SUPPLEMENTARY DATA

Research Design and Methods

Reagents and Antibodies

^{3}H-Glucose was purchased from PerkinElmer (Ontario, Canada). Fatty acid free bovine serum albumin (BSA) was purchased from Sigma (Oakville, Ontario). Saline and 30% dextrose were purchased from Bimrda-MTC Animal Health Inc. (Cambridge, Ontario). Heparin (10,000 units/ml) was from Sandoz Canada Inc. (Quebec, Canada). Insulin (Humulin R) was purchased from Eli Lilly (Toronto, Canada). Polyclonal phosphospecific antibodies to Akt (T308, S473), total Akt2 and horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse-IgG were from Cell Signaling Technology (Beverly, MA) and polyclonal phosphospecific antibodies to insulin receptor substrate-1 (IRS1, Y612) was from Life Technologies (Burlington, ON), peptide affinity purified antibody to PGC1α was from Novus Biological (Oakville, ON), whereas total oxidative phosphorylation (OXPHOS) rodent antibody cocktail was from MitoScience (Eugene, Oregon). Polyvinylidene difluoride (PVDF) membranes were from Bio-Rad (Burlington, ON) and Chemiluminescence Reagent Plus from PerkinElmer (Boston, MA). All other reagents and chemicals used were of the highest purity available. HFD was purchased from Research Diets (New Jersey, USA), which provided 20% kcal from protein, 20% kcal from carbohydrate and 60% kcal from fat compared with the control regular chow diet (LabDiet: Brentwood, MO) which provided 20% kcal from protein, 70% kcal from carbohydrate and 10% kcal from fat.

Glucose tolerance test (GTT) and fasting circulating free fatty acid

To perform GTT animals were starved 5-6 h, being careful to begin at same time of day (~9am) in each case, before receiving a bolus intraperitoneal injection of glucose (2 g/kg body weight). Tail vein blood samples were collected after 15, 30, 60 and 90 min and blood glucose level (mmol/l) determined with a glucometer (Conture, Bayer). Free fatty acid level was assessed using the NEFA-HR(2) kit (Wako, Richmond, VA).

Hyperinsulinemic euglycemic clamp

As described before (1), on the day of experiment animals were starved at ~9am for 5-6 h (same as for GTT studies) and the pre-inserted jugular vein catheter was connected to a swivel for reagent infusion while the intra-artery catheter was used for blood sample collection. During the entire clamp procedure animals were conscious and unrestrained. The protocol consisted of a 90-min tracer equilibration period (infusion of D-^{3}H-glucose at a constant rate of 0.05 ml/hr from Expt = -90 to 0 min) followed by a 120-min experimental period (infusion of the combination of different tracers from Expt = 0 to 120 min: D-^{3}H-glucose and insulin at a constant rate of 0.1 ml/hr to maintain the insulin concentration at 4 mU/kg/min; 30% dextrose at different rates to adjust and maintain the blood glucose level as determined in equilibration period; a bolus of 130 µCi ^{14}C-2-deoxy-glucose was given at Expt = 75 min). Blood samples (~1 µl) were obtained at Expt = -90, -30, -20, -10, 0 min to determine basal glucose levels with a glucometer (Contour, Bayer); additional blood samples (20 µl) were collected at the same time point, serum separated and stored for radioactivity assay. The hyperinsulinemic euglycemic clamp commenced at Expt = 0 min, blood glucose levels measured from blood samples (~1 µl) obtained every 10 min during Expt = 0 – 70 min; 30% dextrose infusion rate (started at 0.05 ml/hr) was adjusted accordingly to maintain blood glucose levels at the predetermined equilibration basal level. The 30% dextrose infusion rate and the corresponding blood glucose level during Expt = 0 – 70 min was used to calculate glucose infusion rate (GIR). The individual infusion rates of the combined infusate including D-^{3}H-glcuose, insulin and 30%

dextrose was maintained at the same rate upon Expt = 70 min and thereafter. Blood samples (~20 µl) collected every 5 min during Expt = 75 – 120 min to measure blood glucose levels and serum samples were obtained for radioactivity analysis. Animals received a constant infusion (0.1 ml/hr) of saline-washed erythrocytes from donor mice throughout the entire procedure from Expt = -90 to 120 min to prevent a fall of > 5% hematocrit. At the end of the clamp, animals were anaesthetized, blood samples collected from cardiac puncture and hind limb skeletal muscles (extensor digitorum longus (EDL), soleus (SOL), Gastrocnemius, tibialis (TA)), were excised, snap frozen in liquid N_2 and stored at –80°C until further analysis. Serum samples collected at Expt = -90, -30, -20, -10, 0, 75, 80, 85, 90, 95, 100, 105, 110, 115 and 120 min were used for radioactivity assay to determine the glucose disappearance rate (Rd), glucose appearance rate (Ra) and glycolytic rate.

Preparation of skeletal muscle homogenates and Western blotting

Skeletal muscle strips snap frozen in liquid nitrogen were first ground into powder then mixed with 100 µl lysis buffer (30 mM Hepes, pH 7.4, 2.5 mM EGTA, 3 mM EDTA, 70 mM KCl, 20 mM β-glycerolphosphate, 20 mM NaF, 1 mM Na_3VO_4, 200 µM PMSF, 1 µM pepstatin A, 10 µM E64, 1 µM leupeptin and 0.1% NP40) per 10 mg tissue and incubated on a rotating rocker for 1 h at 4°C. Samples were centrifuged at 15,000 g for 10 min at 4°C and only supernatants were collected for Western blot analysis. Western blotting was conducted essentially as described by us previously (2) where, after treatments described previously, samples were prepared in 1x Laemmli sample buffer containing 10% (vol/vol) β-mercaptoethanol, passed through a syringe several times, and heated (65°C, 5 min). Lysates were then centrifuged for 5 min in a bench top micro-centrifuge (15,000 g), and approximately 30 µg protein were resolved by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 3% BSA dissolved in 1x wash buffer solution of 50 mM Tris-base, 150 mM NaCl, 1% Triton X-100, and 1% Nonidet P-40 for 1 h. Membranes were incubated overnight with primary antibodies at the following dilutions: phospho-Akt(T308&S473) (1:1,000), total Akt2 (1:1000) and phospho-IRS1(Y612) (1:1,000), PGC1α (1:1,000) and total OXPHOS antibody cocktail (1:2,000). Membranes were then washed four times in 1x wash buffer for 15 min each at room temperature and incubated with appropriate HRP-coupled secondary antibody (1:10,000) for 1 h. Membranes were washed five times in 1x wash buffer for 10 min each and proteins visualized using enhanced chemiluminescence. Quantitative analysis of each band was then determined via densitometric scanning.

Triglyceride (TG) and ATP content assays

TG and ATP content in skeletal muscle was analyzed by colorimetric Triglyceride Quantification Kit (Cat#K622) “ATP Assay Kit” (Cat#K355) purchased from Biovision (California, USA). Gastrocnemius skeletal muscles collected from animals were crushed into powder in liquid nitrogen and prepared for the assay according to manufacturer’s instructions. In short, approximately 100 mg of tissue sample was used for TG extraction and TG content analysis while 10 mg of tissue sample was used for ATP content assay. Both were then measured with a microplate photometric reader set at OD 570 nm as per manufacturer's instructions. Data shown in the results section were normalized by protein concentration of each sample.

Metabolomic and lipidomic analyses

The non-targeted metabolic profiling instrumentation employed for this analysis combined three independent platforms: ultrahigh performance liquid chromatography/tandem mass

spectrometry (UHPLC/MS/MS^2) optimized for basic species, UHPLC/MS/MS^2 optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Samples were processed essentially as described previously (3; 4) and in this case we examined samples extracted from either intact or powdered skeletal muscle sample. For each sample, 100µL was used for analyses. Using an automated liquid handler (Hamilton LabStar, Salt Lake City, UT), protein was precipitated from the tissue water homogenate with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis on the three platforms. Aliquots, dried under nitrogen and vacuum-desiccated, were subsequently either reconstituted in 50µL 0.1% formic acid in water (acidic conditions) or in 50µL 6.5mM ammonium bicarbonate in water, pH 8 (basic conditions) for the two UHPLC/MS/MS^2 analyses or derivatized to a final volume of 50µL for GC/MS analysis using equal parts bistrimethyl-silyl-trifluoroacetamide and solvent mixture acetonitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine at 60°C for one hour. In addition, three types of controls were analyzed in concert with the experimental samples: aliquots of a pooled sample derived from a portion of all experimental samples served as technical replicates throughout the data set, extracted water samples served as process blanks, and a cocktail of standards spiked into every analyzed sample allowed instrument performance monitoring. Experimental samples and controls were randomized across platform run days.

For UHLC/MS/MS^2 analysis, aliquots were separated using a Waters Acquity UPLC (Waters, Millford, MA) and analyzed using an LTQ mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA) which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The MS instrument scanned 99-1000 *m/z* and alternated between MS and MS^2 scans using dynamic exclusion with approximately 6 scans per second. Derivatized samples for GC/MS were separated on a 5% phenyldimethyl silicone column with helium as the carrier gas and a temperature ramp from 60°C to 340°C and then analyzed on a Thermo-Finnigan Trace DSQ MS (Thermo Fisher Scientific, Inc.) operated at unit mass resolving power with electron impact ionization and a 50-750 atomic mass unit scan range. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated MS spectra, and were curated by visual inspection for quality control using software developed at Metabolon (5).

For statistical analyses and data display purposes, any missing values were assumed to be below the limits of detection and these values were imputed with the compound minimum (minimum value imputation). Statistical analysis of log-transformed data was performed using "R" (http://cran.r-project.org/), which is a freely available, open-source software package. Welch's t-tests were performed to compare data between experimental groups. Multiple comparisons were accounted for by estimating the false discovery rate (FDR) using q-values (data not shown) (6).

Additional lipidomic analysis was performed by us, essentially as previously described (7; 8). DAGs were extracted from the skeletal muscle and 1,2-dipentadecanoin was used as an internal standard. Briefly, the tissues were homogenized in PBS and chloroform/methanol (2:1, v/v) was added. The lipids were extracted by vortexing and centrifugation at 3000 x g. The lower phase was collected and samples were evaporated under N_2 gas. After dissolving in hexane/methylene chloride/methyl tert-butyl ether, the samples were loaded onto the diol-bonded solid phase extraction column (Waters, Inc., Milford, MA) under vacuum. DAGs were eluted as described previously (9). Extracted lipids were dried under N_2 and redissolved in

methanol. The contents of DAGs in extracted lipids were quantified by a liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a method described previously (8), using a bench-top tandem mass spectrometry, API 4000 Q-trap (Applied Biosystem, Framingham, MA) interfaced with an atmospheric pressure chemical ionization source and Agilent series 1200 micro-pump equipped with an autosampler. Five species of DAGs (di-16:0, di-18:1, 16:0-18:1, 18:0-18:2, 18:0-20:4) were separated by HPLC with C6-phenyl column, ionized in positive atmospheric pressure chemical ionization mode and quantified by multiple reaction monitoring method. Ceramides were measured by LC-MS/MS employing a previously reported method by Yoo *et al.* (7) with modifications. After tissue homogenization in PBS, C17:0-ceramide was added to the extracts containing 1 mg of proteins as an internal standard. Lipids were extracted by chloroform/methanol (1:2), and phospholipids were saponified by adding KOH and incubation at 37°C for 2 h. Extracts were centrifuged and supernatants were isolated and then dried under N_2. Sphingolipids - ceramides (C14:0, C16:0, C18:0, C18:1, C20:0, C24:0, C24:1), sphinganine, sphingosine, sphingosine-1-phosphate, and sphingomyelins (C16:0, C18:0, C18:1) - were separated by HPLC with C18 columns (XTerra C18, 3.5 □m, 2.1 x 50 mm) and ionized in positive electrospray ionization mode. Metabolites of sphingolipids were monitored for multiple reaction monitoring (MRM) quantification.

Determination of epididymal adipocyte size

Isolated epididymal white adipose tissue was fixed for 24 hr at room temperature in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm before staining with routine hematoxylin and eosin (H&E). To evaluate adipocyte size, sections from 4 mice from each group were used. Cross-sectional areas of at least 300 randomly selected cells from four sections for each group were determined and quantitated by using the NIH ImageJ software.

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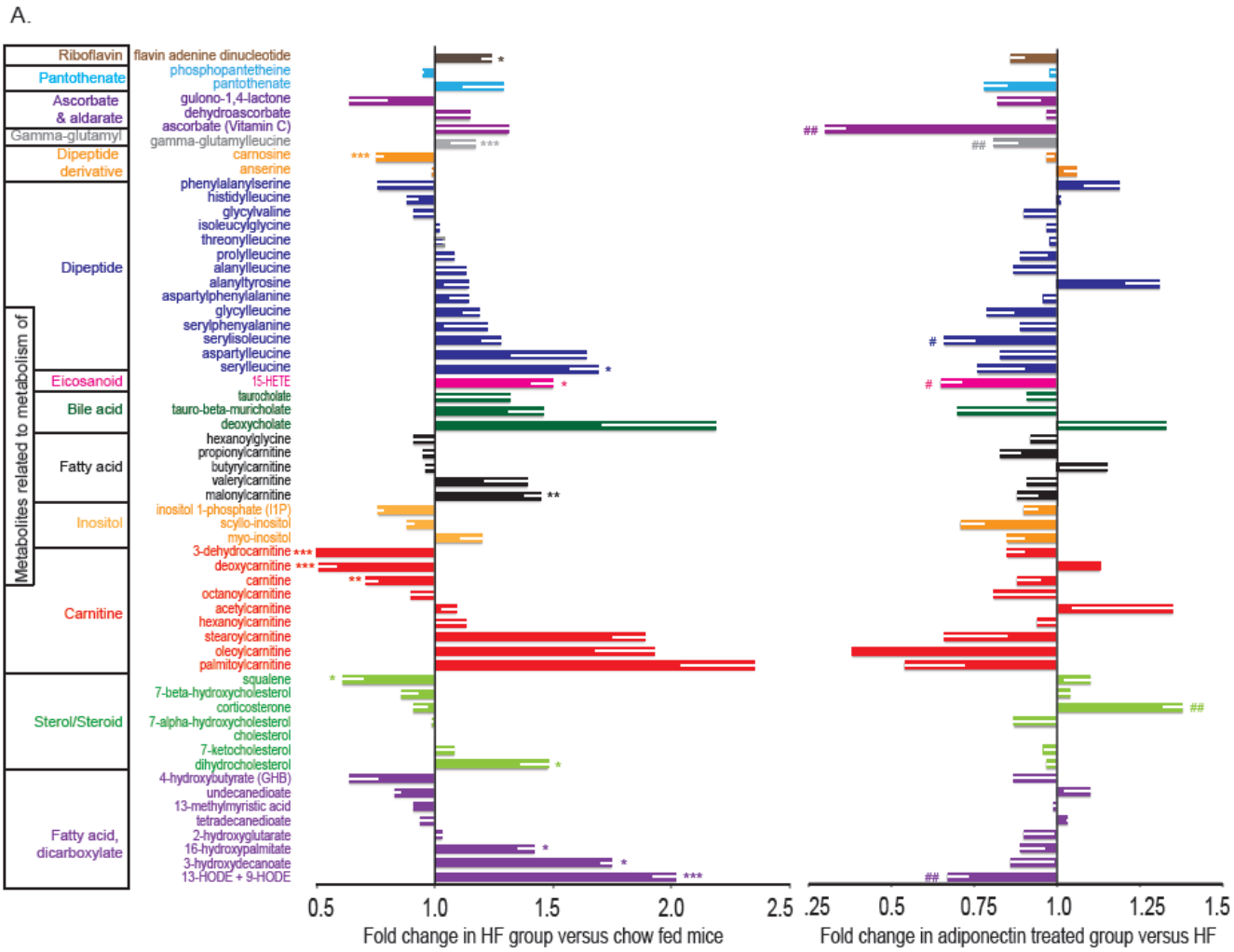
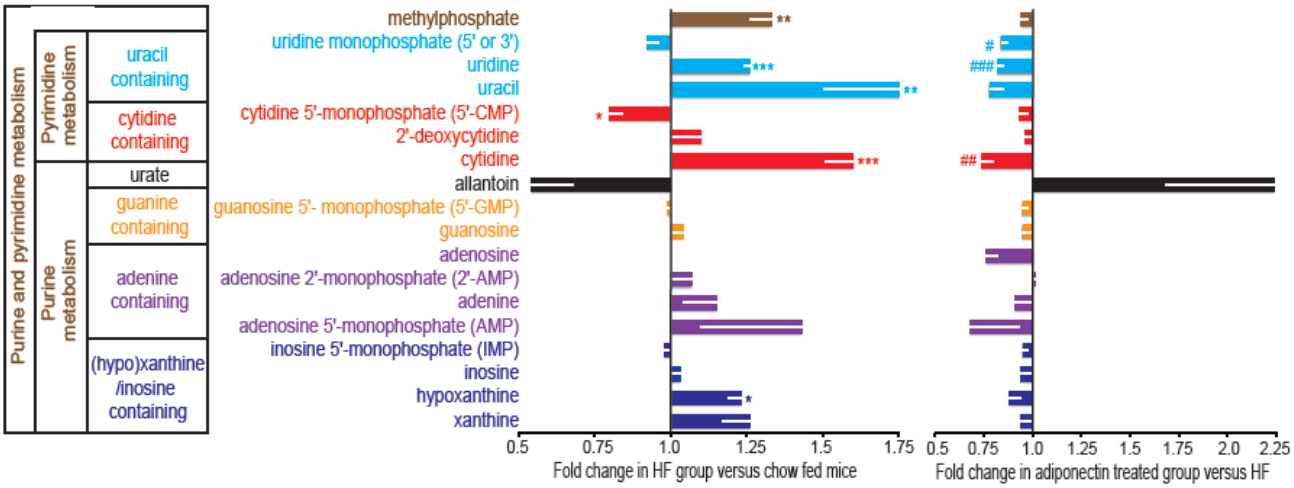
Supplementary Figure 1. Metabolomic analysis of various additional metabolic groups in skeletal muscle.

Metabolomic analyses were performed on skeletal muscle from AdKO mice treated as described in clamp studies (see figure 1) and here data are shown for individual metabolites related to A. peptide and additional lipid-related metabolism; B. purine and pyrimidine metabolism. First the influence of HFD versus chow is shown as fold change observed in HF group versus chow fed mice. In parallel, the right side graph shows fold change in adiponectin treated versus saline treated within HFD group, thus changes indicate a corrective effect of adiponectin. Data represent mean ± SEM; * significant difference between HF diet group and chow diet group; # significant difference between fAd and saline treatment within HF diet group; *,#, P<0.05; **,##, P<0.01; ***,###, P<0.001. n = 5-6.



SUPPLEMENTARY DATA

B.



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