993;34:2147-2158.
9. Nonogaki K, Fuller GM, Fuentes NL, Moser AH, Staprans I, Grunfeld C, Feingold KR. Interleukin-6 stimulates hepatic triglyceride secretion in rats. Endocrinology 1995;136:2143-2149.
10. Memon RA, Grunfeld C, Moser AH, Feingold KR. Tumor necrosis factor mediates the effects of endotoxin on cholesterol and triglyceride metabolism in mice. Endocrinology 1993;132:2246-2253.
11. Yin MJ, Yamamoto Y, Gaynor RB. The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. Nature 1998;396:77-80.
12. Kopp E, Ghosh S. Inhibition of NF-kappa B by sodium salicylate and aspirin. Science 1994;265:956-959.
13. Yuan M, Konstantopoulos N, Lee J, Hansen L, Li ZW, Karin M, Shoelson SE. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikkbeta. Science 2001;293:1673-1677.

14. Hundal RS, Petersen KF, Mayerson AB, Randhawa PS, Inzucchi S, Shoelson SE, Shulman GI. Mechanism by which high-dose aspirin improves glucose metabolism in type 2 diabetes. J Clin Invest 2002;109:1321-1326.
15. Berbee JF, van der Hoogt CC, Sundararaman D, Havekes LM, Rensen PC. Severe hypertriglyceridemia in human APOC1 transgenic mice is caused by apoC-I-induced inhibition of LPL. J Lipid Res 2005;46:297-306.
16. Jong MC, Dahlmans VE, van Gorp PJ, van Dijk KW, Breuer ML, Hofker MH, Havekes LM. In the absence of the low density lipoprotein receptor, human apolipoprotein C1 overexpression in transgenic mice inhibits the hepatic uptake of very low density lipoproteins via a receptor-associated protein-sensitive pathway. J Clin Invest 1996;98:2259-2267.
17. Jong MC, van Ree JH, Dahlmans VE, Frants RR, Hofker MH, Havekes LM. Reduced very-low-density lipoprotein fractional catabolic rate in apolipoprotein C1-deficient mice. Biochem J 1997;321 (Pt 2):445-450.
18. Lee JI, Burckart GJ. Nuclear factor kappa B: important transcription factor and therapeutic target. J Clin Pharmacol 1998;38:981-993.
19. Li N, Karin M. Signaling pathways leading to nuclear factor-kappa B activation. Methods Enzymol 2000;319:273-279.
20. Zambon A, Hashimoto SI, Brunzell JD. Analysis of techniques to obtain plasma for measurement of levels of free fatty acids. J Lipid Res 1993;34:1021-1028.
21. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-917.
22. Rensen PC, van Dijk MC, Havenaar EC, Bijsterbosch MK, Kruijt JK, van Berkel TJ. Selective liver targeting of antivirals by recombinant chylomicrons--a new therapeutic approach to hepatitis B. Nat Med 1995;1:221-225.
23. Rensen PC, Herijgers N, Netscher MH, Meskers SC, van Eck M, van Berkel TJ. 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 1997;38:1070-1084.
24. Jong MC, Rensen PC, Dahlmans VE, van der Boom H, van Berkel TJ, Havekes LM. Apolipoprotein C-III deficiency accelerates triglyceride hydrolysis by lipoprotein lipase in wild-type and apoE knockout mice. J Lipid Res 2001;42:1578-1585.
25. Redgrave TG, Roberts DC, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal Biochem 1975;65:42-49.
26. Belfrage P, Vaughan M. Simple liquid-liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. J Lipid Res 1969;10:341-344.
27. Feingold KR, Marshall M, Gulli R, Moser AH, Grunfeld C. Effect of endotoxin and cytokines on lipoprotein lipase activity in mice. Arterioscler Thromb 1994;14:1866-1872.
28. Desanctis JB, Varesio L, Radzioch D. Prostaglandins inhibit lipoprotein lipase gene expression in macrophages. Immunology 1994;81:605-610.
29. Sommariva D, Bonfiglioli D, Zanaboni L, Fasoli A. Effects of acetylsalicylic acid on plasma lipids and on post-heparin lipase activities. Int J Clin Pharmacol Ther Toxicol 1981;19:112-116.
30. Tsai J, Zhang R, Qiu W, Su Q, Naples M, Adeli K. Inflammatory NF-kappaB activation promotes hepatic apolipoprotein B100 secretion: evidence for a link between hepatic inflammation and lipoprotein production. Am J Physiol Gastrointest Liver Physiol 2009;296:G1287-G1298.
31. van Diepen JA, Wong MC, Guigas B, Bos J, Stienstra R, Hodson L, Shoelson SE, Berbee JF, Rensen PC, Romijn JA, Havekes LM, Voshol PJ. Hepatocyte-specific IKK-{beta} activation enhances VLDL-triglyceride production in APOE*3-Leiden mice. J Lipid Res 2011;52:942-950.
32. Wooles WR, Borzelleca JF, Branham Jr GW. Effect of acute and prolonged salicylate administration on liver and plasma triglyceride levels and diet-induced hypercholesterolemia. Toxicol Appl Pharmacol 1967;10:1-7.
33. Laurell S. Recycling of intravenously injected palmitic acid-1-C14 as esterified fatty acid in the plasma of rats and turnover rate of plasma triglycerides. Acta Physiol Scand 1959;47:218-232.
34. Glatz JF, Luiken JJ, Bonen A. Membrane fatty acid transporters as regulators of lipid metabolism: implications for metabolic disease. Physiol Rev 2010;90:367-417.

35. Stone DB, Brown JD, Steele AA. Effect of sodium salicylate on induced lipolysis in isolated fat cells of the rat. Metabolism 1969;18:620-624.
36. Schonhofer PS, Sohn J, Peters HD, Dinnendahl V. Effects of sodium salicylate and acetylsalicylic acid on the lipolytic system of fat cells. Biochem Pharmacol 1973;22:629-637.
37. Zu L, Jiang H, He J, Xu C, Pu S, Liu M, Xu G. Salicylate blocks lipolytic actions of tumor necrosis factor-alpha in primary rat adipocytes. Mol Pharmacol 2008;73:215-223.
38. Goldfine AB, Silver R, Aldhahi W, Cai D, Tatro E, Lee J, Shoelson SE. Use of salsalate to target inflammation in the treatment of insulin resistance and type 2 diabetes. Clin Transl Sci 2008;1:36-43.