Supplemental Data Mulvihill et al. Diabetes 60:1446–1457, 2011

Supplemental Methods

Immunoprecipitation and Immunoblotting. HepG2 cells were incubated with nobiletin (10μM) or insulin (100nM) for 0-60 min. Cell lysates were isolated, precleared and IR and IRS-1 immunoprecipitated, separated by SDS-PAGE and quantitated by immunoblot analysis using p-TYR antibody or antibodies to IR and IRS-1 (Millipore, Billerica, MA). HepG2 cells were treated for 24h (for apoB100) or 0-30 min (pERK and ERK) with insulin (100nM) or nobiletin (10μM).

PPRE Luciferase Activity. HepG2 cells transfected with 0.01μg/ml human PPARα,γ or δ.SG5 expression vectors and reporter gene plasmids, 0.5μg/ml of pTK-PPRE(X3)-Luc and 0.05μg/ml of the TK promoter-*Renilla* luciferase construct, (tk.pRL) (provided by Dr. John Capone, McMaster University, Hamilton ON). Cells were incubated for 24h with DMSO, nobiletin (10μM) or the appropriate PPAR agonist: PPARα (10nM GW7647, Sigma); PPARγ (3μM Rosiglitazone, Cayman Chemicals, Ann Arbor, MI), PPARδ (20nM GW501516, Calbiochem). Cell lysates were prepared (1) and the Luciferase activity (relative light units (RLU)) measured and normalized to the Renilla activity (2).

Blood and Tissue Collection and Biochemical Determinations. Blood was obtained during the GTT and ITT from the saphenous vein. At sacrifice, blood was obtained by cardiac puncture and tissues harvested as previously described (3). Plasma triglyceride (TG), glycerol, total cholesterol (Roche Diagnostics, Laval, Canada), non-esterified fatty acids (NEFA) (Wako Chemicals, Richmond, VA), glucose (Ascensia Elite, Bayer Healthcare, Toronto, Canada), insulin and leptin (Alpco Diagnostics, Windham, NH) were determined as previously described (3). Lipids were measured in liver and intestine as previously described (4). For atherosclerosis studies, mice were anaesthetized with Avertin and tissues isolated as previously reported (5).

Gene expression by qRT-PCR. Tissue and cellular mRNA levels were determined by quantitative real-time polymerase chain reaction (qRT-PCR) as published previously (3). Primer and probe sets were obtained from Applied Biosystems (Streetsville, CA) inventoried gene expression arrays with the exception of murine *Srebf1c* (*Srebp1c*) which was generated as described previously (3).

Hyperinsulinemic Euglycemic Clamp Procedure. Male *Ldlr*$^{-/-}$ mice, 8-10 per group, were fed the western diet or the western diet + 0.3% nobiletin for 8 weeks. Five days before the experiment, mice were anesthetised under isoflurane and catheters were inserted into the left common carotid artery and right jugular vein for blood sampling and infusions, respectively. Body weight was recorded daily. Mice within 90% of pre-surgery weight by day 5 post-surgery were included in the study. Clamps were performed as described previously (6,7). In brief, on the day of the clamp, mice were fasted for 5 h before the procedure and an intravenous infusion catheter was connected to a swivel 1 h prior to the start of the procedure so that the mice were unrestrained and conscious for the duration of the experiment. A blood sample was obtained to determine initial glucose and insulin concentrations. The protocol consisted of a 90-min tracer equilibration period (t = -90 to 0 min) followed by a 120 min hyperinsulinemic euglycemic clamp (t = 0 to 120 min). A 50μl blood sample was obtained at t = -90 min to determine radioactive-free plasma activity. A 5μCi bolus of [3-^{3}H]-glucose was given at t = -90 min followed by a 0.05 μCi.min^{-1} infusion for 90 min (t = -90 to 0 min). At t = -30, -20, -10 and 0 min, blood samples were taken for the assessment of basal glucose and glucose specific activity. The insulin infusion began at t = 0 min with a primed-continuous infusion of human insulin (16 mU.kg^{-1} bolus followed by a 2.5 mU.kg^{-1}.min^{-1} infusion; Humulin R; Eli Lilly, Indianapolis, IN), to achieve plasma insulin concentrations 6-fold over basal concentrations. At t = 0 min, the [3-^{3}H]-glucose infusion was increased to 0.10 μCi.min^{-1} for the remainder of the experiment to minimize changes in specific activity from the equilibration period. Euglycemia (6.0-7.0mM) was maintained during the clamps by measuring blood glucose every 10 min starting at t = 0 min and infusing 20% glucose as necessary. Blood samples were taken every 10 min from t = 90 to 120 min for the determination of glucose specific activity. Clamp insulin concentrations were determined from samples obtained at t = 120 min. Mice received saline-washed erythrocytes from donor mice throughout the experimental period (5–6 μl.min^{-1}) to prevent a fall of ≥5% hematocrit. Hepatic glucose production (HGP) and peripheral glucose disposal (R_d) were determined using Mari's non-steady-state equations

©2011 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0589/-/DC1

(7). Clamp glucose production was determined by subtracting the glucose infusion rate (GIR) from total glucose production. Clamp studies were performed in the laboratory of Dr. André Marette, Laval University and all protocols were performed in accordance with the Canadian Guide for the Care and Use of Laboratory Animals and were approved by Laval University Council for Animal Care.

VLDL Secretion Rate. Mice were fasted for 4 h before intra-peritoneal (IP) injection with 1000mg/kg Tyloxapol U.S.P. (0.15g/ml in 0.9% NaCl) (Ruger Chemical Company, Irvington, NJ). Mice were conscious and unrestrained for the entire procedure. Tyloxapol coats lipoproteins preventing TG hydrolysis and VLDL clearance. Samples were collected at 0 and 120 min post-injection by cardiac puncture and a combined VLDL and IDL fraction was isolated from 200 µl of plasma by ultracentrifugation (Beckman TLA-120.2; 100,000 rpm, 6 h at $d < 1.019$ g/ml). Plasma and VLDL/IDL fractions were analyzed for TG as described above.

Fatty acid oxidation. Cellular and tissue fatty acid oxidation was performed using minor medications of methods described previously (8). Briefly, HepG2 cells were incubated in 24 well plates in 300 µl media and DMSO, nobiletin (10 µM) or AICAR (500 µM) for 6 h or 17 h at 37°C, followed by the addition of 2.0 µCi/mL [^{3}H]-palmitate in 100 µM palmitate per well for 15 minutes. The media was removed and 10% trichloroacetic acid was added. Unreacted FAs were extracted from the supernatant with n-hexane and the remaining counts determined by scintillation counting. Data was determined as nmol palmitate oxidized/min/mg protein. For tissue, 250 mg of fresh liver tissue was homogenized and incubated with [^{3}H]-palmitate as previously described (8). Reactions were stopped with addition 0.6N perchloric acid and unreacted fatty acids were extracted with n-hexane and $^{3}H_2O$ measured by liquid scintillation counting.

Energy expenditure. Mice were placed in cages for 24 h with free access to food and water. Every 15 min, data on O_2 consumption (VO_2; ml/h/mouse) and CO_2 production ($VCO_{2;}$ ml/h/mouse) were collected (3). The respiratory quotient (RQ) was derived from the ratio of VO_2 to VCO_2, and energy expenditure was determined as: (3.815 + 1.232 X respiratory quotient) X VO_2 and expressed as kcal/h/mouse.

Quantification of atherosclerosis lesions. At sacrifice, hearts were dissected, mounted in Optimum Cutting Temperature media (OCT) and frozen. Frozen serial sections (70-100 per heart, 8µm) of the aortic sinus, initiating at the origin of the aortic valves, were prepared using a Leica CM 3050S cryostat. Sections were stained with Oil-Red-O, counterstained with hematoxylin and lesion area quantitated as previously described (5).

Supplemental References

1. Allister EM, Borradaile NM, Edwards JY, Huff MW: Inhibition of microsomal triglyceride transfer protein expression and apolipoprotein B100 secretion by the citrus flavonoid naringenin and by insulin involves activation of the mitogen-activated protein kinase pathway in hepatocytes. *Diabetes* 54:1676-1683, 2005

2. Argmann CA, Edwards JY, Sawyez CG, O'Neil CH, Hegele RA, Pickering JG, Huff MW: Regulation of macrophage cholesterol efflux through hydroxymethylglutaryl-CoA reductase inhibition: a role for RhoA in ABCA1-mediated cholesterol efflux. *J Biol Chem* 280:22212-22221, 2005

3. Mulvihill EE, Allister EM, Sutherland BG, Telford DE, Sawyez CG, Edwards JY, Markle JM, Hegele RA, Huff MW: Naringenin prevents dyslipidemia, apolipoprotein B overproduction, and hyperinsulinemia in LDL receptor-null mice with diet-induced insulin resistance. *Diabetes* 58:2198-2210, 2009

4. Telford DE, Lipson SM, Barrett PH, Sutherland BG, Edwards JY, Aebi JD, Dehmlow H, Morand OH, Huff MW: A novel inhibitor of oxidosqualene:lanosterol cyclase inhibits very low-density lipoprotein apolipoprotein B100 (apoB100) production and enhances low-density lipoprotein apoB100 catabolism through marked reduction in hepatic cholesterol content. *Arterioscler Thromb Vasc Biol* 25:2608-2614, 2005

5. Mulvihill EE, Assini JM, Sutherland BG, Dimattia AS, Khami M, Koppes JB, Sawyez CG, Whitman SC, Huff MW: Naringenin decreases progression of atherosclerosis by improving dyslipidemia in high-fat-fed low-density lipoprotein receptor-null mice. *Arterioscler Thromb Vasc Biol* 30:742-748

©2011 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0589/-/DC1

6. Ayala JE, Bracy DP, McGuinness OP, Wasserman DH: Considerations in the design of hyperinsulinemic-euglycemic clamps in the conscious mouse. *Diabetes* 55:390-397, 2006

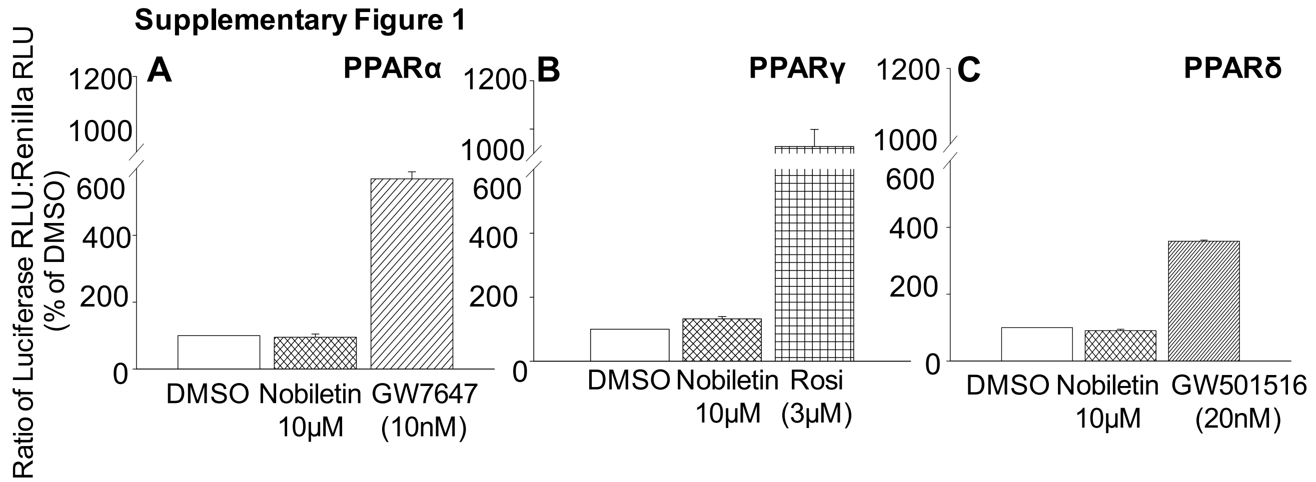
7. Charbonneau A, Marette A: Inducible nitric oxide synthase induction underlies lipid-induced hepatic insulin resistance in mice: potential role of tyrosine nitration of insulin signaling proteins. *Diabetes* 59:861-871, 2010

8. Huss JM, Torra IP, Staels B, Giguere V, Kelly DP: Estrogen-related receptor alpha directs peroxisome proliferator-activated receptor alpha signaling in the transcriptional control of energy metabolism in cardiac and skeletal muscle. *Mol Cell Biol* 24:9079-9091, 2004

©2011 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0589/-/DC1

Supplemental Figure 1

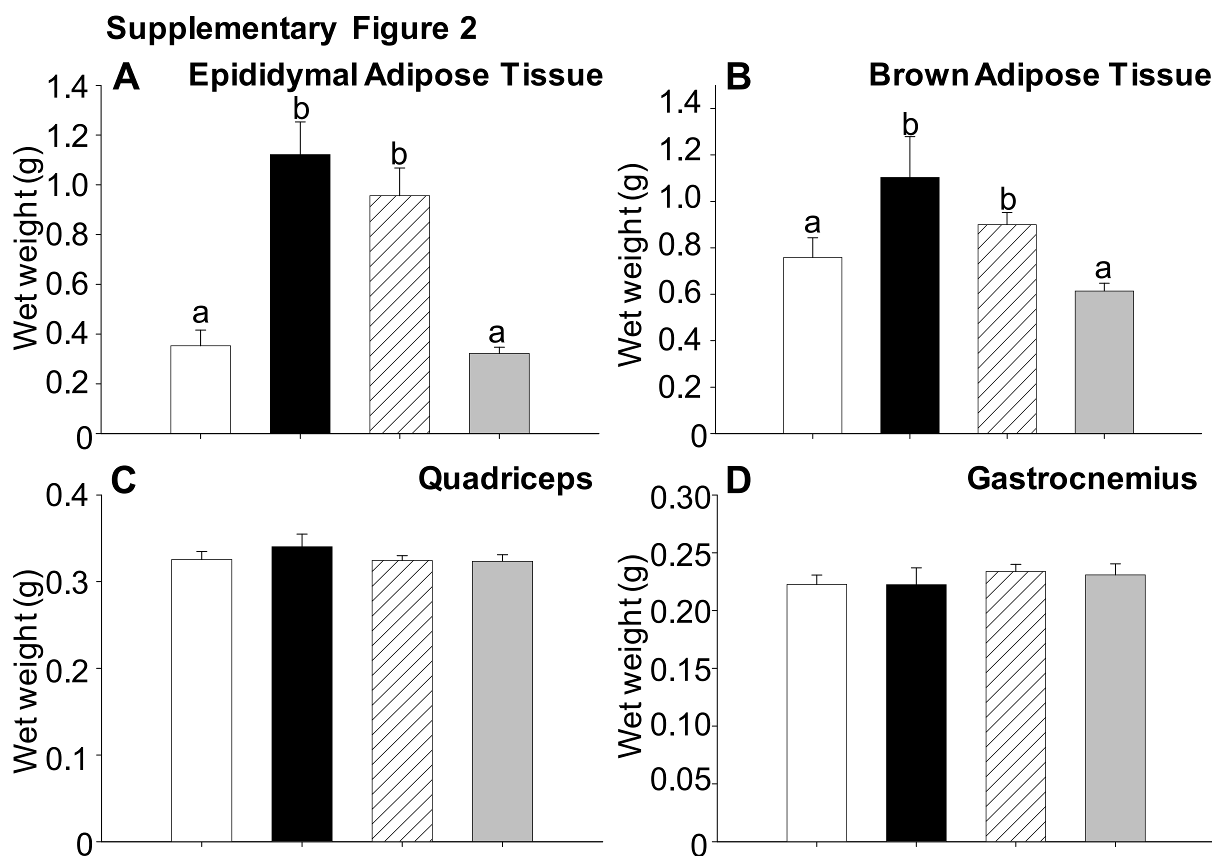
Luciferase activity was measured in HepG2 cells transfected with a PPRE luciferase construct (PPRE X3), control renilla construct and a nuclear receptor expression vector. A: PPARα, B: PPARγ, or C: PPARδ. Following transfection, cells were incubated with DMSO, nobiletin (10μM) or a PPAR agonist A: PPARα (10nM GW7647), B: PPARγ (3μM Rosiglitazone), C: PPARδ (20nM GW501516) for 24h.



©2011 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0589/-/DC1

Supplemental Figure 2

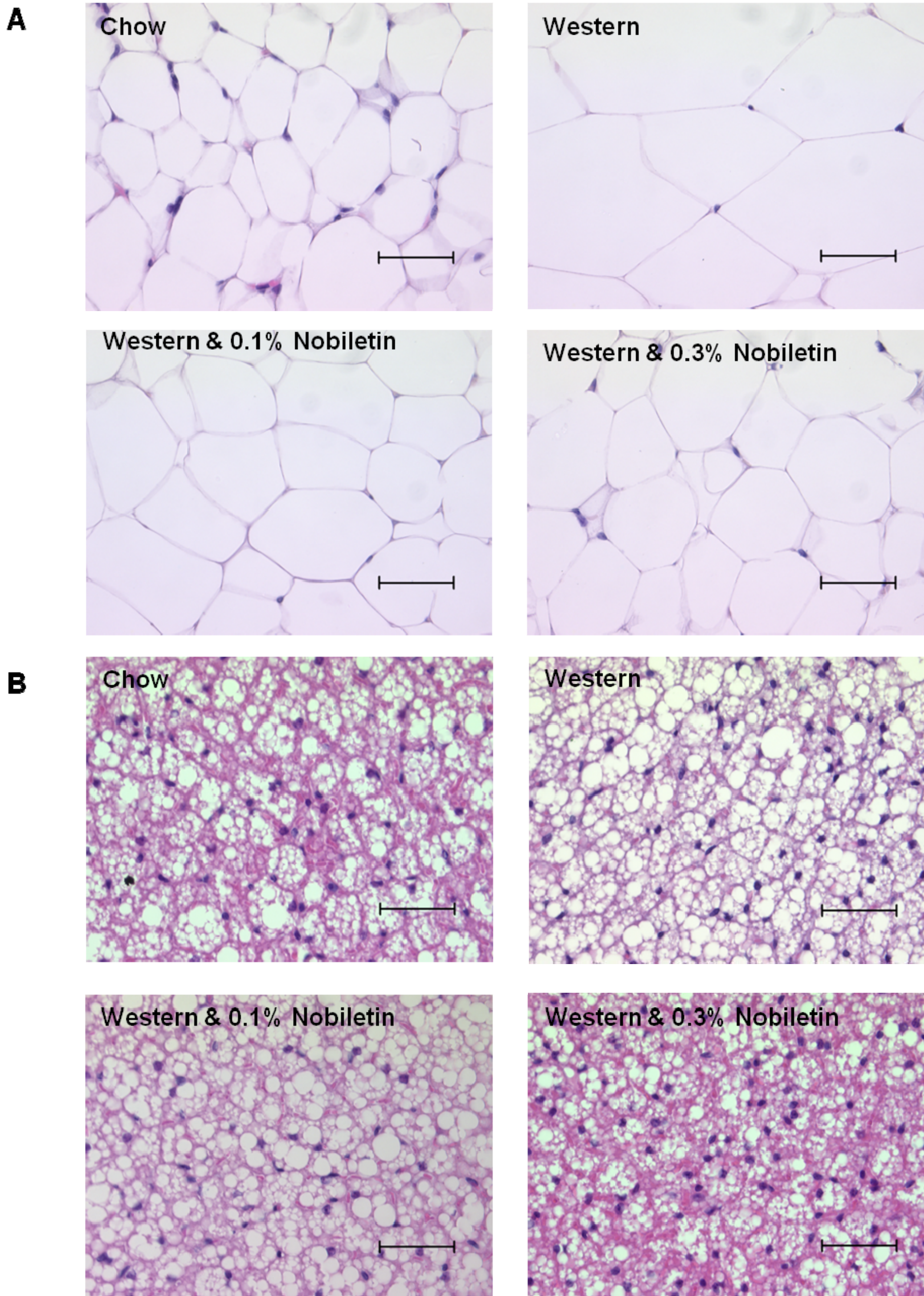
Wet weight mass of A: Epididymal fat pads, B: Brown adipose tissue, C: Quadriceps muscle and D: Gastrocnemius muscle weights from *Ldlr*$^{-/-}$ mice fasted for 6 hours fed chow, a western diet or a western diet and supplemented with 0.1% or 0.3% nobiletin.



©2011 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0589/-/DC1

Supplemental Figure 3

Hematoxylin and Eosin stained Sections of A: Epididymal fat pads and B: Brown adipose tissue, from *Ldlr*$^{-/-}$ mice fasted for 6 hours fed chow, a western diet or a western diet supplemented with 0.1% or 0.3% nobiletin. Size bar = 100µm.



©2011 American Diabetes Association. Published online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0589/-/DC1