Nicotinamide Riboside Promotes Mfn2-mediated Mitochondrial Fusion in Diabetic Hearts through the SIRT1-PGC1α-PPARα Pathway

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Abstract

Background: Myocardial dysfunction is associated with an imbalance in mitochondrial fusion/fission dynamics in patients with diabetes. However, effective strategies to regulate mitochondrial dynamics in the diabetic heart are still lacking. This study investigated whether Nicotinamide riboside (NR) supplementation protects against diabetes-induced cardiac dysfunction by regulating mitochondrial fusion/fission and further explored the underlying mechanisms.

Methods: Obese diabetic (db/db) and lean control (db/+) mice were each given NR oral supplementation in this study. NAD^+ Content was determined in mice hearts and primary neonatal cardiomyocytes. Cardiac function was detected by echocardiography. Mitochondrial dynamics were analyzed by transmission electron microscopy in vivo and by confocal microscopy in vitro.

Results: Here, we show an evident decrease in NAD^+ level and mitochondrial fragmentation in the hearts of leptin receptor-deficient diabetic (db/db) mouse model. NR supplementation significantly increased NAD^+ content in the diabetic heart tissues. Furthermore, NR treatment increased Mfn2 expression, promoted mitochondrial fusion, suppressed oxidative stress, reduced cardiomyocyte apoptosis and consequently improved cardiac function in db/db mice. In neonatal primary cardiomyocytes cultured in a high-glucose/high-fat medium, NR treatment also promoted mitochondrial fusion, suppressed mitochondria-derived ROS production and reduced cardiomyocyte apoptosis, which were all reversed when Mfn2 was knocked down. Mechanistically, chromatin immunoprecipitation (ChIP) and luciferase report assay analysis revealed that PGC1α and PPARα interdependently regulated Mfn2 transcription by binding to its promoter region. NR treatment elevated NAD^+ levels and activated SIRT1, resulting in the deacetylation of PGC1α and promoting the transcription of Mfn2. Furthermore, the inhibition of SIRT1, PGC1α or PPARα blunted the positive effects of NR supplementation on Mfn2 expression and mitochondrial fusion.

Conclusion: NR attenuates the development of diabetes-induced cardiac dysfunction by promoting mitochondrial fusion through the SIRT1-PGC1α-PPARα pathway, with PGC1α and PPARα being the interdependent co-regulatory factors for Mfn2. The promotion of mitochondrial fusion via oral supplementation of NR may be a potential strategy for delaying cardiac complications in patients with diabetes.

Background

Diabetes mellitus (DM) is a major threat to human health worldwide[1]. DM is regarded as a risk equivalent to cardiovascular disease, including coronary heart disease and heart failure (HF)[2]. A Study revealed that the incidence of HF is 2-5 times greater in patients with DM compared to age-matched control subjects[3]. The relationship between diabetes and HF has been widely reported, which mainly implicates the structural and functional abnormalities of the myocardium, leading to diabetic cardiomyopathy (DCM)[4, 5]. Despite significant progress made, the most effective strategy for the

clinical prevention and treatment of DCM is still unclear. Numerous pieces of evidence have revealed that mitochondrial dysfunction is a primary factor leading to cardiomyocyte injury in diabetic heart[6, 7]. Mitochondria provide the ATP to cardiomyocytes for normal heart pumping. However, mitochondria are also major source of reactive oxygen species (ROS) and pro-apoptotic factors[8, 9]. This is supported by a previous report that mitochondria isolated from the atrial tissues of patients with DCM have impaired respiratory state and increased ROS production[10].

Recent studies have underlined the crucial role of imbalanced mitochondria dynamics in its injury[11, 12]. Defective mitochondria fusion is considered a detrimental process because it is always accompanied by decreases mitochondrial function and ATP production[13, 14]. In contrast, proper mitochondria fusion seems to be beneficial because it is associated with decreased ROS generation and elevated mitochondria function[15]. The role of mitochondria dynamics in the pathogenic development of DCM was also investigated in both clinical subjects and laboratory models. Excessive mitochondrial fission was observed in atrial tissues from diabetic patients, which consequently contributed to cardiac contractile dysfunction[10]. Similarly, excessive mitochondrial fission was also detected in prolonged hyperglycemic-cultured primary neonatal cardiomyocytes, leading to ROS accumulation and cell apoptosis[16]. On the other hand, our previous study also demonstrated that inhibiting mitochondrial fusion is pivotal contributor to the occurrence of DCM. The reconstitution of mitochondrial fusion restores the mitochondrial homeostasis and alleviates DCM development[15]. However, specific approaches for restoring mitochondria dynamics are still limited.

NAD^+, a rate-limiting co-substrate for sirtuin enzymes, is emerging as a valuable target to protect against metabolic diseases[17, 18]. Recent studies have shown that NAD^+-related pathway activation protects against aging-related diseases by improving mitochondrial function and energy metabolism[19, 20]. The administration of nicotinic aid (NA), an NAD^+ precursor, has long been considered to have beneficial effects on blood lipids and was shown to protect against type 2 diabetes[21]. However, since NA binds to the GPR109A receptor, it leads to severe flushing, causing poor compliance in patients when administered [22, 23]. NR, a new NAD^+ precursor, was recently found in milk, indicating that it can be a dietary source for NAD^+ production. When NR enters the cell, it is catalyzed by nicotinamide riboside kinases (NRKs) and then metabolized into nicotinamide mononucleotide (NMN)[24]. Compared to NA, NR and NMN have not yet been reported to have any side effects. In both mammalian cells and mice, NR supplementation was found to ameliorate diseases characterized by defective mitochondria function, including obesity, aging-related disorders, neuronal loss, and noise-induced hearing loss. The underlying mechanism is associated with improved mitochondrial function and decreased oxidative stress levels[19, 25-27]. However, the exact mechanism that explaining how NR protects against mitochondrial defects remains poorly understood. Given the important role of mitochondria dynamics imbalance in the diabetic heart, we speculated that NR might alleviate diabetes-induced cardiac dysfunction via the regulation of mitochondria dynamics.

In this study, we used diabetic mice and primary cardiomyocytes to demonstrate that decreased NAD^+ levels and excessive mitochondrial fission are associated with DCM development. We found that NR supplementation significantly increased myocardium NAD^+ content and further promoted Mfn2-mediated mitochondria fusion. Reconstitution of mitochondria dynamics restored mitochondria function and ameliorated the mitochondria-derived oxidative stress. Furthermore, we identified PPARα and PGC1α as co-regulatory factors for Mfn2. NR administration promoted Mfn2 expression through the SIRT1- PGC1α-PPARα pathway.

Materials And Methods

Animal experiments

Animal experiment were performed in accordance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee of Airforce Medical University. Leptin receptor-deficient (db/db) and age-matched male littermates (db/+) were provided by Shanghai Model Organisms (Shanghai, China).

Nicotinamide Riboside (NR) was custom synthesized as previously described[28]. Twelve-week-old diabetic db/db and non-diabetic db/+ animals were each given NR via daily oral gavage at a dose of 400mg/kg/d for 4 weeks. The dosage of NR was chosen based on previous studies about the effect of NR on obese mice[27]. At the end of the experiments (when the mice were 16-weeks-old), the hearts of mice were collected.

Primary culture of neonatal rat cardiomyocytes

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Airforce Medical University. Primary neonatal cardiomyocytes were prepared from neonatal rat (0-3 d) hearts as previously described. According to the results of our previous study[15], HG/HF (25mmol/L glucose and 500umol/L palmitate) medium were employed to induce type 2 diabetes in neonatal primary cardiomyocytes, while HG (25mmol/L) medium were chosen as control medium for all in-vitro experiments in this study. Thereafter, the cardiomyocytes were subjected to normal-glucose medium (25 mmol/L glucose) or high-glucose high-fat medium (25 mmol/L glucose and 500μmol/L palmitate) for 24 hours. For NR treatment, the cells were incubated in NR (2 mmol/L) as previously described[19].

Echocardiography

Echocardiography was performed in M-mode with a Vevo-3100 echocardiography system (Visual sonics Inc. Canada) as previously described[15]. Mice were anesthetized with 2.5% isoflurane. A continuous ECG monitoring system was used to measure mice heart rate of the mice during echocardiography. Left

ventricular fractional shortening (LVFS) and ejection fraction (EF) were calculated from the M-mode images using computer algorithms as previously described [15].

NAD^+ content detection

NAD^+ levels were measured using a NAD/NADH-Glo Assay Kit (Promega). All procedures were conducted strictly according to the manufacturers' instructions.

Transmission electronic microscopy (TEM)

Heart sample for TEM detection were prepared according to a procedure previously described[16]. All images were obtained using a transmission electron microscope (JEM-1230, JEOL Ltd, Tokyo, Japan) at 300kV. Mitochondrial images were analyzed by a technician blinded to the treatment using ImageJ software.

Histological analysis

Mouse hearts were fixed in 4% paraformaldehyde (PH 7.4) overnight, embedded in paraffin as previously described[15]. Hematoxylin and eosin staining was conducted following standard procedures. Collagen content was detected using Masson trichrome staining.

Immunohistochemistry

Immunohistochemistry was performed as previously described[29]. For Mfn2 expression detection in myocardium, primary antibody anti-Mfn2 (1:300, Abcam, USA) were used to incubate with tissue section. At least 10 fields per heart were randomly chosen and analyzed.

Cell apoptosis assay

Terminal deoxynucleotidyl transferase UTP nick end labelling (TUNEL) assay kit (Roche Applied Science, Swiss) were employed to determine apoptosis in heart tissue. All procedures were conducted strictly according to the manufacturers' instruction as previously described[29]. A PE-Annexin V Apoptosis detection kit was used to determine cell apoptosis rate before flow cytometry.

Assessment of Mitochondria morphology in cells

Mitochondria morphology was evaluated in primary cardiomyocytes by staining with Mito-tracker Red CMXRos Probe (Thermofisher, USA) as previously described. Images were obtained with a confocal laser-

scanning microscope (Nikon A1R MP$^+$ Confocal Microscope, Nikon, Japan). Number and morphology of mitochondria were quantified with ImageJ software as previously described[16].

Measurement of cellular ROS and Mito-ROS

Cellular ROS (total ROS) and Mito-ROS were determined using staining with a Fluorometric intracellular ROS Kits (Beyotime, China) and Mito-SOX probe (Invitrogen, USA) respectively as previously described[15]. Thereafter, Images were obtained with a confocal laser-scanning microscope (Nikon A1R MP$^+$ Confocal Microscope, Nikon, Japan).

Western-blotting and quantitative real-time PCR

Mice heart and cultured primary cardiomyocytes were lysed with RIPA buffer containing protease inhibitor cocktail. Western blotting analyze were performed as previously described[29]. The primary antibody against the following proteins were used: β-actin (Proteintech, China, #20536-1-AP), Mfn2 (Abcam, #ab58669), Nox4 (Abcam, #ab13303), cleaved-capase3(Cell signaling technology, #9664), PPARα (Novus, #NBP1-04676), PGC1α (Cell signaling technology, #2178).

Total mRNA was extracted from mice heart and primary cardiomyocytes with RNAisoplus (Takara, Japan) and cDNA were synthesized with a PrimerScript^{TM} RT reagent Kit as previously described. All procedures were conducted following the manufacturers' protocols. The primer sequences are as shown in table S1.

Adenovirus and siRNA transfection

Primary neonatal cardiomyocytes were transfected with adenovirus harboring Mfn2 shRNA, PPARα, PGC1α, SIRT1. Multiplicity of infection (MOI) was 100:1. For siRNA transfection, Lipofectamine RNAiMAX reagent (Invitrogen) were employed as described previously. Cells were transfected with SIRT1 siRNA, SIRT3 siRNA, PPARα siRNA and PGC1α siRNA following the manufactures' instructions. After transfected with adenovirus or siRNA, cells were treated with normal medium or high-glucose high-fat medium for 24h.

Chromatin immunoprecipitation (ChIP) assay

CHIP was carried out with a simpleChIP plus Enzymatic Chromatin IP Kit (Cell Signaling Technology) following the protocol provided by the manufacturer as described previously[15]. Sheared chromatins were incubated with a PPARα antibody or a PGC1α antibody. Then the incubated chromatins were fixed with protein G magnetic beads. DNA eluted from the precipitation was detected by PCR analysis. The

primers specific to the Mfn2 promoter binding region were as follows: Mfn2 promoter forward: 5'-TGATCCGGAAAGGAAAACAG-3' and reverse: 5'-CACCGAAAGGCCACAGTAAT-3'.

Luciferase reporter assay

Full-length of 2kb promoter sequence 5' upstream of the transcription start site of rat Mfn2 were cloned into the PGL3.0-Basic vector upstream of luciferase cassette. For Luciferase reporter assay, a dual-luciferase reporter assay system was used to assess the luciferase activity as previously described[15]. Briefly, HEK-293T cells were transfected with promoter constructs and Renilla luciferase reporter plasmid (Prl-TK). Then the transfected cells were co-infected with PPARα and PGC1α adenovirus or siRNA. The luciferase activity of cell lysis products was measured with a GloMax96 plate reader (Biotek, USA).

Co-immunoprecipitation (Co-IP)

Co-IP were performed using a Pierce classic magnetic IP/co-IP kit (Thermo fisher, USA) according to the manufacturers' instruction as previously described[29]. Lysates samples were incubated with PGC1α antibody or SIRT1 antibody for immunoprecipitation.

Statistical analysis

All values were presented as Mean ± Standard Error (Mean ± SEM). The statistical difference between two groups was assessed with two-tailed Students' t test. For groups of three or more, the data were subjects to ANOVA followed by a Bonferroni correction for a post hoc test. A value of $P<0.05$ were considered as statistically significant difference.

Results

Decreased NAD^+ content and excessive mitochondrial fission were observed in the hearts of db/db mice

Compared with those of db/+ mice, the body weight, blood glucose, serum lipid including triglyceride (TG) and total cholesterol (TC) were significantly increased in db/db mice (Figure S1). In contrast, cardiac NAD^+ content was significantly decreased in db/db mice (Figure S2D). Serial echocardiography was performed in 16-weeks-old db/db mice and db/+ mice. As shown in Figure S2A-C, db/db mice showed impaired cardiac function, as evidenced by the decreased LVEF and LVFS. Moreover, the hearts from db/db mice exhibited excessive mitochondrial fission at 16-weeks-old. Compared with lean control mice, the mean mitochondrial size in the hearts of db/db mice was decreased while the number of mitochondria per μm^2 were increased (FigureS2 G-I). Mfn2, a pivotal mitochondrial fusion protein, was also detected. Western blotting and real-time PCR results showed that Mfn2 expression was reduced at

both protein and mRNA levels in diabetic heart, indicating that Mfn2 downregulation might be responsible for the excessive mitochondrial fission and cardiac dysfunction observed in db/db mice (Figure S2E, F).

NR increased myocardium NAD^+ content and improved cardiac function in db/db mice

To test whether NR can protect against cardiac injury in diabetic hearts, both diabetic db/db mice and lean control db/+ mice were given NR supplementation feeding. NR supplementation significantly increased NAD^+ level in diabetic and control mice (Figure 1B). NR supplementation also alleviated cardiac dysfunction in db/db mice, while there was no significant difference in LVEF and LVFS in db/+ mice (Figure1A, C&D).

Cardiac hypertrophy and fibrosis are important features of diabetic heart. As shown in Figure 1, db/db mice showed significant cardiac fibrosis and hypertrophy compared with lean control mice. NR supplementation restored these pathological changes in db/db mice, as evidenced by the reduced interstitial fibrosis and heart weight-to-tibia length ratios (Figure 1E-H). NR supplementation also reduced the serum lipid level and body weight gain in diabetic db/db mice, as evidenced by the lower body weight index and serum TG and TC levels in NR-supplemented db/db mice compared with those without NR supplementation (Figure S1). These results indicate that NR may function as a regulator of overall metabolism in diabetic mice.

NR reduced cardiomyocytes apoptosis and oxidative stress in db/db diabetic mice

Enhanced cardiac ROS generation and increased cardiomyocytes apoptosis have been reported as major pathological features for cardiac injury in diabetic hearts[4]. Previous research has shown that Nox-4 can induce ROS generation, and it is an important source of oxidative stress in diabetic hearts, leading to cardiomyocytes apoptosis[30]. Here, in diabetic db/db mice hearts, cardiac superoxide species level (detected via DHE fluorescence density), Nox4 expression and MDA levels were significantly higher in diabetic db/db mice hearts (Figure 2A-C). Meanwhile, cardiomyocytes apoptosis was also significantly increased, as evidenced by a higher number of TUNEL-positive cell rate and elevated cleaved-caspase3 expression, which was used as an additional marker of apoptosis. Comparatively, NR treatment inhibited myocardial oxidative stress and further alleviated cardiomyocyte apoptosis in diabetic mice (Figure 2D-G).

NR promoted mitochondrial fusion by elevating Mfn2 expression and transcription

To further investigate the relationship of NR supplementation and Mfn2-mediated mitochondria fusion, Mfn2 expression and mitochondria morphology were determined after NR supplementation. As shown in Figure 3A-E, Mfn2 expression was significantly increased at both protein and mRNA levels. As a result, diabetic mice treated with NR displayed an increased level of mitochondrial fusion compared to non-NR-supplemented diabetic mice. TEM results from myocardium of NR-supplemented db/db mice showed an increased mean mitochondria size and a decreased number of mitochondria per μm^2(Figure 3F-H) These results indicates that NR supplementation promoted mitochondria fusion in diabetic hearts by elevating Mfn2 expression.

NR-driven mitochondrial fusion was also determined in neonatal primary cardiomyocytes in the presence or absence of Mfn2 expression to identify whether the same result will be obtained in humans. Consistent with our results from the *in-vivo* mice experiments, NR supplementation significantly increased NAD^+ content by -2 folds in HG/HF-cultured cardiomyocytes (Figure 4A). Cardiomyocytes cultured in HG/HF medium showed significantly decreased mitochondrial fusion levels. The mitochondria became fragmented (shorter and more spherical) and Mfn2 levels significantly decreased. Notably, NR supplementation dose-dependently increased Mfn2 expression (Figure S3), which further restored mitochondrial morphology, as the mitochondria became more highly interconnected (Figure 4D-G). Moreover, Mfn2 deletion blunted NR-related mitochondria fusion (Figure 4B-G), indicating Mfn2 is a key mediator for NR-driven mitochondria fusion.

NR inhibited mitochondria-derived superoxide production and apoptosis in HG/HF treated cells, which were blunted by Mfn2 deletion

Mitochondria is the major source of superoxide species production in cardiomyocytes. In this study, both cellular (Figure 5A, shown as green) and mitochondria-derived ROS production (Figure 5A, shown as red) were detected. Consistent with our previous study, co-staining results showed that the mitochondria are the major source of superoxide anions in HG/HF treated cardiomyocytes (Figure 5A). NR treatment reduced total cellular ROS and mitochondria-derived ROS levels in HG/HF-treated cardiomyocytes (Figure 5A-C). Moreover, Nox-4 expression was also inhibited upon NR treatment (Figure 5F&G). Silencing of Mfn2 via adenovirus infection blocked the inhibitory effect of NR on ROS production (Figure 5A-C).

Cardiomyocyte apoptosis was also detected via flow cytometry and western-blotting analysis. As shown in Figure 5E and 5F, the percentage of apoptotic cells and the expression of cleaved-caspase3 were significantly increased after cultured in HG/HF for 24h. NR treatment also effectively prevented cardiomyocytes apoptosis induced by HG/HF culturing. Transfection of adenovirus particles encoding Mfn2 shRNA (Ad-Mfn2 shRNA) re-elevated the expression of cleaved-caspase 3 and the percentage of cell apoptosis percentage. These data suggested that NR prevented HG/HF-induced mitochondrial ROS production and cell apoptosis via the up-regulation of Mfn2 protein expression.

NR promoted Mfn2 transcription and regulated its co-regulatory factors PPARα and PGC1α

Previous results showed that HG/HF culturing decreased Mfn2 mRNA levels while NR increased Mfn2 mRNA level, indicating that the expression of Mfn2 was regulated at the transcriptional level. In our previous study, we found for the first time that peroxisome proliferator activator receptor α (PPARα) regulates the transcription of Mfn2 by directly binding to its promoter region[15]. Other studies also defined PGC1α as a positive transcriptional regulator for Mfn2 expression[31]. ChIP analysis revealed that both PGC1α and PPARα bound to the promoter region of the Mfn2 gene in primary cardiomyocytes. We performed a luciferase reporter assay to investigate the specific mechanism of Mfn2 upregulation in cardiomyocytes. As shown in Figure 6B&C, the relative luciferase activity indicated that either Ad-PPARα or Ad-PGC1α could induce a positive and robust response. However, PGC1α silencing blunted the positive response induced by Ad-PPARα while PPARα silencing also reversed the increase in Ad-PGC1α-induced luciferase activity. These data suggest that PGC1α and PPARα are co-transcriptional regulators for Mfn2, while the inhibition of anyone of these two factors would lead to a transcription repression of Mfn2 transcription. In contrast, the activation of PPARα or PGC1α could increase Mfn2 expression in cardiomyocytes. Furthermore, the silencing of PGC1α or PPARα repressed the positive effect of NR supplementation on Mfn2 expression (Figure 6D-F), indicating that the co-regulatory role of PGC1α/PPARα is directly responsible for the effects of NR supplementation on Mfn2-mediated mitochondria fusion.

NR promoted Mfn2 expression and mitochondrial fusion in a SIRT1- PGC1α/PPARα-dependent manner

Canto et al. reported that NR treatment enhanced SIRT1 and SIRT3 activity in a high-fat diet induced obese mice, and that NAD^+ is an important co-substrate for sirtuin family of proteins to catalyze the deacetylation of their target[27]. Previous studies revealed that either SIRT1 or SIRT3 could induce the activation of the PGC1α pathway[32, 33]. To further determine whether SIRT1 or SIRT3 is responsible for the NR-driven PGC1α/PPARα-Mfn2 expression, SIRT1 or SIRT3 was silenced via transfection with small interfering RNA in primary cardiomyocytes (Figure 7A-C). The silencing of SIRT1, but not SIRT3, significantly reduced Mfn2 protein and mRNA levels in the presence or absence of NR supplementation, suggesting that NR promoted Mfn2 transcription in a SIRT1-dependent manner.

To further investigate the mechanism of NR/SIRT1 in regulating Mfn2 transcription, a co-IP assay was performed to detect the potential direct interaction of SIRT1 and PGC1α. The co-IP results showed that SIRT1 directly interacted with PGC1α (Figure 7D&E). SIRT1 was reported to directly deacetylate PGC1α, so we performed another co-IP assay to detect the acetylation level of PGC1α. As shown in Figure 7F-I, culturing in HG/HF medium significantly promoted PGC1α acetylation, while NR supplementation reduced the acetylation level of PGC1α to activate it, further promoting Mfn2 transcription. SIRT1

silencing abolished the NR-induced PGC1α deacetylation and further blocked Mfn2 gene transcription. Moreover, Mfn2 gene transcription and the subsequent mitochondrial elongation were induced by SIRT1 overexpression but were also inhibited by PGC1α silencing (Figure 7J-L). Taken together, these results suggested that NR supplementation induced PGC1α deacetylation, resulting in SIRT1 activation. Deacetylated PGC1α further promoted Mfn2 transcription, which resulted in an increased mitochondria fusion via the co-regulatory factors of Mfn2--PGC1α and PPARα.

Discussion

In this study, we revealed a novel mechanism on how NR supplementation promoted mitochondrial fusion in the diabetic heart. We provided *in vivo* and *in vitro* evidence that NR increased Mfn2 expression and promoted mitochondrial fusion in mice with diabetes via the activation of SIRT1-PGC1α-PPARα signaling. The promotion of mitochondrial fusion suppressed mitochondrial ROS production, reduced cell apoptosis, and further improved cardiac function (Figure 8). Our data demonstrated for the first time that NR supplementation promoted Mfn2-mediated mitochondrial fusion in mice with diabetes in a SIRT1-PGC1α-PPARα-dependent manner.

The homeostasis of mitochondria fusion and fission is now emerging as a pivotal regulator for mitochondrial function, including mitochondrial respiration, ATP production, and ROS generation [11]. Previously, an imbalance in mitochondrial dynamics was observed in myocardium tissue from diabetes patients and *in vitro* HG/HF-cultured cardiomyocytes [10, 15]. The induction of mitochondrial fusion via adenovirus infection inhibited diabetes-induced mitochondrial ROS production, elevated mitochondrial respiration, and reduced cardiomyocyte apoptosis, which could improve cardiac function and protect against diabetes [15]. However, specific strategies for restoring homeostasis in mitochondrial dynamics are still limited. Here in this study, we reported for the first time that NR supplementation promoted Mfn2-mediated mitochondrial fusion and alleviated diabetes-induced cardiac dysfunction. Supporting our results, previous studies also reported that NR administration alleviated cardiac dysfunction in pressure-overload cardiac hypertrophy and dilated cardiomyopathy, possibly by decreasing inflammasome activation and regulating MnSOD signaling [34, 35]. Another study also showed that NR administration prevents sepsis-induced oxidative stress in lung and heart tissue [36]. However, only a few studies have explained the specific subcellular mechanism for NR supplementation and cardiac protection. The present work indicates that NR-driven mitochondrial fusion might be a novel mechanism underlying its cardioprotective effects in the diabetic heart. Mfn2-mediated mitochondria fusion may also be responsible for the reduced oxidative stress observed after cardiac injury under other pathological conditions. Interestingly, another study using mouse livers revealed that NR supplementation promoted mitochondrial biosynthesis and further attenuated alcohol-induced liver injury, which is also related to reduced oxidative stress. These results indicate that NR may also exert its anti-oxidative ability by activating mitochondria biogenesis in diabetic hearts [37]. Further studies are needed to explore the effect

of NR supplementation on the myocardium mitochondria aside from promoting Mfn2-mediated mitochondrial fusion.

NAD^+ is a ubiquitous molecule widely involved in various biological processes in the cell [17]. NAD^+ is a coenzyme for the sirtuin family of proteins, including SIRT1-7, which regulate mitochondrial biogenesis, mitophagy, and cellular stress responses [18, 38]. NAD^+ levels also decrease with age and during obesity-induced pathological conditions induced by oxidative stress [39]. Specific strategies to increase the intracellular concentration of NAD^+ have been demonstrated to protect against age-related metabolic disorders and various other diseases [20, 40, 41]. To examine whether the reduction in NAD^+ levels is involved in the pathogenesis of DCM, we first measured the NAD^+ content in diabetic hearts. We found that NAD^+ levels were significantly decreased in both diabetic heart tissue and cultured cardiomyocytes. NR supplementation elevated intracellular NAD^+ levels and suppressed ROS generation and cell apoptosis, consequently restoring cardiac function and remodeling. These data suggest that NAD^+ shortage might be an important initiator and marker for DCM development. Therefore, the increase in NAD^+ levels after the oral intake of NR could be an effective approach to protect against DCM. There are other lines of evidence supporting the cardiovascular benefit of NR supplementation. A clinical study conducted by Trammell et al. found that a single oral dose of 1000 mg NR can elevate blood NAD^+ as much as 2.7-fold, suggesting the clinical application of NR supplementation for NAD^+ in human patients [42].

Similar to a study in obese mice, we found that NR promoted Mfn2-mediated mitochondrial fusion by activating SIRT1, but not SIRT3. Silencing SIRT1 in neonatal primary cardiomyocytes abolished the NR-driven Mfn2 expression. Our recent study also demonstrated that SIRT1 activation induced by melatonin inhibited the expression of Drp1, a key player involved in mitochondrial fission, can also exhibit a protective effect against oxidative stress and hyperglycemia-induced cardiac injury [16]. Therefore, the suppression of Drp1 provides another explanation for the restored mitochondrial dynamics and decreased ROS generation found in the hearts of NR-administered mice. In addition, the activation of SIRT1 in obese mice also positively impacts the anti-oxidation ability of the molecule SOD2, which can also be beneficial for the mitochondria fitness and metabolic flexibility of NR-supplemented mice [27]. However, in the present study, NR-induced mitochondrial fusion and oxidative stress suppression were reversed mainly via Mfn2 deletion, indicating that Mfn2-mediated mitochondrial fusion functions as a major contributor to the cardioprotective capacity of NR. Interestingly, db/db mice also showed reduced body weights and serum lipid levels, suggesting that NR supplementation can be a potential strategy for regulating overall metabolism aside from cardiac protection. Due to the critical role of lipid metabolism and lipid oxidation found in patients with type 2 diabetic cardiomyopathy [43, 44], the impaired cardiac function might also result from the overall impairment of lipid metabolism recovery. Therefore, further studies are needed to clarify this interesting issue and its connection with the current study.

Another important finding of the present study is that Mfn2 expression in cardiomyocytes is co-regulated by PGC1α and PPARα. If either PGC1α or PPARα is problematic, Mfn2 transcription might be inhibited.

Results of the ChIP assay and the luciferase reporter assay further confirmed the interdependence and indispensable role of PGC1α and PPARα in Mfn2 expression. In our previous study, we concluded that PPARα is a transcription factor of Mfn2 [15], while PGC1α was reported in other studies to promote Mfn2 transcription in hepatic cells [45, 46]. However, the specific mechanism of Mfn2 transcription remains to be clarified. PGC1α and PPARα play important roles in transcription regulation and myocardial metabolism. PPARα always functions as a ligand-activated transcription regulator for fatty acid metabolism, while PGC1α is well-described as a co-activator for PPAR family and metabolic regulation in the mitochondria [47]. In essence, the dysregulation of PGC1α and PPARα could contribute significantly to the changes in the diabetic heart [47]. Here, we distinguished Mfn2 as a novel mitochondria-related target regulated by PGC1α and PPARα. We found that NR-triggered SIRT1-induced Mfn2 expression occurs at both mRNA and protein levels, suggesting that NR supplementation induces SIRT1 to modulate the expression of Mfn2 at the level of transcription and translation. Since SIRT1 is not a transcription factor in the nucleus, a possible way for NR and SIRT1 to regulate Mfn2 expression is that SIRT1 could regulate Mfn2 transcription through its co-transcriptional regulators PGC1α and PPARα. PGC1α, a well-known transcription factor involved in mitochondrial biogenesis, is considered a major downstream target of SIRT1 [48]. In this study, PGC1α was over-acetylated in cardiomyocytes after culturing in a HG/HF medium, showing inhibition. Through the increase in NAD^+ level induced by NR supplementation, SIRT1 removes the acetyl groups from PGC1α, causing its activation and the subsequent transcription of Mfn2. SIRT1 knockdown blocked the deacetylation effect of NR on PGC1α, leading to the suppression of Mfn2 transcription. Interestingly, we also found that SIRT1 overexpression elevated the protein level of PGC1α in HG/HF-cultured cardiomyocytes, which might be another reason for the activation of PGC1α after NR supplementation. Previous studies have demonstrated that PGC1α activation promoted mitochondria biogenesis in pathological conditions [49, 50]. Therefore, there could be multiple effects of NR supplementation on mitochondria function, which might be more than the regulation of Mfn2-mediated mitochondrial fusion. Overall, our study provided the first clue for understanding a regulatory mechanism by which NR could promote mitochondrial fusion in the diabetic heart.

There are some limitations to our study. First, our *in vivo* experiments were merely conducted on diabetic db/db mice. Additionally, a type 2 diabetes model induced by a high-fat diet and STZ injection need to be applied to further confirm the findings in this study. Second, the conclusion that NR promoted Mfn2 transcription by activating SIRT1-PGC1α-PPARα was mostly obtained from *in vitro* experiments. The use of SIRT1 and PGC1α-knockout mice would be beneficial in clarifying the role of the SIRT1-PGC1α axis in Mfn2-mediated mitochondrial fusion. Despite these limitations, we believe that this study has provided novel information for understanding the effects of NR supplementation on mitochondrial dynamics homeostasis.

Conclusion

In summary, our study demonstrated that NAD^+ shortage is responsible for the imbalance in mitochondrial dynamics and excessive oxidative stress found in the diabetic heart. NR supplementation

elevated myocardium NAD^+ content and promoted Mfn2-mediated mitochondrial fusion through the SIRT1-PGC1α-PPARα axis, with PGC1α and PPARα being co-regulatory factors for Mfn2 transcription. These results identify NR-modulated mitochondria fusion as a feasible strategy for the therapeutic intervention of DCM and other cardiac complications of diabetes.

Abbreviations

NR: nicotinamide riboside

NAD^+: nicotinamide adenine dinucleotide

Mfn2: mitofusin2

ROS: reactive oxidation species

ChIP: chromatin immunoprecipitation

PGC1α: α subunit of peroxisome proliferators activated receptor γ coactivator 1

PPARα: peroxisome proliferators-activated receptors α

DM: diabetes mellitus

HF: heart failure

DCM: diabetic cardiomyopathy

NA: nicotinic aid

NMN: nicotinamide mononucleotide

TG: triglyceride

TC: total cholesterol

LVEF: left ventricular ejection fraction

LVFS: left ventricular fraction shortening

MDA: malondialdehyde

STZ: Streptozocin

Declarations

Authors’ contribution

Y.L., F.F. and L.H. conceived and designed the study. L.H., Y.G., N.S., Y.W. and H.W. performed the animal experiments. L.H., Y.G., B.Q., Q.L. and J.G. carried out the cellular experiments. L.H., Q.L., J.G., X.L., and N.S. performed the molecular biology experiments. L.H. L.S. and Y.W. analyzed the data. L.H. drafted the article. F.F., Y.L. and L.S. revised and edited the article. All authors have read and approved the final article.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

Not applicable. The conclusions of the manuscript are based on relevant data sets available in the manuscript.

Consent for publication

Not applicable. This manuscript does not contain data from any individual person.

Ethics approval and consent to participate

This study was performed in compliance with the National Institutes of Health Guidelines on Animal Research, and approved by the Airforce Medical University Ethics Committee.

Author Disclosure Statement

No competing financial interests exist.

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Figures

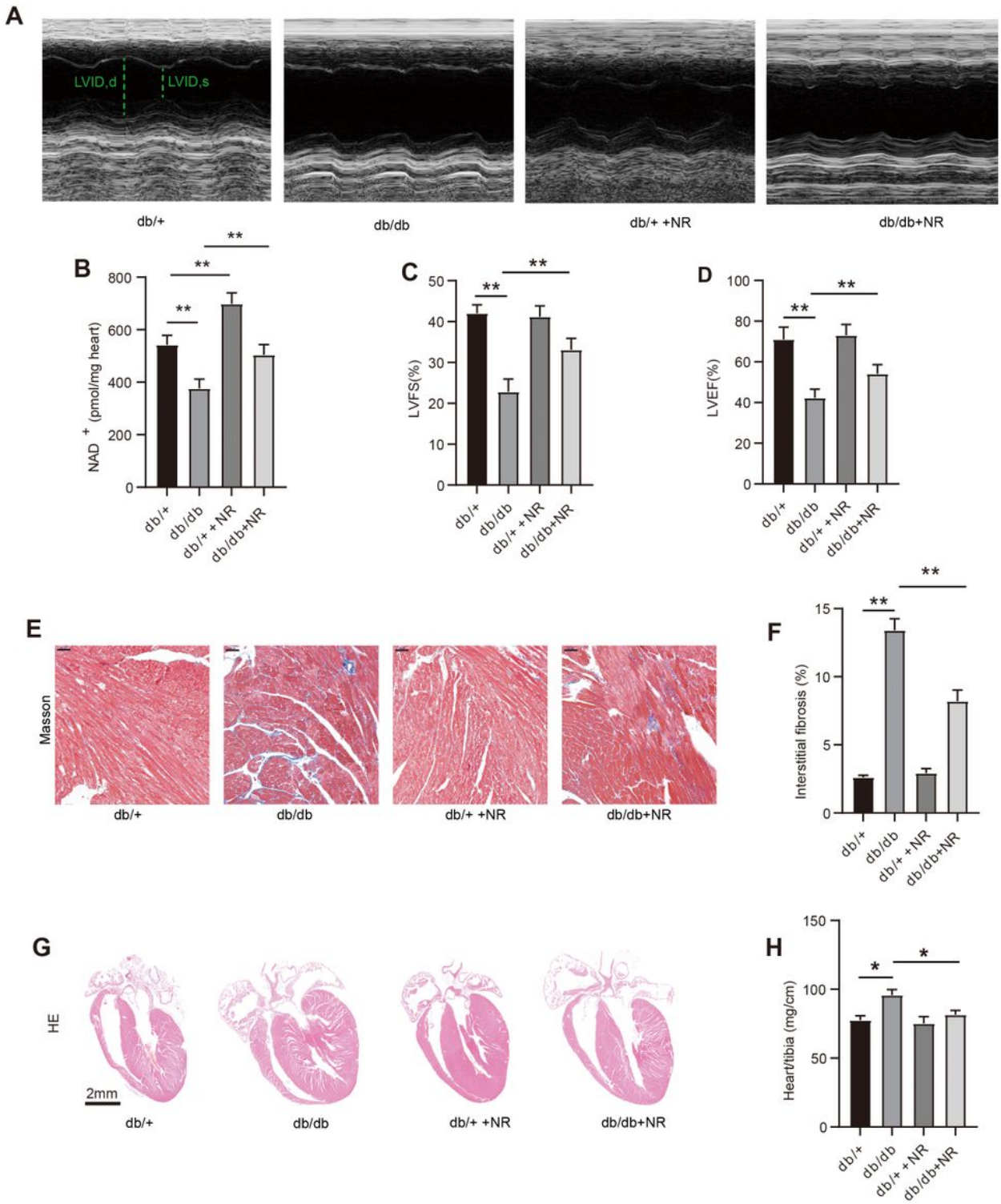

Figure 1

NR increased myocardium NAD+ content and improved cardiac function in diabetic mice. (A) Representative echocardiography images. (B) NAD+ content in heart tissue. (C) Left ventricular ejection fraction (LVEF). (D) Left ventricular fractional shortening (LVFS). (E)&(F) Representative images of Masson trichrome staining of hearts and quantitative analysis of interstitial fibrosis. Scale bar=50 μm. (G) The gross morphology of hearts stained by hematoxylin and eosin staining. Scale bar = 2mm. (H)

The ratio of heart weight to tibia length. LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening. *P<0.05, **P<0.01. n=6-8 in each group.

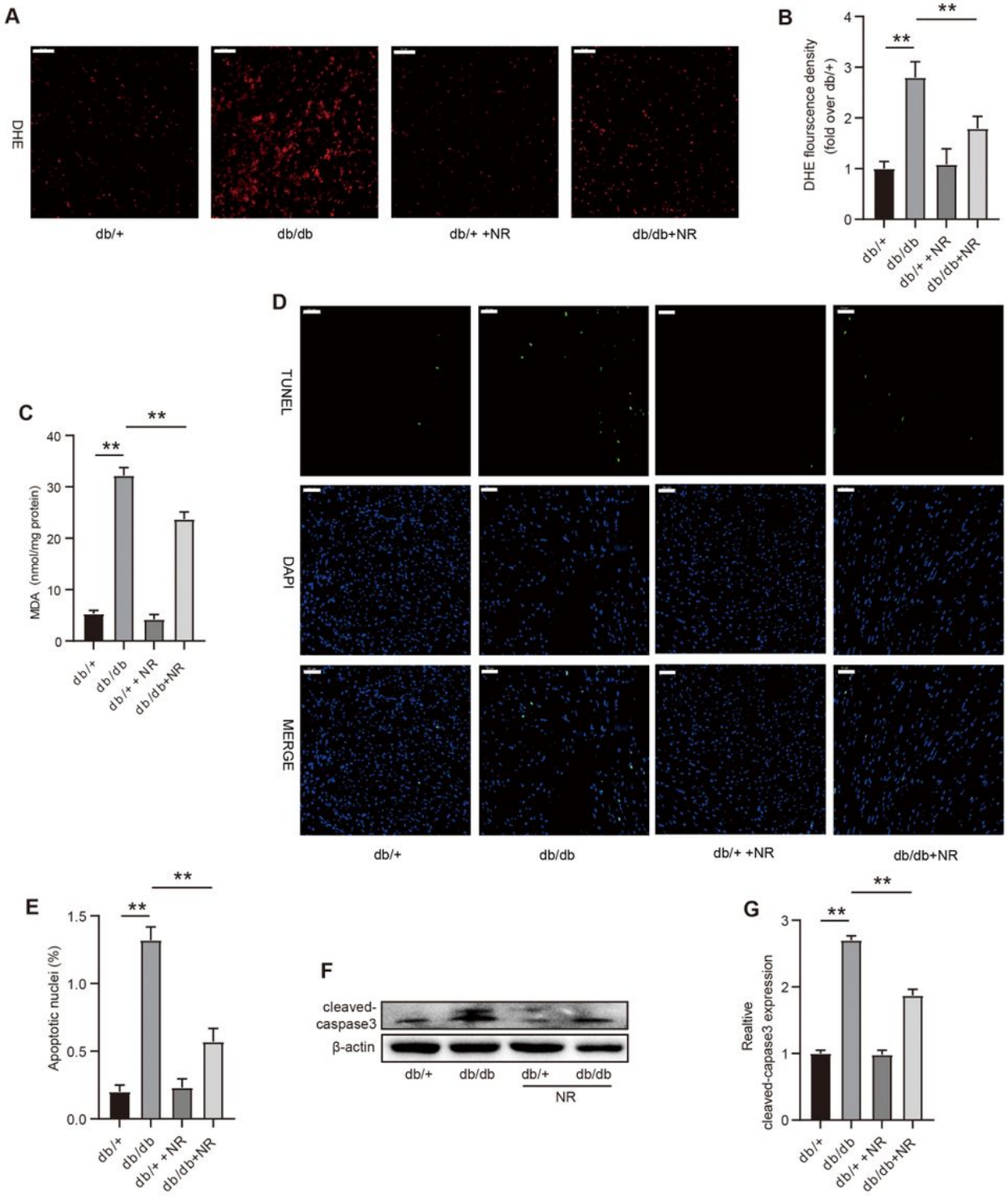

Figure 2

NR reduced cardiomyocytes apoptosis and oxidative stress in db/db diabetic mice. (A) Representative microphotographs of DHE staining in heart sections. Scale bar = 50 μm. (B) Quantitative analysis of DHE

fluorescence density (fold over db/+). (C) Myocardial malondialdehyde (MDA) content. (D) Representative photomicrographs of TUNEL-stained and DAPI-stained heart sections. Green fluorescence shows TUNEL-positive nuclei; Blue fluorescence shows nuclei of total cardiomyocytes (DAPI-positive). Scale bar = 50 μm. (E) Percentage of TUNEL-positive nuclei. (F)&(G) Representative blot images and quantitative analysis of cleaved-caspase 3. **P<0.01. n=6-8 in each group.

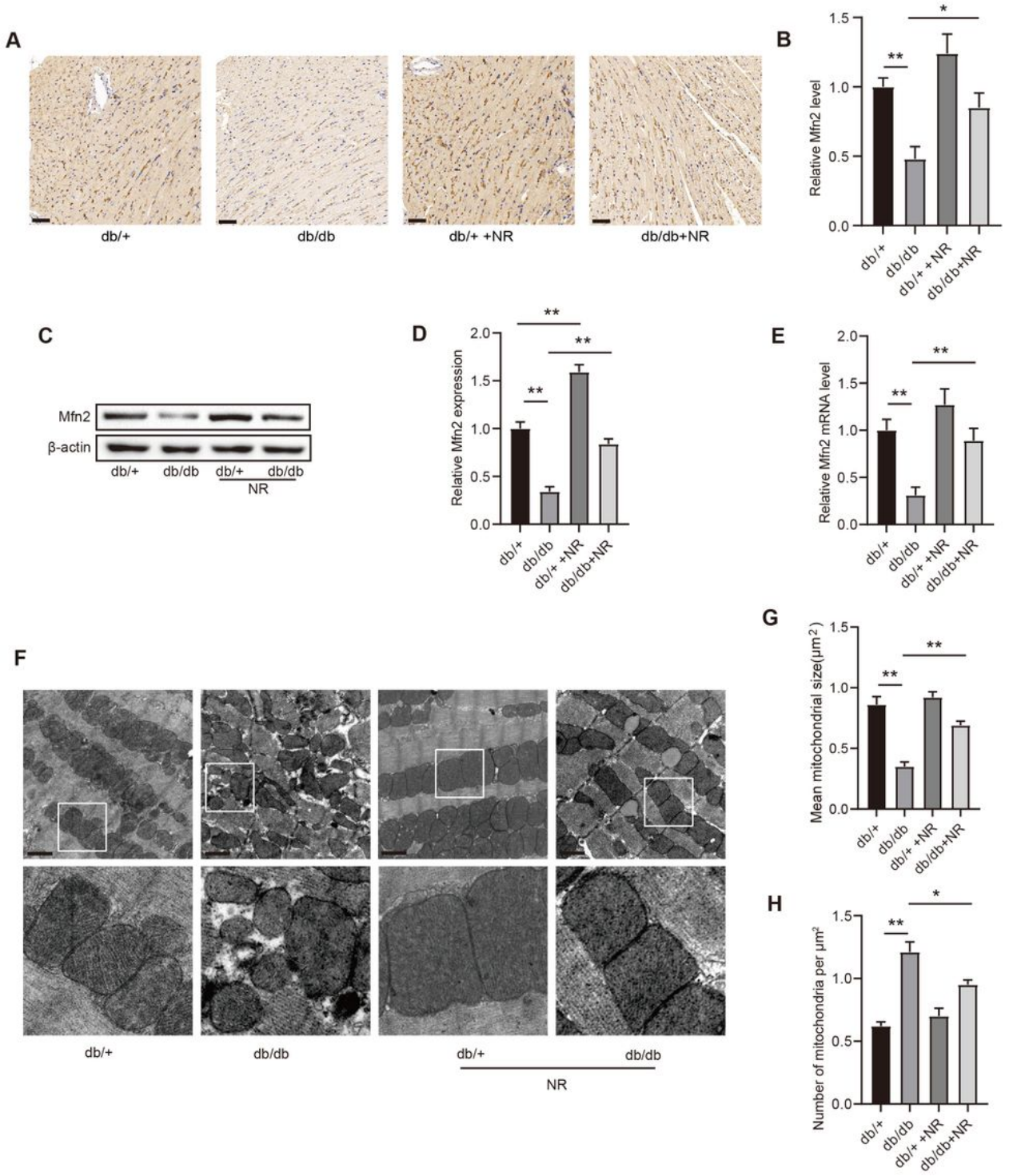

Figure 3

NR promoted mitochondrial fusion by elevating Mfn2 expression in heart of diabetic db/db mice. (A)&(B) Representative immunohistochemical stains and quantitative analysis of Mfn2 expression in mouse hearts. Scale bar = 50 μm. (C)&(D) Representative blot images and quantitative analysis of Mfn2. (E) Real-time PCR analysis of Mfn2 mRNA expression. (F) Representative transmission electron microscopic images of the myocardium. Scale bar = 1 μm. (G) Mean size of mitochondria. (F) The number of mitochondria per μm^2. *P<0.05, **P<0.01. n=6-8 in each group.

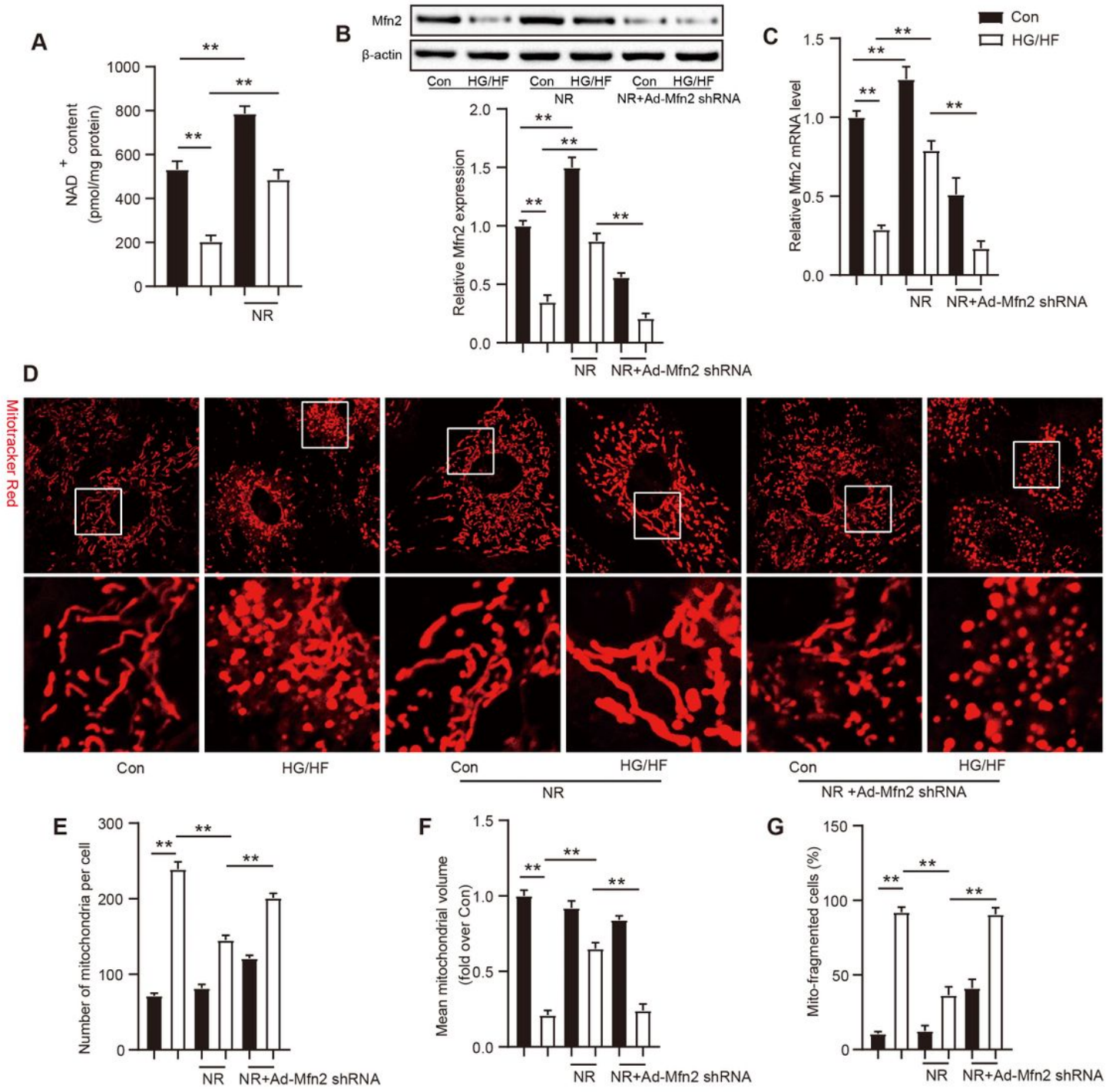

Figure 4

NR promoted mitochondria fusion in HG/HF-cultured cardiomyocytes, which is blunted by Mfn2 deletion. (A) NAD+ content in primary cardiomyocytes. (B) Representative blot images and quantitative analysis of Mfn2. (C) Real-time PCR analysis of Mfn2 mRNA expression. (D) Representative confocal microscope images showing mitochondrial morphology stained by Mito-Tracker Red. Original magnification ×600. (E) The number of mitochondria per cell. (F) Mean volume of mitochondria (fold over Con). (G) The percentage of cells with fragmented mitochondria. HG/HF, high-glucose and high-fat; Ad-Mfn2-shRNA, recombinant adenovirus encoding short hairpin RNA against Mfn2. **P<0.01. n=6 in each group.

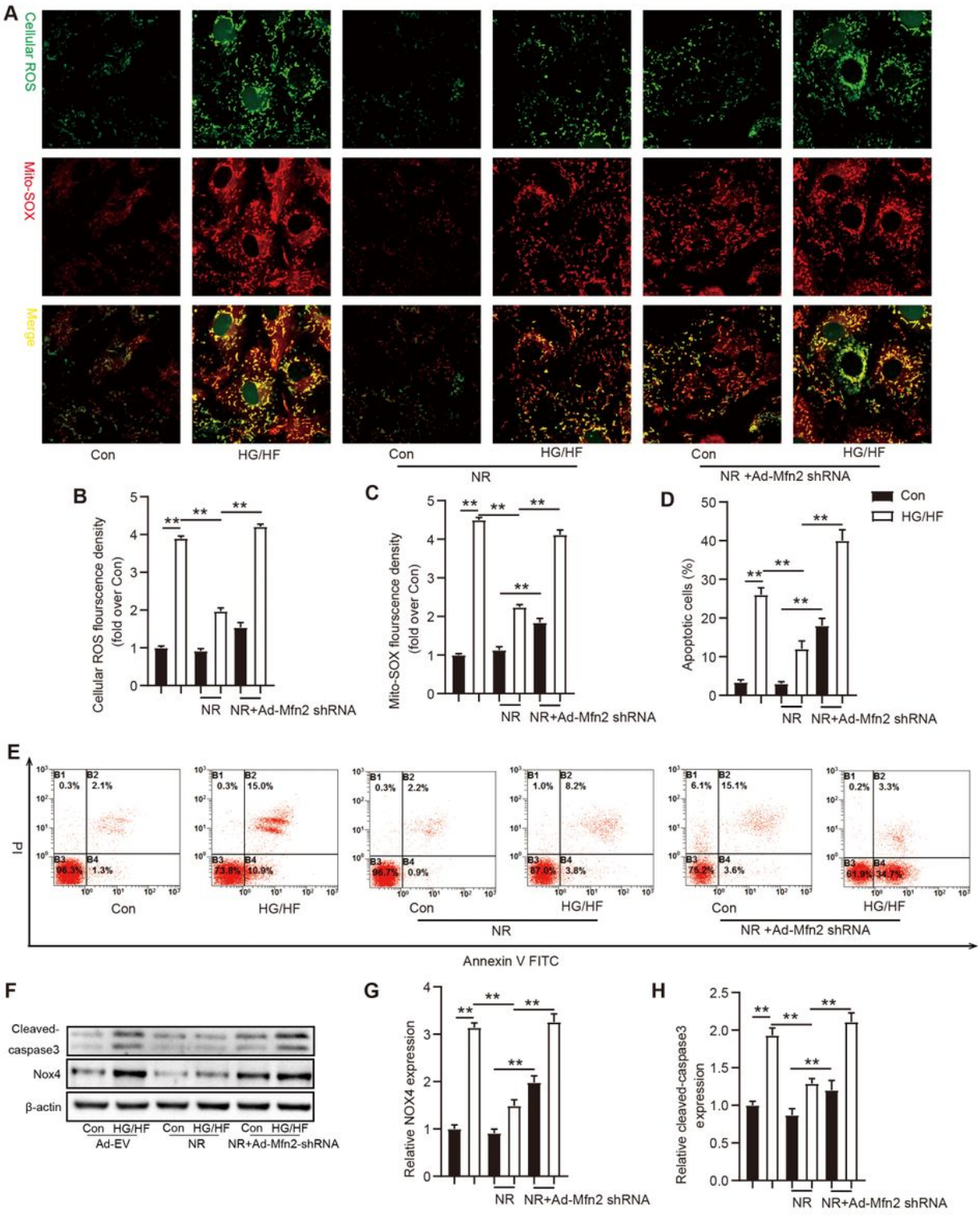

Figure 5

NR inhibited mitochondria-derived superoxide production and apoptosis in HG/HF treated cells, which were blunted by Mfn2 deletion (A) Representative confocal microscope images of intracellular ROS and mitochondria derived superoxide production. Original magnification ×600. (B) Quantitative analysis of intracellular ROS density in primary cardiomyocytes (fold over Con). (C) Quantitative analysis of mitochondria derived superoxide production in primary cardiomyocytes (fold over Con). (D)&(E)Flow

cytometry analysis of apoptosis by annexin V and PI staining and quantification of apoptotic cells in primary cardiomyocytes. (F)-(H) Representative blot images and quantitative analysis of Cleaved-caspase3 and Nox-4. HG/HF, high-glucose and high-fat; Ad-Mfn2-shRNA, recombinant adenovirus encoding short hairpin RNA against Mfn2. **P<0.01. n=6 in each group.

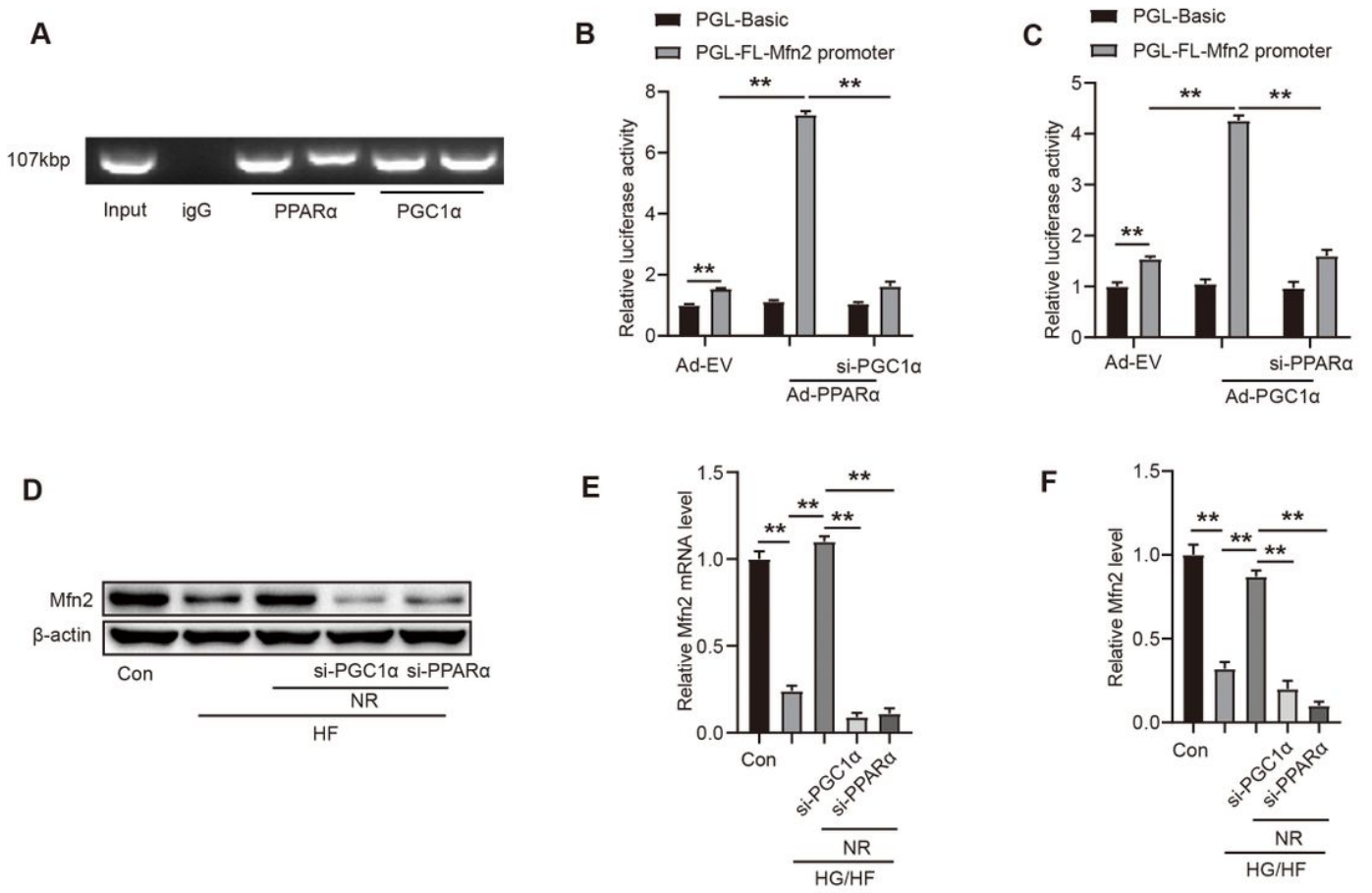

Figure 6

NR promoted Mfn2 transcription by regulating its co-transcriptional factor PPARα and PGC1α. (A) ChIP analysis for PPARα and PGC1α binding to the Mfn2 promoter in primary rat cardiomyocytes. (B) Responses of the Mfn2 promoter reporter to Ad-PGC1α and si-PPARα interference. (C) Responses of the Mfn2 promoter reporter to Ad-PPARα and si-PGC1α. (D)&(E) Representative blot images and quantitative analysis of Mfn2. (F) Real-time PCR analysis of Mfn2 mRNA expression. HG/HF, high-glucose and high-fat; Ad-PPARα, recombinant adenovirus encoding PPARα; Ad-PGC1α, recombinant adenovirus encoding PGC1α; Ad-EV, control adenovirus. **P<0.01. n=4 in each group.

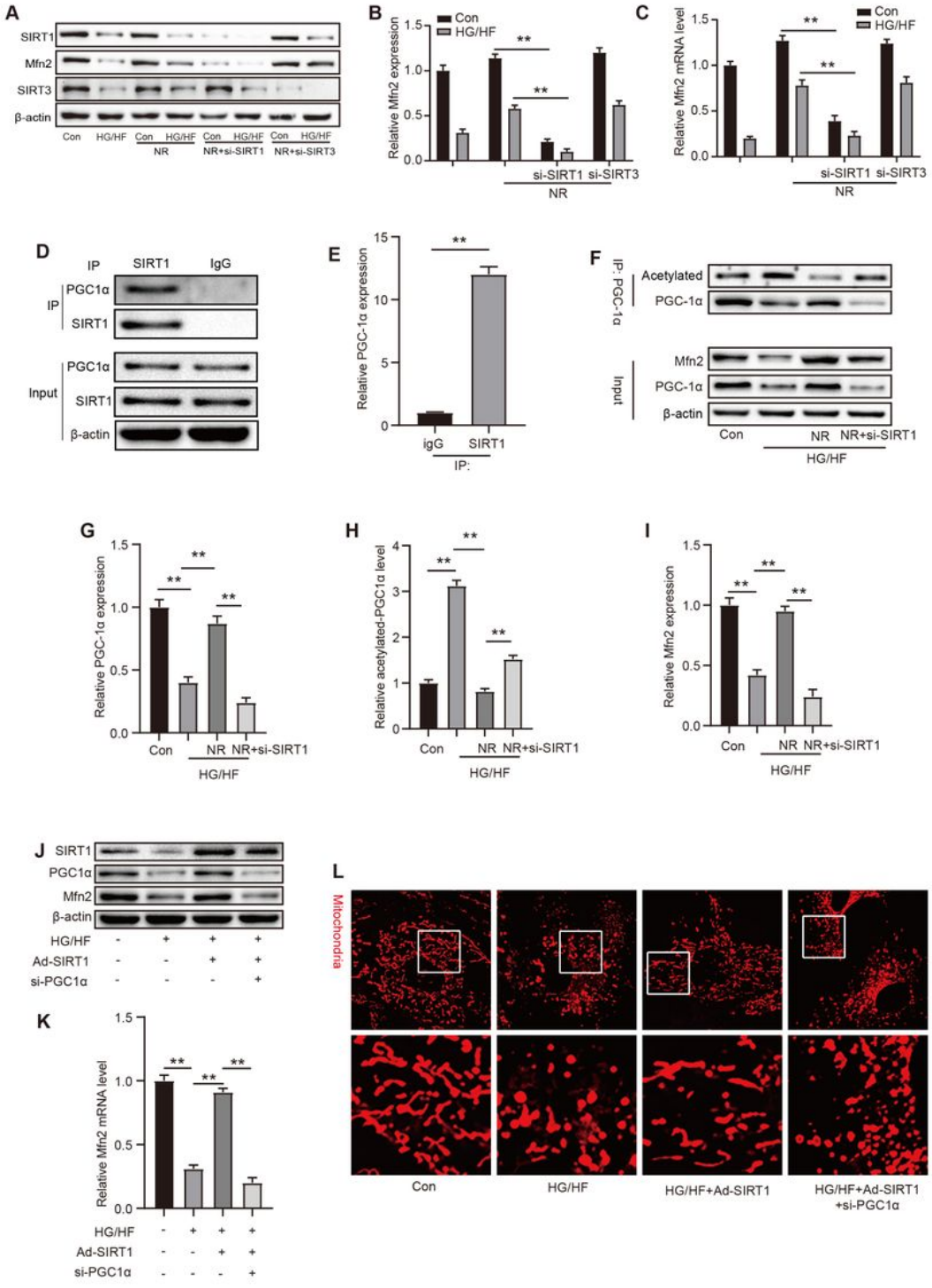

Figure 7

NR promoted Mfn2 expression and mitochondrial fusion in a SIRT1- PGC1α/PPARα dependent manner (A) Representative blot images of SIRT1, SIRT3 and Mfn2. (B) Quantitative analysis of Mfn2 expression. (C) Real-time PCR analysis of Mfn2 mRNA expression. (D)&(E) Interaction between SIRT1 and PGC1α was demonstrated via Co-IP. (F) Mfn2 expression, PGC1α expression and PGC1α acetylation level were determined by Western-blotting analysis. (G) Quantitative analysis of PGC1α expression. (H) Quantitative

analysis of PGC1α acetylation level. (I) Quantitative analysis of Mfn2 expression. (J)&(K) Representative blot images and quantitative analysis of SIRT1, PGC1α and Mfn2. (L) Representative confocal microscope images showing mitochondrial morphology stained by Mito-Tracker Red. HG/HF, high-glucose and high-fat; Ad-SIRT1, recombinant adenovirus encoding SIRT1. **P<0.01. n=3-4 in each group.

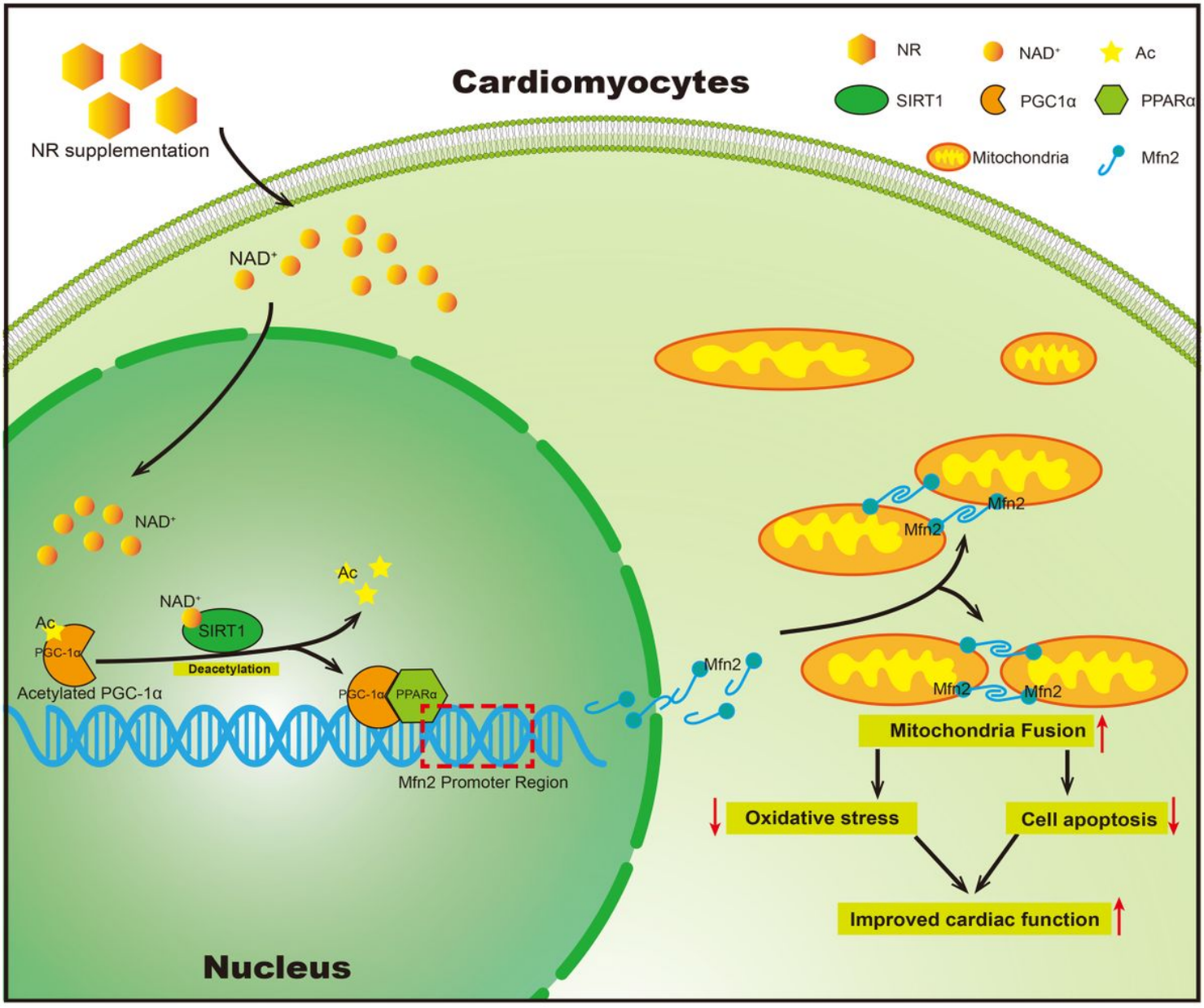

Figure 8

Schematic Figure illustrating that NR promoted Mfn2-mediated mitochondrial fusion and prevented diabetes-induced cardiac dysfunction through SIRT1-PGC1α/PPARα pathway. NR supplementation activated SIRT1 by elevating NAD^+ content in diabetic hearts. SIRT1 activation removed the acetyl group from PGC1α to activate PGC1α, which further promoted Mfn2 transcription directly, with PGC1α and PPARα being the co-regulatory factors for Mfn2 transcription. As a result, NR promoted Mfn2-mediated

mitochondrial fusion, suppressed mitochondria-derived ROS production, reduced cardiomyocytes apoptosis and consequently protected against diabetic-induced cardiac dysfunction.

Supplementary Files

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