Supplemental Materials

Nicotinamide phosphoribosyltransferase potentiates antioxidant defense in diastolic heart failure associated with obesity.

Running title: Nampt attenuates oxidative stress via NADPH production.

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Expanded Materials & Methods

The author declare that all supporting data are available within the article. In addition, any raw data that support the findings of this study are available from the corresponding author upon reasonable request.

Animal experiments

Nampt transgenic mice (Tg-Nampt) were generated using the α-myosin heavy chain promoter to achieve cardiac-specific expression of Nampt on an FVB background [15]. Non-transgenic mice (NTg) were used as control mice. Systemic Nampt heterozygous knockout mice with a C57BL/6 genetic background were provided by Dr. Yamanaka (Kyoto University, Japan). Both male and female mice were used. Age- and gender-matched littermates were randomly assigned to either normal diet (ND) or HFD feeding group. Echocardiographic measurements, PV loop and histological analyses were performed in blind fashion. Measurement of Number of animal used are shown in figure legends. No animals were excluded from analyses. All procedures involving animals were performed in accordance with protocols approved by Rutgers Biomedical and Health Sciences.

Echocardiography

Echocardiography was conducted as described [36]. Mice were anesthetized using 12 μL/g body weight of 2.5% Avertin (Sigma-Aldrich), and echocardiography was performed using ultrasonography (Acuson Sequoia C256; Siemens Medical Solutions). A 13-MHz linear ultrasound transducer was used. The mouse chest was shaved. Electrocardiographic leads were attached to each limb using needle electrodes. Two-dimensional images and M-mode tracings (sweep speed = 100 - 200 mm/s) were recorded from the parasternal short-axis view at the mid-papillary muscle level. Care was taken not to apply too much pressure to the chest wall.

The images were recorded and analyzed.

Pressure-volume (PV) loop analysis

PV loop analysis was performed using the Millar PV system MPVS300/400. Mice were anesthetized using 0.06 mg/g body weight of pentobarbital or 12 µL/g body weight of 2.5% Avertin. After the chest opening, the PV probe was inserted into the left ventricle. The left ventricular pressure and volume were measured.

Glucose tolerance test

In order to perform an intraperitoneal glucose tolerance test (IGTT), mice were fasted for five hours and body weight was measured. Glucose (2 g/Kg body weight) was injected intraperitoneally and blood was collected by retro-orbital bleeding (50 µL) using heparinized microhematocrit tubes. The blood glucose level was measured at 0, 30, 60 and 120 mins after glucose loading with a glucometer (ACCU-CHECK Compact Plus, Roche).

Lipidomics analysis

Chemicals: LC-MS-grade solvents and mobile phase modifiers were obtained from Honeywell Burdick & Jackson (Morristown, NJ) (acetonitrile, isopropanol, formic acid), Fisher Scientific (Waltham, MA) (methyl *tert*-butyl ether (MTBE)) and Sigma–Aldrich/Fluka (St. Louis, MO) (ammonium formate, ammonium bicarbonate). Lipid Standards: Mouse SPLASH LIPIDOMIX and Deuterated Ceramide LIPIDOMIX were obtained from Avanti Polar Lipids (Alabaster, AL) and labeled carnitine standards (NSK-B and NSK-B-G) were obtained from Cambridge Isotope Laboratories (Cambridge, MA). Sample Preparation: Extraction of lipids was carried out using a biphasic solvent system of cold methanol, MTBE, and water [37] with some modifications. In a randomized sequence, tissue lipids were extracted in bead-mill tubes (ceramic 1.4 mm, Mo-Bio, Qiagen, Germantown, MD) containing a solution of 225 µL MeOH, 750 µL MTBE, and internal

standards (Avanti Mouse SPLASH LIPIDOMIX, Deuterated Ceramide LIPIDOMIX, and labeled carnitine standards (NSK-B and NSK-B-G), all at 10 µL per sample). Samples were homogenized in one 30 sec cycle and then rested on ice for 1 hr with occasional vortexing. Then, 188 µL of PBS was added, followed by a brief vortex. Samples were then centrifuged at 14,000 x *g* for 10 minutes at 4°C, and the upper phases were collected. Another aliquot of 750 µL MTBE was added to the bottom aqueous layer, followed by a brief vortex. Samples were then centrifuged again at 14,000 x *g* for 10 minutes at 4°C, and the upper phases were collected. The collected upper phases were combined and evaporated to dryness in a Speedvac. Lipid extracts were reconstituted in 250 µL of mobile phase B and transferred to an LC-MS vial for analysis. Concurrently, a process blank sample was prepared and then a pooled quality control (QC) sample was prepared by taking equal volumes (~50 µL) from each sample after final resuspension. LC-MS Analysis: Lipid extracts were separated on an Acquity UPLC CSH C18 1.7 µm 2.1 x 100 mm column maintained at 65°C and connected to an Agilent HiP 1290 Sampler, Agilent 1290 Infinity pump, and Agilent 6490 triple quadrupole (QqQ) mass spectrometer. Sphingolipids were detected using dynamic multiple reaction monitoring (dMRM) in positive ion mode. Source gas temperature was set to 150°C, with a gas (N_2) flow of 17 L/min and a nebulizer pressure of 20 psi. Sheath gas temperature was 200°C, sheath gas (N_2) flow was 10 L/min, capillary voltage was 3500 V, nozzle voltage was 500 V, high pressure RF was 190 V and low pressure RF was 120 V. Injection volume was 10 µL and the samples were analyzed in a randomized order with the pooled QC sample injected eight times throughout the sample queue. Mobile phase A consisted of ACN:H_2O (60:40 *v/v*) in 10 mmol/L ammonium formate and 0.1% formic acid, and mobile phase B consisted of IPA:ACN:H_2O (90:9:1 *v/v/v*) in 10 mmol/L ammonium formate and 0.1% formic acid. The chromatography gradient started at 15% mobile phase B, increased to 30% B over 0.7 min, increased to 60% B from 0.7-1.4 min, increased to 80% B from 1.4-7.0 min, and increased to 99% B from 7.0-7.14 min, where it was held until 10.2 min and then returned to starting conditions at 10.3 min. Post-time was 4 min and

the flowrate was 0.4 mL/min throughout. Collision energies and cell accelerator voltages were optimized using sphingolipid standards with dMRM transitions of $[M+H]^+ \rightarrow [m/z = 284.3]$ for dihydroceramides, $[M-H_2O+H]^+ \rightarrow [m/z = 264.2]$ for ceramides, and $[M-H_2O+H]^+ \rightarrow [m/z = 271.3]$ for isotope labeled ceramides. All carnitines were monitored with dMRM transitions of $[M+H]^+ \rightarrow [m/z = 85.1]$. Sphingomyelins and phosphatidylcholines were monitored with dMRM transitions of $[M+H]^+ \rightarrow [m/z = 184.1]$. Glycerolipids were monitored with dMRM transitions of $[M+NH_4]^+ \rightarrow$[m/z = neutral loss of acyl chain]. Lipids without available standards were identified based on HR-LC/MS, quasi-molecular ions and characteristic product ions. Their retention times were either taken from HR-LC/MS data or inferred from the available sphingolipid standards. LC-MS Data Processing: For data processing, Agilent MassHunter (MH) Workstation and software packages MH Qualitative and MH Quantitative were used. The pooled QC (n=8) and process blank (n=4) were injected throughout the sample queue to ensure the reliability of acquired lipidomics data. Data exported from MH Quantitative was evaluated using Excel, where initial lipid targets were parsed based on the following criteria. Only lipids with relative standard deviations (RSD) less than 30% in QC samples were used for data analysis. Additionally, only lipids with background AUC counts in process blanks that were less than 30% of QC were used for data analysis. The parsed Excel data tables were normalized based on the ratio to class-specific internal standards, then to tissue mass prior to statistical analysis. Statistical Analysis and Data Visualization: Multivariate analysis was performed using MetaboAnalyst [38]. Statistical models were created for the normalized data after logarithmic transformation (base 10) and Pareto scaling. The initial pass for the volcano plot used a fold change (FC) cut-off of 1.5 and raw p-value cut-off of 0.05.

Histological analyses

Heart specimens were fixed with 10% formalin, embedded in paraffin, and sectioned at 6-μm thickness. The sections were incubated in 3% H_2O_2 in PBS to prevent endogenous peroxidation

and blocked with 5% BSA in PBS. Cardiomyocyte (CM) cross-sectional area was measured with ImageJ software (http://rsbweb.nih.gov/ij/) from images captured of sections stained with wheat germ agglutinin (WGA) [39]. The outlines of 200 CMs were traced in each section. Sections were stained with Masson's Trichrome to evaluate interstitial fibrosis according to the manufacturer's protocol (K037, Poly Scientific R & D, Bay Shore, NY). Fibrotic areas within sections were measured by visualizing blue-stained areas, exclusive of staining that colocalized with perivascular or intramural vascular structures, the endocardium, or LV trabeculae. Using ImageJ software (http://rsbweb.nih.gov/ij/), blue-stained areas and non-stained myocyte areas from each section were determined using color-based thresholding. The total percentage of the area of fibrosis was calculated as the sum of blue-stained areas divided by total ventricular area. Apoptosis was evaluated *in situ* using TUNEL. Briefly, deparaffinized LV sections were incubated with Proteinase K and fragmented DNA in the nucleus was labeled with fluorescein-conjugated dUTP using TdT (Roche Molecular Biochemicals). The presence of TUNEL signal in the nucleus was determined by co-localization with DAPI-staining. n = 10 - 15 fields for each section using the 40 X magnification objective, and the number of TUNEL-positive nuclei was determined by examining the entire section using the same power objective.

NAD^+ and NADH measurements

NAD^+ and NADH were measured using the EnzyChrom™ NAD^+/NADH Assay Kit according to the manufacturer's protocol (ECND-100, Bioassay Systems, Hayward, CA). The ratio of NAD^+/NADH was calculated by dividing the value of NAD^+ by NADH.

$NADP^+$ and NADPH measurements

$NADP^+$ and NADPH were measured using the EnzyChrom™ $NADP^+$/NADPH Assay Kit according to the manufacturer's protocol (ECND-100, Bioassay Systems, Hayward, CA).

Mitochondrial oxygen consumption assay to determine fatty acid oxidation (FAO) activity

A Seahorse XF96 extracellular flux analyzer was employed to evaluate FAO via measurement of oxygen consumption rate (OCR). OCR was measured in the presence and absence of Etomoxir, an inhibitor of carnitine palmitoyltransferase-1 (CPT-1), which is essential for FAO and is located at the inner face of the outer mitochondrial membrane. Cardiomyocytes ($1x10^5$ per well) were plated on an XF96 plate. After 24 hours, the cells were transduced with adenoviral particles with a multiplicity of infection of 10 (MOI 10) for shNampt and 25 for Nampt. After 3 days of adenovirus transduction, the growth medium was replaced with substrate limited medium- DMEM (Sigma, D-5030) supplemented with 0.5 mmol/L glucose, 1 mmol/L glutamine, 0.5 mmol/L carnitine and 1% FBS, and the cells were maintained overnight at 37°C as per the manufacturer's FAO assay protocol (Seahorse, Agilent). On the following day, 45 mins prior to the assay, the culture medium was replaced with KHB buffer (110 mmol/L NaCl, 4.7 mmol/L KCl, 1.25 mmol/L $CaCl_2$, 2 mmol/L $MgSO_4$, and 1.2 mmol/L Na_2HPO_4) supplemented with 2.5 mmol/L glucose, 0.5 mmol/L carnitine and 5 mmol/L HEPES (pH 7.4) and the cells were incubated in a non-CO_2 incubator. The Mito Stress Test was performed in the presence of Etomoxir (40 µmol/L) and Palmitate-BSA (150 µmol/L) or control-BSA, each of which were added at 15 and 0 mins prior to the assay, respectively. FAO was determined by calculating the difference in the OCR value between cells treated with and without Etomoxir. OCR values were expressed as pmol/L/min/µg protein.

GSH and GSSG measurements

GSH and GSSG were measured using the EnzyChrom™ GSH/GSSG Assay Kit according to the manufacturer's protocol (EGTT-100, Bioassay Systems, Hayward, CA).

Immunoblot analyses

Heart homogenates or cardiomyocyte lysates were prepared in RIPA lysis buffer containing 50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1% IGEPAL CA-630 (Sigma Aldrich), 0.1% SDS, 0.5% deoxycholic acid, 10 mmol/L $Na_4P_2O_7$, 5 mmol/L EDTA, 0.1 mmol/L Na_3VO_4, 1 mmol/L NaF, 0.5 mmol/L 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.5 µg/mL aprotinin, and 0.5 µg/mL leupeptin. Equal amounts of protein (5-10 µg, BCA quantification) were subjected to 10–15% SDS–PAGE. After proteins were transferred to a PVDF membrane, immunoblots were probed with the indicated primary antibodies. Horseradish peroxidase linked anti-rabbit IgG (Cell Signaling Technology (CST), 7074) and anti-mouse IgG (CST, 7076) were used for secondary antibodies.

Antibodies

The following antibodies were used for immunoblots: Nampt (Bethyl, A700-058 and Abcam, ab58640), NADK (CST, 55948), phosphorylated IKK (CST, 2697), IKKα (CST, 2682), IKKβ (CST, 2678), phosphorylated mTOR at Ser 2481 (CST, 2774), mTOR (CST, 2797), phosphorylated p70 S6 Kinase (S6K) (CST, 9205), S6K (CST, 9202), phosphorylated AMPKα (CST, 2535), AMPKα2 (CST, 2757), phosphorylated JNK (CST, 9251), JNK (CST, 9252), phosphorylated TAK1 (CST, 4351), TAK1 (CST, 5206), TLR4 (Novus Biologicals, NB100-56579), tubulin (Sigma, T6199), Prdx1 (Abcam, ab41906), Prdx1-SO_3H (Abcam, ab16830), Glucose-6-Phosphate Dehydrogenase (CST, 8866), DiTyrosine (StressMarq, SMC-521D), Gapdh (CST, 2118), Trx1 (CST, 2429), Collagen 3a1 (Novus, NB600-594), TGFβ1 (Abcam, 92486) and MitoProfile® (Abcam, 110413), containing antibodies against Atp5a, Uqcrc2, Sdhb, and Ndfub8. In all Western blot images shown in figures, individual lanes correspond to individual mice or independent samples from cell culture experiments. Molecular weights (MW) associated with the bands are shown. The signal intensity of Western blot signals was quantified using the ImageJ program. The signal intensity of a specific protein with modification such as phosphorylation or sulfonic acid formation was normalized with the total protein without

modification. The signal intensity of other proteins was normalized by a loading control such as tubulin or Gapdh.

Primary cultures of neonatal rat ventricular myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl: (WI) BR-Wistar rats (Harlan). A cardiac myocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient. Cells were cultured in complete medium for 1 - 2 days. Cells were then treated with reagents or adenovirus for further studies. The complete medium contains Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 5% horse serum, 4 μg/mL transferrin, 0.7 ng/mL sodium selenite (Life Technologies, Inc.), 2 g/L bovine serum albumin (fraction V), 3 mmol/L pyruvic acid, 15 mmol/L Hepes, 100 μmol/L ascorbic acid, 100 μg/mL ampicillin, 5 μg/mL linoleic acid, and 100 μmol/L 5-bromo-2'-deoxyuridine (Sigma).

Adenovirus vectors

Adenovirus vectors used in this study were described previously [5, 15, 36, 40]. After adenovirus Nampt transduction (10 MOI), cardiomyocytes were cultured with 100 μmol/L nicotinamide for 1 day before harvest to ensure Nampt-induced NAD production, since DMEM/F-12 medium contains a limited amount of nicotinamide (1.6 μmol/L) [41].

Knockdown with shRNA

Small hairpin RNA (shRNA) for rat NADK (SI04748191), rat Sirt3 (SI01584779) and control shRNA (1022076) were obtained from Qiagen. The shRNA was transfected using Lipofectamine RNAiMax (Invitrogen). Four and a half μl of Lipofectamine RNAiMax was diluted with 125 μL OPTI-MEM. Forty pmol of shRNA was diluted with 125 μL OPTI-MEM. The diluted

Lipofectamine RNAiMax and shRNA were then mixed and incubated at room temperature for 20 minutes. The mixture was added to cardiomyocytes plated on 3.5 cm dishes with 2 ml DMEM/F-12 medium. The cells were used after 1-2 days of transfection.

Evaluation of autophagy and mitophagy in cardiomyocytes

To evaluate autophagic flux *in vitro*, cardiomyocytes were transduced with adenovirus vectors expressing GFP-LC3 and Nampt. After 2 days of transduction, the cells were treated with 10 μM chloroquine for 4 hours. GFP-LC3 dots in the cells were visualized by fluorescence microscopy and counted. To evaluate mitophagy, cardiomyocytes were transduced with an adenovirus vector harboring Mito-Keima, a fluorescence-based mitochondria-localized pH-indicator protein. The excitation wavelength shifts from 440 nm at natural pH to 586 nm at acidic pH, indicating fusion of mitochondria-containing autophagosomes to lysosomes, while its emission remains the same at 620 nm. The area showing fluorescence/emission at 620 nm excited by 586 nm was visualized by fluorescence microscopy and defined as the mitophagy area [5].

Statistical analysis

All values in graphs are expressed as the mean ± S.E. Normality was tested with the Shapiro-Wilk normality test. If the data exhibited a normal distribution, pairwise testing was performed with the Student's *t* test or multiple group comparisons were performed by 2-way ANOVA, followed by Tukey post-test. If the data failed normality testing or N<6, pairwise testing was performed with the non-parametric Mann-Whitney *U* test and multiple group comparisons were performed by the non-parametric Kruskal-Wallis test, followed by Dunn's post-test. Priori power calculations were performed based on data from published studies [5, 16, 17] and pilot experiments. The effect size in this study was 1-5 with an alpha = 0.05 and power = 0.80. Microsoft Excel 2016 was used for Student's *t* tests and GraphPad Prism 9 was used for ANOVA and Kruskal-Wallis tests. Precise p-values and N are shown in **Table II in Data Supplement.** Dot plot was

used except where it visually interferes with interpretation (Fig. 1D, 8B and 8F). All quantified Western blot and cell viability data are shown as values relative to control.

Online Table I

	Normal Diet (3.85 Kcal/g)	**High-Fat Diet (5.24 Kcal/g)**
Nutrition Information	% Kcal from	% Kcal from
Protein	20	20
Carbohydrate	70	20
Fat	10	60
Total	100	100
Ingredient	g/Kg	g/Kg
Casein, 30 Mesh	200	200
L-Cystine	3	3
Corn Starch	315	0
Maltodextrin 10	35	125
Sucrose	350	68.6
Cellulose, BW200	50	50
Soybean oil	25	25
Lard	20	245
Mineral Mix S10026	10	10
DiCalcium Phosphate	13	13
Calcium Carbonate	5.5	5.5
Potassium Citrate	16.5	16.5
Vitamin Mix V10001	10	10
Choline Bitartrate	2	2
FD&C Blue Dye #1	0.05	0.05
Total	1055.55	773.85

Online Table II

Statistics Data Set

Online Table III

Echocardiographic analyses for Tg-Nampt mice under HFD consumption.

Group	*n*	DSEP WT	LVEDD	DPW WT	LVESD	EF (%)	%FS
Wild ND	**5-7**	0.91±0.13	2.92±0.41	0.79±0.04	1.46±0.28	78.36±9.88	37.84±4.1
Wild HFD	**5-6**	1.04±0.18	2.8±0.61	0.96±0.31	1.59±0.16	76.34±7.06	40.15±6.42
TG ND	**5**	0.89±0.0	3.22±0.57	0.77±0.16	2.0±0.54	72.09±5.68	37.59±1.88
TG HFD	**4-5**	0.87±0.11	2.99±0.34	0.76±0.09	1.84±0.21	68.94±5.41	33.78±4.52

HFD: 3 months. Value are means ± SD. n, number; DSEP WT, diastolic septal wall thickness, mm; LVEDD, left ventricular end diastolic dimension, mm; DPW WT, diastolic posterior wall thickness, mm; LVESD, left ventricular end systolic dimension, mm; EF, left ventricular ejection fraction, %; FS, fractional shortening, %. Statistical significance was determined with the Kruskal Wallis test. There is no statistical significance between NTg and TG in the identical feeding conditions.

Online Table IV

Organ weight data of Tg-Nampt mice under HFD consumption.

	Wild ND (21)	Wild HFD (31)	TG ND (26)	TG HFD (24)
Body weight (BW), g	29.05±3.6	40.04±7.74	29.63±3.71	36.79±7.58
Tibia length (TL), mm	20.16.8±1.09	20.12±1.24	20.15±1.05	20.36±1.09
Heart weight (HW), mg	105.36±14.54	140.75±20.38	112.41±21.20	125.10±16.15
LV weight (LVW), mg	86.38±12.03	112.49±16.07	91.59±16.84	102.51±14.00
Lung weight (LUW), mg	138.67±12.53	161.90±26.34	142.87±16.87	149.5±15.96
HW/TL	5.25±0.86	7.03±1.11	5.59±1.06	6.15±0.80**
LVW/TL	4.30±0.69	5.61±0.83	4.46±0.66	5.04±0.62*
LUW/TL	6.90±0.77	8.06±1.27	7.11±0.96	7.36±0.83

HFD: 3 months. Data are mean ± SD. LV: Left Ventricle. Statistical significance was determined by the Mann Whitney U Test (Lung weight). Besides this, statistical significance was determined with the ANOVA (HW/TL, LVW/TL and LUW/TL) and the Kruskal Wallis test (BW, TL, HW, LV, Lung). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ vs Wild HFD. Parenthesis indicates N.

Online Table V

Lipidomics Results

Online Table VI

Echocardiographic analyses for Nampt$^{+/-}$ mice under HFD consumption.

Group	*n*	DSEP WT	LVEDD	DPW WT	LVESD	EF (%)	%FS
Wild ND	**7**	0.74±0.08	3.61±0.27	0.67±0.28	2.47±0.36	67.17±9.99	31.6±6.79
Wild HFD	**8**	0.82±0.12	3.65±0.26	0.80±0.13	2.34±0.45	72.32±11.9	36.02±9.43
KO ND	**7**	0.81±0.06	3.75±0.32	0.76±0.12	2.47±0.30	69.9±10.8	33.82±8.07
KO HFD	**8**	0.80±0.08	3.64±0.25	0.75±0.06	2.37±0.33	71.8±9.21	35.07±701.

HFD: 3 months. Value are means ± SD. n, number; DSEP WT, diastolic septal wall thickness, mm; LVEDD, left ventricular end diastolic dimension, mm; DPW WT, diastolic posterior wall thickness, mm; LVESD, left ventricular end systolic dimension, mm; EF, left ventricular ejection fraction, %; FS, fractional shortening, %. Statistical significance was determined with the ANOVA (DSEP WT, LVEDD, LVESD, EF and FS) and Kruskal Wallis test (DPW WT). There is no statistical significance between Wild and Nampt$^{+/-}$ mice in the identical feeding conditions.

Online Table VII

Organ weight data of Nampt$^{+/-}$ mice under HFD consumption.

	Wild ND (10)	Wild HFD (11)	KO ND (10)	KO HFD (12)
Body weight (BW), g	33.89±2.72	47.82±4.10	29.22±2.12	48.25±6.73
Tibia length (TL), mm	17.96±0.22	17.92±0.33	17.71±0.30	17.69±0.28
Heart weight (HW), mg	131.7±10.77	156.09±15.82	129.7±9.20	156.75±24.89
LV weight (LVW), mg	102.00±7.20	121.00±11.54	103.3±10.92	120.67±20.17
Lung weight (LUW), mg	151.90±5.80	166.73±20.27	151.3±14.05	160.83±14.74
HW/TL	7.33±0.55	8.71±0.83	7.32±0.53	8.85±1.32
LVW/TL	5.68±0.37	6.75±0.59	5.83±0.63	6.81±1.07
LUW/TL	8.46±0.37	9.31±1.20	8.55±0.80	9.09±0.79

HFD: 3 months. Data are mean ± SD. LV: Left Ventricle. Statistical significance was determined with the ANOVA (TL and LUW) and the Kruskal Wallis test (BW, HW, LV, HW/TL, LV/TL and LUW/TL). There is no statistical significance between Wild and KO in the identical feeding conditions. Parenthesis indicates N.