Mice deficient in Group VIB phospholipase A2 (iPLA2γ) exhibit relative resistance to obesity and metabolic abnormalities induced by a Western diet

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Submitted 4 January 2010; accepted in final form 17 February 2010

Song H. Wohltmann M. Bao S. Ladenson JH. Semenkovich CF. Turk J. Mice deficient in group VIB phospholipase A2 (iPLA2γ) exhibit relative resistance to obesity and metabolic abnormalities induced by a Western diet. Am J Physiol Endocrinol Metab 298: E1097–E1114, 2010. First published February 23, 2010; doi:10.1152/ajpendo.00780.2009.—Phospholipases A2 (PLA2s) play important roles in metabolic processes, and the Group VI PLA2 family is comprised of intracellular enzymes that do not require Ca2+ for catalysis. Mice deficient in Group VIA PLA2 (iPLA2β) develop more severe glucose intolerance than wild-type (WT) mice in response to dietary stress. Group VIB PLA2 (iPLA2γ) is a related enzyme distributed in membranous organelles, including mitochondria, and iPLA2γ knockout (KO) mice exhibit altered mitochondrial morphology and function. We have compared metabolic responses of iPLA2γ-KO and WT mice fed a Western diet (WD) with a high fat content. We find that KO mice are resistant to WD-induced increases in body weight and adiposity and in blood levels of cholesterol, glucose, and insulin, even though WT and KO mice exhibit similar food consumption and dietary fat digestion and absorption. KO mice are also relatively resistant to WD-induced insulin resistance, glucose intolerance, and altered patterns of fat vs. carbohydrate utilization. KO skeletal muscle exhibits impaired mitochondrial β-oxidation of fatty acids, as reflected by accumulation of larger amounts of long-chain acylcarnitine (LCAC) species in KO muscle and liver compared with WT in response to WD feeding. This is associated with increased urinary excretion of LCAC and much reduced deposition of triacylglycerols in liver by WD-fed KO compared with WT mice. The iPLA2γ-deficient genotype thus results in a phenotype characterized by impaired mitochondrial oxidation of fatty acids and relative resistance to the metabolic abnormalities induced by WD.

insulin resistance; glucose intolerance; diabetes; lipids; mass spectrometry

PHOSPHOLIPASES A2 (PLA2s) were once considered to be primarily digestive enzymes and noxious venom components, but the discovery of new members of this superfamily since 1991 has revealed that they play myriad roles in regulating biological processes (15, 27, 31, 37, 38, 41, 48, 70, 77, 79). PLA2s catalyze hydrolysis of phospholipids to yield a free fatty acid, e.g., arachidonic acid (AA), and a 2-lysophospholipid, e.g., 2-lysophosphatidylcholine (LPC), and can generate signals by both product generation and substrate loss or modification. For example, AA has intrinsic mediator functions (36) and can also be converted to bioactive eicosanoids (26), and lysophospholipids can also serve as signaling molecules (57, 80, 81) or as biosynthetic intermediates (66). In addition, substrate hydrolysis can compromise the integrity of membranous organelles, e.g., mitochondria, and lead to release of their contents and initiation of intracellular signaling cascades (7).

At least 15 mammalian PLA2 groups are recognized, which fall into five main categories (15, 70). Secretory PLA2s (sPLA2, Groups I–III, V, IX–XIV) were the first recognized and have low (<14–18 kDa) molecular masses, require millimolar [Ca2+] for catalysis, and play roles in nutrient digestion, inflammation, and pathophysiological states, e.g., cancer and atherosclerosis (41, 70). Various members of the platelet-activating factor (PAF) acetylhydrolase (Groups VII and VIII) PLA2 category have anti-inflammatory and/or proatherogenic properties or may participate in brain development and spermatogenesis (15, 70). The recently recognized lysosomal PLA2 (Group XV) is important in cellular phospholipid clearance, and mice deficient in its expression develop macrophage phospholipidosis (31).

The first recognized cytosolic PLA2 is now designated cPLA2α (Group IVA), and five other Group IV family members have been identified (27). The cPLA2α requires micromolar [Ca2+] to associate with its membrane substrates and is also regulated by phosphorylation. It prefers substrates with sn-2 arachidonoyl substituents and provides AA for conversion to eicosanoids in many signaling and inflammatory processes. Mice deficient in its expression exhibit female reproductive abnormalities and modified responses in several disease models (27).

The Group VI PLA2s (iPLA2s) are also intracellular enzymes and do not require Ca2+ for catalysis (37, 48, 77, 79). They are now recognized to belong to a “patatin-like phospholipase domain-containing lipase” (PNPLA) family with nine members that share a GXSXG lipase consensus motif (37, 38). PNPLA2 is also designated adipose triglyceride lipase (ATGL) and catalyzes the first step in triacylglycerol (TAG) hydrolysis. Mice deficient in its expression exhibit massive TAG accumulation in multiple tissues and cardiomyopathy, although they have improved insulin sensitivity and glucose tolerance (38). PNPLA3 is also designated adiponutrin and is expressed in adipocytes, where its levels increase during differentiation and decrease with fasting, and PNPLA5 exhibits similar properties. PNPLA4 hydrolyzes keratinocyte retinyl esters, and PNPLA6 and PNPLA7 are lysophospholipases expressed in brain and in muscle and fat, respectively. Human mutations in the former are associated with neurodegenerative disease (38).

PNPLA9 and 8 correspond to Group VIA and VIB PLA2, respectively (38), and are also designated iPLA2β and iPLA2γ (37, 48). The former plays an important role in glucose homeostasis (6, 8). Male iPLA2β-null mice exhibit impaired glucose tolerance, but transgenic male mice that overexpress iPLA2β in insulin-secreting β-cells of pancreatic islets exhibit...
improved glucose tolerance (6). Female iPLA2β-null mice exhibit normal glucose tolerance when fed standard chow (SC) but develop more severe glucose intolerance than wild-type female mice when fed a Western diet (WD) (8). In each of those cases, glucose tolerance correlates with insulin secretion by pancreatic islets isolated from the animals (6, 8), and a similar relationship is observed in insulinoma cells, in which iPLA2β expression is manipulated genetically (5, 46). This appears to reflect the effect of iPLA2β reaction products to attenuate β-cell Kv2.1 currents and thereby to amplify glucose-induced insulin secretion (6, 36). In addition, iPLA2β deficiency affects insulin sensitivity in a sex-dependent manner (6, 8).

iPLA2γ-null mice have also been generated (49, 50) and exhibit phenotypic abnormalities that include altered mitochondrial morphology, function, and lipid composition associated with reduced exercise tolerance, cardiac dysfunction, and hippocampal degeneration, among other features, although their glucose tolerance is not obviously perturbed when fed SC (50). Here, we have examined the metabolic responses of these animals to a WD and find that, in contrast to iPLA2β-null mice (8), iPLA2γ-null mice exhibit remarkable resistance to obesity and metabolic abnormalities that consumption of the WD induces in wild-type littermates, suggesting that the enzyme plays an important and previously unrecognized role in nutritional effects on metabolic regulation.

EXPERIMENTAL PROCEDURES

Materials. [14C]palmitic acid (50 mCi/mmol) was obtained from American Radiolabeled Chemicals, St. Louis, MO; [3H]triolein from PerkinElmer (Waltham, MA); enhanced chemiluminescence (ECL) reagents from GE Healthcare (Piscataway, NJ); sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) supplies from Bio-Rad Laboratories (Richmond, CA); ATP, common reagents, and salts from Sigma Chemical Co. (St. Louis, MO); [3H]triolein from American Radiolabeled Chemicals, St. Louis, MO; [3H]triolein from CEA (Cambridge, MA). Solvents for sample preparation and for separations, among other features, although their glucose tolerance is not obviously perturbed when fed SC (50). Here, we have examined the metabolic responses of these animals to a WD and find that, in contrast to iPLA2β-null mice (8), iPLA2γ-null mice exhibit remarkable resistance to obesity and metabolic abnormalities that consumption of the WD induces in wild-type littermates, suggesting that the enzyme plays an important and previously unrecognized role in nutritional effects on metabolic regulation.

Generating and genotyping iPLA2γ-null mice. Mice with iPLA2γ-null alleles were obtained from the laboratory of Richard Gross, and their generation is described elsewhere (50). This had involved preparing an iPLA2γ knockout construct, introducing it into 129/SvJ mouse embryonic stem (ES) cells, their selection with G418, characteristic by Southern blotting, injection into C57BL/6 mouse blastocysts, production of chimeras and then heterozygotes, and mating of heterozygotes to yield wild-type, heterozygous, and iPLA2γ-null mice, which were genotyped by Southern blotting of tail clipping genomic DNA (50). Genotyping was also performed by PCR with tail clipping DNA as template, using two pairs of primers. The primer “NEO forward” (AAAGGCTACCCGTCCTACCTTGCA) was used with a common primer (CCATCTAATCACCTACCACTGC) to amplify a 608-nt product from the knockout allele. The common primer was used with the primer “iPLA2 FOR” (CTGGTCAAGAGGTGTTGTTGC) to amplify a 435-nt product of the wild-type allele. The genetic background of the resultant mice is mixed 129/SvJ-C57BL/6 (50). All experiments were performed using sex- and age-matched littermate controls.

General animal studies. Animal protocols were in accordance with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the Animal Care Committee of Washington University.

WD feeding studies. Mice were housed in a pathogen-free barrier facility with unrestricted access to water and standard mouse chow (Purina Mills Rodent Chow 5053) containing 6% fat, which is designated Standard Chow (SC) hereafter. For dietary intervention studies, mice were fed SC until age 8 wk and thereafter were randomized into groups that were fed either SC or a WD with a high fat content, as described (8, 21). The composition of the WD (Harlan Teklad catalog no. TD88137) is energy from fat 42%, casein 195 g/kg, sucrose 341 g/kg, corn starch 150 g/kg, cellulose 50 g/kg, anhydrous milk fat 210 g/kg, cholesterol 1.5 g/kg, and caloric content 18.95 kJ/g. Body weights of iPLA2γ-knockout (KO) and wild-type (WT) mice were determined gravimetrically at weekly to monthly intervals, as described (6), and blood samples were also obtained after a 4-h fast for determination of cholesterol, insulin, and glucose concentrations.

Blood concentrations of cholesterol, insulin, leptin, glucose, TAG, free fatty acid, β-hydroxybutyrate, and glycerol. As described (6, 8), blood samples were obtained from the lateral saphenous vein in heparinized capillary tubes, and glucose concentrations were measured in whole blood with a blood glucose monitor (Becton-Dickinson, Franklin Lakes, NJ) or an Ascensia ELITE XL blood glucose meter. As described (9, 18), plasma was prepared from heparinized blood by centrifugation, and cholesterol, TAG, free fatty acids, β-hydroxybutyrate, and glycerol were measured by enzymatic assays with kits from Sigma (St. Louis, MO), insulin by ELISA with a kit from Crystal Chem (Downer’s Grove, IL), and leptin and resistin by ELISA with kits from Millipore (Billerica, MA).

Body composition. Mice were anesthetized with pentobarbital sodium (40 mg/kg ip) and weighed. Body composition determinations were obtained by dual-energy X-ray absorptiometry (DEXA Lunar, Madison, WI) scanning, as described (9, 67), to determine percentage of body fat.

Food consumption. Individually housed age- and sex-matched mice were given preweighed amounts of food, and the amount of food consumed was determined over a 24-h period for 5 days, as described (35), and expressed as grams consumed per day.

Stool weight and extractable lipid content and composition. Mice of a given genotype were housed three to a cage with unrestricted access to food and water. Stool was collected at 24-h intervals, and both initial (wet) weight and weight after drying (1 h, 70°C, LabConco CentriVap Concentrator) were determined as described (35). Dried fecal samples (60 mg) were homogenized in water, and lipids were extracted with 4 ml of chloroform-methanol (1:1, vol/vol) and 1.8 ml of water as described (36, 70). Extractable lipid content was determined as described (72) in a process involving vortex mixing, centrifugation, and collection of the chloroform (lower) phase. Additional chloroform (2 ml) was added and extraction repeated. Combined chloroform extracts were concentrated to dryness under nitrogen, and the residue was reconstituted in chloroform and again extracted. Resultant chloroform extracts were placed in preweighed test tubes and concentrated to dryness. The residue was further desolvated (1 h, 70°C, CentriVap Concentrator), and tubes were again weighed (72). Dried lipid residues were reconstituted in chloroform (100 μl), and aliquots (5 μl) were analyzed by TLC with standard lipids in parallel lanes as described (30, 31). Lipid species were visualized by charring (11, 45) and analyzed by densitometry (AlphaInnotech FluorChem 8900 apparatus with AlphaEaseFC software).

Postprandial fat absorption. Triolein absorption was measured as described (35). Briefly, mice were fasted overnight, and [3H]trioliene (1 μCi, PerkinElmer) in olive oil was administered by gavage. Blood samples were obtained 1, 2, 4, and 6 h thereafter by tail bleeding.
Radioactivity appearing in plasma was determined by liquid scintillation counting.

**Glucose and insulin tolerance tests.** As described (6, 8, 62), intraperitoneal glucose tolerance tests were performed in mice fasted overnight from which a baseline blood sample was obtained followed by intraperitoneal injection of D-glucose (2 mg/g) and subsequent (30, 60, and 120 min) collection of blood for glucose measurement. Insulin tolerance tests were performed in mice with free access to water and chow until intraperitoneal administration of human regular insulin (0.75 U/kg; Lilly, Indianapolis, IN). Blood was collected at 0, 30, 60, and 120 min for glucose measurements (6, 8, 62).

**Insulin secretion in vivo.** Mice were fasted overnight, and baseline blood samples were obtained from saphenous vein, followed by intraperitoneal injection of D-glucose (3 mg/g), and a blood sample was obtained 15 min thereafter for measurement of plasma insulin levels.

**Insulin secretion by isolated pancreatic islets.** Islets were isolated from pancreata of mice by collagenase digestion after mincing, followed by Ficoll step density gradient separation, and manual selection under stereomicroscopic visualization to exclude contaminating tissues, as described (6, 8, 54). Mouse islets were counted and used to measure ex vivo secretion of insulin (30 islets per incubation), as described (6, 8). Islets were rinsed with KRB medium containing 3 mM glucose and 0.1% BSA and placed in silanized tubes (12 × 75 mm) in that buffer, through which 95% air-5% CO2 was bubbled before incubations. Tubes were capped and incubated (37°C, 30 min, shaking water bath). Buffer was then replaced with KRB medium containing 3, 8, or 20 mM glucose and 0.1% BSA without or with forskolin (2.5 μM), and samples were incubated for 30 min. Secreted insulin was measured by radioimmunoassay.

**Indirect calorimetry and determination of the respiratory exchange ratio (RER) and measurements of activity by infrared sensor monitoring.** SC-fed or WD-fed mice (age 6 mo) were studied in the fed state in a single-chamber indirect calorimetry system for 24 h (Columbus Instruments, Columbus, OH), as described (9), to determine V\(\text{O}_2\), V\(\text{CO}_2\), heat generation, and RER. Activity was measured with an infrared sensor in an InfraMot apparatus (TSE Systems, Midland, MI).

**Tissue glycerol oxidation by gastrocnemius muscle.** SC-fed or WD-fed mice (age 6 mo) were euthanized with pentobarbital sodium, and the gastrocnemius muscle was excised. Oxidation of [1-\(\text{14C}\)]Palmitate and of [1-\(\text{14C}\)]glucose by muscle homogenate was then performed as described (16). Briefly, gastrocnemius specimens (70 mg) were minced with scissors in homogenization buffer (200 μl: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, and 2 mM ATP, pH 7.4), diluted 20-fold, and transferred to a 3-ml Teflon-on-glass homogenization vessel. Homogenization was performed twice (on ice, 12 passes, 30 s, 1,500 rpm), and the resultant homogenates were used for oxidation experiments after removal of an aliquot for protein determination. To measure \(\text{14C}\)O\(_2\) production from [1-\(\text{14C}\)]palmitate, palmitate (100 μM) was bound to 0.5% BSA (final concentration), and acetyl-CoA and glucose were added. Each substrate was dissolved in reaction buffer (in mM: 100 sucrose, 10 Tris-HCl, 10 KPO\(_4\), 100 KCl, 1 MgCl\(_2\)-6H\(_2\)O, 1 L-carnitine, 0.1 malate, 2 ATP, 0.05 coenzyme A, 1 dithiothreitol, pH 7.4). Aliquots (80 μl, in triplicate) of diluted muscle homogenates were plated in a 100-well trapping device. Reaction buffer (325 μl) was placed in the bottom of each well. In addition, 1 N NaOH (400 μl) was placed in a 500-μl uncapped microcentrifuge tube, which was also placed in each well to trap CO\(_2\). The trapping device was then sealed with parafilm, a siliconized rubber gasket, and the trapping device lid and allowed to incubate (with shaking, 37°C, 1 h). Reactions were terminated by adding 70% perchloric acid (100 μl) via injection ports in the trapping device lid. The trapping device was again placed in the shaking incubator, and released \(\text{14C}\)O\(_2\) was trapped (1 h, room temperature), quantitated by liquid scintillation spectrometry, and expressed as CO\(_2\) produced per milligram of protein (16).

**Electron microscopy of gastrocnemius muscle sections.** Electron microscopic examination of muscle tissue was performed as described (47). Briefly, euthanized mice were perfused via cardiac puncture with modified Karnovsky’s fixative buffer containing 3% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium cacodylate (pH 7.4). The gastrocnemius was excised and cut into strips (1 × 1 mm) along the fiber length and immersed in fixative buffer (24 h). Muscle strips were washed, placed in postfixative solution (1% osmium tetroxide, 1 h), dehydrated with alcohols, and embedded in PolyBed 812 (Polysciences, Warrington, PA). Sections (90 nm) were prepared and viewed with a JEOL model 1200EX electron microscope.

**Immunostaining and fluorescence microscopy of gastrocnemius muscle sections.** Immunocytochemical analyses with mouse antibody MS502 (MitoSciences, Eugene, OR) directed against mitochondrial ATP synthase (complex V) α-subunit (57) were performed essentially as described (44). Briefly, euthanized mice were perfused via cardiac puncture with PBS (20 ml) and then with 4% paraformaldehyde (20 ml). Gastrocnemius was then excised and immersed in 30% sucrose (4°C, overnight). Fixed tissue was frozen in embedding medium and cut into 4-μm sections (LEICA CM1850), which were mounted on l-polylysine-coated glass slides, allowed to dry overnight, and washed with PBS. Microwave-assisted (power level 2, 5 min) antigen retrieval was performed in 1 mM EDTA buffer, and the slides were allowed to cool to room temperature, washed (PBS, 5 min, thrice), fixed (4% paraformaldehyde, 1 h, room temperature), permeabilized (0.1% Triton X-100 and 0.1% sodium citrate in PBS), again washed (PBS, 5 min, twice), and blocked (5% goat serum and 3% BSA in TBS-T). Slides were incubated with primary antibody directed against mitochondrial ATP synthase (complex V) α-subunit (20 μl; MS502, Mitoscientce) at 1:25 dilution (overnight, 4°C), washed (PBS, 20 min, 4 times), and incubated (1 h) with secondary antibody (20 μl, 1:100 dilution, goat anti-mouse IgG-TR, SC-2781; Santa Cruz Biotechnology, Santa Cruz, CA), and again washed (PBS, 10 min, thrice). A drop of Prolong Gold antifade reagent with DAPI (P36935, Invitrogen) was applied, and tissue sections were covered with glass coverslips. Sealed (Nail oil) slides were examined with a Nikon TE300 microscope.

**Tissue cardiolipin content.** Euthanized mice were perfused via cardiac puncture with PBS (20 ml), and liver and gastrocnemius muscle were excised and frozen by immersion in liquid nitrogen. Tissues were processed essentially as described (43) with a tissue pulverizer apparatus (Spectrum Laboratories, Rancho Dominguez, CA). Tissue powder (20–100 mg) was homogenized in 1 ml of ice-cold mitochondrial isolation buffer (20 mM HEPES-KOH buffer, pH 7.4, containing 100 mM KCl, 5 mM MgCl\(_2\), 1 mM EGTA, 250 mM sucrose) containing protease inhibitors (Sigma) in a Teflon-on-glass homogenization vessel. Homogenate was centrifuged (1,000 g, 5 min), and the supernatant was collected and its protein content determined (Coomasie kit; Pierce, Rockford, IL). Supernatant was again centrifuged (10,000 g, 10 min), and the mitochondria-containing pellet was suspended in 20 mM LiCl (1.8 ml), to which cardiolipin internal standard was added. Mitochondrial lipids were extracted by a modified Bligh-Dyer method (12) by vortex mixing in methanol-chloroform (1:1, vol/vol, 4 ml) followed by centrifugation (2,500 rpm, 10 min). The organic (lower) phase was concentrated to dryness under nitrogen, and the residue was reconstituted in methanol-chloroform (1:1, vol/vol, 200 μl). Appropriate dilutions of the resultant solution were analyzed by ESI-MS on a Finnigan TSQ 7000 instrument as described below. Cardiolipin species were quantified relative to internal standard by integration from a standard curve, normalized to sample protein content, and expressed as nanomoles of cardiolipin per milligram of protein.

**Tissue acylcarnitine content.** Acylcarnitines were measured by direct injection tandem mass spectrometry as described (3), with minor modification. Briefly, gastrocnemius muscle was harvested, frozen, and pulverized as above. Tissue powder (20–100 mg) was homogenized in H\(_2\)O (1 ml, ice-cold, double distilled) in a Teflon-on-glass homogenization vessel, and the homogenate was sonicated (15 s, twice, on ice) and centrifuged (14,000 rpm, 10 min). An aliquot
of supernatant was removed to measure protein content, and the rest was diluted appropriately. To an aliquot (400 μl) of diluted supernatant was added an internal-standard solution (2 μl, 0.2 nmol/μl [2H3]acetylcarnitine). Protein was precipitated with acetonitrile (1,600 μl) and the mixture centrifuged (2,000 g, 5 min). The supernatant was collected, and two aliquots (400 μl) were transferred to two centrifuge tubes (1.5 ml), and the samples were concentrated to dryness (50°C, 20 min, under nitrogen). The residues were incubated with either 3 M methanol-HCl (200 μl, 50°C, 15 min, Supelco) or 3 M n-butanol-HCl (200 μl, 65°C, 15 min; Regis Chemical, Morton Grove, IL) to form the corresponding acylcarnitine methyl or n-butyl ester derivatives. Samples were concentrated to dryness (50°C, 20 min, under nitrogen) and the residues reconstituted in methanol-water (200 μl, 85:15, vol/vol), diluted (1:3, vol/vol) with 85% methanol, and analyzed by ESI-MS-MS scanning for precursors of [M – H]− ions by negative ion ESI-MS-MS, acylcarnitines as methyl or n-butyl ester derivatives (3, 22) by positive ion ESI-MS scanning for precursors of m/z 99 or 85, respectively, and TAG (33) as Li+ adducts by positive ion ESI-MS-MS on a Finnigan (San Jose, CA) TSQ-7000 triple-stage quadrupole MS with an ESI source controlled by Finnigan ICIS software operated on a DEC Alpha work station.

For acylcarnitines, methyl or butyl ester derivatives in 85% methanol were infused (1.2 μl/min) into the ESI source with skimmer set at ground potential, electrospray needle at 4.5 kV, and heated capillary temperature 250°C. The first quadrupole (Q1) was scanned in negative mode from m/z 1200 to 1600 (rate 3 s/scan). Mass spectra were accumulated (5 min) in profile mode for quantitation. To identify molecular species, ESI-MS-MS spectra were obtained by selecting precursor [M – H]− ions in Q1, accelerating them (46–50 eV) into the rf-only second quadrupole (Q2) containing Ar (2.3 mTorr) to induce collisionally activated dissociation (CAD), and analyzing product ions in the third quadrupole (Q3). Both Q1 and Q3 were tuned to unit mass resolution and scanned at a rate of 3 s/scan. Tandem mass spectra were accumulated (20 min) in profile mode. For acylcarnitines, methyl or butyl ester derivatives in 85% methanol were infused (1.2 μl/min) into the ESI source, using instrumental parameters specified above. The mass spectrometer was operated in positive ion scan mode. Q1 scanned from m/z 150 to 600 (3 s/scan), and precursor ions were accelerated (30 eV for butyl esters or 40 eV for methyl esters) into Q2 to induce CAD. For acylcarnitine butyl ester derivatives, Q3 was set to detect the product ion m/z 85, and for methyl ester derivatives Q3 was set to detect the product ion m/z 99. For quantitation, the peak intensity of each acylcarnitine species was normalized to that of internal-standard [2H3]acetylcarnitine (m/z 221 for methyl esters or m/z 263 for butyl esters). That ratio was then used to homogenized in a Dounce apparatus in extraction buffer (in mM: 20 HEPES-KOH, 100 KCl, 5 MgCl2, 1 EGTA, 250 sucrose, pH 7.4, phosphatase inhibitor at 1:200). An aliquot was removed to measure protein (Coomassie kit, ThermoScientific). Samples were extracted as above and the organic residue was suspended in chloroform-methanol (1:1). Quantitation of TAG was performed as described (33), using Infinity Triglyceride Reagent (ThermoScientific) in an assay based on enzymatic triglyceride hydrolysis and colorimetric measurement of liberated glycerol by coupled enzymatic reactions (25, 55).
iPLA2γ-KO mice are thus relatively resistant to diet-induced adiposity, as reflected by much smaller food consumption by WT and iPLA2γ-KO mice, as shown in Figure 1. Individually housed mice age 12 wk were given preweighed amounts of food, and the amounts consumed per 24 h were determined daily for 5 days and expressed as grams consumed per day, as described in EXPERIMENTAL PROCEDURES and elsewhere (35).

**RESULTS**

To determine whether genetic deficiency of iPLA2γ affects dietary influences on metabolic processes, we compared effects of feeding an SC or a WD with a high fat content to iPLA2γ knockout (KO) mice and their wild-type (WT) littermates. All mice were fed SC from the time of weaning until age 8 wk and were then separated into groups of male or female WT or KO mice that were fed SC or WD thereafter.

**Differential effects of WD on weight gain and body fat content of WT and iPLA2γ-KO mice.** Figure 1 illustrates that on this dietary regimen weight gain for a given sex was similar for WT and KO mice up to the age of 8 wk (Fig. 1). Thereafter, WD-fed WT mice gained more weight than SC-fed WT mice, and both groups gained more weight than did KO mice fed either diet. By the end of the 6 mo differential feeding interval, the mean body weight of WD-fed males (38.61 ± 0.95 g) was 22% greater than that of SC-fed males (31.75 ± 0.95 g), and both exceeded the body weights of KO males fed SC (20.94 ± 0.61 g) or WD (21.82 ± 0.42 g) (Fig. 1A).

Although female body weights were lower than those of males in each genotype/diet group, comparisons among groups were similar to those with males, and weights of WD-fed (31.75 ± 1.20 g) and SC-fed (25.92 ± 0.76 g) WT females were significantly different from each other and from the weights of WD-fed (18.44 ± 0.27 g) and SC-fed (17.54 ± 0.33 g) KO females. The body weights of SC-fed and WD-fed KO mice did not differ from each other for males (20.94 ± 0.62 vs. 21.87 ± 0.43 g) or females (17.54 ± 0.33 vs. 18.44 ± 0.027 g) by the end of the differential feeding interval (Fig. 1).

The 22% greater body weight of WD-fed compared with SC-fed male WT mice was associated with a 21% greater body fat content (37.89 ± 1.20% for WD vs. 16.77 ± 1.0% for SC; Fig. 2A), as determined by DEXA measurements. The body fat percentage of male SC-fed KO mice (16.2 ± 1.22%) was not significantly different from either WD-fed KO (19.92 ± 1.68%) or SC-fed WT mice (Fig. 2A). Females exhibited similar trends of smaller magnitude (Fig. 2B).

**Table 1. Food consumption by WT and iPLA2γ-KO mice fed standard chow or a Western diet**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
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Male (M) or female (F) Group VIB phospholipase A2 (iPLA2γ)-knockout (KO) and wild-type (WT) mice were fed standard chow (SC) or a Western diet (WD) as in Figure 1. Individually housed mice age 12 wk were given preweighed amounts of food, and the amounts consumed per 24 h were determined daily for 5 days and expressed as grams consumed per day, as described in EXPERIMENTAL PROCEDURES and elsewhere (35).

**Table 2. Stool weight and extractable lipid content for WT and iPLA2γ-KO mice fed SC or WD**

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<td>WD</td>
<td>WD</td>
<td>SC</td>
<td>SC</td>
<td>WD</td>
<td>WD</td>
</tr>
<tr>
<td>Stool lipid extract</td>
<td>3.08</td>
<td>2.31</td>
<td>3.68</td>
<td>3.26</td>
<td>2.19</td>
<td>2.19</td>
<td>1.81</td>
<td>1.43</td>
</tr>
<tr>
<td>SE</td>
<td>0.24</td>
<td>0.15</td>
<td>0.45</td>
<td>0.21</td>
<td>0.20</td>
<td>0.26</td>
<td>0.12</td>
<td>0.17</td>
</tr>
<tr>
<td>P WT vs. KO</td>
<td>0.02</td>
<td>0.42</td>
<td>0.98</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>8</td>
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<td>12</td>
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<tr>
<td>Stool weight</td>
<td>3.38</td>
<td>3.97</td>
<td>1.28</td>
<td>1.32</td>
<td>3.12</td>
<td>3.48</td>
<td>0.75</td>
<td>1.06</td>
</tr>
<tr>
<td>SE</td>
<td>0.43</td>
<td>0.26</td>
<td>0.14</td>
<td>0.10</td>
<td>0.40</td>
<td>0.09</td>
<td>0.26</td>
<td>0.10</td>
</tr>
<tr>
<td>P WT vs. KO</td>
<td>0.30</td>
<td>0.83</td>
<td>0.43</td>
<td>0.33</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Male or female iPLA2γ-KO and WT mice were fed SC or WD as in Table 1. Individually housed mice age 12 wk had unrestricted access to food and water, and all stool was collected for 24 h intervals. Stool wet (expressed in grams and tabulated) and dry weights and extractable lipid content (expressed in mg lipid per 60 mg stool dry weight) were determined as in EXPERIMENTAL PROCEDURES and elsewhere (35, 72).
rises in body weight (Fig. 1) and body fat percentage (Fig. 2) when fed a WD. There were also smaller diet-induced rises in adipokines and body brown fat content in KO compared with WT mice. WD-fed WT male and female mice had higher serum leptin levels than did SC-fed WT mice (Fig. S1; supplementary materials are found in the online version of this article). There was no significant WD-induced rise in serum leptin levels for KO males (Fig. S1), and that for KO females was significantly lower than for WT. Similar trends for dietary and genotype effects were observed for serum resistin levels and body brown fat content with some sex heterogeneity (not shown).

**Similarity of food consumption and fat digestion and absorption of WT and iPLA2γ-KO mice.** The possibilities were considered that the relative resistance of iPLA2γ-KO mice to diet-induced adiposity might be attributable to failure to consume the WD or to absorb its fat content. Food consumption by SC-fed or WD-fed KO mice was found not to differ significantly from that of WT littermates (Table 1). Unabsorbed dietary fat is reflected by stool-extractable lipid content, and neither this parameter nor stool weight was significantly different for WT or KO mice fed SC or WD in the case of males or females (Table 2), with the single exception of a slightly lower stool lipid content (reflecting more complete fat absorption) for SC-fed KO male mice compared with WT. In addition, TLC analyses of stool lipid components revealed no significant differences between WD-fed WT and KO mice in stool content of free fatty acids, cholesterol-diacylglycerol, cholesterol esters, or total lipids (Table 3). Although the SC-fed KO mice had a slightly lower stool content of some of these lipids, this again reflects absorption that is more (rather than less) complete for KO than for WT mice.

That KO mice digested and absorbed dietary fat content as efficiently as did WT mice was also reflected by the time course of appearance of [3H]oleate in plasma after administration of [3H]triolein into the gastrointestinal tract by gavage (Fig. 3). Data in Tables 1–3 and Fig. 3 thus indicate that differences between iPLA2γ-KO mice and their WT littermates in metabolic responses to a WD do not reflect impaired consumption of the diet or impaired digestion and absorption of its fat content.

**Differential effects of a WD on blood levels of cholesterol, insulin, glucose, and other analytes for wild-type and iPLA2γ-knockout mice.** Expected sequelae of consuming a WD include hypercholesterolemia and insulin resistance, and fasting serum

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**Table 3. TLC analyses of stool lipid species for WT and iPLA2γ-KO mice fed SC or WD**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT</th>
<th>KO</th>
<th>WT</th>
<th>KO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>324</td>
<td>304</td>
<td>420</td>
<td>435</td>
</tr>
<tr>
<td>WD</td>
<td>304</td>
<td>324</td>
<td>435</td>
<td>420</td>
</tr>
<tr>
<td>n</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE</td>
<td>11</td>
<td>19</td>
<td>23</td>
<td>41</td>
</tr>
<tr>
<td>P WT vs. KO</td>
<td>0.39</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol/DAG</td>
<td>588</td>
<td>485</td>
<td>541</td>
<td>474</td>
</tr>
<tr>
<td>SE</td>
<td>28</td>
<td>27</td>
<td>71</td>
<td>77</td>
</tr>
<tr>
<td>P WT vs. KO</td>
<td>0.02</td>
<td>0.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>315</td>
<td>152</td>
<td>216</td>
<td>213</td>
</tr>
<tr>
<td>SE</td>
<td>20</td>
<td>23</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>P WT vs. KO</td>
<td>&lt;0.01</td>
<td>0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total stool lipids</td>
<td>1,227</td>
<td>941</td>
<td>1,177</td>
<td>1,123</td>
</tr>
<tr>
<td>SE</td>
<td>40</td>
<td>54</td>
<td>85</td>
<td>82</td>
</tr>
<tr>
<td>P WT vs. KO</td>
<td>&lt;0.01</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>16</td>
</tr>
</tbody>
</table>

Stool lipid extracts obtained as in Table 2 were analyzed by TLC, and amounts of separated lipid classes were determined by densitometry after charring as described in EXPERIMENTAL PROCEDURES and elsewhere (11) and expressed in IDV units.
cholesterol levels in WD-fed WT male mice rose with time after initiation of differential feeding (Fig. 4). After 22 wk on the diet, serum cholesterol levels were significantly higher for WD-fed WT (368 ± 45 mg/dl) than for KO (194 ± 12 mg/dl) male mice (Fig. 4A). Cholesterol levels of both WD-fed groups were significantly higher than those of the corresponding SC-fed groups of the same genotype (170 ± 21 mg/dl for WT and 140 ± 9 mg/dl for KO), although the levels for SC-fed WT and KO male mice did not differ significantly from each other.

Similar trends of smaller magnitude were observed with females (Fig. 4B).

Similarly, rises in fasting serum insulin levels reflecting diet-induced insulin resistance were observed in WD-fed male (Fig. 5A) and female (Fig. 5B) WT mice, and the levels were significantly higher than those for SC-fed WT mice and for SC-fed or WD-fed KO mice of the corresponding sex. Interestingly, no significant WD feeding-induced rise in fasting serum insulin levels was observed in male or female KO mice (Fig. 5), suggesting that the development of diet-induced insulin resistance is attenuated with the iPLA2 KO genotype.

Fig. 5. Diet effects on plasma insulin concentrations of iPLA2-KO and WT mice. Insulin concentration (ng/ml) of plasma from 4-h-fasted WT (circles) or iPLA2-KO (squares) male (A) and female (B) mice fed SC (open symbols) or WD (closed symbols) as in Fig. 1 was determined by ELISA as described in EXPERIMENTAL PROCEDURES and elsewhere (6, 9). Mean values ± SE (n = 21) are displayed. *Significantly (P < 0.05) greater insulin level in genotype and diet comparisons.

Fig. 6. Diet effects on blood glucose concentrations of iPLA2-KO and WT mice. Glucose concentration (mg/dl) of blood from 4-h-fasted WT (circles) or iPLA2-KO (squares) male (A) and female (B) mice fed SC (open symbols) or WD (closed symbols) as in Fig. 1 was determined enzymatically as described in EXPERIMENTAL PROCEDURES and elsewhere (6, 9). Mean values ± SE (n = 33) are displayed. *Significantly (P < 0.05) greater glucose level in genotype and diet comparisons.

Fig. 7. Glucose tolerance tests of iPLA2-KO and WT mice fed SC or a WD. Male WT (A) or iPLA2-KO (B) WT (circles) mice were fed SC (○) or WD (■), as in Fig. 1. At age 6 mo, a baseline (time 0) blood sample was obtained; a glucose (2 mg/kg ip) solution was administered, and blood samples were collected 30, 60, and 120 min thereafter, as described in EXPERIMENTAL PROCEDURES and elsewhere (6, 9). Glucose concentrations were determined as in Fig. 6. Mean values ± SE (n = 23) are displayed. *Significantly (P < 0.05) greater glucose level in diet and genotype comparisons.

Fig. 8. Diet effects on glucose tolerance tests area under the curve (AUC) for iPLA2-KO and WT mice. Experiments were performed as in Fig. 7, and the area under the glucose concentration vs. time curve was calculated as described (8). Delta (gray bars) represents the difference between AUC values for mice of a given sex and genotype fed SC (light bars) or WD (dark bars) as in Fig. 1. Mean values ± SE are indicated (n = 23). *Significantly (P < 0.05) greater value in genotype and diet comparisons; x, significantly greater value only in genotype comparisons.
Rising fasting blood glucose levels reflecting diet-induced deterioration in glucose tolerance were also observed in WD-fed male (Fig. 6A) and female (Fig. 6B) WT mice, and these levels were also significantly higher than those for SC-fed WT mice and for SC-fed or WD-fed KO mice of the corresponding sex. As with fasting insulin levels, no significant WD feeding-induced rise in fasting blood glucose levels was observed in male or female KO mice (Fig. 6), suggesting that diet-induced deterio-

Fig. 9. Insulin secretion in vivo and from pancreatic islets isolated from iPLA\textsubscript{2γ}-KO and WT mice fed SC or WD. A (in vivo secretion): experiments were performed as in Fig. 7, except that the glucose dose was 3 mg/kg ip, and blood samples for insulin determinations were collected at baseline (time 0) and 15 min after glucose administration as described in EXPERIMENTAL PROCEDURES and elsewhere (8, 62). Mean values ± SE are indicated (n = 45). *Significantly (P < 0.05) greater value in genotype and diet comparisons; x, significantly greater value only in genotype comparisons.

B: islets isolated from pancreata of WT (1st and 3rd bars in each set) or iPLA\textsubscript{2γ}-KO (2nd and 4th bars in each set) male mice were incubated (30 min, 37°C) in buffer containing 3, 8, or 20 mM d-glucose without or with 2.5 μM forskolin, and an aliquot of medium was then removed for measurement of insulin, as described in EXPERIMENTAL PROCEDURES and elsewhere (6, 8, 62). Mean values ± SE are indicated (n = 39). *Significantly (P < 0.05) greater value in diet comparisons.
Dietary effects on insulin secretion and sensitivity. To examine the relative contributions of altered insulin secretion and insulin sensitivity to diet-induced glucose intolerance in the mouse WD feeding model, insulin secretion in vivo was assessed by the rise in serum insulin concentration after intraperitoneal glucose administration (Fig. 9A), and insulin secretion in vitro from isolated pancreatic islets was also determined (Fig. 9B). Insulin sensitivity was evaluated by insulin tolerance testing (ITT; Fig. 10).

SC-fed WT mice exhibited an increase in serum insulin levels 15 min after glucose administration of about twofold for females and nearly threefold for males (Fig. 9A). These responses were significantly reduced in WD-fed WT mice of the corresponding gender (Fig. 9A) by the time that diet-induced deterioration in glucose tolerance had occurred (Figs. 7 and 8). Serum insulin levels in KO mice did not change appreciably in response to diet (Fig. 9B), but no significant diet-induced deterioration in insulin secretion was observed for male KO mice (Fig. 7B). Similar trends of smaller magnitude were observed with female mice (Fig. 8). Compared with SC-fed mice, WD-fed male (Fig. 8A) and female (Fig. 8B) mice experienced a significant increase in GTT area under the curve (AUC), but significant diet-induced increases in GTT AUC were not observed for male (Fig. 8A) or female (Fig. 8B) KO mice, again indicating that diet-induced deterioration in glucose tolerance is attenuated with the iPLA2γ-KO genotype.

Dietary effects on glucose tolerance. Glucose tolerance testing (GTT) confirmed the diet-induced deterioration in WD-fed WT compared with SC-fed male mice (Fig. 7A), but no significant diet-induced deterioration in glucose tolerance was observed for male KO mice (Fig. 7B). Similar trends of smaller magnitude were observed with female mice (Fig. 8). Compared with SC-fed mice, WD-fed male (Fig. 8A) and female (Fig. 8B) mice experienced a significant increase in GTT area under the curve (AUC), but significant diet-induced increases in GTT AUC were not observed for male (Fig. 8A) or female (Fig. 8B) KO mice, again indicating that diet-induced deterioration in glucose tolerance is attenuated with the iPLA2γ-KO genotype.

The relative contributions of altered insulin secretion and insulin sensitivity to diet-induced glucose intolerance in the mouse WD feeding model were assessed by the rise in serum insulin concentration after intraperitoneal glucose administration (Fig. 9A), and insulin secretion in vitro from isolated pancreatic islets was also determined (Fig. 9B). Insulin sensitivity was evaluated by insulin tolerance testing (ITT; Fig. 10).

SC-fed WT mice exhibited an increase in serum insulin levels 15 min after glucose administration of about twofold for females and nearly threefold for males (Fig. 9A). These responses were significantly reduced in WD-fed WT mice of the corresponding gender (Fig. 9A) by the time that diet-induced deterioration in glucose tolerance had occurred (Figs. 7 and 8). Serum insulin levels in KO mice did not change appreciably in this model and were not significantly affected by diet. Insulin secretion by isolated islets tended to be lower for KO than for WT mice and lower for WD-fed than for SC-fed mice (Fig. 9B). These differences were not statistically significant, with the single exception of the greater secretion of SC-fed compared with WD-fed KO islets stimulated with 20 mM glucose and 2.5 μM forskolin (Fig. 9B).
Genotype-dependent differences in insulin secretion thus do not appear to explain the differential WD diet-induced deterioration of glucose tolerance for WT compared with KO mice, and this suggests that diet-induced changes in insulin sensitivity differ between the genotypes. In ITT, insulin administration resulted in statistically indistinguishable time courses of decline and recovery of blood glucose levels for SC-fed WT and KO mice (Fig. 10A). In contrast, WD-fed WT mice exhibited a significantly smaller decline in blood glucose concentrations than KO mice after insulin administration, and WD-fed WT but not KO mice exhibited rebound hyperglycemia at the ITT 120 min time point (Fig. 10B). WT mice thus experience a greater WD feeding-induced decrease in insulin sensitivity than do KO mice.

Dietary effects on diurnal variation in utilization of carbohydrate and fat as fuels. Indirect calorimetric measurements performed with an Oxymax apparatus demonstrated that SC-fed WT mice exhibit the expected diurnal increase in RER in their active (dark cycle) phase compared with their resting (light cycle) phase (Fig. 11), which reflects increased metabolism of carbohydrate compared with fat in the active phase. This diurnal variation was greatly reduced for WD-fed WT mice (Fig. 11), reflecting greater metabolism of fat and lower RER during the active phase for WD-fed than for SC-fed mice. SC-fed KO mice exhibited diurnal variations in RER that were indistinguishable from that of SC-fed WT mice, but WD-fed KO mice exhibited a much smaller diminution in dark cycle RER compared with SC-fed mice than did WT mice (Fig. 11). This indicates that the WD feeding-induced shift in active-phase fuel metabolism from carbohydrate to fat is attenuated with the iPLA2γ/H9253-KO genotype.

WD-fed WT mice exhibited a significant increase in heat production compared with SC-fed WT mice, and there was little diurnal variation in this parameter (Fig. S2). KO mice generated less heat than WT mice, but WD-fed KO mice increased heat production, V\textsubscript{O\textsubscript{2}}, and V\textsubscript{CO\textsubscript{2}} during their active phase compared with SC-fed KO mice (Fig. S2). There were trends for dark cycle activity to decline in WD-fed compared with SC-fed WT mice and for dark cycle activity to be higher in WD-fed KO compared with WT mice, but these trends did not achieve statistical significance (Fig. S2).
Oxidation of palmitate and glucose by skeletal muscle mitochondria and their morphology and distribution within myocytes. Skeletal muscle is a principal site of insulin action and substrate oxidation and is rich in mitochondria. Examination of mitochondrial β-oxidation of [14C]palmitate to [14C]O2 by excised gastrocnemius muscle specimens in vitro revealed lower oxidation rates with KO than with WT muscle for SC-fed and WD-fed male and female mice (Fig. 12). β-Oxidation rates were higher for male than for female muscle specimens, and muscle from both WD-fed WT and KO male mice exhibited higher β-oxidation rates than did specimens from SC-fed male mice of the corresponding genotype (Fig. 12). In contrast, rates of glucose oxidation were similar for skeletal muscle specimens from both SC-fed and WD-fed WT and KO mice (Table S2).

The overall reduction in mitochondrial β-oxidation of palmitate by KO compared with WT muscle specimens was associated with differences in mitochondrial morphology and distribution within muscle cells. KO mitochondria were generally larger (Fig. S3) than WT mitochondria, as reported by others (49), and KO mitochondria appeared to be distributed more homogeneously throughout muscle fibers than did WT (Fig. S4).

Cardiolipin composition of mitochondria and tissue content. Cardiolipins (CL) are phospholipids that occur virtually exclusively within mitochondria in mammalian tissues and are important in mitochondrial function. Several CL molecular species with different [M − H]− ion m/z values were observed upon ESI-MS analyses of lipid extracts from gastrocnemius skeletal muscle (Fig. 13) and liver (Fig. 14) of WT and KO mice (Table S3). Liver from SC-fed WT (Fig. 14A) and KO (Fig. 14B) mice exhibited a predominant CL molecular species with an [M − H]− ion of m/z 1448 (Fig. 14, A and C) that corresponded to (18:2)4-CL, as demonstrated by tandem MS (Fig. S5A). CL from gastrocnemius muscle (hereafter designated only “muscle”) of SC-fed WT (Fig. 13A) and KO (Fig. 13C) mice comprised a more heterogeneous mixture of several species of comparable abundance.

ESI-MS signal for endogenous CL species relative to internal standard (14:0)4-CL tended to be greater for tissues from WD-fed compared with SC-fed mice (Figs. 13 and 14 and Table S3), and tissue total CL contents were significantly greater for WD-fed compared with SC-fed KO mice by ~2-fold for muscle and 1.5-fold for liver (Fig. 15). There were no significant differences between WD-fed and SC-fed WT mouse
tissue CL content, but the heterogeneity of CL molecular species tended to increase in tissues from WD-fed compared with SC-fed mice (Figs. 13 and 14 and Table S3). In liver from WD-fed mice, the [M – H]⁻ ion m/z values for the most abundant CL species shifted from 1448 to 1450/1452, and satellite clusters centered around m/z 1424 and 1476 became more prominent than in CL spectra from SC-fed mouse livers (Fig. 14). Ions of m/z 1450/1452 represent CL species that contain 18:1 fatty acid substituents(s) in addition to the usual 16:0 fatty acid substituents, respectively, as indicated by tandem MS (Fig. S5). The abundance of these species also increased in muscle of WD-fed compared with SC-fed KO mice (Fig. 13).

Dietary and genotype effects on tissue profiles and urinary excretion of LCAC species. Mitochondrial β-oxidation of long-chain fatty acyl-CoA esters yields shorter-chain species that equilibrate with pools of corresponding carnitine esters, and tissue acylcarnitine profiles reflect mitochondrial oxidative activity. ESI-MS signal for endogenous LCAC species relative to the internal standard tended to be greater for muscle (Fig. 16 and Table S4) and liver (Fig. S6) from WD-fed than from SC-fed mice, and amounts of tissue total LCAC were significantly greater for WD-fed than for SC-fed KO mice by 3.8-fold in muscle and 2.2-fold in liver (Fig. 17, A and C). Trends for tissue LCAC content to be greater in WD-fed compared with SC-fed WT mice did not achieve statistical significance, and LCAC contents of muscle and liver of WD-fed KO mice were significantly greater than those of WD-fed WT mice (Fig. 17, A and C).

The mean carbon chain length of LCAC in both liver and muscle of WD-fed KO mice was also significantly greater than that of SC-fed KO mice, and this was true for liver but not muscle of WT mice (Fig. 17, B and D). The increasing length of the LCAC carbon chain is likely to reflect less complete mitochondrial β-oxidation of fatty acid substrates in WD-fed mice. The increased tissue content of LCAC in WD-fed compared with SC-fed KO mice was accompanied by significantly greater urinary excretion of LCAC in WD-fed compared with SC-fed KO mice (Fig. 18). WT mice exhibited a similar trend of smaller magnitude that did not achieve statistical significance. Increased urinary excretion of LCAC may represent one means by which KO mice dispose of the excess caloric and fat load of the WD and the possibility that tissue TAG deposition might represent another was considered.

Dietary and genotype effects on tissue accumulation and molecular composition of TAG. The TAG content of liver from WT WD-fed mice was 8- to 10-fold higher than that of WT SC-fed mice and 4- to 6-fold higher than that of WD-KO mice (Fig. 19). Gastrocnemius muscle TAG content was comparable to that of liver for SC-fed WT and KO mice but did not increase appreciably upon WD feeding. Despite the differing tissue contents of TAG, ESI-MS profiles of WT and KO TAG molecular species in a given tissue, i.e., liver (Fig. 20) or muscle (Fig. S7), were similar, and there was a tendency for tissues of WD-fed mice to contain more saturated TAG species than those for SC-fed mice, as indicated by the relative abundances of members of the ion pairs m/z 863/865 and of m/z 889/891 (Fig. 20, Fig. S7, and Table S5). Tandem MS analyses revealed that this reflected the presence of more saturated fatty acid substituents, e.g., 16:0, and fewer polyunsaturated substituents, e.g., 18:2, in TAG from tissues from WD-fed mice (Fig. S8).

Oil red O staining of liver sections to visualize neutral lipid content (Fig. S9) confirmed conclusions from quantitative TAG analyses (Fig. 19) and indicated that there is a low level of staining in liver from SC-fed mice and much greater staining in sections from WD-fed WT mice, reflecting greater neutral lipid content. Staining is also greater in sections from WD-fed compared with SC-fed KO mice but is much less intense than that in sections from WD-fed WT mice (Fig. S9). Deposition of TAG in nonadipose tissues is thus another metabolic effect of WD-feeding in WT mice that is attenuated in the iPLA2γ-KO genotype, similar to increased body weight and adiposity, insulin resistance, glucose intolerance, and disruption of the normal diurnal variation in RER that reflects preferential active-phase oxidation of fatty acid over carbohydrate substrates by WD-fed WT mice.

**DISCUSSION**

Male WT mice that consume a WD achieve a body weight 23% greater than that of littermates fed SC (39), increased adipose tissue mass (18, 21), and hepatic steatosis (18). This is associated with insulin resistance reflected by hyperinsulinemia, elevated fasting blood glucose levels, impaired glucose tolerance, reduced insulin responsiveness, and rebound hyperglycemia after insulin administration (39). WD-fed mice also lose the normal ability to switch from utilizing fat as fuel during periods of fasting and inactivity to utilizing carbohydrate during periods of activity and food consumption, as reflected by a loss of normal diurnal variation in RER (18, 39). Our observations with WD-fed WT mice agree with these reports, but iPLA2γ-null mice clearly differ in their responses to WD consumption.
Events similar to those for WD-fed WT mice occur in human obesity-related insulin resistance and metabolic syndrome (59, 60), precursors of overt type 2 diabetes precipitated by pancreatic islet β-cell failure to maintain compensatory hypersecretion of insulin (61). Whole body knockout of iPLA2-y yields a complex phenotype affecting multiple organ systems and physiological abnormalities that include growth retardation, neurodegeneration, and cognitive dysfunction. This makes it difficult to determine the precise mechanisms that defend against diet-induced insulin resistance, but published evidence suggests that iPLA2-y might play important physiological roles in tissues involved in obesity and glucose homeostasis (47, 49, 50, 74).

Among tissues affected by WD consumption are adipose tissue, liver, and skeletal muscle. Increased adipose tissue in WD-fed WT mice accounts for most of the diet-induced increase in body weight and is greatly reduced in iPLA2-y-null mice, as are blood levels of the adipokine leptin, and this might reflect an impaired ability of iPLA2-y-null mice to expand their adipocyte pool. When 3T3-L1 preadipocytes are induced to differentiate into adipocytes, iPLA2-y mRNA levels rise, and differentiation is prevented by iPLA2-y siRNA (74). This suggests that iPLA2-y facilitates adipocyte differentiation, and iPLA2-y-deficiency might impair it. The reduced whole body adiposity and tissue TAG content of iPLA2-y-null mice, despite normal food intake, suggests energetic inefficiency that results in resistance to weight gain. Such leanness may protect against the adverse effects of obesity, including tissue lipid accumulation, inflammation, and endoplasmic reticulum stress, and this may enhance insulin sensitivity.

Human type 2 diabetes, the metabolic syndrome, and animal models of insulin resistance are marked by hepatic steatosis and TAG deposition (14, 23, 42, 69), and this also occurs in our WD-fed WT mice. Liver TAG content increased 8- to 10-fold in WD-fed compared with SC-fed WT mice, and this was greatly attenuated in iPLA2-y-null mice. For both genotypes, there was a shift in TAG molecular species distribution toward a higher content of saturated fatty acid substituents, which also occurs with myocardial TAG in mice with streptozotocin-diabetes (28).

Increased hepatic lipogenesis is a classical insulin response, and its occurrence in the setting of insulin resistance has been considered paradoxical (63). Potential explanations include...
selective insulin resistance (13). Insulin-resistant humans with insulin receptor (IR) mutations and mice with liver-specific IR deletion exhibit hyperglycemia and hyperinsulinemia but not hepatic steatosis (10, 73), suggesting that insulin-resistance does not involve all signaling pathways and that those governing lipid synthesis are preserved. Hyperinsulinemia from H9252\textsuperscript{-cell} compensation for muscle insulin resistance might thus drive hepatic lipid deposition, which is consistent with our findings that WD-fed iPLA\textsubscript{2-}null mice, unlike WT mice, do not develop hyperinsulinemia and experience much less diet-induced hepatic steatosis.

A converse effect occurs with transgenic mice that overexpress iPLA\textsubscript{2-} in myocardium, in which diet-induced cardiac TAG accumulation is augmented and precipitates cardiac dysfunction (47). That is attributed to iPLA\textsubscript{2-}-mediated accumulation of endocannabinoid precursors (76) and signaling molecules, e.g., (1-lyso, 2-arachidonoyl)-GPC (47, 82), that drive expression of the lipid biosynthetic enzyme diacylglycerol acyltransferase-1 (DGAT1), whose mRNA increases several-fold in myocardium of iPLA\textsubscript{2-} transgenic mice subjected to dietary stress (47). Genetic ablation of iPLA\textsubscript{2-} might thus result in reduced tissue levels of these signaling lipids and of DGAT1, and deficiency of endocannabinoid receptors (40) or of DGAT1 (20) also protects mice from diet-induced obesity (20, 40). These findings suggest that iPLA\textsubscript{2-} plays a physiological role in regulating tissue lipid deposition that might relate to effects on mitochondrial bioenergetics and fat oxidation.

Protection of iPLA\textsubscript{2-}-KO mice against diet-induced obesity and glucose intolerance is associated with a sparing of skeletal

![Fig. 17. Acylcarnitine content and mean carbon chain length in gastrocnemius muscle and liver of iPLA\textsubscript{2-}KO and WT mice fed SC or WD. Gastrocnemius muscle (A and B) and liver (C and D) specimens were obtained as in Figs. 12 and 14, respectively, from WT (light bars) and iPLA\textsubscript{2-}-KO (dark bars) male mice age 6 mo fed SC or WD as in Fig. 1, and acylcarnitine species in homogenates were quantitated by ESI-MS as in Fig. 16. A and C: values represent the sum of moles of acyl chain carbon for endogenous long-chain acylcarnitine (LCAC) species/mg tissue protein normalized to value for WTmice fed SC. B and D: values represent mean carbon chain length as computed from the ratio moles of acyl chain carbon relative to moles of acylcarnitine. Mean values ± SE (n = 10) are indicated. *Significantly (P < 0.05) higher value in genotype comparisons; x, significantly higher value in diet comparisons.](http://ajpendo.physiology.org/)

![Fig. 18. Acylcarnitine content of urine from iPLA\textsubscript{2-}KO and WT mice fed SC or WD. Urine was collected for 24 h on each of 4 consecutive days from male (A) or female (B) iPLA\textsubscript{2-}KO (dark bars) and WT (light bars) mice age 6 mo fed SC or WD as described in Fig. 1, and urine acylcarnitine content/g body wt was measured as in Fig. 17. Mean values ± SE are indicated (n = 25). *Significantly (P < 0.05) higher value in genotype comparisons; x, significantly higher value in diet comparisons.](http://ajpendo.physiology.org/)

![Fig. 19. Triacylglycerol (TAG) content of liver and gastrocnemius muscle of iPLA\textsubscript{2-}KO and WT mice fed SC or WD. Gastrocnemius muscle and liver specimens were obtained as in Figs. 12 and 14, respectively, from WT and iPLA\textsubscript{2-}KO male (A) and female (B) mice age 6 mo fed SC (light bars) or WD (dark bars) as in Fig. 1, and TAG content of lipid extracts was measured enzymatically as described in EXPERIMENTAL PROCEDURES and elsewhere (25, 53, 55) and expressed as μg TAG/mg tissue protein. Mean values ± SE are indicated (n = 15). *Significantly (P < 0.05) higher value in genotype comparisons; x, significantly higher value in diet comparisons.](http://ajpendo.physiology.org/)
muscle from WD feeding-induced deterioration in insulin responsiveness observed in WT mice, as reflected by greater responsiveness in insulin tolerance tests for WD-fed KO compared with WT mice. KO skeletal muscle resistance to WD-induced deterioration in insulin sensitivity is associated with impaired mitochondrial fatty acid oxidation reflected by reduced \([14C]\)palmitate oxidation ex vivo and by muscle accumulation of LCAC in vivo. Altered fatty acid processing by KO mitochondria is associated with morphological abnormalities and an altered distribution of mitochondria in muscle cells.

Cardiolipin (CL) is a mitochondrial inner membrane phospholipid with two negative charges at physiological pH that is enriched in linoleate (18:2) and has a large aliphatic chain to polar head group volume. These features allow CL to interact with electron transport chain proteins, e.g., cytochrome c oxidase (31, 64, 71, 84). Streptozotocin-diabetes induction in mice results in a decline in myocardial CL content, an increase in CL longer-chain fatty acid substituents, and cardiac dysfunction (29, 74). CL alterations and cardiac dysfunction also occur in iPLA2γ-null mice and transgenic mice that overexpress iPLA2γ in myocardium, and this may reflect a role for iPLA2γ in CL biosynthesis/remodeling that affects mitochondrial bioenergetics and cardiac physiology (47, 50). A neurophysiological role of iPLA2γ is also suggested by the increased hippocampal CL content and altered CL fatty acid composition of iPLA2γ-null mice, which is associated with mitochondrial degeneration and cognitive dysfunction (49).

We find that muscle and liver CL content increases in WD-fed compared with SC-fed iPLA2γ-null mice, and this rise is greater in KO than in WT mice. WD feeding is also associated with an increase in muscle and liver CL heterogeneity, and this is more pronounced in WT than in KO livers. Such diet-induced changes in CL content and composition may contribute to the differential effects of WD feeding on fuel selection in mitochondria of WT vs. iPLA2γ-null mice and in the impaired ability of the latter to process fatty acids oxidatively.

Such impaired processing is reflected by increased WD-induced accumulation of LCAC in iPLA2γ-null mouse skeletal muscle compared with WT. Moreover, the average muscle LCAC chain length increases significantly in WD-fed com-
pared with SC-fed iPLA\(_{2\gamma}\)-null mice. This indicates that muscle mitochondria of iPLA\(_{2\gamma}\)-null mice are less effective than those of WT mice in dietary fatty acid \(\beta\)-oxidative processing. LCAC also accumulate in myocardium of mice with streptozotocin-induced diabetes (74).

Muscle insulin resistance has been thought to involve impaired carnitine palmitoyltransferase-1 (CPT 1)-mediated mitochondrial fatty acid uptake and oxidation (58, 68) and preferential channeling of fatty acids into synthesis of the lipid mediators diacylglycerol and ceramide, and of TAG storage molecules (58, 68) that activate stress kinases, which interfere with insulin signaling (32, 83). Genetic manipulations that increase tissue levels of such lipids have been found not to induce insulin resistance (3, 56), however, and emerging evidence indicates that obesity-associated glucose intolerance arises from increased rather than decreased flux of fatty acids through mitochondrial \(\beta\)-oxidation (3, 39, 59–61).

Nutritional, pharmacological, and genetic maneuvers that suppress mitochondrial \(\beta\)-oxidation can protect against lipid-induced insulin resistance, and dietary fat is less damaging to skeletal muscle metabolic function when \(\beta\)-oxidation is constrained (39). Overexpressing malonyl-CoA decarboxylase results in increased levels of the CPT 1 inhibitor malonyl-CoA, which suppresses mitochondrial fatty acid import and oxidation, but this reverses muscle insulin resistance (3). In contrast, PPAR\(\alpha\) transgenic overexpression increases expression of genes involved in lipid oxidation and produces glucose intolerance and insulin resistance, and this phenotype is reversed by pharmacologic CPT 1 blockade (24). Moreover, PPAR\(\alpha\)-null mice have diminished muscle fatty acid \(\beta\)-oxidation and resist lipid-induced diabetes (78).

This has led to the hypothesis that WD consumption results in excessive mitochondrial \(\beta\)-oxidation and lipid-induced mitochondrial stress and consequent insulin resistance and glucose intolerance (39). With inborn mitochondrial diseases, acylcarnitine production is considered a detoxification mechanism that permits mitochondrial efflux of excess acyl groups (4). Muscle accumulation of these intermediates could represent a means to relieve redox stress imposed by mitochondrial overload (4), and their subsequent urinary excretion could represent a means to eliminate excess fat and caloric load.

Consistent with the hypothesis that inhibiting muscle fat oxidation protects against diet-induced metabolic dysregulation is a recent report that mice with genetic disruption of fatty acid \(\beta\)-oxidation intermediates (e.g., 3-hydroxyoctanoic acid) and shorter-chain hydroxyacarboxylic acids are ligands of a subfamily of G protein-coupled receptors that regulate adipocyte lipolysis (1, 2). Such compounds might be circulating mediators that coordinate metabolic activities of different tissues (1, 2) and represent candidate mitochondrial-derived signals of excessive \(\beta\)-oxidation. Since the average chain length of fatty acid \(\beta\)-oxidation intermediates that accumulate in muscle of WD-fed iPLA\(_{2\gamma}\)-null mice is substantially greater than that of WT mice, it is possible that a specific set of shorter-chain signaling molecules generated by WT mitochondria are not produced by iPLA\(_{2\gamma}\)-null mitochondria because of their less complete fatty acid \(\beta\)-oxidation.

ACKNOWLEDGMENTS

We thank Alan Bohrer and Min Tan for excellent technical assistance. Dr. Trey Coleman for helpful technical advice, Drs. Sasanka Ramanadham and Kevin Yarasheski for a critical reading of the manuscript, and Drs. Richard Gross and David Mancuso for providing breeder mice that we used to establish our colony of iPLA\(_{2\gamma}\)-null mice and wild-type littermates.

GRANTS

This work was supported by United States Public Health Service Grants R37 DK-34388, P41 RR-00954, P60 DK-20579, P30 DK-56341, and 1 R01 DK-07629 (C. F. Semenkovich).

DISCLOSURES

No conflicts of interest are reported by the author(s).

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AJP-Endocrinol Metab • VOL 298 • JUNE 2010 • www.ajpendo.org


