Exogenous NADPH exerts a positive inotropic effect and enhances energy metabolism via SIRT3 in pathological cardiac hypertrophy and heart failure

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Summary

Background Therapies are urgently required to ameliorate pathological cardiac hypertrophy and enhance cardiac function in heart failure. Our preliminary experiments have demonstrated that exogenous NADPH exhibits a positive inotropic effect on isolated heart. This study aims to investigate the positive inotropic effects of NADPH in pathological cardiac hypertrophy and heart failure, as well as the underlying mechanisms involved.

Methods Endogenous plasma NADPH contents were determined in patients with chronic heart failure and control adults. The positive inotropic effects of NADPH were investigated in isolated toad heart or rat heart. The effects of NADPH were investigated in isoproterenol (ISO)–induced cardiac hypertrophy or transverse aortic constriction (TAC)–induced heart failure. The underlying mechanisms of NADPH were studied using SIRT3 knockout mice, echocardiography, Western blotting, transmission electron microscopy, and immunoprecipitation.

Findings The endogenous NADPH content in the blood of patients and animals with pathological cardiac hypertrophy or heart failure was significantly reduced compared with age-sex matched control subjects. Exogenous NADPH showed positive inotropic effects on the isolated normal and failing hearts, while antagonism of ATP receptor partially abolished the positive inotropic effect of NADPH. Exogenous NADPH administration significantly reduced heart weight indices, and improved cardiac function in the mice with pathological cardiac hypertrophy or heart failure. NADPH increased SIRT3 expression and activity, deacetylated target proteins, improved mitochondrial function and facilitated ATP production in the hypertrophic myocardium. Importantly, inhibition of SIRT3 abolished the positive inotropic effect of NADPH, and the anti-heart failure effect of NADPH was significantly reduced in the SIRT3 Knockout mice.

Interpretation Exogenous NADPH shows positive inotropic effect and improves energy metabolism via SIRT3 in pathological cardiac hypertrophy and heart failure. NADPH thus may be one of the potential candidates for the treatment of pathological cardiac hypertrophy or heart failure.

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Keywords: NADPH; Heart failure; Cardiac hypertrophy; Positive inotropic effect; SIRT3; Acetylation

Introduction

Heart failure is the end stage of disease progression due to contractile or/and diastolic dysfunction of the heart. According to the latest epidemiological data, the prevalence of heart failure among adults in developed countries is 1–2%, with the prevalence exceeding 10% among

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Research in context

Evidence before this study

NADPH serves as a crucial hydrogen donor for numerous anabolic metabolic processes. The authors have previously reported that exogenous administration of NADPH confers protection against ischemic stroke and cardiac ischemia/reperfusion injury.

Added value of this study

1. Endogenous NADPH content in the blood of patients with pathological cardiac hypertrophy or heart failure is reduced
2. Exogenous NADPH shows positive inotropic effect in heart failure.
3. Exogenous NADPH improves cardiac function and inhibits pathological cardiac hypertrophy in heart failure.
4. Exogenous NADPH improves energy metabolism via SIRT3 in heart failure.

Implications of all the available evidence

NADPH may become one of the potential candidates for the treatment of pathological cardiac hypertrophy or heart failure.

people over 70 years of age.[1,2] The mortality of heart failure is not only much higher than other cardiovascular diseases, but 75% of the patients exhibit varying degrees of workforce loss, severely affecting the quality of life.[3]

The development of heart failure is often accompanied by pathological cardiac hypertrophy, which is characterized by an increase in area and protein synthesis of cardiomyocytes, proliferation of connective tissue cells (fibroblasts) and myocardial fibrosis.[4] The early compensatory cardiac hypertrophy helps to maintain cardiac function. However, the late pathological cardiac hypertrophy, accompanied by a decrease in contractile function of the hypertrophic heart, electrophysiological abnormalities and myocardial fibrosis, prompts the gradual conversion of cardiac function from compensatory to decompensated state and is involved in the development of heart failure.[5] Therefore, there is an urgent need to develop new drugs that ameliorate pathological cardiac hypertrophy and improve cardiac function with mild adverse effects for the treatment of heart failure.

NADPH (Nicotinamide adenine dinucleotide phosphate hydrogen), also known as coenzyme II, is an endogenous reactive substance mainly produced by the pentose phosphate pathway and acts as a hydrogen donor for many anabolic metabolisms[6,7] NADPH is also an important component of the antioxidant system in the body, protecting cells from oxidative stress. In addition, NADPH can deliver hydrogen to NAD to become NADH, which enters the mitochondria to produce ATP.[8] In 2016, our laboratory reported that administration of exogenous NADPH exhibited neuroprotection against ischemic stroke in rodents and monkeys.[9] NADPH significantly increased intracellular reduced glutathione (GSH) and ATP, while decreased reactive oxygen species (ROS), suggesting that NADPH can protect neuronal ischemia/reperfusion injury through antioxidant effects.[10–12] Furthermore, exogenous NADPH also protected against cardiac ischemia/reperfusion injury by activating the AMP-dependent protein kinase (AMPK)–mammalian target of rapamycin (mTOR) pathway in cardiomyocytes[13] and endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) system in endothelial cells.[14]

Based on the above research, the potential value of NADPH in cardiovascular diseases has attracted great interest to us. Recently, we unexpectedly discovered the positive inotropic action of NADPH in the isolated toad heart. NADPH can increase the myocardial contractility of isolated heart both in normal and low calcium Ringer's solution. We then established isoproterenol (ISO)–induced cardiac hypertrophy and transverse aortic constriction (TAC)–induced heart failure models in mice to investigate the effects of NADPH in pathological cardiac hypertrophy and heart failure and the underlying mechanisms.

Methods

Ethics

The research protocol of human blood specimens was approved by the Ethics Committee of Suzhou Municipal Hospital (Approval ID: IEC-C-008-A07-V1.0), and the participants or their legal representatives signed a written informed consent. All animals were used following the Soochow University guidelines for laboratory animal care and use, and the research protocol was approved by the Ethics Committee of Soochow University (Approval number 201806A056). All animals used in this study were purchased from the Experimental Animal Center of Soochow University. To avoid the influence of hormones and ovulatory cycles on drug action in female animals, male animals were exclusively utilized in the study. Animals were housed under a 12h light/dark cycle, with ad libitum access to food and water, and maintained at a controlled temperature range of 20–25 °C.

Measurement of NADPH level in human blood samples

Patients with heart failure in Suzhou Municipal Hospital from June 1, 2018 to December 30, 2021 were recruited. The recruitment criteria were as follows:

chronic heart failure (NYHA functional class II-IV) was diagnosed; there was no serious primary disease of other organ systems; the patient or his family members agreed and signed an informed consent form (n = 12). The sex-age-matched people with normal cardiac function who came to Suzhou Municipal Hospital for physical examination from November 19, 2018 to December 30, 2021 were also recruited as the control group (n = 12). One ml of plasma from controls or patients with heart failure were collected, and the plasma NADPH levels were measured using an assay kit (BioAssay, ECNP-100). The demographic and clinical characteristics of the controls and patients with heart failure in the study were listed in Table 1 and Supplemental Table S1. There were no significant differences in terms of sex distribution and mean age between patients with heart failure and control adults.

Perfusion of isolated heart

The isolated toad heart perfusion was established as per described.[15,16] Briefly, male toad (50–100 g) was pithed and sacrificed, and then the left and right aorta and the inferior vena cava were dissected. The venous cannula was inserted into the inferior vena cava, while the arterial cannula was inserted into the left aorta, and the right aorta was ligated. The toad heart was then anteriorly perfused with Ringer's solution (mmol/L: NaCl 111, KCl 1.9, $CaCl_2$ 1.1, $NaHCO_3$ 2.4, NaH_2PO_4 0.08, Glucose 11; ioninc strength 117.0), at a fixed venous pressure and a fixed afterload, in a non-recirculating fashion. The apex of the heart was clamped with a clip, which was connected to a biomedical signal processing system (Anhui Zhenghua Biologic apparatus facilities Co. Ltd. MD3000) through a tension transducer (Beijing Xinhangxingye Technology& trade Co. Ltd. JZ100) to record the myocardial contraction curve and heart rate. The outflow per unit time from left aortic cannula was measured as cardiac output. In the isolated toad heart failure model, the heart was perfused with low calcium Ringer's solution (mmol/L: NaCl 111, KCl 1.9, $CaCl_2$ 0.54, $NaHCO_3$ 2.4, NaH_2PO_4 0.08, Glucose 11; ioninc strength 118.7). The heart was stable for 20 min before treatment.

To investigate the positive inotropic effects of NADPH on normal hearts, toad hearts were perfused with Ringer's solution and randomly assigned to treatment groups of 3 μM, 6 μM and 12 μM NADPH (Roche 11195140), respectively (n = 6). To examine the inotropy of NADPH on heart failure, toad hearts were perfused with low calcium Ringer's solution and randomly allocated to treatment groups of 0 μM, 6 μM and 12 μM NADPH, respectively (n = 6). To study the effects of Coenzyme I, Coenzyme II and ATP on toad hearts, they were perfused with Ringer's solution and randomly treated with NADPH (6 μM), NAD (Sigma N0632, 6 μM), NADH (Sigma N4505, 6 μM), NADP (Sigma 93205, 6 μM) or ATP (Selleck S1985, 50 μM), respectively (n = 6). To determine the mechanism underlying the positive inotropic action of NADPH, toad hearts were perfused with Ringer's solution and randomly assigned to designated groups (n = 6). The heart was pretreated with suramin (MCE HY-B0879, an ATP P2Y11 receptor non-selective antagonist, 30 μM),[17,18] or NF340 (sc-361274, an ATP P2Y11 receptor selective antagonist, 10 μM),[19,20] or 3-acetylpyridine (3-AP, Sigma 350-03-8a, a NAD inhibitor, 5 mM),[21–23] or diltiazem (Shanghai Dibo Chemicals Technology Co. Ltd., 33286-22-5, a calcium channel blocker, 0.1 μM),[24] or 3-TYP (Selleck S8628, a SIRT3 inhibitor, 68 μM),[25] or probenecid (MCE HY-B0545, 200 μM) and carbenoxolone (MCE, HY-B1588S, both Pannexin-1 antagonists, 10 μM),[26] or SQ22536 (AdooQ, A16116, an adenylate cyclase blocker, 30 μM), or U73122 (AdooQ A14339, a phospholipase C blocker, 1 μM)[27] for 20 min. Then NADPH (6 μM) or NAD (6 μM) was added to the perfusion solution. NADPH, NAD, NADH was dissolved in a $NaHCO_3$ buffer (pH 9.0) to prepare the stock solution and then diluted with perfusion solution to the required concentration.

Male SD rats (300–350 g) were anesthetized and sacrificed. Then the hearts were quickly taken out. The isolated rat heart was retrogradely perfused through the aorta on a Langendorff device with a perfusion solution (mmol/L: NaCl 117, KCl 5.7, $CaCl_2$ 1.8, $MgCl_2$ 1.7, NaH_2PO_4 1.5, HEPES 20, Glucose 11 gassed with 95% O_2 plus 5% CO_2, pH 7.4) at 37.5 ± 0.5 °C. The pressure

Control			Heart Failure		
Age	Sex	NYHA class	Age	Sex	NYHA class
49	M	N	45	M	IV
53	F	N	52	F	II
57	F	N	57	F	II
55	M	N	62	M	III
54	M	N	62	M	III
68	F	N	63	F	II
56	M	N	58	M	IV
58	M	N	63	M	II
64	M	N	56	M	II
64	M	N	69	M	III
67	F	N	52	F	II
68	M	N	65	M	II
59.4 ± 5.98			58.7 ± 6.16		

N: Normal LV thickness and function. According to the New York Heart Association (NYHA) Functional Classification: I: No limitation of physical activity. Ordinary physical activity does not cause undue fatigue, palpitation, dyspnea (shortness of breath). II: Slight limitation of physical activity. Comfortable at rest. Ordinary physical activity results in fatigue, palpitation, dyspnea (shortness of breath). III: Marked limitation of physical activity. Comfortable at rest. Less than ordinary activity causes fatigue, palpitation, or dyspnea. IV: Unable to carry on any physical activity without discomfort. Symptoms of heart failure at rest. If any physical activity is undertaken, discomfort increases.

Table 1: **Demography of human subjects.**

transducer filled with the perfusion solution was inserted into the left ventricle through the left atrium, and connected to the RM6240 multi-channel signal processing system to record the left ventricular contraction curve. When the isolated heart contraction reached stable, the intraventricular pressures were measured. The isolated rat hearts were randomly allocated to treatment groups of 3 μM, 6 μM and 12 μM NADPH, respectively (n = 3).

Assessment of cardiac function with intracardiac catheterization

To evaluate the cardiac function in vivo, the left common carotid artery was dissected in rat after anesthesia, and a cardiac catheter filled with normal saline containing heparin was inserted into the left ventricle through the left common carotid artery, which was connected to a cardiac function analysis system ((ALC-BIO, Shanghai Alcott Biotech CO., LTD).[28] The rats were randomly allocated to the control and experimental groups (n = 5). The maximum up rate of left ventricular pressure (+dp/dtmax), and the maximum down rate of left ventricular pressure (–dp/dtmax) were recorded at baseline and after the intravenous administration of NADPH (16 mg/kg). Subsequently, the rats were euthanized under anesthesia.

Isoproterenol model and transverse aortic constriction (TAC) model in mice

Adult male ICR mice (8–12 weeks old, 25–30 g), SIRT3 Knockout (KO) mice (*SIRT3*–/–, 8–12 weeks old, 20–25 g) and age-matched wild-type (WT) C57BL/6 J mice were used. SIRT3 KO mice were kindly gifted by Professor Wei-Li Shen from Shanghai Jiaotong University.[29] The identification of SIRT3 KO was performed by cutting the tail tip to extract genomic DNA. Then the following primers were used for quantitative PCR: wild type forward: 5′-CTT CTG CGG CTC TAT ACA CAG-3′, common: 5′-TGC AAC AAG GCT TTA TCT TCC-3′, mutation reverse: 5′-TAC TGA ATA TCA GTG GGA ACG-3′. All animals were kept in an environment with a light/dark cycle of 12 h, room temperature at 20–25 °C and humidity at 40–70%, and were fed ad libitum. Mice were randomly assigned to each group.

For isoproterenol (ISO) model, mice received subcutaneous injection of ISO (Shanghai Yuanye Biological Technology Co. Ltd., 51-30-9) 1 mg/kg twice a day in every morning and evening for 14 consecutive days. Medication was administered at noon every day for 14 consecutive days.[30]

For transverse aortic constriction (TAC) model, mice underwent the TAC surgery as described previously. In brief, mice were anesthetized with isoflurane (1.5%). After the mouse underwent thoracotomy, the transverse aortic arch between the right innominate and left common carotid arteries was dissected. Then the aorta was constricted using a 27-gauge blunted needle (diameter 0.4 mm), which was ligated with aorta with a 6-0 silk suture and removed afterwards. The aorta was isolated without ligation in sham-operated mice. One day after surgery, medication was administered daily for 4 weeks.[31,32]

In the ISO model, mice were randomly assigned to control, ISO, ISO + captopril group, ISO + NADPH 1 mg/kg, ISO + NADPH 2 mg/kg, and ISO + NADPH 4 mg/kg groups based on computer-generated sequence (n = 10). To compare the effects of NADPH and NAD, mice were randomly assigned to the control, ISO, ISO + NAD 2 mg/kg (2.4 μmol/kg), ISO + NADPH 1.6 mg/kg (2.4 μmol/kg) groups (n = 4–6). In the TAC model, mice were randomly assigned to sham, TAC, TAC + captopril, TAC + NADPH 1 mg/kg, TAC + NADPH 2 mg/kg and TAC + NADPH 4 mg/kg groups (n = 10). To investigate the relationship between the anti-heart failure effect of NADPH and SIRT3, age-matched WT and SIRT3 KO mice were randomly assigned to WT sham, WT TAC, WT TAC + NADPH, KO sham, KO TAC, KO TAC + NADPH groups (n = 4–5). Mice were intraperitoneally administered daily with NADPH (1, 2, 4 mg/kg) or NAD (1.6 mg/kg) or captopril (Shanghai Yuanye Biological Technology Co. Ltd. 62571-86-2) for either 2 weeks (ISO) or 30 days (TAC). The mice in the sham group and the model group received an equal volume of vehicle injection. The doses of NADPH used in isolated heart perfusion or in vivo ISO or TAC models were determined based on preliminary experiments and our previous research.[13]

Exercise exhaustion test

The exercise exhaustion test was measured blindedly using mouse wheel fatigue meter (wheel radius = 10 cm, Jinan Yiyan Technology Development Co., Ltd YLS-10B). Three days before the test, the mice ran for 10 min every day with a speed of 10 rpm to get familiar with the running on the wheel. For test, the initial speed of 19 rpm/min was increased by 2 rpm every 2 min. The mice kept running until they were exhausted. Exhaustion was defined as the mice were unable to keep up with the wheel and did not respond to electrical stimulation. Total running time was recorded as the stating time to exhaustion time and then converted to distance.[32,33]

Echocardiography

Mice were anesthetized with isoflurane (1.5%) and placed on a heating pad in a supine position. Echocardiography was performed using Vevo 2100 Imaging System with a probe frequency of 30 MHz (Visual Sonics, Toronto, ON, Canada). Standard left ventricular papillary short and long axial view was inspected with M-mode Doppler ultrasound. Left ventricular internal diameter at diastole (LVIDd) and systole (LVIDs), left ventricular volume at diastole (LVVd) and systole (LVVs), fraction shortening (FS) and ejection fraction

(EF) was measured using Vevo2100 software. The average values of 3 consecutive cardiac cycles were taken.

Measurement of heart weight index and lung weight index

After the mice were sacrificed after anesthesia, the hearts and lungs were dissected to determine the lung weight (LW), heart weight (HW) and left ventricular weight (LW). The lung weight index (LW/BW), heart weight index (HW/BW) and left ventricular weight index (LVW/BW) were calculated.

Measurement of biochemical indices in serum and myocardium

Left ventricle and serum were collected, followed by measurement of the cardiac and serum levels of MDA (Beyotime, S0131), H_2O_2 (Beyotime, S0038-3), GSH (Beyotime, S0053), ATP (Beyotime, S0026), ATPase (Na^+-K^+, Ca^{2+}-Mg^{2+}, T-ATPase, Nanjing Jiancheng, A070-6), NADPH/NADP (BioAssay, ECNP-100) according to the manufacturer's instructions. Refer to the Supplemental Method for detailed procedures.

Measurement of SIRT3 activity

The SIRT3 activity was evaluated using the SIRT3 activity assay kit (fluorometric, ab156067, Abcam) according to the manufacturer's instructions. Briefly, the myocardial tissue was homogenized with lysis buffer lacking protease inhibitors and then centrifuged at 4 °C, 12500 rpm for 10 min to obtain supernatant. The lysate (1 mg) was then incubated with anti-SIRT3 antibody (3 μL, Cell Signaling technology 2627S) and protein A/G magnetic beads (20 μL, Selleckchem B23202) at 4 °C for 4 h. Following immunoprecipitation, the beads were washed with SIRT3 Assay Buffer and incubated with a reaction mixture containing Fluoro-Substrate Peptide, NAD, and developer for an hour to serve as "Enzyme sample". Subsequently, fluorescence intensity was measured by exciting at 360 nm and detecting emission at 460 nm to determine SIRT3 activity.[33]

Histology

The apex of the left ventricle was fixed in 4% paraformaldehyde PBS, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE). The cross-sectional area of at least 100 cardiomyocytes were measured in four or more random areas in each slice using Image J.

Assessment of oxygen consumption

The High Resolution Respirometry (Oxygraph-2k, Oroboros, Innsbruck, Austria) chamber was added with 2 ml myocardial tissue homogenates. After the oxygen flux was stabilized, the oxygen consumption rate (OCR) was measured with continuous addition of different reagents. Mitochondrial complex I substrates 5 mM pyruvate (Pyr), 10 mM glutamic acid (Glu) and 2 mM malic acid (Mal) were added to record the leak of mitochondrial complex I. The maximum oxidative phosphorylation of complex I was recorded by adding 2.5 mM of ADP. Cytochrome c (Cyt c) 10 μM was added to verify mitochondrial membrane integrity. The maximum oxidative phosphorylation of complex I + II (OXPHOS CI + CII) was obtained by adding complex II substrate succinate 10 mM. The maximum electron transport capacity of complex I + II (ETS CI + CII) was recorded by adding 0.05 μM of Carbonyl cyanide p-trifluoro-methoxyphenyl hydrazone (FCCP). The maximum electron transport capacity of complex II was obtained by adding 0.5 μM of rotenone (Rot), a complex I inhibitor. The residual non-mitochondrial respiration was obtained by adding 2.5 μM Antimycin A (Ama), a complex III inhibitor. Finally, the complex IV respiratory function was evaluated by adding 2 mM Ascorbate (Asc) and 0.5 mM TMPD as complex IV substrates.[34]

Electron microscopy

About 1 cubic millimeter of myocardium in the apex of the left ventricle was double fixed with 4% glutaraldehyde and 1% osmium acid. After acetone dehydration, resin embedding, sectioning, double staining with uranyl acetate and lead citrate, the ultrastructure of myocardium was observed using transmission electron microscope (Hitachi HT7700). Mitochondrial damage was evaluated in a blinded manner. Damaged mitochondria show mitochondrial vacuolization, and mitochondrial cristae are ruptured, perforated, or disappeared.[35,36]

Western blotting

Left ventricles were lysed and homogenized in lysate (10 mM Tris. HCl pH 7.4, 150 mM NaCl, 1% Triton-100, 1% sodium deoxycholate, 10% SDS, and 5 mM EDTA), and total protein was extracted.[37] A BCA Protein Assay Kit (Thermo Scientific, MA, USA) was used to quantify the protein concentrations. Proteins were separated on SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk dissolved in TBST (0.05% Tween-20). The membranes were then incubated with primary antibodies against P-AKT (Cell Signaling Technology 4060, 1:1000), AKT (Cell Signaling Technology 4685, 1:1000), P-ERK (Santa Cruz sc7383, 1:500), ERK (Santa Cruz sc81457, 1:500), SIRT3 (Cell Signaling Technology 2627, 1:5000), SIRT1 (Santa Cruz, sc74594, 1:1000), Pan-acetylation (Cell Signaling Technology 9441, 1:1000), P-LKB1 (Cell Signaling Technology 3055, 1:1000), LKB1 (Proteintech, 10746-1-AP, 1:1000), P-AMPK (Cell Signaling Technology 2535, 1:1000), AMPK (Cell Signaling Technology 5832, 1:1000), SOD2 (Santa Cuz sc-137254, 1:200), AcSOD2 (K68) (Abcam ab137037, 1:1000), GAPDH (Abcam ab8245, 1:5000) overnight at 4 °C. Next, the membranes were incubated with secondary anti-mouse IgG or anti-rabbit IgG

(1:10000; Li-Cor Bioscience, Lincoln, NE, USA) for 2 h in the dark at room temperature. Images of the protein–antibody interactions were captured using an Odyssey infrared imaging system (Li-CorBioscience). GAPDH was used as the loading control. The RRID tags and antibody validation are presented in the Supplementary Materials.

Immunoprecipitation

The left ventricle was homogenized with cold RIPA lysis buffer. After centrifugation at 14,000 g for 15 min at 4 °C, the supernatant was collected and incubated with protein A/G agarose (Bimake B23202), and shaken on a horizontal shaker for 10 min to eliminate non-specific binding proteins. After centrifugation at 14,000 g for 15 min at 4 °C, the supernatant was collected and incubated with the acetylated-lysine antibody (Kac, CST 9441, 1:1000) overnight at 4 °C, and continued to be incubated with an appropriate amount of Protein G/A for 4 h. After centrifugation at 500 g for 3 min, the pellet was washed 3 times with pre-cooled PBS, and then the beads were boiled in 2 × loading buffer (100 mM Tris-HCl pH6.8, 4% SDS, 1% bromophenol blue, 20% glycerol, and 2% β-mercaptoethanol). Then, the supernatants were collected and subjected to Western blot analysis of ATP5A1 (Santa Cruz sc136178, 1:2000) or SDHA (Abcam ab137040, 1:2000) or LKB1.

Statistical analysis

The data were expressed as mean ± S.D. In all clinical and animal experiments, N used for statistical analysis was the number of subjects or animals under each condition. The sample size was determined based on power analysis and adhered to the principles of the 3Rs. No exclusions of experimental units or data points were made during the analysis for each experimental group. GraphPad Prism 8.0 was used for statistical analysis. Student's t test was used to compare two groups of data. Multiple groups of data were compared using one-way ANOVA or two-way ANOVA followed with Newman–Keuls post-hoc analysis. P values less than 0.05 were considered statistically significant.

Role of funders

The funders played no role in study design, data collection, data analyses, interpretation, or writing of report.

Results

Reduced NADPH content in blood of patients and animals with pathological cardiac hypertrophy or heart failure

We collected blood samples from patients with chronic heart failure (NYHA functional class II-IV, Table 1, Supplemental Table S1) and sex-age-matched control adults. Besides, we established mice models of pathological cardiac hypertrophy induced by isoproterenol (ISO) or heart failure induced by transverse aortic constriction (TAC). NADPH contents in the blood samples were measured. Patients with heart failure may present with various etiologies, including ischemic heart disease, hypertensive heart disease, valvular and rheumatic heart failure, cardiomyopathy, etc. However, these causes are not mutually exclusive and can coexist.[38] Due to the challenge of obtaining a sufficient sample size of clinical patients with a single cause of heart failure, this study included patients with heart failure (NYHA functional class II-IV) who had multiple co-morbidities such as coronary artery disease, hypertension, dilated cardiomyopathy, or pulmonary hypertension. Some patients also exhibited arrhythmias such as atrial flutter, atrial fibrillation, ventricular premature beat or short ventricular tachycardia (Supplemental Table S1). Nevertheless, the results suggest that in comparison to sex-age-matched healthy individuals, patients with heart failure (NYHA class II-IV) exhibited a significant reduction in plasma NADPH levels by 24.4% (Fig. 1a). Furthermore, we observed a decline in serum NADPH concentrations in two distinct mouse models: ISO-induced cardiac hypertrophy (27.8% decrease, Fig. 1b) and TAC-induced heart failure (24.1% decrease, Fig. 1c). These results suggest that endogenous NADPH contents in blood were significantly reduced in patients and animals with pathological cardiac hypertrophy or heart failure.

The cardiac positive inotropic action of NADPH

The isolated toad hearts perfused with normal Ringer's solution were treated with 3, 6 and 12 μM NADPH, and the effects of NADPH on isolated toad hearts were investigated. The results showed that 6 μM and 12 μM NADPH increased the force of contraction of toad heart (Fig. 2a). The positive inotropic action of NADPH took effect in about 20 min and maintained for more than 2 h (Fig. 2b), while NADPH showed no significant effect on heart rate (Supplemental Fig. S1a). Next, the isolated toad hearts were perfused with low-calcium Ringer's solution to establish an isolated heart failure model. Following 20 min of perfusion, the toad hearts were treated with 0 μM, 6 μM or 12 μM of NADPH. Low calcium Ringer's perfusion significantly decreased force of contraction and cardiac output, while treatment with 6 μM or 12 μM of NADPH increased force of contraction and cardiac output of failing heart compared to pre-administration or 0 μM NADPH (Fig. 2d, Supplemental Fig. S1b). The positive inotropic effect of NADPH on failure heart maintained for more than 2 h. To further confirm the positive cardiac inotropic effect of NADPH, the Langendorff device was used to measure the left ventricular inotropy of isolated rat hearts. Results showed that

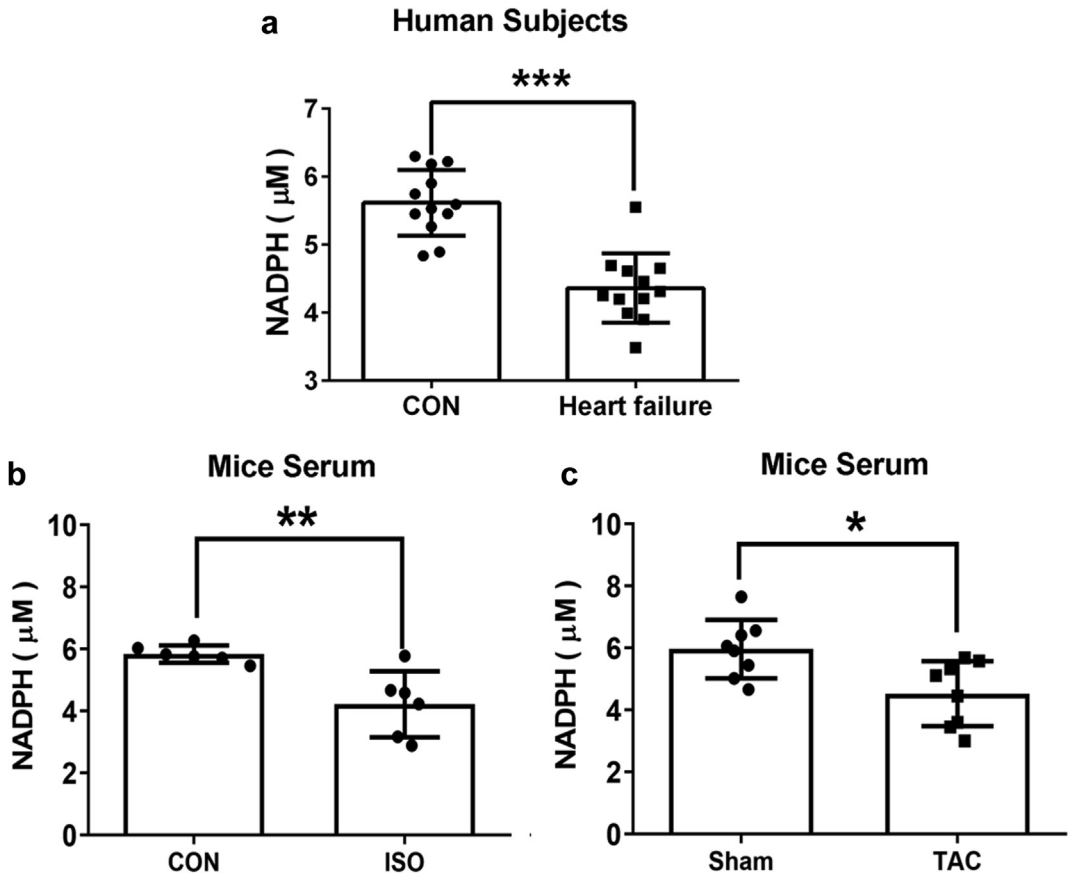

Fig. 1: **NADPH content in blood of patients and animals with pathological cardiac hypertrophy or heart failure. (a).** The plasma concentration of NADPH is downregulated in patients with heart failure. The blood samples were collected from sex-age-matched control subjects and patients with chronic heart failure (Table 1). The content of NADPH in plasma was analyzed. Control: N = 12. Heart failure: N = 12. **(b).** Serum NADPH levels were reduced in ISO model. Cardiac hypertrophy was induced by subcutaneous injection with ISO (1 mg/kg, b. i.d.) for 2 weeks. **(c)**. Serum NADPH levels were reduced in TAC model. Heart failure was induced by transverse aortic constriction (TAC) in mice for 30 days. The content of NADPH in serum was examined. N = 6–8 mice. Bar represents mean ± SD. (*P < 0.05, **P < 0.01, ***P < 0.001, the significance of differences was analyzed by unpaired t test.)

NADPH (3 μM, 6 μM, and 12 μM) increased left ventricular systolic pressure in a concentration dependent manner in the non-pacing mode (Supplemental Fig. S2a and b). The cardiac function in rat was further measured by intracardiac catheterization.[28] A cardiac catheter was inserted into the left ventricle via the left carotid artery. The maximum up rate of left ventricular pressure (+dp/dtmax) and the maximum down rate of left ventricular pressure (–dp/dtmax) were measured at baseline and after the administration of NADPH. The results showed that +dp/dt was significantly increased while the –dp/dt was decreased within 60 min after NADPH administration, suggesting that NADPH can enhance the cardiac function of rats in vivo (Supplemental Fig. S2c and d). All these results indicate that NADPH showed a positive inotropic effect on normal and failing heart.

NADPH may exert positive inotropic effects through the P2Y11 receptor–adenylate cyclase/phospholipase pathway

Coenzyme I and II are closely related and can be transformed into each other in cells,[6] while ATP is the direct supplier of energy. ATP can activate the P2Y11 receptor to produce positive inotropic effects on the heart.[27] We thus applied NAD, NADH, NADP, NADPH and ATP on the toad heart to observe their effects of myocardial contractility. After treatment with equimolar concentrations of NAD, NADH, NADP and NADPH, NAD and NADPH showed positive inotropic effects on toad hearts instead of NADP and NADH. ATP also showed a positive inotropic effect (Fig. 3a). Next, we evaluated the time course of NADPH, NAD, and ATP's effects on toad heart respectively. NADPH took effect in about 20 min and maintained for more than 1 h. NAD's

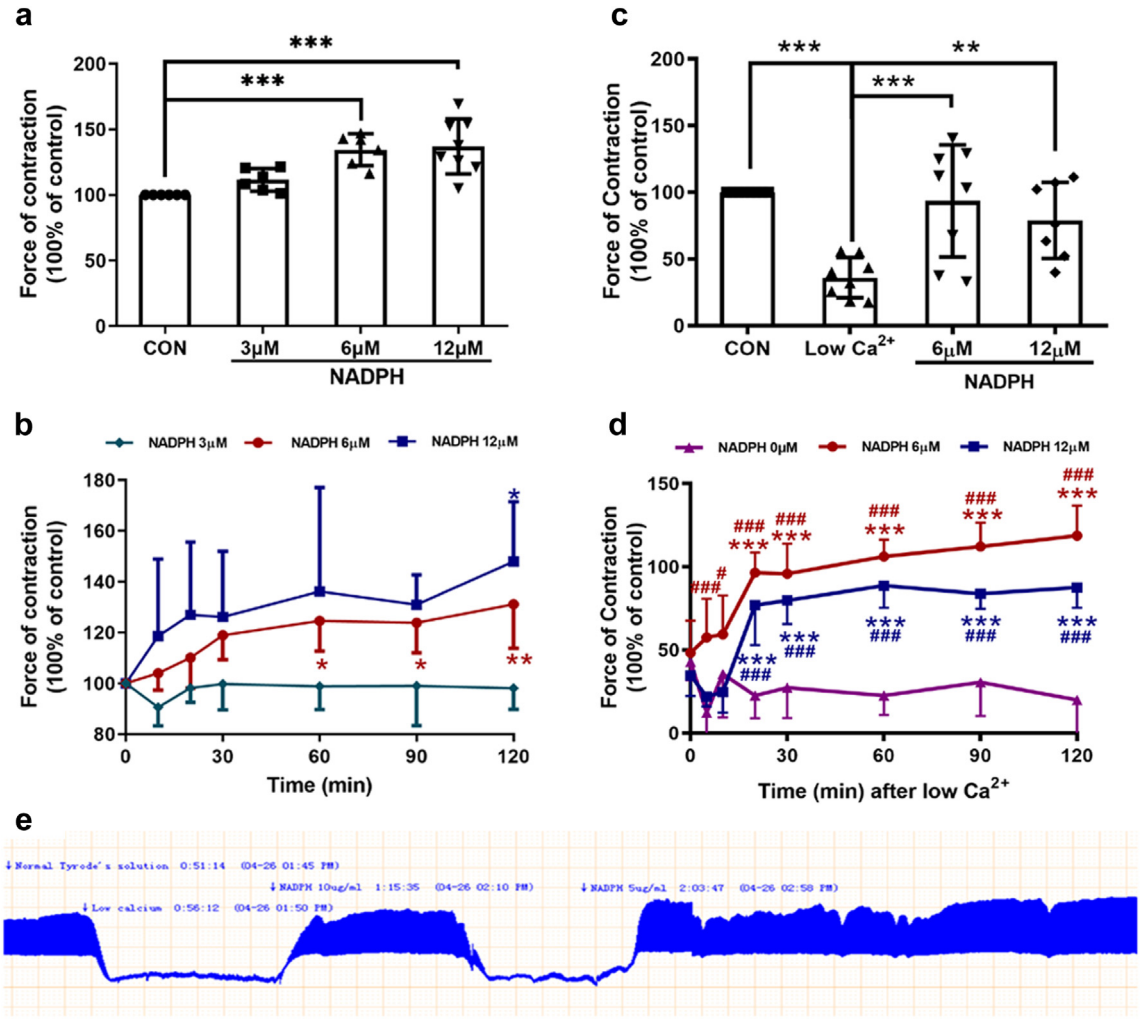

Fig. 2: **The positive inotropic action of NADPH on isolated toad heart.** The toad heart was perfused with normal calcium Ringer's solution (a, b) or low calcium Ringer's solution (c, d) and then was treated with NADPH. **(a, c).** NADPH increased cardiac contractility. **(b, d).** Time course of NADPH on force of contraction. Values were expressed as the percentage increase in force of contraction after administration compared to before administration (CON) in the same toad heart. The difference of force of contraction at each time point after administration and before administration was compared. **(e).** Representative curve showed the positive inotropic action of NADPH on the cardiac contractility. Bar represents mean ± SD. (N = 6–8 toads. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, one-way ANOVA followed by Newman–Keuls post-hoc test, (d) #*P* < 0.05, ###*P* < 0.001 compared with 0 μM NADPH time-control We have now incorporated the statement in the Results according to the comment., two-way ANOVA followed by Newman–Keuls post-hoc test).

effect was slightly weaker than NADPH with the maintenance time similar to NADPH. ATP increased force of contraction stronger than NADPH, but only maintained for about 15 min (Fig. 3b). To investigate whether the positive inotropic effect of NADPH is related to ATP or NAD, toad hearts were pretreated with NAD inhibitor 3-acetylpyridine (3-AP), ATP receptor antagonist suramin or P2Y11R selective antagonist NF340[19,20] before NADPH treatment. 3-AP can cancel the positive inotropic effect of NAD (Supplemental Fig. S3a), but it has no effect on the positive inotropic effect of NADPH (Fig. 3c). Importantly, both suramin and NF340 abolished the positive inotropic effect of NADPH (Fig. 3d and e). In addition, Ca^{2+} is also an important factor in myocardial contraction.[24] Toad hearts were thus also pretreated with calcium channel blocker diltiazem before NADPH, but NADPH still increased the cardiac contractility after treatment with diltiazem (Supplemental Fig. 3b). The above results suggest that the positive inotropic effect of NADPH on isolated toad hearts might be independent of NAD, NADH, NADP or Ca^{2+}, but depend on ATP receptor.

Apelin-13 can stimulate the opening of Pannexin-1 hemichannel to elevate extracellular ATP levels.[26] To

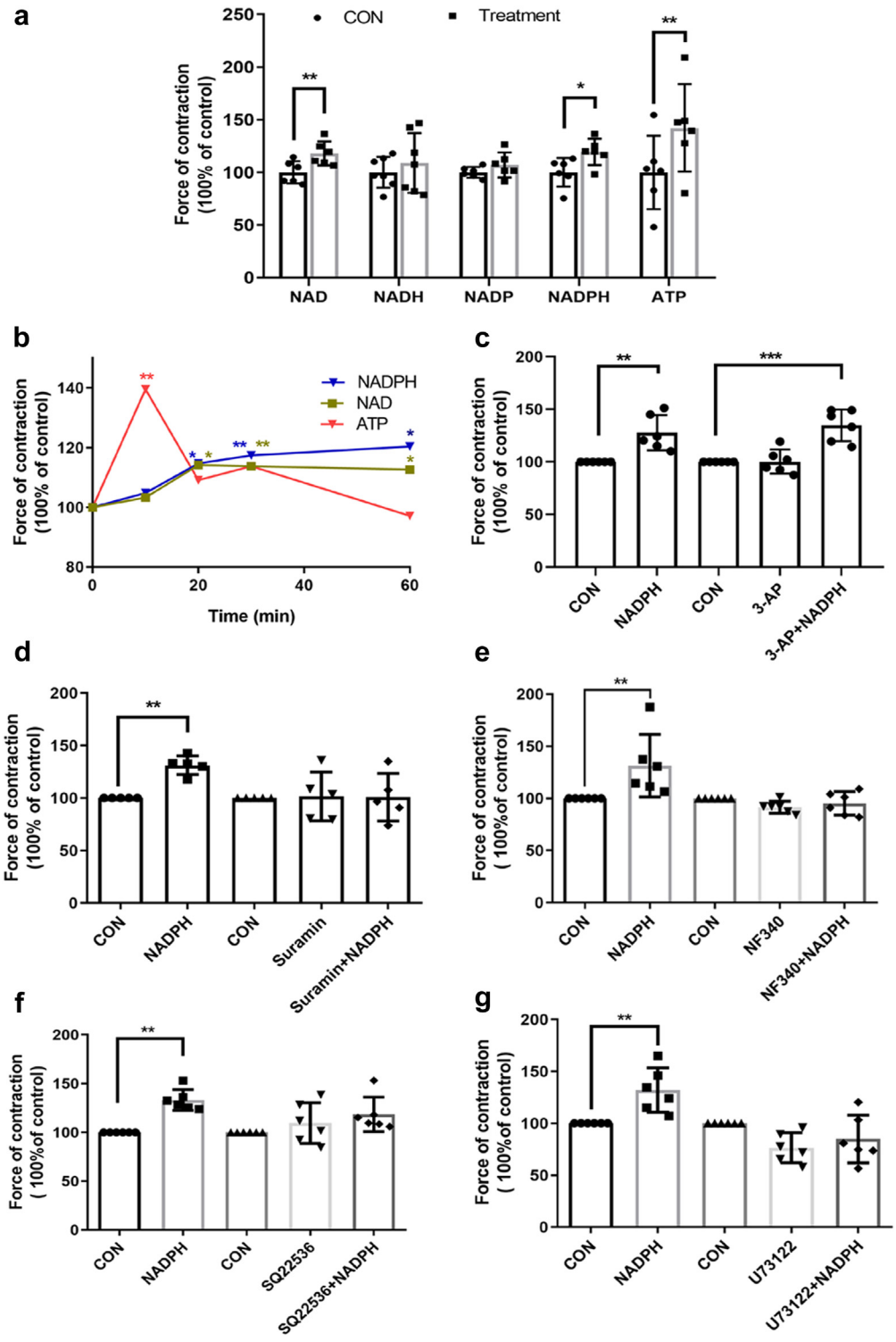

***Fig. 3:* NADPH may exert positive inotropic effects through the P2Y11 receptor-adenylate cyclase/phospholipase pathway. (a).** Effect of NAD (6 μM), NADH (6 μM), NADP (6 μM), NADPH (6 μM) and ATP (50 μM) on cardiac contractility of toad heart. **(b).** The time course of NADPH, NAD, ATP's positive inotropic action. Values were expressed as the percentage increase in force of contraction after administration compared to before administration (CON) in the same toad heart. The difference of force of contraction at each time point after administration and before administration was compared. **(c).** 3-Acetylpyridine (3-AP, 5 mM), a NAD inhibitor, had no effect on the action of NADPH.

investigate whether NADPH stimulates the opening of Pannexin-1 hemichannel to produce positive inotropic effects by increasing extracellular ATP, we treated isolated toad hearts with probenecid and carbenoxolone, both Pannexin-1 antagonists. The results showed that the exposure to probenecid and carbenoxolone did not affect the positive inotropic action of NADPH, indicating that the inotropy of NADPH might not be dependent of Pannexin-1 hemichannels (Supplemental Fig. S3c and d). Previous studies have demonstrated that the P2Y11 receptor agonist AR-C67085 induces positive inotropic effects in cardiomyocytes through adenylate cyclase and phospholipase C.[27] To further investigate the mechanisms underlying NADPH's positive inotropic effects via P2Y11 receptor, we treated the isolated toad hearts with either an adenylate cyclase blocker (SQ22536) or a phospholipase C blocker (U73122). The results showed that the inotropic response of NADPH was absent under the treatment with SQ22536 and U73122, indicating that adenylate cyclase and phospholipase C were involved in the inotropic effect of NADPH (Fig. 3f and g). These findings suggest that NADPH may exert positive inotropic effects through the P2Y11 receptor-adenylate cyclase/phospholipase pathway.

NADPH attenuated isoproterenol (ISO)-induced cardiac hypertrophy in mice

Next, we ask whether exogenous NADPH is effective in rodent pathological cardiac hypertrophy or heart failure models. We thus established isoproterenol (ISO) -induced cardiac hypertrophy model in mice. Mice were injected subcutaneously with ISO (1 mg/kg) every morning and evening for two weeks to induce pathological cardiac hypertrophy. The mice were also administered intraperitoneally with saline or NADPH 1, 2 and 4 mg/kg for 2 weeks. ISO treatment significantly increased the heart weight indices (HWI) and left ventricular indices (LVWI) in mice, while NADPH decreased HWI and LVWI relative to the ISO group (Fig. 4a and b). HE staining showed that NADPH can reduce the cross-sectional areas of cardiomyocytes in hypertrophic myocardium (Fig. 4c). M-mode echocardiography showed that the ejection fraction (EF) and left ventricular fraction (FS) of the ISO group were reduced compared with the control group, indicating that the ISO model deteriorated the cardiac function of mice. NADPH showed a trend to improve cardiac function in mice, although this increase did not reach statistical significance (Supplemental Fig. S4, Supplemental Table S2). We further examined the effect of NADPH on ATP and ATPase in the hypertrophic myocardium induced by ISO. The ATP content was significantly decreased after ISO administration, while the ISO + NADPH could increase the ATP content in the myocardium compared to ISO group (Fig. 4d). Significantly elevated total ATPase activity was also found after NADPH administration (Fig. 4e). These results suggest that NADPH attenuated isoproterenol (ISO)-induced cardiac hypertrophy and improved ATP and ATPase in the hypertrophic myocardium.

Exogenous NADPH increased serum and cardiac NADPH in isoproterenol (ISO)-induced cardiac hypertrophy

Since exogenous NADPH attenuated ISO-induced cardiac hypertrophy in mice, and both NAD and NADPH showed positive inotropic effects in isolated toad hearts, we next intend to investigate whether the effect of NADPH on pathological cardiac hypertrophy is dependent on NAD. We thus administered NAD and NADPH at equimolar concentrations to the ISO-treated mice. The results showed that exogenous NADPH reduced HWI and LVWI, but NAD failed to decrease HWI and LVWI (Fig. 5a and b). Our previous studies have demonstrated that intravenous or intraperitoneal injection of NADPH (2.5–16 mg/kg) in normal mice or rats significantly elevates NADPH concentrations in the blood, brain, or heart.[9,13,39,40] The half-life of NADPH is approximately 6 h in blood and 7 h in brain tissues.[9] In line with these findings, the data presented here indicate that exogenous administration of NADPH increases the NADPH content both in serum (Fig. 5c) and myocardium (Fig. 5d) of ISO mice. However, NAD did not significantly increase NADPH content in either serum or myocardium. Therefore, the lack of inhibitory effect on cardiac hypertrophy by NAD at this dose may be partially attributed to its inability to increase NADPH content in serum or myocardium. Further investigations are warranted to explore the impact of higher doses of NAD on cardiac hypertrophy as well as its effects on serum and myocardial NAPDH content. The above data indicate that in the ISO- induced cardiac hypertrophy model, equimolar dose of NADPH might be superior in efficacy to NAD. Exogenous administration of NADPH increased NADPH content in myocardium and serum of mice.

(d). Suramin (30 μM), an ATP receptor antagonist, abolished the positive inotropic action of NADPH. **(e).** NF340 (10 μM), a P2Y11 receptor selective antagonist, partly abolished the positive inotropic action of NADPH. **(f).** SQ22536 (30 μM), an adenylate cyclase blocker, abolished the positive inotropic action of NADPH. **(g).** U73122 (1 μM), a phospholipase C blocker, abolished the positive inotropic action of NADPH. Bar represents mean ± SD. N = 5–7 toads. (*P < 0.05, **P < 0.01, ***P < 0.001. Paired t test was used to compare the difference after single treatment in the same frog, and one-way ANOVA followed by Newman–Keuls post-hoc test to compare the effect of different regulators on NADPH.)

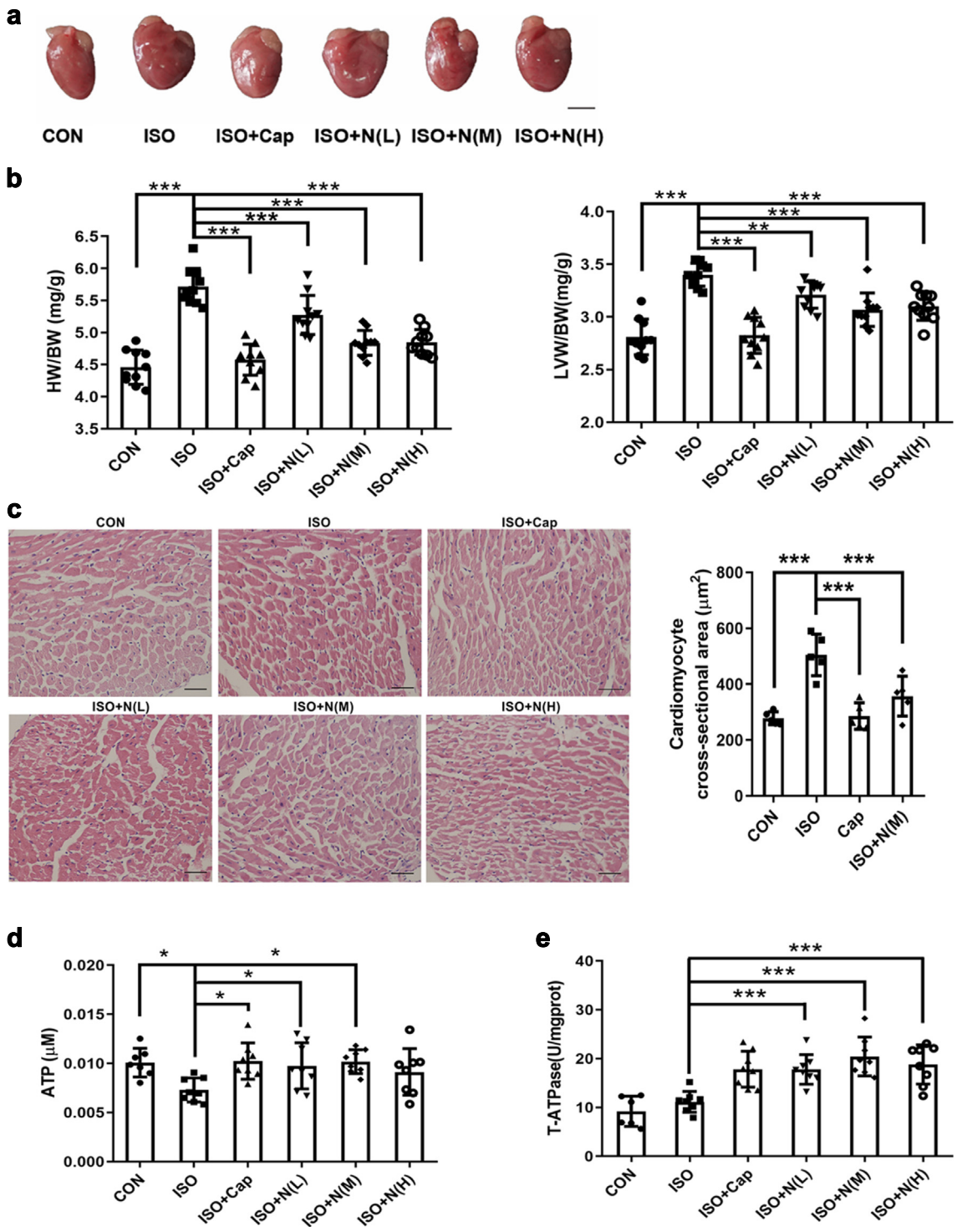

Fig. 4: **NADPH attenuated isoproterenol (ISO)-induced cardiac hypertrophy in mice.** Cardiac hypertrophy was induced by subcutaneous injection with ISO (1 mg/kg, b. i.d.) for 2 weeks. Captopril 100 mg/kg, NADPH 1, 2 and 4 mg/kg were administered intraperitoneally for 2 weeks. N (L) = 1 mg/kg, N (M) = 2 mg/kg, N (H) = 4 mg/kg. **(a).** Representative photographs of hearts showed effects of NADPH on ISO model. Scale = 5.0 mm. **(b).** Effects of NADPH on heart weight indices in ISO induced cardiac hypertrophy. The wet weights of the whole heart and left ventricle were measured. The degree of cardiac hypertrophy was estimated by measuring HWI (HWI = HW/BW) and LVWI (LVWI = LVW/BW).

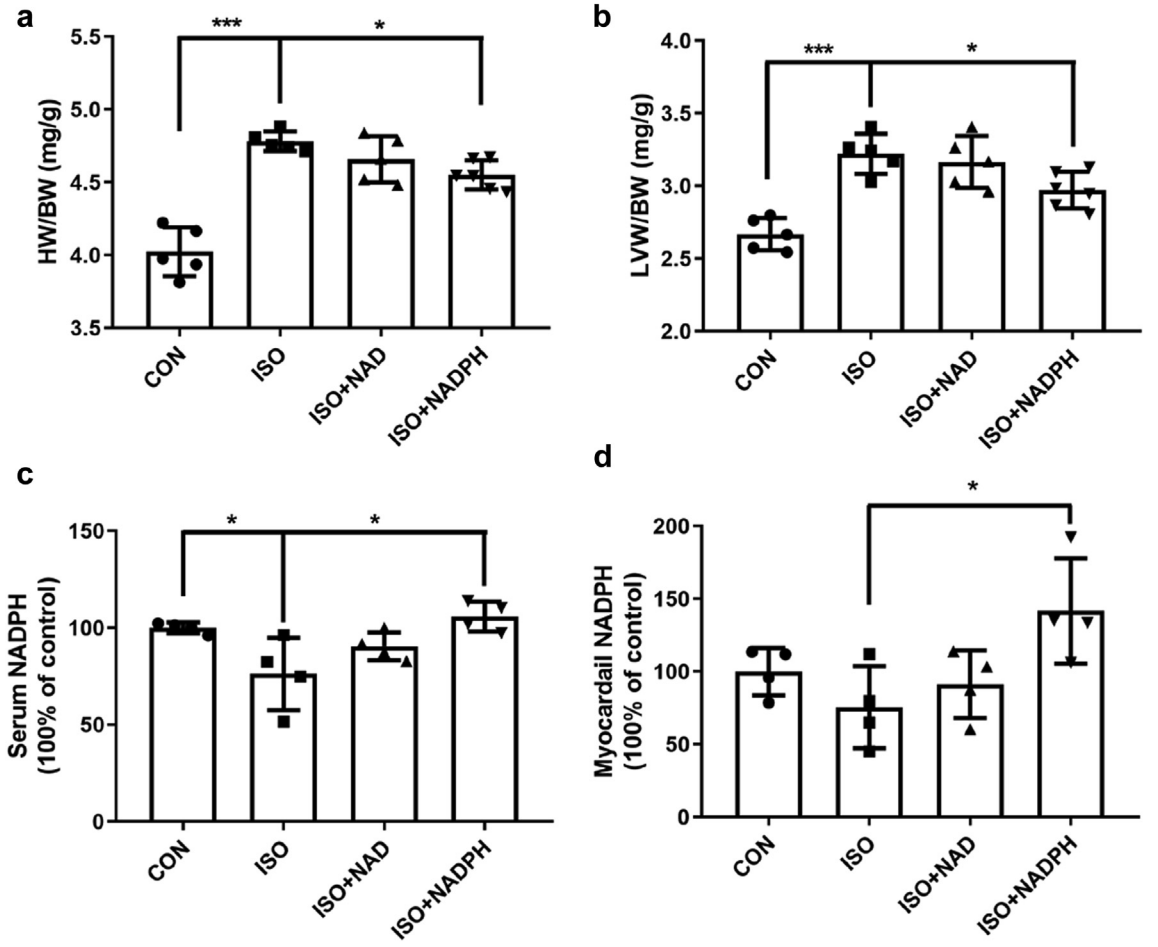

Fig. 5: **Exogenous NADPH increased serum and cardiac NADPH in isoproterenol (ISO)- induced cardiac hypertrophy.** Cardiac hypertrophy was induced by subcutaneous injection with ISO (1 mg/kg, b. i.d.) for 2 weeks. NADPH 2 mg/kg (2.4 μmol/kg) and NAD 1.6 mg/kg (2.4 μmol/kg) were administered intraperitoneally for 2 weeks. **(a, b).** Effects of NADPH on heart weight indices in ISO induced heart failure. The wet weights of the whole heart and left ventricle were measured. The degree of cardiac hypertrophy was estimated by measuring the HWI (HWI = HW/BW) **(a)** and LVWI (LVWI = LVW/BW) **(b)**. **(c, d).** The level of NADPH in serum and myocardium were examined. **(c).** Exogenous NADPH increased NADPH content in serum. **(d).** Exogenous NADPH increased NADPH content in myocardium. Bar represents mean ± SD. (N = 4–6 mice, $^{*}P < 0.05$, $^{***}P < 0.001$, one-way ANOVA followed by Newman–Keuls post-hoc test).

NADPH prevented heart failure induced by transverse aortic constriction (TAC) in mice

Pressure overload-induced cardiac hypertrophy tends to progress into heart failure in the late stage.[5] Mice with heart failure exhibited reduced cardiac function, pulmonary congestion and decreased exercise tolerance.[41] We established a pressure overload-induced cardiac hypertrophy and heart failure in mice through transverse aortic constriction (TAC). The TAC mice were intraperitoneally administered with NADPH (2 mg/kg) daily for 30 consecutive days. The maximal exercise capacity of mice was assessed through forced wheel running.[41,42] The data indicated a significant decrease in forced running time and distance of TAC mice at 18 and 28 days post-surgery, while NADPH treatment significantly improved these parameters (Supplemental Fig. S5a and b). Furthermore, TAC mice exhibited increased HWI, LVWI, and lung weight index compared

N = 10 mice. **(c).** Representative sections showing NADPH reduced cardiomyocyte cross-sectional area. The left ventricles from each group were stained with hematoxylin and eosin. Scale = 50 μm. N = 5 mice. **(d, e).** The levels of ATP and T-ATPase in myocardium were examined. **(d).** Effects of NADPH on ATP level. **(e).** Effects of NADPH on the activity of total ATPase. N = 6–8 mice. Bar represents mean ± SD. ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, one-way ANOVA followed by Newman–Keuls post-hoc test).

to the sham group; however, NADPH administration effectively reduced heart weight indices and lung weight index relative to the TAC group (Fig. 6a and b, Supplemental Fig. S5c and d). Echocardiographic assay showed that the cardiac ejection fraction (EF) and left ventricular fraction shortening (FS) of TAC mice decreased by about 30%, indicating a significant decrease in cardiac function, while TAC + NADPH treatment significantly increased the EF and FS relative to TAC group (Fig. 6d, Supplemental Table S3). HE staining showed that NADPH significantly improved the histopathological changes in myocardium and reduced the cross-sectional area of cardiomyocytes (Fig. 6c). It has been reported that AKT and ERK1/2 signaling involved in protein synthesis, are significantly upregulated in pressure overload cardiac hypertrophy.[43,44] Consistently, Western blotting assay revealed that the phosphorylation of AKT and ERK1/2 was significantly increased in the myocardium of TAC mice, while NADPH reduced the phosphorylation of AKT and ERK1/2 induced by TAC (Supplemental Fig. S6a and b). The above results indicate that NADPH efficiently attenuated TAC –induced cardiac hypertrophy and heart failure.

NADPH improved oxidative stress and energy metabolism in heart failure induced by TAC

Reactive oxygen species (ROS) are mainly derived from free radicals produced by mitochondrial respiration, and effective removal of ROS might relieve the progression of pathological cardiac hypertrophy to heart failure.[4,45] We thus examined the lipid peroxidation product malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) in the serum and myocardium of TAC mice to indicate ROS. The MDA and H_2O_2 in the serum or myocardium were found to be significantly increased in the TAC group, while the TAC + NADPH(H) group could reduce TAC-induced increase in MDA or H_2O_2 (Fig. 7a and b). Reduced glutathione as a product of NADPH can effectively eliminate ROS. Consistently, myocardial GSH was significantly decreased in the TAC group, while TAC + NADPH treatment increased GSH in myocardium compared to TAC group (Fig. 7c). Moreover, similar to the effect of NADPH in ISO induced cardiac hypertrophy, the TAC + NADPH treatment could alleviate TAC-induced decrease in myocardial ATP content (Fig. 7d). All these results suggest that NADPH could improve oxidative stress and energy metabolism in heart failure induced by TAC.

NADPH alleviated mitochondrial damage and improved mitochondrial respiration in hypertrophic myocardium

Mitochondrial dysfunction elicited by heart failure leads to increased mitochondrial ROS production, and the dysfunctional mitochondria cannot efficiently synthesize and transfer energy.[46] Mitochondria produce ATP for energy mainly through the mitochondrial respiratory chain, and the stability and integrity of the mitochondrial respiratory chain reflect mitochondrial function.[35,46] We thus measured myocardial mitochondrial respiration and oxygen consumption in TAC mice using a High Resolution Respirometry O2K.[34] The results showed a significant reduction in maximum phosphorylated respiratory capacity (OXPHOS CI + CII), maximum electron transport system-uncoupled respiratory capacity (ETS CI + CII), and mitochondrial complex IV respiratory function in hypertrophic myocardium, whereas NADPH treatment significantly upregulated OXPHOS CI + CII, ETS CI + CII, and mitochondrial complex IV respiratory function (Fig. 8a and b). These findings suggest that NADPH can improve the mitochondrial electron respiratory chain complex and mitochondrial oxygen consumption. Additionally, we observed the effect of NADPH on mitochondrial ultrastructure in the myocardium of TAC mice by electron microscope. The myocardium in the sham-operated group showed continuous and regularly spaced myofilaments with serially arranged normal mitochondria. In contrast, the sarcomere of the myocardium of TAC mice exhibited disorganized arrangement and discontinuous rupture. Some mitochondrial cristae were broken, perforated, or even disappeared. However, exogenous administration of NADPH significantly improved myofilament damage and reduced mitochondrial damage in hypertrophy myocardium (Fig. 8c). These results indicate that NADPH alleviated mitochondrial damage and improved mitochondrial respiration in hypertrophic myocardium.

NADPH increased SIRT3 expression and deacetylated target proteins in TAC-induced heart failure

The dysregulation of SIRT3 located in mitochondria and its downstream effectors are involved in the pathogenesis of pathological cardiac hypertrophy and heart failure.[47] To determine whether NADPH acts on mitochondrial SIRT3 to regulate mitochondrial function, we examine the protein expression and activity of SIRT3 in the myocardium of TAC mice. The data showed that TAC treatment significantly decreased SIRT3 expression and activity compared to sham group, while SIRT3 protein levels and activity were upregulated in TAC + NADPH(H) group (Fig. 9a and b). SIRT1 is also known to have high abundance in the heart.[48] However, there was no significant difference in SIRT1 expression among all groups (Supplemental Fig. 7). Since SIRT3 is mainly located in mitochondria and is responsible for the deacetylation of many proteins,[49] we thus examined the degree of pan acetylation of cardiac proteins in different groups. Compared with the sham group, TAC treatment significantly increased the level of protein acetylation. However, the degree of protein acetylation in the TAC + NADPH(H) group decreased

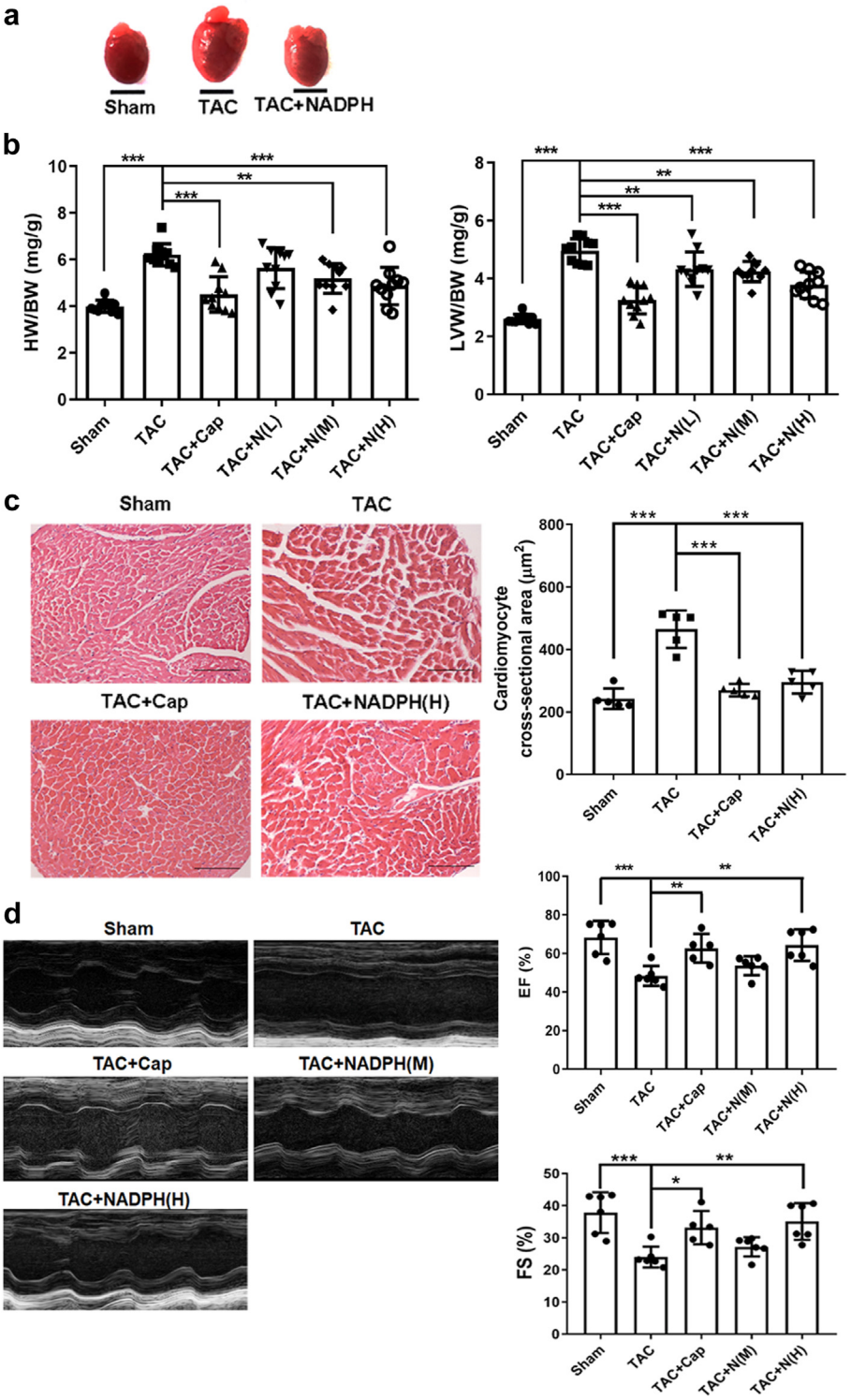

Fig. 6: **NADPH prevented heart failure induced by transverse aortic constriction (TAC) in mice.** Mice were subjected to TAC model for 30 days. NADPH 1, 2 and 4 mg/kg and Cap 15 mg/kg were administered intraperitoneally for 30 days. N (L) = 1 mg/kg, N (M) = 2 mg/kg, N (H) = 4 mg/kg. **(a).** Representative photographs of hearts showed effects of NADPH(H) on TAC model. Scale = 5.0 mm. **(b).** Effects of NADPH on heart weight indices in TAC model. The wet weights of the whole heart and left ventricle were measured. The degree of cardiac hypertrophy was estimated by measuring the HWI (HWI = HW/BW) and LVWI (LVWI = LVW/BW). N = 10 mice. **(c).** Representative sections showing NADPH

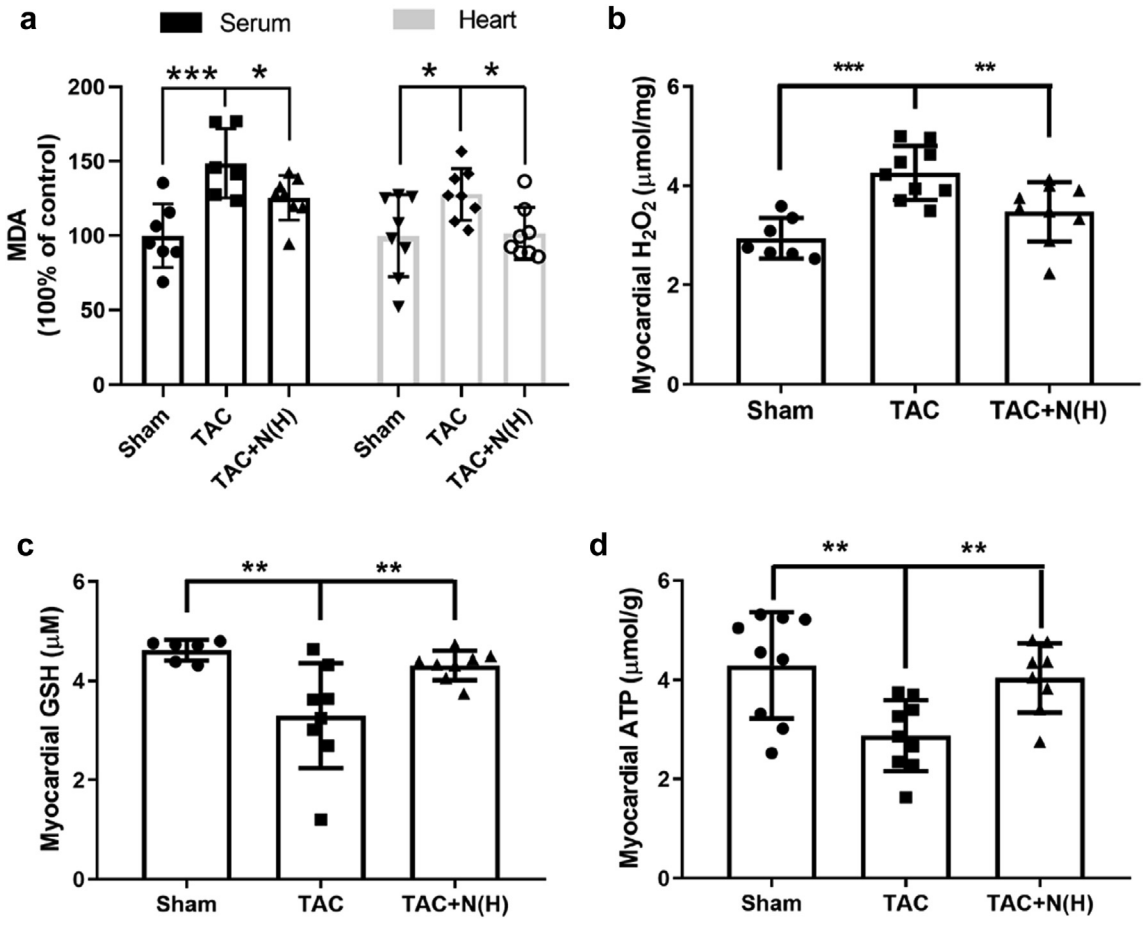

Fig. 7: **NADPH improved oxidative stress and energy metabolism in TAC-induced heart failure in mice.** Mice were subjected to TAC model for 30 days. NADPH 4 mg/kg were administered intraperitoneally for 30 days. N (H) = 4 mg/kg. **(a).** Effects of NADPH on malondialdehyde (MDA) level in serum and myocardium. **(b).** Effects of NADPH on H_2O_2 level in myocardium. **(c).** Effects of NADPH on GSH level in myocardium. **(d).** Effects of NADPH on ATP level in myocardium. Bar represents mean ± SD, N = 6–9 mice. (*P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Newman–Keuls post-hoc test).

(Fig. 9c), indicating that NADPH can effectively reduce protein acetylation induced by TAC.

SOD2, a superoxide dismutase located on mitochondria, whose activity is mainly regulated by reversible post-translational acetylation, is one of the substrates of SIRT3.[50] Western blotting assay showed that the acetylation of SOD2 (K68) was significantly increased in the TAC group, while TAC + NADPH downregulated the acetylation of SOD2 (Fig. 9d). The results suggest that NADPH may activate SIRT3 to deacetylate SOD2, thereby preventing oxidative stress during pathological cardiac hypertrophy.

ATP5A1 is a subunit of ATP synthase and SDHA is the subunit of succinate dehydrogenase in the tricarboxylic acid cycle, both of which are substrates of SIRT3 and play important roles in the oxidative phosphorylation of mitochondrial respiration.[51,52] The immunoprecipitation assay showed that the acetylation of ATP5A1 and SDHA were significantly increased in the TAC group compared to sham group, while TAC + NADPH (H) treatment significantly downregulated the acetylation of ATP5A1 and SDHA (Fig. 9e and f).

Liver kinase B1 (LKB1) is one of the substrates of SIRT3. SIRT3 can deacetylate LKB1, which is then

reduced cardiomyocyte cross-sectional area. The left ventricles from each group were stained with hematoxylin and eosin. Scale = 50 μm. N = 5 mice. **(d).** Representative M-mode echocardiography showing that NADPH significantly increased the LV ejection fraction (EF) and LV fraction shortening (FS). N = 5–6 mice. Bar represents mean ± SD. (*P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Newman–Keuls post-hoc test).

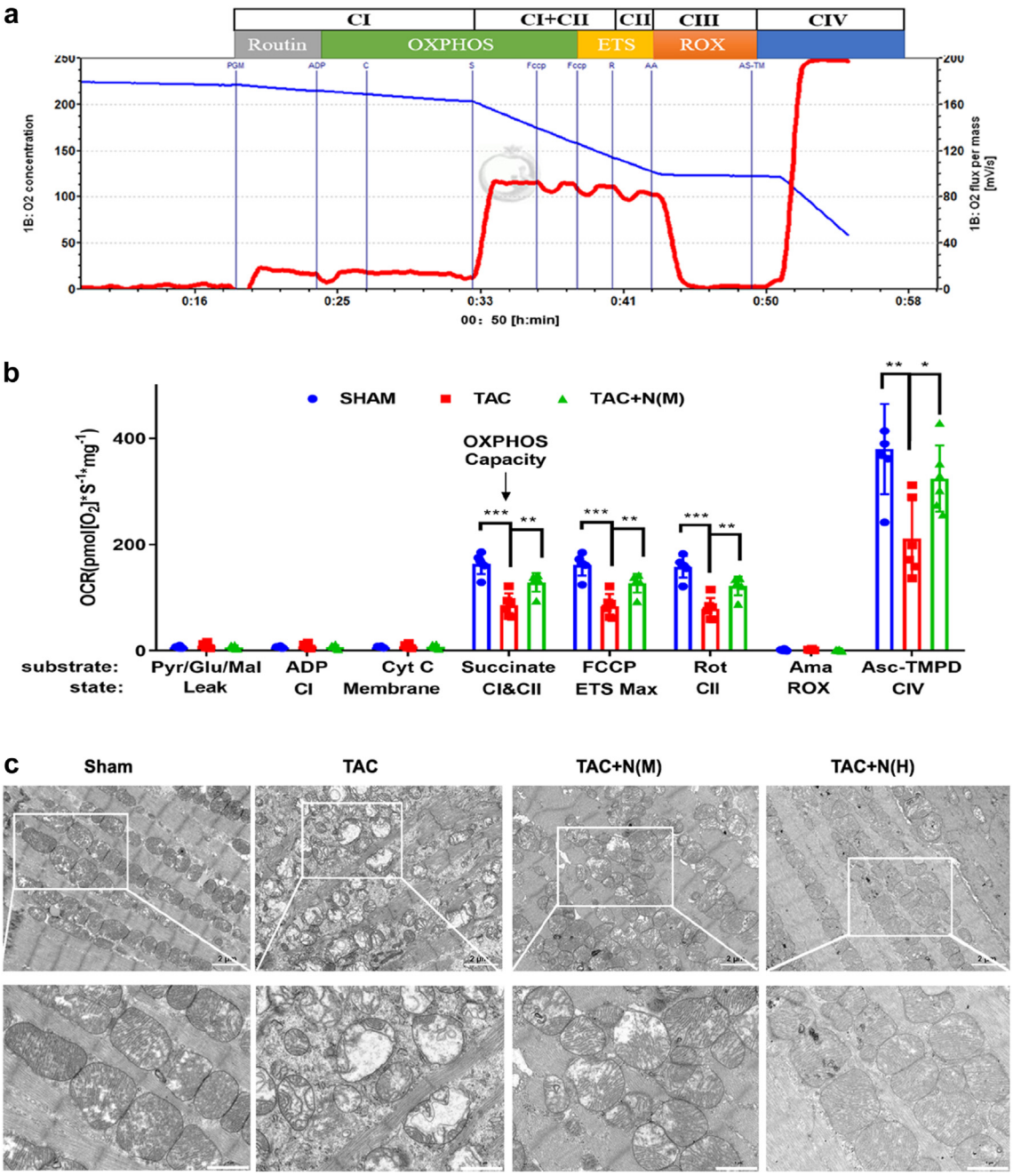

Fig. 8: **NADPH alleviated mitochondrial damage and improved mitochondrial respiration in hypertrophic myocardium.** Mice were subjected to TAC model for 30 days. NADPH 2 and 4 mg/kg were administered intraperitoneally for 30 days. N (M) = 2 mg/kg, N (H) = 4 mg/kg. Mitochondrial respiratory function was assessed by O2K. **(a).** Representative trace of a respirometry assay. Chamber O_2 content is represented in blue while the rate of O_2 consumption per mg tissue is in red. **(b).** Mitochondrial respiratory function and O_2 consumption rate were evaluated as indicated in the Method. n = 6 mice. Bar represents mean ± SD. (*P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Newman–Keuls post-hoc test). **(c).** Transmission electron microscopy analysis of mitochondria in heart showing that NADPH ameliorated mitochondrial damage. Scale bar = 1 μm. n = 3 mice.

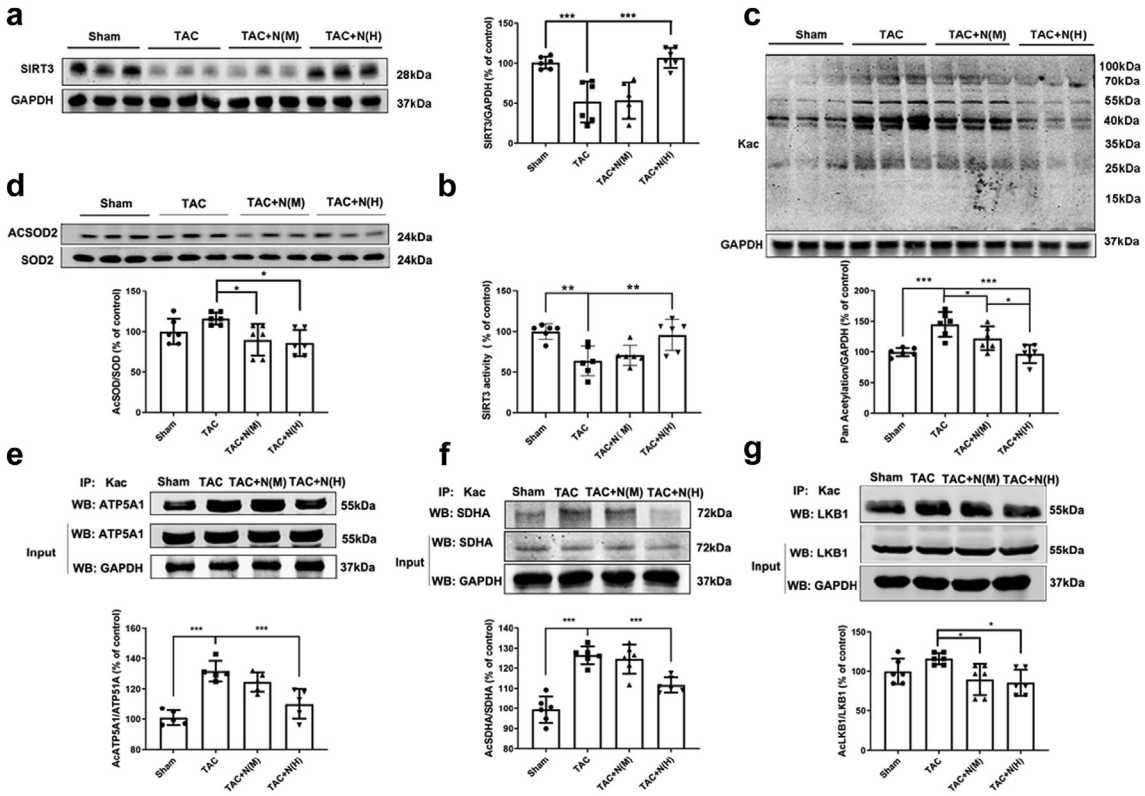

Fig. 9: **NADPH increased SIRT3 expression and deacetylated target proteins in TAC-induced heart failure.** Mice were subjected to TAC model for 30 days. NADPH were administered intraperitoneally for 30 days. N (M) = 2 mg/kg, N (H) = 4 mg/kg. **(a).** NADPH upregulated the protein expression of SIRT3. **(b).** NADPH upregulated SIRT3 activity. **(c).** NADPH reduced the pan acetylation. **(d).** NADPH reduced SOD2 acetylation. Protein levels of SIRT3, pan acetylation, SOD2 and AcSOD2 in myocardium were determined by Western blotting analysis. Levels of GAPDH were used as the loading control. **(e).** NADPH downregulated ATP5A1 acetylation. **(f).** NADPH downregulated SDHA acetylation. **(g).** NADPH downregulated LKB1 acetylation. The left ventricle lysates were immunoprecipitated with acetylated-lysine antibody (Kac). Then the immunoprecipitates were analyzed by Western blot analysis with anti-ATP5A1, anti-SDHA or anti-LKB1. Bar represents mean ± SD. N = 5–6 mice. (*P < 0.05, **P < 0.01, ***P < 0.001, one-way ANOVA followed by Newman–Keuls post-hoc test).

phosphorylated to activate the downstream AMPK,[53,54] and the activation of AMPK could prevent pathological cardiac hypertrophy via improvement of myocardial energy metabolism and inhibition of protein synthesis.[55] We therefore investigated the effect of exogenous NADPH administration on the LKB1-AMPK pathway in TAC mice. Compared with the sham group, the LKB1 protein acetylation in the TAC group was increased, while the LKB1 acetylation in the TAC + NADPH groups was significantly downregulated relative to TAC group (Fig. 9g). Consistently, the phosphorylation of LKB1 in the TAC group was significantly reduced, while TAC + N(H) treatment inhibited the downregulation of LKB1 phosphorylation induced by TAC (Supplemental Fig. S8a). In addition, AMPK phosphorylation was significantly reduced in the TAC group, whereas the TAC + NADPH treatment increased the AMPK phosphorylation (Supplemental Fig. S8b). These results imply that exogenous NADPH administration may prevent pathological cardiac hypertrophy and heart failure by activating the SIRT3-LKB1-AMPK pathway in hypertrophic myocardium.

The above results indicate that NADPH upregulates SIRT3 expression and activity, and deacetylates the target proteins including SOD2, ATP5A1, SDHA and LKB1, which may then improve oxidative stress and energy metabolism during pathological cardiac hypertrophy and heart failure.

The positive inotropic action and anti-heart failure effect of NADPH might depend on SIRT3

We then ask whether the cardiotonic and anti-heart failure effects of NADPH depend on SIRT3. We applied 3-TYP, a specific inhibitor of SIRT3,[56] on the isolated toad heart. Consistent with the previous results, NADPH increased the myocardial contractility of isolated toad heart, but 3-TYP pretreatment abolished the positive inotropic effect of NADPH (Supplemental Fig. 9), indicating that the positive inotropic effect of NADPH may depend on SIRT3. We also introduced

SIRT3 KO mice, and constructed TAC-induced heart failure model in WT and SIRT3KO mice. The mice were intraperitoneally injected with 2 mg/kg of NADPH daily for 30 days. Similar to the previous results, the hearts of WT and SIRT3KO mice undergoing TAC showed significant cardiac hypertrophy, and HWI and LVWI increased significantly. NADPH administration prevented TAC-induced cardiac hypertrophy and reduced HWI and LVWI in WT mice, but this phenotype was not observed in the SIRT3KO + TAC + NADPH group (Fig. 10a and b). The HE staining results showed that NADPH significantly improved the pathological damage of myocardium induced by TAC in WT mice, and reduced the cross-sectional area of cardiomyocytes, but NADPH could not reduce the cross-sectional area of cardiomyocytes in SIRT3 KO mice (Fig. 10c). The data of echocardiography showed that pressure overload significantly reduced ejection fraction (EF) and fraction shortening (FS) in both WT and SIRT3 KO mice. After administration of NADPH, the EF and FS of WT mice increased significantly. However, NADPH could not improve the reduction of EF and FS induced by TAC in SIRT3 KO mice (Fig. 10d, Supplemental Table S4). The above results show that SIRT3 plays a key role in the positive inotropic action and anti-heart failure effects of NADPH.

Discussion

Our lab previously reported that exogenous NADPH administration exhibits neuroprotection in rodent and monkey cerebral ischemia-reperfusion models. NADPH not only reduces the cerebral infarct volume, but also improves the long-term survival and neurobehavioral function of ischemic stroke animals.[9–12] Our recent work further demonstrated that exogenous NADPH protects against myocardial ischemia/reperfusion injury in rats.[13] Based on these studies, we thus investigate the effects of exogenous NADPH on pathological cardiac hypertrophy and heart failure. First, we collected blood samples from age and sex-matched normal people and patients with heart failure. The plasma NADPH content in patients with reduced cardiac function were much lower than that in normal people. Similarly, a decline in serum NADPH was also found in the ISO or TAC-induced cardiac hypertrophy or heart failure. We then investigate the effects of NADPH on isolated hearts and pathological cardiac hypertrophy and heart failure models. Exogenous NADPH treatment exhibited a relative long-lasting cardiac positive inotropic action both in isolated hearts and intact animals. Exogenous NADPH administration also significantly alleviated ISO and TAC induced cardiac hypertrophy or heart failure, reduced the heart weight indices, improved the cardiac function, and improved pathological injury in hypertrophic myocardium. These data indicate that exogenous NADPH administration might effectively prevent pathological cardiac hypertrophy or heart failure.

NADPH plays a dual role in regulating oxidative stress and energy metabolism.[6] NADPH functions not only as a reducing agent for the removal of reactive oxygen species (ROS) through the generation of reduced glutathione (GSH), but it also indirectly contributes to energy production by facilitating ATP synthesis.[57] Consistently, our results found that NADPH and ATP both increased cardiac contractility, while ATP receptor antagonists abolished the positive inotropic action of NADPH. NADPH may exert positive inotropic effects through the P2Y11 receptor-adenylate cyclase/phospholipase pathway. Additionally, NADPH significantly decreased the serum and cardiac content of MDA or H_2O_2, while NADPH increased the levels of GSH, ATP and ATPase in the hypertrophic myocardium. These data suggest that the administration of exogenous NADPH to stressed cells may maintain the amount of cofactor necessary to the metabolic processes, thereby helping the antioxidant defense, and sparing high-energy phosphates that in turn can improve contractile function.

Energy metabolism, ATP and ROS production are mainly derived from mitochondrial respiration.[58] Pathological cardiac hypertrophy or heart failure causes significant mitochondrial damage and dysfunction, manifested by disorganized cristae and vacuolization of mitochondria, decreased mitochondrial enzyme activity, impaired mitochondrial electron transport chain, reduced ATP production and increased ROS formation, leading to energy deficiency and oxidative stress.[31,55,59,60] Based on our current data, NADPH increased the maximum phosphorylated respiratory capacity (OXPHOS CI + CII), maximum electron transport system-uncoupled respiratory capacity (ETS CI + CII), and mitochondrial complex IV respiratory function in hypertrophic myocardium. Additionally, NADPH significantly mitigated mitochondrial damage in myocardium. Collectively, these results suggest that NADPH can ameliorate mitochondria damage, improve mitochondrial oxygen consumption rate and mitochondrial respiratory function to enhance energy metabolism in hypertrophic myocardium.

Sirtuins (SIRT) is a class III histone deacetylase with seven members in mammals (SIRT1-SIRT7), and SIRT1 and SIRT3 are predominantly found in heart.[48] SIRT1 is mainly present in the cytoplasm and nucleus, while SIRT3 is mainly located in the mitochondria.[61] Cardiac SIRT3 activity is reduced in heart failure, and mitochondrial proteins are highly acetylated and inactivated.[62,63] Therefore, the deregulation of SIRT3 and its downstream effectors may be the key link between mitochondrial dysfunction and heart failure. We thus investigated whether the anti-heart failure effect of NADPH is related to SIRT3. The data showed that the expression and activity of SIRT3 was significantly downregulated in the myocardium of TAC mice, whereas exogenous administration of NADPH could

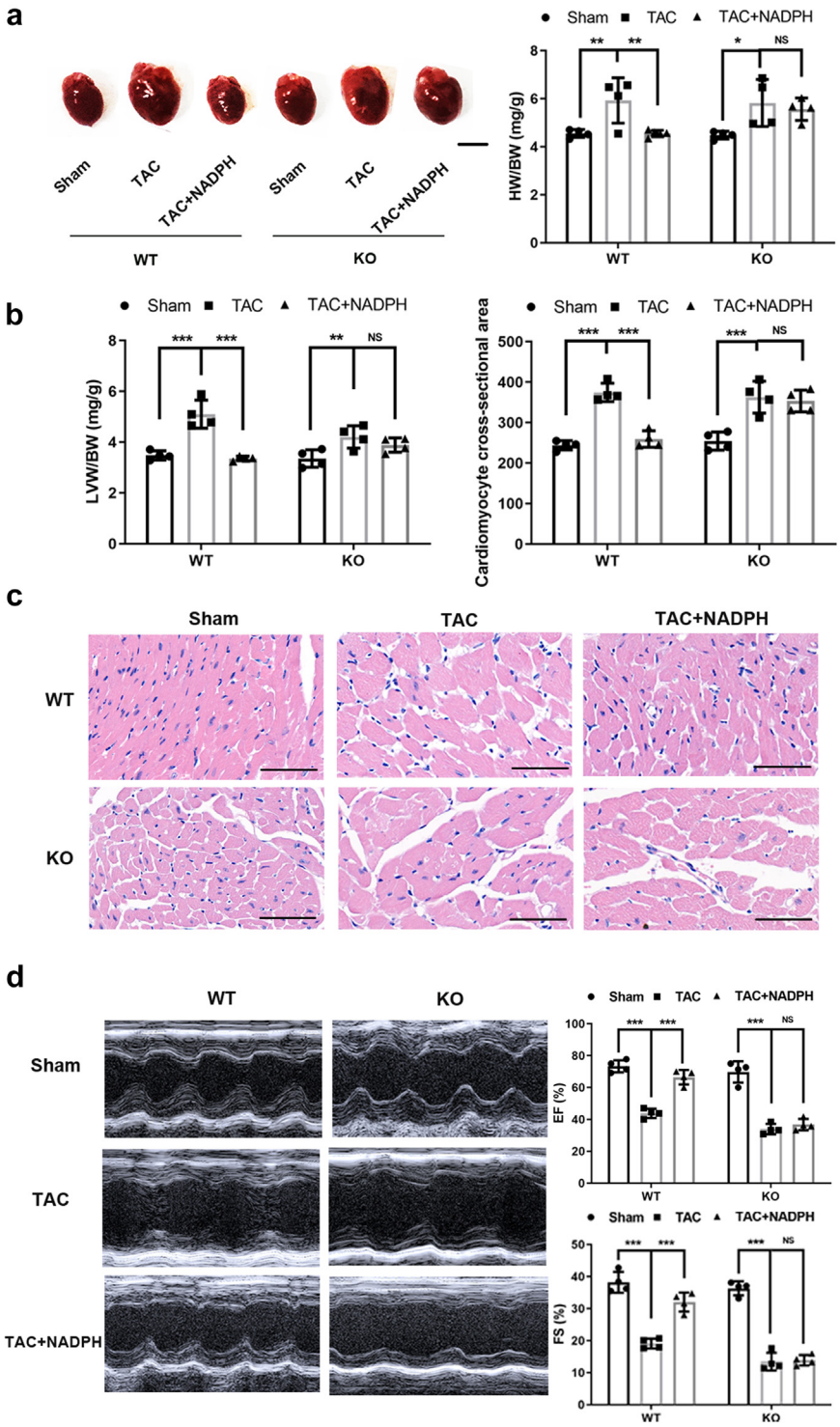

***Fig. 10*: The anti-heart failure effect of NADPH might depend on SIRT3.** Wild-type (WT) and SIRT3KO mice were subjected to TAC model for 30 days. NADPH 2 mg/kg were administered intraperitoneally for 30 days. **(a).** Representative photographs showed the hearts of WT and SIRT3KO mice undergoing TAC model and NADPH administration. Scale = 5.0 mm. **(b).** Effects of NADPH on heart weight indices in WT and SIRT3KO mice subjected to TAC model. The wet weights of the whole heart and left ventricle were measured. The degree of cardiac hypertrophy was estimated by measuring the HWI (HWI = HW/BW) and LVWI (LVWI = LVW/BW). **(c).** Representative sections showing NADPH reduced cardiomyocyte cross-sectional area. The left ventricles from each group were stained with hematoxylinand eosin. Scale = 50 μm. **(d).**

increase the expression and activity of SIRT3 and reduce pan-acetylation.

The upregulation of SIRT3 by NADPH may increase ATP production in two ways. First, SIRT3 can deacetylate some key enzymes in the mitochondria involved in respiratory chain such as NADH dehydrogenase ubiquinone subcomplex subunit 9 (NDUF9A), succinate dehydrogenase A (SDHA), pyruvate dehydrogenase (PDH), ATP5A1 and other ATP synthase subunits to improve mitochondrial respiration efficiency and maintain ATP production.[31,64–67] The data showed that, SDHA and ATP5A1 were highly acetylated in the TAC group, while NADPH could significantly reverse this phenotype. These results indicate that NADPH may enhance ATPase activity and increase ATP synthesis through SIRT3-mediated deacetylation of SDHA and ATP5A1. Second, NADPH may increase ATP content by activating SIRT3-LKB1-AMPK pathway. AMPK (AMP-dependent protein kinase), an intracellular energy sensor, is activated in the presence of ATP deficiency and increased AMP concentration during nutrition deprivation or hypoxia.[68,69] The activated AMPK then regulates the balance of multiple decomposition and synthesis signal pathways, ultimately elevating ATP content. Our previous study demonstrated that NADPH can activate the AMPK-mTOR pathway to protect the heart from ischemia-reperfusion injury.[13] LKB1 with the serine/threonine kinase activity is one of the upstreams of AMPK.[70,71] Post-translational modifications of LKB1 include phosphorylation and acetylation, and LKB1 is one of the substrates of SIRT3.[53] In the present study, we examined the degree of acetylation and phosphorylation of LKB1, respectively. The results showed that NADPH decreased the acetylation of LKB1, but increased the phosphorylation of LKB1 and AMPK. We thus tentatively conclude that NADPH initiates the SIRT3-LKB1 pathway, which then activates AMPK, thus balancing decomposition and synthesis reactions to increase ATP.

Upregulation of SIRT3 via NADPH also serves to enhance ROS clearance. The accumulation of ROS and its metabolites, leading to oxidative stress, is a significant contributor to pathological cardiac hypertrophy or heart failure,[4,45] while SIRT3 is one of the pivotal regulators of oxidative stress.[50] Manganese-dependent superoxide dismutase (MnSOD), also known as SOD2, is an enzyme that scavenges excess ROS in mitochondria. The activity of SOD2 is regulated by reversible post-translational modification-acetylation by SIRT3.[72,73] Honokiol can activate SIRT3, which in turn deacetylate SOD2 into an activated state capable of performing the function of ROS clearance.[63] Similarly, our findings indicate that NADPH significantly upregulates SIRT3 expression, which is downregulated under pressure overload. Furthermore, NADPH reduces the degree of acetylation of SOD2 at the K68 position. These results indicate that the exogenous administration of NADPH may protect the failing heart from oxidative stress by upregulating SIRT3 to deacetylate SOD2.

We further applied pharmacological and genetic approaches of SIRT3 to validate whether the anti-heart failure effect of NADPH depends on SIRT3. In isolated toad heart, the inhibitor of SIRT3, 3-TYP, abolished the positive inotropic action of NADPH on heart, suggesting that exogenous administration of NADPH many provide inotropic action via SIRT3. We then investigated the effect NADPH on pressure overload induced heart failure in SIRT3KO mice. The TAC model resulted in significant cardiac hypertrophy and reduced cardiac function, while exogenous administration of NADPH effectively reversed pathological cardiac hypertrophy and partially restored cardiac function in wild-type mice. However, in SIRT3 Knockout mice, exogenous administration of NADPH could not rescue TAC induced cardiac hypertrophy and heart failure. These data suggest that NADPH may exert positive inotropic action and anti-heart failure effect via SIRT3.

Although this study indicates that exogenous administration of NADPH may prevent pathological cardiac hypertrophy or heart failure by activating SIRT3, it should be noted that there are still some limitations in this work. First, patients with heart failure may present with various etiologies, including ischemic heart disease, hypertensive heart disease, valvular and rheumatic heart failure, cardiomyopathy, etc. However, these causes are not mutually exclusive and can coexist.[38] Due to the challenge of obtaining a sufficient sample size of clinical patients with a single cause of heart failure, this study included patients with heart failure (NYHA functional class II-IV) who had multiple co-morbidities such as coronary artery disease, hypertension, dilated cardiomyopathy, or pulmonary hypertension. Some patients also exhibited arrhythmias such as atrial flutter, atrial fibrillation, ventricular premature beat or short ventricular tachycardia. Conducting of large-scale clinical case studies to differentiate alterations in plasma NADPH levels among patients with heart failure of diverse etiologies would be highly valuable and significant for future investigations.

Representative M-mode echocardiography showing the effects of NADPH on LV ejection fraction (EF) and LV fraction shortening (FS) of WT and SIRT3KO mice subjected to TAC model. N = 4–5 mice. Bar represents mean ± SD. (*P < 0.05, **P < 0.01, ***P < 0.001, two-way ANOVA followed by Newman–Keuls post-hoc test).

Secondly, although our previous[9,13,39,40] and current studies have shown the exogenous administration of NADPH significantly increased the NADPH content in serum and heart, it is unclear how exogenous NADPH penetrates cell membrane. Purinoreceptors such as cell membrane P2X (ion channels) and P2Y (G-protein coupled) receptors are known to bind extracellular ATP and initiate downstream signaling cascades that play a crucial role in cardiac hypertrophy and heart failure pathology.[74] Recent studies have demonstrated that NAD can penetrate cell membranes via P2X7-R[75] or connexin 43.[76] Therefore, it is plausible to speculate that NADPH may enter cells through a similar mechanism as NAD, or potentially an unknown transporter or receptor on the cell membrane. Our unpublished preliminary data indicates that antagonism or knockdown of P2X7-R effectively abolishes the entry of NADPH into HEK 293-T cells (data not shown). Interestingly, this study also revealed that antagonists of P2Y11 receptor such as suramin and NF340, along with the adenylate cyclase blocker SQ22536 and the phospholipase C blocker U73122, abolished the positive inotropic effects of NADPH. This suggests that NADPH may exert positive inotropic effects through the P2Y11 receptor-adenylate cyclase/phospholipase pathway. However, since small molecule inhibitors may have off-target effects, future genetic intervenes targeting P2Y11 or P2X7 are necessary for further verification of these purinoreceptors' involvement in transmembrane transport of NADPH as well as its positive inotropy and anti-cardiac hypertrophy effects.

Furthermore, the investigation of mitochondrial function in SIRT3KO mice was not conducted in both sham and TAC models due to the observed higher mortality rates in the TAC model. However, previous studies have indicated impaired cardiac function and mitochondrial respiration in SIRT3KO mice with TAC or angiotensin II-induced cardiac hypertrophy.[77,78] Further exploration is warranted to examine the impact of NADPH on mitochondrial function in SIRT3KO mice subjected to TAC model. Furthermore, it remains unclear whether the anti-cardiac hypertrophy effect of NADPH is a result of direct or indirect regulation of SIRT3. The mechanism by which NADPH activates and increases SIRT3 expression in mice with cardiac hypertrophy requires further investigation. A recent study has demonstrated that exogenous NADPH can directly interact with histone deacetylase 3 (HDAC3) to inhibit its activation in adipocytes and tumor cells.[79] In our follow-up experiments, we intend to employ isotope or biotin-labeled NADPH, DARTS (Drug Affinity Responsive Target Stability) or CETSA (Cellular Thermal Shift Assay) assay to analyze the interaction between NADPH and SIRT3. Through these experiments, we aim to elucidate the specific target of NADPH in cardiomyocytes.

Taken together, these results indicate that exogenous NADPH exerts a positive inotropic action through the P2Y11 receptor-adenylate cyclase/phospholipase pathway. Additionally, NADPH prevents pathological cardiac hypertrophy or heart failure. NADPH may improve mitochondrial function to increase ATP

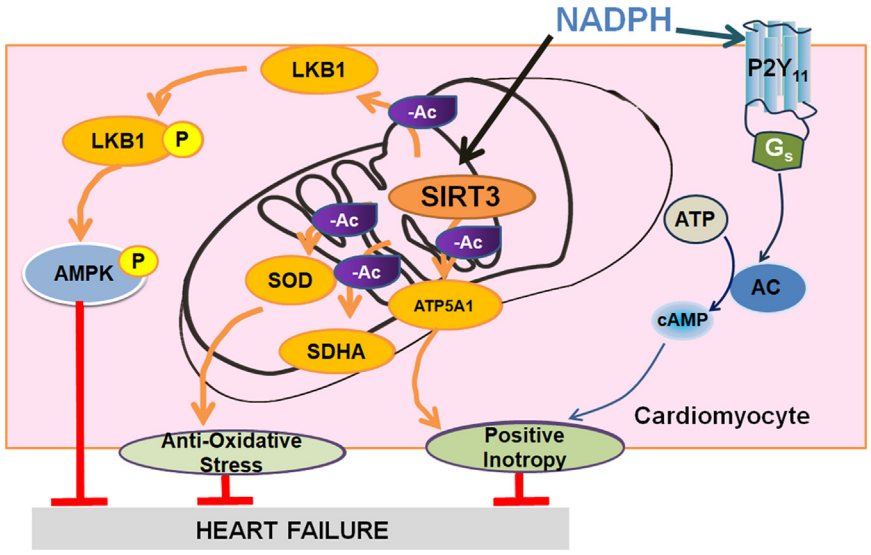

***Fig. 11:* Exogenous NADPH improves energy metabolism and exerts a positive inotropic effect via SIRT3 in pathological cardiac hypertrophy and heart failure.** Exogenous NADPH activates SIRT3 and deacetylates LKB1, ATP5A1, SDHA and SOD2 to increase ATP production and reduce oxidative stress. NADPH also exerts a positive inotropic action through the P2Y11 receptor-adenylate cyclase/phospholipase pathway. NADPH thus exerts a positive inotropic effect in pathological cardiac hypertrophy or heart failure. The -Ac represents deacetylation and the P represents phosphorylation.

production and to reduce oxidative stress via activation of SIRT3 to deacetylate the target proteins (Fig. 11). This study suggests that exogenous NADPH administration may be an effective strategy for the prevention and treatment of pathological cardiac hypertrophy or heart failure.

Contributors

R.S. conceived and supervised the study. K.Q., J.T., Y.J.L, M.Z. L.J.Z. and Y. X. performed the experiments. K.Q., J.T., Y.J.L, M.Z. K.N. collected, analyzed and interpreted the data. X.X.Y. and K.Y.S. recruited and collected the human plasma for analysis. R.S. and K.Q. wrote the manuscript. Z.H.Q. and R.S. revised the manuscript. R.S., K.Q., J.T., Y.J.L and M.Z. verified the data. All authors read and approved the final version of the manuscript.

Data sharing statement

The data for this study are available upon reasonable request to the corresponding author.

Declaration of interests

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2023.104863.

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