SUPPLEMENTAL DATA

Detailed Methods

Animals and diets

Male *Ldlr*$^{-/-}$ mice on a C57BL/6J background (Jackson Laboratory), 8-12 weeks of age, were housed in pairs, on a 12-hour light/dark cycle, at 23°C and handled in accordance with the Canadian Guide for the Care and Use of Laboratory Animals and approved by the University of Western Ontario Animal Care Committee (protocol #2008-071). Mice were fed *ad libitum* for 12 weeks (n= 10-12/group) either a standard chow diet (14% kcal fat, TD8604, Harlan Teklad, Madison, WI), a high-fat, high-cholesterol diet (HFHC, 42% kcal fat, 0.2% cholesterol, TD09268, Harland Teklad), the HFHC diet supplemented with 3% wt/wt naringenin (Sigma-Aldrich, St. Louis, MO), a low-fat, semi-synthetic diet (LF, 12.4% kcal fat, 52% kcal sucrose, AIN-76A, Harlan Teklad), the low-fat, high-cholesterol diet (LFHC, 12.4% kcal fat, 52% kcal sucrose, 0.2% cholesterol, TD.09801, Harlan Teklad) or the LFHC diet supplemented with 3% wt/wt naringenin. Food intake and body weight measurements were conducted three times per week. Caloric intake was calculated as weight of food eaten (g) multiplied by the caloric content of each diet (Chow; 3.1 kcal/g, HFHC; 4.5 kcal/g, LF and LFHC; 3.8 kcal/g).

Blood and tissue collection

Mice were fasted for 6 hours before sacrifice. At sacrifice, animals were anesthetized with Avertin (0.015-0.017 mL/g body weight) and blood was collected via cardiac puncture in syringes containing 80 µl of 7% Na_2-EDTA. Blood was centrifuged at 14,000 rpm for 5 minutes at 4°C to separate the plasma, which was stored at -20°C for further use. To dissect the heart for histological analysis, the left ventricle was perfused with PBS (10 units/mL) and the right atrium was cut to drain the perfusate. The heart and full-length aorta were dissected together. The top half of the heart was removed, placed in Optimum Cutting Temperature (OCT) medium and frozen on dry ice. The full-length aortae were dissected, rinsed and cleaned using a dissecting microscope to remove any adipose tissue. A subset was snap frozen in liquid nitrogen and stored at -80°C, for mRNA and lipid measurements. Remaining aortae were fixed in 10% formalin for 24 hours for histology. All other tissues including the liver, muscle and adipose tissue, were removed, weighed, snap frozen in liquid nitrogen and stored at -80°C.

Plasma and tissue analysis

Plasma concentrations of insulin, leptin (ALPCO Diagnostics, Windham, NH) and SAA (Invitrogen, Life Technologies, Mississauga, ON) were determined in plasma samples by

mouse-specific ELISA as per manufacturer's instructions. Plasma concentrations of triglyceride and total cholesterol were measured enzymatically (Roche Diagnostics, Laval, QC) on a Cobas Mira S autoanalyzer (Roche Diagnostics).

Fresh-EDTA plasma (50 µl) was separated by Fast Performance Liquid Chromatography (FPLC) using an AKTA purifier and a Superose 6 column. A constant flow rate of 0.4 mL/min was used to collect 500 µl fractions. A 50 µl aliquot was used to measure cholesterol and triglyceride enzymatically in both samples and standards on a microtitre plate with 150 µl reagent (triglyceride, Roche Diagnostics; cholesterol, Wako, Richmond, VA).

Hepatic and aortic lipids were extracted from 100 mg pieces of liver and whole aortas weighing approximately 20 mg, using the method of Folch et al.(1). Quantitation of hepatic and aortic lipids was determined as described previously(2). Briefly, [^{3}H] cholesteryl oleate (Amersham, GE Healthcare, Oakville, ON) was added to lipid extracts to assess recovery. A combined 200 µg/mL triolein and 200 µg/mL cholesterol standard (0-64 µg) was prepared in isopropanol and aliquoted for standard curves. Aliquots of standards and samples were dried under N_2 and 400 µl of chloroform added. Samples and standards were dried under N_2 and 500 µl of a 1% Triton X-100 solution in chloroform added. Following solublization, samples and standards were capped, left at room temperature for 1 hour, and dried under N_2. Fifty microliters of deionized water was added and incubated at 37°C for 15 minutes. Triglyceride, total cholesterol, and free cholesterol were determined by enzymatic, colorimetric assays (triglyceride, Roche Diagnostics; total cholesterol and free cholesterol, Wako) as per manufacturer's instructions. Cholesteryl ester was determined as the difference between total cholesterol and free cholesterol. Gallbladder bile was collected from mice fasted for 4 hours prior to sacrifice and snap-frozen in liquid nitrogen. A measured volume (5–10 µl) of bile was extracted with 2:1 CHCl3/MeOH. The aqueous phase was analyzed for BA content using an enzymatic assay employing hydroxysteroid dehydrogenase as described previously (3).

Fatty acid and cholesterol synthesis were measured following *i.p.* injection of radiolabeled acetic acid (Amersham Canada Ltd). Briefly, mice fasted for 6 hours were injected with 20 µCi of [1-^{14}C]-acetic acid and sacrificed 1 hour later. Tissues (500 mg) were extracted in chloroform:methanol and incorporation of [1-^{14}C]-acetic acid into fatty acid and cholesterol were assayed as described previously (4,5).

For fatty acid oxidation, fresh liver and muscle (soleus) tissue was homogenized in 0.1 M phosphate buffer containing 0.25 M sucrose and 1 mM EDTA. Homogenates were incubated for 30 min (liver) and 45 min (muscle) at 37ºC in a buffer containing 150 mM KCl, 10 mM Hepes

(pH 7.2), 5 mM Tris malonate, 10 mM $MgCl_2$, 1 mM carnitine, 0.15% fatty acid free bovine serum albumin (FAF-BSA), 5 mM ATP and 50 μM ^{3}H-palmitate (Perkin Elmer, Waltham, MA) complexed with FAF-BSA. Reactions were stopped with 200 μL 0.6 N perchloric acid and unreacted fatty acids extracted with n-hexane. ^3H_2O in the aqueous phase was measured by liquid scintillation counting (4,6).

Glucose and insulin tolerance tests

Glucose tolerance tests (GTT) were conducted in mice fasted for 6 hours and injected intraperitoneally (*i.p*) with a 15% D-glucose solution in 0.9% NaCl at a dose of 1 g/kg body weight. Blood was collected from the saphenous vein and blood glucose was measured using a hand-held glucometer (Acenscia Elite, Bayer Healthcare, Toronto, ON) at 0, 15, 30, 45, 60, 90, and 120 minutes post-injection. For insulin tolerance tests (ITT), after a 5 hour fast, mice were injected *i.p.* with 0.6 IU/kg Novolin ge Toronto (Novo Nordisk, Mississauga, ON). Blood was collected from the saphenous vein and blood glucose was measured as stated above every 15 minutes for one hour. Glucose utilization and insulin sensitivity were calculated based on the absolute area under the curve (AUC).

Triglyceride and apoB100 secretion; triglyceride and cholesterol absorption.

Triglyceride and apoB100 secretion into plasma was determined in mice fed the experimental diets (6 mice per group) for 4 weeks. The rate of triglyceride secretion into plasma was measured in mice fasted for 4 hours and injected *i.p.* with 1 g/kg Tyloxapol USP (0.1 g/mL in 0.9% saline) (Ruger Chemical Company, Linden, NJ). Following the injection, mice were sacrificed by CO_2 inhalation; blood was collected at 0, 60 and 120 min by cardiac puncture and analyzed for triglyceride. For apoB100 secretion measurements, mice were injected *i.p.* with 1 g/kg Tyloxapol USP and 200 μCi of Tran ^{35}S-label (1000 Ci/mmol, L-[^{35}S]-methionine and L-[^{35}S]-cysteine, MP Biomedicals Inc., Irvine, CA). Following sacrifice by CO_2 inhalation, plasma samples were obtained by cardiac puncture at 60 and 120 minutes (7). A combined VLDL/IDL fraction (density <1.019 g/mL) was isolated from 250 μl of fresh plasma by ultracentrifugation at 120,000 rpm for 4 hours using a Beckman TLA 120.2 rotor. VLDL/IDL fractions were separated on 4.5% SDS-PAGE gels, fixed, dried and placed on a phosphorimager screen. Band volumes corresponding to apoB48 and apoB100 were quantitated using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA) (8).

Triglyceride and cholesterol absorption was determined in mice fed the experimental diets for 4 weeks as described previously (4,9). Mice were administered, by gavage, 150 μL of medium chain triglyceride (MCT) oil (Novartis Medical Nutrition, Fremont, MI) containing 1 μCi of [4-^{14}C]-Cholesterol (Perkin Elmer) or 2.5 μCi of glycerol tri[1-^{14}C]oleate (Amersham, Piscataway NJ)

and 2.0 µCi of [5,6-^{3}H]-β-sitostanol (American Radiolabeled Chemicals Inc., St Louis. MO). Mice were housed individually in metabolic cages for 72 hours and feces collected every 24 hours. Fecal samples were saponified in 95% ethanol/10N KOH and neutral lipids extracted with hexane. The ratio of [^{14}C] to [^{3}H] was used to calculate the percentage of lipid absorbed. In all animals, the recovery of radiolabeled sitostanol was 75-90%.

Energy expenditure

Energy expenditure, respiratory quotient, and activity (ambulatory and steriotypic) were assessed by using the Comprehensive Laboratory Animal Monitoring system (CLAMS) (Columbus Instruments, Columbus, OH). Mice were placed in cages with free access to food and water and acclimatized for 24 hours. For the subsequent 24 hours, every 10 minutes, data on O_2 consumption (VO_2; mL/hour/kg lean body weight) and CO_2 production (VCO_2; mL/hour/kg lean body weight) were collected. The respiratory exchange ratio (RQ) was derived from the ratio of VCO_2 to VO_2, and energy expenditure (EE) was determined as: (3.815 + 1.232 X respiratory quotient) X VO_2 and expressed as kcal/hour/kg lean body mass. Lean body mass was calculated from whole body composition analysis conducted by micro-CT imaging using a *Locus Ultra* micro-CT scanner (GE Healthcare, London, ON) (10).

Tissue sectioning and histology

Immediately after dissection, a small piece of liver was placed in OCT medium and frozen at -80°C. Frozen liver samples were sectioned at 8 µm using a Leica CM3050S cryostat (Leica Microsystems, Concord ON). Liver sections were then stained with Oil Red O. Briefly, sections were fixed with 40% formaldehyde for 1 minute. A 0.5% stock solution of Oil Red O dissolved in isopropanol was prepared, and a working solution (30 mL of stock solution and 20 mL of PBS) was used to stain liver sections for 10 minutes, followed by a rinse in deionized water and counter-stained using hematoxylin. Liver sections were also stained with Hoechst 33258 (2.5 µg/mL, Sigma-Aldrich). Lipid droplets (Oil Red O) and nucleus (Hoechst) were visualized by fluorescence microscopy at 493 nm excitation.

Immunohistochemistry (IHC) was conducted on liver sections (frozen) for the macrophage marker MAC-2. Briefly, slides were fixed in acetone, rinsed in PBS and quenched with peroxidase blocking agent (3% H_20_2 in methanol). Slides were blocked in 10% BSA and incubated with rat anti-mouse MAC-2 antibody (1:1000) (Cedarlane, Burlington, ON) for 1 hour, followed by an incubation with a secondary antibody (biotinylated goat anti-rat IgG antibody, 1:200) (Vector, Burlington, ON) for 30 minutes. Sections were incubated in ABC reagent (ABC Elite Standard Kit, Vector) followed by a short incubation in DAB substrate (DAB peroxidase

substrate kit, Vector) and counterstained in hematoxylin. Slides were cover-slipped using Cytoseal xylene-based mounting medium (Thermo Fisher Scientific, Nepean, ON). All photomicrographs were generated using an Olympus BX50 microscope with a QImaging Retiga EXi FAST camera (QImaging, Surrey, BC).

White adipose tissue (WAT) was fixed in 10% formalin, embedded in paraffin, sectioned using a Microm HM335E Microtome (Thermo Fisher Scientific) and stained with hematoxylin and eosin. Adipocyte diameter was calculated using Northern Eclipse 7.0 software on 20x magnification photomicrographs.

Frozen aortic sinus was prepared in cross-section by serial sectioning (90-100 sections per heart, 10μm) beginning at the aortic valves using a cryostat. In a similar protocol as described above for MAC-2 staining, IHC was performed on sections of the aortic sinus for the presence of macrophages using the rat anti-mouse monocyte/macrophage antibody-2 (MOMA-2, Accurate Chemical and Scientific Corporation, Westbury, NY). In other sections of the aortic sinus, a modified Verhoff and Masson's Trichrome stain was used to detect the presence of collagen (green) and elastin (black), and IHC was performed to detect the presence of smooth muscle cells (SM α-actin antibody, Clone 1AH, Sigma-Aldrich) at the Robarts Research Institute, Molecular Pathology Core Facility. The aortic sinus was also stained with Oil Red O to detect the presence of lipid and plaque area was quantitated (3-4 serial sections per aortic sinus) using Axiovision 4.8 software (Carl Zeiss Canada Ltd). For morphometric analysis of atherosclerotic plaques the relative area of lesions that stained positive for MOMA-2, collagen (trichrome) and SM α-actin was determined relative to the total area of the respective plaque using Axiovision 4.8 software. Lesion analysis began at the origin of the aortic valves to ensure that a standard region was measured in each mouse.

Full-length aortas were stained with Sudan IV (Sigma-Aldrich). In brief, the aorta was washed in distilled water, incubated in 70% ethanol and placed in Sudan IV for 15 minutes, followed by de-staining in 80% ethanol. A longitudinal slice from the aortic arch to the abdominal aorta was made to expose the plaque on the intimal surface (*en face* preparation). Aortas were mounted using glycerol gelatin (Sigma-Aldrich). Stained lesion area was quantitated using Axiovision 4.8 software by dividing the stained plaque area by the total area of the aortic arch (ascending and descending aorta), expressed as a percentage.

Gene expression analysis

RNA was isolated from liver, adipose tissue, aorta and peritoneal macrophages using the TRIzol reagent (Invitrogen, Grand Island, NY) and mRNA concentrations were quantitated using an

ND-1000 nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcribed total RNA at 10-50 ng/uL from each mouse were assayed in triplicate in 20 µL reactions using a two-step quantitative real time-PCR protocol. Gene-specific mRNA quantitation was determined for all genes; (*Ccl3 , Ccl2, Saa1/2, Tnfa, F4/80, Il1b, Il6, Soat2, Acox, Srebf2, Srebf1c, Hmgcr, Pgc1a, Cpt1a, Fgf21, Abcg5, Abcg8, Abca1, Abcg1* and *Cyp7a1*) on an ABI Prism (model 7900 HT) Sequence Detection System (Applied Biosystems, Streetsville, ON) according to the manufacturer's instructions. All primer probes sets were manufactured by Taqman® Assays-on-Demand (Applied Biosystems) except *Srebf1c* which was designed from the *Mus musculus Srebf1* sequence (Genbank accession no. AL669954) as described previously (4). The probe for *Srebf1c* (Applied Biosystems) overlapped the boundary between exon 1 specific for *Srebf1c* and exon 2. *Srebf1c* forward primer: CAGGCCCGGGAAGTCACT, reverse primer: GACCACGGAGCCATGGATT, and probe: ATTTGAAGACATGCTCCA (Gene is on the minus strand and primers have been designed as such).

Peritoneal macrophages

In a separate study, male *Ldlr*$^{-/-}$ mice 8-12 weeks of age (n= 16/group) were fed either standard chow or the HFHC diet ± 3% (wt/wt) naringenin. After 12 weeks, mice were injected *i.p.* with 2.5 mL 4% Brewer's Thioglycollate Medium (Sigma-Aldrich), and 5 days later mice were sacrificed via CO_2 inhalation. Each mouse was dissected down the midline to expose the intact peritoneal cavity and 10 mL of cold PBS was injected, followed by light gentle massage of the abdomen. The PBS mixture was carefully removed and centrifuged at 1500 rpm for 10 minutes at 4°C. Cells were resuspended in serum-free Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with gentamycin (40 mg/mL, Merck-Frosst, Kirkland, QC) and fungizone (20 µg/mL, Invitrogen). Cells were placed in 35mm (6-well plates) at a density of 2.76-3.3x10^6 per well and were allowed to adhere for approximately 2 hours, after which cells were washed 3 times with PBS.

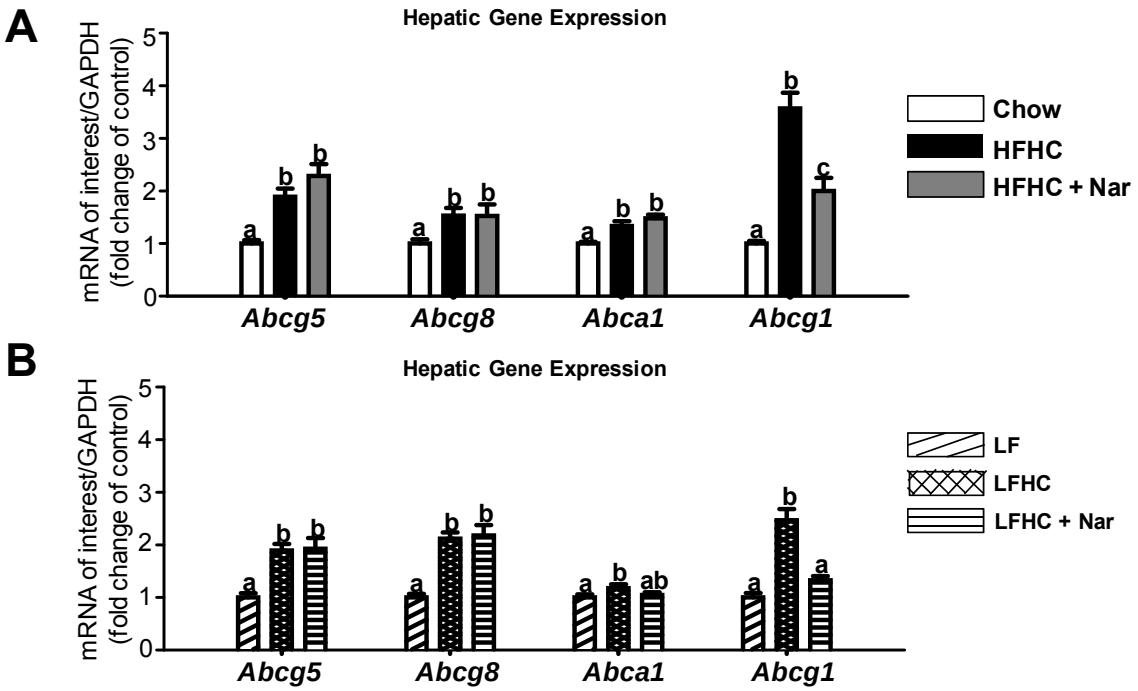
A subset of peritoneal macrophages were plated into wells containing cover slips at the same density as described above and allowed to adhere for 2 hours. Cells were washed with PBS and stained with Oil Red O. After washing 3 times in PBS, the cover slip was removed from the well and mounted on a glass slide using Aquatex aqueous mounting medium (VWR, Bridgeport NJ). Photomicrographs were generated using an Olympus BX50 microscope with a QImaging Retiga EXi FAST camera.

Cellular total cholesterol, cholesteryl ester and triglyceride mass in peritoneal macrophages were determined in cells extracted with 3:2 (vol:vol) hexane:isopropanol (11). Cellular protein

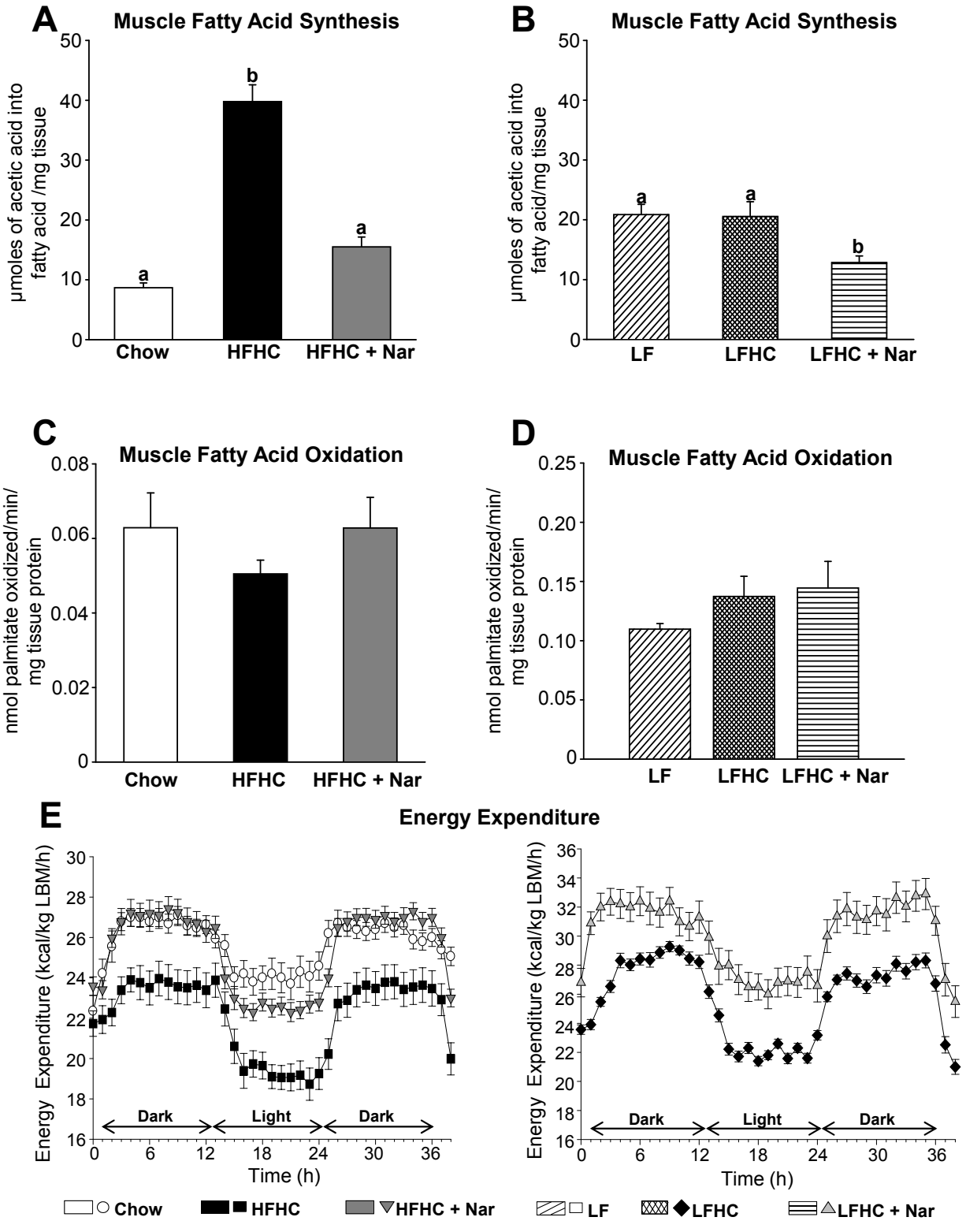
was extracted by 0.1N NaOH and was determined using a modified Lowry method (12). Standard solutions were made from 200 µg/mL cholesterol and 200 µg/mL triolein in isopropanol at concentrations of 1-20 µg. Samples and standards were dried under N_2. For standards, 1 mL of chloroform and 1 mL of 1% Triton X-100 was added. For samples 300 µl of $CHCl_3$ and 300 µl of 1% Triton were added. Samples and standards were dried under N_2 and were resolubilized in 500 µl (standards) and 150 µl (samples) of deionized water. Triglyceride, total cholesterol, and free cholesterol were determined by enzymatic, colorimetric assays (triglyceride, Roche Diagnostics; total cholesterol and free cholesterol, Wako) as per manufacturer's instructions. Cellular cholesterol ester was determined by subtracting free cholesterol from total cholesterol. Cellular lipids were normalized to cell protein.

Statistical analysis

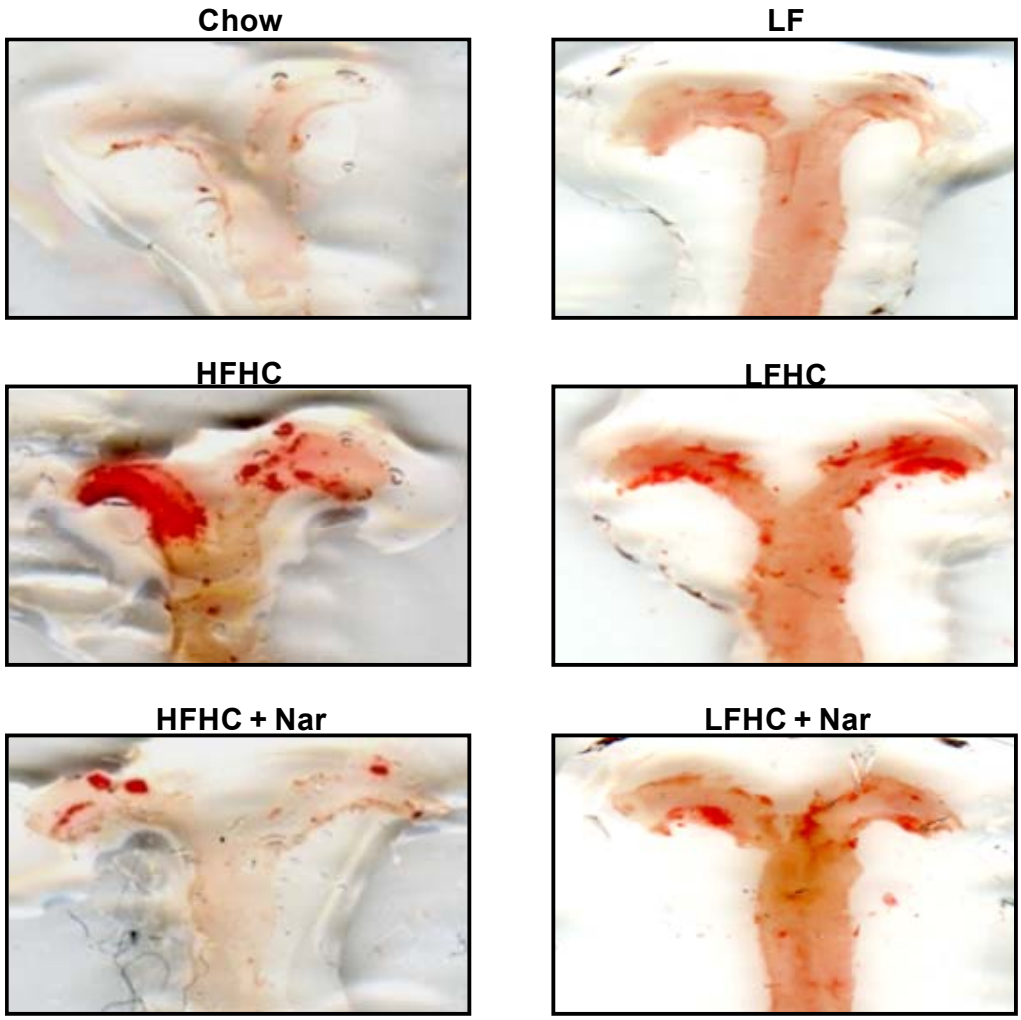
Data is presented as the mean ± SEM. A one-way ANOVA and post hoc Tukey test was performed using Sigma Plot version 14.0 to determine statistical significance. Different letters indicate statistical significance within each separate study ($P<0.05$); Chow, HFHC, and HFHC + Nar or LF, LFHC, and LFHC + Nar. A two-sample t-test was also employed to test the pre-specified hypothesis that there is a difference between Chow and LF groups ($P<0.05$).



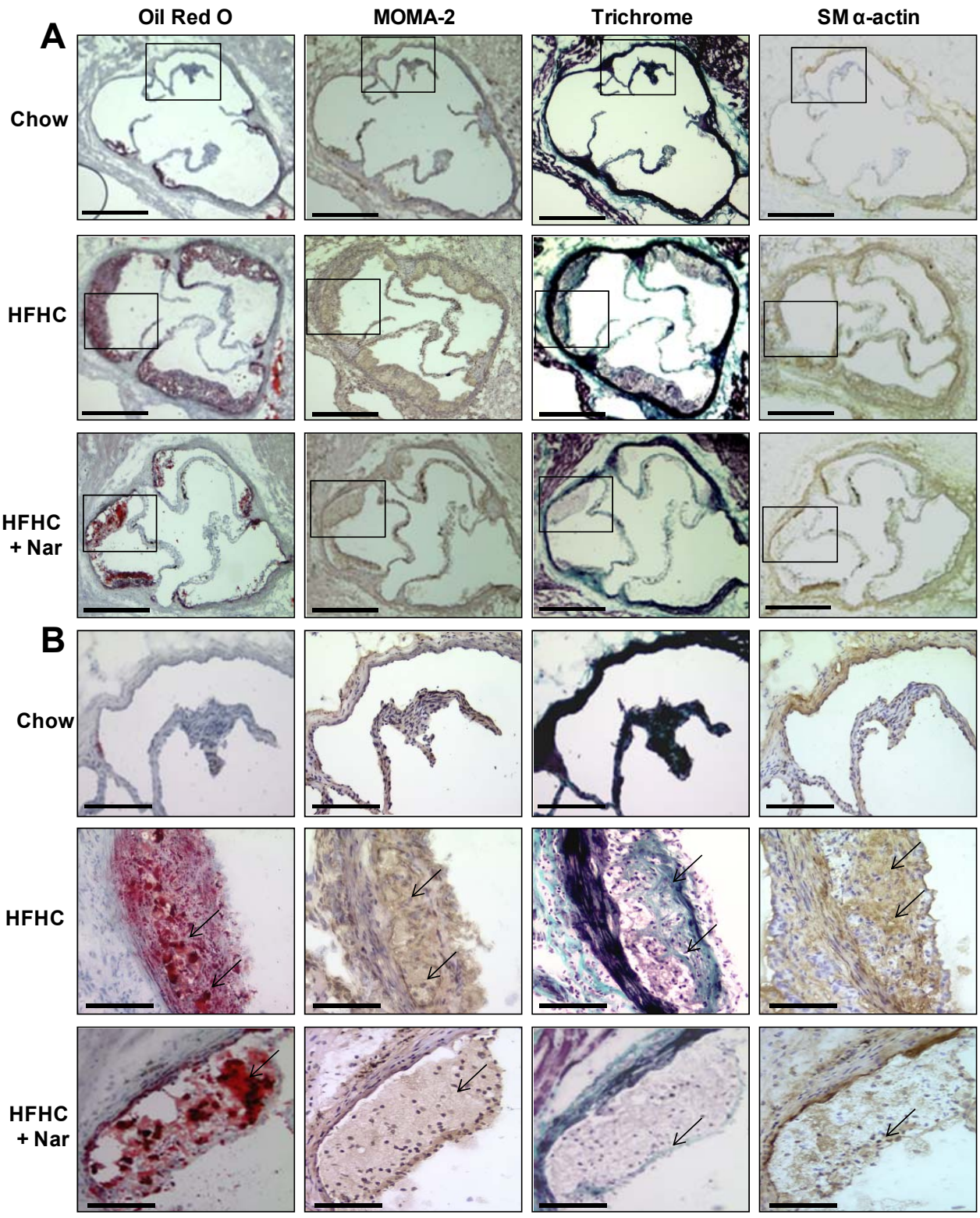
Supplementary Figure I. Hepatic gene expression in cholesterol-fed mice treated with naringenin. (A) and (B) Hepatic mRNA expression (n= 10-12/group). Values are the mean ± SEM. Different letters are statistically different (*P* <0.05).


A
Muscle Fatty Acid Synthesis
µmoles of acetic acid into fatty acid /mg tissue
50
40
30
20
10
0
a
b
a
Chow
HFHC
HFHC + Nar
B
Muscle Fatty Acid Synthesis
µmoles of acetic acid into fatty acid/mg tissue
50
40
30
20
10
0
a
a
b
LF
LFHC
LFHC + Nar
C
Muscle Fatty Acid Oxidation
nmol palmitate oxidized/min/ mg tissue protein
0.08
0.06
0.04
0.02
0
Chow
HFHC
HFHC + Nar
D
Muscle Fatty Acid Oxidation
nmol palmitate oxidized/min/ mg tissue protein
0.25
0.20
0.15
0.10
0.05
0
LF
LFHC
LFHC + Nar
E
Energy Expenditure
Energy Expenditure (kcal/kg LBM/h)
30
28
26
24
22
20
18
16
Dark
Light
Dark
0
6
12
18
24
30
36
Time (h)
Energy Expenditure (kcal/kg LBM/h)
36
34
32
30
28
26
24
22
20
18
16
Dark
Light
Dark
0
6
12
18
24
30
36
Time (h)
Chow
HFHC
HFHC + Nar
LF
LFHC
LFHC + Nar

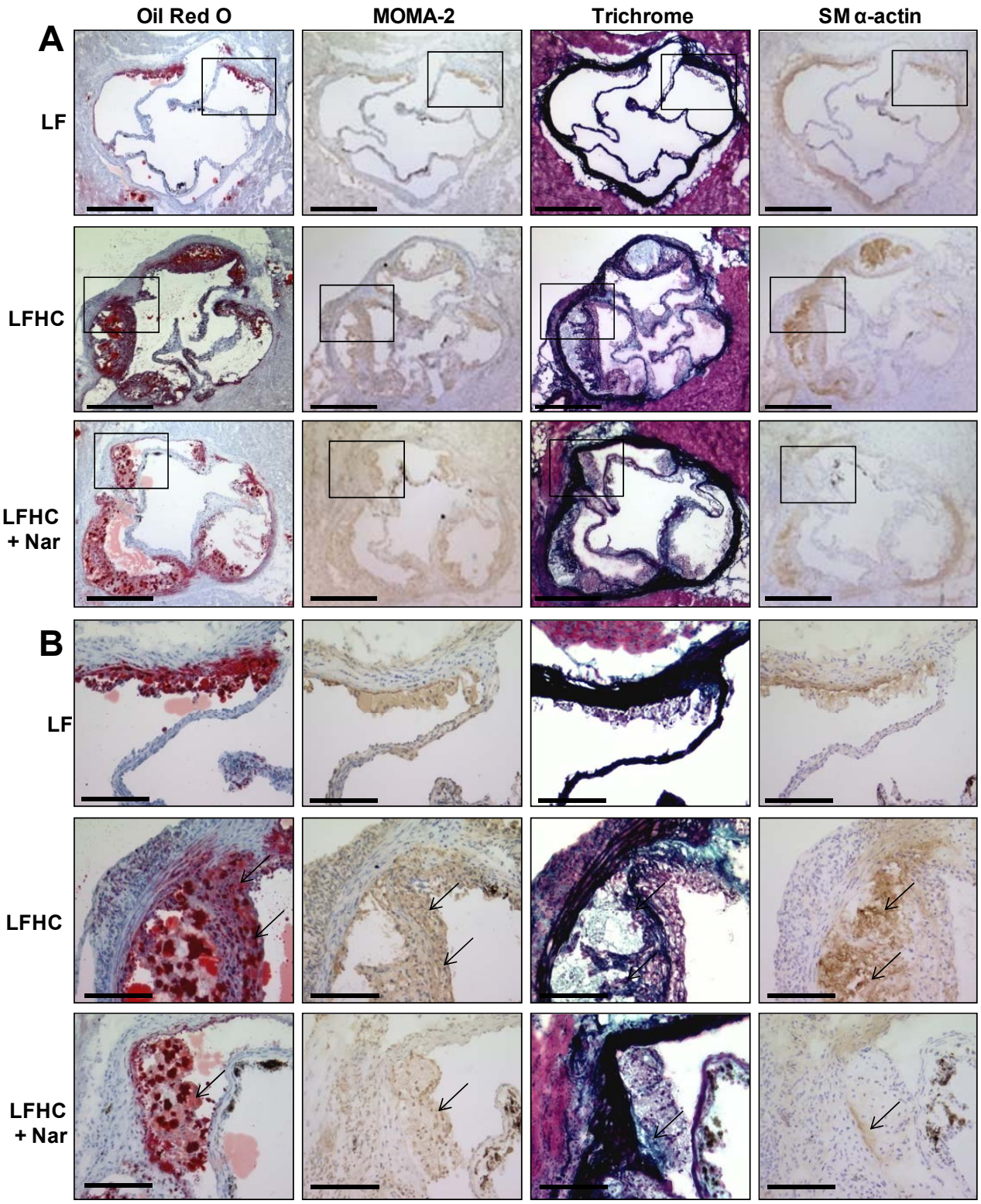
Supplementary Figure II. Muscle fatty acid metabolism and energy expenditure in cholesterol-fed mice. (A) and (B) FA synthesis in the soleus muscle obtained 60 min post *i.p.* injection with [^{14}C]acetic acid (n = 6-8/group). (C) and (D) FA oxidation in the soleus muscle (n = 6-8/group) determined by [^{3}H]palmitate conversion to H_2O. Values are the mean ± SEM. (E) Energy expenditure determined by indirect calorimetry (CLAMS) during both the light and dark cycles (7:00 pm – 7:00 am). Measurements were collected every 10 minutes and each data point, expressed as kcalories per kg lean body mass (LBM)/hour, represents the average of six-10 minute measurements, and plotted as the mean ± SEM for all mice (n=6/group). Different letters are statistically different (P <0.05).



Supplementary Figure III. Atherosclerotic plaque in the aortic arch of cholesterol-fed animals supplemented with naringenin. Representative photomicrographs of atherosclerotic lesions in the *en face* preparation of the aortic arch stained with Sudan IV.


A
Oil Red O
MOMA-2
Trichrome
SM α-actin
Chow
HFHC
HFHC
+ Nar
B
Chow
HFHC
HFHC
+ Nar

Supplementary Figure IV. Atherosclerotic plaque morphology in HFHC-fed animals supplemented with naringenin. (A) and (B) Photomicrographs of serial sections of the aortic sinus stained with Oil Red O and hematoxylin, monocyte/macrophage antibody-2 (MOMA-2), trichrome or antibody to smooth muscle (SM) α-actin. Size bar = 500 μm (A) and 250 μm (B). Arrows indicate positive stain for lipid (Oil Red O), macrophages (MOMA-2), collagen (trichrome) and smooth muscle cells (SM α-actin).


Oil Red O
MOMA-2
Trichrome
SM α-actin
A
LF
LFHC
LFHC
+ Nar
B
LF
LFHC
LFHC
+ Nar

Supplementary Figure V. Atherosclerotic plaque morphology in LFHC-fed animals supplemented with naringenin. (A) and (B) Photomicrographs of serial sections of the aortic sinus stained with Oil Red O and hematoxylin, monocyte/macrophage antibody-2 (MOMA-2), trichrome or antibody to smooth muscle (SM) α-actin. Size bar = 500 μm (A) and 250 μm (B). Arrows indicate positive stain for lipid (Oil Red O), macrophages (MOMA-2), collagen (trichrome) and smooth muscle cells (SM α-actin).

Supplementary Table I

Supplementary Table I. Diet Composition

Formula	Chow Diet TD 8604	High-Fat, High-Cholesterol (HFHC) Diet TD 09268	Low-Fat (LF) Diet TD AIN-76A	Low-Fat, High-Cholesterol (LFHC) Diet TD 09801
		g/kg	g/kg	g/kg
Casein	*See represented ingredients, exact amounts not specified	195	200	200
DI-Methionine		3	3	3
Sucrose		341.46	497.99	497.99
Cornstarch		150.75	150	150
Corn Oil			50	50
Anhydrous Milk fat		160		
Lard, (Pork)		50		
Cellulose		35.13	50	50
Mineral Mix		50	35	35
Zinc Carbonate		0.04		
Vitamin Mix		10	10	10
Choline Bitartrate		3	2	2
Ethoxyquin		0.02	0.01	0.01
Macronutrients by % by weight (% calories)				
Protein	24.3 (32)	17.3 (15.2)	17.7 (18.8)	17.7 (18.8)
Carbohydrate	40.2 (54)	48.7 (42.8)	64.9 (68.8)	64.9 (68.8)
Fat	4.7 (14)	21.2 (42.0)	5.2 (12.4)	5.2 (12.4)
Kcal/g	3.0	4.5	3.8	3.8
Cholesterol	0.005%	0.2%	0.0%	0.2%

*Represented Ingredients: Dehulled soybean meal, wheat middlings, flaked corn, ground corn, fish meal, soybean oil, brewers dried yeast, cane molasses, dried whey, dicalcium phosphate, calcium carbonate, iodized salt, choline, chloride, magnesium oxide, Vitamin A acetate, Vitamin D3, Vitamin E, niacin, pyridoxine hydrochloride, menadione, sodium bisulfite, Vitamin B, 12, manganous oxide, ferrous ,sulfate copper sulfate, zinc oxide, calcium, iodate, cobalt carbonate, chrominum, potassium sulfate, kaolin

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