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Nrf2 expression and function, but not MT expression, is indispensable for sulforaphane-mediated protection against intermittent hypoxia-induced cardiomyopathy in mice

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ABSTRACT

We reported previously that nuclear factor erythroid 2-related factor 2 (Nrf2) and metallothionein (MT) play critical roles in preventing intermittent hypoxia (IH)-induced cardiomyopathy. In addition, positive feedback regulation between Nrf2 and MT is required for the efficient compensative responses of the heart to IH. As an activator of Nrf2, sulforaphane (SFN) has attracted attention as a potential protective agent against cardiovascular disease. Here, we investigated whether SFN can up-regulate cardiac Nrf2 expression and function, as well as MT expression, to prevent IH-induced cardiomyopathy, and if so, whether Nrf2 and MT are indispensable for this preventive effect. Nrf2-knock-out (Nrf2-KO) or MT-KO mice and their wild-type (WT) equivalents were exposed to IH for 4 weeks with or without SFN treatment. SFN almost completely prevented IH-induced cardiomyopathy in WT mice, and this preventive effect was abolished in Nrf2-KO mice but retained in MT-KO mice. In IH-exposed WT mice, SFN induced significant increases in the expression levels of Nrf2 and its downstream antioxidant target genes, as well as those of MT, but these effects were not seen in IH-exposed Nrf2-KO mice. By contrast, KO of MT did not affect the ability of SFN to up-regulate the expression of Nrf2 and its downstream antioxidant targets. These results suggest that SFN-induced MT expression is Nrf2-dependent, and SFN prevents IH-induced cardiomyopathy in a Nrf2-dependent manner, for which MT is dispensable. This study provides important information that is relevant to the potential use of SFN to prevent IH-induced cardiomyopathy.

1. Introduction

Obstructive sleep apnea (OSA) is a public health problem owing to its high prevalence and adverse consequences, including excessive daytime somnolence [1] and cognitive impairment [2], as well as increased cardiovascular and metabolic morbidity [3]. OSA is characterized by short repetitive cycles of oxygen desaturation followed by rapid reoxygenation, termed intermittent hypoxia (IH), a process that plays a pivotal role in the development of cardiovascular diseases [4]. Although animal experiments have shown that short-term exposure to IH can have beneficial effects on cardiovascular disorders [5,6], longer-term exposures cause left ventricular (LV) remodeling and dysfunction [7,8], and promote ventricular arrhythmias and sudden cardiac death [9].

Extensive animal studies have shown that oxidative stress is a major cause of chronic IH-induced cardiac damage [10–13]. Oxidative stress represents an imbalance between the production and antioxidant buffering of reactive oxygen and/or nitrogen species [11]. Metallothioneins (MTs) are a family of cysteine-rich, low molecular weight proteins that have binding capacity for physiological and xenobiotic heavy metals through their cysteine thiol groups [14]. We reported previously that exposure of mice to IH for 3 days significantly increases cardiac MT expression, whereas prolonged exposure for 4 weeks has the opposite effect [15–17]. Furthermore, we found that cardiac-specific overexpression of MT protects against chronic IH-induced cardiomyopathy in mice, whereas global knock-out (KO) of MT renders mice more susceptible to IH-induced oxidative and inflammatory aortic injury [15–17]. These findings suggest that cardiac MT plays an important role

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as a potent antioxidant in the prevention of chronic IH-induced cardiomyopathy.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a master transcription factor that has a critical function in the regulation of antioxidant defense genes [18]. Nrf2 regulates the expression of its target genes by binding to antioxidant response elements in their promoter regions [19]. MT was identified as a potential Nrf2 downstream target a long time ago [20], but there is lack of real supportive evidence. As seen for MT in our earlier studies [15–17], our recent study showed that exposure of mice to IH for 3 days significantly increases cardiac Nrf2 expression and function, whereas exposure for 4 weeks has the opposite effect [21]. We also found that cardiac overexpression of Nrf2 or global KO of the *Nrf2* gene (Nrf2-KO) renders mice highly resistant or highly susceptible, respectively, to IH-induced cardiomyopathy and increased MT expression. Furthermore, we found that cardiac overexpression of MT prevents chronic IH-induced down-regulation of Nrf2 expression and function. Taken together, these findings suggest that cardiac Nrf2 and MT are under beneficial feedback control [21]. Notably, MT has also been identified as a downstream target of Nrf2 under diabetic conditions [22,23].

In the current study, we examined whether stimulation of endogenous Nrf2 expression increases the expression of its multiple downstream genes, including that encoding MT, to protect against chronic IH-induced cardiomyopathy. To this end, we used sulforaphane (SFN), a phytochemical that efficiently induces Nrf2 expression [24,25]. We also used Nrf2-KO and MT-KO mice to determine whether these proteins are indispensable for SFN-mediated protection against IH-induced cardiomyopathy.

2. Materials and methods

2.1. Experimental animals and IH exposures

All experiments were performed using male mice. MT-KO (stock number: 002211) and Nrf2-KO (stock number: 017009) mice and their wild-type (WT) equivalents (129S1 and C57BL/6 J, respectively) were purchased from The Jackson Laboratory. Groups of 20 C57BL/6 J, 129S1, Nrf2-KO, and MT-KO mice were randomly assigned into two experimental groups of 10 mice, which were exposed to room air or IH during sleep, as described previously [26,27]. Because deoxygenation-reoxygenation episodes occur in moderate to severe OSA patients, the 4 week IH paradigm consisted of alternating cycles of 20.9% O_2/8% O_2 FiO_2 (30 episodes per hour), with 20 s at the nadir FiO_2, during the 12 h light phase. The IH-exposed and age-matched control mice were treated with SFN (Sigma-Aldrich) or vehicle (phosphate buffered saline; PBS) as described in Section 2.2. All animal 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. After IH exposure, the mice were transferred to room air and echocardiography was performed to detect cardiac function. The mice were then sacrificed for tissue collection.

2.2. In vivo administration of SFN

SFN was given subcutaneously at a dose of 0.5 mg/kg for 5 days in each week. The dose of SFN used was based on a previous study [28]. Vehicle control mice were given an equivalent volume of PBS containing 1% dimethyl sulfoxide.

2.3. Echocardiography

To assess cardiac function, transthoracic echocardiograms were performed on mice using a Visual Sonics Vevo 770 high-resolution imaging system, as described previously [29]. Briefly, mice were sedated with Avertin (240 mg/kg, intraperitoneally) and placed in a supine position on a heating pad. Two-dimensional and M-mode echocardiography was used to assess wall motion, chamber dimensions, and cardiac function.

2.4. Sirius-Red staining

After anesthesia, mouse hearts were isolated and fixed in 10% buffered formalin, and then dehydrated in a graded alcohol series, cleared with xylene, embedded in paraffin, and sectioned at 5 μm thickness. Cardiac fibrosis was examined by staining collagen with 0.1% Sirius-Red F3BA and 0.25% Fast Green FCF, as described in our previous study [30].

2.5. Western blotting

Western blots were performed as described previously [31]. Briefly, the heart tissue and nuclei were homogenized in lysis buffer and proteins were collected by centrifuging at 12,000 g in a Beckman GS-6R centrifuge for 15 min at 4 °C. The protein concentrations were measured using a Bradford assay. Samples of total protein and nuclear protein were diluted in loading buffer, heated at 98 °C for 5 min, and then subjected to electrophoresis on a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, which was rinsed briefly in Tris-buffered saline, blocked in blocking buffer (5% milk and 0.5% bovine serum albumin) for 1.5 h, and then washed three times with Tris-buffered saline containing 0.1% Tween 20 (TBST). The membranes were incubated with primary antibodies overnight at 4 °C, washed with TBST, and then incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. The antigen-antibody complexes were visualized using an ECL kit (Amersham). MT expression was detected using a modified western blotting protocol [32].

The primary antibodies used in the study were as follows: anti-3-nitrotyrosine (3-NT, 1:1000; Chemicon), anti-4-hydroxynonenal (4-HNE, 1:2000; Calbiochem), anti-plasminogen activator inhibitor type 1 (PAI-1, 1:2000; BD Biosciences), and anti-NRF2 (1:1000, Abcam). The primary antibodies against vascular cell adhesion molecule (VCAM, 1:1000), connective tissue growth factor (CTGF, 1:1000), NAD(P)H:quinone oxidoreductase 1 (NQO1, 1:1000), and superoxide dismutase 2 (SOD2, 1:5000) were purchased from Santa Cruz Biotechnology. GAPDH was used as a loading control.

2.6. Quantitative real-time PCR (RT-qPCR)

Total RNA was extracted using TRIzol Reagent (Invitrogen). RNA concentrations and purities were quantified using a NanoDrop ND-1000 spectrophotometer. First-strand complementary DNA (cDNA) was synthesized from total RNA according to the manufacturer's protocol (Promega). Reverse transcription was performed using a Mastercycler Gradient (Eppendorf) at 42 °C for 50 min followed by 95 °C for 5 min, with 0.5 μg of total RNA in a final volume of 20 μl (4 μl of 25 mM $MgCl_2$, 4 μl of AMV reverse transcriptase 5 × buffer, 2 μl of dNTPs, 0.5 μl of RNase inhibitor, 1 μl of AMV reverse transcriptase, 1 μl of dT primer, and nuclease-free water). Primers targeting MT1, NQO1, and SOD2 were purchased from Applied Biosystems. Quantitative PCR was performed in a 20 μl solution (10 μl of TaqMan Universal PCR Master Mix, 1 μl of primers, and 9 μl of cDNA) using the ABI 7300 Real-Time PCR system. Data were expressed as fold increases relative to controls, using the ΔΔCt method and GAPDH as a reference gene.

2.7. Statistical analysis

Data are expressed as the mean ± SD (n = 5) for normally distributed variables. Comparisons of treatments (air vs. 8% O_2) in mice with different genotypes were performed using a two-way analysis of variance, and the overall *F*-test was performed to determine the significance of the models. Multiple comparisons were performed using

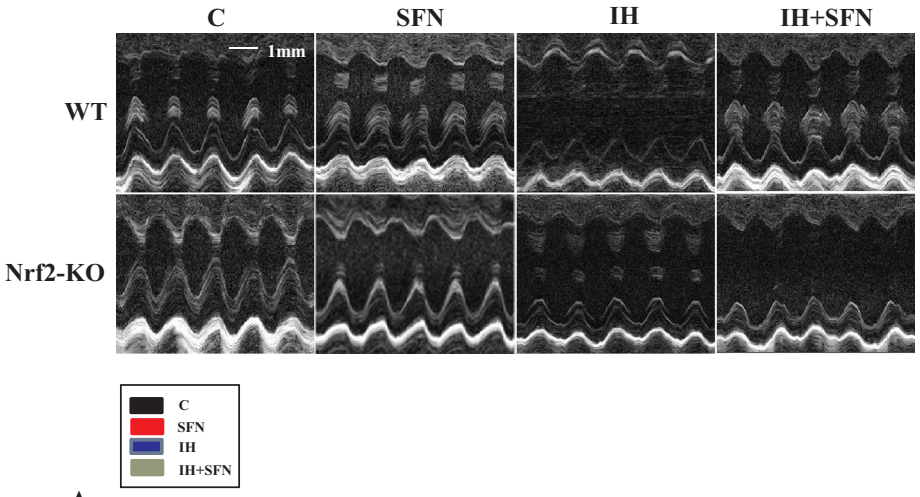

Fig. 1. SFN protects against IH-induced cardiac dysfunction in WT but not Nrf2-KO mice. Nrf2-KO and C57BL/6 J WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. Cardiac function was measured by echocardiography, for which representative M-mode echocardiograms from 4 groups of both WT and Nrf2-KO were provided (A). By echocardiography LVFS % (B) and LVEF % (C) were measured. Data are presented as the mean ± SD (n ≥ 5). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

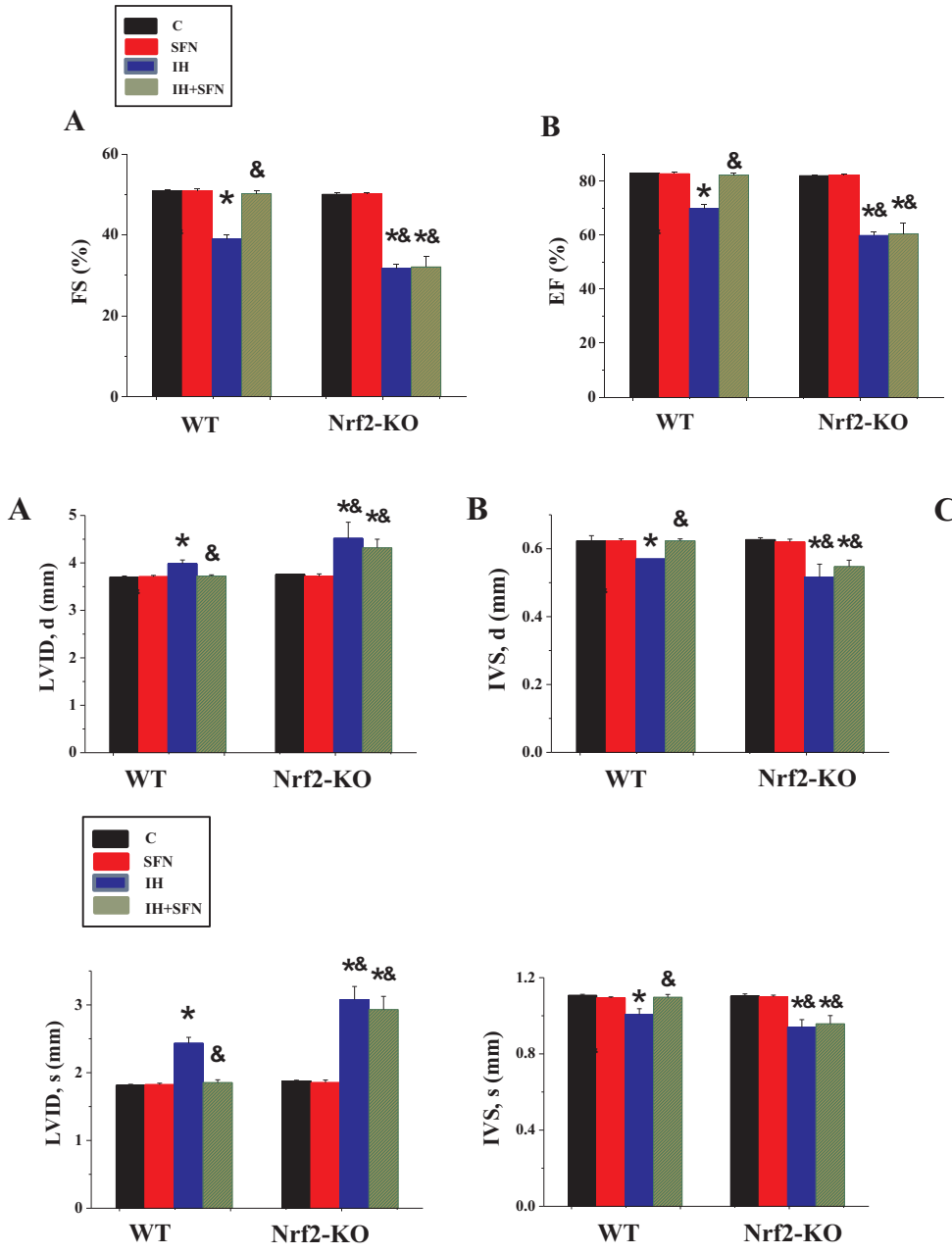

Fig. 2. SFN protects against additional IH-induced cardiac dysfunctions in WT but not Nrf2-KO mice. As described in Fig. 1, Nrf2-KO and C57BL/6 J WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. By echocardiography, LVID,d and LVID, s (A), IVS,d and IVS, s (B), and LVPW, d and LVPW, s (C) were measured. Data are presented as the mean ± SD (n ≥ 5). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

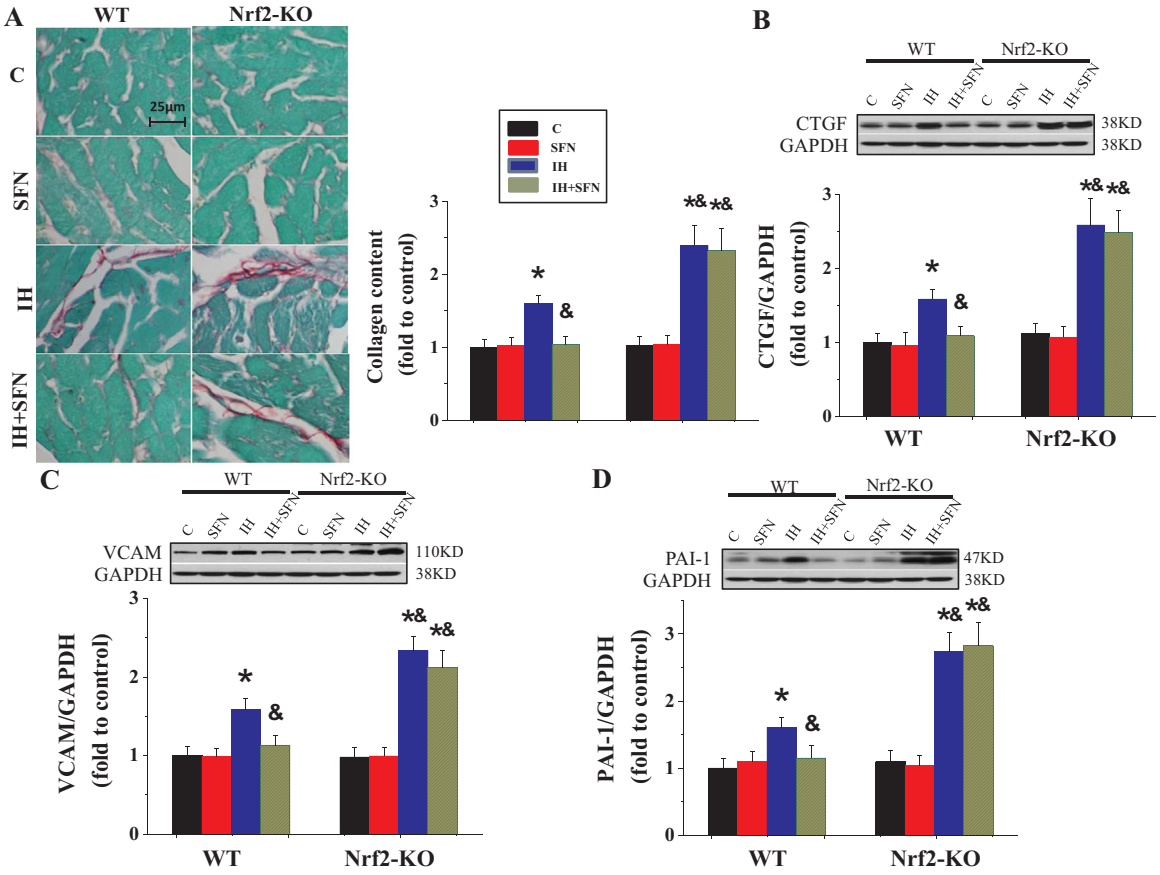

Fig. 3. SFN protects against IH-induced inflammation and fibrosis in WT but not Nrf2-KO mice. Nrf2-KO and C57BL/6 J WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. Cardiac fibrosis was measured by Sirius-Red staining (A, ×400) and western blot analysis of CTGF (B). Cardiac inflammation was measured by western blot analyses of VCAM (C) and PAI-1 (D). Data are presented as the mean ± SD (n ≥ 5). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

the Bonferroni test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. SFN-mediated protection against IH-induced cardiomyopathy is Nrf2-dependent

First, we examined whether chronic IH-induced cardiomyopathy can be prevented by treatment of mice with the Nrf2 inducer SFN, and whether the preventive effect is dependent on Nrf2. WT and Nrf2-KO mice (n = 10 per group) were exposed to room air or IH for 4 weeks, and administered SFN or vehicle subcutaneously. The body weights of the IH-exposed WT and Nrf2-KO mice were slightly lower than those of the corresponding controls exposed to room air ($P > 0.05$), but there was no significant effect of IH exposure on the heart weight in either group (Supplemental Figure 1).

An echocardiographic analysis showed that exposure to IH for 4 weeks significantly decreased the LV fractional shortening (LVFS%) and ejection fraction (LVEF%) in WT and Nrf2-KO mice (Fig. 1). These IH-induced decreases were larger in Nrf2-KO mice than WT mice. SFN treatment completely prevented the IH-induced decreases in LVFS% and LVEF% in WT mice but not Nrf2-KO mice, suggesting that cardiac protection by SFN is Nrf2-dependent. Similarly, the IH-induced changes in other echocardiographic measurements, including increased LV internal diameter at end-diastole and end-systole (LVID,d and LVID,s, respectively; Fig. 2A), decreased inter-ventricular septum at end-systole and end-diastole (IVS,d and IVS,s, respectively; Fig. 2B), and decreased LV posterior wall thickness at end-systole and end-diastole (LVPW,d and LVPW,s, respectively; Fig. 2C), were all prevented by SFN treatment in WT but not Nrf2-KO mice, providing further evidence that SFN can prevent IH-induced cardiac dysfunction in a Nrf2-dependent manner.

Consistent with the observed changes in cardiac function, exposure of WT or Nrf2-KO mice to IH for 4 weeks also promoted cardiac fibrosis and inflammation, as shown by increased cardiac expression of Picro Sirius-Red staining materials (Fig. 3A), CTGF (Fig. 3B), VCAM (Fig. 3C), and PAI-1 (Fig. 3D). These IH-induced changes were larger in the WT mice than the Nrf2-KO mice. SFN treatment almost completely prevented the IH-induced fibrotic and inflammatory responses in WT but not Nrf2-KO mice (Fig. 3).

3.2. SFN-mediated protection against IH-induced oxidative damage is dependent on Nrf2 and its downstream antioxidant genes

IH-exposed WT and Nrf2-KO mice displayed signs of cardiac oxidative stress and damage, as indicated by increased accumulation of 3-NT (Fig. 4A) and 4-HNE (Fig. 4B). These changes were more robust in the Nrf2-KO mice than the WT mice. SFN treatment significantly prevented chronic IH-induced cardiac oxidative damage (3-NT and 4-HNE accumulation) in the WT mice but not the Nrf2-KO mice (Fig. 4). This finding suggests the indispensability of Nrf2 for SFN-mediated protection against IH-induced cardiac oxidative stress.

As a nuclear transcription factor, Nrf2 is unable to reduce oxidative stress directly; therefore, we examined the effects of IH and SFN on the mRNA and protein levels of two antioxidant targets (SOD2 and NQO1) located downstream of Nrf2. We showed recently that, as another

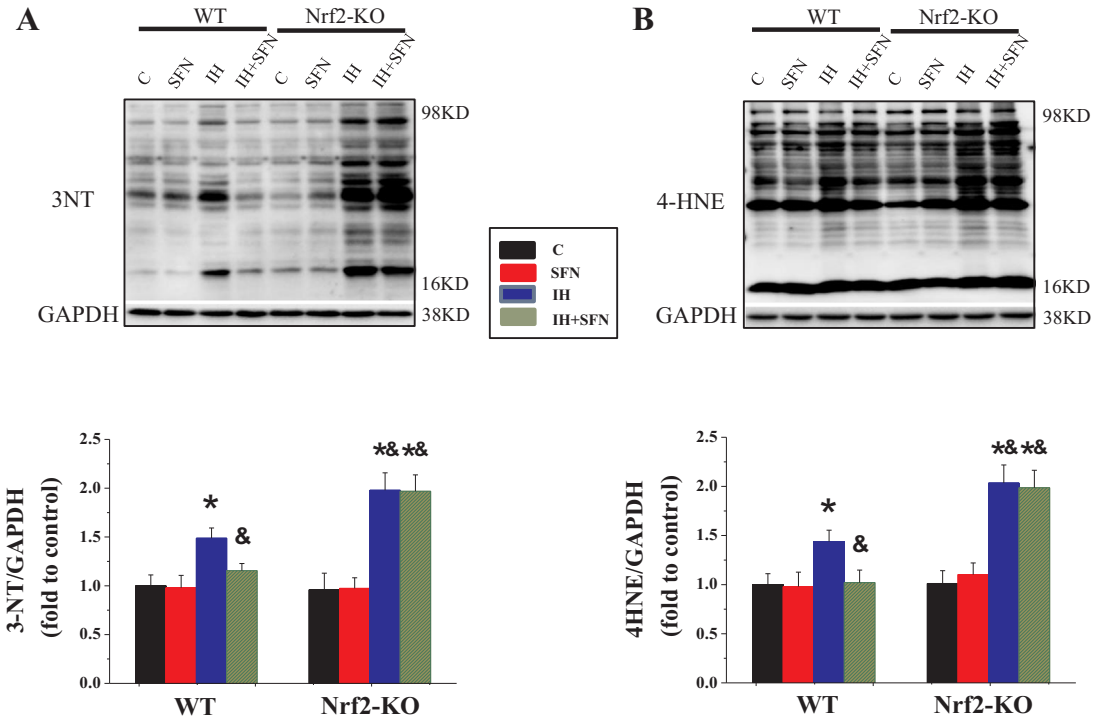

Fig. 4. SFN protects against IH-induced oxidative stress in WT but not Nrf2-KO mice. Nrf2-KO and C57BL/6 J WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. Cardiac oxidative damage was measured by western blot analyses of 3-NT (A) and 4-HNE (B). Data are presented as the mean ± SD (n ≥ 5). * $P < 0.05$ vs. WT/C. & $P < 0.05$ vs. WT/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

downstream target of Nrf2, MT plays an important role in preventing cardiac damage induced by IH [15–17] or diabetes [22,23]; therefore, we also examined the effects of IH and SFN on the mRNA and protein levels of MT. Exposure of WT mice to IH reduced cardiac expression of the Nrf2 protein, but this decrease was reversed by SFN treatment (Fig. 5A). As expected, Nrf2 expression was not detected in any of the Nrf2-KO groups (Fig. 5A). In WT mice, exposure to IH for 4 weeks significantly reduced the expression levels of the mRNAs encoding SOD2, NQO1, and MT (Fig. 5B). By contrast, treatment of control WT mice with SFN for 4 weeks significantly increased cardiac expression of the mRNAs encoding SOD2 and NQO1, but did not affect that of the mRNA encoding MT (Fig. 5B). In addition, treatment of WT mice with SFN reversed the inhibitory effect of IH and maintained the cardiac expression levels of the mRNAs encoding SOD2, NQO1, and MT at control levels (Fig. 5B).

In Nrf2-KO mice, the basal levels of the mRNAs encoding SOD2 and NQO1 were very low, regardless of room air (control), IH, or SFN exposure (Fig. 5B). The expression levels of the mRNA encoding MT were similar in control Nrf2-KO and WT mice, and were reduced by IH exposure in both mouse models. However, unlike in WT mice, SFN treatment was unable to reverse this IH-induced reduction in MT expression in the Nrf2-KO mice (Fig. 5B). Western blot analyses confirmed that the expression levels of the SOD2 (Fig. 5C), NQO1 (Fig. 5D), and MT (Fig. 5E) proteins in the WT and Nrf2-KO groups were comparable to those of their corresponding mRNAs.

3.3. SFN-mediated protection against IH-induced cardiomyopathy is independent of MT

As a downstream antioxidant target of Nrf2, MT plays an important role in SFN-mediated protection against diabetic cardiomyopathy and nephropathy [22,23]. To examine whether MT is also required for SFN-mediated protection against IH-induced cardiac damage, MT-KO and WT 129S1 mice were exposed to IH for 4 weeks with or without SFN treatment. Exposure to IH decreased the body weights of the WT and MT-KO mice slightly, but did not affect the heart weights in either group (Supplemental Figure 2).

As seen in the experiments using C57BL/6 J mice (Figs. 1–5), IH exposure induced cardiac dysfunction and structural changes (Figs. 6 and 7), remodeling and inflammation (Fig. 8), and oxidative stress (3-NT and 4-HNE accumulation, Fig. 9) in 129S1 WT mice, and these effects were prevented by concomitant SFN treatment. IH exposure had more severe effects on cardiac function, structure, oxidative stress, and damage in MT-KO mice than in WT mice (Figs. 6–9), suggesting that MT plays an important protective role against IH-induced cardiac damage. However, deletion of the gene encoding MT did not affect the preventive effect of SFN on IH-induced cardiac dysfunction and pathogenic changes (Figs. 6 and 7), fibrotic and inflammatory responses (Sirius-Red staining materials, CTGF, VCAM, and PAI-1 levels; Fig. 8), or oxidative stress (3-NT and 4-HNE levels, Fig. 9). Furthermore, KO of MT did not affect the SFN-induced increases in Nrf2 expression (Fig. 10) or function (levels of the mRNAs encoding SOD2 and NQO1) in mice exposed to room air or IH (Fig. 10A–C). As expected, MT protein expression was not detected in the MT-KO mice (Fig. 10D).

4. Discussion

Based on our previous findings that Nrf2 and MT play important roles in preventing chronic IH-induced cardiomyopathy [15–17,21], and that MT expression is regulated in part by Nrf2 under certain conditions [20–23], we speculated that the potent Nrf2 activator SFN could prevent chronic IH-induced cardiomyopathy. We found that (1) SFN protects against IH-induced cardiomyopathy in C57BL/6J and 129S1 WT mice by up-regulating Nrf2 expression and function, as well as MT expression; (2) Nrf2 is required for SFN-induced MT expression under IH conditions; and (3) SFN-mediated protection against IH-induced cardiomyopathy is Nrf2-dependent, for which MT expression is dispensable (Fig. 11).

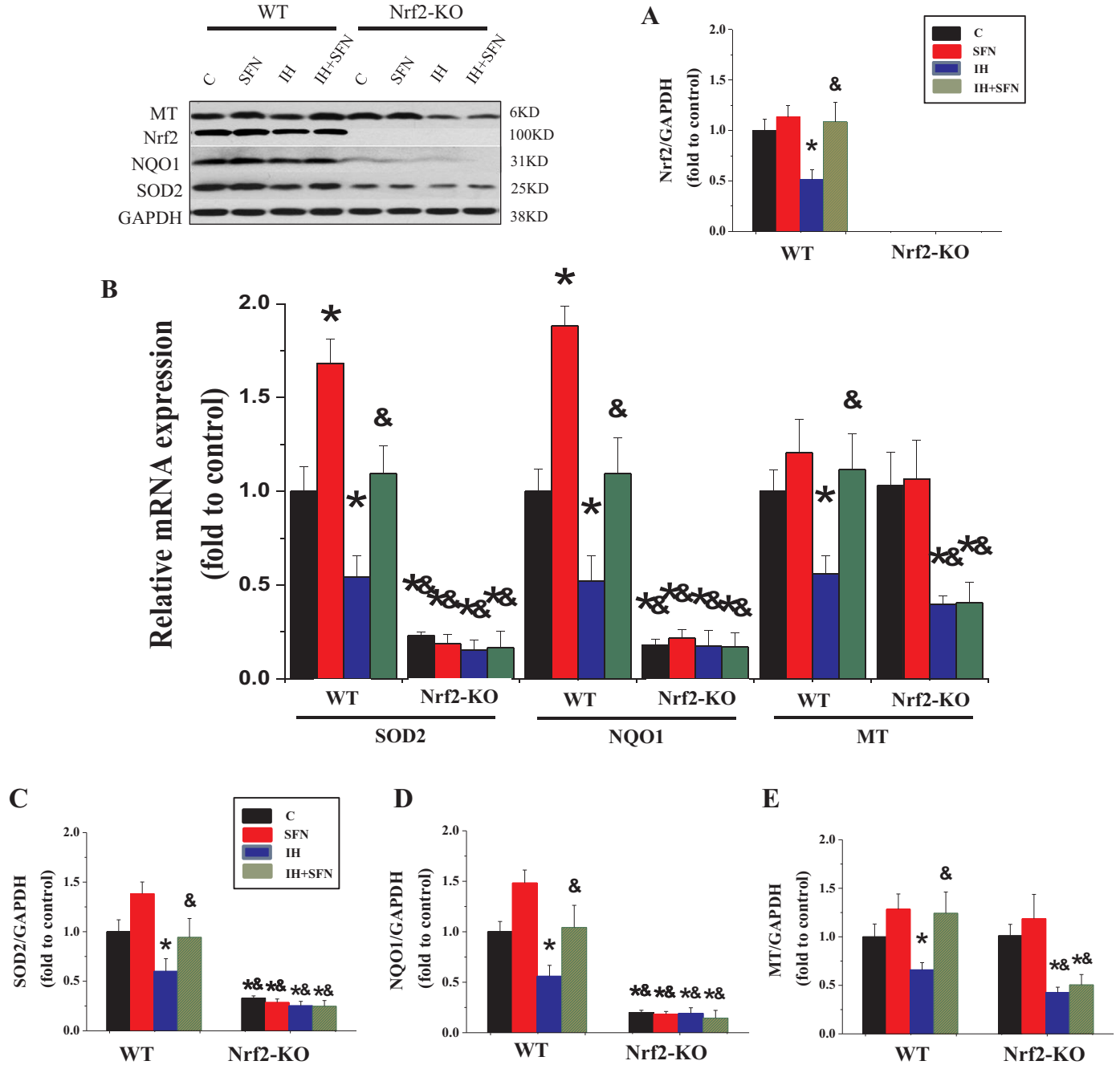

Fig. 5. SFN induces MT expression in WT and Nrf2-KO mice. Nrf2-KO and C57BL/6 J WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. The expression levels of Nrf2 (A), SOD2 (C), NQO1 (D), and MT (E) were measured by western blotting. The expression levels of the mRNAs encoding SOD2, NQO1, and MT were determined in each group by RT-qPCR (B). Data are presented as the mean ± SD (n ≥ 5). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

In a previous study, chronic IH exposure increased the sensitivity of the heart to infarction in rats [12]. In that study, the rats were exposed to IH for 14 days, and then hearts from IH-exposed and normoxic rats were subjected to ischemia-reperfusion using a Langendorff system. The ischemia-reperfusion-induced infarct sizes were significantly larger in the IH-exposed group than the normoxic group, and concomitant administration of the antioxidant tempol or melatonin prevented the IH-induced increase in infarction aggravation. Exposure to IH also induced myocardial oxidative stress and damage, and these effects were abolished by tempol or melatonin treatment. These findings suggest that oxidative stress may mediate the deleterious cardiovascular effects of IH and, in particular, the increased susceptibility to myocardial infarction [12]. As an activator of Nrf2, SFN has received intense attention for its potential protective effects against cardiovascular disease [33–35]. In fact, we recently reported a protective role of Nrf2 against chronic IH-induced cardiomyopathy (21).

Under normal conditions, Nrf2 is degraded via Kelch-like ECH associated protein 1 (Keap1)-dependent and -independent ubiquitination-mediated proteasomal pathways [36,37]. Nrf2 inducers, including SFN, inhibit Nrf2 ubiquitination and phosphorylation at Ser40, resulting in accumulation of Nrf2 and increased transactivation activity in vitro [24]. Therefore, although SFN did not increase cardiac Nrf2 expression

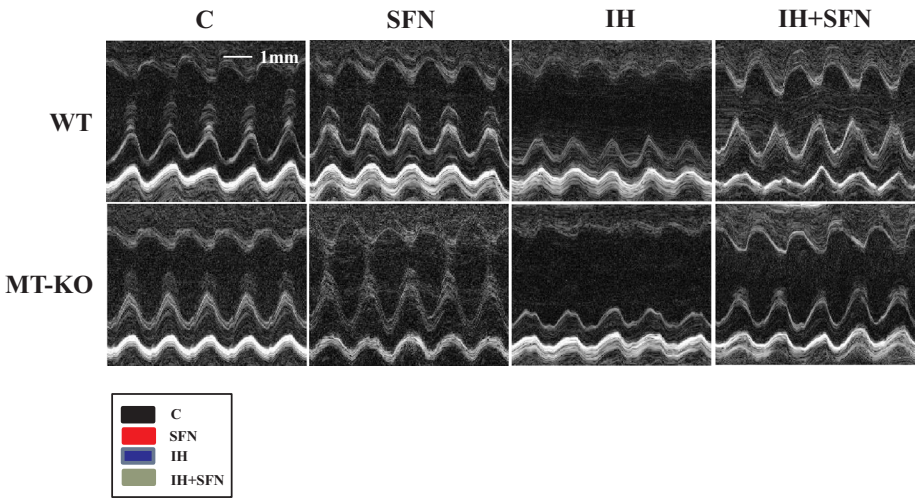

Fig. 6. SFN protects against IH-induced cardiac dysfunction in WT and MT-KO mice. MT-KO and 129S1 WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. Cardiac function was measured by echocardiography, for which representative M-mode echocardiograms from 4 groups of both WT and Nrf2-KO were provided (A). By echocardiography LVFS % (B) and LVEF % (C) were measured. Data are presented as the mean ± SD ($n \geq 5$). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. $^{\#}P < 0.05$ vs. MT-KO/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

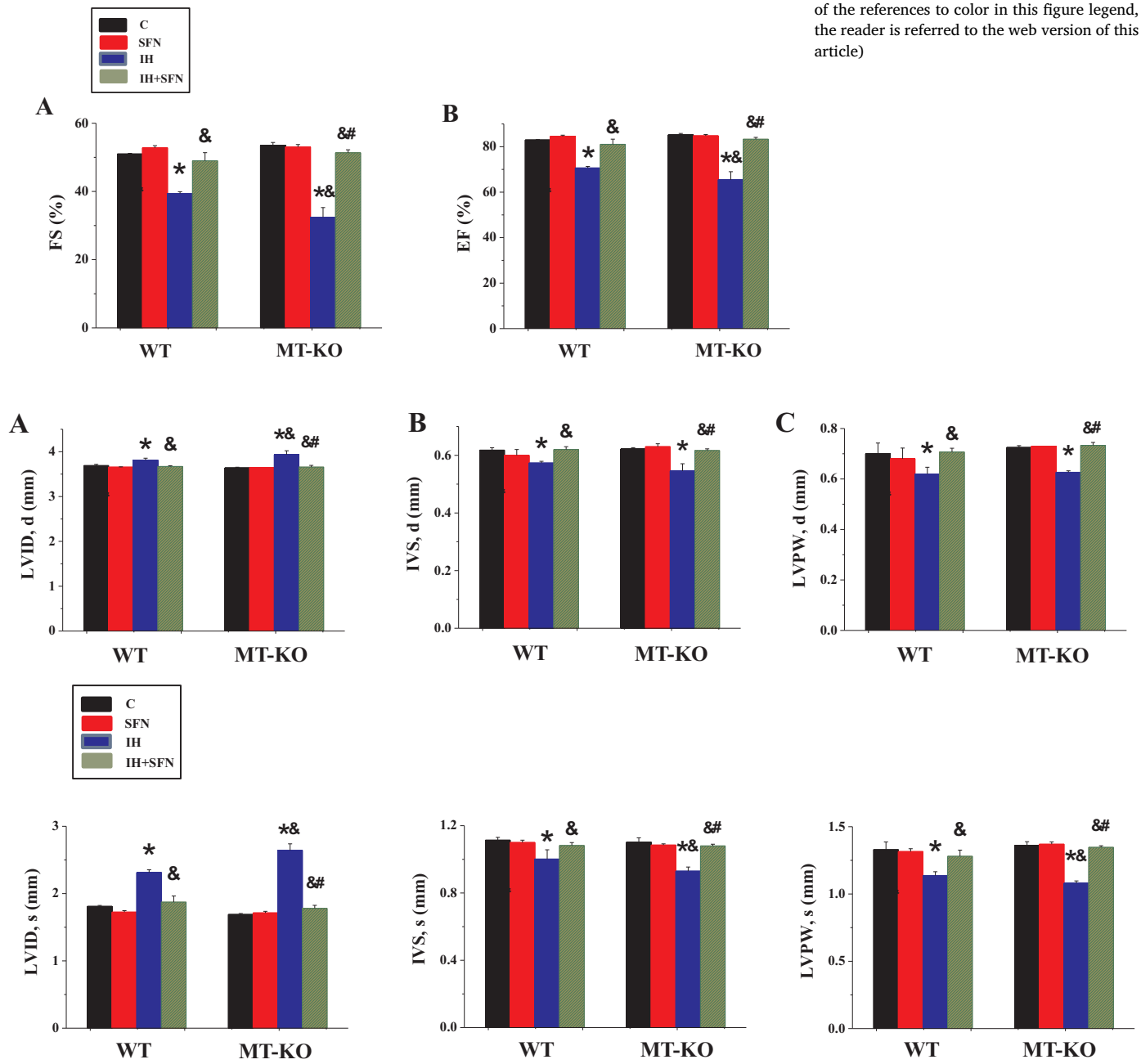

Fig. 7. SFN protects against IH-induced cardiac dysfunction in WT and MT-KO mice. As described in Fig. 6, MT-KO mice and 129S1 WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. By echocardiography LVID, d and LVID, s (A), IVS,d and IVS, s (B), and LVPW, d and LVPW, s (C) were measured. Data are presented as the mean ± SD ($n \geq 5$). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. $^{\#}P < 0.05$ vs. MT-KO/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

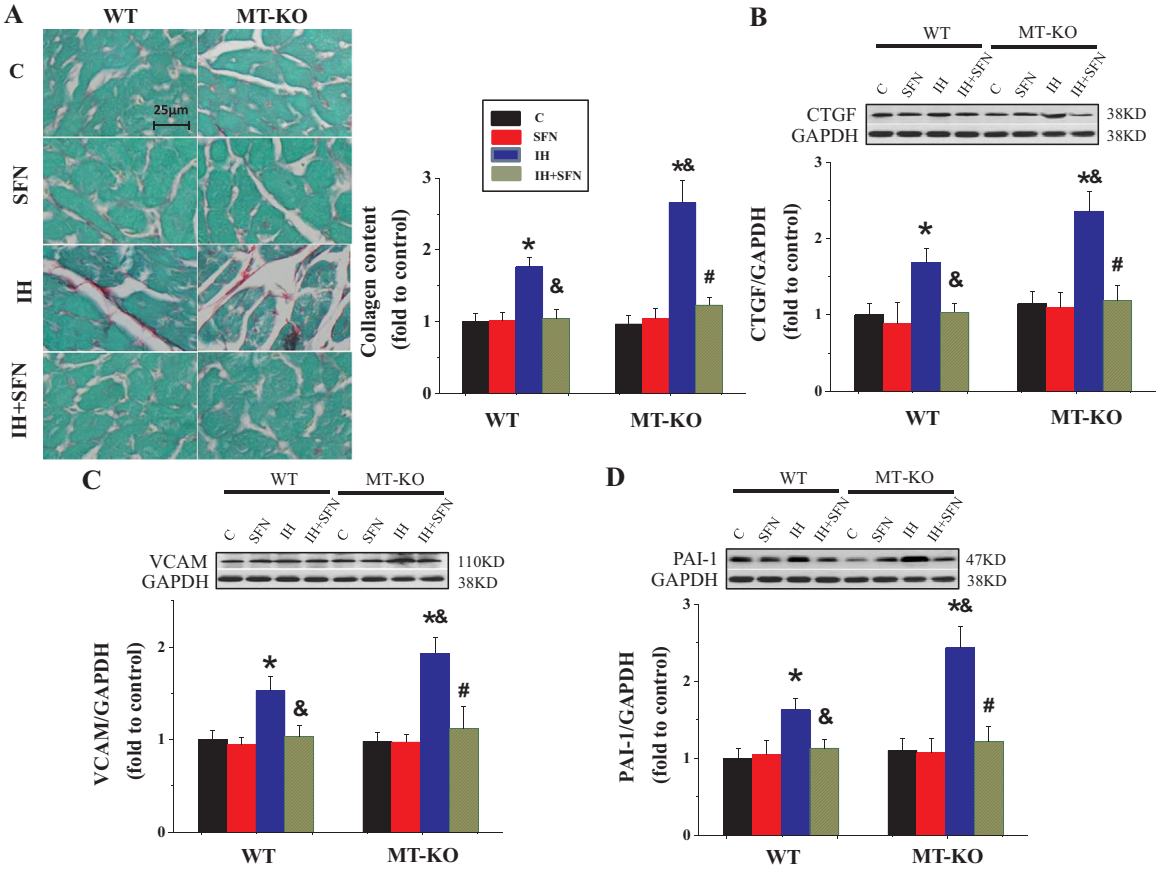

Fig. 8. SFN protects against IH-induced inflammation and fibrosis in WT and MT-KO mice. MT-KO and 129S1 WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. Cardiac fibrosis was measured by Sirius-Red staining (A, ×400) and western blot analysis of CTGF (B). Cardiac inflammation was measured by western blot analyses of VCAM (C) and PAI-1 (D). Data are presented as the mean ± SD (n ≥ 5). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. $^{\#}P < 0.05$ vs. MT-KO/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

significantly in the current study, it may have induced Nrf2 to dissociate from Keap1 and enter the nucleus, where it binds to antioxidant response elements in the promotor regions of its downstream targets. We found that SFN increased the expression levels of Nrf2-downstream antioxidants in both control and IH-exposed mice, preserved cardiac MT expression following chronic IH exposure, and prevented IH-induced cardiac oxidative damage and dysfunction in WT but not Nrf2-KO mice. These findings suggest a potential therapeutic use of SFN for the prevention of IH-induced cardiomyopathy via activation of Nrf2-mediated antioxidant responses.

MT is a potent antioxidant that is able to protect the heart against oxidative stress-related cardiomyopathies induced by alcohol [38], anticancer drugs [39], and diabetes [40]. In our previous studies, we highlighted the importance of MT in the prevention of IH-induced cardiomyopathy, using cardiomyocyte-specific MT transgenic or global MT-KO mouse models [15–17]. To further determine whether cardiac MT, as a downstream target of Nrf2, is also indispensable for SFN-mediated cardiac protection against chronic IH, we exposed MT-KO and WT mice to IH for 4 weeks with and without simultaneous SFN treatment. Our results showed that SFN can prevent the development of IH-induced cardiac remodeling and dysfunction in both WT and MT-KO mice. This finding is consistent with the results of a previous study showing that SFN protects against diabetic cardiomyopathy in WT and MT-KO mice, albeit to a lesser extent in the latter [22]. Taken together, these findings indicate that SFN can almost completely prevent IH-induced cardiomyopathy, and Nrf2, but not MT, is indispensable for this function.

It is worth noting that SFN increased the basal levels of Nrf2 and prevented the chronic IH-induced reduction in these levels in the hearts of WT (C57BL/6 J in Fig. 5A and 129S1 in Fig. 10A) and MT-KO mice (Fig. 10A), but not Nrf2-KO mice (Fig. 5A). In WT control mice, SFN did not affect the basal level of MT, but completely prevented the chronic IH-induced decrease in MT expression (Figs. 5B, 5E, and 10D), suggesting that the basal level of cardiac MT is not regulated by Nrf2. By contrast, the basal cardiac mRNA and protein levels of two other downstream targets of Nrf2 (SOD2 and NQO1) were increased significantly following SFN treatment of control mice (Figs. 5B–D, 10B, and 10C). These results indicate that, although MT mRNA expression was higher in the IH/SFN group than in the IH group, SFN does not induce MT mRNA expression under IH conditions, but may prevent IH-induced reductions in MT mRNA and protein expression (Figs. 5B and 5E) via other mechanisms. The exact mechanism by which SFN prevents the IH-induced reduction in MT expression requires further investigation.

Our own and other groups have identified a number of polymorphisms in the human *Nrf2* gene [41–43]. Using SFN to prevent chronic IH-induced cardiomyopathy via stimulation of Nrf2-mediated MT expression may be impossible for individuals with certain *Nrf2* mutations; therefore, methods of inducing MT via Nrf2-independent pathways should be examined. Zinc has been used extensively to induce MT and prevent various cardiomyopathies [32,44,45]. Indeed, the importance of zinc availability to protect against IH-induced cardiopathogenesis has been reported recently [46–48]. Therefore, examining whether zinc can be used to induce cardiac MT and protect the heart in patients subject to chronic IH due to OSA may be a worthwhile study.

In summary, our results reveal a preventive effect of SFN on IH-

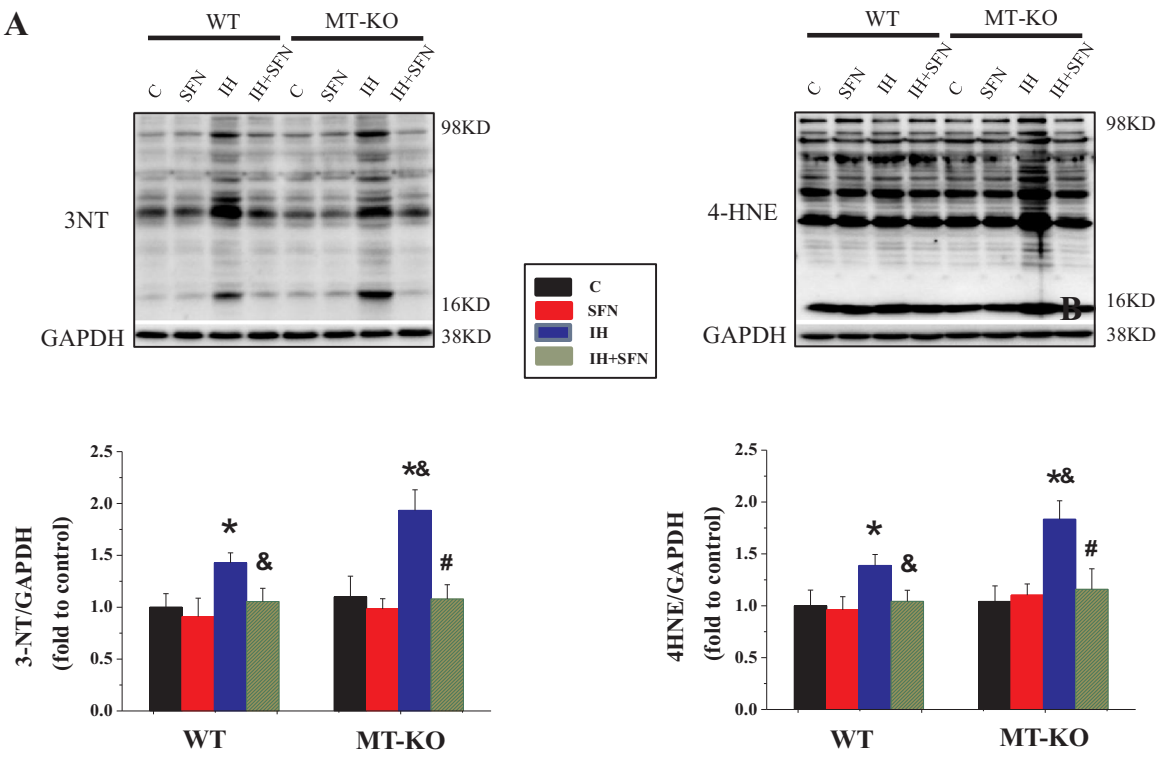

Fig. 9. SFN protects against IH-induced oxidative stress in WT and MT-KO mice. MT-KO and 129S1 WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. Cardiac oxidative damage was measured by western blot analyses of 3-NT (A) and 4-HNE (B). Data are presented as the mean ± SD (n ≥ 5). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. $^{\#}P < 0.05$ vs. MT-KO/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

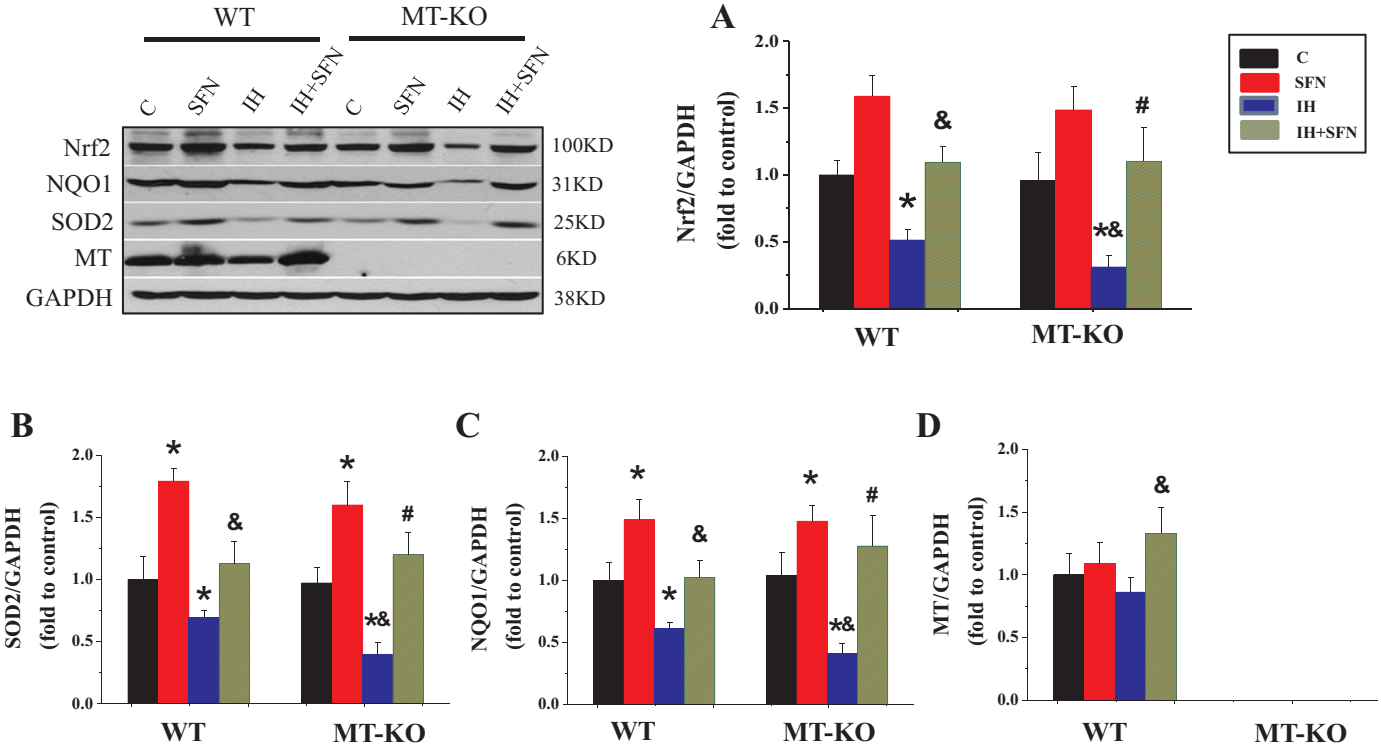

Fig. 10. SFN induces Nrf2 expression in WT and MT-KO mice. MT-KO mice and 129S1 WT mice were exposed to room air (black and red bars) or IH (green and blue bars) for 4 weeks, and SFN (red and green bars) or vehicle (black and blue bars) was administered simultaneously. The expression levels of Nrf2 (A) and its target genes, SOD2 (B), NQO1 (C), and MT (D), were measured by western blotting. Data are presented as the mean ± SD (n ≥ 5). $^{*}P < 0.05$ vs. WT/C. $^{\&}P < 0.05$ vs. WT/IH. $^{\#}P < 0.05$ vs. MT-KO/IH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

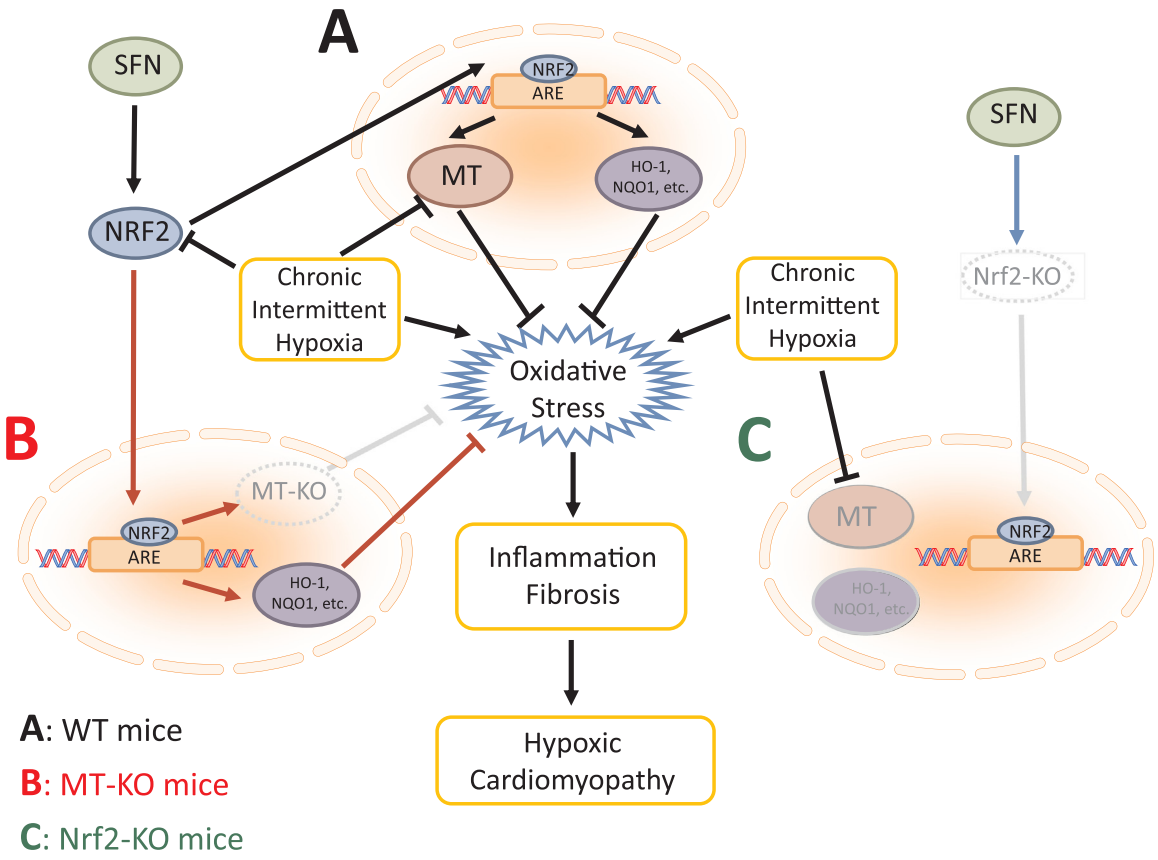

Fig. 11. Schematic illustration of SFN-mediated protection against IH-induced cardiomyopathy and its dependence on Nrf2 and MT.

induced cardiomyopathy that is mediated by Nrf2 and MT. Given that the safety and efficiency of SFN in preventing other chronic diseases, such as cancer and metabolic syndrome [49–51], has been established, and zinc is commonly used in clinical practice, these agents have great potential to be considered as clinical therapies for the prevention and treatment of IH-induced cardiovascular diseases, including cardiomyopathy.

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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 doi:10.1016/j.redox.2018.07.014.

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