SUPPLEMENTAL MATERIAL

DETAILED METHODS

Animals

Male and female mice with cardiac specific overexpression of ACS1 linked to the α-myosin heavy chain promoter (MHC-ACS1) were used for this study[1]. The low-overexpressing J3 line was used for breeding pairs since these mice do not display overt cardiac dysfunction or poor survivability[1]. Dr. Jean E. Schaffer generously donated breeders. Mice were bred on the FVB/N background and backcrossed for 10 generations with outbred FVB/N mice, strain code 207, obtained from Charles Rivers Laboratory. Thereafter the colony has been maintained by using transgenic males and FVB/N females. Isolated heart perfusions and subsequent LC-MS/MS analysis was performed on mice at 12-14 weeks of age. The University of Illinois at Chicago Animal Care Committee (IACUC) approved all animal protocols, surgical and non-surgical. Age matched non-transgenic littermates were used as controls.

Ovariectomy (OVX)

Surgeries were performed on female mice at 8-10 weeks of age. An initial dose of sustained-release buprenorphine (0.1 mg/kg SC) was given at the time of anesthesia prior to conducting surgery. Anesthesia was maintained with 1.5% isoflurane via inhalation. Depth of anesthesia was assessed prior to and monitored during surgery with toe pinch and palpebral reflex. Reduction of uterus weight 3 weeks post-op at the time of isolated heart perfusion was measured in OVX females to ensure loss of female sex hormones.

Isolated heart perfusions

Heparin intraperitoneal injection was administered 30 minutes prior to excision to prevent formation of coronary artery thrombi. Mice were anesthetized with ketamine (80 mg/kg) and xylazine (12 mg/kg) both intraperitoneal injections. Anesthesia was confirmed by the loss of palpebral reflex and then toe-pinch response. Mouse hearts were isolated and perfused through a retrograde Langendorff perfusion, as described [2–4]. Hearts were supplied a modified Krebs-Henseleit buffer (118.5 mmol/L NaCl, 4.7 mmol/L KCl, 1.5 mmol/L $CaCl_2$, 1.2 mmol/L $MgSO_4$, and 1.2 mmol/L KH_2PO_4) equilibrated with 95/5% O_2/CO_2 and sustained at 37 °C. Buffer contained 0.4 mmol/L ^{12}C palmitate/albumin complex (3:1 molar ratio), 10 mmol/L glucose, and 1 mmol/L lactate. Perfused hearts were situated in a 10 mm broadband probe in a vertical wide-bore (89mm) 14.1 T NMR magnet and maintained at 37 °C. After 15 min metabolic equilibration, ^{31}P NMR signal was acquired for phosphocreatine:β-ATP ratio verification of baseline energetics to ensure viable isolated heart preparation. Background ^{13}C natural abundance signal was then collected for later digital subtraction from ^{13}C-enriched signals. Perfusate was then switched to buffer containing 0.4 mmol/L [U-^{13}C] palmitate, 10 mmol/L glucose, and 1 mmol/L for all perfusions during acquisition of dynamic-mode ^{13}C NMR spectra over 30 minutes. At the end of the perfusion, hearts were rapidly frozen in liquid N_2 for subsequent in vitro analysis.

Hemodyanmics were obtained from the water-filled intraventricular balloon connected to a pressure transducer (Power Lab, AD Instruments) during isolated heart perfusion. Rate pressure, an index of cardiac workload, was calculated from heart rate (beats per minute) x left ventricle developed pressure (mm Hg). Contractility was assessed from positive and negative dP/dt obtained from the derivative of the pressure trace. Mean

values of RPP, +dP/dt, and –dP/dt were compared over the course of the entire ^{13}C enrichment protocol.

Nuclear Magnetic Resonance (NMR) spectroscopy and kinetic analysis of ^{13}C NMR data

During the course of isolated heart perfusion inside the 14.1 T magnet (Bruker Instruments), continuous dynamic-mode ^{1}H decoupled ^{13}C NMR spectra were acquired using a WALTZ sequence at 151 MHz with a 45-degree flip angle and 2-second pulse duration for 32 scans and signal averaged over two-minute intervals [2–4]. Triglyceride (TAG) dynamics are calculated from integrating the ^{13}C resonance signal at 30.5 ppm, corresponding to the methylene ($-CH_2-$) carbon of TAG, from the dynamic-mode ^{13}C NMR spectroscopy. The esterification rate of exogenously supplied ^{13}C palmitate into TAG was calculated from the apparent linear phase slope of the integrated 30.5 ppm peak growth during the course of the isolated heart perfusion. Steady-state turnover of TAG = (TAG ^{13}C enrichment linear phase slope x endpoint TAG ^{13}C enrichment obtained by LCMS x TAG content) [5,2,3,6,7].

A subset of hearts from each group was examined to assess the contribution of palmitate to oxidative metabolism in the citric acid cycle (Figure 4E, main text). Oxidation of palmitate to acetyl CoA was determined in isolated hearts perfused with buffer containing 0.4 mmol/L [2, 4, 6, 8, 10, 12, 14, 16-^{13}C] palmitate/albumin complex (3:1 molar ratio), 10 mmol/L glucose, and 1 mmol/L lactate until isotopic steady state was reached at 20 minutes. Glutamate was extracted from frozen tissue samples with 7% perchloric acid and neutralized to pH 7.0 using KOH. Samples were reconstituted in 500 µL of D_2O. In-vitro high-resolution ^{13}C NMR of reconstituted samples in a 5 mm broadband probe (Bruker Instruments) enabled glutamate isotopomer and isotopologue analysis for substrate selection. Analysis was performed to determine the fractional contribution of ^{13}C palmitate to [2-^{13}C] acetyl CoA formation, as previously described in detail [6,8–10].

Myocardial lipid analysis

All quantified lipids were normalized to tissue protein content (BCA assay, Thermo Scientific. TAG was isolated from 30 mg of frozen heart tissue and homogenized in 500 µL PBS. Lipids were separated from tissue homogenates via a modified Folch extraction and re-suspended in 500 µL chloroform [2,3,5,7]. A 200 µL aliquot was set aside, dried down, re-suspended in 3:2 t-butyl alcohol/triton X-100 for TAG content quantification (Wako Pure Chemical Industries). Phospholipids were removed from remaining 300 µL of total lipids with NH_2 column preconditioned with hexane. The column was rinsed with 1 mL of 1:2 2-propanol/chloroform to pull TAG off the column. The effluent was evaporated and re-suspended in 470 µL of methanol and saponified in 30 µL of 1 M KOH for one hour to release the fatty acids and later neutralized with 10% formic acid. Fatty acids were separated from glycerol with two successive hexane extraction washes. Hexane was dried down and fatty acids were re-suspended in 300 µL of 1:1 chloroform/methanol in preparation for LCMS. End-point ^{13}C TAG enrichment was calculated as percentage of ^{13}C enriched fatty acids from isolated TAG. Fatty acids were analyzed from MS2 scans scanning for m/z 100-400 obtained by LC-ESI-MS using instrument configurations described [5,7]. HPLC timetable was as follows: 0-2 minutes isocratic hold 25% A (water with 0.05% formic acid) and 75% B (methanol), 2-20 minutes gradient to 15% A and 85% B, 20-35 minutes gradient to 0% A and 100% B, 35-40 minutes isocratic hold 0% A and 100% B, 40-42 minutes gradient to 25% A and 75%

B, and 42-49 minutes isocratic hold 25% A and 75% B. Flow rate was set to 0.2 mL/minute.

Ceramide species were isolated using extraction techniques previously described [5,7,11]. A 30 mg sample of frozen heart tissue was homogenized in 300 µL PBS and 1 mL of 2:1 methanol/chloroform was used to extract ceramides from tissue homogenate. A known quantity of C17 ceramide was added to each sample as an internal standard to ensure 2 pmol of C17 ceramide was injected into the LC-MS/MS along with each sample. Samples were vortexed, sonicated, and then incubated overnight at 48 °C. Upon allowing to cool to room temperature, samples were saponified with 150 µL of 1 M KOH for two hours and later neutralized with glacial acetic acid. Following neutralization, samples were extracted with 1 mL of 1:2 chloroform/water. Lower layer was removed following vortexing and 10 minutes of centrifugation. Upper layer was washed twice with 1 mL of chloroform, vortexed, and centrifuged. Lower layer was removed and added to previous lower layer. Samples were dried down and dissolved in 300 µL of mobile phase A (60:40 2-propanol/acetonitrile with 0.2% formic acid and 1 mM ammonium formate). Ceramide species were separated by liquid chromatography (LC), ionized by electrospray-ionization (ESI), and then quantified from precursor-product ion scans from multiple-reaction monitoring (MRM) using described LC-ESI-MS/MS instrument configurations, with some modifications [11,12] Ceramide samples were injected into a EC125/2 Nucleodur 100-3 C8 Sec column (Macherey-Nagel).. Ceramide HPLC timetable was as follows: 0-2 minutes isocratic hold 75% A and 25% mobile phase B (8:1:1 water/2-propanol/acetonitrile with 0.2% formic acid and 2 mM ammonium formate), 2-18 minutes gradient to 90% A and 10% B, 18-20 minutes gradient to 100% A and 0% B, 20-22 minutes gradient to 75% A and 25% B, 22-25 minutes isocratic hold at 75% A and 25% B. A standard curve was generated for each ceramide carbon chain including C14, C16, C18, C18:1, C20, C22, C24, and C24:1. Flow rate was set to 0.3 mL/minute. A standard curve was generated for each species against a fixed quantity of C17 ceramide in each standard mix. The slope of the standard curve was factored into the final ceramide concentration to account for matrix effects in the samples.

Acyl CoA species were isolated from frozen heart tissue using the extraction from Haynes et al with slight modifications [13]. A 30 mg tissue sample was homogenized in 500 µL PBS. Methanol with 1 mmol/L EDTA was added to each sample along with a volume of C17 acyl CoA internal standard to ensure 2 pmol of C17 acyl CoA was injected along with each sample into the LC-MS/MS. Samples were sonicated and then incubated overnight at 50 °C with 250 µL of chloroform. Upon cooling to room temperature, 250 µL of chloroform then water was added to each sample, followed by vortexing and then centrifugation for 5 minutes. Upper layer containing the acyl CoAs was removed. Lower layer was washed twice with 500 µL of 50:45:5 methanol/water/chloroform and each wash was followed by vortexing and then centrifugation for 5 minutes at 3000 rpm. Before storing samples, 180 µL of 50:25:25 methanol/butanol/chloroform was added. For mass spec preparation, samples were dried down and re-suspended in 1:1 water/acetonitrile. Acyl CoA species were separated by liquid chromatography (LC), ionized by electrospray-ionization (ESI), and then quantified from precursor-product ion scans from multiple-reaction monitoring (MRM) using described LC-ESI-MS/MS instrument configurations [13]. Acyl CoA samples were injected into a XTerra MS C18 Column 125Å 3.5 µm 2.1 mm X 100 mm (Waters). HPLC solvents consisted of mobile phase A (85:15 water/acetonitrile with 0.5% triethlyamine (TEA)) and mobile phase B (90:10 acetonitrile/water with 0.05% TEA). LC-MS/MS settings as described [13] with modifications to the HPLC timetable as

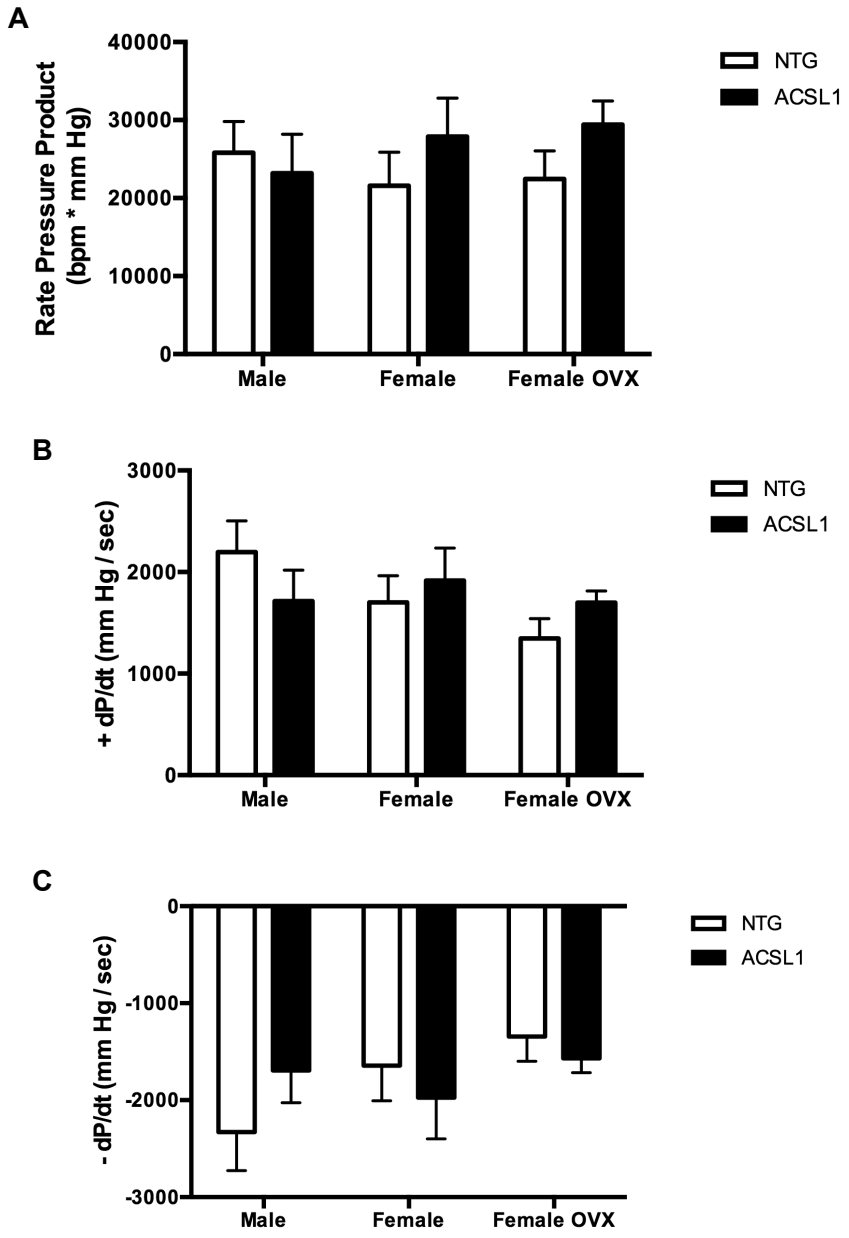
follows: 0-5 minutes hold 100% A and 0% B, 5-19 minutes gradient to 50%A and 50% B, 19-20 minutes gradient to 100% B, 20-25 minutes isocratic hold at 0%A and 100% B, 25-26 minutes gradient to 100% A and 0% B, and 26-30 minutes isocratic hold at 100% A and 0% B. Flow rate was set to 0.2 mL/minute. A standard curve was generated for each acyl CoA carbon chain including C14, C16, C18, C18:1, C18:2, C20, C20:4, C22, and C24. A standard curve was generated for each species against a fixed quantity of C17 acyl CoA in each standard mix. The slope of the standard curve was factored into the final acyl CoA concentration to account for matrix effects in the samples.

Western blot

Protein expression was measured by Western immunoblots on whole cell lysates from frozen un-perfused heart tissue as described [4,5,7]. SDS-PAGE was used to separate proteins followed by transfer to PVDF membranes. Western blots were probed with acyl CoA synthetase-1 (ACSL1, rabbit Cell Signaling Technology #4047), fatty acid transport protein 1 (FATP1, rabbit, Abcam ab103668) fatty acid transport protein 6 (FATP6, rabbit, Thermo Scientific PA5-34544) and CD36 (mouse, Cascade Bioscience ABM-5525). Expression changes were quantified and normalized to intensity of loading control band calsequestrin (calseq, rabbit, Pierce Thermo Scientific PA1-913) by band densitometry measured with NIH ImageJ.

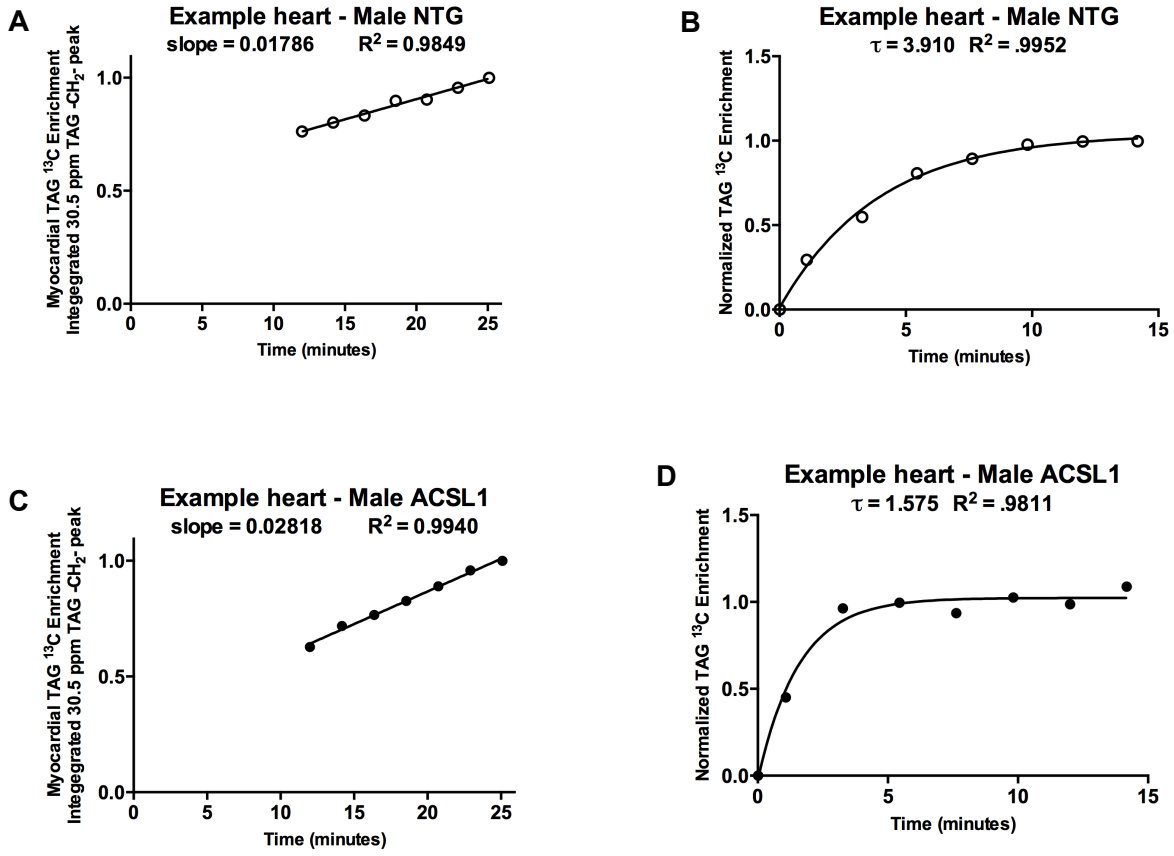
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Supplemental Figure 1



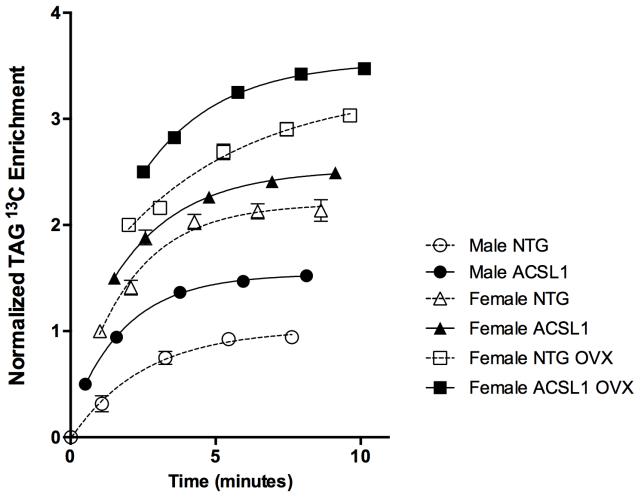
Supplemental Figure 1. Hemodyanmics of isolated perfused hearts. Hemodynamic functional parameters obtained from pressure trace of water-filled balloon in left ventricle during isolated heart perfusion. Graphs display mean ± SEM of **(A)** rate pressure product, **(B)** positive dP/dt, and **(C)** negative dP/dt. Male NTG, male ACSL1, and female ACSL1 n = 6. Female ACSL1 n = 5. Female NTG OVX and ACSL1 OVX n = 4.

Supplemental Figure 2



Supplemental Figure 2. Example fits of the exponential and linear phase of myocardial TAG ^{13}C enrichment data from isolated perfused hearts. (A and B) Linear regression analysis is used to fit the integrated NMR resonance at 30.5 ppm, the -CH_2- of TAG. The slope of the linear regression fit, in combination with TAG content (Figure 4A), and final TAG ^{13}C enrichment, quantified by LC-MS, were used to calculate turnover of the intramyocardial TAG pool (Figure 4B). **(C and D)** The linear phase data points are subtracted from the overall NMR ^{13}C enrichment data to obtain normalized ^{13}C enrichment. This remaining apparent exponential phase of ^{13}C enrichment is fitted with a non-linear regression analysis and used to calculate the time constant, τ. Representative fits from a male NTG (A and B) and a male ACSL1 (C and D) heart are displayed.

Supplemental Figure 3



Supplemental Figure 3. Waterfall plot of Figure 2A, the initial exponential component of ^{13}C enrichment data normalized to enrichment level.

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