Sulforaphane alleviates muscular dystrophy in mdx mice by activation of Nrf2

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Sulforaphane (SFN), one of the most important isothiocyanates in the human diet, is known to have chemo-preventive and antioxidant activities in different tissues via activation of nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated induction of antioxidant/phase II detoxifying enzymes, such as heme oxygenase-1 and NAD(P)H quinone oxidoreductase 1. However, its effects on muscular dystrophy remain unknown. This work was undertaken to evaluate the effects of SFN on Duchenne muscular dystrophy. Four-week-old mdx mice were treated with SFN by gavage (2 mg·kg body wt−1·day−1 for 8 wk), and our results demonstrated that SFN treatment increased the expression and activity of muscle phase II enzymes NAD(P)H quinone oxidoreductase 1 and heme oxygenase-1 with a Nrf2-dependent manner. SFN significantly increased skeletal muscle mass, muscle force (~30%), running distance (~20%), and GSH-to-GSSG ratio (~3.2-fold) of mdx mice and decreased the activities of plasma creatine phosphokinase (~45%) and lactate dehydrogenase (~40%), gastrocnemius hypertrophy (~25%), myocardial hypertrophy (~20%), and malondialdehyde levels (~60%). Furthermore, SFN treatment also reduced the central nucleation (~40%), fiber size variability, and inflammation and improved the sarcolemmal integrity of mdx mice. Collectively, these results show that SFN can improve muscle function and pathology and protect dystrophic muscle from oxidative damage in mdx mice associated with Nrf2 signaling pathway, which indicate Nrf2 may have clinical implications for the treatment of patients with muscular dystrophy.

Sulforaphane: Duchenne muscular dystrophy; Nrf2; oxidative stress

Duchenne muscular dystrophy (DMD), one of the most common lethal genetic neuromuscular disorder in children, occurs in 1 in 3,500 live male births (5). It is caused by loss-of-function mutations in the gene that encodes a large-scale cytoskeletal protein dystrophin and is characterized in humans and mdx mice by severe oxidative stress, inflammation, progressive destruction of muscle fibers, and cell death (13). It usually leads to gastrocnemius (GAS) hypertrophy, increase of plasma creatine phosphokinase (CK) activity, progressive paresis, respiratory failure, and premature death (14). Presently, there is no cure for this disaster disease, and the only medications proven to favorably alter its natural history are corticosteroids (33). There is evidence from randomized controlled trials that glucocorticoid therapeutics in DMD treatment improve muscle function and strength in the short-term period of 6 mo to 2 yr. However, the long-term curative effect of corticosteroids in DMD with randomized placebo-controlled trials will likely never be studied in consideration of its obvious side effects and orphan disease state (24, 31). Recent studies show corticosteroids are associated with plentiful side effects, particularly weight gain, diabetes, hypertension, stunted height/growth, and mood/behavioral changes (24, 31, 60). Thus better treatments to augment or supplant corticosteroid use in DMD would be of immense value.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is the most important endogenous transcription factor, which widely locates in vivo and regulates the expression of many antioxidant/detoxification enzymes, stress response proteins, molecular chaperones, as well as proteasome subunits (29). Researches demonstrate Nrf2 protects a variety of tissues and cells against oxidative stress and electrophiles through antioxidant response element (ARE)-mediated induction of diverse antioxidant and phase II detoxification enzymes, such as heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1) (63, 64). Nrf2 can also protect skeletal muscle against damage induced by exhaustive exercise in rats through its antioxidative effects (30). However, whether Nrf2 can protect dystrophic muscle from oxidative damage is still unknown.

Sulforaphane [SFN; 1-isothiocyanate-(4R)-(methylsulfinyl) butane], presenting as glucoraphanin in plants of the brassicae family, is one of the most important natural isothiocyanates in the human diet (22). Accumulating evidence suggests that SFN is a promising chemo-preventive agent that can counteract with oxidative stress via Nrf2-dependent induction of phase II detoxifying enzymes HO-1 (20) and NQO1 (51). Moreover, recent studies suggest that the chemo-preventive effect of SFN is mediated by multiple mechanisms, including inhibition of angiogenesis and metastasis and induction of cell cycle arrest and apoptosis (7, 9, 62). Despite its proven chemo-preventive efficacy, the potential effects of SFN on muscular dystrophy have not been evaluated yet. We assume that SFN has a favorable role in treatment of DMD.

Dystrophin, one scaffolding protein located in the sarcolemmal cytoskeleton, is extremely important in maintaining cytomembrane structural integrity and regulating growth and repair of muscle cell (muscle fiber). The dystrophin-deficient myotubes are highly susceptible to oxidative damage (12). Loss of dystrophin compromises the muscle fiber membrane, leading to cycles of muscle fiber degeneration and regeneration, chronic inflammation, and accumulation of fibrotic tissue (32). Previous studies showed that oxidative stress plays a major role in the pathogenesis of DMD. Skeletal muscles of mdx mice demonstrate an increase in the quantities of oxidative damage markers involving the side-products of lipid peroxidation and carbonyls (17, 49). Several comorbidities in DMD, including muscle fatigue and cardiomyopathy, are associated with increased oxidative stress (3, 8). To prevent and counteract reactive oxygen species (ROS) generation and oxidative stress, many studies have been focused on the identification of natural compounds, which act as direct antioxidants with ROS.
scavenging properties, and find that various antioxidants, such as resveratrol (16, 18), polyphenol (39), and curcumin (48), can alleviate dystrophic muscle pathology in mdx mice via reducing oxidative stress. However, the effects of using antioxidants to prevent oxidative-stress-related diseases are compromised (27, 36). Hori et al. find that resveratrol, a typical antioxidant, can decrease oxidative damage in mdx mice; however, it cannot reverse the high-serum levels of CK and lactate dehydrogenase (LDH), which indicates that muscle injuries are still proceeding in the resveratrol-treated mdx mice (18). Recently, interest has been diverted to the possibility of counteracting oxidative stress by inducing endogenous antioxidant/detoxifying enzymes known as phase II enzymes. The induction of these enzymes by molecules known as chemical inducers (4) involves the activation of ARE. The transcription of ARE-driven genes is regulated by Nrf2, and SFN is an effective inducer of Nrf2. Compared with resveratrol, polyphenol, and curcumin, SFN is much milder, safer, and more effective as an antioxidant, since it can activate Nrf2, the endogenous transcription factor, which regulates the expression of many detoxification and antioxidant enzymes.

Mdx is the most commonly used mouse model for the study of pathogenesis of DMD. Mdx mice experience a crisis phase of muscle necrosis followed by regeneration at ∼3–6 wk of postnatal age (5). Thus the mice are frequently studied only during the first 6 wk of their lives. In this study, we assessed the ability of SFN to improve muscle function and protect dystrophic muscle from oxidative damage in mdx mice undergoing an acute exercise protocol. We found the muscle function and running ability of mdx mice were enhanced by SFN treatment. Moreover, all of the dystrophic pathology markers, such as central nucleation, muscle mass loss, ROS levels, inflammation, and membrane infiltration, are improved. We also confirmed the effects of SFN on muscular dystrophy were attributed to Nrf2 activation and Nrf2-mediated upregulation of phase II enzymes NQO1 and HO-1. Our data suggest that Nrf2 may be represented as a new therapeutic target for muscular dystrophy.

MATERIALS AND METHODS

Reagents and antibodies. δ-Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)-butane], Evans blue dye (EBD), Trolox, menadione, DMSO, isopentane, dicumarol, 1-chloro-2,4-dinitrobenzene, 5,5′-di-thiobis (2-nitrobenzoic) acid, reduced glutathione (GSH), oxidized glutathione (GSSG), mammalian protease inhibitor mixture, digitonin, glucose-6-phosphate, sodium piruvate, glucose-6-phosphate dehydrogenase, NADP, NADPH, NADH, FAD, and 2,2′-azino-bis(3-ethylbenothiazoline-6-sulfonic acid) diaminonitric acid salt were all purchased from Sigma-Aldrich (St. Louis, MO). Optimum cutting temperature compound was from Sakura Finetek (Torrance, CA). Trizol reagent was purchased from Invitrogen (Carlsbad, CA). Polyvinylidene difluoride (PVDF) Western blotting membrane was from Bio-Rad (Hertfordshire, UK), anti-Nrf2 and anti-GAPDH were from Affinity Biologicals (Hamilton, ON, Canada). Anti-HO-1, anti-CD45, and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). NQO1 and HO-1 ELISA kit were from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China).

Animals. C57BL1/10ScSn-Dmdmdx/Nju mice were obtained from Nanjing Biomedical Research Institute of Nanjing University and bred in our facility. Twelve 4-wk-old male mdx mice and six 4-wk-old C57/black 10 mice were housed in a controlled environment with a 12:12-h light-dark cycle at 22°C and were provided with mice chow and water ad libitum. Body weight was measured every week. All experiments were approved and conducted in accordance with the guidelines of the Animal Care Committee of Wuhan University. The experimental procedures were approved by the Ethics Committee of Wuhan University. Animals were randomly divided into the following three groups: C57/black 10 mice (BL10), Mdx control mice (Mdx), and SFN-treated mdx mice (SFN).

Four-week-old male mdx mice in the SFN group were treated with SFN by gavage (2 mg·kg body wt day −1 ), solubilized in 0.5 ml of DMSO, and then diluted in 49.5 ml corn oil (0.1 mg/ml) for 8 wk. At the same time, mice of corn oil and BL10 groups were treated with corn oil (contain 1% DMSO) with the same doses. The 2 mg/kg body wt dose was a little higher than that previously reported by Souza et al. (1 mg/kg body wt was used by gavage) (53). Moreover, de Souza et al. (11) used a 0.1–0.5 mg/kg body wt in a SFN gavage feeding protocol. Since DMD is a more serious disease than the two in the above studies, we chose a higher SFN dose in our study.

Grip strength assay. Force measurements were performed by the same technician. Briefly, mice were placed in a tensile tester (YiYan Tek, JiNan, China), and their forelimbs were put on the horizontal bar, which was the inductor of tensile tester. Then the mice’s tail was pulled against horizontal bar until the mice’s forelimb left the bar, the value in the screen was recorded, the above steps were repeated for four times, and the mean of the three upper values were calculated as the forelimb grip force of the mouse.

Mice exercise capacity assay. After 8 wk of treatment, mice exercise capacity was assessed by the exercise protocol as described before (1) with slight modifications. Three days before the acute exhaustive exercise protocol, all animals were familiarized to the treadmill (ZT treadmill): day 1, unmoving flat treadmill 2 min, unmoving −15° treadmill for 5 min, then 5 m/min on −15° treadmill for 15 min (total 22 min); day 2, unmoving flat treadmill 2 min, −15° treadmill at 5 m/min for 5 min, then increase to 10 m/min for 10 min (total 22 min); day 3, unmoving flat treadmill 2 min, on −15° treadmill, 5 m/min for 5 min, then 10 m/min for 10 min (total 22 min). The day of the experiment, mice were placed on the treadmill at −15° slope, and the speed was rapidly increased up to 25 m/min. Animals were allowed to run until exhaustion, which was defined as the point at which the animals failed to get off the shock grid and thus had to be manually repositioned to the front of the treadmill for three consecutive occasions.

Sample collection. Blood was collected from the eye socket every week in the fasted condition and then centrifuged (1,500 g, 10 min, 4°C) to separate plasma, which was stored at −80°C for further analyses (1). Two hours after exhaustive exercise bout, the mice were anesthetized with chloral hydrate (400 mg/kg body wt). Tibial anterior (TA), extensor digitorum longus (EDL), GAS, soleus (SOL), triceps brachii (TB) muscle, and heart samples were removed, frozen in liquid nitrogen, and stored at −80°C. In this study, we used TA muscle as a representative muscle for making muscle homogenates and the following assays on account of the fact that the status of TA was usually treated as an indicator of DMD status.

Measurement of reduced (GSH) to oxidized (GSSG). The ratio of GSH to GSSG (GSH/GSSG) was frequently applied as an indicator of oxidative stress. GSH/GSSG were measured on muscle (TA) homogenates by the spectrophotometric method, as reported by Ren et al. (50) with slight modifications. Muscle (TA) homogenates were centrifuged, and supernatant fractions were collected for GSH and GSSG assay. One-half of each sample was used for GSH determination, and the other one-half for GSSG. Samples (50 µl) were incubated at room temperature with 1 µl 4-vinyl pyridine for 1 h to conjugate GSH for determination of GSSG. The GSSG was then subtracted from the total glutathione to determine the GSH levels.

ELISA. The muscle (TA) protein expression levels of phase II enzymes, namely, NQO1 and HO-1, were quantified using ELISA kits (from Nanjing Jiancheng Bioengineering Institute, Jiangsu, China), according to the manufacturer’s instructions. Muscle (TA) tissues
were prepared as 10% tissue homogenates and centrifuged at 3,000 rpm at 4°C for 10 min. The supernatant was collected for ELISA assays. First, 10 μl of supernatant of each sample and 40 μl of sample diluent were added, and 50 μl of horseradish peroxidase (HRP) labeled detection antibody were added. Then the sample was incubated at 37°C for 1 h, and washed 5 times each for 1 min. Substrates A and B were then added, 50 μl each. The sample was incubated at 37°C for 15 min in dark condition, then the stop solution, 50 μl, was added. Absorbance was measured at 450 nm and compared with a standard curve constructed with known concentrations of NQO1 and HO-1. Values were expressed as units per liter muscle homogenates.

**CK activity assay.** Blood was collected from eye socket one day before exercise and 2 h after exercise bout in the fasted condition. CK activity was assayed in plasma samples by a spectrophotometric method based on the phosphorylation of ADP to ATP coupled to the reduction of NADP⁺ to NADPH, as catalyzed by glucose-6-phosphate dehydrogenase. The increase in absorbance at 340 nm was monitored at 37°C as reported by Noda et al. (44). Data are presented as U/L plasma. One unit of CK activity is defined as the amount of enzyme that transfers 1.0 μmol of phosphate from phosphocreatine to ADP per minute at pH 7.4 at 30°C.

**LDH activity assay.** Blood was collected from the eye socket 1 day before exercise and 2 h after the exercise bout in the fasted condition. LDH activity was assayed in plasma samples by a spectrophotometric method based on the reduction of pyruvate to lactic acid coupled to NADH oxidation, as reported by Korzeniewski and Callewaert (26). The decrease in absorbance at 340 nm was monitored at 37°C. Data are presented as units per liter plasma. One unit of LDH activity is defined as the amount of enzyme that catalyzes the reduction of 1.0 μmol of pyruvate to d-lactate per minute at pH 7.0 at 25°C.

**TBARS assay.** Thiobarbituric acid-reactive substances (TBARS) were measured on muscle (TA) homogenates by the spectrophotometric method as reported by Ohkawa et al. (45) with slight modifications. A mixture of 0.1 ml of tissue homogenate, 0.1 ml of 30 g/l SDS, and 4 ml of color reagent containing 7 g/l of thiobarbituric acid was incubated in boiling water for 60 min. After the mixture had cooled, the organic layer was collected after centrifuging at 1,000 rpm for 10 min at 4°C. Absorbance was measured at 532 nm and compared with a standard curve constructed with known concentrations of malondialdehyde (MDA). Data are reported as nanomoles of MDA per milligram of protein.

**Western blot analysis.** Muscles lysates were prepared as described before (18). Protein concentrations were determined by the BCA protein assay. Muscles lysates were run on 10% SDS-polyacrylamide gels (30 μg/lane), and proteins were transferred to PVDF membranes (Hertfordshire, UK) by semidyed electroblotting (1.5 mA/cm²). PVDF membranes were then incubated in blocking buffer [Tris-buffered saline supplemented with 0.05% (vol/vol) Tween 20 (TBST)] containing 5% (wt/vol) skimmed milk powder for 120 min at room temperature, followed by three 10-min washes in TBST. The PVDF membranes were then incubated with anti-Nrf2 (1:1,000), anti-hO-1 (1:500), anti-CD45 (1:1,000), and anti-β-actin (1:5,000) or anti-GADPH (1:5,000) as internal normalizers in TBST containing 5% (wt/vol) skimmed milk powder (antibody buffer) overnight at 4°C on a three-dimensional rocking table. Then the membranes were washed three times for 10 min in TBST and then incubated with goat anti-rabbit IgG conjugated with HRP (1:12,000 dilutions) in antibody buffer for 120 min. Finally, membranes were washed three times for 10 min in TBST and exposed to ECL Advance reagent (GE Healthcare Biosciences, Buckinghamshire, UK) for 2 min, as described in the manufacturer’s protocol. Then membranes were exposed to Hyperfilm-ECL (GE Healthcare Bio-Sciences) for 2–5 min and visualized using a Fluor S Multimager and Quantity One 4.1 (Bio-Rad Laboratories, Hercules, CA). The molecular weights of the bands were calculated by a comparison with prestained molecular weight markers (molecular weight range: 6,500–175,000) that were run in parallel with the samples. Semiquantitative analysis of specific immunolabeled bands was performed using a Fluor S image analyzer and Quantity One 4.1.

**Quantitative RT-PCR analyses.** Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions from muscles (TA). Two micrograms of total RNA were reversely transcribed using the First Strand cDNA synthesis kit (Takara, Tokyo, Japan), according to the manufacturer’s protocol. Quantitative (q) RT-PCR was carried out using the Power SYBR Green PCR Master Mix with the StepOnePlus real-time PCR System (Applied Biosystems, Warington, UK). Gene-specific primer pairs used in qRT-PCR analysis are shown in Table 1. All assays, including no template controls, were done in triplicate. The expression levels of GAPDH were used as internal controls, and the normalized values were subjected to a 2^−ΔΔCT formula to calculate the fold change. The formula and its derivations were obtained from the ABI Prism 7300 sequence detection system user guide.

**Histological and morphometric analyses.** Samples of skeletal muscle tissues (TA) were collected, then embedded with optimum cutting temperature compound (Sakura Finetek, Torrance, CA), and fixed in precooled isopentane with liquid nitrogen. Serial cross sections (10 μm thick) from the midbelly of frozen TA muscle tissues were cut on a standard cryostat with a clean blade and mounted on poly-L-lysine precoated (C to C Laboratory Supplies, Chicago, IL) glass slides. The unfixed sections were immediately stored at −80°C. The frozen sections were thawed at room temperature for 30–60 s without drying and immersed immediately in cold acetone (5 min). After fixation, the slides were rinsed briefly in 1 × phosphate-buffered saline (pH 7.4), and stained with hematoxylin and eosin as Nakaso et al. (40) described with some modulations. They were finally observed under light microscopy (Olympus 600 auto-biochemical analyzer, Tokyo, Japan) using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) to record images and make muscle fiber morphometric analysis. Pictures of the whole muscle sections were captured, and the percentage of centrally nucleated fibers was counted in the entire muscle section (total 300–500 fibers). All subsequent image analysis was performed in a blinded fashion. To quantify the variation in fiber size, fiber cross-sectional area was measured for every fiber in each section using Image-Pro Plus software. The muscle fiber size distribution was analyzed using SPSS version 17, specifically testing the frequency with the x² test.

**Immunohistochemistry/immunofluorescence.** Immunohistochemistry and immunofluorescence of the muscle tissues [TA, TB, GAS, quadriceps (QUAD)] was performed as described previously (21). Ten-micrometer muscle sections were incubated with commercial rabbit polyclonal antibodies against CD45 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1/100 dilution overnight at 4°C. In immunohistochemistry, sections were conjugated with HRP antibody (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 2 h, then covered by diaminobenzidine (Vector Laboratories), nuclei were counterstained in hematoxylin, and slides were mounted with Vectashield mounting medium (Vector Laboratories). In immunofluorescence, sections were visualized using a secondary

**Table 1. Primer sequences for quantitative RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’—3’)</th>
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<tbody>
<tr>
<td>Mouse GAPDH</td>
<td>For: CGGATTCCTCTTGCAAGT  &lt;br&gt; Rev: GGGACAACTCTCAGACTTGCC</td>
</tr>
<tr>
<td>Mouse Nrf2</td>
<td>For: TCTGGCTTCTGGAAGAAGAA  &lt;br&gt; Rev: AATGTGCTGGCTGTGCTTTA</td>
</tr>
<tr>
<td>Mouse NQO1</td>
<td>For: CAGGGTTCGCTCCTGTTGG  &lt;br&gt; Rev: AAGGTCGGCCATTGATAGT</td>
</tr>
<tr>
<td>Mouse HO-1</td>
<td>For: AAACAGGCAAACCCAGTATGCG  &lt;br&gt; Rev: AGGTTAGGGGGGTATAGGGGG</td>
</tr>
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Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NADPH quinone oxidoreductase 1; HO-1, heme oxygenase-1.
antibody coupled to a fluorescent marker, Cy3, at room temperature for 2 h. Then slides were mounted using Vectashield with 4',6-diamidino-2-phenylindole for detection of nuclei (Vector Laboratories, Burlingame, CA). Subsequently, all fields were observed under light (immunohistochemistry) and fluorescence (immunoﬂuorescence) microscopy (Olympus 600 auto-biochemical analyzer, Tokyo, Japan) at ×20 magnification. Control experiments without primary antibody demonstrated that the signals observed were specific. CD45-positive areas, marking inﬂamed areas, were semi-quantified and represented as the percentage of total muscle area.

**EBD staining.** EBD injections were performed as described by Straub et al. (56) with slight modifications. Briefly, 100 μl/10 g (body wt) of EBD were injected via the tail vein of the mice. Six to twelve hours later, muscles were isolated and frozen in precooled isopentane with liquid nitrogen. Before observing the sections under the microscope, 10-μm muscle sections were incubated in ice-cold acetone for 10 min, washed three times for 10 min with phosphate-buffered saline, and mounted with Vectashield mounting medium (Vector Laboratories). The presence of EBD in myofibers was observed under fluorescence microscopy (Olympus 600 auto-biochemical analyzer, Tokyo, Japan), and the intensity level was determined using northern Eclipse software by converting images to 8-bit gray scale and determining the total and average gray intensity taken as a measure of EBD fluorescence in the entire area of each ×20 cross section. EBD-positive areas were semi-quantified and represented as the percentage of total muscle area. Noninjected mice were used to determine the threshold values for all mice. The average gray intensity was then compared between groups with n = 6 animals per group. A noninjected mouse muscle was used to determine the threshold for all mice. All subsequent image analysis was performed in a blinded fashion. Three ×20 cross sections per animal were used to obtain average intensities.

**Statistics.** All values are reported as means ± SE of six animals. To check whether difference was statistically significant, given the small sample sizes and the independence of samples, we adopted the one-way ANOVA, which was statistically informative, despite the

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**Fig. 1.** Sulforaphane (SFN) increased muscle weight and improved gastrocnemius (GAS) hypertrophy and myocardial hypertrophy in mdx mice. Four-week-old male mdx mice were separately treated with SFN (2 mg/kg body wt by gavage) or corn oil, and 4-wk-old C57/black 10 (BL10) mice were treated with corn oil in the same dose. Body weights of the mice were checked every week. After 8 wk of treatment, mice were killed, and their hearts and related muscles were weighed. **A:** body weight at different ages. **B:** weight of skeletal muscles. **C:** weight of the heart. Values are means ± SE of three independent experiments; n = 6 mice for each group. BL10, BL10 control mice; Mdx, Mdx control mice; SFN, SFN-treated mdx mice; TA, tibial anterior; SOL, soleus; EDL, extensor digitorum longus. *P < 0.05 vs. BL10 mice. #P < 0.05 vs. mdx control mice. Statistical analysis was conducted using one-way ANOVA.
small number of subjects in each group. The one-way ANOVA is based on the null hypothesis that all groups have the same mean. To test for potential significant differences between the groups, the least significant difference test was performed (Prism 5, GraphPad Software, San Diego, CA). Fiber size distribution was analyzed by χ² test (SPSS version 17). Values of P < 0.05 were considered as statistically significant.

RESULTS

SFN treatment increased muscle mass, improved GAS and myocardial hypertrophy in mdx mice. Compared with the BL10 mice, mdx control mice gained less body weight from 4 wk old to 9 wk old, but with no statistical difference from 9 wk old to 11 wk old at equal tempo. However, when treated with SFN, the weights of mdx mice were significantly higher than that of BL10 and mdx counterparts (Fig. 1A). As described before, DMD usually exhibited a serious GAS and myocardial hypertrophy (14). To evaluate the potential of SFN in ameliorating pseudohypertrophy of mdx mice, we isolated and weighted the mass of hearts, GAS, TA, EDL, and SOL (Fig. 1, B and C), and found that mdx control mice showed a much heavier heart and GAS than the BL10 controls, but SFN treatment significantly decreased the weights of heart (~20%) and GAS (~25%) in mdx mice. The TA, EDL, and SOL muscles were all lighter in mdx control mice than those in the BL10 mice, but, when treated with SFN with the muscle mass increased for TA, EDL, and SOL muscles compared with mdx control counterparts. These results demonstrate that SFN treatment increases the body weight and skeletal muscle mass and improves GAS and myocardial hypertrophy in mdx mice.

SFN treatment improved muscle function and increased exercise ability in mdx mice. To determine whether SFN can improve muscle function of mdx mice, we assessed the effect of SFN on the forelimb grip force of the mice. The forelimb grip force of mdx control mice was less than that of BL10 (Fig. 2A), but it was significantly increased (~30%) by SFN treatment compared with mdx control counterparts and normalized to the level of BL10 mice. To test if SFN can increase the exercise ability of mdx mice, we ran the mice on a treadmill with an exhaustive exercise protocol (1). As shown in Fig. 2B, the running distance of mdx control mice was much shorter than that of BL10 mice, but, when treated with SFN, the running distance of mdx mice was significantly increased (~20%) compared with mdx control counterparts. Taken together, SFN improves muscle function and increases exercise ability in mdx mice.

SFN treatment alleviated the oxidative stress of mdx mice and protected dystrophic muscle from exercise-induced damage. CK and LDH have been used as markers for membrane damage. The CK and LDH will be released from muscle cells if the muscle membrane is damaged. Patients with DMD and mdx mice regularly display high levels of CK and LDH activities (34, 47). To test if SFN can protect dystrophic muscle from oxidative damage, we first tested the plasma CK (Fig. 3A) and LDH activities (Fig. 3B) and found that the activities of CK and LDH in mdx control mice were significantly higher than that of BL10 mice, but, when treated with SFN, the activities of both enzymes were decreased by ~45% and ~40% separately compared with mdx control counterparts. Besides, we compared the CK and LDH activities in mice pre- and postexercise. Our results demonstrated exercise induced significant damage to the muscle, as indicated by the elevated CK and LDH activities in the postexercise mice. While SFN treatment significantly reduced CK and LDH activities to the preexercise level in postexercise mdx mice. This demonstrated SFN treatment can protect dystrophic muscle from exercise-induced damage. To determine whether SFN could mitigate the oxidative stress of mdx mice, we checked the muscle (TA) TBARS levels. TBARS is one of the most widely used markers of lipid peroxidation in published literature (57). Increased levels have been demonstrated in animal models of muscular dystrophy (15, 46) and humans (23). Muscle TBARS levels were indicated by MDA levels (Fig. 3C). The results showed that the muscle MDA levels of mdx control mice were remarkably higher than that of BL10 mouse; however, after SFN treatment, the MDA levels of mdx mice were significantly decreased compared with mdx control counterparts by ~60% and normalized to the levels of BL10 mice. Furthermore, we also evaluated the muscle (TA) GSH/GSSG (Fig. 3D). It is widely known that the decrease of GSH/GSSG is a symbol of oxidative stress (38) and is found decreased in mdx muscles compared with the normal controls (54). Our results showed

Fig. 2. Increase of the forelimb grip and running distance in SFN-treated mdx mice. Four-week-old male mdx mice were separately treated with SFN (2 mg/kg body wt by gavage) or corn oil, and 4-wk-old BL10 mice were treated with corn oil in the same dose. After 8 wk of treatment, the forelimb grip (A) and running distance (B) were tested. Values are means ± SE of three independent experiments; n = 6 mice for each group. #P < 0.05 vs. BL10 mice. *P < 0.05 vs. mdx control mice. Statistical analysis was conducted using one-way ANOVA.
that the GSH/GSSG of mdx control mice were significantly lower than that of BL10, but, after SFN treatment, the ratio was much increased (~3.2-fold) compared with mdx control counterparts and reached the level of BL10 mice. These results indicate that SFN alleviates the oxidative stress of mdx mice.

**SFN treatment activated Nrf2/ARE signal pathway in mdx mice.** To examine if SFN really activated Nrf2/ARE signaling pathway, we assessed the protein and mRNA expression of Nrf2/ARE downstream phase II detoxifying enzymes NQO1 and HO-1. Western blot and qRT-PCR analyses confirmed that SFN successfully increased the protein (~40%) (Fig. 4A) and mRNA (~13-fold) (Fig. 4B) expression of Nrf2. ELISA (Fig. 4C) and qRT-PCR analyses (Fig. 4D) showed NQO1 was upregulated by SFN treatment. Additionally, as shown in Fig. 4E (ELISA), Fig. 4F (Western blot), and Fig. 4G (qRT-PCR), SFN treatment significantly increased the protein (~90%) and mRNA (~10-fold) expression of HO-1. Moreover, we also found that the expressions of NQO1 and HO-1 in mdx control mice were less than those in the BL10 mice, which indicated oxidative stress was much more serious in mdx mice. These results strongly indicate that SFN successfully activates Nrf2/ARE signaling pathway in mdx mice.

**SFN treatment enhanced the sarcolemmal integrity of mdx mice.** EBD is a vital dye that is unable to penetrate the sarcolemma of normal muscle fibers, and it has been used to evaluate sarcolemmal integrity in mouse models of muscular...
dystrophy (56). Accordingly, we used EBD penetration into the muscle fiber cytoplasm as an index of sarcolemmal disruption resulting from oxidative stress induced by DMD. SFN-treated mdx mice decreased the levels of EBD staining in TB, TA, and GAS muscles by 4.1-, 4.2-, and 2.5-fold, respectively (Fig. 5, A and B), compared with mdx control counterparts. This finding demonstrates SFN enhances the sarcolemmal integrity of mdx mice.

SFN treatment improved morphological features in mdx muscles. To determine whether stimulation of the Nrf2/ARE signaling pathway could result in an attenuation of the dystrophic pathology, we examined several morphological parameters in muscles from BL10 and mdx mice. Hematoxylin and eosin staining of muscle sections revealed a healthier appearance (decrease in both necrotic and angulated fibers) of SFN-treated mdx muscle fibers compared with mdx control counterparts (Fig. 6A). An assessment of the percentage of central nucleation seen in SFN-treated mdx muscles showed a ~40% reduction of this parameter (Fig. 6A), in agreement with other studies that used different paradigms to improve the dystrophic phenotype (2, 55, 59). Importantly, the reduction in the percentage of central-nucleated fibers reflects the enhanced survival of myofibers in SFN-treated mdx muscle (6). Among the several abnormal morphological indexes, dystrophic muscle fibers are known to display an increased variability of skeletal muscle fiber cross-sectional area (5). The average area of muscle fibers was similar among BL10 control mice (2,751 ± 858 μm²), mdx control mice (2,059 ± 647 μm²), and SFN-treated mdx mice (2,356 ± 815 μm²). However, there was a difference in distribution with mdx control mice displaying a greater number of smaller fibers, in the 1,100- to 2,400-μm² fiber size range, than BL10 control mice (Fig. 6B), whereas, in accordance with our expectations, when mdx mice were treated with SFN, the distribution showed a greater homogeneity with BL10 control mice. This correction in the morphological features of muscles from SFN-treated mdx mice is similar to those reported by others using different paradigms and correlates favorably with the functional improvements seen in dystrophic muscle (59). Accordingly, SFN significantly improves morphological features in mdx muscles.

SFN treatment ameliorated the inflammation of mdx mice. CD45, which could detect all lymphocytes, was frequently used as an indicator of inflammation (21). To determine whether activation of the Nrf2/ARE signaling pathway could result in an attenuation of the inflammation in dystrophic muscle, we examined the expression of CD45 in TA muscle from BL10 and mdx mice. Results of Western blot (Fig. 7C), immunohistochemistry (Fig. 7A), and immunofluorescence
(Fig. 7, D–G) showed mdx mice exhibited a more serious inflammation than the normal BL10 mice, which was indicated by the increase in the expression of CD45. However, when treated with SFN, the expression of CD45 was remarkably reduced in mdx mice. These results indicate that SFN significantly ameliorates inflammation in mdx muscle.

**DISCUSSION**

Presently, there is no cure for DMD, and the only medications proven to favorably alter its natural history are corticosteroids. Considering its serious side effects for long-term use, it is imperative to find a safer and more effective succedaneum. In this study, we demonstrate that SFN treatment is able to improve skeletal muscle function and attenuate muscular dystrophy in mdx mice through the modulation of a scope of Nrf2 downstream detoxifying phase II enzymes.

Increasing evidence shows that oxidative stress is involved in the pathogenesis of DMD. The continuous membrane damage of muscle fibers due to oxidative stress and lipid peroxidation can lead to an alteration of the structure and function of these membranes, which, in turn, lead to further acceleration of the disorder (61). It is assumed that cell death in muscular...
dystrophy may be attributed to free radical-mediated injury (37), because oxidative stress contributes to similarities between the pathology of DMD and muscle injury (19). Nrf2 is a member of the capncollar family of basic leucine-zipper transcription factors (35) and protects a variety of tissues and cells against oxidative stress and electrophiles through ARE-mediated induction of diverse antioxidant and phase II detoxification enzymes, such as HO-1 and NQO1 (41), and SFN is known to increase the cellular antioxidant capacity through Nrf2-induced enzymes, such as HO-1 (20) and NQO1 (51). Nrf2 can also protect skeletal muscle against damage induced by exhaustive exercise in rats through its antioxidative effects (30), but, to our knowledge, no studies have investigated the role of SFN on skeletal muscle of \textit{mdx} mice. In this study, we first checked the effect of SFN on oxidative stress and lipid peroxidation in dystrophic \textit{mdx} mouse model. Results showed significant decreases in the plasma activity of the intracellular enzyme CK, LDH, and muscle MDA levels, and remarkable increase in GSH/GSSG, which all supported the decreased oxidative stress and lipid peroxidation in SFN-treated \textit{mdx} mice. Furthermore, the exercise-induced damage was decreased in dystrophic muscles by SFN treatment. Other studies show that resveratrol (16, 18), polyphenol (39), and curcumin (48) can also alleviate dystrophic muscle pathology in \textit{mdx} mice via reducing oxidative stress, but their antioxidative abilities are not stronger than SFN. Resveratrol cannot reverse the high level of CK activity in \textit{mdx} mice (18): although polyphenol and curcumin decrease the level of CK activity to some degree, SFN decreases more than them. Additionally, the antioxidative role of SFN is attributed to Nrf2-induced endogenous antioxidant/detoxifying enzymes known as phase II enzymes. Accordingly, compared with some antioxidants applying in DMD treatment, SFN is a much safer, milder, and more effective antioxidant.

However, these antioxidants may ameliorate dystrophic pathology through other mechanisms, except for their antioxidative...
Fig. 7. SFN ameliorates inflammation (CD45) in mdx muscle fibers [TA, TB, GAS, and quadriceps (QUAD)]. A: shown are representative photomicrographs of muscle cross sections processed for immunohistochemistry (IHC) (CD45) from BL10 control, mdx control, and SFN-treated mdx mice. B: statistics of IHC (CD45) show a reduction of CD45 expression in SFN-treated mdx mice. C: Western blot shows SFN reduced CD45 expression in mdx mice. D–H: immunofluorescence of CD45 shows SFN attenuated inflammation (CD45) in mdx muscle fibers (photomicrographs: GAS (D), TB (E), TA (F), and QUAD (G); statistics of IHC (CD45) (H)). Values are means ± SE of three independent experiments; n = 6 for each group. *P < 0.05 vs. BL10 mice. #P < 0.05 vs. mdx control mice. Bar = 100 μm. Magnification: ×20. Statistical analysis was conducted using one-way ANOVA.
tive abilities, such as reduction of inflammation, decrease of degeneration and necrosis in myofiber, inhibition of fibrosis, switch of fiber type, and so on. While Hori et al. (18) found that long-term treatment of mdx mice with resveratrol failed to suppress the infiltration of inflammatory cells or upregulation of cytokines in the muscle of mdx mice, consistent with their results, Gordon et al. (16) also discovered resveratrol did not affect total immune cell infiltration at 12 wk of age and had no effect on oxidative capacity. We examined the role of SFN on the inflammation in mdx mice. Interestingly, we found that SFN significantly attenuated the expression of CD45, an indicator of inflammation, which may contribute to the improvement of dystrophic pathology. Thus, compared with resveratrol (18), SFN may ameliorate muscular dystrophy by anti-inflammatory ability. However, resveratrol inhibited fibrosis and myofibroblast differentiation in the muscle of mdx mice (18); we will further detect the role of SFN on muscle fibrosis in mdx mice.

Our results showed that the morphology of dystrophic muscle was normalized by SFN treatment, indicated by reduction of central nucleation and fiber size variability. This correction in the morphological features of muscles from SFN-treated mdx mice is similar to those reported by others using different paradigms and correlates favorably with the functional improvements seen in dystrophic muscle (59). Moreover, our EBD staining showed a favorable role of SFN in ameliorating

Fig. 7—Continued
muscular sarcolemmal integrity of mdx mice, which could contribute to the reduction of CK and LDH activities as well. Furthermore, mdx mice show reductions in body weight and specific force of EDL muscles (28). In our study, increases in the body weight, muscle mass of some skeletal muscles, forelimb grip, and running distance were observed in SFN-treated mdx mice, demonstrating the increase in motor function, probably due to reduced muscle damage or increased regeneration. Alterations in muscle fiber-type proportions are related to many compound-induced preservation of muscle-specific force in mdx mice and may contribute to SFN-induced increase of forelimb grip as well. Additionally, SFN significantly improved GAS muscle hypertrophy and myocardial hypertrophy in mdx mice. These results demonstrate SFN exerts a strong favorable role on DMD treatment.

NQO1 is a cytosolic flavoprotein and acts as an antioxidant enzyme protecting cells against the toxicity of quinones by catalyzing the two-electron reduction of these compounds and generating the corresponding hydroquinone. NQO1 also catalyzes the regeneration of oxidized vitamin E, contributing to the maintenance of these important antioxidants (43). It has been demonstrated that NQO1 is strongly induced by SFN in different tissues, such as the rat bladder and animal and human mammalian tissues (10, 63) and cell lines, such as rat aortic...
smooth muscle cells and cardiac fibroblasts (64, 65). HO-1 exhibits multiple beneficial properties, including antioxidant, anti-inflammatory, and cytoprotective functions, and is often endogenously induced by various stress stimuli and SFN (25, 52, 58). To our knowledge, no studies have evaluated whether NQO1 or HO-1 expression varies in mdx mice. In this research, mdx control mice demonstrate decreases of NQO1 and HO-1 expression in TA muscle compared with BL10 mice. SFN treatment was able to increase NQO1 and HO-1 expression in mdx mice, suggesting strong implications of NQO1 and HO-1 in the protection of skeletal muscles from oxidative stress and inflammation-induced muscle damage. However, in our research, there exist translational/protein degradation events following SFN treatment; it may be caused by changes in the level of posttranscriptional regulation. The cumulative effects of SFN treatment reported in this study suggest that SFN contributes to the increase of antioxidant capacity of muscle tissue by inducing important antioxidant and phase II enzymes. Phase II enzymes are induced partially through the Nrf2/ARE pathway in many tissues and cells (64, 65). Our results indicate Nrf2 expression is strongly induced by SFN, suggesting that the antioxidative and anti-inflammatory activities of SFN on DMD are, at least partly, attributed to its activation of Nrf2-induced antioxidant and phase II detoxification enzymes, such as HO-1 and NQO1. Nrf2 phosphorylation by protein kinases, such as Akt kinase, ERK, JNK, and PKC, influences the activation of the Nrf2/ARE pathway (42). Further studies on the role of Nrf2 in improving muscle regeneration, differentiation, inflammation, and fibrosis are warranted.

Taken together, we have effectively shown SFN can ablate the core mechanistic hallmarks of dystrophic progression and improve muscle function of mdx mice associated with activating Nrf2/ARE pathway (upregulation of downstream two-phase enzymes HO-1 and NQO1). These effects could explain the overall protection of SFN against DMD. Nrf2 activation may be a useful approach for the patients with muscular dystrophy.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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