

Supplemental Information

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

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Short Title: Recovery of PDH activity after a ketogenic diet

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Supplemental Figures

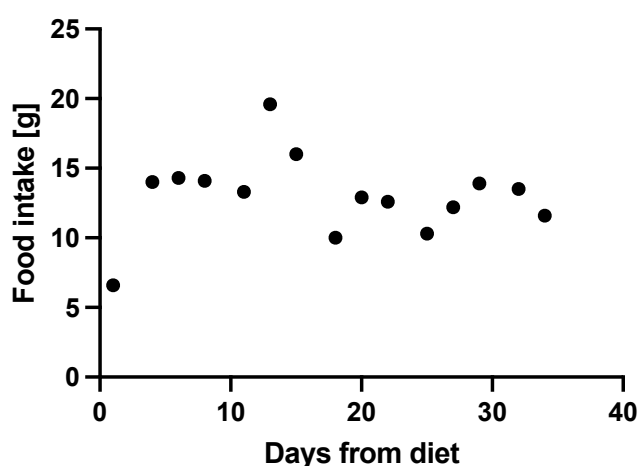


Figure S1. Daily intake of KD. Rats were fed a ketogenic diet (Teklad Custom Diet TD.96355; 90.5% kcal from fat [vegetable shortening and corn oil], 0.3% kcal from carbohydrates, and 9.1% kcal from protein; 6.7 kcal/g). The average daily intake per rat was consistent throughout the 5-week feeding period, except for the first day (6.6 g), after which consumption stabilized.

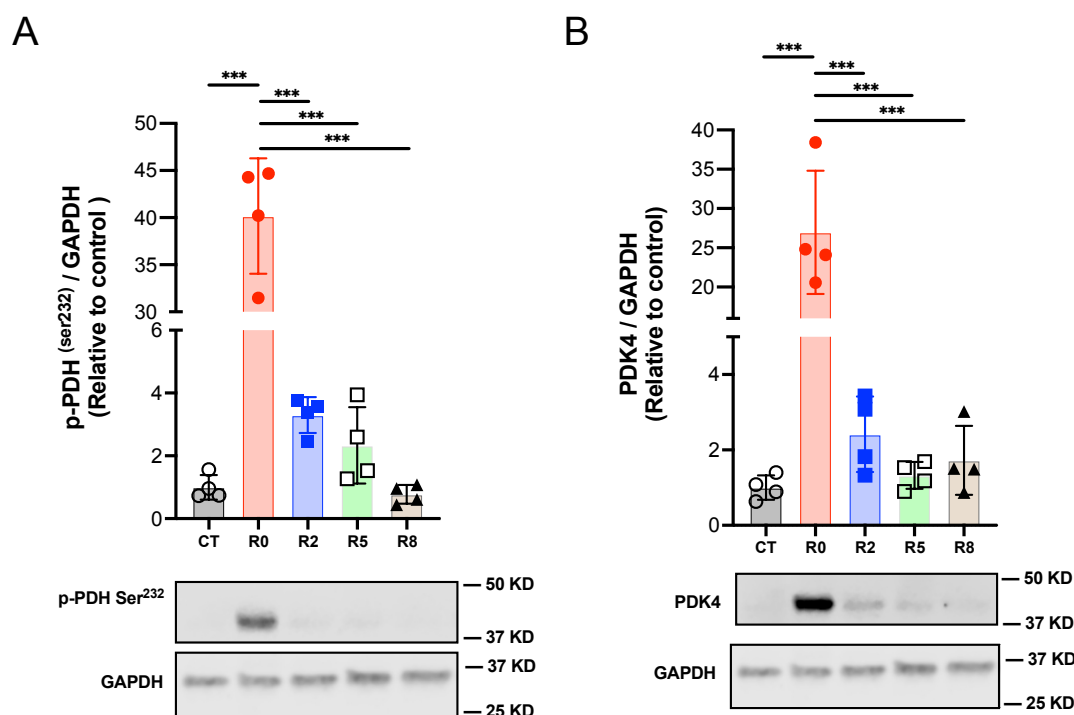
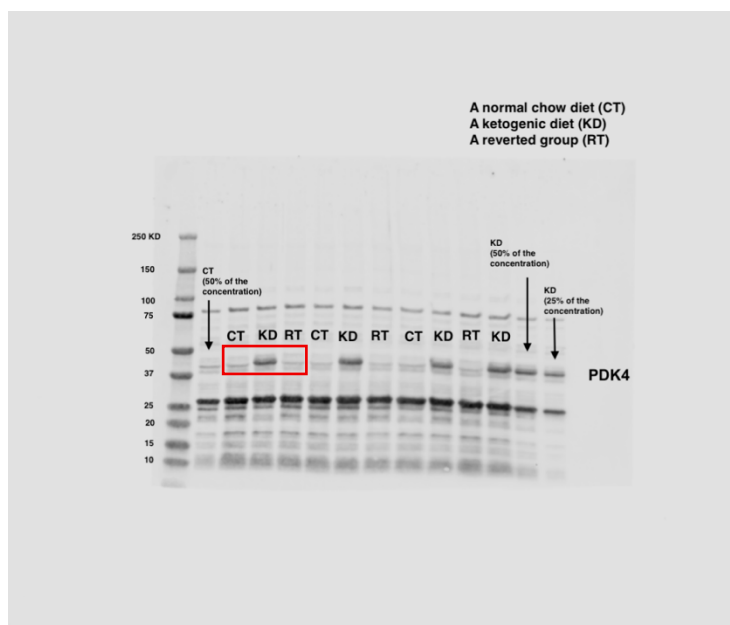
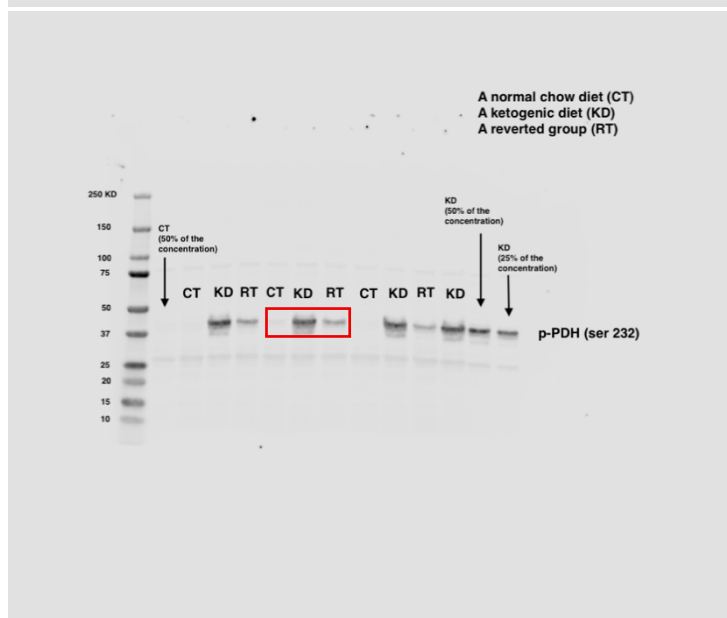


Figure S2. Recovery PDK4 and p-PDH after 2 weeks of KD. (A) PDK4 and (B) phosphorylated PDH (p-PDH) were measured *ex vivo* from the cardiac tissue of the control group (CT, $n = 4$), the ketogenic diet group (KD, 2 weeks, $n = 4$), and the KD-to-ND reverted (RT, 2 weeks of KD followed by ND) groups, measured at 2-day ($n = 4$), 5-day ($n = 4$), and 8-day ($n = 8$) from the reversion. Tukey's multiple comparison tests were used ($\alpha = 0.05$). *** indicates $P < 0.001$. PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase.

(A)



(B)



(C)

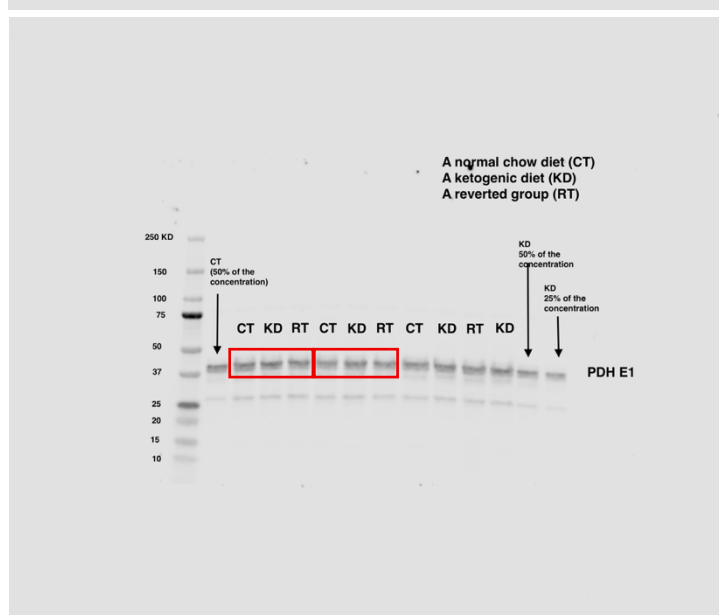
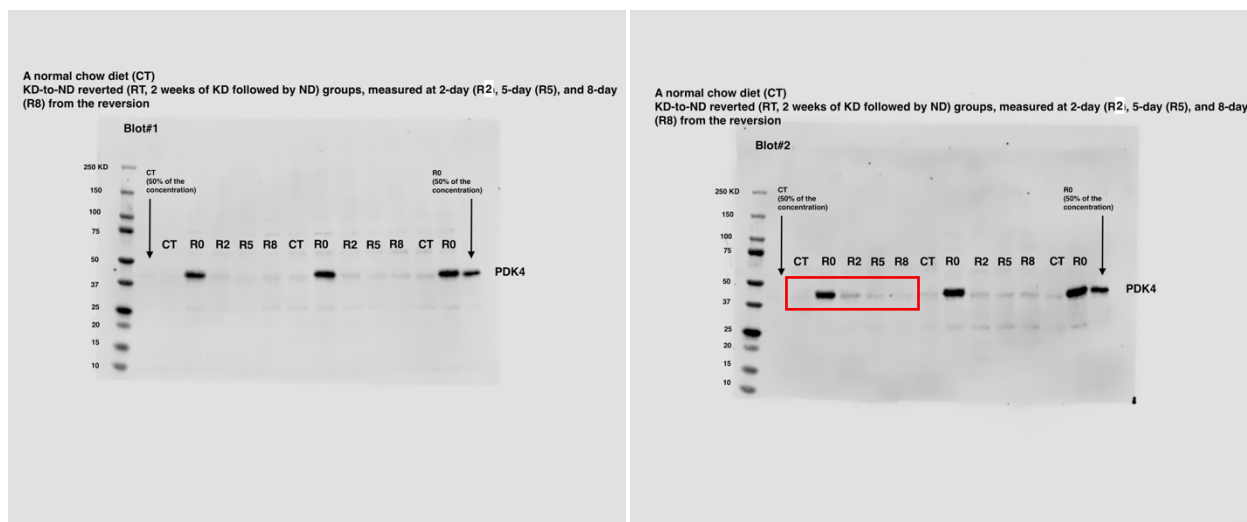
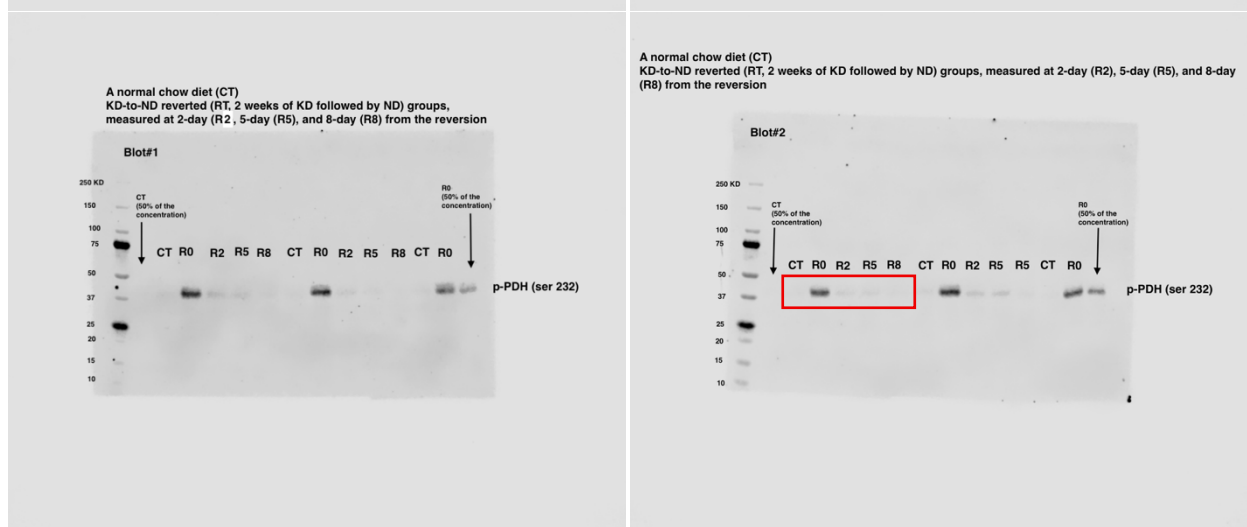


Figure S3. Complete gels of Figure 4. (A) PDK4, (B) p-PDH Ser²³², (C) PDH E1. Red boxes indicate the cropped blot images.

(A)



(B)



(C)

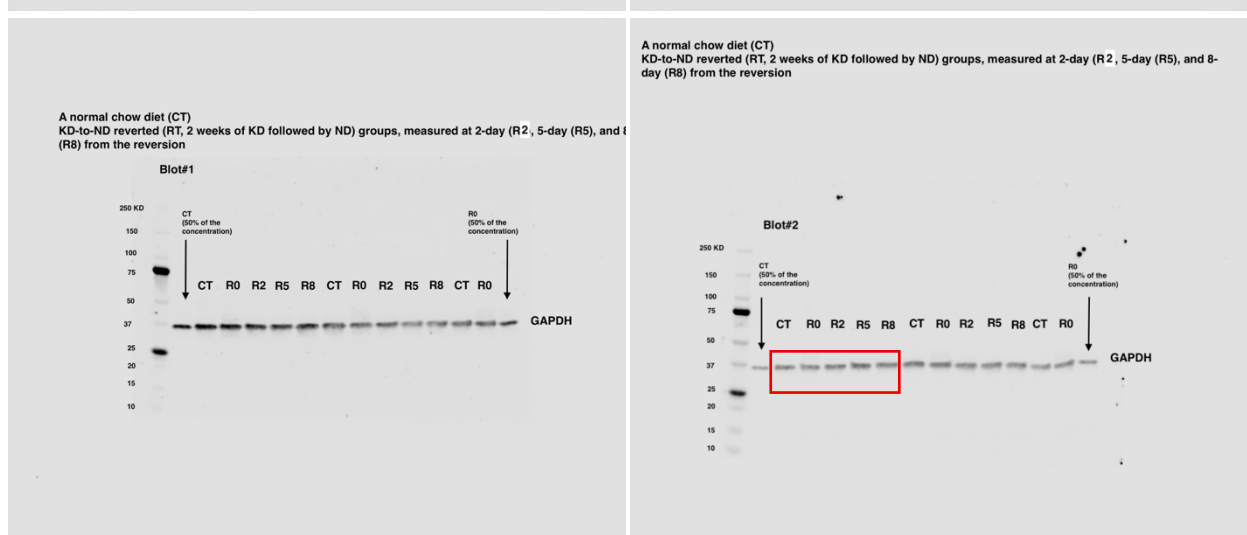


Figure S4. Complete gels of Figure S2. (A) PDK4, (B) p-PDH Ser²³², (C) GAPDH. Red boxes indicate the cropped blot images.

Supplemental Tables

Table S1. Animal usage. KD: ketogenic diet; ND: normal diet; RT: reverted diet (5 weeks of KD followed by 8 days of ND).

ID	In vivo imaging										Ex vivo		
	Baseline	ND			KD		RT				NMR	PDH analysis	
		2w	5w	5w + 8d	2w	5w	1d	2d	5d	8d			
01	×	-	-	-	×	×	×	×	×	×	RT	-	
02	×	-	-	-	×	×	×	×	×	×	RT	-	
03	×	-	-	-	×	×	×	×	×	×	RT	-	
04	×	-	-	-	×	×	×	-	×	×	RT	-	
05	-	-	-	-	-	-	-	-	-	-	KD	-	
06	-	-	-	-	-	-	-	-	-	-	KD	-	
07	-	-	-	-	-	-	-	-	-	-	KD	-	
08	-	-	-	-	-	-	-	-	-	-	KD	-	
09	-	-	-	-	-	-	-	-	-	-	ND	-	
10	-	-	-	-	-	-	-	-	-	-	ND	-	
11	-	-	-	-	-	-	-	-	-	-	ND	-	
12	-	-	-	-	-	-	-	-	-	-	-	ND	
13	×	×	×	×	-	-	-	-	-	-	ND	-	
14	×	×	×	×	-	-	-	-	-	-	-	-	
15	×	×	×	×	-	-	-	-	-	-	-	-	
16	×	×	×	×	-	-	-	-	-	-	-	-	
17	-	-	-	-	-	-	-	-	-	-	ND	-	
18	-	-	-	-	-	-	-	-	-	-	-	KD	
19	-	-	-	-	×	×	-	-	-	-	-	KD	
20	-	-	-	-	-	-	-	-	-	-	-	KD	
21	-	-	-	-	-	-	-	-	-	-	-	KD	
22	-	-	-	-	-	×	×	×	×	×	-	RT	
23	-	-	-	-	-	×	×	×	×	×	-	RT	
24	-	-	-	-	-	-	-	-	-	-	-	RT	
25	-	-	-	-	-	×	-	×	×	×	-	-	
26	×	×	×	×	-	-	-	-	-	-	-	ND	
27	-	×	×	×	-	-	-	-	-	-	-	ND	
28	-	-	×	×	-	-	-	-	-	-	-	-	
ID	Blood analysis												
	Baseline	ND						KD			RT		
		2w	3.5 w	5w	5w+ 2d	5w+ 5d	5w+ 8d	2w	3.5 w	5w	5w+ 2d	5w+ 5d	5w+ 8d
29-33	×	×	×	×	×	×	×	-	-	-	-	-	-
34-38	×	-	-	-	-	-	-	×	×	×	×	×	×

Table S2. Longitudinal HP ¹³C metabolite levels during KD and ND.

Diet	Metabolite	Baseline	2 weeks	5 weeks
KD	HCO ₃ ⁻ /TP (%)	8.56 ± 2.29	0.37 ± 0.34	0.46 ± 0.27
	Lactate/TP (%)	58.21 ± 3.95	59.56 ± 2.73	65.46 ± 5.28
	Alanine/TP (%)	33.23 ± 2.73	40.07 ± 1.78	34.08 ± 0.38
ND	HCO ₃ ⁻ /TP (%)	9.28 ± 2.31	9.10 ± 0.81	9.30 ± 1.27
	Lactate/TP (%)	61.61 ± 3.48	46.67 ± 7.84	51.04 ± 5.42
	Alanine/TP (%)	29.11 ± 3.89	44.23 ± 7.85	39.67 ± 4.58

Table S3. Longitudinal HP ¹³C metabolite levels measured when reverted from KD to ND.

Diet	Metabolite	Day 0	Day 1	Day 2	Day 5	Day 8
RT	HCO ₃ ⁻ /TP (%)	0.40 ± 0.26	2.94 ± 1.29	4.02 ± 2.16	7.51 ± 2.07	8.40 ± 1.47
	Lactate/TP (%)	64.58 ± 4.94	57.56 ± 5.41	56.61 ± 7.61	53.83 ± 6.89	53.36 ± 9.01
	Alanine/TP (%)	35.02 ± 1.28	39.50 ± 4.75	39.37 ± 7.24	38.66 ± 4.36	38.24 ± 6.59
CT	HCO ₃ ⁻ /TP (%)	9.36 ± 1.17	-	-	-	8.69 ± 2.10
	Lactate/TP (%)	50.67 ± 5.04	-	-	-	47.54 ± 9.29
	Alanine/TP (%)	39.98 ± 4.26	-	-	-	43.76 ± 8.42

Table S4. Ex vivo NMR measurements. KD: ketogenic diet; ND: normal diet; RT: reverted diet (5 weeks of KD followed by 9 days of ND).

	CT	KD	RT
[2- ¹³ C]acetyl-CoA/total acetyl-CoA	0.2757 ± 0.0947	0.0412 ± 0.0246	0.2273 ± 0.0182
[1,2- ¹³ C ₂]acetyl-CoA/total acetyl-CoA	0.3016 ± 0.0537	0.3161 ± 0.0170	0.2718 ± 0.0352
[2- ¹³ C]acetyl-CoA/[1,2- ¹³ C ₂]acetyl-CoA	0.9478 ± 0.3600	0.1291 ± 0.0717	0.8444 ± 0.1026
¹³ C-enrichment in lactate	0.1482 ± 0.0283	0.1482 ± 0.0588	0.1257 ± 0.0376
¹³ C-enrichment in alanine	0.4710 ± 0.0475	0.5497 ± 0.0436	0.4345 ± 0.0544

Table S5. Ex vivo PDH analysis and metabolite levels. KD: ketogenic diet; ND: normal diet; RT: reverted diet (5 weeks of KD followed by 9 days of ND).

	CT	KD	RT
PDH activity (mmol NADH/min/mg)	90.97 ± 5.40	1.60 ± 0.12	36.80 ± 6.07
p-PDH ser232 /E1 (relative to control)	1.00 ± 0.56	33.39 ± 0.42	11.80 ± 1.58
PDK4 / E1 (relative to control)	1.00 ± 0.12	3.48 ± 0.37	0.87 ± 0.10
CoASH (nmol/mg)	0.618 ± 0.011	0.444 ± 0.069	0.585 ± 0.044
Acetyl-CoA (nmol/mg)	0.040 ± 0.011	0.244 ± 0.056	0.124 ± 0.055
Acetyl-CoA/CoASH	0.064 ± 0.019	0.550 ± 0.117	0.212 ± 0.093

Supplementary Methods

In Vivo MR Protocol

Prior to *in vivo* imaging, each rat was anesthetized with 2 – 3 % isoflurane in 1.5 L/min oxygen, and the tail vein was cannulated with a catheter, which is connected to an extension line. Anesthetized rats were placed in a $^{13}\text{C}/^1\text{H}$ dual-tuned birdcage RF coil (inner diameter = 80 mm) in the MRI scanner. The respiration and the body temperature were monitored throughout the experiment. An integrated $^1\text{H}/^{13}\text{C}$ MRI protocol (~20 minutes) was used. Following a three-plane localizer scan, spatial B_0 inhomogeneity around the heart was minimized using ^1H point-resolved spectroscopy (PRESS) sequence by adjusting the shim currents. For ^{13}C MRS, an axial slice (slice thickness = 10 mm) that included majority of the heart was prescribed, then was tilted to an oblique plane, if needed, to exclude other major organs such as liver. Acquisition of ^{13}C MRS (spectral width = 5,000 Hz, #spectral points = 2,048) started with a bolus injection of 120-mM HP [$1\text{-}^{13}\text{C}$]pyruvate (1.5 mmol/kg body weight, injection rate = 0.25 mL/s, up to 4 mL) via the tail vein catheter and repeated every 3 seconds for 4 minutes. A 10° slice-selective RF pulse was used and the center frequency was set to [$1\text{-}^{13}\text{C}$]pyruvate by calculating from the water frequency of the ^1H PRESS.¹

Tissue Preparation for NMR Isotopomer Analysis

For the NMR metabolomic analysis, frozen heart tissue (~0.5 g) was extracted with 4 times sample size 5% perchloric acid and then lyophilized at -84°C using a FreeZone™ benchtop freeze dryer (Labconco Corp. Kansas City, MO, USA). Each sample was resuspended in 300 mL of D_2O solution that contained 1-mM DSS and 1-mM ethylenediaminetetraacetate (EDTA). The ^{13}C NMR spectra were acquired using a 14.1-T Avance III HD NMR spectrometer (Bruker, Billerica, MA, USA), equipped with a 10-mm cryoprobe (Bruker; spectral width = 36,057 Hz, acquisition time = 2 s, D1 = 6 s, #repetition = 8,000).

Western Blot Analysis

Anti-PDH-E1 α (ab168379) and anti-phospho-PDH-E1 α (pSer²³² AP1063) were purchased from Abcam and EMD Millipore, respectively. Rabbit polyclonal antisera to PDK4 was produced by Biosynthesis, Inc. to a mixture of following peptide sequences: CIPSREPKNLAKEKLA, DLVEFHEKSPEDQKALSE, and EFVDTLVKVRNRHHNVVPT linked to KLH. Specificity of antibody binding was validated by Western blot detection of protein at the appropriate molecular weight (Precision Plus Dual Color Standards, Bio-Rad, 1610374) and the requirement of primary antibody for secondary antibody binding. For analysis of mitochondrial proteins, mitochondria prepared in isolation buffer containing 20 mM NaF and 1 mM dichloroacetate were suspended in Laemmli sample buffer (Bio-Rad, 1610747) containing protease inhibitor cocktail (Sigma-Aldrich, P2714). Proteins (approximately 6 – 12 $\mu\text{g}/\text{lane}$, within the linear range) were resolved using 4 – 20% SDS-PAGE gradient gels (Bio-Rad, 4568096) and transferred to nitrocellulose membranes (0.2 μm). Membranes were incubated with primary antibodies at optimized dilution, washed, then incubated with fluorescent secondary antibodies (LI-COR, 925-32211). Proteins of interest were visualized and quantified using an Odyssey scanner and Image Studio Lite software (LI-COR). To reevaluate Western blots using a different antibody, membranes were stripped using NewBlot Nitro stripping buffer (LI-COR, 928-40030), checked for complete absence of prior signal, and then reprobed and reprocessed with appropriate primary and secondary antibodies.

CoASH Analysis by HPLC-UV/Vis

CoASH was extracted from snap frozen cardiac tissue crushed over liquid nitrogen. Approximately 30 – 50 mg of crushed frozen tissue was suspended in ice cold 0.35-M perchloric acid, 10-mM DTT by sonication. After 10 min on ice, proteins were pelleted by centrifugation at $16,000 \times g$ and supernatants were filtered (0.45 μm PVDF). Protein pellets were resuspended in 150-mM KOH and protein content determined using the BCA assay. CoASH in the supernatants (100 μL , equivalent to approximately 0.3-0.5 mg protein) was resolved by ion-pair reverse phase HPLC (Waters XBridge C18 column, $150 \times 4.5 \text{ mm}^2$, 5 μm) and detected by UV/Vis absorbance at 254 nm (Shimadzu LC-20AD HPLC system equipped with SIL-20A autosampler and photodiode array M20A UV/Vis detector). The mobile phase consisted of acetonitrile vs. 100 mM KH_2PO_4 , 1 mM tetrabutylammonium sulfate, pH 6 using the following elution method with a flow rate of 1 mL/min: 0-10 min, 0-15% acetonitrile; 10-15 min, 15% acetonitrile; 15-17 min, 15-20% acetonitrile; 17-25 min, 20% acetonitrile;

25-30 min, 0% acetonitrile. CoASH levels were quantified using a standard curve constructed with known concentrations of CoASH (Sigma-Aldrich). Metabolite content is expressed as nmol/mg protein.