Extended Materials and Methods

Tracer infusion protocol.

Infusion of labeled palmitate was based Blachnio-Zabielska et al.(Blachnio-Zabielska, et al. 2013). Uniformly labeled potassium [U-$_{13}$C]-palmitate (Sigma-Aldrich, St. Louis, MO, USA) was prepared in fatty acids free albumin (Sigma-Aldrich) as albumin-bound potassium salt according to Guo et al. (Guo and Jensen 1998) . Firstly, appropriate amount of potassium palmitate tracer dissolved in chloroform/methanol (2:1) was dried under nitrogen stream and subsequently suspended in hot albumin solution (essentially fatty acids and globulin free in 10x PBS stock, both from Sigma-Aldrich) in 1:10 ratio (palmitate/albumin, wt./wt.). Suspension was sonicated in hot bath (60°C) until clear, diluted to a final concentration of 1nmol of [U-$_{13}$C]-palmitate/µl and stirred for 1 hour on heater. Infusate was filter-sterilized with 0.2µm syringe filter and stored in sterile falcon tubes -20°C until use. At the day of experiment the food was withdrawn 6 hours prior to infusion. Animals received pentobarbital anesthesia intraperitoneally at a dose of 80~~50~~ mg/kg and were placed on a heating blanket. Pentobarbital anesthesia compared to other anesthetics has lowest impact on whole body and tissue glucose metabolism (Saha, et al. 2005; Sano, et al. 2016). Tail was shaved off and tail blood circulation was stimulated mild irradiation with IR heating lamp. Elastic infusion catheter (MTV 1, Braintree Scientific, Braintree, MA) was inserted through integrated 23Ga needle into lateral tail vein and the catheter was secured with small drop of cyanoacrylic adhesive and piece of fabric tape. Infusion line was connected to syringe pump (NE-1000 model, New Era Pump Systems, Farmingdale, NY). To ensure quick isotopic equilibration of fatty acids pool, animals received 0.5 µmol/kg bolus of [U-^{13}C]-palmitate tracer during the first 10 seconds followed by continuous infusion at 3µmol/kg/h for the next 2 hours. To prevent increase in total body fluids, the infused volume did not exceeded 17% of total plasma volume (calculated according to Lee et al. , (Lee and Blaufox 1985)) and was lower than 2-hour urine output, according to the values for Wistar rats from Rat Phenome Database (Serikawa, et al. 2009). Every 15 min, a 50 µl blood sample was taken from saphenous vein into a heparinized Microvette capillary tube (Stardstedt, Numbrecht, Germany). Plasma was obtained by centrifugation for analysis of palmitate concentration and enrichment. The tracer infusion had no effect on the level of plasma FFA. The pre- and postinfusion concentration of total plasma FFA and FFA palmitate did not differ

significantly (274 μmol/l versus 271 μmol/l for total FFA; 64 μmol/l versus 64 μmol/l for palmitate). During the last 30 minutes, insulin (0.5 U/kg) was administrated through the catheter to measure the insulin-stimulated protein phosphorylation. The rats were anaesthetized by intraperitoneal injection of pentobarbital in a dose of 80 mg/kg of body weight and the last blood sample was collected from inferior vena cava. Rats were euthanised by heart incision. The muscle samples were cleaned off connective tissue and fat, frozen in liquid nitrogen and stored at -80°C until analysis.

Plasma fatty acids concentration and enrichment.

Plasma fatty acid profile and palmitate enrichment was measured according to Persson et al. (Persson, et al. 2010), with the use of Agilent 1290 UHPLC and Agilent 6460 MS/MS. Briefly, 200μl (for concentration) or 20 μl (for enrichment) of plasma was spiked with internal standard (C17:0 heptadecanoate, 60μg/ml in 2% albumin in PBS). Fatty acids were extracted with acidic Dole solution (isopropyl alcohol:heptane:1N H_2SO_4; 40:10:1; v/v/v), evaporated to dryness under nitrogen and re-suspended in HPLC Buffer A. Fatty acids were separated on Zorbax C18, 1.8 μm, 2.1 ×150 mm UHPLC column (Agilent) with appropriate buffer system (Buffer A: 80% acetonitrile/0.5 mM ammonium acetate; Buffer B: 99% acetonitrile, 1% 0.5 mM ammonium acetate). Negative ESI ionization and SIM mode was used to monitor ions corresponding to individual fatty acids as their $[M\text{-}H]^-$ ions. To measure palmitate enrichment ions were monitored as $[M+2\text{-}H]^-$ for palmitate and $[M+16\text{-}H]^-$ for [U-^{13}C]-palmitate. Plasma fatty acids concentration was calculated with the use of appropriate concentration curves against heptadecanoate internal standard. Palmitate enrichment was measured against appropriate enrichment curve, taking into account isotopic purity of both palmitate and [U-^{13}C]-palmitate tracer. All standards were from Sigma-Aldrich. The area under palmitate enrichment curve was identical in all experimental groups (Figure S1). Measured fatty acid species are listed in Table S2.

Fatty acid-CoAs tissue concentration and enrichment.

Malonyl-CoA, LCACoA content and [$^{13}C_{16}$]-CoA enrichment was measured according to Blachnio-Zabielska et al. (Blachnio-Zabielska, et al. 2011), with the use of LC/MS/MS. Briefly, tissues were pulverized in LN2 and approx. 20 mg was placed in 250ul of 100mM KH_2PO_4, pH

4.9. Subsequently, 20ng of internal standard (heptadecanoyl-CoA, Avanti Polar Lipids) was added in 250ul of ACN:2-propanol:methanol (3:1:1, v/v/v). Samples were homogenized on ice, vortexed and sonicated for 3 minutes on ice. After centrifugation for 10 min@16000g (4°C) the supernatant was collected and the pellet re-extracted with 250ul of ACN:2-propanol:methanol (3:1:1, v/v/v, without ISTD). Combined supernatants were dried under nitrogen, re-suspended in 50 µl of methanol:water 1:1 (v/v), centrifuged for 10minutes@14000 (4°C) and subjected for UPLC/MS/MS. Individual LCACoAs were separated with Agilent 1290 UHPLC on Zorbax C8, 1.8 µm, 2.1 ×150 mm UHPLC column, using binary solvent system of 15mM NH_4OH as solvent A and 15mM NH_4OH in ACN as solvent B. Precursors were generated as $[M+H]^+$ ions using +ESI. Multiple reaction monitoring (MRM) was used to generate acyl-pantetheine product ions which contain fatty acid necessary for enrichment measurement. All LCACoA precursors were monitored as $[M+H]^+$ ions, except of palmitoyl-CoA and $[U^{13}C]$ palmitoyl-CoA monitored as the $[M+2+H]^+$ and $[M+16+H]^+$ ions, respectively. Quantified LCACoA species are listed in Table S3. All standards were purchased from Avanti Polar Lipids (Alabaster, Al, USA) whereas $[U^{13}C]$palmitoyl-CoA was purchased from Sigma-Aldrich (Isotec).

Tissue ceramide concentration and enrichment.

The ceramide content and isotopic enrichment was measured using a UHPLC/MS/MS approach according to Blachnio-Zabielska et al. (Blachnio-Zabielska, et al. 2012). Briefly, tissue samples were pulverized in LN_2. Approx. 20mg of tissue powder was spiked with internal standards (d17:1-Sph, d17:1-Spa, d17:1-S1P, C17:0-Cer and C25:0-Cer, Avanti Polar Lipids Alabaster, AL) and homogenized on ice in cold homogenization buffer (50mM Tris/HCl, pH 7.4, 0.25 M sucrose, 25 mM KCl and 0.5mM EDTA). After sonication for 30s on ice, the homogenate was extracted twice with 2-propanol/water/ethyl acetate (30:10:60; v/v/v). Organic phase was evaporated under nitrogen and suspended in HPLC Buffer A. Sphingolipids were resolved on Zorbax SB-C8, 1.8 µm, 2.1 ×150 mm UHPLC column with the use of Agilent 1290 UHPLC and two buffer system (Buffer A: 2mM ammonium formate, 0.1% formic acid in LC/MS grade methanol, Buffer B: 1 mM ammonium formate, 0.1% formic acid in water). Eluting sphingolipids were monitored on Agilent 6460 MS in +ESI mode as $[M+H]^+$ ions except C16:0-Cer and $[^{13}C_{16}]$16:0-Cer which were monitored as $[M+2+H]^+$ and $[M+16+H]^+$ ions, respectively.

The entire analysis was performed in MRM mode. Concentration of individual ceramides and isotopic enrichment of palmitoyl-ceramide was calculated with the use of calibration curves prepared on commercially available standards (Avanti Polar Lipids Alabaster, AL). Ceramide enrichment curve was prepared on synthetic natural abundance C16:0-Cer (Avanti Polar Lipids) and [$^{13}C_{16}$]palmitoyl-Cer (Lipidomics Core, Medical University of South Carolina) taking into account isotopic purity of both the natural abundance and enriched species. Measured Cer molecular species are listed in Table S5.

Tissue diacylglycerol concentration and enrichment.

The content and isotopic enrichment of DAG was measured using a UHPLC/MS/MS approach according to Blachnio-Zabielska et al (Blachnio-Zabielska et al. 2013). Diacylglycerols were extracted together with ceramides, using the same method and solvent system as in the case of ceramides. The 50 ng of 1,3 15/15-DAG (Avanti Polar Lipids) was added as internal standard. Individual diacylglycerols were separated with the use of Agilent 1290 UHPLC on Zorbax SB-C8 column 2.1 × 150 mm, 1.8 µm using binary gradient of 2 mM ammonium formate, 0.15% formic acid in LC/MS grade methanol as solvent A and 1.5 mM ammonium formate, 0.1% formic acid in water as solvent B. Samples were suspended in 100 µl of buffer A. Individual DAG species were analyzed without derivatization as $[M+NH_4]^+$ ammonium adducts in +ESI mode. Low levels of isotopic enrichment in 16:0/16:0-DAG and 16:0/18:1-DAG species required monitoring of $[M+2+NH_4]^+$ ions for unenriched species and $[M+16+NH_4]^+$ ions for enriched ones. Analysis was performed in MRM mode with collision energy of 10 eV which preserved [$^{13}C_{16}$]palmitate fatty acid moiety and part of glycerol backbone as product ion, permitting calculation of isotopic enrichment. Concentration of individual diacylglycerols was calculated with the use of calibration curves prepared on commercially available standards (Avanti Polar Lipids Alabaster, AL). Isotopic enrichment curve was prepared on synthetic natural abundance 16:0/16:0-DAG and 16:0/18:1-DAG (Avanti Polar Lipids) and [$^{13}C_{16}$]palmitate labeled species (Lipidomics Core, Medical University of South Carolina). Monitored diacylglycerols molecular species are listed in S6.

Tissue acyl-carnitine concentration and enrichment.

Acyl-carnitine concentration and isotopic enrichment of $^{13}C_{16}$-palmitoyl carnitine was measured according to Sun et al. (Sun, et al. 2006) with minor modifications. Briefly, 20mg of LN_2 pulverized tissue was suspended in 50μl of 1M KH_2PO_4 and 1ml of extraction solution of ACN/MeOH (3:1; v/v) with 20ng of heptadecanoyl-carnitine as internal standard. Samples were vortexed for 2 minutes and sonicated on ice for 30s. After centrifugation for 20min@14000g (4°C) the supernatant was dried under nitrogen, re-suspended in 100μl of ACN/MeOH (3:1; v/v) and subjested to LC-MS analysis. Samples were resolved on on Zorbax SB-C8 column 2.1 × 150 mm, 1.8 μm and Agilent 1290 UHPLC using binary gradient of 0.05% HFBA in water as solvent A and acetonitrile as solvent B. Positive electrospray (+ESI) and SIM mode was used on Agilent 6460 MS/MS to monitor $[M+H]^+$ ions of palmitoyl- and oleoyl-carnitine for concentration measurements and $[M+2+H]^+$ and $[M+16+H]^+$ ions of palmitoyl-carnitine for isotopic enrichment measurements. Concentration curves were prepared on commercial standards (Avanti Polar Lipids). Labeled [13C16]palmitoyl-carnitine from Isotec (Sigma-Aldrich) was used for enrichment curve. Table S4 lists measured acyl-carnitine species.

Calculation of lipids fractional synthesis rate.

Primed continuous infusion of labeled palmitate produced quick isotopic equilibration in each of the experimental groups (Supplement Figure S1A,B), thus the rate of appearance of palmitate (palmitate Ra) was calculated using steady-state equations (Kim, et al. 2016; Wolfe 1984):

$$Palmitate\ Ra = \frac{\left[U - {}^{13}C\right] palmitate\ infusion\ rate\ (\frac{nmol}{min}/kg)}{Mean\ plasma\ \left[U - {}^{13}C\right] palmitate\ enrichment\ (MPE)}$$

Total fatty acid Ra (FFA Ra) was calculated by dividing the palmitate Ra by the proportional contribution of palmitate to the total concentration of fatty acids as measured by LC/MS/MS. After reaching steady-state plasma fatty acids rate of disappearance (FFA Rd) balanced plasma fatty acids rate of appearance (FFA Ra), thus FFA Ra was equal to FFA turnover rate. For estimation of Acyl-CoA turnover rate, we assumed quick isotopic equilibration of acyl-CoA pool as shown by Zhang et al. (Zhang, et al. 2012). We used palmitoyl-CoA enrichment in muscle to calculate Ra. Total LCACoA Ra was calculated by dividing the palmitoyl-CoA Ra by the proportional contribution of palmitoyl-CoA to the total concentration of LCACoA as measured by LC/MS/MS.

Fractional synthesis rate of acyl-CoA, acyl-carnitine, C16:0-Cer and C16:0/C16:0-DAG and C16:0/C18:1-DAG lipids was calculated by the tracer incorporation method, which is based on the precursor-product principle.

$$Lipid\ FSR\ (\%/h) = \frac{(LipidE_{t2h} - LipidE_{t0})}{\int_{t0}^{t2h} EA\Delta t}$$

Where $(LipidE_{t2} - LipidE_{t1})$ is the enrichment increment of palmitate in analyzed from the beginning (t0) to the end (t2h) of tracer infusion and $\int_{t0}^{t2h} EA\Delta t$ is the integral of plasma palmitate enrichment versus time function (the area under plasma precursor enrichment curve) approximated using trapezoidal rule. Values $LipidE_{t2h}, LipidE_{t0}$ and $\int_{t0}^{t2h} EA\Delta$t were calculated individually for each animal.

Protein and RNA isolation: Total RNA and protein was isolated from40mg of LN2 pulverized muscle samples using NucleoSpin ®RNA/Protein isolation kit (Macherey-Nagel, Bethlehem, PA), according to procedure by Bahn et al (Bahn, et al. 2008). RNA and proteins were separated on NucleoSpin columns according to manufacturer guidelines.

Western Blot: Pulverized tissue samples weighting approx. 40mg were homogenized on ice in RIPA buffer supplemented with protease and phosphatase inhibitors (CompleteUltra and

Phospho-Stop, Roche). After 1 minute of sonication, samples were centrifuged for 10min@1000g to sediment cellular debris. Supernatant protein was measured with Poerce660nm protein assay kit (Thermo-Scientific). Proteins were prepared in Laemmli buffer with 5mMTCEP, normalized to 2µg/µl of protein, thermally denaturated for 10min@85°C and stored in -20°C for further analysis. Prepared samples were loaded on gradient AnykD Criterion gels and resolved using Criterion Cell electrophoresis unit. Proteins were blotted onto PVDF membraned with the use of TransBlott SD semi-dry transfer unit. Membranes were blocked with Blotto blocking grade blocker or BSA (in case of phosphoproteins) and incubated overnight with primary antibody. Following target proteins were targeted using primary antibodies: GLUT4(Abcam, Cambridge, MA, ab33780), FABP (Santa Cruz Biotechnology, sc-135181), CD36 (Abcam, ab64014), FATP1 (Santa Criz Biotechnology, sc-25541), CPT1A (Abcam, ab83862), ACC (Abcam, ab45174), ACS (Abcam, ab66038), SPT (Abcam, ab23696), PAP2B (Abcam, ab52581), Akt (Cell Signaling Technology, #4691), pAktSer473 (Cell Signaling Technology, #9271), pAktThr308 (Cell Signaling Technology, #9275), AS160 (Invitrogen, MA5-14840), AS160Thr642 (Invitrogen, 44-1071G), and GAPDH (Santa Cruz Biotechnology, sc-32233). After incubation with appropriate HRP-conjugated secondary antibody protein bands were visualized by chemiluminescence using ChemiDoc XRS+ system. Values were normalized to GAPDH protein expression measured from parallel runs and expressed as fold changes over control group values. All chemicals and equipment used for immunoblotting were purchased from Bio-Rad (Hercules, CA).

RT-PCR: The RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) with oligo(dT)$_{18}$. Specific primers for the ACC, ACS, CD36, CPT1, FABPpm, FATP1, GLUT4, SPT and GAPDH were designed using the Beacon Designer Software. The housekeeping gene GAPDH was used as the reference gene for quantification. Quantitative real-time PCR was performed with SYBR Green Supermix Kit (Bio-Rad) using a Bio-Rad Chromo4 system.

Isolation of muscle microsomes:

Muscle microsomes were isolated from LN2 pulverized muscle samples by ultracentrifugation. Briefly, approx. of 50mg of LN_2 pulverised muscle tissue was homogenized on ice in 25mM HEPES, pH 7.4, 0.25M sucrose, 2mM EDTA, 2mM DTT and in the presence of CompleteUltra protease inhibitors (Roche). Homogenate was centrifuged for 10min@22000g (4°C) to sediment cellular debris. Supernatant was transferred to cold ultracentrifuge tubes and microsomes were pelleted by ultracentrifugation (40min@150000g, 4°C). Isolated microsomes were suspended in storage buffer (20% glycerol in homogenization buffer) and stored in -20°C for further analysis.

Acyl-coenzyme A synthetase (ACSs) activity in microsomal fraction: Acyl-coenzyme A synthetase (ACSs) activity was assayed in muscle microsomes according de Jong et al. (de Jong, et al. 2007), with the use of radiolabeled [9,10-3H(N)]-palmitate (American Radiolabeled Chemicals, Inc. Saint Louis, MO) and cold potassium palmitate (Sigma-Aldrich). Approx. 20µg of microsomal proteins were incubated for 10 minutes (37°C) in 200µl of ACS assay buffer (175mM Tris-HCl, pH 7.4, 8mM $MgCl_2$, 5mM DTT, 1mM ATP, 0.2mM CoA-SH and 50µM of [9,10-3H(N)]-palmitate (specific activity adjusted with cold palmitate to 1250DPM/pmol). Reaction was stopped by addition of 1ml of acidic Dole solution, and unreacted palmitate was extracted with 2ml of heptane and 0.5ml of H_2O. Upper palmitate-containing phase was discarded and lower, aqueous phase was washed twice with 2ml of heptane and transferred to 4ml scintillation liquid. Radioactivity was measured with Packard TriCarb 1900TR liquid scintillation counter. ACS activity was expressed in nmol/min/mg of microsomal protein.

Phosphatidate phosphatase 2 (PAP2) activity in microsomal fraction: Activity of PAP2 in skeletal muscle microsomes was measured according to Martin et al. (Martin, et al. 1991), with the use of radiolabeled L-a-dioleoyl [oleoyl-1-^{14}C] –glycerolo-3-phosphate (American Radiolabeled Chemicals, Inc. Saint Louis, MO) and cold 1,2-dioleoyl-sn-glycero-3- phosphate (Sigma-Aldrich). Briefly, approx. 30 µg of microsomal proteins were incubated for 15 minutes (37°C) in 100µl of 100mM Tris:maleate, pH6.5, 5mM $MgCl_2$, 0.05% Tween 20, 2mg/ml essentially fatty acids free albumin, 0.4mM phosphatidycholine, 1mM EDTA, 0.2mM Orlistat (lipase inhibitor) and 0.4mM L-a-dioleoyl [oleoyl-1-^{14}C] –glycerolo-3-phosphate (specific

activity adjusted to 2100DPM/nmol with cold cold 1,2-dioleoyl-sn-glycero-3- phosphate). Reaction was stopped with 2ml of $CHCL_3$/MeOH (19:1, with 0.08% addition of olive oil). After vortexing for 30s, 1g of basic alumina oxide was added to bind unreacted substrate. After centrifugation for 10min@2000g, upper organic phase containing radioactive product (dioleoyl-diacylglycerol) was transferred to scintillation vial, evaporated under stream of nitrogen and suspended in 5ml of scintillation cocktail. Radioactivity was measured with Packard TriCarb 1900TR liquid scintillation counter. Each sample was measured twice, without the presence of NEM (N-Ethylmaleimide, PAP1 inhibitor, Sigma-Aldrich) to measure total phosphatidate phosphatase activity (PAP1 and PAP2), and in the presence of 5mM NEM to measure PAP2 activity. PAP1 activity was established by subtraction of PAP2 activity from total PAP activity. The activity was expressed in nmol/min/mg of protein.

Oral Glucose Tolerance Test (OGTT), Insulin Tolerance Test (IPTT), plasma insulin and HOMA-IR: Both OGTT and IPTT were performed on animals which were fasted for 6 hours. For OGTT animals received oral gavage of glucose at a dose of 3g/kg. For IPTT animals received intraperitoneal injection of insulin at dose of 0.75U/kg body weight. Blood samples from tail vein were measured at given intervals by glucometer (AccuCheck, Roche. Germany). Plasma insulin was measured by ELISA (Rat/Mouse Insulin Millipore). HOMA-IR index value was calculated according to Cacho et al. (Cacho, et al. 2008).

Statistical significance estimation and principal component analysis: Statistical significance between groups was estimated using ANOVA with the Tukey honestly significant difference post-hoc test for equal n-numbers. Significance level was set to $p<0.05$. PCA was performed using Statistica 10.0 software package using noniterative partial least squares algorithm (NIPLAS) as described earlier (Zabielski, et al. 2014). Maximum number of iterations and convergence criterion were set at 50 and 0.0001, respectively. The number of principal components was determined using the Krzanowski cross-validation method. To prevent an artificial increase in the PCA model strength, we excluded a majority of closely interdependent

variables. The results of PCA analysis and the list of variables is included in supplementary table S7 and S8, respectively.

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