



Published in final edited form as:

Cardiovasc Res. 2026 April 28; 122(6): 723–733. doi:10.1093/cvr/cvag053.

***In vivo* assessment of the recovery of myocardial pyruvate dehydrogenase activity following a ketogenic diet**

Jun Chen, Ph.D.¹, Zohreh Erfani, M.D.¹, Abdallah Elnwasany, M.D.^{2,3}, Sarah Al Nemri, B.A.¹, Joseph Park, B.A.¹, Mai T. Huynh, Ph.D.¹, Maheen Zaidi, B.S.¹, Crystal E. Harrison, Ph.D.¹, Xiaodong Wen, M.D.¹, Luke I. Szveda, Ph.D.^{2,4}, Jae Mo Park, Ph.D.^{1,5,6,7,*}

¹Advanced Imaging Research Center, UT Southwestern Medical Center, Dallas TX 75390

²Division of Cardiology, Department of Internal Medicine, UT Southwestern Medical Center, Dallas TX 75390

³Department of Biology, University of Dallas, Irvin TX 75062

⁴Department of Medicine, University of Arizona, Tucson AZ 85719

⁵Department of Biomedical Engineering, UT Southwestern Medical Center, Dallas TX 75390

⁶Department of Radiology, UT Southwestern Medical Center, Dallas TX 75390

⁷Charles and Jane Pak Center for Mineral Metabolism and Clinical Research, UT Southwestern Medical Center, Dallas TX 75390

Abstract

Aims: A ketogenic diet (KD) can suppress cardiac carbohydrate utilization, which may adversely impact heart function. However, the reversibility of KD-induced metabolic changes is poorly understood. This study aims to characterize myocardial PDH flux during the transition from a prolonged KD to a normal chow diet (ND).

Methods and Results: Cardiac metabolism was longitudinally assessed in rats using hyperpolarized [1-¹³C]pyruvate at baseline, during a KD (2 and 5 weeks), and a subsequent ND (1, 2, 5, 8 days) after the 5-week KD. Hyperpolarized ¹³C products were compared between the KD group and age-matched ND controls. In parallel, NMR isotopomer analysis of cardiac tissue with an injection of [3-¹³C]pyruvate and [1,2-¹³C₂]acetate was performed along with *ex vivo* enzymatic analysis of PDH activity. Myocardial [¹³C]bicarbonate production relative to total ¹³C products decreased from 8.56±2.29% at baseline to 0.46±0.27% after 5 weeks of KD. Reverting to ND gradually restored PDH flux (8.40±1.47% by day 8) to control levels (8.69±2.10%). *Ex vivo* NMR analysis of glutamate C4 showed reduced pyruvate contribution to acetyl-CoA during KD (4.1±2.5%), which recovered upon reverting to ND (22.7±1.82% vs. control: 27.6±9.5%).

*Correspondence to: Jae Mo Park, Ph.D., The University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd. Dallas TX 75390, jaemo.park@utsouthwestern.edu.

Author Contributions: J.C. and J.M.P. designed the study; J.C., Z.E., S.A.N., M.T.H., C.E.H., and J.M.P. performed *in vivo* experiments; A.E., S.A.N., M.Z., X.W. and L.I.S. contributed tissue collection and *ex vivo* analysis; J.M.P. analyzed the NMR isotopomer results; Z.E. and J.P. performed blood analysis; J.C., S.A.N., L.I.S., and J.M.P. wrote the draft; All authors contributed data interpretation and manuscript editing.

Disclosures: None.

Although PDK4 expression normalized, PDH activity remained partially impaired in the reverted group (36.80 ± 6.07 mmol NADH/min/mg) compared to controls (90.97 ± 5.40 ; $P = 0.00007$).

Conclusions and Translational Perspective: KD-induced suppression of myocardial PDH flux is reversible, but its recovery requires significant time, with prolonged metabolic inflexibility persisting after transitioning to an ND. These findings highlight the value of *in vivo* assessment of cardiac PDH activity, complemented by conventional enzymatic analyses, to identify persistent metabolic inflexibility following ketogenic interventions. Such PDH inactivation may have clinical implications for heart failure or diabetes, where PDH regulation is impaired. Translating these results to humans requires consideration of slower metabolic rates, greater substrate flexibility, and higher reliance on carbohydrate oxidation, compared to rodents.

Category:

Original Article

INTRODUCTION

Fatty acids and glucose are principal substrates for cardiac metabolism to support adenosine triphosphates (ATP) production. Fractional contributions can rapidly adapt to substrate availability, workload, circadian rhythm, pathological conditions, and nutritional states.¹ Glucose metabolism plays a key role in providing the metabolic flexibility, which is strongly linked to cardiac damage and dysfunction.² For instance, the utilization of fatty acids increases in the heart under a fasted condition due to limited glucose availability. The diminished glucose oxidation is, in part, driven by increased expression of pyruvate dehydrogenase kinase 4 (PDK4) and phosphorylation and inhibition of pyruvate dehydrogenase (PDH).^{3, 4} Since PDH is the rate limiting step in the oxidation of glucose-derived pyruvate for ATP production within the mitochondria, measuring the PDH flux is essential for evaluating cardiac glucose metabolism.

Considering the sensitive enzymatic activities to multiple physiological factors and complex regulatory pathways, glucose oxidation in the heart needs to be understood *in vivo*. In particular, the PDH activity is sensitive to various factors such as plasma substrate composition and insulin level,^{5, 6} it is more reliable to measure the activity *in vivo*. ¹³C magnetic resonance spectroscopy (MRS) with a bolus injection of hyperpolarized (HP) [1-¹³C]pyruvate enables *in vivo* assessment of PDH flux via the detection of [¹³C]bicarbonate ($\text{H}^{13}\text{CO}_3^-$), which is formed from ¹³CO₂ generated during the conversion from [1-¹³C]pyruvate to acetyl-CoA (Figure 1, A).^{7, 8} One of the most exciting motivations of cardiac MRI/MRS with HP [1-¹³C]pyruvate is its capability of assessing PDH flux *in vivo* with systemic factors present. The utility of HP $\text{H}^{13}\text{CO}_3^-$ as a noninvasive biomarker of *in vivo* PDH activity has been demonstrated in both preclinical studies under various nutritional and pathological conditions^{9–12} and translational studies with patients.^{13–16} These studies reported that HP $\text{H}^{13}\text{CO}_3^-$ is sensitive to physiological perturbations in PDH flux,^{9, 17} correlates with cardiomyopathies with manifest alteration in PDH activity,^{9, 10, 18} and reflects drug-induced changes in PDH flux.^{17, 19}

A ketogenic diet (KD) also alters cardiac glucose metabolism, inducing a metabolic shift towards the use of ketones for energy production.²⁰ It has been reported that a KD has therapeutic effects on diseases such as epilepsy,^{21, 22} PDH deficiency,^{23, 24} and traumatic brain injury.²⁵ Beyond patient managements, following a KD has become a trend to the general public, driven by its positive effects on weight loss for obese individuals²⁶ and enhanced performance for endurance athletes.^{27, 28} As a result, approximately 12.9 millions of Americans follow the KD each year.²⁹ However, a KD increases low-density lipoprotein cholesterol in the blood^{30, 31} and insulin resistance,³² having risks of developing metabolic syndrome and potential adverse effects on the function of the heart. Importantly, the long-term cardiovascular effects are unknown.³³ Therefore, the reversibility of specific metabolic changes, induced by KD, needs to be investigated.

Metabolic effects and reprogramming associated with a KD have been studied,^{34–36} primarily based on *ex vivo* experiments. It was reported that a KD induces an increase in the content and activity of PDK4 and inhibition of PDH and glucose oxidation.³⁷ However, reversibility and temporal dynamics of altered PDH activity induced by a KD is underexplored. In this study, we investigated the reversibility of myocardial PDH flux, suppressed by a KD, and its recovery dynamics when switching back to a normal diet (ND), using ¹³C MRS with HP [1-¹³C]pyruvate *in vivo*, and the *in vivo* results were compared with *ex vivo* enzymatic analysis.

METHOD

Overall Study Design and Feeding Condition

A total of 38 healthy male Sprague-Dawley rats (Charles River Laboratories, Houston, TX, USA) were used in this study and randomly assigned to subgroups. The animals were used for *in vivo* MRS, tissue isotopomer analysis, enzymatic analysis, or combination of these. The detailed usage of the animals is summarized in the Supplemental Information (Table S1). Inclusion of only male rats was to avoid biological variability that complicates the interpretation of metabolic adaptations to dietary interventions.³⁸ Body weight was measured twice a week throughout the study. To minimize any confounders, all animals were ordered at the same age and housed in the same room. All studies were approved by the UT Southwestern Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Two types of diet (Envigo Rms, Inc., Indianapolis, IN, USA) were used for the rats: the standard ND (Teklad Global Diet 2014, 13% kcal from fat, 67% kcal from carbohydrates and 20% kcal from protein; 2.9 kcal/g) and the KD (Teklad Customer Diet, TD.96355, 90.5% kcal from fat [vegetable shortening and corn oil], 0.3% kcal from carbohydrates and 9.1% kcal from protein; 6.7 kcal/g), which elevates levels of circulating b-hydroxybutyrate and ketones.^{39, 40} Cardiac metabolism under three nutritional conditions was investigated: 1) ND, 2) KD for 5 weeks, and 3) reverted from a 5-week KD to ND. The KD was replenished three times a week. For each cage, the amount of diet consumed was calculated as 25g × (number of rats per cage) × (days until the next refill). On each feeding day, both the total weight of the remaining food and the total weight of the newly added food were recorded to determine food intake. Although no animals met the criteria, those showing hypoactivity,

dehydration, or severe weight loss (>20%) would have been excluded. Cardiac function was not monitored in this study, based on the weak correlation between KD-induced metabolic changes and function in the absence of cardiovascular disease.^{41, 42}

In addition, 20 healthy male rats were investigated for cross-sectional *ex vivo* enzymatic analyses of KD rats following a shorter KD duration (2 weeks) and subsequent diet reversal (2, 5, 8 days) as well as age-matched controls.

In Vivo MR Experiments

Longitudinal imaging studies were performed to monitor KD-associated metabolic changes. Fifteen of the rats were used for *in vivo* experiments. The switch of diet started at 10 weeks of age (242 – 278g). All control animals (CT group, n = 7) were fed with normal rodent chow, whereas the other group (KD group, n = 8) were fed with the KD (TD96355) meal for 5 weeks, immediately followed by the ND for 8 days. For imaging, each rat was continuously anesthetized under fed condition using a vaporizer with 2–3 % isoflurane in 1.5 L/min oxygen and the tail vein was cannulated with a catheter before positioning in the MRI scanner. Imaging data was collected between 8 am and 10 am to normalize the metabolic state. An integrated ¹H/¹³C MRI protocol that includes a localizer, B₀ shimming, and a slice-selective, time-resolved ¹³C MRS with a bolus injection of 120-mM HP [1-¹³C]pyruvate (1.5 mmol/kg body weight) via the tail vein catheter was used. Description on the MR protocol is included in the Supplemental Information. Missing datapoints (Rat IDs: 4, 27, 28) were due to equipment failure or maintenance.

Euthanasia

The rat was placed in home cage, which was used as the chamber for euthanasia. CO₂ was delivered by turning on the CO₂ tank and adjusting the flow rate to 3–11 L/min. The animal remained in the chamber for a minimum of 3–5 minutes, during which signs of cessation of vital functions, including limb movement, twitching, breathing, or pink skin color, were observed. After removing the animal from the chamber, death was confirmed by the absence of breathing and a heartbeat. To ensure the animal's death, a secondary method of euthanasia, thoracotomy, was performed.

Hyperpolarization of [1-¹³C]Pyruvate

For dynamic nuclear polarization (DNP), a sample that contains 55.8 µL of neat 14-M [1-¹³C]pyruvic acid (Sigma-Aldrich, St. Louis, MO, USA) and 15-mM OX063 trityl radical (GE Healthcare, Waukesha, WI) was prepared in a sample vial. The sample vial was assembled to a research fluid path (GE Healthcare) and 16 mL of dissolution media (0.1 g/L EDTA-Na₂) was loaded in the dissolution syringe of the fluid path. Each sample was polarized using a SPINlab™ DNP polarizer (GE Healthcare) for 3–4 hours, rapidly dissolved with the preheated dissolution media, and neutralized with NaOH immediately before injecting to animals.⁴³ The final solution was ~6 mL of 120-mM [1-¹³C]pyruvate with pH of ~7.5.

NMR Isotopomer Analysis

For cross-sectional tissue NMR analyses, three groups of Sprague-Dawley rats were used: CT group (ND for 5 weeks, $n = 5$), KD group (KD for 5 weeks, $n = 4$), and KD-to-ND reverted (RT) group (5-week KD followed by ND for 9 days, $n = 4$). To mimic the *in vivo* experiment, each rat was anesthetized with 2–3 % isoflurane and the tail vein was catheterized (same parameters as *in vivo* studies). The heart was collected between 9 and 10 am, 2 minutes after a bolus injection (injection rate = 0.25 mL/s) of a non-hyperpolarized mixture of 120-mM [1,2- $^{13}\text{C}_2$]acetate (1.5 mmol/kg) and 120-mM [3- ^{13}C]pyruvate (1.5 mmol/kg) through tail vein. The harvested heart tissue was immediately freeze-clamped and prepared for NMR isotopomer analysis as described in the Supplemental Information.

Measurement of Mitochondrial PDH Activity, Phosphorylated PDH, and PDK4

Three groups of rats were used for tissue analysis: CT group ($n = 3$), KD group ($n = 4$), and RT group ($n = 3$). Rats were anesthetized using a vaporizer with 3% isoflurane in 1.5 L/min and hearts were excised between 9 and 10 am and homogenized in ice-cold isolation buffer 10 mM MOPS, 1.0 mM EDTA, 210 mM mannitol, 70 mM sucrose, 20 mM NaF, and 5 mM dichloroacetate at pH 7.4) using a Polytron homogenizer. The homogenate was centrifuged at $500 \times g$ for 5 min (4°C) and the supernatant was filtered through cheese cloth. The mitochondrial pellet was obtained by centrifugation of the supernatant at $12,000 \times g$ for 10 min (4°C).⁴⁴ Mitochondria were resuspended in homogenization buffer at a final concentration of 20 – 30 mg/mL. Protein determinations were made using the bicinchoninic acid method (PierceTM; Thermo Fisher Scientific, Waltham, MA, USA) with bovine serum albumin as standard. Mitochondria were then diluted to 0.05 mg/mL in buffer containing 25 mM MOPS and 0.05% Triton X-100 at pH 7.4. Solubilization of mitochondria with 0.05% Triton X-100 inhibits complex I of the respiratory chain preventing consumption of NADH. PDH activity was measured spectrophotometrically (Agilent, 8452A) as the rate of NAD^+ reduction to NADH (340 nm, $\epsilon = 6,200 \text{ M}^{-1}\text{cm}^{-1}$) upon addition of 2.5 mM pyruvate, 0.1 mM CoASH, 0.2 mM thiamin pyrophosphate, 1.0 mM NAD^+ , and 5.0 mM MgCl_2 at pH 7.4.⁴⁴

Western Blot and CoASH Analysis

PDH-E1 α , phospho-PDH-E1 α , and PDK4 antibodies were validated by Western blot detection of protein at the appropriate molecular weight and the requirement of primary antibody for secondary antibody binding.⁴⁵ For CoASH analysis, approximately 30–50 mg of frozen tissue was extracted with perchloric acid, proteins were pelleted, and CoASH in the supernatants was analyzed by ion-pair reverse-phase HPLC with UV/Vis detection at 254 nm. CoASH levels were quantified using a standard curve and expressed as nmol/mg protein. Detailed procedures are described in the Supplemental Information.

Post-Processing and Quantification of Metabolites

In vivo ^{13}C raw data from the time-resolved slice-selective spectroscopy were processed using MATLAB (Mathworks, Natick, MA, USA; version 9.11.0.1809720). The data were first apodized by a 10-Hz Gaussian filter, followed by zero-filling by a factor of 4 and a fast Fourier transform. HP $\text{H}^{13}\text{CO}_3^-$, [1- ^{13}C]lactate, [1- ^{13}C]alanine, [1- ^{13}C]pyruvate-hydrate

and [1-¹³C]pyruvate signals were quantified from the cumulative spectrum, summed over 0 – 90 s, by integrating the respective peak in absorption mode after a 0th order phase correction. The integrated metabolite peaks were normalized to the total HP ¹³C products (TP), which is the sum of HP H¹³CO₃⁻, [1-¹³C]lactate, and [1-¹³C]alanine. The normalized HP metabolites were used to evaluate metabolic changes between groups or time points. Time-courses of H¹³CO₃⁻, [1-¹³C]lactate, and [1-¹³C]alanine were measured in a similar manner from time-resolved spectra, then normalized to the TP (Figure 1). For display, each cumulative spectrum was normalized by the pyruvate peak (Figure 1 and 2). For a clear visual comparison of HP spectra between groups, the rolling baseline was subtracted by fitting a spline function to the signal-free regions of each spectrum (Figure 2).

NMR data were processed using TopSpin (Bruker, Billerica, MA, USA; version 4.3.0). After 0th and 1st order phase corrections, lactate and alanine peaks were measured from ¹H spectra and glutamate C3 and C4 resonances were measured from ¹³C spectra. Fractional enrichments of lactate (1.31 ppm) and alanine (1.45 ppm) were calculated from J_{CH}-coupled outer doublets relative to the central doublets. In ¹³C spectra, relative contribution of pyruvate towards acetyl-CoA was calculated by estimating the fractional concentration of [2-¹³C]acetyl-CoA (*F_{C2}*), which was generated from [3-¹³C]pyruvate, from C3 and C4 glutamate resonances.⁴⁶ Similarly, relative contribution of acetate to acetyl-CoA ([1,2-¹³C₂]acetyl-CoA) was calculated from the glutamate peaks by estimating the fractional concentration of [1,2-¹³C₂]acetyl-CoA (*F_{C12}*). The following equations were used from glutamate peaks:

$$F_{C2} = \int C4 \, D34 \cdot \frac{\int C4}{\int C3} \quad \text{- eq (1)}$$

$$F_{C12} = \int C4 \, Q \cdot \frac{\int C4}{\int C3} \quad \text{- eq (2)}$$

Plasma Analysis

For plasma analysis, separate cohorts of a KD group (n = 5) and a control group (n = 5) were prepared. As in the HP study, the KD group was maintained on the KD for 5 weeks and then switched back to the standard diet. Blood samples were collected from both groups under fed condition at 10:30–11:30 am at baseline, and at 2, 3.5, and 5 weeks on the assigned diets. After reverting the KD, samples were obtained on the day of switching and at 2, 5, 8 days, as well as 2 weeks post-switch. Whole blood (1 mL) was collected from the tail vein of each rat into Eppendorf tubes, containing 20 µL of heparin (1,000 U/mL) to prevent coagulation. Samples were immediately placed on ice and centrifuged at 2,000 × *g* for 10 minutes at 4°C to separate plasma. The resulting plasma was analyzed for circulating levels of ketone bodies, glucose, and free fatty acids. Total blood ketone levels were measured using the Wako Autokit Total Ketone Bodies assay (415–73301;

Wako Chemicals, Lexington, MA). Non-esterified fatty acids (NEFAs) were quantified using the Wako NEFA kit (999–34691, 995–34791, 991–34891, 993–35191, 276–76491; Wako Chemicals). Glucose concentrations were measured using a VITROS 350 clinical analyzer (microchemical slides technology; Ortho Clinical Diagnostics, Raritan, NJ).

Data Analysis and Statistical Evaluation

The results are reported as mean $\pm$ standard deviation. All data were analyzed blinded to the group assignment of each animal. J.C., S.A.N., and Z.E. were aware of the group allocation until the interpretation and analysis of the results. For evaluating statistical significance, GraphPad Prism software (version 10.4.10) was used. Two-way ANOVA tests were performed to evaluate longitudinal metabolic differences between CT and KD groups at corresponding age. For comparing CT and RT and for evaluating *ex vivo* data of CT, KD, and RT groups, unpaired t-tests between the groups were used with two-tailed distributions and equal variances ($\alpha = 0.05$), assuming normal distribution.⁴⁷ A *P* value smaller than 0.05 was considered statistically significant and marked as a single asterisk (*). *P* values smaller than 0.01 are indicated with double asterisks (**).

Data Availability

The corresponding author has full access to all the data in the study and takes responsibility for its integrity and the data analysis. The data that support the findings of this study are available from the corresponding author upon reasonable request.

RESULTS

KD-fed rats consumed 13.0 ± 2.9 g (87.1 ± 19.4 kcal) of food daily, which is comparable to the intake of rats with a normal diet,⁴⁸ except the first day of KD (6.6 g), Figure S1. The KD group experienced short-term weight loss ($P = 1.1 \times 10^{-3}$, Figure 1, B), which was consistent with other KD studies.^{49–51} After a two-week KD, HP $\text{H}^{13}\text{CO}_3^-$ produced in the heart decreased from 8.56 ± 2.29 % of the total HP products (TP) at baseline ($P = 0.97$ vs. ND group) to 0.37 ± 0.34 % ($P = 1.9 \times 10^{-9}$ vs. ND group), with little change observed with extended diet durations (5 weeks; 0.46 ± 0.27 %, $P = 1.5 \times 10^{-9}$ vs. ND group), Figure 1, C–E. Conversely, *in vivo* HP $[1-^{13}\text{C}]\text{lactate}/\text{TP}$ was higher in the KD at 2 weeks ($P = 6.2 \times 10^{-3}$) and 5 weeks ($P = 1.9 \times 10^{-3}$) compared to the ND group, Figure 1, F. Within the KD group, lactate/TP remained relatively stable (58.21 ± 3.95 % at baseline, 59.56 ± 2.73 % at 2 weeks, 65.46 ± 5.28 % at 5 weeks), whereas in the ND group it decreased with age (61.61 ± 3.48 % at baseline, 46.67 ± 7.84 % at 2 weeks, 51.04 ± 5.42 % at 5 weeks). This pattern is consistent with an age-related decline in myocardial LDH activity.⁵² It is worth noting that the overall large proportion of lactate is partly due to the significant contribution of extracellular HP lactate signals originating from plasma or other organs such as the liver^{53, 54} in addition to accelerated isotope exchange between HP pyruvate and unlabeled lactate pool.⁵⁵ HP $[1-^{13}\text{C}]\text{alanine}/\text{TP}$ did not show significant differences between CT and KD groups ($P = 0.86$ at baseline, $P = 0.76$ at 2 weeks, and $P = 0.48$ at 5 weeks), Figure 1, G. Longitudinal changes of *in vivo* HP metabolites during KD and ND are summarized in Supplemental Information (Table S2).

Reverting to ND did not show noticeable change in body weight, maintaining the weight difference induced by the KD, Figure 2, B. However, diet reversal gradually recovered the PDH flux ($\text{H}^{13}\text{CO}_3^-/\text{TP}$) from 0.40 ± 0.26 % (day 0) to 2.94 ± 1.29 % (day 1), 4.02 ± 2.16 % (day 2), 7.51 ± 2.07 % (day 5), and 8.40 ± 1.47 % (day 8), Figure 2, C–E. Age-matching CT group had non-significant changes from 9.36 ± 1.17 % to 8.69 ± 2.10 % ($P = 0.36$) during the same period. In contrast, $[1-^{13}\text{C}]\text{lactate}/\text{TP}$ in the KD group showed the opposite but similar dynamic patterns, likely reflecting the redirected metabolic fate of pyruvate towards PDH, Figure 2, F. Again, $[1-^{13}\text{C}]\text{alanine}/\text{TP}$ did not show noticeable changes and was relatively unchanged after reverting from KD to ND, Figure 2, G. Longitudinal changes of *in vivo* HP product levels after reverting to ND are summarized in Supplemental Information (Table S3).

Separately, a snapshot of *in vivo* metabolism was assessed via isotopomer NMR analysis from freeze-clamped cardiac tissues of KD, RT, and CT groups to confirm the *in vivo* measurements. Bolus-injected $[3-^{13}\text{C}]\text{pyruvate}$ and $[1,2-^{13}\text{C}_2]\text{acetate}$ generated $[2-^{13}\text{C}]\text{acetyl-CoA}$ and $[1,2-^{13}\text{C}_2]\text{acetyl-CoA}$, respectively (Figure 3, A). Isotopomer analysis of glutamate C4 resonance showed that relative concentration of $[2-^{13}\text{C}]\text{acetyl-CoA}$ isotopomer to the total acetyl-CoA was reduced in the KD group and recovered in the RT group. Relative contribution of the injected pyruvate to acetyl-CoA was 4.1 ± 2.5 % for KD, 22.7 ± 1.82 % for RT, and 27.6 ± 9.5 % for CT (Figure 3, B and C). Relative contribution of the injected acetate to acetyl-CoA was comparable between groups (31.6 ± 1.7 % for KD, 37.2 ± 6.4 % for RT, 30.2 ± 5.4 % for CT). Fractional ^{13}C enrichment in lactate, measured from ^1H NMR, was also comparable between groups (14.8 ± 5.9 for KD, 12.6 ± 3.8 % for RT, 14.8 ± 2.8 % for CT). Two pairs of outer doublets observed in the lactate resonance region represent the three-bond J_{CH} spin-spin coupling between methyl protons and the methyl ^{13}C , confirming the presence of injected lactate in the metabolite pool. Fractional ^{13}C enrichment in alanine was higher in the KD group (55.0 ± 4.4 %) as compared with RT (43.5 ± 5.4 %, $P = 0.016$) and CT (47.1 ± 4.8 %, $P = 0.038$) as summarized in Figure 3, B and D, likely due to elevated circulating alanine aminotransferase.^{56, 57} The NMR measurements are summarized in the Supplemental Information (Table S4).

Enzymatic analysis was performed from cardiac tissues of KD, RT, and CT groups to compare with the *in vivo* observation. The *ex vivo* analysis revealed that PDK4 level in the myocardium was elevated with KD (3.48 ± 0.37 , relative to control), then returned to the normal level (0.87 ± 0.10) at day 9 after reverting to ND (Figure 4, B). On the contrary, PDH activity and phosphorylated PDH level were not fully recovered to the normal level, Figure 4, C and D. PDH activity in the cardiac tissue increased from 1.60 ± 0.12 mmol NADH/min/mg in KD group ($n = 4$) to 36.80 ± 6.07 in RT group ($n = 3$), which was significantly lower than PDH activity in CT group ($n = 3$, 90.97 ± 5.40 , $P = 7.1 \times 10^{-5}$). Similarly, phosphorylated PDH (p-PDH), the inactive form of PDH, was 33.39 $\pm$ 0.42-fold higher in KD as compared to CT but remained much higher level in RT (11.80 ± 1.58). The CoASH level was lower in the KD group (0.444 ± 0.069 nmol/mg) as compared to the CT group (0.618 ± 0.011 , $P = 0.008$) and the RT group (0.585 ± 0.044 , $P = 0.028$). The acetyl-CoA level and the acetyl-CoA-to-CoASH ratio showed the opposite pattern, Figure 4, E–G. The PDH measurements and the metabolite levels are summarized in the Supplemental Information (Table S5). These results contrasted with the complete recovery of both PDK4

and phosphorylated PDK at day 8 after reverting to ND following a 2-week KD, Figure S2, suggesting a strong dependence of PDH recovery dynamics on the duration of KD exposure.

Circulating profiles of ketones, glucose, and NEFAs in blood were shifted with the KD, Figure 5. The plasma ketone level increased by 12.10 ± 8.09 -fold after two weeks of KD (baseline: $163.0 \text{ mmol/L} \pm 89.4$, 2 weeks: $2057.8 \pm 2061.2 \text{ mmol/L}$) while the control group showed no significant change ($P = 0.13$). The elevated ketone level in the KD group remained significant at 3.5 (3177.5 ± 1217.1 , $P = 4.7 \times 10^{-3}$) and 5 weeks of KD (2180.0 ± 1508.9 , $P = 0.038$) compared with the control group (84.6 ± 29.1 at 3.5, 84.3 ± 23.5 at 5 weeks). In contrast, glucose level showed the opposite trend, decreasing by 34.3 ± 14.9 % at 2 weeks ($110.0 \pm 7.0 \text{ mg/dL}$) relative to baseline ($174.5 \pm 32.0 \text{ mg/dL}$). The reduced glucose level remained lower in the KD group at 3.5 weeks (113.5 ± 9.1 , $P = 1.4 \times 10^{-4}$) and 5 weeks (135.5 ± 17.7 , $P = 5.7 \times 10^{-3}$) than in the controls (191.5 ± 18.1 at 3.5, 192.5 ± 13.5 at 5 weeks). The KD also elevated plasma NEFAs by 5.53 $\pm$ 2.95-fold from baseline ($0.262 \pm 0.113 \text{ mEq/L}$) to 2 weeks (1.206 ± 0.330), which then gradually decreased over time to 0.764 ± 0.320 at 3.5 and 0.603 ± 0.057 at 5 weeks. Nevertheless, NEFA levels remained significantly higher in the KD group than in controls ($P = 2.5 \times 10^{-3}$ at 2 weeks, 1.5×10^{-2} at 3.5 weeks, 6.0×10^{-6}). Upon reversion to the ND, both ketone and NEFA levels returned to control levels within 2 days. However, glucose level recovered more slowly, remaining lower than controls at 2 days ($P = 0.011$) but fully recovering thereafter ($P > 0.4$).

DISCUSSION

In this study, we show that myocardial PDH flux, measured by HP [$1\text{-}^{13}\text{C}$]pyruvate *in vivo*, requires approximately one week to fully recover from a 5-week KD. Cross-sectional isotopomer analysis of freeze-clamped tissue using ^{13}C NMR with [$3\text{-}^{13}\text{C}$]pyruvate and [$1,2\text{-}^{13}\text{C}_2$]acetate supports this full recovery of myocardial PDH flux one week after reverting the diet. In contrast, *ex vivo* enzymatic analysis shows incomplete recovery of PDH activity during the same period.

Prolonged exposure to a KD induces a state of metabolic inflexibility in the heart, where cardiac ketolysis and β -oxidation predominate over carbohydrate oxidation. This metabolic shift reflects functional adaptations of associated transporters and enzymes for sustained utilization of ketones and fatty acids, consistent with the elevated plasma ketones and fatty acid levels and the reduced glucose concentrations observed here. Similar changes have been reported in KD-fed mice for 3 weeks, showing increased palmitate oxidation, unaltered β -hydroxybutyrate oxidation and impaired glucose oxidation.⁴¹ Gallop, *et al.* also reported that long-term KD also elevated circulating lipids and suppressed insulin secretion.⁵⁸

The discrepancies between the ^{13}C results and enzymatic analyses regarding the extent of recovery one week after reverting from a KD to an ND likely arise from the methodological differences. Due to complex metabolic cross talks and signaling pathways, metabolic fluxes need to be assessed under strict, well-controlled environments. *Ex vivo* enzyme assay measures the maximum enzyme activity. However, in physiological settings, metabolic systems typically operate below their maximal capacity, allowing for metabolic flexibility to maintain homeostasis. For instance, the activity of PDH depends on multiple factors,

including malonyl-CoA levels, CoASH availability, PDK regulation, redox state, and alternative substrate pathways such as fatty acid oxidation.⁵⁹ Enzymatic analyses of isolated cardiac mitochondria post-KD reversion revealed a reduction in PDK4 levels to those seen in control diet-fed rats. Despite this, PDH activity remained attenuated. Similarly, while acetyl-CoA levels were significantly lower, free CoASH levels returned to normal, suggesting that while acetyl-CoA synthesis or utilization was altered, the pool of available CoASH was restored.

In contrast, isotopomer analysis offers a more direct method of measuring *in vivo*-like metabolic fluxes. Using freeze-clamped tissues and substrates labeled with stable isotopes, NMR or mass spectrometry can capture metabolism under conditions closer to physiological states. In our study, we employed ¹³C-pyruvate and NMR to assess PDH flux, and the results showed fully recovered PDH flux, consistent with *in vivo* findings using HP [1-¹³C]pyruvate. Together, these findings suggest that, about one week after reverting the KD, myocardial PDH flux reaches the restored maximum PDH activity. However, the persistence of altered metabolic flexibility indicates incomplete recovery of the broader metabolic network. The discordance between enzymatic analyses and *in vivo* ¹³C NMR results highlights the necessity of complementary approaches. Therefore, measuring metabolic fluxes *in vivo* under physiological conditions provides critical insights into dynamic metabolic states, which are often missed by *ex vivo* analyses. This integrative approach is essential for capturing the complexity of metabolic recovery.

Sustained enhancement of fatty acid oxidation may partly explain the delayed PDH recovery observed here. We previously showed that high-fat diet-induced declines in cardiac PDH activity plateau within one day, coinciding with increased PDK4 expression and PDH E1a phosphorylation.⁴⁴ PDK4 is selectively degraded by the LON protease in a CoA-dependent manner upon dissociation from the PDH complex.⁴⁵ However, even after normalization of PDK4, PDH can remain sensitized to fatty acid-induced inhibition due to persistent upregulation of β -oxidation enzymes, including very long chain acyl-CoA dehydrogenase, carnitine palmitoyltransferase 2, and β -hydroxyacyl-CoA dehydrogenase.⁶⁰ Moreover, elevated acetyl-CoA and ketone levels during KD can increase protein acetylation, which in turn may influence transcriptional regulation of metabolic enzymes. Such epigenetic or acetylation-mediated remodeling may also prolong metabolic reprogramming beyond the time course of PDH phosphorylation changes.

The slow recovery of PDH flux and metabolic flexibility resonates the challenges of accurately characterizing disease-induced metabolic profiles, particularly when dietary interventions introduce significant metabolic shifts. Noninvasive assessment of disease-induced metabolic changes is crucial for diagnosing and managing cardiovascular diseases. Understanding how dietary conditions affect metabolism is important for developing clinically applicable imaging protocols. Clinically applicable *in vivo* imaging methodologies that measure carbohydrate metabolism, such as [¹⁹F]FDG-PET,⁶¹ deuterium metabolic imaging (DMI),⁶² and HP [¹³C]pyruvate MRI⁶³ are highly sensitive to special diets that suppress glycolysis and pyruvate oxidation, such as KD.⁶⁴ Consequently, the metabolic shifts induced by a KD often present challenges for assessing disease-specific cardiac metabolism. A common strategy is to administer a standardized diet to the subject prior

to imaging. For instance, clinical protocols using FDG attempt to standardize glucose uptake by oral glucose loading protocol, which consists of 6–12 h fasting, glucose loading, and insulin administration prior to PET imaging.⁶⁵ This is to maximize physiological PDH flux from a standardized (fasted) minimum baseline. Similarly, for human cardiac imaging studies with HP ^{13}C -pyruvate, an oral glucose load after an overnight fast has been employed as a standardized preparation procedure^{13–15} due to its effectiveness in maximizing HP $\text{H}^{13}\text{CO}_3^-$ production.⁵⁴ However, the metabolic recovery rate of suppressed PDH activity has been unknown.⁶⁶ Although diet-induced increases in PDK4 content are rapidly reversible,⁴⁴ returning to a carbohydrate-based diet after a period of consuming a KD has post-diet effects on caloric intake, body weight gain, and insulin levels.⁶⁷ Our study results confirms that the PDH flux, suppressed by a long-term KD, exhibited a protracted recovery in the myocardium after the diet was reverted, and therefore, an oral glucose challenge may not be sufficient to normalize the dietary component.

The prolonged PDH inactivation highlights the potential for lingering metabolic inflexibility following ketogenic interventions, which may have clinical relevance in conditions such as heart failure or diabetes, where PDH regulation is already impaired. However, recovery kinetics may differ in humans, given their slower basal metabolic rate⁶⁸ and greater substrate flexibility, and higher reliance on carbohydrate oxidation,^{69, 70} compared to rodents. Moreover, the abundance and turnover rate of mitochondrial enzymes, including PDH, are lower in humans,⁷¹ suggesting a slower onset but potentially faster recovery metabolic reprogramming once established.

Limitations were identified in this study, which suggest opportunities for future investigations. First, HP $[1-^{13}\text{C}]$ pyruvate was limited to assess PDH flux only up to acetyl-CoA formation since the labeled carbon is decarboxylated by PDH. Downstream metabolic fate of acetyl-CoA and contributions from other substrates were not explored. To link the PDH flux to pyruvate oxidation and to assess how acetyl-CoA is metabolized in the heart *in vivo*, HP $[2-^{13}\text{C}]$ pyruvate can be considered.^{19, 72} Alternatively, HP $[1-^{13}\text{C}]$ acetyl-L-carnitine can be utilized for probing acetyl-CoA metabolism by detecting $[5-^{13}\text{C}]$ glutamate.⁷³ Integrating HP $[1-^{13}\text{C}]$ pyruvate with additional HP substrates such as $[1-^{13}\text{C}]$ butyrate and $[3-^{13}\text{C}]$ acetoacetate may further delineate substrate competition.⁷⁴ In addition, a fluxomic analysis using ^{13}C -glucose could elucidate how glucose uptake and glycolysis recover relative to PDH flux since glucose fate in the myocardium can be influenced by elevated ketone availability.^{75, 76} Second, each HP product was quantified as a relative amount compared to total HP ^{13}C products. This metric is not sensitive to changes in the total product amounts. Although not done in our study due to the absence of polarization measurements, a potential solution for more consistent quantification between the modalities is normalization of each HP signals to its polarization level at the time of injection.⁷⁷ Third, inclusion of only males limits translational relevance as sex-specific differences in PDH regulation are significant.^{38, 78} Finally, while the current study focused on PDK4-mediated PDH regulation, other mechanisms that could diminish PDH activity in response to high dietary fat were not investigated. For instance, Ca^{2+} is known to activate PDH by stimulating pyruvate dehydrogenase phosphatase 1 and dephosphorylation of the enzyme. Future studies combining transcriptomic and acetyl-proteomic profiling will clarify these longer-term mechanisms.

In conclusion, KD-induced suppression of myocardial PDH flux is reversible but recovers slowly upon reintroduction of a ND. *In vivo* measurements of restored PDH flux aligned with PDK4 normalization but were not consistent with tissue level PDH activity, implying persistent metabolic inflexibility in the myocardium. These findings highlight the value of *in vivo* assessments of PDH activity for assessing cardiac carbohydrate metabolism and emphasize the complementary insights gained from integrating *in vivo* HP ^{13}C MR and *ex vivo* biochemical analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

The authors thank the UT Southwestern Metabolic Phenotyping Core for performing blood analyses and Anna Karki for assistance with tissue sample preparation and analysis.

Sources of Funding:

This study was supported by the National Institutes of Health of the United States (R01 NS107409, R01 HL170039, P41 EB015908, S10 OD018468, S10 OD028490, P30 DK127984), U.S. Army Medical Research Acquisition Activity (W81XWH2210485, HT94252510616), Muscular Dystrophy Association (MDA963281), and the Cancer Prevention and Research Institute of Texas (RP210099).

References

1. Lopaschuk GD, Ussher JR, Folmes CD, Jaswal JS, Stanley WC. Myocardial fatty acid metabolism in health and disease. *Physiol Rev* 2010;90:207–258. [PubMed: 20086077]
2. Tran DH, Wang ZV. Glucose Metabolism in Cardiac Hypertrophy and Heart Failure. *J Am Heart Assoc* 2019;8:e012673. [PubMed: 31185774]
3. Gertz EW, Wisneski JA, Stanley WC, Neese RA. Myocardial substrate utilization during exercise in humans. Dual carbon-labeled carbohydrate isotope experiments. *J Clin Invest* 1988;82:2017–2025. [PubMed: 3198763]
4. Wieland O, Siess E, Schulze-Wethmar FH, von Funcke HG, Winton B. Active and inactive forms of pyruvate dehydrogenase in rat heart and kidney: effect of diabetes, fasting, and refeeding on pyruvate dehydrogenase interconversion. *Arch Biochem Biophys* 1971;143:593–601. [PubMed: 4326833]
5. Cooper RH, Randle PJ, Denton RM. Stimulation of phosphorylation and inactivation of pyruvate dehydrogenase by physiological inhibitors of the pyruvate dehydrogenase reaction. *Nature* 1975;257:808–809. [PubMed: 171583]
6. Caruso M, Maitan MA, Bifulco G, Miele C, Vigliotta G, Oriente F, Formisano P, Beguinot F. Activation and mitochondrial translocation of protein kinase Cdelta are necessary for insulin stimulation of pyruvate dehydrogenase complex activity in muscle and liver cells. *J Biol Chem* 2001;276:45088–45097. [PubMed: 11577086]
7. Golman K, in 't Zandt R, Thaning M. Real-time metabolic imaging. *Proc Natl Acad Sci U S A* 2006;103:11270–11275. [PubMed: 16837573]
8. Merritt ME, Harrison C, Storey C, Jeffrey FM, Sherry AD, Malloy CR. Hyperpolarized ^{13}C allows a direct measure of flux through a single enzyme-catalyzed step by NMR. *Proc Natl Acad Sci U S A* 2007;104:19773–19777. [PubMed: 18056642]
9. Schroeder MA, Cochlin LE, Heather LC, Clarke K, Radda GK, Tyler DJ. In vivo assessment of pyruvate dehydrogenase flux in the heart using hyperpolarized carbon-13 magnetic resonance. *Proc Natl Acad Sci U S A* 2008;105:12051–12056. [PubMed: 18689683]

10. Golman K, Petersson JS, Magnusson P, Johansson E, Akeson P, Chai CM, Hansson G, Mansson S. Cardiac metabolism measured noninvasively by hyperpolarized ¹³C MRI. *Magn Reson Med* 2008;59:1005–1013. [PubMed: 18429038]
11. Le Page LM, Rider OJ, Lewis AJ, Ball V, Clarke K, Johansson E, Carr CA, Heather LC, Tyler DJ. Increasing Pyruvate Dehydrogenase Flux as a Treatment for Diabetic Cardiomyopathy: A Combined ¹³C Hyperpolarized Magnetic Resonance and Echocardiography Study. *Diabetes* 2015;64:2735–2743. [PubMed: 25795215]
12. Timm KN, Perera C, Ball V, Henry JA, Miller JJ, Kerr M, West JA, Sharma E, Broxholme J, Logan A, Savic D, Dodd MS, Griffin JL, Murphy MP, Heather LC, Tyler DJ. Early detection of doxorubicin-induced cardiotoxicity in rats by its cardiac metabolic signature assessed with hyperpolarized MRI. *Commun Biol* 2020;3:692. [PubMed: 33214680]
13. Cunningham CH, Lau JY, Chen AP, Geraghty BJ, Perks WJ, Roifman I, Wright GA, Connelly KA. Hyperpolarized ¹³C Metabolic MRI of the Human Heart: Initial Experience. *Circ Res* 2016;119:1177–1182. [PubMed: 27635086]
14. Rider OJ, Apps A, Miller J, Lau JYC, Lewis AJM, Peterzan MA, Dodd MS, Lau AZ, Trumper C, Gallagher FA, Grist JT, Brindle KM, Neubauer S, Tyler DJ. Noninvasive In Vivo Assessment of Cardiac Metabolism in the Healthy and Diabetic Human Heart Using Hyperpolarized (¹³C) MRI. *Circ Res* 2020;126:725–736. [PubMed: 32078413]
15. Park JM, Reed GD, Liticker J, Putnam WC, Chandra A, Yaros K, Afzal A, MacNamara J, Raza J, Hall RG, Baxter J, Derner K, Pena S, Kallem RR, Subramanian I, Edpuganti V, Harrison CE, Muthukumar A, Lewis C, Reddy S, Unni N, Klemow D, Syed S, Li H, Cole S, Froehlich T, Ayers C, de Lemos J, Malloy CR, Haley B, Zaha VG. Effect of Doxorubicin on Myocardial Bicarbonate Production From Pyruvate Dehydrogenase in Women With Breast Cancer. *Circ Res* 2020;127:1568–1570. [PubMed: 33054563]
16. Apps A, Lau JYC, Miller J, Tyler A, Young LAJ, Lewis AJM, Barnes G, Trumper C, Neubauer S, Rider OJ, Tyler DJ. Proof-of-Principle Demonstration of Direct Metabolic Imaging Following Myocardial Infarction Using Hyperpolarized ¹³C CMR. *JACC Cardiovasc Imaging* 2021;14:1285–1288. [PubMed: 33582059]
17. Dodd MS, Ball V, Bray R, Ashrafian H, Watkins H, Clarke K, Tyler DJ. In vivo mouse cardiac hyperpolarized magnetic resonance spectroscopy. *J Cardiovasc Magn Reson* 2013;15:19. [PubMed: 23414451]
18. Merritt ME, Harrison C, Storey C, Sherry AD, Malloy CR. Inhibition of carbohydrate oxidation during the first minute of reperfusion after brief ischemia: NMR detection of hyperpolarized ¹³CO₂ and H¹³CO₃. *Magn Reson Med* 2008;60:1029–1036. [PubMed: 18956454]
19. Josan S, Park JM, Hurd R, Yen YF, Pfefferbaum A, Spielman D, Mayer D. In vivo investigation of cardiac metabolism in the rat using MRS of hyperpolarized [1–¹³C] and [2–¹³C]pyruvate. *NMR Biomed* 2013;26:1680–1687. [PubMed: 23904148]
20. Peterman MG. The Ketogenic Diet. *J Am Med Assoc* 1928;90:1427–1429.
21. Wilder RM. The effect of ketonemia on the course of epilepsy. *Mayo Clinic Proceedings* 1921;2:307–308.
22. Ułamek-Kozioł M, Czuczwar SJ, Januszewski S, Pluta R. Ketogenic Diet and Epilepsy. *Nutrients* 2019;11.
23. Sofou K, Dahlin M, Hallbook T, Lindefeldt M, Viggedal G, Darin N. Ketogenic diet in pyruvate dehydrogenase complex deficiency: short- and long-term outcomes. *J Inher Metab Dis* 2017;40:237–245. [PubMed: 28101805]
24. Wexler ID, Hemalatha SG, McConnell J, Buist NR, Dahl HH, Berry SA, Cederbaum SD, Patel MS, Kerr DS. Outcome of pyruvate dehydrogenase deficiency treated with ketogenic diets. *Studies in patients with identical mutations. Neurology* 1997;49:1655–1661. [PubMed: 9409363]
25. Deng-Bryant Y, Prins ML, Hovda DA, Harris NG. Ketogenic diet prevents alterations in brain metabolism in young but not adult rats after traumatic brain injury. *J Neurotrauma* 2011;28:1813–1825. [PubMed: 21635175]
26. Muscogiuri G, El Ghoch M, Colao A, Hassapidou M, Yumuk V, Busetto L, Obesity Management Task Force of the European Association for the Study of O. European Guidelines for Obesity

- Management in Adults with a Very Low-Calorie Ketogenic Diet: A Systematic Review and Meta-Analysis. *Obes Facts* 2021;14:222–245. [PubMed: 33882506]
27. Greene DA, Varley BJ, Hartwig TB, Chapman P, Rigney M. A Low-Carbohydrate Ketogenic Diet Reduces Body Mass Without Compromising Performance in Powerlifting and Olympic Weightlifting Athletes. *J Strength Cond Res* 2018;32:3373–3382. [PubMed: 30335720]
 28. Cao J, Lei S, Wang X, Cheng S. The Effect of a Ketogenic Low-Carbohydrate, High-Fat Diet on Aerobic Capacity and Exercise Performance in Endurance Athletes: A Systematic Review and Meta-Analysis. *Nutrients* 2021;13. [PubMed: 35010888]
 29. Council IFI. 2021 Food & Health Survey. 2021.
 30. Buren J, Ericsson M, Damasceno NRT, Sjodin A. A Ketogenic Low-Carbohydrate High-Fat Diet Increases LDL Cholesterol in Healthy, Young, Normal-Weight Women: A Randomized Controlled Feeding Trial. *Nutrients* 2021;13.
 31. Hengist A, Davies RG, Walhin JP, Buniam J, Merrell LH, Rogers L, Bradshaw L, Moreno-Cabanas A, Rogers PJ, Brunstrom JM, Hodson L, van Loon LJC, Barton W, O'Donovan C, Crispie F, O'Sullivan O, Cotter PD, Proctor K, Betts JA, Koumanov F, Thompson D, Gonzalez JT. Ketogenic diet but not free-sugar restriction alters glucose tolerance, lipid metabolism, peripheral tissue phenotype, and gut microbiome: RCT. *Cell Rep Med* 2024;101667. [PubMed: 39106867]
 32. Kosinski C, Jornayvaz FR. Effects of Ketogenic Diets on Cardiovascular Risk Factors: Evidence from Animal and Human Studies. *Nutrients* 2017;9.
 33. O'Neill B, Raggi P. The ketogenic diet: Pros and cons. *Atherosclerosis* 2020;292:119–126. [PubMed: 31805451]
 34. Matsuura TR, Puchalska P, Crawford PA, Kelly DP. Ketones and the Heart: Metabolic Principles and Therapeutic Implications. *Circ Res* 2023;132:882–898. [PubMed: 36996176]
 35. Mohammadifard N, Haghighatdoost F, Rahimlou M, Rodrigues APS, Gaskarei MK, Okhovat P, de Oliveira C, Silveira EA, Sarrafzadegan N. The Effect of Ketogenic Diet on Shared Risk Factors of Cardiovascular Disease and Cancer. *Nutrients* 2022;14.
 36. Schulze PC, Wu JMF. Ketone bodies for the starving heart. *Nat Metab* 2020;2:1183–1185. [PubMed: 33106691]
 37. Rinnankoski-Tuikka R, Silvennoinen M, Torvinen S, Hulmi JJ, Lehti M, Kivela R, Reunanen H, Kainulainen H. Effects of high-fat diet and physical activity on pyruvate dehydrogenase kinase-4 in mouse skeletal muscle. *Nutr Metab (Lond)* 2012;9:53. [PubMed: 22682013]
 38. Reue K, Wiese CB. Illuminating the Mechanisms Underlying Sex Differences in Cardiovascular Disease. *Circ Res* 2022;130:1747–1762. [PubMed: 35679362]
 39. Granados-Rojas L, Jeronimo-Cruz K, Juarez-Zepeda TE, Tapia-Rodriguez M, Tovar AR, Rodriguez-Jurado R, Carmona-Aparicio L, Cardenas-Rodriguez N, Coballase-Urrutia E, Ruiz-Garcia M, Duran P. Ketogenic Diet Provided During Three Months Increases KCC2 Expression but Not NKCC1 in the Rat Dentate Gyrus. *Front Neurosci* 2020;14:673. [PubMed: 32733191]
 40. Drabinska N, Juskiewicz J, Wiczowski W. The Effect of the Restrictive Ketogenic Diet on the Body Composition, Haematological and Biochemical Parameters, Oxidative Stress and Advanced Glycation End-Products in Young Wistar Rats with Diet-Induced Obesity. *Nutrients* 2022;14.
 41. Ho KL, Karwi QG, Wang F, Wagg C, Zhang L, Panidarapu S, Chen B, Pherwani S, Greenwell AA, Oudit GY, Ussher JR, Lopaschuk GD. The ketogenic diet does not improve cardiac function and blunts glucose oxidation in ischaemic heart failure. *Cardiovasc Res* 2024;120:1126–1137. [PubMed: 38691671]
 42. Murphy NE, Carrigan CT, Margolis LM. High-Fat Ketogenic Diets and Physical Performance: A Systematic Review. *Adv Nutr* 2021;12:223–233. [PubMed: 32865567]
 43. Hackett EP, Shah BR, Cheng B, LaGue E, Vemireddy V, Mendoza M, Bing C, Bachoo RM, Billingsley KL, Chopra R, Park JM. Probing Cerebral Metabolism with Hyperpolarized (13)C Imaging after Opening the Blood-Brain Barrier with Focused Ultrasound. *ACS Chem Neurosci* 2021;12:2820–2828. [PubMed: 34291630]
 44. Crewe C, Kinter M, Szweda LI. Rapid inhibition of pyruvate dehydrogenase: an initiating event in high dietary fat-induced loss of metabolic flexibility in the heart. *PLoS One* 2013;8:e77280. [PubMed: 24116221]

45. Crewe C, Schafer C, Lee I, Kinter M, Szweda LI. Regulation of Pyruvate Dehydrogenase Kinase 4 in the Heart through Degradation by the Lon Protease in Response to Mitochondrial Substrate Availability. *J Biol Chem* 2017;292:305–312. [PubMed: 27856638]
46. Malloy CR, Thompson JR, Jeffrey FM, Sherry AD. Contribution of exogenous substrates to acetyl coenzyme A: measurement by ¹³C NMR under non-steady-state conditions. *Biochemistry* 1990;29:6756–6761. [PubMed: 1975750]
47. Lahti A, Hyltoft Petersen P, Boyd JC, Fraser CG, Jorgensen N. Objective criteria for partitioning Gaussian-distributed reference values into subgroups. *Clin Chem* 2002;48:338–352. [PubMed: 11805016]
48. Evans SA, Messina MM, Knight WD, Parsons AD, Overton JM. Long-Evans and Sprague-Dawley rats exhibit divergent responses to refeeding after caloric restriction. *Am J Physiol Regul Integr Comp Physiol* 2005;288:R1468–1476. [PubMed: 15695322]
49. Woods SC, D'Alessio DA, Tso P, Rushing PA, Clegg DJ, Benoit SC, Gotoh K, Liu M, Seeley RJ. Consumption of a high-fat diet alters the homeostatic regulation of energy balance. *Physiol Behav* 2004;83:573–578. [PubMed: 15621062]
50. Geary N, Scharrer E, Freudlsperger R, Raab W. Adaptation to high-fat diet and carbohydrate-induced satiety in the rat. *Am J Physiol* 1979;237:R139–146. [PubMed: 474786]
51. Kephart WC, Mumford PW, Mao X, Romero MA, Hyatt HW, Zhang Y, Mobley CB, Quindry JC, Young KC, Beck DT, Martin JS, McCullough DJ, D'Agostino DP, Lowery RP, Wilson JM, Kavazis AN, Roberts MD. The 1-Week and 8-Month Effects of a Ketogenic Diet or Ketone Salt Supplementation on Multi-Organ Markers of Oxidative Stress and Mitochondrial Function in Rats. *Nutrients* 2017;9.
52. Prathima S, Devi SA. Adaptations in lactate dehydrogenase and its isozymes in aging mammalian myocardium: interaction of exercise and temperature. *Mech Ageing Dev* 1999;108:61–75. [PubMed: 10366040]
53. Wespi P, Steinhauser J, Kwiatkowski G, Kozerke S. Overestimation of cardiac lactate production caused by liver metabolism of hyperpolarized [1-(13) C]pyruvate. *Magn Reson Med* 2018;80:1882–1890. [PubMed: 29607535]
54. Larson PEZ, Tang S, Liu X, Sinha A, Dwork N, Sivalokanathan S, Liu J, Bok R, Ordovas KG, Slater J, Gordon JW, Abraham MR. Regional quantification of cardiac metabolism with hyperpolarized [1-(13)C]-pyruvate CMR evaluated in an oral glucose challenge. *J Cardiovasc Magn Reson* 2023;25:77. [PubMed: 38093285]
55. Kettunen MI, Hu DE, Witney TH, McLaughlin R, Gallagher FA, Bohndiek SE, Day SE, Brindle KM. Magnetization transfer measurements of exchange between hyperpolarized [1–13C]pyruvate and [1–13C]lactate in a murine lymphoma. *Magn Reson Med* 2010;63:872–880. [PubMed: 20373388]
56. Jornayvaz FR, Jurczak MJ, Lee HY, Birkenfeld AL, Frederick DW, Zhang D, Zhang XM, Samuel VT, Shulman GI. A high-fat, ketogenic diet causes hepatic insulin resistance in mice, despite increasing energy expenditure and preventing weight gain. *Am J Physiol Endocrinol Metab* 2010;299:E808–815. [PubMed: 20807839]
57. Anekwe CV, Chandrasekaran P, Stanford FC. Ketogenic Diet-induced Elevated Cholesterol, Elevated Liver Enzymes and Potential Non-alcoholic Fatty Liver Disease. *Cureus* 2020;12:e6605. [PubMed: 32064187]
58. Gallop MR, Vieira RFL, Mower PD, Matsuzaki ET, Liou W, Smart FE, Roberts S, Evason KJ, Holland WL, Chaix A. A long-term ketogenic diet causes hyperlipidemia, liver dysfunction, and glucose intolerance from impaired insulin secretion in mice. *Sci Adv* 2025;11:eadx2752. [PubMed: 40971428]
59. Zhang S, Hulver MW, McMillan RP, Cline MA, Gilbert ER. The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. *Nutr Metab (Lond)* 2014;11:10. [PubMed: 24520982]
60. Schafer C, Young ZT, Makarewich CA, Elnwasany A, Kinter C, Kinter M, Szweda LI. Coenzyme A-mediated degradation of pyruvate dehydrogenase kinase 4 promotes cardiac metabolic flexibility after high-fat feeding in mice. *J Biol Chem* 2018;293:6915–6924. [PubMed: 29540486]

61. Al-Mallah MH, Sitek A, Moore SC, Di Carli M, Dorbala S. Assessment of myocardial perfusion and function with PET and PET/CT. *J Nucl Cardiol* 2010;17:498–513. [PubMed: 20379862]
62. Wang T, Zhu XH, Li H, Zhang Y, Zhu W, Wiesner HM, Chen W. Noninvasive assessment of myocardial energy metabolism and dynamics using in vivo deuterium MRS imaging. *Magn Reson Med* 2021;86:2899–2909. [PubMed: 34231258]
63. Schroeder MA, Clarke K, Neubauer S, Tyler DJ. Hyperpolarized magnetic resonance: a novel technique for the in vivo assessment of cardiovascular disease. *Circulation* 2011;124:1580–1594. [PubMed: 21969318]
64. Scholtens AM, Verberne HJ, Budde RP, Lam MG. Additional Heparin Preadministration Improves Cardiac Glucose Metabolism Suppression over Low-Carbohydrate Diet Alone in (1)(8)F-FDG PET Imaging. *J Nucl Med* 2016;57:568–573. [PubMed: 26659348]
65. Mhlanga J, Derenoncourt P, Haq A, Bhandiwad A, Laforest R, Siegel BA, Dehdashti F, Gropler RJ, Schindler TH. (18)F-FDG PET in Myocardial Viability Assessment: A Practical and Time-Efficient Protocol. *J Nucl Med* 2022;63:602–608. [PubMed: 34503961]
66. Timm KN, Apps A, Miller JJ, Ball V, Chong CR, Dodd MS, Tyler DJ. Assessing the optimal preparation strategy to minimize the variability of cardiac pyruvate dehydrogenase flux measurements with hyperpolarized MRS. *NMR Biomed* 2018;31:e3992. [PubMed: 30040147]
67. Honors MA, Davenport BM, Kinzig KP. Effects of consuming a high carbohydrate diet after eight weeks of exposure to a ketogenic diet. *Nutr Metab (Lond)* 2009;6:46. [PubMed: 19925676]
68. Agoston DV. How to Translate Time? The Temporal Aspect of Human and Rodent Biology. *Front Neurol* 2017;8:92. [PubMed: 28367138]
69. Glatz JF, Jacobs AE, Veerkamp JH. Fatty acid oxidation in human and rat heart. Comparison of cell-free and cellular systems. *Biochim Biophys Acta* 1984;794:454–465. [PubMed: 6430348]
70. Bugger H, Byrne NJ, Abel ED. Animal Models of Dysregulated Cardiac Metabolism. *Circ Res* 2022;130:1965–1993. [PubMed: 35679363]
71. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, Bain J, Stevens R, Dyck JR, Newgard CB, Lopaschuk GD, Muoio DM. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab* 2008;7:45–56. [PubMed: 18177724]
72. Schroeder MA, Atherton HJ, Ball DR, Cole MA, Heather LC, Griffin JL, Clarke K, Radda GK, Tyler DJ. Real-time assessment of Krebs cycle metabolism using hyperpolarized ¹³C magnetic resonance spectroscopy. *FASEB J* 2009;23:2529–2538. [PubMed: 19329759]
73. Chen J, Singh TK, Al Nemri S, Zaidi M, Billingsley KL, Park JM. Hyperpolarized [1-(¹³C)]Acetyl-L-Carnitine Probes Tricarboxylic Acid Cycle Activity In Vivo. *ACS Sens* 2023;8:2927–2932. [PubMed: 37578472]
74. Abdurrachim D, Teo XQ, Woo CC, Ong SY, Salleh NF, Lalic J, Tan RS, Lee PTH. Cardiac metabolic modulation upon low-carbohydrate low-protein ketogenic diet in diabetic rats studied in vivo using hyperpolarized (¹³) C pyruvate, butyrate and acetoacetate probes. *Diabetes Obes Metab* 2019;21:949–960. [PubMed: 30536560]
75. Schnelle M, Chong M, Zoccarato A, Elkenani M, Sawyer GJ, Hasenfuss G, Ludwig C, Shah AM. In vivo [U-(¹³C)]glucose labeling to assess heart metabolism in murine models of pressure and volume overload. *Am J Physiol Heart Circ Physiol* 2020;319:H422–H431. [PubMed: 32648823]
76. Zoccarato A, Smyrnias I, Reumiller CM, Hafstad AD, Chong M, Richards DA, Santos CXC, Visnagri A, Verma S, Bromage DI, Zhang M, Zhang X, Sawyer G, Thompson R, Shah AM. NRF2 activation in the heart induces glucose metabolic reprogramming and reduces cardiac dysfunction via upregulation of the pentose phosphate pathway. *Cardiovasc Res* 2025;121:339–352. [PubMed: 39657243]
77. Park JM, Harrison CE, Ma J, Chen J, Ratnakar J, Zun Z, Liticker J, Reed GD, Chhabra A, Haller RG, Jue T, Malloy CR. Hyperpolarized (¹³C) MR Spectroscopy Depicts in Vivo Effect of Exercise on Pyruvate Metabolism in Human Skeletal Muscle. *Radiology* 2021;300:626–632. [PubMed: 34156298]
78. Murphy E, Amanakis G, Fillmore N, Parks RJ, Sun J. Sex Differences in Metabolic Cardiomyopathy. *Cardiovasc Res* 2017;113:370–377. [PubMed: 28158412]

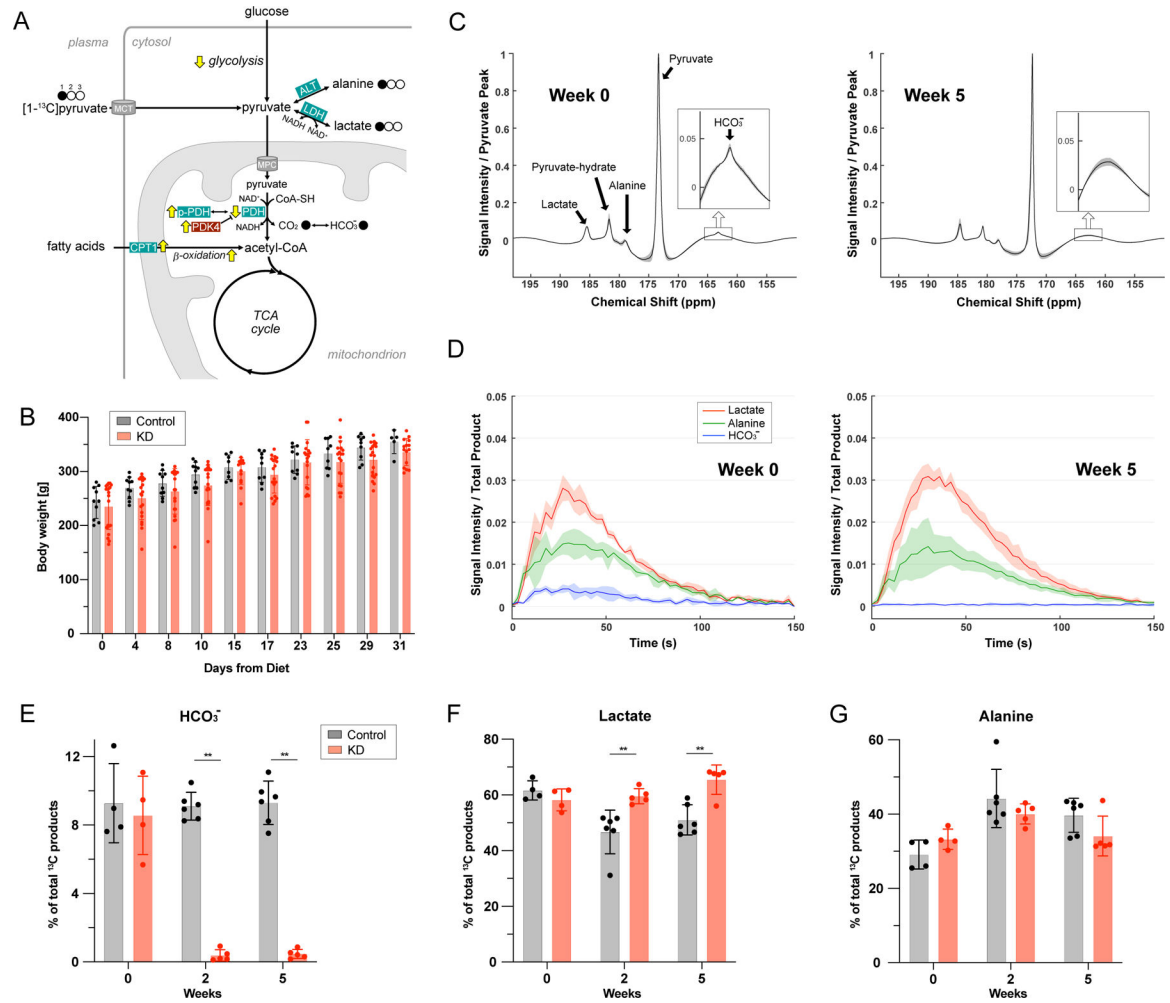


Figure 1. Longitudinal monitoring of skewed cardiac pyruvate metabolism by KD using HP [1-¹³C]pyruvate.

(A) Cardiac metabolism altered by KD and the pathway of labeled carbon when HP [1-¹³C]pyruvate is injected. The solid circle and empty circle represent labeled (¹³C) and unlabeled (¹²C) carbons, respectively. The yellow arrow indicates metabolic changes induced by a KD. (B) Body weights of the rats during a KD and a ND. (C) Accumulative ¹³C spectra averaged from the KD group at baseline and after 5 weeks of KD. (D) Time-courses of HP [1-¹³C]lactate (red), [1-¹³C]alanine (green), and H¹³CO₃⁻ (blue) at baseline and after 5 weeks of KD. The shaded spectra in (C) and shaded time-courses in (D) indicate +/- standard deviation. Changes of relative metabolite levels to total HP products from the CT (black, n = 6) and KD groups (red, n = 5): (E) H¹³CO₃⁻/TP, (F) [1-¹³C]lactate/TP, and (G) [1-¹³C]pyruvate/TP. Two-way ANOVA tests were used (α = 0.05). ** indicates *P* < 0.01. TP, total HP ¹³C products; KD, ketogenic diet; ND, normal diet.

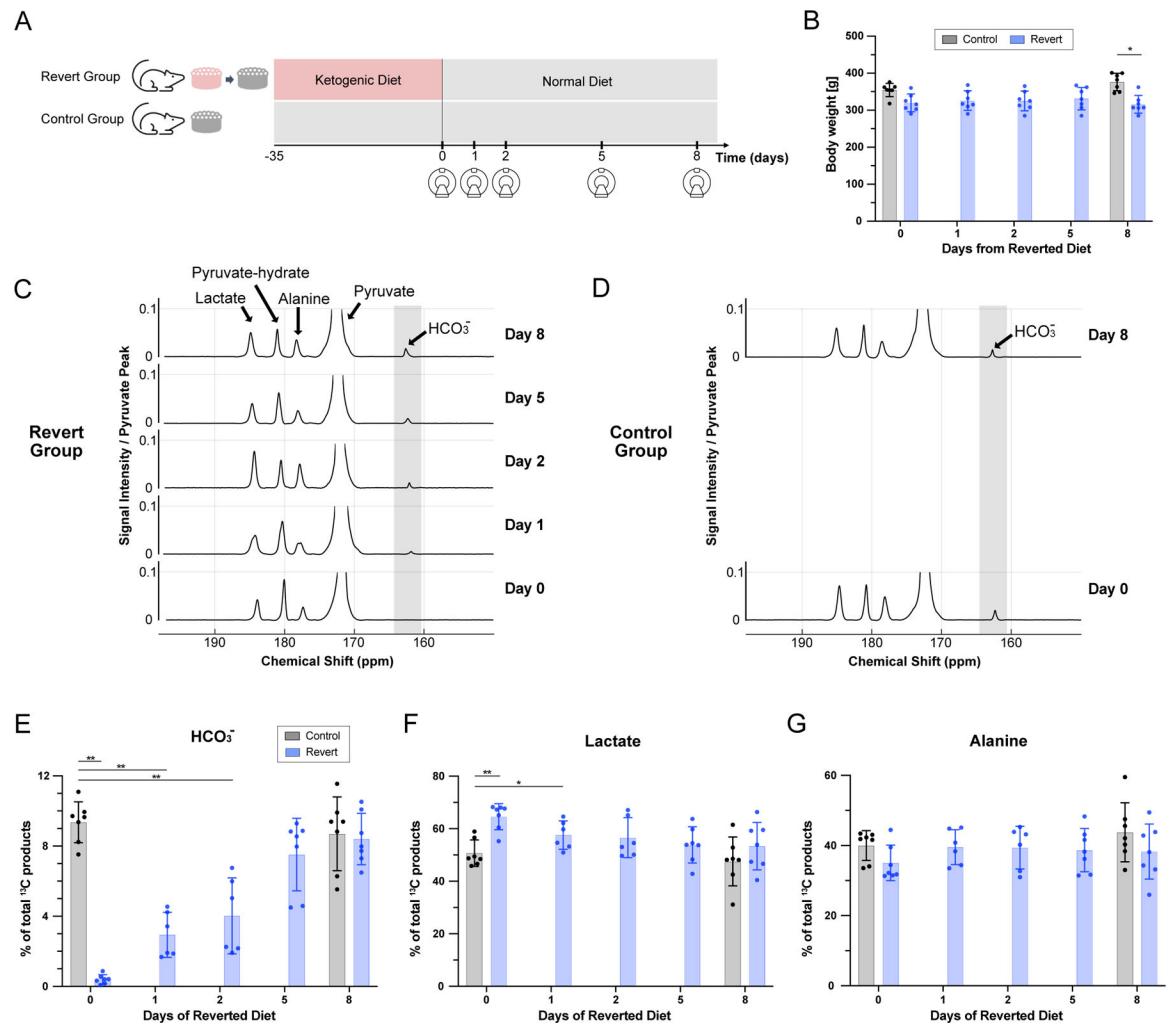


Figure 2. Recovery of *in vivo* myocardial PDH flux by reverting KD to ND.

(A) *In vivo* imaging timeline for the KD-to-ND revert group (n = 7) and the ND-fed control group (n = 7). (B) Body weight monitored during the timeframe. (C) Time-averaged cardiac ^{13}C spectra acquired from a representative rat at day 0, 1, 2, 5, and 8 after changing the diet. Reverting to ND gradually recovered the PDH flux ($\text{H}^{13}\text{CO}_3^-/\text{TP}$). (D) Age-matching CT group had non-significant changes. (E) HP $\text{H}^{13}\text{CO}_3^-/\text{TP}$, (F) $[1-^{13}\text{C}]\text{lactate}/\text{TP}$, and (G) $[1-^{13}\text{C}]\text{alanine}/\text{TP}$ were longitudinally monitored. Unpaired t-tests were used (two tailed, $\alpha = 0.05$). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively. TP, total HP ^{13}C products.

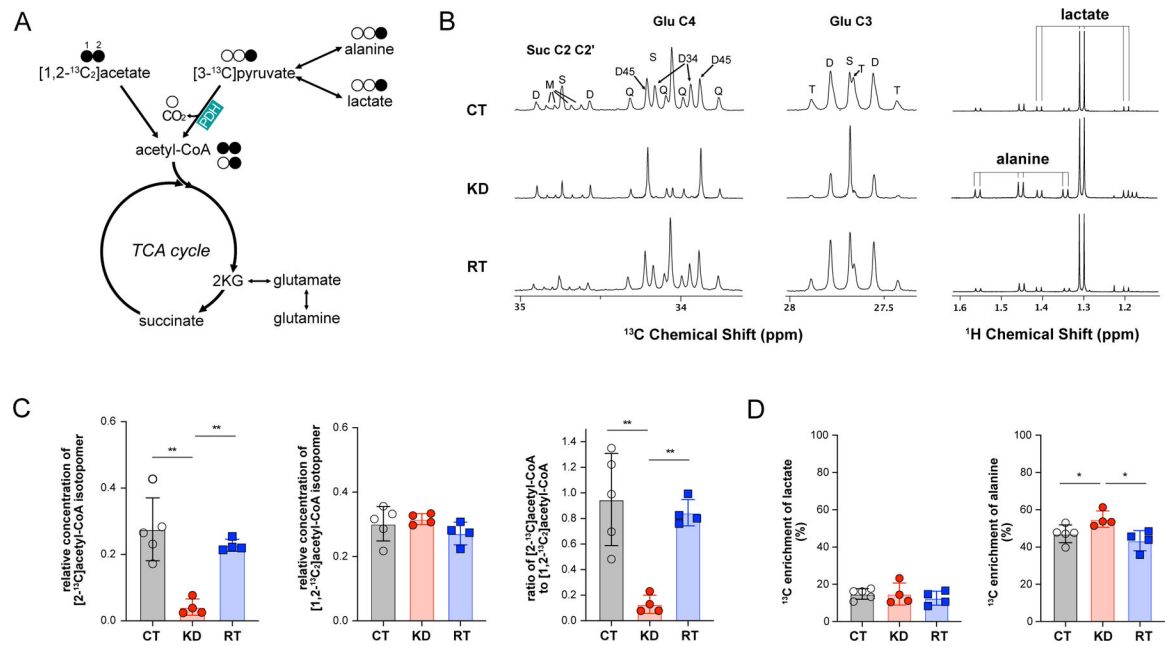


Figure 3. NMR Isotopomer analysis using [3-¹³C]pyruvate and [1,2-¹³C₂]acetate.

(A) Metabolic pathways of labeled substrates. (B) Representative ¹³C (27.0 – 35.0 ppm) and ¹H (1.1 – 1.6 ppm) NMR spectra from control (CT, n = 5), ketogenic diet (KD, n = 4), and reverted (RT, n = 4) rat hearts. (C) Relative concentrations of [2-¹³C]acetyl-CoA isotopomer and [1,2-¹³C₂]acetyl-CoA were calculated from ¹³C NMR. (D) ¹³C enrichments of lactate and alanine were calculated from ¹H NMR. Unpaired t-tests were used (two tailed, α = 0.05). * and ** indicate *P* < 0.05 and *P* < 0.01, respectively. Suc, succinate; Glu, glutamate; CT, control; KD, ketogenic diet; RT, reverted diet.

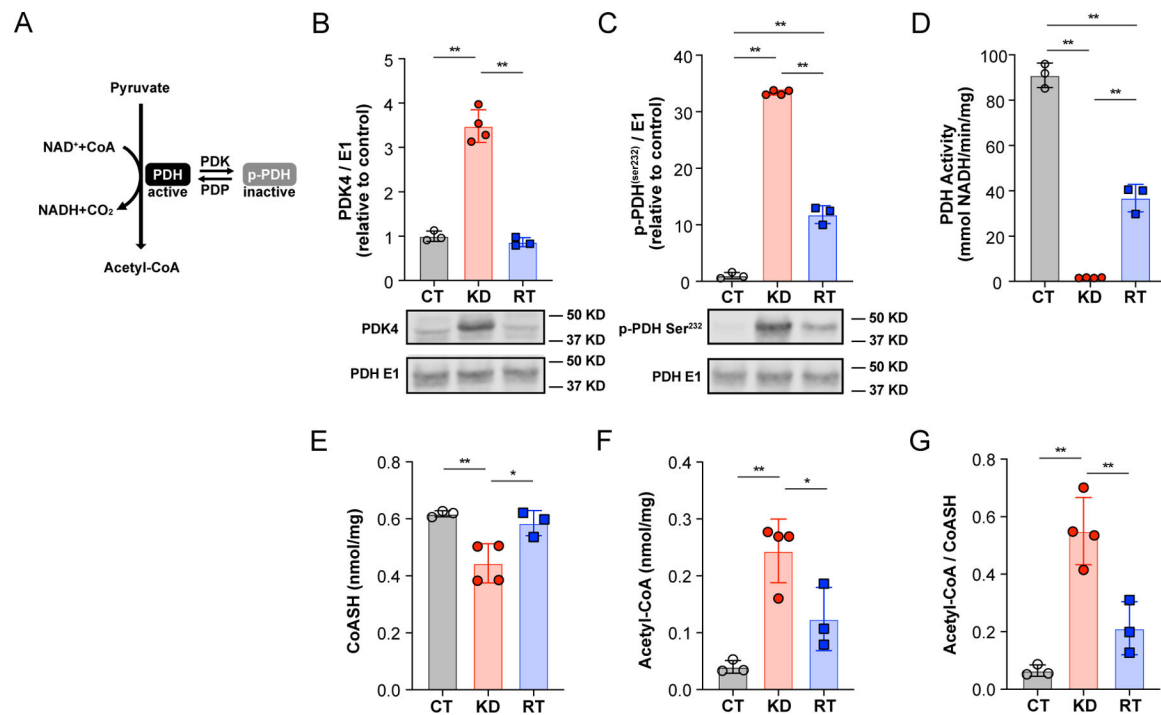


Figure 4. Enzymatic analysis of PDH.

(A) Active and inactive forms of PDH are regulated by PDK and PDP. (B) PDK, (C) PDH activity, (D) phosphorylated PDH (p-PDH), (E) CoASH, (F) Acetyl-CoA, and (G) Acetyl-CoA/CoASH levels were measured *ex vivo* from the cardiac tissue of the control (CT, n = 3), ketogenic diet (KD, 5 weeks, n = 4), and KD-to-ND reverted (RT, 5 weeks of KD followed by 9 days of ND, n = 3) groups. Unpaired t-tests were used (two tailed, $\alpha = 0.05$). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively. PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PDP, pyruvate dehydrogenase phosphatase.

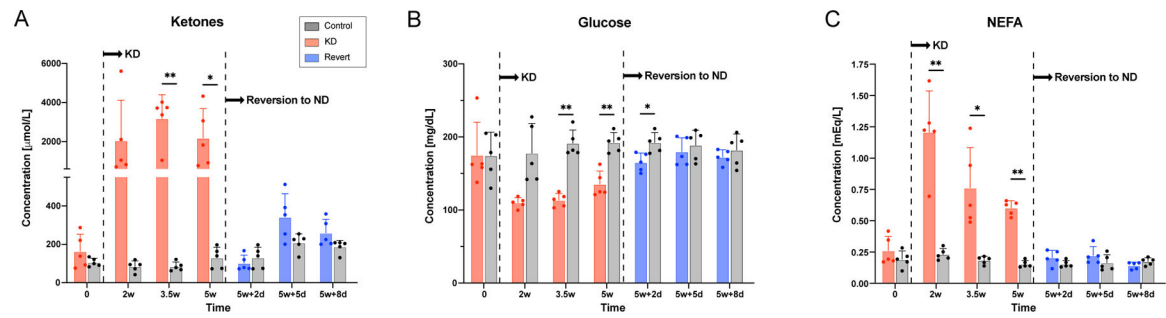


Figure 5. Plasma levels of ketones, glucose, and NEFA.

Circulating ketones, glucose, and NEFA were measured from blood longitudinally at baseline, during KD up to 5 weeks, and after reversion to ND up to 8 days. (A) The elevated level of ketones by a KD remained consistent during the KD and decreased to the control level within 2 days of reverted diet. (B) Glucose level dropped with a KD, then recovered to the control level within 2 days of reverted diet. (C) The NEFA level peaked at 2 weeks of KD then gradually decreased over the period of KD, then decreased to the control level within 2 days of reverted diet. Two-way ANOVA tests were used ($\alpha = 0.05$). * and ** indicate $P < 0.05$ and $P < 0.01$, respectively. KD, ketogenic diet; ND, normal diet; NEFA, non-esterified fatty acids.