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Sulforaphane prevents angiotensin II-induced cardiomyopathy by activation of Nrf2 via stimulating the Akt/GSK-3ß/Fyn pathway

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ABSTRACT

Aims: Activation of nuclear factor erythroid 2-related factor 2 (Nrf2) by sulforaphane (SFN) protects from, and deletion of the *Nrf2* gene exaggerates, diabetic cardiomyopathy. Angiotensin II (Ang II) plays a critical role in the development of diabetic cardiomyopathy. Therefore, whether SFN prevents Ang II-induced cardiomyopathy through activation of Nrf2 was examined using wild-type, global deletion of *Nrf2* gene (Nrf2-KO) and cardiomyocyte-specific overexpression of *Nrf2* gene (Nrf2-TG) mice.

Methods and results: Administration of a subpressor dose of Ang II to wild-type mice induced cardiac oxidative stress, inflammation, remodeling and dysfunction, all of which could be prevented by SFN treatment with Nrf2 up-regulation and activation. Nrf2-KO mice are susceptible, and Nrf2-TG mice are resistant, respectively, to Ang II-induced cardiomyopathy. Meanwhile, the ability of SFN to protect against Ang II-induced cardiac damage was lost in Nrf2-KO mice. Up-regulation and activation of Nrf2 by SFN is accompanied by activation of Akt, inhibition of glycogen synthase kinase (GSK)-3β, and accumulation of Fyn in nuclei. *In vitro* up-regulation of Nrf2 by SFN was abolished and nuclear Fyn accumulation was increased when cardiac cells were exposed to a PI3K inhibitor or GSK-3β-specific activator.

Conclusion: These results suggest that Nrf2 plays a central role in the prevention of Ang II-induced cardiomyopathy, and SFN prevents Ang II-induced cardiomyopathy partially via the Akt/GSK-3β/Fyn-mediated Nrf2 activation.

1. Introduction

Cardiovascular diseases rank high among the most common disorders in adults and are the number one cause of death globally. Pathological activation of the renin–angiotensin system (RAS) is a pivotal contributor to several cardiovascular disorders [1,2]. Angiotensin II (Ang II) is a primary effector peptide of the RAS. It exists not only in systemic circulation but also locally within numerous organs, such as the brain, vasculature, kidney, and heart [3,4]. Multiple lines of experimental and clinical evidence indicate that in addition to the induction of hypertension, increased Ang II in circulation and heart tissue also directly promotes cardiac cell death, hypertrophy, and remodeling [4–7]. Cardiac Ang II has been proven to play a critical role in the pathophysiology of various cardiac diseases, such as acute myocardial infarction [8], alcoholic cardiomyopathy [9], and diabetic cardiomyopathy [7].

It is established that the pathophysiological effects of Ang II in cardiac diseases are mediated by its induction of oxidative stress [10]. Ang II interacts with its receptors (AT), predominantly AT1, to induce nicotinamide adenine dinucleotide phosphate oxidase-mediated generation of reactive oxygen species (ROS), which overwhelms the endogenous antioxidant defenses, leading to oxidative stress [10]. Ang II-induced oxidative stress rapidly activates apoptotic signaling pathways to cause myocardial apoptosis or necrosis that leads to the late

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development of cardiomyopathy [7]. Therefore, oxidative stress plays a central role in Ang II-associated cardiomyopathies, and strategies to reduce the elevated levels of ROS have been intensely investigated [1]. However, failure of ROS scavengers such as antioxidant vitamins or a single antioxidant to improve cardiovascular diseases in large clinical trials indicated the lack of suitable exogenous antioxidants [11,12]. All of those results highlight a unique regulatory role of intrinsic antioxidant defenses in cardiovascular homeostasis and indicate that an approach to up-regulating multiple endogenous antioxidants may be an effectively and safely therapeutic option for cardiovascular patients.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that up-regulates a battery of antioxidant genes and cytoprotective enzymes that constitute the defense against oxidative stress [13]. Activation of Nrf2 protects the heart against ischemia-reperfusion injury [14] and diabetic cardiomyopathy [15]. In our previous studies, we showed that mice with Nrf2 gene deletion are susceptible to Ang II-induced cardiac hypertrophy independent of blood pressure (BP) [16]. In contrast, activation of Nrf2 is associated with the suppression of Ang II-induced oxidative stress in cardiac cells in vitro [17]. However, all of these results did not sufficiently clarify whether up-regulation of Nrf2 can prevent Ang II-induced cardiomyopathies in vivo.

Therefore, the aims of the present study are to determine whether Nrf2 plays a central role in the development of Ang II-induced cardiomyopathy and whether sulforaphane (SFN), a strong Nrf2 activator, can prevent Ang II-induced cardiomyopathy, and to dissect the underlying mechanisms connecting Nrf2 function and cardiac protection in Ang II-induced cardiomyopathy.

2. Materials and methods

2.1. Animals

Male cardiomyocyte-specific Nrf2-gene-overexpressing transgenic (Nrf2-TG) mice with FVB background were generated and described previously [18]. Male FVB mice, mice with global Nrf2 knockout (Nrf2-KO) and their wild-type (WT) C57BL/6 J control mice were purchased from Jackson Laboratory. Mice were housed in the University of Louisville Research Resources Center at 22 °C with a 12/12-h light/dark cycle and free access to rodent chow and tap water. All mice were kept under these conditions for 1 week before each experiment. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, revised 2011). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which is certified by the American Association for Accreditation of Laboratory Animal Care.

For the first animal study, nine-week-old FVB male mice were randomly allocated to four groups (n = 14 per group): control, SFN, Ang II, and Ang II plus SFN treatment (Ang II/SFN). Mice were subcutaneously injected with a subpressor dose of Ang II (0.5 mg/kg body weight, Sigma-Aldrich, St. Louis, MO) based on our previous study [7] every other day for 2 months, with or without subcutaneous injection of SFN (0.5 mg/kg body weight, Sigma-Aldrich) 5 days of each week for 3 months. At the end of the 3-month SFN treatment (3 M), seven mice in each group were euthanized, and the remaining seven mice in each group were kept for additional 3 months (6 M) without SFN treatment.

The second animal study aimed to define whether the prevention of Ang II-induced cardiomyopathy by SFN is Nrf2-dependent or not. Nrf2-KO and WT C57BL/6 J mice as their controls from a separate colony were randomly divided (n = 6 per group) to receive Ang II treatment at 0.5 mg/kg body weight every other day for 2 months, with or without SFN (0.5 mg/kg body weight) 5 days per week for 3 months.

The third animal study aimed to define the direct role of Nrf2 in the prevention of Ang II-induced cardiomyopathy. Therefore, 10 Nrf2-TG and 10 FVB WT mice as their controls from a separate colony were equally divided (n = 5 per group) to receive either Ang II or vehicle treatment for 2 months and then kept for additional 1 month without treatment, creating the WT, WT/Ang II, Nrf2-TG, and Nrf2-TG/Ang II groups.

Three months after the first injection of SFN, the BP of these mice was measured. At the end of each experiment, the animals were euthanized under anesthesia induced by intraperitoneal injection of 1.2% 2,2,2-Tribromoethanol (avertin, ip) at the dose of 240 mg/kg body weight and all efforts were made to minimize suffering, and the hearts were collected for protein, mRNA, and histopathological analyses after cardiac function examination by echocardiography (echo).

2.2. Measurements of non-invasive BP and cardiac function

BP was measured by tail-cuff manometry using a CODATM non-invasive BP monitoring system (Kent Scientific, Torrington, CT) as previously described [9]. Mice were restrained in a plastic tube restrainer and adapted for 5 min prior to BP measurement. Occlusion and volume-pressure recording (VPR) cuffs were placed over the tail of mice to measuring the BP by 20 measurement cycles. Mice were warmed by heating pads during the acclimation cycles to ensure sufficient blood flow to the tail. After 3 days of training for the BP measurement, formal measurements were performed to collect the BP data.

Under sedation of mice with avertin (240 mg/kg, ip), mice were placed in a supine position on a heating pad to maintain body temperature at 36–37 °C that was continuously monitored using a rectal thermometer. Under these conditions, the animal's heart rate ranged between 400 and 550 beats per min, and cardiac function were measured using a high-resolution imaging system (Vevo 770, Visual Sonics, Canada) equipped with a high-frequency ultrasound probe (RMV-707B) as previously described [15]. The animals were monitored closely throughout the measurement protocol, and removed from heating pad immediately upon completing the measurement protocol, and were put back into the home cage after complete recover from the avertin sedation. Echo analysis include the indices of left ventricle (LV) diameters in diastole (LVID;d), LV posterior wall thickness in diastole (LVPW;d), systolic function by ejection fraction (EF, %), and fractional shortening (FS, %).

2.3. Cell culture

Embryonic rat heart-derived cells (H9c2) were purchased from ATCC (CRL-1446, MD, Manassas, VA), and the neonatal cardiomyocytes were isolated from FVB mice as described previously [19]. Both H9c2 cells and neonatal cardiomyocytes were maintained in high-glucose Dulbecco's modified Eagle's medium (4.5 g/L glucose) supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). In the mechanistic study, cells were exposed to a PI3K inhibitor (Ly294002 at 10 μmol/l, Sigma-Aldrich) [20] or a GSK-3β activator [sodium nitroprusside (SNP) at 2 mmol/l, Sigma-Aldrich, based on a pilot study] with or without the presence of SFN (100 nmol/l, Sigma-Aldrich) for 24 h [15].

2.4. Western blot analysis

Heart tissues and harvested cell pellets were homogenized in RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA) to obtain total protein or nuclear protein extracted using a nuclei isolation kit (NUC201, Sigma-Aldrich). Western blot assay was performed for target protein quantification, as described previously [7,9,15]. The total or nuclear proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. Membranes were blocked with 5% non-fat milk for 1 h and incubated overnight at 4 °C with the following antibodies from Santa Cruz Biotechnology: anti-atrial natriuretic peptide (ANP), β-actin, transforming growth factor (TGF)-β1, connective tissue growth

factor (CTGF), tumor necrosis factor (TNF)-α, Nrf2, catalase (CAT), NAD(P)H: quinone oxidoreductase (NQO1), Histone 3, and heme oxygenase 1 (HO-1), all of which were used at a dilution of 1:1000. The primary antibodies also included those against phosphorylated Akt (Ser473, p-Akt), phosphorylated GSK-3β (Ser9, p-GSK-3β), Akt, GSK-3β, and Fyn (Cell Signaling Technology, Danvers, MA, 1:1000 dilution), as well as 3-nitrotyrosine (3-NT, 1:3000 dilution, Millipore, Billerica, MA), 4-hydroxy-2-nonenal (4-HNE, 1:4000, Alpha Diagnostic International, San Antonio, TX), plasminogen activator inhibitor-1 (PAI-1, 1:2000, BD Biosciences, San Jose, CA), AT1 (1:1000, Abcam, Cambridge, MA), and phosphorylated Nrf2 at Ser40 antibody (p-Nrf2; 1:10,00, Abcam, Cambridge, MA). After washing off the unbound antibodies, membranes were incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000, Santa Cruz Biotechnology) for 1 h at room temperature. Specific bands were visualized using an enhanced chemoluminescence detection kit (ECL, Thermo Scientific, Waltham, MA). Quantitative densitometry was performed on the identified bands using Image Quant 5.2 software (Molecular Dynamics, Inc., Sunnyvale, CA).

2.5. Cardiac pathological examination and immunostaining

Heart tissue was fixed in 10% formalin for 24 h, embedded in paraffin, and sectioned at 5 μm. The heart sections were deparaffinized, rehydrated, and then stained with hematoxylin and eosin (H&E).

Cardiac fibrosis was examined by Sirius-red staining for collagen deposition as described previously. [21] The proportion of fibrosis (collagen) was quantitated using a Nikon Eclipse E600 microscopy system as described previously [21].

Standard immunohistochemical and immunofluorescent staining protocols were performed as described previously [22]. For immunohistochemical staining, heart tissue sections were stained with p-Nrf2 (1:10,000). After washing, sections were incubated with HRP-conjugated secondary antibody, developed with a DAB (3,3-diaminobenzidine) developing system (Vector Laboratories, Inc., Burlingame, CA), and counterstained with hematoxylin. For immunofluorescent staining, anti-Fyn antibody (1:500, Cell Signaling Technology) was used. The secondary antibodies Cy3-conjugated immunoglobulin G (IgG; at 1:200, Abcam) were applied and counterstained with 4,6-diamidino-2-phenylindole (DAPI, 0.0002% solution, Sigma-Aldrich).

2.6. RNA isolation and real-time PCR

Total RNA was extracted from heart tissues using Trizol reagent (RNA STAT 60 Tel-Test Ambion, Austin, TX). The cDNA was synthesized from 1 μg of total RNA according to the manufacturer's protocol for the RNA RCR kit (Promega, Madison, WI). The mRNA expression of target genes was detected as described previously [7]. The primers (Nrf2: Mm00477784, HO-1: Mm00516005, NQO1: Mm01253561; CAT: Mm00437229; β-actin: Mm00607939; ANP: Mm01313000; AT1: Mm Mm00616371) were purchased from Applied Biosystems (Foster City, CA). Comparative cycle time (Ct) was used to determine fold differences between samples and normalized to an endogenous reference (β-actin).

2.7. Biochemical measurement of lipid peroxides

Lipid peroxide accumulation in heart was detected by measuring thiobarbituric acid (TBA) reactivity, which is reflected by the amount of malondialdehyde (MDA) formed during acid hydrolysis of the lipid peroxide compound. The assay procedure was described previously [22]. The lipid peroxide level in heart is expressed in units of nanomole of MDA per milligram of tissue.

2.8. Enzyme immunoassay for Ang II

Ang II levels in the plasma and cardiac tissue were analyzed with enzyme immunoassay as described previously [9]. Briefly, heart tissues were boiled in 0.1 mol/L acetic acid for 20 min at 100 °C and then homogenized in an ice bath. The homogenate was centrifuged at 13,000 × *g* for 30 min at 4 °C to collect the supernatant. Then extraction of peptides from plasma or supernatant of heart tissues was performed using a kit including a SEP-COLUMN and Buffer A and B (Pack Phoenix Pharmaceuticals, Inc., Burlingame, CA) based on the manufacturer's instructions. Ang II levels in the dried samples from plasma were measured with the Ang II Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions. Ang II concentration is expressed in units of picogram Ang II per milliliter plasma or picogram Ang II per milligram cardiac protein.

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD) values (n = 5–7 per group). Comparisons were performed by two-way analysis of variance (ANOVA) for the different groups, followed by Tukey's test in pair-wise repetitive comparisons with Origin 7.5 software (OriginLab Corporation, Northampton, MA). A P *value* < 0.05 was considered statistical significant.

3. Results

3.1. SFN prevents Ang II-induced cardiac dysfunction and hypertrophy

To define whether SFN protects against Ang II-induced cardiomyopathy, mice were given a subpressor dose of Ang II every other day for 2 months with or without treatment with SFN 5 days per week for 3 months (3 M) and then kept until 6 months (6 M). Ang II treatment significantly increased the level of Ang II in plasma at 3 M and in hearts at both 3 M and 6 M (Fig. 1**A**). These increases were accompanied by elevated expression of the *AT1* gene in hearts (Online Fig. S1). While BP was not significantly changed at 1 month after Ang II administration (3 M), indicating the lack of a persistent high BP effect at the dose used (Fig. 1**B**). However, the subpressor dose of Ang II induced cardiac dilation (enlargement of LV chamber diameter, LVID), systolic dysfunction (reduced EF and FS), and hypertrophy (increased LVPW at diastole), as examined by echo (Fig. 1**C**). Ang II-induced cardiac hypertrophy was also demonstrated by an increased ratio of heart weight to tibia length (Fig. 1**D**) and enlarged and thickened myocardial cells with large nuclei or multi-nuclei as observed by H&E staining (Fig. 1**E**). Furthermore, expression of the molecular hypertrophic marker ANP was also progressively increased at both protein (Fig. 1**F**) and mRNA (Fig. 1**G**) levels from 3 M to 6 M. However, all these changes related to cardiac dysfunction and hypertrophy were significantly prevented by SFN treatment for 3 months (Fig. 1**C–G**), which did not change plasma and cardiac Ang II (Fig. 1**A**) levels and AT1 expression (Online Fig. S1).

3.2. SFN prevents Ang II-induced cardiac inflammation, oxidative damage, and fibrosis

Because inflammation and oxidative stress have been suggested to play important roles in Ang II-induced cardiomyopathy [7], cardiac expression of the inflammatory cytokine TNF-α and PAI-1 was examined by Western blot. Treatment with Ang II for 2 months induced a persistent elevation of these inflammatory factors in the heart, an effect that is prevented by SFN-treatment (Fig. 2**A**). Ang II treatment also significantly increased the accumulation of 3-NT (an index of nitrosative damage) and 4-HNE (an index of lipid peroxidation) in the hearts of mice at both 3 M and 6 M, which was almost completely prevented by SFN treatment (Fig. 2**B**).

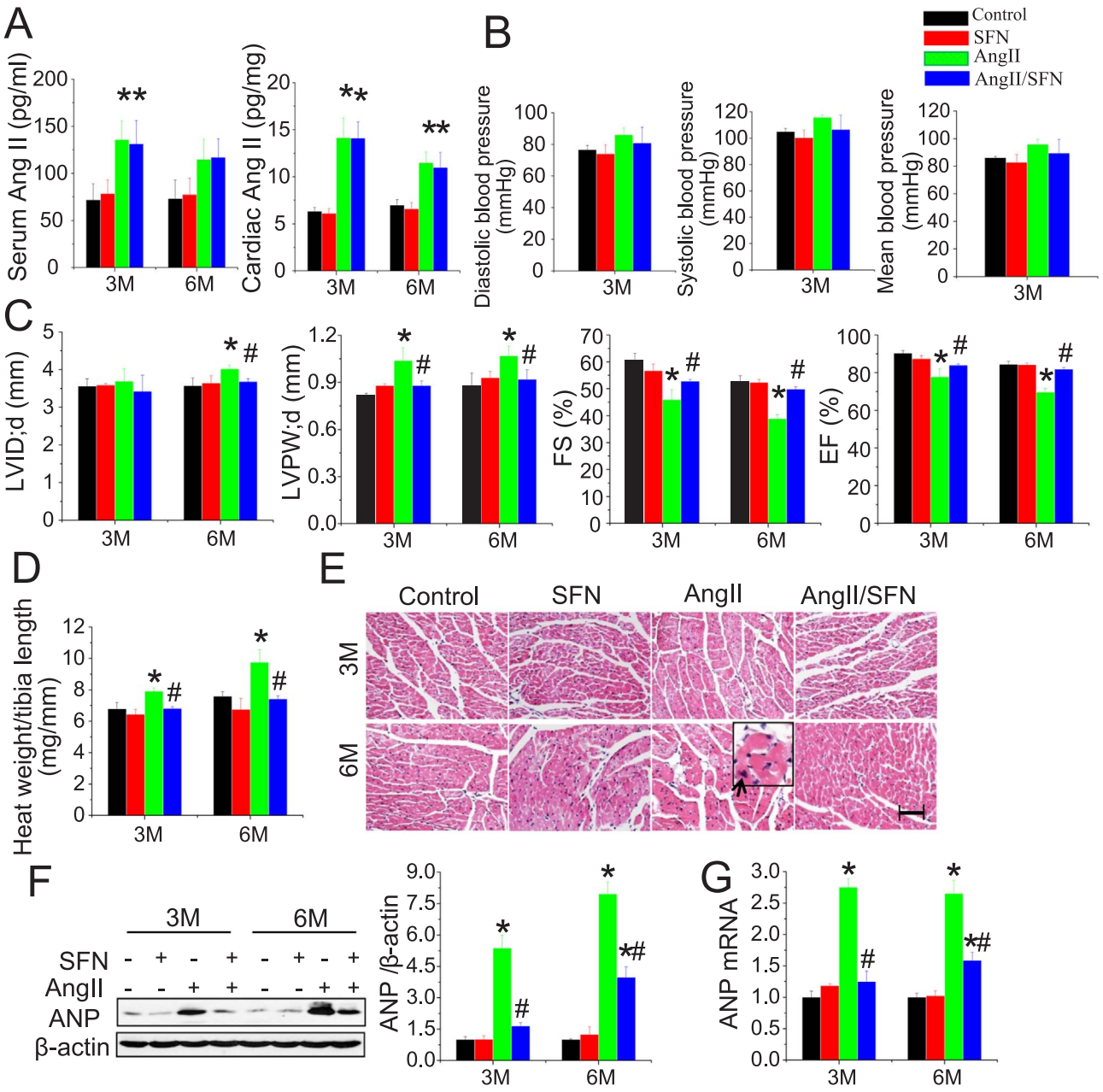

Fig. 1. SFN prevents Ang II-induced cardiac dysfunction and hypertrophy in the heart. Mice were given a subcutaneous injection of angiotensin (Ang II) at 0.5 mg/kg body weight every other day for 2 months, with or without SFN (0.5 mg/kg body weight) 5 days per week for 3 months (3 M) and then kept until 6 months (6 M). At both 3 M and 6 M, Ang II levels in the plasma and cardiac tissue (**A**) were examined by enzyme-linked immunosorbent (ELISA) assay, and cardiac function was measured by Echo (**C**). At 3 M, blood pressure was detected by a CODA™ non-invasive blood monitoring system (**B**). The ratio of heart weight to tibia length (**D**) was calculated, and cardiac morphological changes were examined by hematoxylin and eosin staining (**E**, bar = 100μm). Expression of a cardiac hypertrophic marker, atrial natriuretic peptide (ANP) at protein (**F**) and mRNA (**G**) levels was detected by Western blot and real-time PCR. Data are presented as the mean ± standard deviation (SD, n = 7). *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. Ang II. LVID;d = Left ventricular end-diastolic diameter; LVPW; d = Left ventricular end-diastolic posterior wall thickness; FS = fractional shortening; EF = ejection fraction.

It has been proven that Ang II-induced cardiac inflammation, cell death, and oxidative damage led to the development of cardiac remodeling and dysfunction at later stages [7]. Therefore, whether SFN prevents Ang II-induced cardiac remodeling was examined by Western blot analysis, which revealed a time-dependent increase in the expression of pro-fibrotic factors CTGF and TGF-β1 (Fig. 2**C**) and the accumulation of collagen as shown by Sirius red staining (Fig. 2**D**) in Ang II group from 3 M to 6 M, but not in Ang II/SFN group.

3.3. SFN up-regulates the expression and function of Nrf2 in the heart

The results described above indicate that SFN can prevent Ang II-induced cardiomyopathy. Therefore, whether SFN prevents Ang II-induced this effect by activating Nrf2 was examined first by measuring Nrf2 expression and its transcription in the heart. The expression of Nrf2 at both mRNA (Fig. 3**A**) and protein (Fig. 3**B**) levels was significantly increased in SFN group at both 3 M and 6 M. Ang II treatment did not affect cardiac Nrf2 expression at 3 M but significantly decreased it at 6 M. However, compared with that in the Ang II group, cardiac Nrf2 expression was significantly increased in the Ang II/SFN group at 3 M and even at 6 M (Fig. 3**A&B**).

Given that Nrf2 phosphorylation at Ser40 (p-Nrf2) represents its activation, immunohistochemical staining for p-Nrf2 was performed and showed that p-Nrf2 expression was predominantly located in the

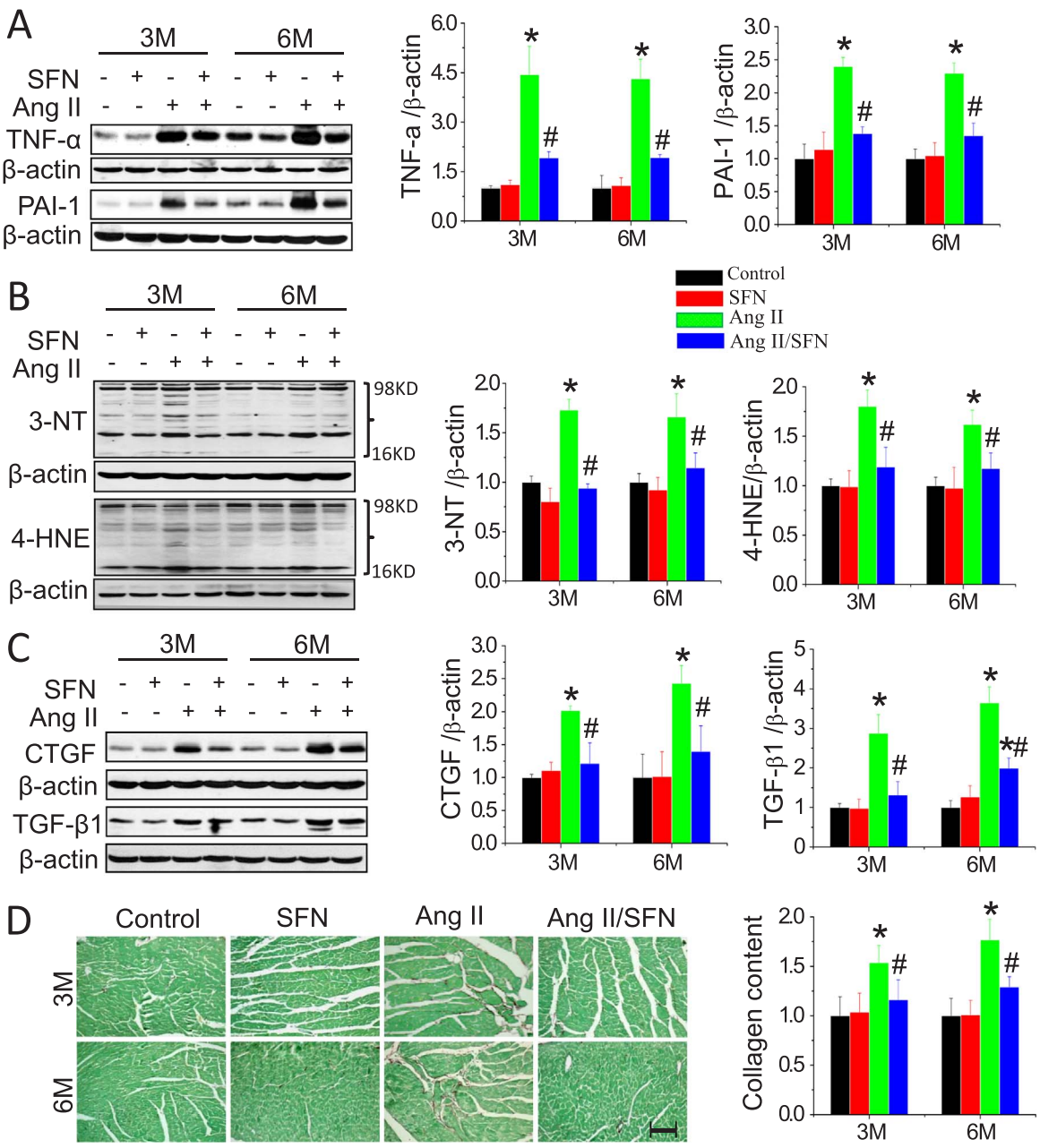

Fig. 2. SFN prevents Ang II-induced cardiac inflammation, oxidative stress, and fibrosis in the heart. Mice were treated as described in Fig. 1. Western blot was used to detect the expression of inflammatory factors tumor necrosis factor-α (TNF-α) and plasminogen activator inhibitor-1 (PAI-1) (**A**), the accumulation of oxidative stress markers 3-nitrotyrosine (3-NT) and 4-hydroxy-2-nonenal (4-HNE) (**B**), and the cardiac fibrotic markers connective tissue growth factor (CTGF) and transforming growth factor-β1 (TGF-β1) (**C**). Sirius red staining was used to detect the deposition of collagen (**D**, bar = 100μm). Data are presented as the mean ± SD (n = 7). *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. Ang II.

nuclei of cardiomyocytes in SFN-treated control and Ang II groups (Fig. 3**C**). Quantitative analysis of p-Nrf2 expression by Western blot confirmed the significant increase in p-Nrf2 expression in both SFN and Ang II/SFN groups at both 3 M and 6 M (Fig. 3**D**).

Nrf2 transcriptional function was confirmed by increased expression of its downstream antioxidant genes, CAT, NQO1, and HO-1 at the mRNA (Fig. 3**E**) and protein (Fig. 3**F**) levels. Ang II did not affect the expression of these genes at 3 M, but significantly decreased their expression at 6 M (Fig. 3**E&F**). Compared with that in Ang II group, cardiac expression of these genes was significantly increased in Ang II/SFN group at both 3 M and 6 M (Fig. 3**E&F**). These results suggest that SFN-mediated prevention of Ang II-induced cardiac damage is associated with the activation of Nrf2.

3.4. Knockout of Nrf2 gene abolishes the protection of SFN against Ang II-induced cardiomyopathy

To determine the direct role of Nrf2 in SFN-mediated cardiac protection against Ang II, Nrf2-KO and WT mice were treated with Ang II and SFN for 2 and 3 months, respectively. In Nrf2-KO mice, Western blot analysis revealed almost no Nrf2 expression regardless of SFN administration (Fig. 4**A**). Expression of Nrf2 downstream genes, NQO1 and HO-1, was significantly decreased in Nrf2-KO control or SFN-treated mice (Fig. 4**B**). These results indicate that deletion of Nrf2 gene abolishes the SFN-induced activation of antioxidant genes downstream of Nrf2.

In C57BL/6J (WT) mice, consistent with the changes in FVB mice (Figs. 1–3), Ang II treatment caused significant cardiac systolic dysfunction (reduced EF and FS) and cardiac hypertrophy (increased LVID

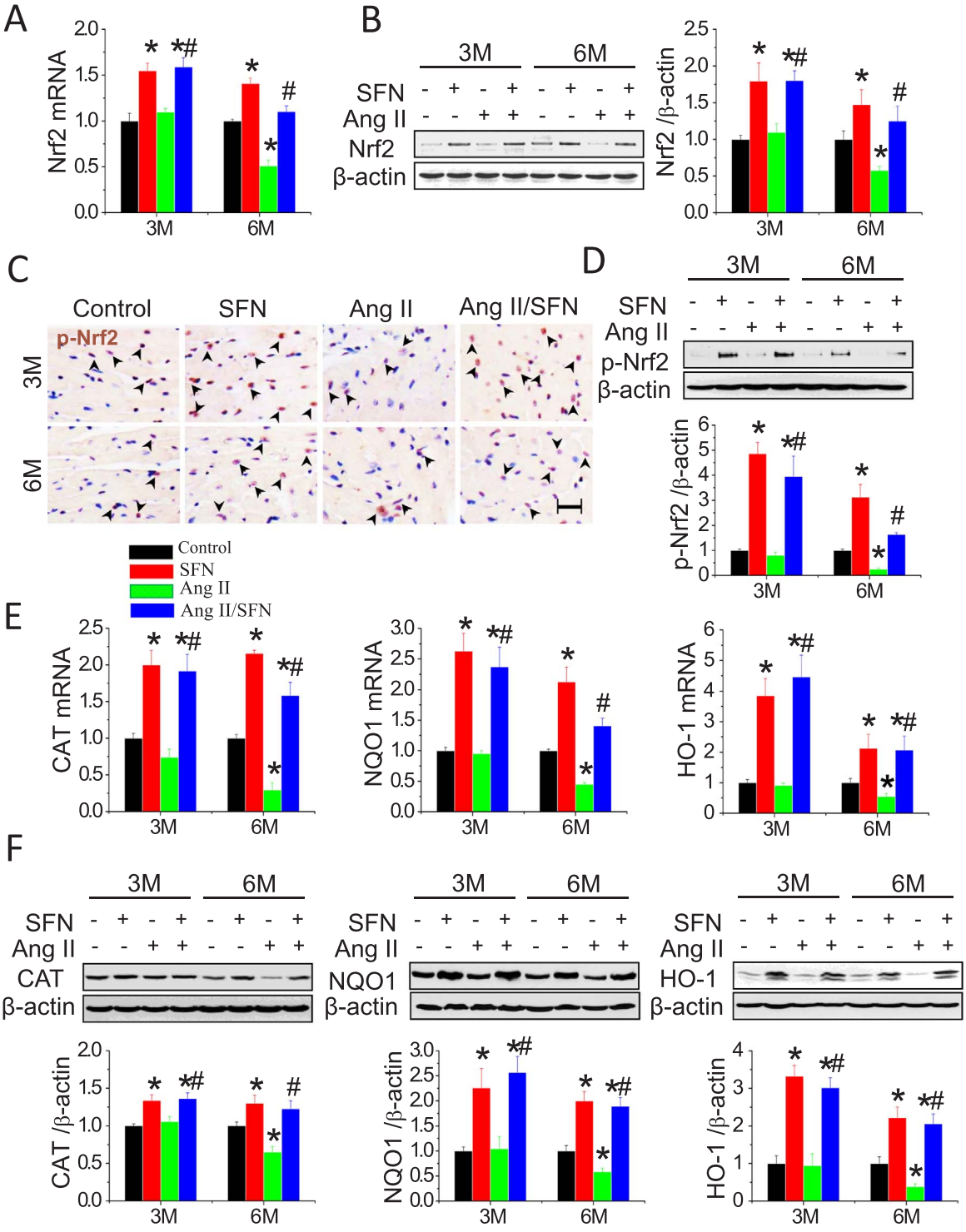

Fig. 3. SFN up-regulates the expression and function of Nrf2 in the heart. Mice were treated as described in Fig. 1. Nrf2 expression at mRNA and protein levels was detected by real-time PCR (**A**) and Western blot (**B**), respectively. The activation of Nrf2 was examined by immunohistochemical staining (**C**, counter stain with hematoxylin, bar = 25μm) and Western blot (**D**) for Nrf2 phosphorylation at Ser40 (p-Nrf2). Nrf2 function was measured by determining the expression of Nrf2 downstream genes, catalase (CAT), NAD(P)H: quinone oxidoreductase (NQO1), and heme oxygenase 1 (HO-1), at both mRNA (**E**) and protein (**F**) levels with real-time PCR and Western blot, respectively. Data are presented as the mean ± SD (n = 7). *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. Ang II.

and LVPW at diastole, elevated heart weight to tibia length ration and ANP expression) without affecting BP (Fig. 4**C–F**). All of these cardiac changes were prevented by SFN treatment. Compared with WT mice, however, Nrf2-KO mice treated with Ang II showed further decreases in EF and FS and further increases in the ratio of heart weight to tibia length and ANP expression. Meanwhile, SFN treatment could not prevent Ang II-induced cardiac dysfunction and cardiac hypertrophy in Nrf2-KO mice (Fig. 4**C–F**).

Ang II significantly induced cardiac fibrosis [increased collagen accumulation (Fig. 5**A**) and CTGF expression (Fig. 5**B**)], inflammation (elevated TNF-α and PAI-1 expression, Fig. 5**C**), and oxidative damage [increased accumulation of 3-NT (Fig. 5**D**) and formation of MDA (Fig. 5**E**)] in both WT and Nrf2-KO mice. CTGF, TNF-α, and PAI-1 as fibrotic and inflammatory factors were further increased in the Nrf2-

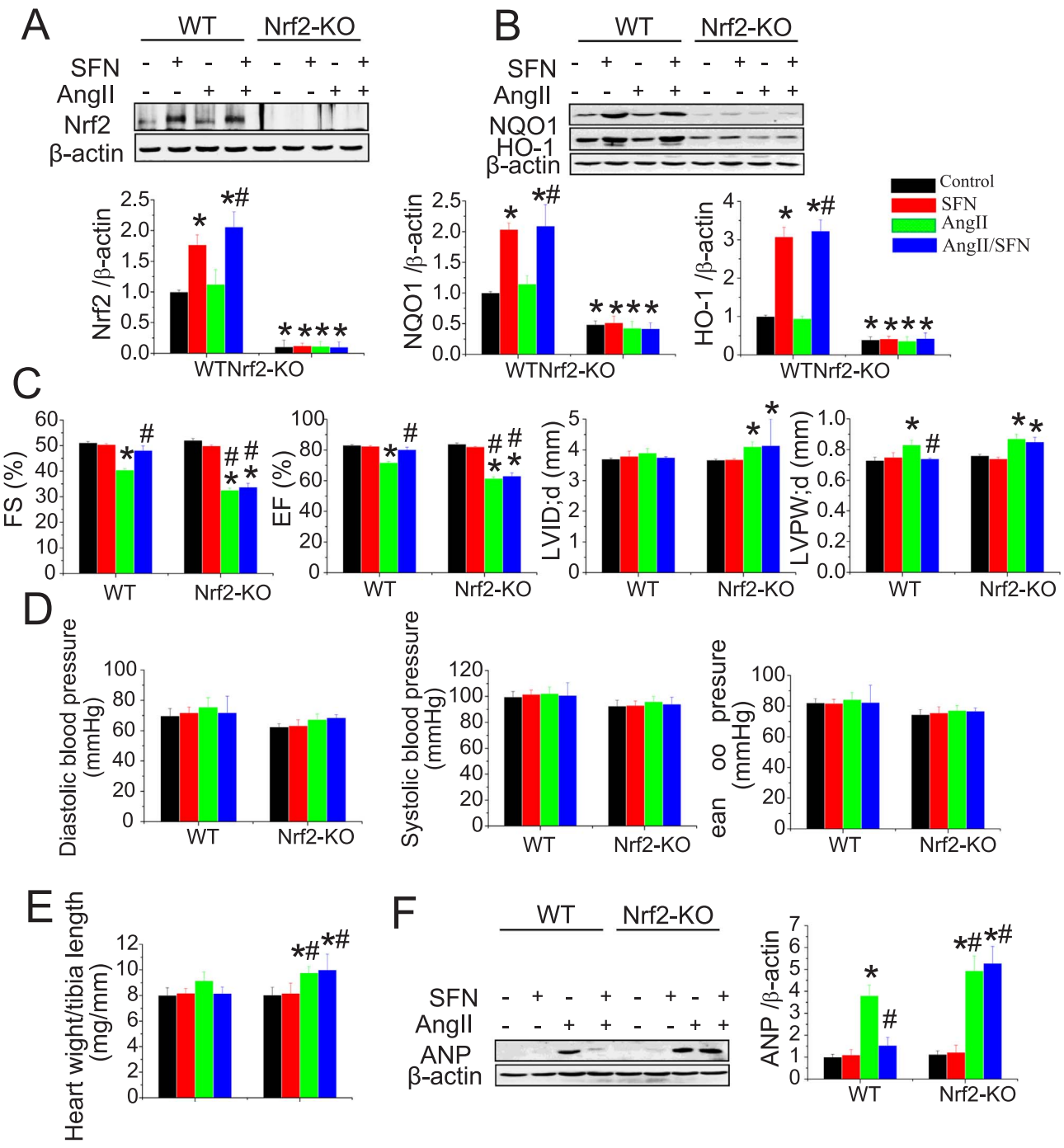

Fig. 4. Knockout of Nrf2 gene abolishes the protection of SFN against Ang II-induced cardiac dysfunction and hypertrophy. Nrf2-knockout (Nrf2-KO) and wild-type (WT) control mice were subcutaneously injected with Ang II at 0.5 mg/kg body weight every other day for 2 months, with or without SFN (0.5 mg/kg body weight) 5 days per week for 3 months. Expression of Nrf2 (**A**) and its downstream genes NQO1 and HO-1 (**B**) were examined by Western blot. Cardiac function (**C**) and blood pressure (**D**) were measured as described in Fig. 1. Cardiac hypertrophy was determined by calculating the ratio of heart weight to tibia length (**E**) and evaluating ANP expression by Western blot (**F**). Abbreviations are the same as in Figs. 1–3. Data are presented as the mean ± SD (n = 6). *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. WT/Ang II.

KO/Ang II mice compared with WT/Ang II mice. However, SFN treatment prevented these pathological changes in WT mice, but not in Nrf2-KO mice (Fig. 5**A–E**). Hence, deletion of *Nrf2* gene exacerbates Ang II-induced cardiac damage and dysfunction and abolished SFN's cardiac protection against Ang II-induced cardiac damage.

3.5. Cardiac overexpression of Nrf2 prevents Ang II-induced cardiomyopathy

Although we discovered the exacerbation of Ang II-induced cardiac pathological damage and dysfunction in Nrf2-KO mice compared to WT mice, it's still difficult to rule out the systemic effect of the global deletion of *Nrf2* gene. To define the critical role of Nrf2 in the prevention of Ang II-induced cardiomyopathy, cardiomyocyte-specific Nrf2-TG mice and their counterpart WT mice were treated with Ang II under the same conditions as used for Nrf2-KO mice. Western blot analysis showed that compared to WT mice, Nrf2-TG mice exhibited significantly higher expression of cardiac Nrf2 and its downstream genes NQO1 and HO-1 (Fig. 6**A**). WT mice exposed to Ang II for 2 months showed significant cardiac systolic dysfunction, reflected by the

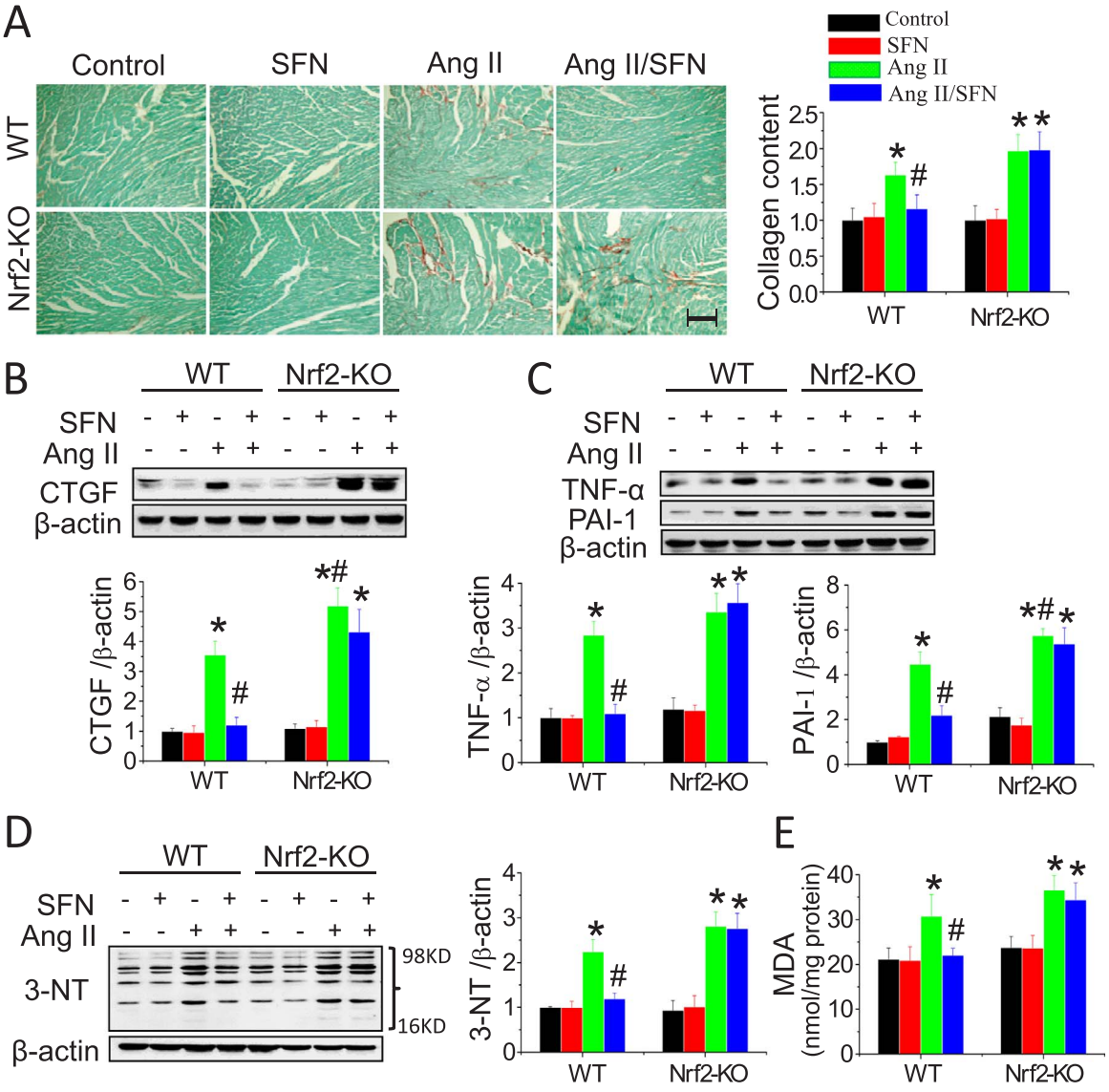

Fig. 5. Knockout of Nrf2 gene abolishes the protection of SFN against Ang II-induced cardiac inflammation, oxidative stress, and fibrosis. Mice were treated as described in Fig. 4. Cardiac fibrosis was determined by Sirius red staining of collagen deposition (A, bar =100μm) and Western blot analysis of CTGF expression (B). The cardiac inflammation was assessed by Western blot analysis of TNF-α and PAI-1 expression (C). Cardiac oxidative stress was evaluated by 3-NT accumulation (D) and malondialdehyde (MDA) production (E). Data are presented as the mean ± SD (n = 6). *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. WT/Ang II.

reduced EF and FS, but without significant effects on other echo measurements and BP (Fig. 6**B**). Exposure to Ang II also significantly induced cardiac fibrosis (increased CTGF expression and Sirius red staining, Fig. 6**C&D**), cardiac inflammation (elevated TNF-α expression, Fig. 6**D**), and cardiac oxidative damage (increased accumulation of 3-NT and formation of MDA, Fig. 6**E&F**). As expected, all of these pathogenic changes and cardiac dysfunction were not observed in Nrf2-TG/Ang II group (Fig. 6**B–F**).

3.6. Up-regulation and activation of Nrf2 by SFN is achieved partially through stimulating the Akt/GSK-3β/Fyn pathway

Reportedly, the function of Nrf2 can be regulated by the Akt/GSK-3β/Fyn pathway via controlling Fyn-mediated export and degradation of nuclear Nrf2 [23]. To explore the mechanism by which SFN up-regulates Nrf2 activity, activation of the Akt/GSK-3β/Fyn pathway upon SFN treatment was examined. SFN treatment significantly increased the activation of Akt (increased p-Akt expression, Fig. 7**A**), accompanied by a decrease in GSK-3β activity (increased GSK-3β phosphorylation) in the hearts (Fig. 7**B**).

Immunofluorescent staining (Fig. 7**C**) and Western blot analysis of nuclear proteins from hearts (Fig. 7**D**) revealed that SFN significantly reduced the nuclear accumulation of Fyn in control mice at both 3 M and 6 M. Ang II did not affect Akt or GSK-3β activation and nuclear translocation of Fyn at 3 M, but significantly inactivated Akt and activated GSK-3β at 6 M as well as increased nuclear accumulation of Fyn (Fig. 7**A–D**). However, SFN could preserve Akt activation and inhibit GSK-3β activation and nuclear translocation of Fyn in Ang II/SFN group at both 3 M and 6 M (Fig. 7**A–D**). These in vivo results imply that SFN activation of Nrf2 may involve Akt, GSK-3β, and Fyn.

To directly demonstrate that Akt, GSK-3β, and Fyn are involved in the regulation of Nrf2 activation by SFN, H9c2 cells were exposed to a PI3K inhibitor (Ly294002) or a GSK-3β activator (SNP) with or without SFN treatment, and then the changes in Nrf2 activation were examined. Ly294002 significantly attenuated Akt activation and enhanced GSK-3β activation and nuclear accumulation of Fyn (Online Fig. S2A), which resulted in the down-regulation of Nrf2 and expression of its downstream genes HO-1 and NQO1in H9c2 cells treated with SFN (Online Fig. S2B). To explore the role of GSK-3β in activating Nrf2 in response to SFN, GSK-3β phosphorylation inhibitor, SNP, was used to evaluate its

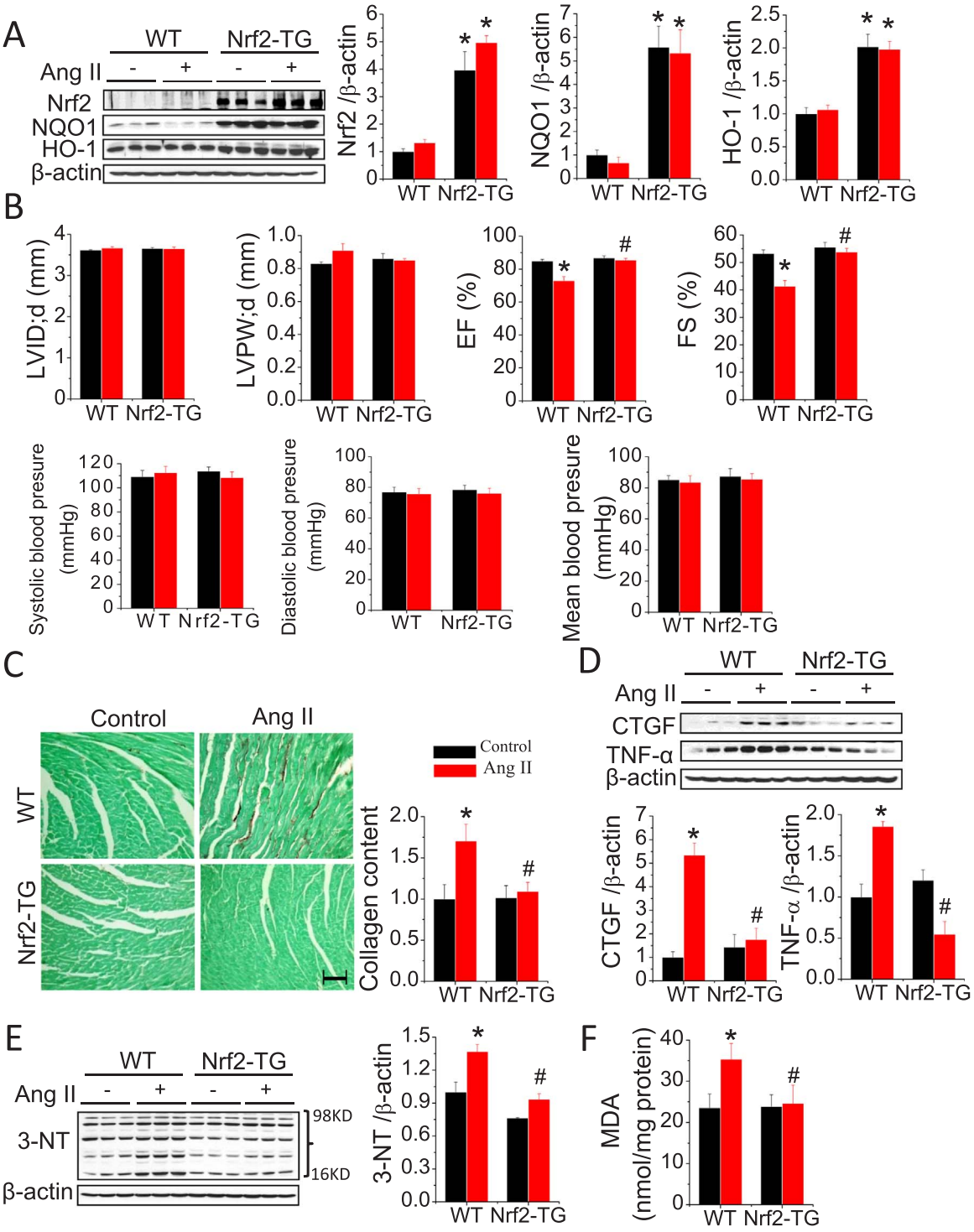

Fig. 6. Cardiac overexpression of Nrf2 renders mice resistant to Ang II-induced cardiomyopathy. Transgenic mice with cardiomyocyte overexpression of Nrf2 (Nrf2-TG) and FVB wild type (WT) mice were treated with or without Ang II at 0.5 mg/kg body weight every other day for 2 months. At 3 months after the first dose of Ang II, the expression of Nrf2 and its downstream genes NQO1 and HO-1 was detected by Western blot (**A**). Cardiac function and blood pressure were examined as described in Fig. 1 (**B**), and cardiac fibrosis was determined by Sirius red staining of collagen deposition (**C**, bar = 100μm) and Western blot analysis of CTGF expression (**D**). Cardiac inflammation was assessed by Western blot analysis of TNF-α expression (**D**). Cardiac oxidative stress was determined by 3-NT accumulation (**E**) and MDA production (**F**). Data are presented as the mean ± SD (n = 5). *, $p < 0.05$ vs. WT; #, $p < 0.05$ vs. WT/Ang II.

inhibiting efficiency for GSK-3β phosphorylation at the dose (2 mmol/L) without cytotoxic effect (Online Fig. S3**A&B**). Furthermore, activation of GSK-3β by SNP at 2 mmol/L significantly induced the nuclear accumulation of Fyn in the cells exposed to SFN (Online Fig. S4A), decreased expression of Nrf2 in nuclei, and decreased expression of Nrf2 downstream genes HO-1 and NQO1 (Online Fig. S4B). Most importantly, the responses to Ly294002 or SNP seen in H9c2 cells were also observed in the primary culture of neonatal cardiomyocytes (Online Fig. S5-S6). These in vitro results prove that either inactivation of Akt or activation of GSK-3β causes the nuclear translocation of Fyn to block the activation of Nrf2 by SFN in cardiomyocytes.

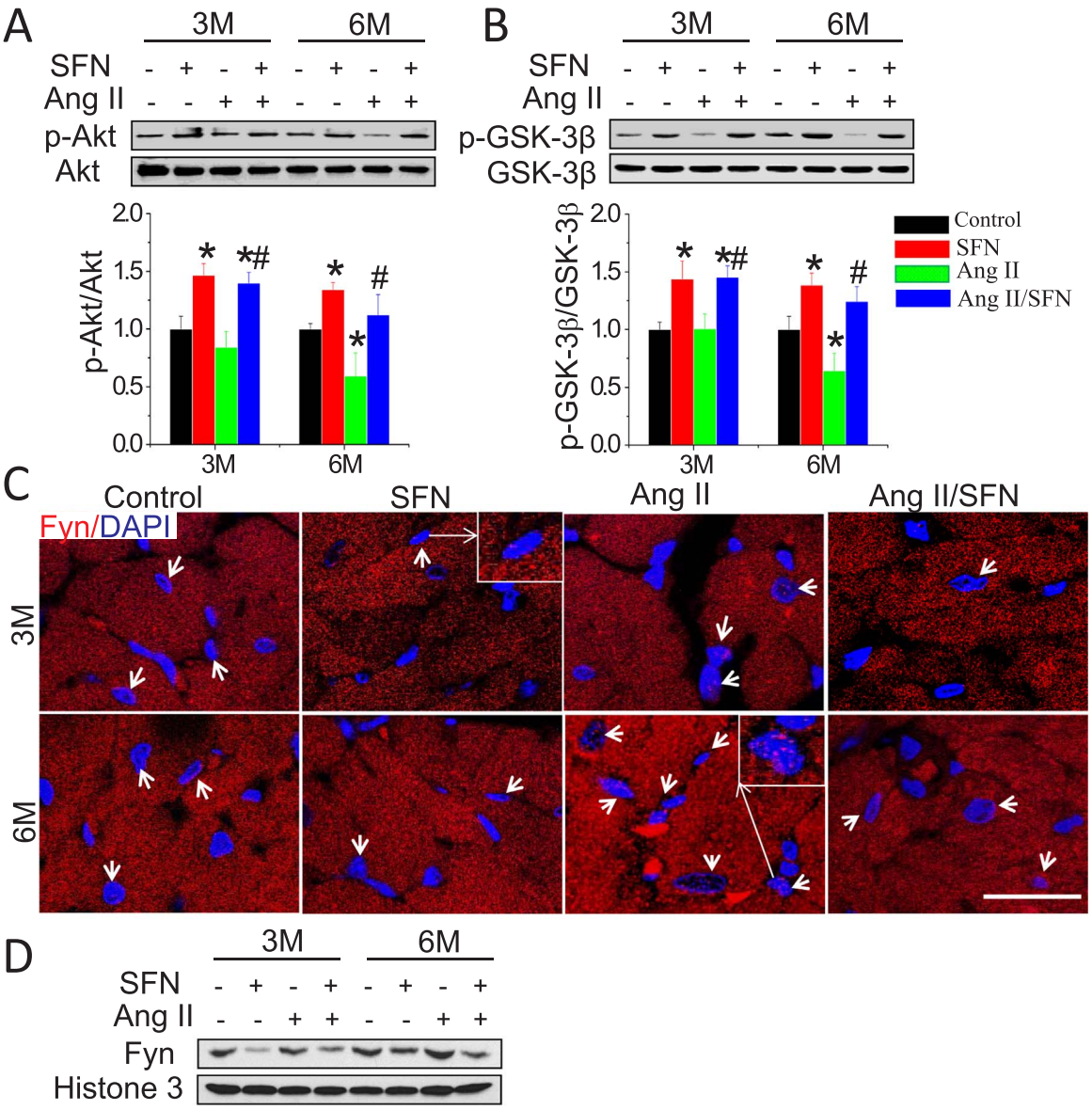

Fig. 7. Up-regulation of Nrf2 by SFN is partially achieved through the AKT/GSK-3β/Fyn pathway. Cardiac tissues were collected as described in Fig. 1. Cardiac Akt (**A**) and GSK-3β (**B**) phosphorylation was examined by Western blot. The nuclear translocation of Fyn was determined by immunofluorescent staining of Fyn nuclear accumulation (red) on heart tissue sections (**C**, bar = 100μm) and Western blot assay of Fyn expression in cardiac nuclear fraction (**D**). Data are presented as the mean ± SD (n = 7). *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. Ang II. DAPI = 4,6-diamidino-2-phenylindole. (**E**) Schematic illustration of mechanism by which SFN protects against Ang II-induced cardiomyopathy: Ang II-induced cardiac inflammation, oxidative stress, and fibrosis lead to cardiac remodeling and dysfunction via increasing the generation of reactive oxygen and/or nitrogen species (ROS and/or RNS). SFN can attenuate Ang II-induced cardiac oxidative stress, inflammation, and dysfunction by up-regulating and activating Nrf2 via the Akt/GSK-3β/Fyn pathway.

4. Discussion

We had shown that SFN can prevent diabetic cardiomyopathy in both type 1 and type 2 diabetic mice via up-regulation of Nrf2 [15,24,25]. It has also been established that Ang II plays a critical role in the development of diabetic cardiomyopathy [7]. However, there was no evidence to show the direct role of Nrf2 in the protection from Ang II-induced cardiomyopathy in vivo. Moreover, the underlying mechanisms connecting Nrf2 function and Ang II-induced cardiomyopathy remain unknown. Therefore, the present study shows for the first time that: (1) SFN can prevent Ang II-induced cardiomyopathy; (2) Nrf2 activation plays a pivotal role in the protective effect of SFN on Ang II-induced cardiac damage; (3) SFN stabilizes Nrf2 protein and preserves its activation through the Akt/GSK-3β/Fyn pathway in the heart. Therefore, this study provides direct evidence that administration of Nrf2 activator, SFN, is an effective and safe strategy to prevent Ang II-associated cardiomyopathy.

Oxidative stress has been defined as a key player for Ang II's pathogenic effect on the inflammatory and fibrotic responses, leading to the development of cardiomyopathy. [7] In the present study, administration of a subpressor dose of Ang II for 2 months without pressure overload induced a late cardiomyopathy at 3 M and 6 M, reflected by progressive increases in cardiac dysfunction and cardiac remodeling following the significant increases in cardiac inflammation and oxidative damage (Figs. 1 and 2).

Nrf2 is a master redox regulator that controls the basal and inducible expression of a battery of intrinsic antioxidant genes and phase II detoxifying enzymes. Up-regulation of antioxidant enzymes, most of which are regulated by Nrf2, has been shown to inhibit Ang II-induced oxidative stress and cardiac hypertrophy [26], and knockout of Nrf2 exaggerates Ang II-induced pathological changes [16]. These results indicate the important role of the precise interplay between Ang II and Nrf2-mediated endogenous antioxidant defense in the development of Ang II-induced cardiomyopathies. Here we found that the expression

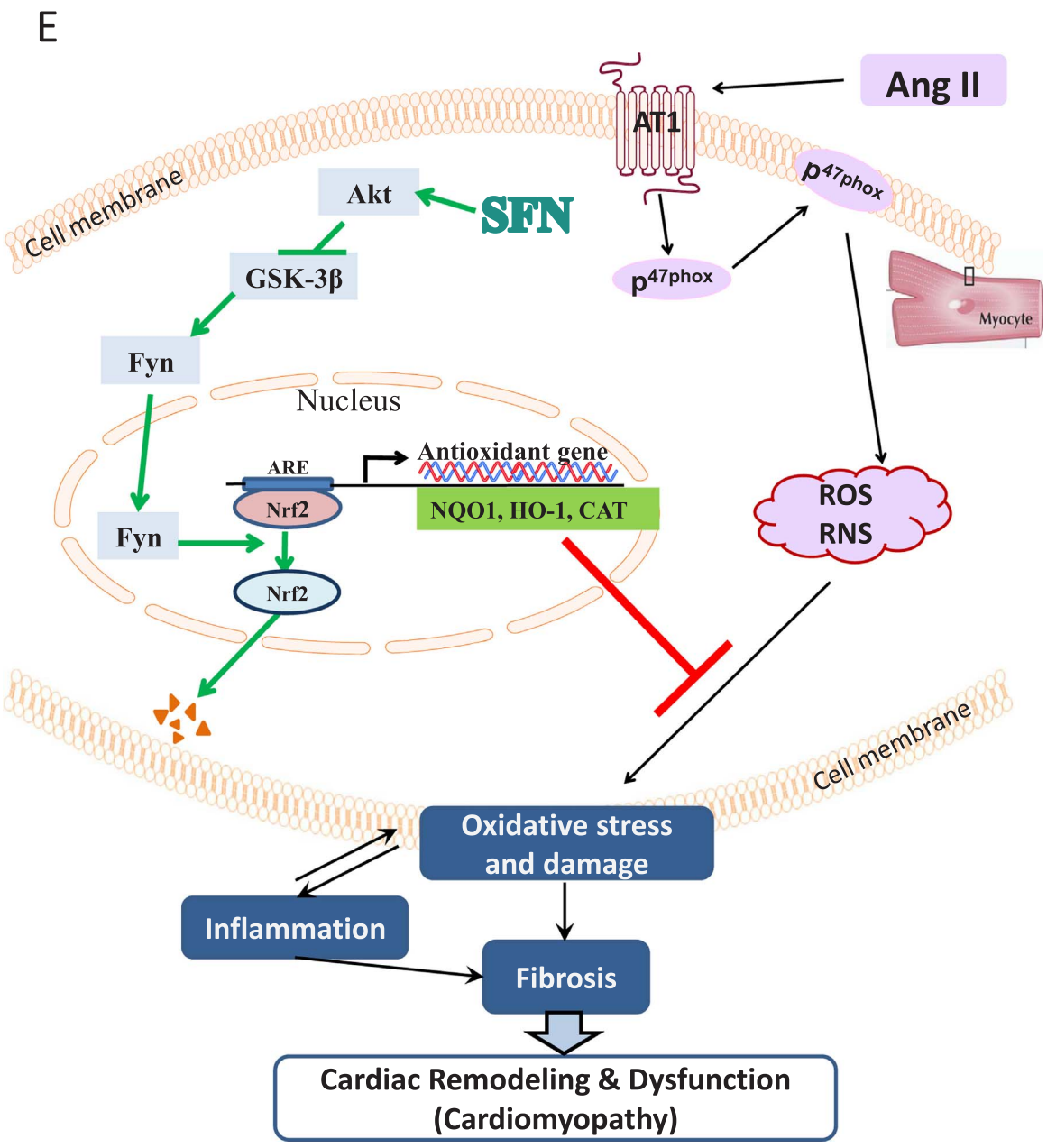

Fig. 7. (*continued*)

and function of Nrf2 was not changed at 3 M, but significantly decreased at 6 M in the heart of Ang II-treated mice along with cardiac pathological changes and dysfunction (Fig. 3). Notably, Nrf2 function was up-regulated in the heart after 2 weeks in an Ang II-induced hypertension model [16]. The same pattern of cardiac Nrf2 expression also was seen in diabetes-induced oxidative damage and cardiomyopathy [15,20]. Down-regulation of Nrf2 function is also found to be responsible to redox-sensitive vascular dysfunction under hypertension condition [27]. Therefore, we speculate that like diabetes, the increase in Nrf2 expression and function during the early stage is an important compensatory response trying to overcome Ang II-induced oxidative stress, whereas long-term oxidative stress impairs Nrf2 function, which exaggerates cardiac oxidative damage at late stages. In further support of our speculation and to directly clarify the central role of cardiac Nrf2 in the prevention of Ang II-induced damage, the present study provides the first evidence that further up-regulated cardiac Nrf2 expression and function can completely prevent Ang II-induced cardiomyopathy (Fig. 6). Moreover, these results also suggest that an approach to targeting the activation of Nrf2 could achieve protection against Ang II-associated cardiomyopathy. It should be mentioned that Nrf2 might play multifactorial roles in cardiac function under different pathophysiological conditions. For example, one recent study found that when autophagy is intact, Nrf2 is required for cardiac adaptive responses; however, autophagy impairment turns off Fyn-operated Nrf2 nuclear export thus activating Nrf2-driven angiotensinogen transcription, which exacerbates cardiac maladaptation leading to dysfunction [28]. Another study also found that stimulation of Nrf2 using SFN negated Akt2 ablation-offered beneficial effect against paraquat whereas inhibition of Nrf2 using luteolin mimicked Akt2 ablation-induced beneficial effect against paraquat challenge [29]. So far, the accurate mechanism of the detrimental effects of Nrf2 on myocardium remains largely unknown, which needs to be studied in the future.

A synthetic triterpenoid derivative has been used as Nrf2 activator to prevent diabetic nephropathy in a phase III trial, but withdrawn early due to its potential cardiovascular toxicity [30]. Therefore, dietary sources of Nrf2 activators have been investigated due to their many

advantages [1]. SFN, as an organosulfur compound, has received intense attention for its unique features, such as its origin in naturally consumed vegetables, the commercially available SFN supplement and the safety for human consumption [31,32]. Most importantly, SFN has shown potential protective effects on heart disease [33]. However, no study has revealed the protective effect of SFN on Ang II-induced cardiomyopathy. Instead, previous studies only demonstrated that its cardiac protection is associated with up-regulated Nrf2 expression or function, which lack of definite evidence. The current study revealed that treatment with SFN for 3 months significantly attenuated Ang II-induced cardiac inflammation (Fig. 2**A**), oxidative stress (Fig. 2**B**), and fibrotic response (Fig. 2**C&D**), resulting in the prevention of cardiac hypertrophy and dysfunction (Fig. 1**C–G**). Furthermore, Nrf2-KO mice showed more severe cardiac hypertrophy and dysfunction than WT mice exposed to Ang II, and SFN treatment could not up-regulate Nrf2 and its downstream antioxidants to prevent Ang II-induced pathological changes in Nrf2-KO mice (Figs. 4 and 5). These results clarify the pivotal role of the Nrf2-mediated exogenous antioxidant defense in the heart and that SFN prevents Ang II-induced cardiomyopathy through up-regulation of Nrf2 expression and function. Meanwhile, SFN treatment did not affect serum and cardiac Ang II levels (Fig. 1**A**) or AT1 expression in the heart (Online Fig. S1), suggesting that cardiac protection offered by SFN is not attributed to its inhibition of Ang II generation or AT1 expression (Fig. 7**E**).

Then the question arises of how SFN up-regulates Nrf2 expression and activation. In addition to evidence showing that SFN directly decreases Nrf2 degradation by promoting Nrf2 phosphorylation and translocation into nuclei to initiate transcription of downstream antioxidant genes via modification of Keap1 [34], several studies have provided evidence that the GSK-3β/Fyn pathway also prevents the nuclear export of Nrf2, thus maintaining Nrf2 transcriptional action in nuclei [23,35]. To elucidate this mechanism in the heart, we examined cardiac GSK-3β and nuclear Fyn expression. We found that down-regulated Nrf2 expression and activation (Fig. 3**A–D**) in Ang II-treated mice at 6 M was accompanied by GSK-3β activation (Fig. 7**B**) and increase in nuclear accumulation of Fyn (Fig. 7**C&D**); however, SFN activated Nrf2 as well as suppressed GSK-3β and nuclear accumulation of Fyn (Fig. 7**B–D**). *In vitro*, the up-regulation of Nrf2 by SFN in H9c2 cells and neonatal cardiomyocytes was abolished by exposure to a GSK-3β-specific activator, SNP (Online Figs. S4 and S6), which fully proves the participation of the GSK-3β/Fyn pathway in the regulation of cardiac Nrf2 by SFN. These results are consistent with previous findings that inactivation of GSK-3β prevents diabetes-induced cardiac damage [36] and methylglyoxal-derived advanced glycation endproduct-induced cardiomyocyte dysfunction [37].

Akt is a well-known up-stream regulator of GSK-3β, and up-regulation of Nrf2 by SFN is related to activation of Akt [38]. Whether SFN suppression of GSK-3β/Fyn signaling is mediated by Akt is unclear. Meanwhile, recent in vitro work in H9c2 cells indicates that SFN suppresses Akt to attenuate Ang II-induced hypertrophy. Therefore, it remains controversial whether the predominant role of Akt in vivo with SFN and Ang II treatment is to either stimulate hypertrophy or to reduce oxidative stress via Nrf2 stimulation. In the present study, the trend of Akt activation (Fig. 7**A**) was consistent with Nrf2 expression and activation (Fig. 3**A&B**) in both the Ang II- and SFN-treated mice. However, Ang II-induced cardiac hypertrophy appeared at 3 M without significant Akt activation (Figs. 1**D–G and** 7**A**). These results imply that the predominant role of Akt may be related to the SFN-mediated Nrf2 activation in vivo. To verify this hypothesis, we treated H9c2 cells and neonatal cardiomyocytes with a PI3K-specific inhibitor and SFN, and found that up-regulation of Nrf2 by SFN in H9c2 cells and neonatal cardiomyocytes was abolished and GSK-3β activation and nuclear accumulation of Fyn were enhanced (Online Figs. S2A and S5A), which resulted in the down-regulation of Nrf2 and expression of its downstream genes, NQO1 and HO-1 (Online Figs. S2B and S5B). All of these results provide the evidence that Akt is involved in the activation of Nrf2 by SFN through the GSK-3β/Fyn pathway (Fig. 7**E**).

Another innovative finding of the present study is that treatment with SFN for the first 3 months resulted in persistent up-regulation of Nrf2 transcription and expression for another 3 months at least after SFN treatment was stopped (Fig. 3**A&B**). The underlying mechanism remains unclear; however, based on recent reports, epigenetic regulation of Nrf2 by SFN may be involved. Yu et al. first revealed that Nrf2 expression can be regulated by modifying the methylation status of the first five CpGs in the Nrf2 promoter in prostate cancer in TRAMP mice [39]. Later it is further reported that SFN directly enhances Nrf2 transcription and expression by decreasing methylation and elevating histone 3 acetylation in the Nrf2 promoter in prostate cancer cells and mouse skin cells [40]. Therefore, we assume that Nrf2 transcription and expression are up-regulated and retained at elevated levels by SFN via epigenetic regulation, which results in indirect Nrf2 translocation into nuclei and activation, providing a sustained prevention of Ang II-induced oxidative damage and subsequent cardiomyopathy.

In conclusion, the present study first provides evidence that SFN can prevent Ang II-induced cardiomyopathy via activating Nrf2-mediated exogenous antioxidant defenses, and that up-regulation and activation of Nrf2 by SFN is achieved partially through the Akt/GSK-3β/Fyn pathway as illustrated in Fig. 7**E**.

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Declarations of interest

None.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2017.12.016.

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