Methods

Animals and diets

Male apoE$^{-/-}$ mice (stock number: 002052), backcrossed to C57BL/6J mice for at least 10 generations, and C57BL/6J mice (wild-type, WT, stock number: 000664) were purchased from the Jackson Laboratories (Bar Harbor, ME). After weaning, mice were fed a normal diet (ND) containing 4.5% (w/w) fat. At 6 weeks of age, they were switched to either a high-fat diet (HFD) consisting of 21% (w/w) fat (Dyets Inc, Bethlehem, PA) or maintained on ND ad libitum for an additional 12 weeks, during which body weight was monitored weekly. Mice were housed in a pathogen-free facility with a 12-hour light/12-hour dark cycle. Total body fat mass was determined with a PIXI-mus Small Animal Densitometer (LUNAR, Madison, WI) 1 week before sacrifice. All procedures were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine.

Biochemical analytes

Blood was collected from mice after a 6-hour fast. Plasma levels of glucose, insulin, cholesterol, triglycerides (TGs) and nonesterified fatty acids (NEFAs) were measured by enzymatic methods at the Mouse Metabolic Phenotyping Center, University of Cincinnati Medical Center. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated using the formula: fasting insulin (μIU/mL)×fasting glucose (mmol/L)/22.5.

Glucose and insulin tolerance tests (GTT and ITT)

After a 6-hour fast, mice were injected intraperitoneally (i.p.) with glucose (1 g/kg body weight; dextrose in water) for GTT or human insulin (1.5 units/kg body weight; humulin R, Eli Lilly, Indianapolis, IN) for ITT. Glucose levels were measured in blood from tail veins with a glucometer (Bayer, Elkhart, IN) before and 15, 30, 45, 60, 90 and 120 minutes after injection.

Insulin-stimulated phosphorylation of Akt

After a 6-hour fast, mice were injected i.p with humulin (1.5 units/kg body weight) or sterile phosphate-buffered saline and 10 minutes later, mice were sacrificed and quadriceps femoris and perigonadal adipose tissue were excised. Proteins were extracted from the tissues using a lysis buffer of 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, Triton X-100, 10% glycerol, 0.1% SDS and 0.5% deoxycholate supplemented with Complete Mini, a protease inhibitor (Roche Applied Science, Indianapolis, IN).

Tissue-phosphorylated–Ser^{473}Akt was estimated by immunoblotting using equal amounts of tissue proteins, which were separated by polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After blocking with 5% bovine serum albumin (BSA) or nonfat milk, the membranes were incubated with specific antibodies against phosphorylated-Ser^{473} Akt or total Akt (Cell Signaling Technology, Inc., Danvers, MA) at 4^{o}C overnight. After washing, the membranes were incubated with a horseradish peroxidase–conjugated secondary antibody (Cell Signaling Technology) in tris-saline–Tween buffer containing 5% BSA for 1 hour. Phosphorylated-Ser^{473}Akt and total Akt were determined by electrochemiluminescence reagent and visualized with a STORM 840 scanner (GE Healthcare Bioscience, Sweden).

Histology

Perigonadal adipose depots were excised, fixed in Z-fix (Anatech Ltd, Battle Creek, MI) for up to 24 hours, embedded in paraffin and sectioned. Sections were stained with hematoxylin–eosin (H&E). Stained sections were photographed with a Leitz Camera (Orthomat2) mounted on a microscope. Adipocyte diameter was calculated from ≥200 cells/sample using Image J (1.43u) software. Crown-like structures were counted from ≥5 fields/sample, and the average number/field was calculated.

RNA isolation and quantitative RT-PCR (qPCR)
To determine mRNA levels of selected molecules, quantitative reverse-transcription polymerase chain reaction (qPCR) was performed using premade specific primers and probes from Applied Biosystems (Carlsbad, CA) on total RNA isolated from adipose tissue and quadriceps femoris using Trizol reagent (Invitrogen, Carlsbad, CA).[1,2] Serial dilutions of a representative sample were used to generate standard curves for relative quantification, which was normalized to that of 18S ribosomal RNA.

Analysis of plasma lipoprotein profile
Blood was collected by cardiac puncture from apoE$^{-/-}$ mice fed with ND or HFD. Plasma, isolated from blood by low-speed centrifugation, was adjusted to a d = 1.21 g/mL by adding KBr and centrifuged at 40,000 rpm in a Beckman SW 40 rotor. The infranatant was collected and analyzed by passing over a pair of Superose HR6 columns in tandem and the absorbance (280 nm) of the effluent monitored.

Flow cytometric analysis
Stromal/vascular cells (S/Vs) were isolated from adipose tissue with collagenase digestion as described previously.[2] After Fc receptor blocking with Mouse BD Fc Block (BD Biosciences), S/Vs were stained with FITC-conjugated anti-mouse CD11c (BD Biosciences) and PE-conjugated anti-mouse F4/80 antibodies (eBioscience, Inc., San Diego, CA), or with an FITC-conjugated anti-mouse CD3 antibody (BD Biosciences), with appropriate isotype antibodies as negative controls. Data were collected from the stained samples by a BD FACScan Flow Cytometer and analyzed with CellQuest (BD Biosciences) or FlowJo software as described previously for quantitation of macrophages/dendritic cells and T cells in adipose tissue.[3]

Tissue uptake of dietary [^{3}H]palmitic acid
After fasting overnight, mice were gavaged with 10 μCi [^{3}H]palmitic acid (PA; MP Biomedicals, Inc. Solon, OH) dispersed in 200 μL triolein. Blood was collected through retro-orbital puncture at various timepoints and plasma ^{3}H radioactivity measured by β-counting. At 6 hours post gavage, perigonadal adipose tissue, quadriceps femoris, diaphragm and liver were harvested, a portion of each was homogenized and total lipids were extracted with hexane/isopropanol (3:2). Tissue [^{3}H]PA was expressed as cpm/mg.[4]

Effect of intravenously injected VLDL on adipose tissue inflammation
VLDL was isolated by density-gradient ultracentrifugation of plasma from apoE$^{-/-}$ mice fed HFD. WT and apoE$^{-/-}$ mice on ND were intravenously injected with VLDL (from apoE$^{-/-}$ mice on HFD) at 15 μg/g body weight once a day for 3 days. At 6 hours after the final injection, mice were sacrificed. Perigonadal adipose tissue was collected and analyzed for expression of inflammatory markers by qPCR.

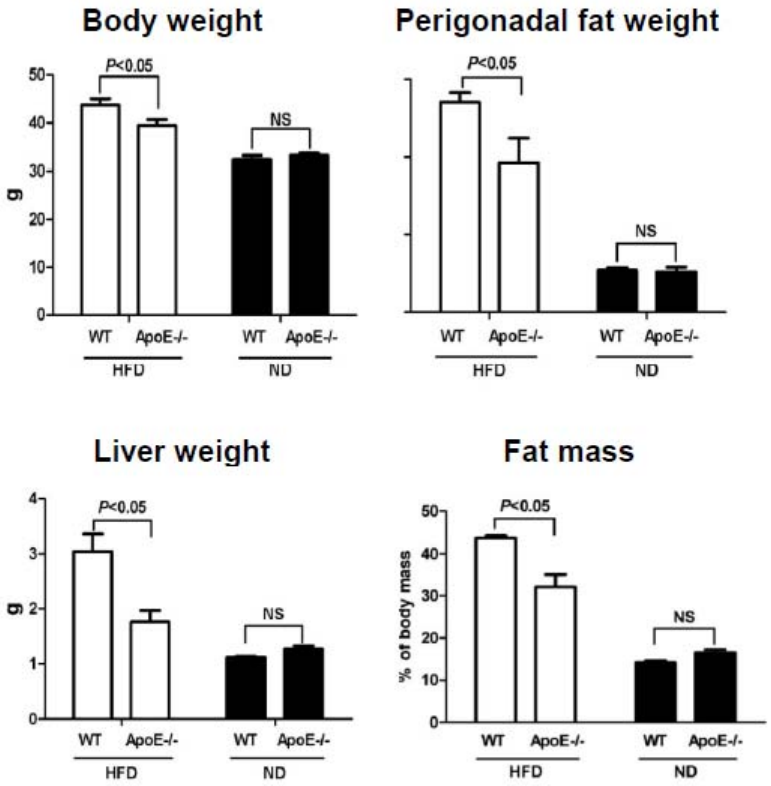
Lipid peroxidation assay

Lipid peroxidation was measured in adipose tissue homogenate using the Thiobarbituric acid-reactive substances (TBARS) Assay kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instruction. In brief, 25 mg of adipose tissue was homogenized in 250 μl of RIPA buffer with 1mM EDTA using 1.0mm zirconia/silica beads. Tissue homogenate was collected by spinning the sample at 1,600 g for 10min. Malondialdehyde (MDA) in tissue homogenate was reacted with thiobarbituric acid (TBA) to form MDA-TBA adduct, which was detected colorimetrically.[5] The amount of MDA was normalized to protein content in the tissue.

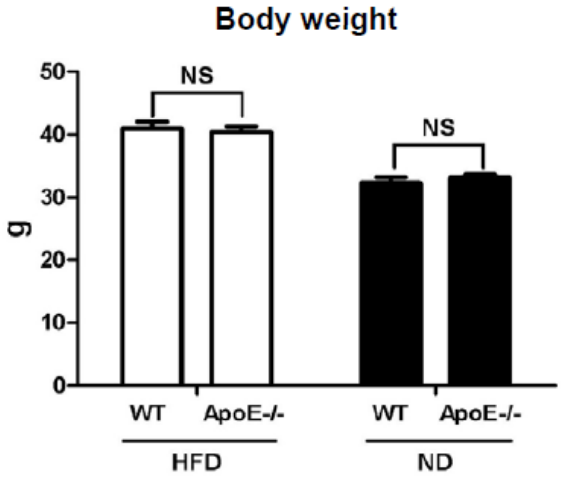
Tissue TG content
Quadriceps femoris were homogenized, and total lipids were extracted with hexane/isopropanol, dried under nitrogen and dissolved in isopropanol. TG content was determined by an enzymatic method (Wako Diagnostics, Richmond, VA) and presented as milligrams of TGs per gram of tissue.

Statistical analysis
Data are expressed as mean±SEM. Unpaired two-tailed Student's *t* tests (for comparison between 2 groups) or one-way ANOVA followed by Tukey post hoc pairwise tests (for comparison of ≥3 groups) were performed for statistical analysis using GraphPad Prism 5 (San Diego, CA). Differences were considered significant when $P<0.05$.

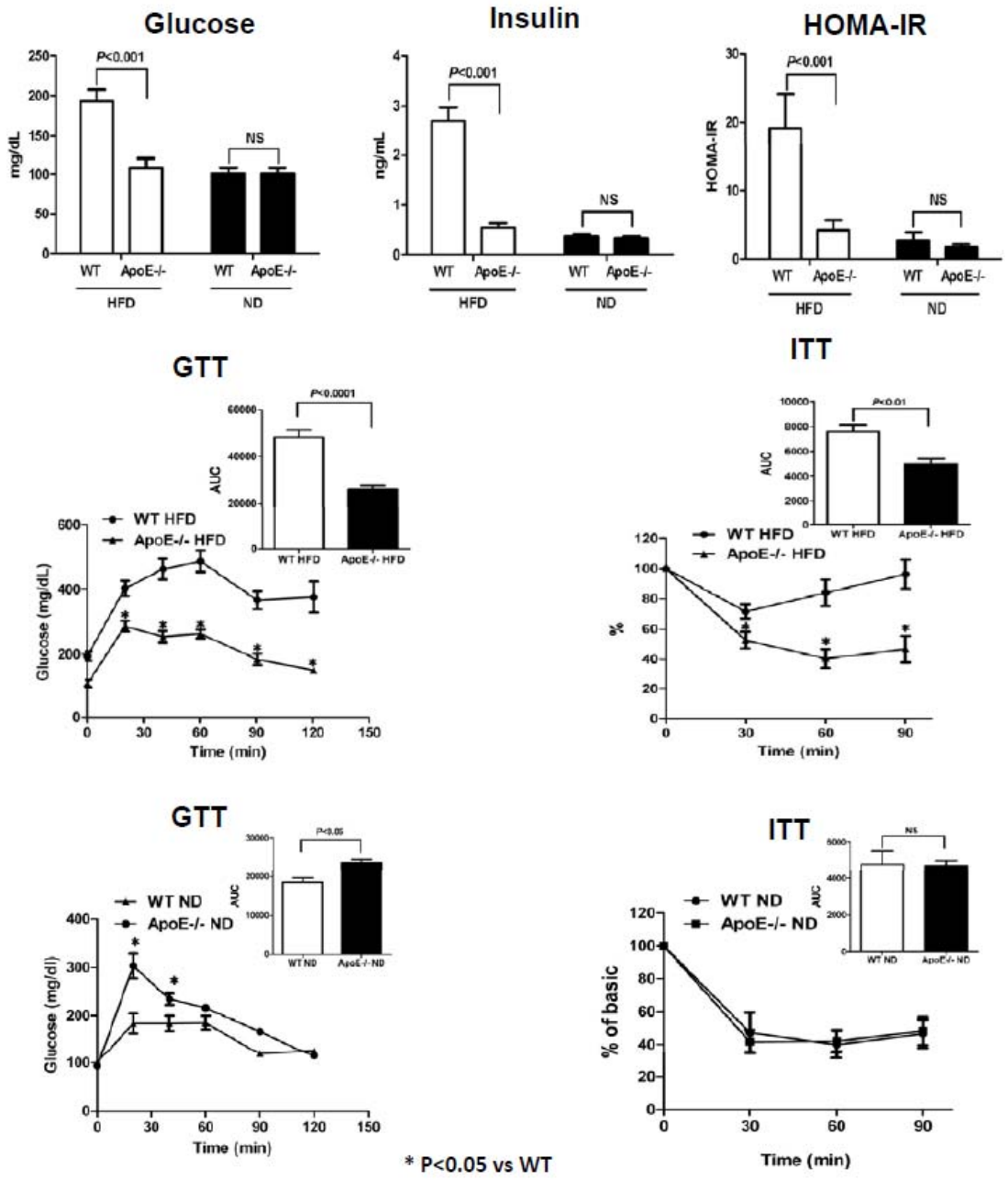
Results:



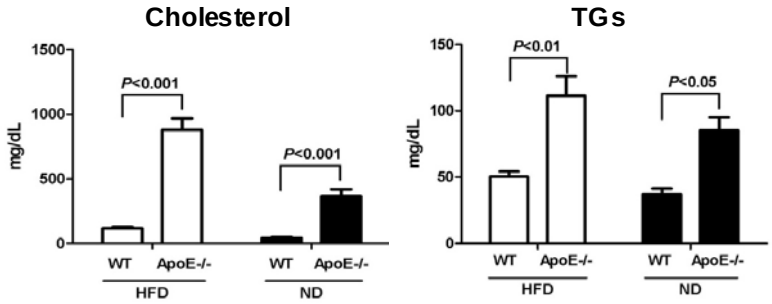
Supplemental Figure I: Body weights, weights of perigonadal fat pads and liver and total fat mass of all examined mice.



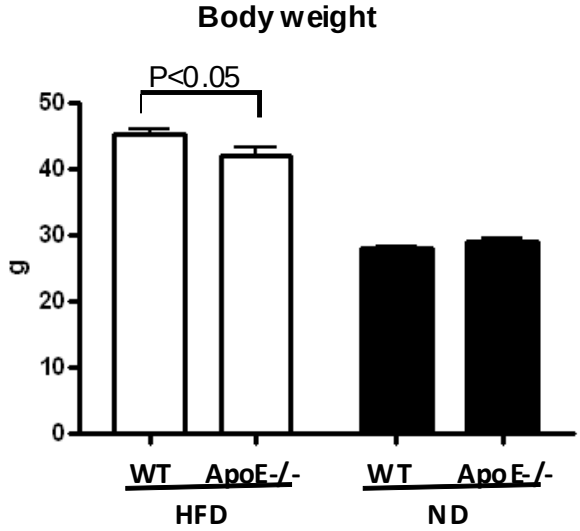
Supplemental Figure II: Body weights of selected apoE$^{-/-}$ mice and WT mice with comparable body weights.



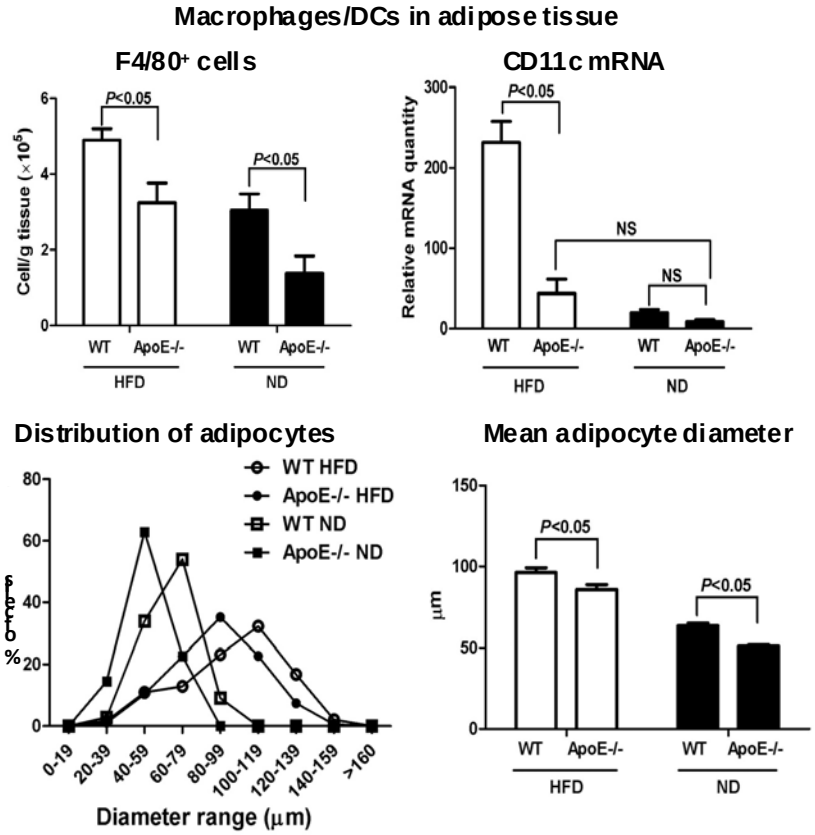
Supplemental Figure III: Plasma levels of glucose and insulin, calculated HOMA-IR, GTT and ITT in all examined WT and apoE$^{-/-}$ mice on HFD or ND.



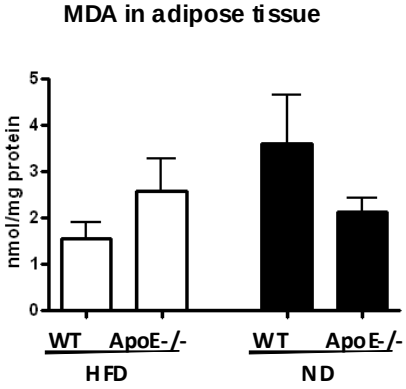
Supplemental Figure IV: Plasma levels of cholesterol and TGs in WT and apoE$^{-/-}$ mice on HFD or ND.



Supplemental Figure V: Body weights of apoE$^{-/-}$ mice and WT mice for adipose tissue inflammation assays.



Supplemental Figure VI: Macrophage/dendritic cell (DC) markers, including numbers of F4/80+ cells and mRNA levels of CD11c in adipose tissue, distribution of adipocytes based on cell diameters and mean adipocyte diameters of apoE$^{-/-}$ mice and WT mice on HFD or ND.



Supplemental Figure VII: MDA levels in adipose tissue of apoE$^{-/-}$ mice and WT mice on HFD or ND

References

1. Wu H, Ghosh S, Perrard XD, Feng L, Garcia GE, Perrard JL, Sweeney JF, Peterson LE, Chan L, Smith CW, Ballantyne CM. T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. *Circulation* 2007;115:1029-38.
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3. Wu H, Gower RM, Wang H, Perrard XY, Ma R, Bullard DC, Burns AR, Paul A, Smith CW, Simon SI, Ballantyne CM. Functional role of CD11c+ monocytes in atherogenesis associated with hypercholesterolemia. *Circulation* 2009;119:2708-17.
4. Goudriaan JR, Espirito Santo SM, Voshol PJ, Teusink B, van Dijk KW, van Vlijmen BJ, Romijn JA, Havekes LM, Rensen PC. The VLDL receptor plays a major role in chylomicron metabolism by enhancing LPL-mediated triglyceride hydrolysis. *J Lipid Res* 2004;45:1475-81.
5. Sohet FM, Neyrinck AM, Dewulf EM, Bindels LB, Portois L, Malaisse WJ, Carpentier YA, Cani PD, Delzenne NM. Lipid peroxidation is not a prerequisite for the development of obesity and diabetes in high-fat-fed mice. *British Journal of Nutrition* 2009;102:462-9.