NAD^+ Redox Imbalance in the Heart Exacerbates Diabetic Cardiomyopathy

Ying Ann Chiao, PhD[1], Akash Deep Chakraborty, PhD[1,2], Christine M. Light, BS[2], Rong Tian, MD PhD[3], Junichi Sadoshima, MD PhD[4], Xiaojian Shi, PhD[5], Haiwei Gu PhD[5], Chi Fung Lee PhD[2,6*]

[1]Aging and Metabolism Research Program, [2]Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK

[3]Mitochondria and Metabolism Center, University of Washington, Seattle, WA

[4]Department of Cell Biology and Molecular Medicine, Rutgers New Jersey Medical School, Newark, NJ

[5]Arizona Metabolomics Laboratory, College of Health Solutions, Arizona State University, Scottsdale, AZ

[6]Department of Physiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

*** Address correspondence to:**

Chi Fung Lee, PhD
Cardiovascular Biology Research Program, MS 45
Oklahoma Medical Research Foundation
825 NE 13th Street
Oklahoma City, OK 73104, USA
Tel: 405-271-1703
Fax: (405) 271-7417
chifung-lee@omrf.org
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Supplemental methods

Animal care and experiments

All animal care and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Oklahoma Medical Research Foundation and the University of Washington. All procedures were performed in accordance with IACUC regulations. Cardiac-specific Ndufs4 (cKO) mice were generated from the breeding of $Ndufs4^{flox/flox}$ mice (control) with alpha-MHC-Cre expressing mice. Expression of NAMPT in the heart was achieved by crossing a cardiac-specific NAMPT mouse line with cKO and control mice. Three to four-month-old littermate mice were used at the beginning of this study. STZ was used to induce beta-cell and insulin depletion, and mice receiving STZ or vehicle were randomly chosen in different cages. STZ in citrate buffer was given to mice intraperitoneally at 50 mg/kg for five days in chronic diabetic experiments. One STZ administration at 50 mg/kg was given in acute STZ experiment. Body weights were measured before, 8 weeks or 15 weeks after STZ injection in different experiments. Blood glucose levels were measured to confirm diabetic phenotypes. STZ-treated mice without hyperglycemia were to be excluded. All diabetic mice reported showed the expected hyperglycemia and no exclusion was needed.

Systolic function and cardiac geometry were assessed by echocardiography with parasternal long axis view and M-mode images were recorded using VEVO 2100 system (VisualSonics) on lightly anesthetized mice. Parameters such as fractional shortening (FS), and left ventricular internal diameter at diastole (LVID;d) were measured, blindly analyzed and calculated by using the software package in the VEVO 2100 system when the heart rate was within 500-600 beats per minute. Using the same system, parameters of diastolic function were measured. E'/A' ratio was measured by tissue doppler imaging

of mitral annulus, e velocity measured by transmitral pulse-wave doppler, and e/E' ratio was also calculated. Myocardial performance index (MPI) was calculated as the sum of isovolumetric contraction time (IVCT) and isovolumetric relaxation time (IVRT) divided by ejection time (ET). These parameters were measured and calculated by averaging three cardiac cycles.

Tissue harvest and processing

On the day of tissue harvest, mice were subjected to fasting for 6 hours and anesthetized using pentobarbital. Rib cages were cut open to expose hearts for blood collection by cardiac puncture. Heart weight, wet and dry lung weights, and tibia length were measured post-mortem. Cardiac tissue samples were collected and snap-frozen with liquid nitrogen. Frozen tissues were pulverized using a Tissuelyzer II (Qiagen) for biochemical assays.

NAD^+ assay and AMPK activity assay

10-15 mg of pulverized cardiac tissue were used for measurements of NAD^+ and NADH levels (BioAssay) and AMPK activity (MBL life science) using commercially available kits under the manufacturer's instructions.

Analysis of mRNA levels

Total RNA was extracted from pulverized cardiac tissues using RNeasy fibrous tissue mini kit (Qiagen). RNA concentrations were quantified by nanodrop. To assess expression of genes related to fibrosis, cDNA samples were synthesized, and quantitative PCR reactions were performed using RT2 Profiler PCR Array for mouse extracellular matrix and adhesion molecules genes (Qiagen, Cat. No. 330231 ID: PAMM-013Z) according to manufacturer's instructions.

Western blotting

Pulverized cardiac tissues were homogenized in RIPA buffer (Sigma) with protease and phosphatase inhibitor cocktail (Halt, ThermoFisher) and deacetylase inhibitors (10 mM nicotinamide, 10 uM trichostatin A). Protein concentrations of samples were determined by BCA assay (ThermoFisher), and equal amounts of protein (20 ug per sample) were loaded for SDS-PAGE using the Criterion system. Proteins were transferred onto PVDF membrane using Criterion Blotter (Biorad). Blots were blocked in 5% BSA-TBST. Primary antibodies were diluted using 5% BSA-TBST. Antibodies from the following companies were used for Western blot analysis: acetyl-lysine (1:1000, 9441-Cell signaling), SDHA (1:10000, ab14715-Abcam), SOD2 (1:3000, PA5-30604-Thermo Fisher) SOD2-K68Ac (1:10000, ab137037-Abcam), TnI-S150Pi (1:1000, PAS-35410-Thermo), TnI-S23/24Pi (1:1000, 4004S-Cell Signaling), TnI (1:1000, 4002S-Cell Signaling), MyBPC-S282Pi (1:2000, ALX-215-057-R050), MyBPC (1:1000, SC-137237, Santa Cruz), AMPKa (1:1000, 2532S-Cell Signaling), AMPKa-T172Pi (1:1000, 2535S-Cell Signaling) and AMPK-S485Pi (1:1000, 4185S-Cell Signaling). Protein bands were visualized with chemiluminescence assay (Pierce) with secondary antibodies coupled with HRP using the G-Box imaging system. The protein abundance was analyzed by densitometry with ImageJ.

Sample analyses by LC-MS/MS

Mouse blood was collected using cardiac puncture at tissue harvest. Blood samples were incubated in EDTA collection tube (BD Diagnostics). After thorough mixing, blood samples were centrifuged at 300 g for five minutes at room temperature. Plasma samples were collected as supernatant and snap-frozen by liquid nitrogen. Glucose levels

in plasma were measured by test strips (AlphaTRAK 2 blood glucose tests). Plasma insulin levels were measured by a commercially available kit (Crystalchem).

In metabolite analysis, acetonitrile (ACN), methanol (MeOH), ammonium acetate, and acetic acid, all LC-MS grade, were obtained from Fisher. Ammonium hydroxide was bought from Sigma-Aldrich. PBS was bought from GE Healthcare. The standard compounds were purchased from Sigma-Aldrich and Fisher.

Frozen plasma samples were thawed overnight under 4^{o}C. 500 µL of MeOH and 50 µL of internal standard solution were added to each sample (50 µL) for protein precipitation and metabolite extraction (containing 1,810.5 µM $^{13}C_3$-lactate and 142 µM $^{13}C_5$-glutamic acid). The mixture was vortexed and stored at -20^{o}C, followed by centrifugation at 14,000 RPM. The supernatants were collected and dried using a CentriVap Concentrator (Labconco). The dried samples were reconstituted with 40% PBS/60% ACN. A pooled sample was used as the quality-control (QC) sample.

The targeted LC-MS/MS method was used in a growing number of studies. Briefly, all LC-MS/MS experiments were performed on an Agilent 1290 UPLC-6490 QQQ-MS system. Each sample was injected twice for analysis using negative and positive ionization mode. Both chromatographic separations were performed in hydrophilic interaction chromatography (HILIC) mode on a Waters XBridge BEH Amide column (Waters). The mobile phase was composed of Solvents A (10 mM ammonium acetate, 10 mM ammonium hydroxide in 95% H_2O/5% ACN) and B (10 mM ammonium acetate, 10 mM ammonium hydroxide in 95% ACN/5% H_2O). After the initial 1 min isocratic elution of 90% B, the percentage of Solvent B decreased to 40% at t=11 min. The composition

of Solvent B maintained at 40% for 4 min (t=15 min), and then the percentage of B gradually went back to 90%, to prepare for the next injection.

The mass spectrometer is equipped with an electrospray ionization (ESI) source. Targeted data acquisition was performed in multiple-reaction-monitoring (MRM) mode. The extracted MRM peaks were integrated using Agilent MassHunter Quantitative Data Analysis.

Histology

One to two-millimeter thick heart tissues were cut cross-sectionally at the mid-section and fixed with 4% paraformaldehyde for 24 hours. The tissues were changed to 70% ethanol and fixed tissues were processed for paraffin embedding and sectioning. Tissue sections were stained with trichrome for collagen analysis. The trichrome stained slides were scanned with an Aperio Scanscope AT2 (Leica) at 40x. Quantitative image analysis of whole stained sections was blindly performed using Aperio Brightfield Image Analysis. Toolbox software and a color deconvolution algorithm was used to measure RGB color vectors of blue (positive trichrome stain) and total stained tissue area. The quantitative analysis of cardiac fibrosis for the percentages of collagen (trichrome-blue stain component) and total stained tissue area (mm^2) was performed. Color images of H&E stained sections were selected at 10X zoom mode using ImageScope and were exported as an image file. Cardiomyocyte sizes were measured using ImageJ: a masking algorithm was utilized to select cells with clear boundaries. Cross-section fields of cardiomyocytes were chosen and tangential cardiomyocytes were avoided. Further, thresholding of the masked image was used to reduce saturation and set the background level. The threshold was kept similar across all images analyzed. To enhance the cell

selection procedure, particle size threshold was set to select cells within a range to exclude bad cells/debris. This helped eliminate cells sectioned tangentially.

Statistical analysis

For comparisons involving two groups, unpaired 2-tailed t-tests were used. For analysis of sex-dependent changes, two-way ANOVA was used. All analyses were performed using GraphPad Prism 8.0. All data are expressed as mean ± SEM, and a $p<0.05$ was considered significant. Metabolite levels were statistically analyzed by MetaboAnalyst 4.0. Adjusted P-value (FDR) cutoff for the dataset was set at 0.05. Raw data are available as supplementary materials. Heatmaps were generated by Morpheus.

Supplemental Table I. Baseline cardiac function of control or cKO mice.

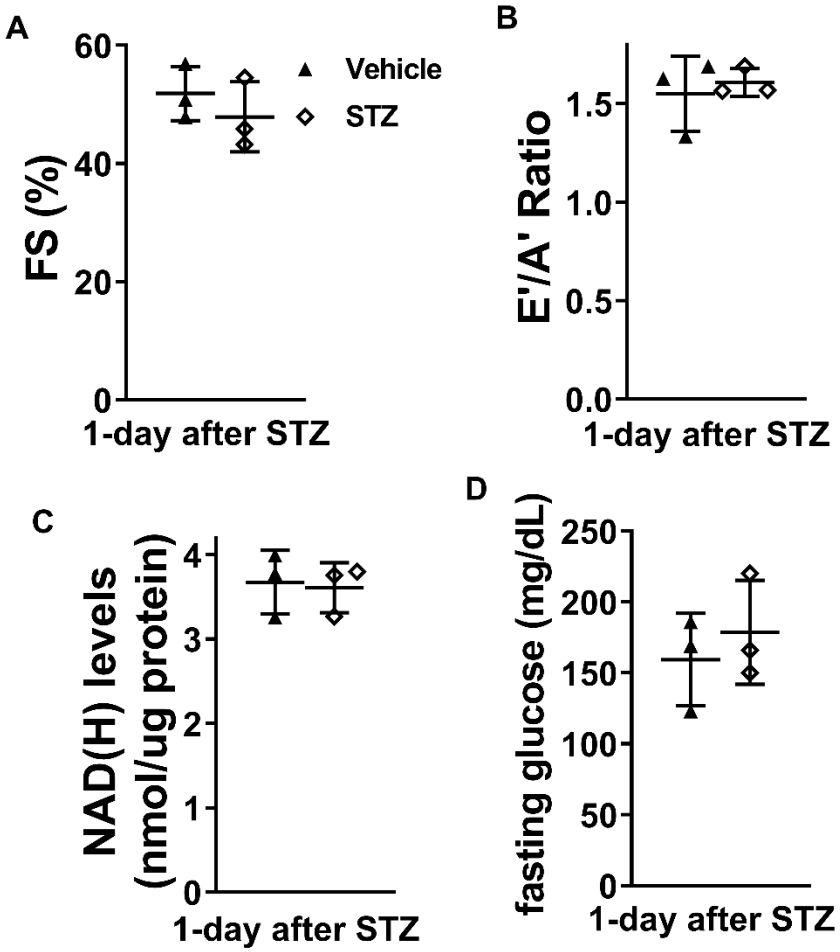
Baseline cardiac function	Male-control	Female-control	cKO
FS	49.7 +/- 2.0	51.1 +/- 2.1	49.9+/- 2.7
E'/A'	1.41 +/- 0.03	1.39 +/- 0.05	1.35+/- 0.04

Supplemental Table II. Cardiac function of male and female, diabetic control and diabetic cKO mice.

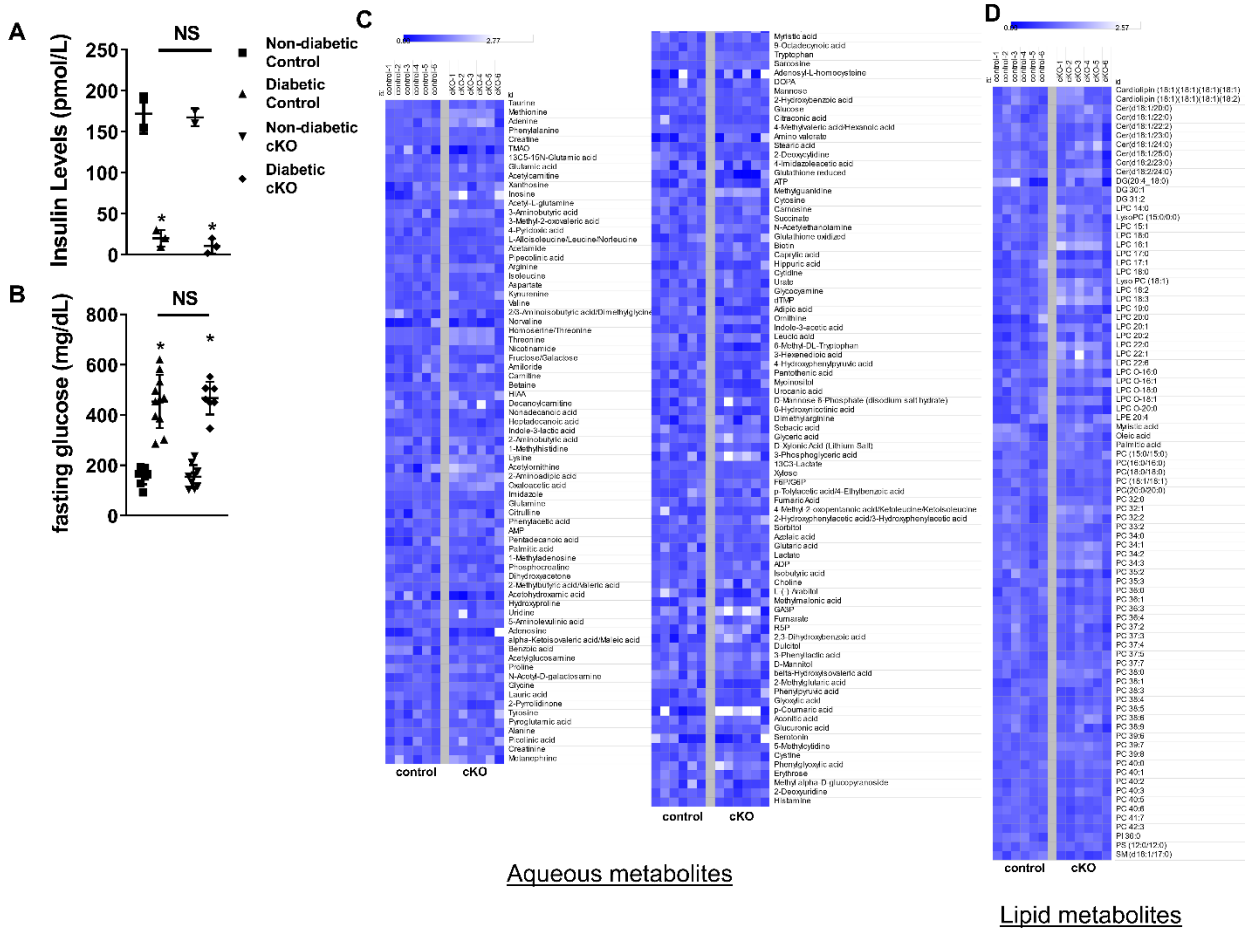
8-week diabetic stress	Male		Female	
	Diabetic Control	Diabetic cKO	Diabetic Control	Diabetic cKO
FS	36.2 +/- 1.4	24.4 +/- 2.7*	39.1 +/- 3.7	24.5 +/- 2.7*
E'/A'	1.17 +/- 0.05	0.98 +/- 0.08*	1.17 +/- 0.04	0.91 +/- 0.09*
e/E'	24.5 +/- 2.8	40.6 +/- 2.9*	22.7 +/- 2.8	31.5 +/- 3.7*
IVRT	14.1 +/- 1.5	22.6 +/- 1.9*	19.9 +/- 2.1	23.1 +/- 1.4
MPI	0.71 +/- 0.05	0.98 +/- 0.09*	0.76 +/- 0.04	0.79 +/- 0.03
LVID;d	3.83 +/- 0.07	3.94 +/- 0.11	3.13 +/- 0.14#	3.40 +/- 0.19#

*: P<0.05 vs diabetic cKO

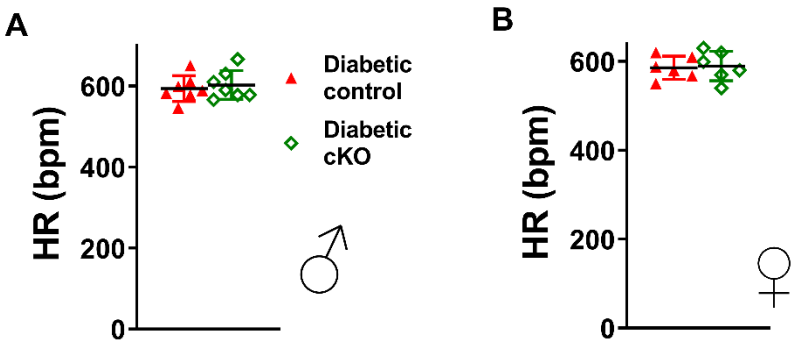
#: P<0.05 vs male counterpart



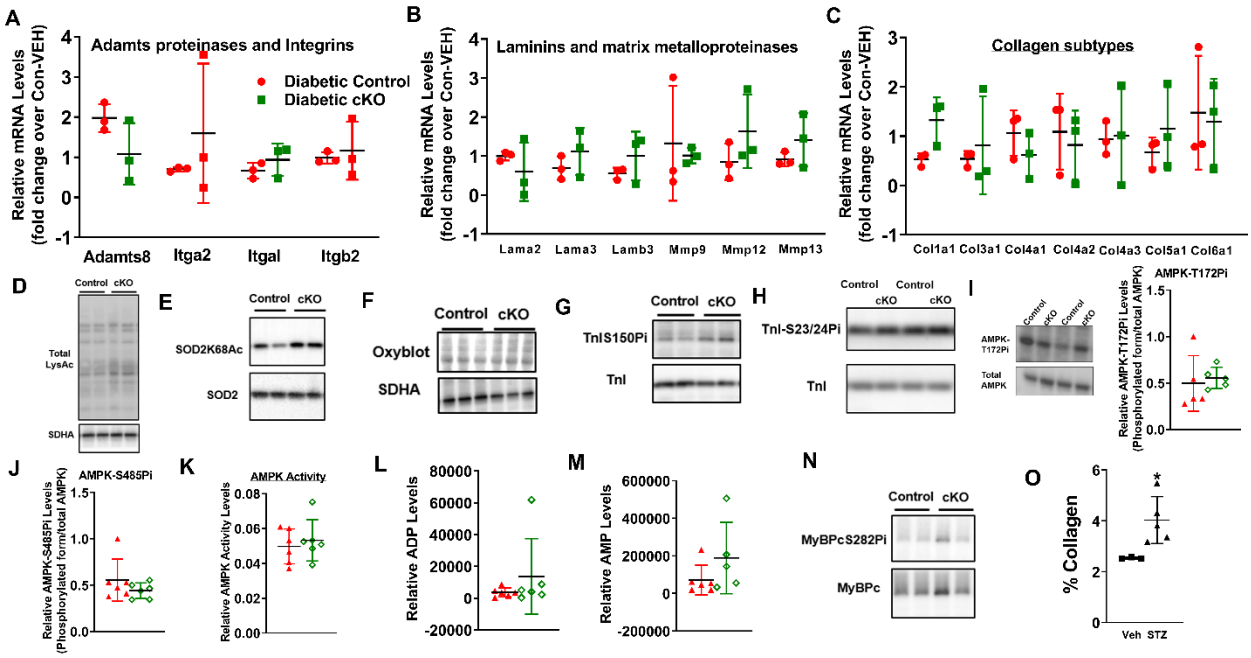
Supplemental Figure I. (A) FS, **(B)** E'/A' ratio and **(C)** NAD(H) pool and **(D)** fasting glucose levels were measured in mice 1-day after STZ injection.



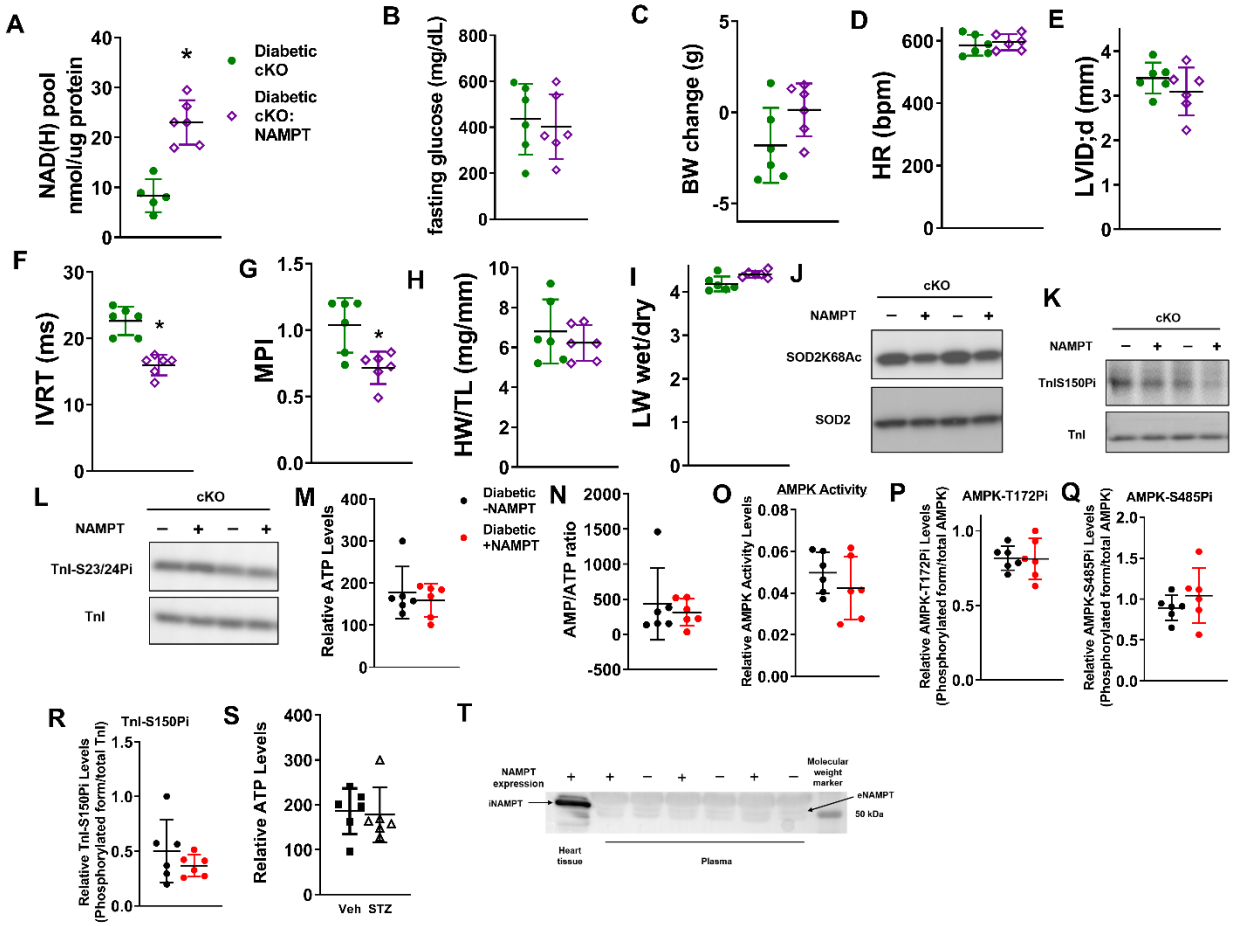
Supplemental Figure II. (A) Plasma insulin levels and **(B)** fasting glucose levels of male mice with indicated treatment were measured. Heatmaps of **(C)** aqueous and **(D)** lipid metabolite levels in plasma of diabetic control and diabetic cKO mice. Heatmaps were generated using Morpheus. Raw data can be found in supplementary Table 2. N=6 male mice. *: P<0.05 to corresponding non-diabetic group.



Supplemental Figure III. Heart rates (HR) of **(A)** male, **(B)** female cohorts of diabetic control and diabetic cKO mice. N=6-7.



Supplemental Figure IV. Additional gene expression analysis of **(A)** Adamts proteinases and integrins, **(B)** laminins and MMPs, and **(C)** collagen subtypes in diabetic control and diabetic cKO hearts were performed. N=3 male mice. **(D-H)** Representative Western blots for results shown in Figure 5. Phosphorylation levels of **(I)** AMPK-T172 and **(J)** AMPK-S485 levels were measured. **(K)** AMPK activity of indicated hearts were measured. **(L)** ADP and **(M)** AMP levels were quantified in control diabetic and cKO diabetic hearts. **(N)** Representative blot of MyBPc-S282 protein levels. **(O)** Collagen levels in non-diabetic and STZ-induced diabetic hearts. *: P<0.05 to vehicle-treated mice.



Supplemental Figure V. (A) NAD^+ pool of diabetic cKO and diabetic cKO:NAMPT mice 8 weeks after STZ treatment were measured. N=5 male mice. **(B)** Fasting glucose levels, **(C)** body weight (BW) change, **(D)** heart rate (HR) at the time acquiring echocardiography data, **(E)** LV dilation, **(F)** IVRT, **(G)** MPI, **(H)** hypertrophy and **(I)** lung edema of indicated mice were measured at 8-week endpoint. **(J-L)** Representative Western blots for results shown in Figure 6. **(M)** ATP levels and **(N)** AMP/ATP ratio of indicated hearts were measured. **(O)** AMPK activity, **(P)** phosphorylation of AMPK-T172, **(Q)** AMPK-S485 and **(R)** TnI-S150, were measured. **(S)** ATP levels of vehicle or STZ-treated diabetic control mice were measured. **(T)** Levels of plasma NAMPT protein were measured by Western blots. *: P<0.05 to diabetic cKO mice.