Supplemental Material

PDK4 inhibits cardiac pyruvate oxidation in late pregnancy

Short title: Cardiac pyruvate oxidation in late pregnancy

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Supplemental Experimental Methods:

Reagents and Materials. Unless otherwise stated, all reagents were from Sigma. \(^{13}\)C-labeled substrates were from Cambridge Isotope Laboratories. PDK4 KO, PDK2 KO, and DKO mouse tissues were generously provided by Dr. Adam Wende. Dr. Robert Harris kindly gave us the PDK4 antibody.

Plasma metabolite assays. Blood was collected from non-fasted mice at mid- or late pregnancy via cheek puncture (mid-pregnancy) or terminal cardiac puncture (late pregnancy). Plasma triglyceride (Cat. No. 100010303, Cayman, MI), glucose (Cat. No. 10009582 Cayman, MI), and free fatty acids (Cat. No. 700310, Cayman, MI) were measured. Tissue triglycerides were measured using a kit (Abcam, ab65336) per manufacturer instructions, using normalized amounts of heart weights.

In vivo fatty acid bioluminescence. Bioluminescence studies were conducted at the Longwood Small Animal Imaging Facility (SAIF). Protocol for injection and uptake was described briefly.\(^1\) Mice were sedated with isoflurane and then intravenously injected (retro-orbital) with 100μL of 20 μM FFA-luciferin (Intrace Medical). Mice were placed in the supine position in the imaging chamber of the Xenogen IVIS-50 Bioluminescence Imaging System (Perkin Elmer). Flux analyses were performed and region of interest (ROI) were chosen with the Living Image Software from Xenogen.

Langendorff perfusion. Non-circulating, Langendorff perfusion was performed as described previously.\(^2\) Briefly, hearts were removed and immediately placed in cold saline. Then, a cannula was placed into the aorta to undergo perfusion with non-labeled buffer for 20-25
minutes. The modified Krebs-Henseleit buffer (KHB) used contained 118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.1 mM EDTA, 50 μM L-carnitine, 5 mM glucose, 0.4 mM palmitic acid conjugated to 2% BSA, 1.5 mM lactate, and 0.2 mM pyruvate, with the pH adjusted to 7.35. The buffer composition was chosen due to previously published reports. The buffer was gassed with 95% CO₂/5% O₂ and maintained at 37°C throughout the entirety of the experiment. Hearts were paced at 420 beats per minute, and perfusion pressure was set at 80 mmHg for the whole study. After stable perfusion for 20 minutes, perfusate was switched to labeled substrates for roughly 30 minutes.

**Tissue preparations for mass spectrometry.** After 25 minutes of labeling, the heart was immediately freeze clamped with tongs pre-chilled in liquid nitrogen. The tissue was pulverized with mortar and pestle, and the weight recorded. Metabolites were extracted with 80% methanol in H₂O with 0.1% formic acid. For the second and third extractions, a 40:40:20 ratio of methanol:acetonitrile: H₂O solution was used. All three extractions steps were completed with centrifugations at maximum speed for 5 minutes at 4°C. Then, the samples were dried under nitrogen gas before processing by mass spectrometry.

**Measurement of ¹³C-labeling pattern of cellular metabolites using liquid chromatography mass spectrometry (LC-MS).** Samples were sent to the Metabolomics Core at the Penn Diabetes Research Center (DRC) for further processing. In brief, samples were analyzed via reverse phase ion-pairing chromatography coupled to an Exactive orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA). The data analyses were performed using MAVEN software. Data were corrected to remove the contribution from the nature isotope ¹³C as described before. Labeling percentages were calculated from signal intensity using Excel.
**Isolated mitochondria.** Mitochondria were isolated from mouse hearts as previously described (Readnower et al., 2012). Mitochondria was quantified using Bradford and approximately 2 µg of protein was loaded onto the Seahorse plate and spun at 2000 rpm for 10 minutes at room temperature.

**Seahorse experiments.** Mitochondrial respiratory function experiments were performed on a XF Flux Analyzer (Seahorse Biosciences). Isolated mitochondria was plated at 2µg per well and isolated cardiomyocytes were plated at 20,000 cells per well. To assay complex I respiration, 10mM pyruvate and 2mM malate was used. State 3 respiration was induced with 4mM ADP, followed by state 4_o respiration with 3µM oligomycin. Then, 4µM of the uncoupler FCCP produced maximal respiration and lastly 2µM of rotenone and antimycin A was utilized to inhibit respiration. Fatty acid oxidation respiration rates were determined with 50µM palmitoylcarnitine and 0.5mM malate, which preceded the addition of the complex I respiration substrates. For isolated cardiomyocytes, 10µg/mL saponin was used to permeabilize the membrane, and then the inhibitors described were treated on cells.

**Gene expression studies.** Upon harvesting, mouse tissues were immediately frozen in liquid nitrogen. Total RNA was isolated from with the TRIzol (Invitrogen) and chloroform method.

**Western blots.** Protein was isolated in RIPA Lysis Buffer (VWR 97063-270) supplemented with protease inhibitor (11836153001 ROCHE) and phosphotase inhibitor (PHOSS-RO ROCHE). Western blot was performed for 20ug protein/sample loaded per well. The primary antibodies were for immunoblot were: GLUT4 (Millipore), CD36 (Novus), PDK4 (a kind gift from Dr. Robert Harris), phospho-PDH at serine 293 (Novus), PDH (Cell signaling), Na⁺/K⁺ ATPase (Novus), Total OXPHOS Cocktail (MitoSciences), pAKT (Ser473) (CST 9271S) and pan AKT (CST
4691S). Primary antibodies were incubated overnight at 1:1000 and secondary antibodies at 1:10,000. Vinculin (Cell signaling) and GAPDH (Santa Cruz) were used as loading controls.

**Insulin injection studies.** 20-week-old female C57BL/6 mice (Jackson Laboratories), pregnant (e18.5) versus nonpregnant, were fasted overnight (~16 hours) and then injected IV with 2mU insulin (Novolin R) per gram bodyweight and harvested after 15 minutes. Blood was collected from the heart and serum was isolated. Liver and heart samples were snap frozen in liquid nitrogen.

**Progesterone studies in vivo.** Mice were exposed to Estradiol (E2, 5 mg) and progesterone (P4, 1 mg), versus vehicle (n=3) or untreated (n=3) control, daily for 14 days (controls were pooled). The hormones were dissolved in sesame oil, and delivered subcutaneously.

**Histological studies.** Hearts were excised from mice and rinsed in cold phosphate-buffer saline (PBS). Tissue was embedded in optimal cutting temperature (OCT) and then 10 µm sections were cut. Fixation with 4% PFA was carried out for 15-30 minutes. Blocking was with 3% BSA/PBS + 0.1% Triton-X for 1 hour at room temperature. Primary antibodies used were GLUT4 (Santa Cruz, 1:100 and 1:500) and CD36 (Cayman, 1:100 and 1:500).

**Subcellular fractionation.** Subcellular fractionation was carried out as previously described\(^8,9\). Briefly, heart tissue was placed chilled HES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA) with protease inhibitors and then homogenized. Samples were then centrifuged at 2000g for 10 minutes at 4°C. The supernatant is transferred to a new tube, where it is then centrifuged at 9000g for 20 minutes at 4°C. The pellet (P1) is the plasma membrane fraction. The supernatant is ultracentrifuged at 180,000g for 90 minutes. The supernatant is the cytosol component and the pellet is then resuspended in PBS and protease inhibitors and then loaded
onto a 10-30% continuous sucrose gradient. It is then centrifuged further at 48,000 rpm for 55 minutes. The pellet (P2) is the plasma membrane, whereas the supernatant was separated into 10-15 fractions and make up the intracellular vesicles.

**Neonatal cardiomyocyte isolations.** Neonatal cardiomyocyte isolations were carried out as previously described.\(^\text{10}\) Briefly, hearts of 1-2 day old Sprague Dawley neonates were excised and ventricles were isolated. Sequential incubations with collagenase type II (Worthington) were carried out in a 37°C shaker. Cells were purified from non-cardiomyocytes via a crude, 1 hour pre-plating step. Cells were then incubated in DMEM, 10% HS, 5% FBS, and 1% penicillin-streptomycin (PS).

**Chromatin Immunoprecipitation (ChIP)-Sequence and expression quantitative trait loci (eQTL) data analyses.** ChIP-seq data sets, derived from HUVECs expressing progesterone receptor and treated with progesterone versus vehicle control, followed by chromatin immunoprecipitation with PR-specific antibodies versus IgG control, were obtained from GEO (GSE43786)\(^\text{11}\) as BAM alignment files. The software package deepTools\(^\text{2}\) was then used to create University of California Santa Cruz (UCSC) genome browser ‘wiggle’ tracks of ChIP signal density, using the GRCh37/hg19 human genome assembly. The tracks were then aligned with DNase I hypersensitivity sites and histone modification information (H3K4M3 and H3K27ac for promoter activity and enhancer activity markers, respectively) from ENCODE\(^\text{13}\), also obtained from the UCSC browser. PDK4 eQTL were identified via the ExSNP integrated eQTL database (http://www.exsnp.org/)\(^\text{14}\). Alignment was then performed with consensus progesterone-binding sites derived from Yin et al.\(^\text{15}\)
Supplemental References:


Online Figures:

Online Figure I: Absence of appreciable change in pyruvate carboxylase (PC)-mediated anapleurosis in late gestational mouse hearts. (A) Schematic depicting approach to estimate relative PC and PDH flux from [U-\(13\)C] Glucose. (B) Relative PC activity identified by comparing the M+3/M+2 ratio of succinate to that of aspartate, in the presence of labeled glucose (left) versus palmitate (right). Data shown as mean ± SEM, (n≥3 for all groups). Data are represented as mean ±SEM; *, p < 0.05 compared with control.
Online Figure II: Insulin signaling, and GLUT4 and CD36 expression and translocation are unaltered in late gestational mouse hearts. (A) Quantification of immunoblots from Figure 3A. Data are represented as mean ±SEM; *, p < 0.05 compared with control. (B) Immunoblots and quantification of phospho-Akt/Akt signaling for skeletal muscle from non-pregnant and late pregnant mice. Data are represented as mean ±SEM. (C) Immunoblots of phospho-Akt/Akt signaling in heart (top) and liver (bottom) of nonpregnant (NP) and late pregnant (LP) mice after injection with insulin versus vehicle. (D) Plasma insulin levels in NP versus LP mice. Data are represented as mean ±SEM; *, p < 0.05 compared with control. (E) Quantification of immunoblots from Figure 3C. Data are represented as mean ±SEM. (F) Subcellular fractionation of mouse hearts from nulliparous control mice and late pregnant mice. The plasma membrane (PM, P1 and P2) fractions were separated from the intracellular vesicle fractions (IV, 1-14) by ultracentrifugation. Na/K ATPase was used as a PM marker, and all fractions were blotted with GLUT4 to examine localization. Quantification of the abundance of GLUT4 on the PM versus IV (PM/IV) is shown.
Online Figure III: Mitochondrial gene expression is unaltered in late gestational mouse hearts. (A) qRT-PCR of nuclear encoded mitochondrial genes from late pregnant and control mouse hearts. Data are represented as mean ±SEM. (F) qRT-PCR of mitochondrial encoded mitochondrial genes from late pregnant and control mouse hearts. Data are represented as mean ±SEM. (G) qRT-PCR of genes pertaining to mitochondrial dynamics from late pregnant and control mouse hearts. n=6 for all groups. Data are represented as mean ±SEM, *, p < 0.05 compared with control.
Online Figure IV: PDK4 is induced in a muscle-specific manner by progesterone. (A) qRT-PCR analysis in hearts from pregnant mice (n=7 for all groups). Data are represented as mean ±SEM; *, p < 0.05 compared with control. (B) Immunoblot of PDK2/4 antibody with hearts from PDK2- and PDK4-null mice. “S”: starvation; “F”: fed. (C and D) Immunoblot and quantification of hearts from mice at different stages of pregnancy blotted against the PDK2/4 antibody and vinculin control antibody. Data are represented as mean ±SEM; *, p < 0.05 compared with control. (E) Relative PDH activity in cardiac tissue from control and pregnant mice (n=3 per
Regulation of PDK4 by progesterone receptor. Top: chromatin immunoprecipitation-sequencing (ChipSeq) signal density tracks in the PDK4 regulatory region, with antibody to progesterone receptor in cells receiving progesterone (PR + P4), cells receiving vehicle (PR only), control cells (Neg.), or with IgG control antibody (IgG control). Tracks below indicate DNAsel hypersensitivity sites (HS), histone H3K4M3 methylation, and H3K27ac acetylation status. PDK4 gene is indicated, with three expression quantitative trait loci (eQTLs) in the upstream region indicated in red. One of these eQTL, rs3807891, modifies the consensus PR binding site and is outlined in detail at bottom. (G) qRT-PCR of PDK4 gene expression in hearts from male and female mice after 14 days of treatment with Progesterone + Estrogen (PE) versus control (Ctr). Data are represented as mean ±SEM; n>=3 per group; *, p < 0.05.
Online Figure V: PDK4 inhibition reverses the endogenous substrate switch of decreased glucose and increased fatty acid oxidation in hearts from late gestational mice. (A) qRT-PCR of Langendorff perfused non-pregnant or late pregnant mouse hearts, treated with or without DCA (n=4 for each group). (B) Sample echocardiographs of non-pregnant or late pregnant mice, treated with or without DCA. (C) Morphometric parameters including heart weight (HW) and heart weight to tibia length (HW:TL) ratio in non-pregnant controls and late pregnant mice, with and without DCA treatment (n>=5 per group). Data are represented as mean ±SEM. *, p < 0.05.