Sulforaphane Attenuates Muscle Inflammation in Dystrophin-deficient mdx Mice via NF-E2-related Factor 2 (Nrf2)-mediated Inhibition of NF-κB Signaling Pathway*

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Background: Sulforaphane attenuates inflammation in different tissues via activation of Nrf2.

Results: Sulforaphane-induced Nrf2 ameliorates muscle inflammation in mdx mice and inhibits NF-κB signaling pathway.

Conclusion: Sulforaphane-mediated Nrf2 mitigates muscle inflammation in mdx mice via inhibition of NF-κB signaling pathway.

Significance: Nrf2 may represent a new and promising therapeutic target for dystrophic muscle inflammation.

Inflammation is widely distributed in patients with Duchenne muscular dystrophy and ultimately leads to progressive deterioration of muscle function with chronic muscle damage, oxidative stress, and reduced oxidative capacity. NF-E2-related factor 2 (Nrf2) plays a critical role in defending against inflammation in different tissues via activation of phase II enzyme heme oxygenase-1 and inhibition of the NF-κB signaling pathway. However, the role of Nrf2 in the inflammation of dystrophic muscle remains unknown. To determine whether Nrf2 may counteract inflammation in dystrophic muscle, we treated 4-week-old male mdx mice with the Nrf2 activator sulforaphane (SFN) by gavage (2 mg/kg of body weight/day) for 4 weeks. The experimental results demonstrated that SFN treatment increased the expression of muscle phase II enzyme heme oxygenase-1 in an Nrf2-dependent manner. Inflammation in mice was reduced by SFN treatment as indicated by decreased infiltration of immune cells and expression of the inflammatory cytokine CD45 and proinflammatory cytokines tumor necrosis factor-α, interleukin-1β, and interleukin-6 in the skeletal muscles of mdx mice. In addition, SFN treatment also decreased the expression of NF-κB(p65) and phosphorylated IκB kinase-α as well as increased inhibitor of κB-α expression in mdx mice in an Nrf2-dependent manner. Collectively, these results show that SFN-induced Nrf2 can alleviate muscle inflammation in mdx mice by inhibiting the NF-κB signaling pathway.

Duchenne muscular dystrophy (DMD), a the most frequent and lethal form of muscular dystrophy, is an X-linked genetic disease caused by a mutation in the gene encoding dystrophin (1) and affects 1 of 3,500 newborns (2). This mutation leads to the loss of the functional protein dystrophin, the critical member of the dystrophin glycoprotein complex that creates a direct link between the intracellular cytoskeleton and the extracellular matrix of skeletal muscle (3). The loss of this connection leaves the muscle fibers highly susceptible to damage, resulting in continuous rounds of muscle degeneration/regeneration (4). This process is coupled with and exacerbated by chronically elevated muscle inflammation, which is thought to contribute to the dystrophic pathology (5). The muscle fibers eventually lose the ability of regeneration and will be replaced with fibrous and fatty tissue (3), resulting in a decrease in muscle function, loss of ambulation, and death in their mid to late 20s (1). Although reintroducing a functional dystrophin gene could alleviate or cure the disease, this technology is still in optimization. Corticosteroids are the only currently prescribed treatment, and their therapeutic strategies for DMD are largely based on symptomatic treatment with their anti-inflammatory effects, but they only show modest improvements in muscle function with many undesirable side effects such as bone loss, diabetes, hypertension, and behavioral changes (6, 7). Therefore, alternative therapies are needed to help alleviate the disease pathology.

The dystrophin-deficient mdx mouse is the most widely used animal model of DMD for studying this disease. Although the long term muscle pathology is not as severe as that in human, mdx mice exhibit a muscle pathology similar to humans between 3 and 8 weeks of age. During this time, there is wide-

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‡1 The abbreviations used are: DMD, Duchenne muscular dystrophy; Nrf2, NF-E2-related factor 2; HO-1, heme oxygenase-1; SFN, sulforaphane; IKK, IκB kinase; IκB, inhibitor of κB; qRT, quantitative real time; p-IKK, phosphorylated IKK; p-IκB, phosphorylated IκB; TA, tibialis anterior; GAS, gastrocnemius; TB, triceps brachii; ANOVA, analysis of variance; NF-κB, nuclear factor κ-light-chain-enhancer of activated B-cell.
spread muscle necrosis accompanied by infiltration of damaging inflammatory cells (8). By the age of 12 weeks, the necrosis is replaced by regeneration of damaged muscle fibers, and the immune cell infiltration subsides with a corresponding change in macrophage phenotype, which promotes the regeneration (8). Therefore, studies on the effect of a therapy on muscle pathology should be carried out between 3 and 12 weeks of age when the pathology is most similar to that in humans (5, 9). Studies on mdx mouse showed that reducing inflammation through a variety of interventions improves muscle morphology and muscle function, indicating that inflammation may be a viable therapeutic target.

Nrf2, a member of the cap ‘ n’ collar family of redox-sensitive basic leucine zipper proteins, is the major transcriptional regulator of the expression of genes encoding phase II detoxifying enzymes, including heme oxygenase-1 (HO-1) (10). Recent research indicates that Nrf2 plays a strong anti-inflammatory role in many different tissues via inhibition of the NF-κB signaling pathway. Nrf2 plays a protective role in inflammation-mediated colonic tumorigenesis (11), allergen-mediated airway inflammation (12), cigarette smoke-induced emphysema (13), and dextran sulfate sodium-mediated colitis (14). However, the role of Nrf2 in the inflammation of dystrophic muscles and whether Nrf2 may inhibit NF-κB signaling pathway in patients with DMD are still unknown. SFN, an activator of Nrf2, is found in cruciferous vegetables such as broccoli, Brussels sprouts, and cabbages (15). Accumulating evidence suggests that SFN is a promising chemopreventive agent in various cancers and has antidiabetic and antimicrobial properties via Nrf2-dependent induction of the phase II detoxifying enzyme HO-1 (16, 17). SFN also has a strong anti-inflammatory effect in many tissues. Nallasamy et al. (18) have found that dietary supplementation of SFN prevents TNF-α-induced vascular endothelial inflammation of mice. Besides, Nguyen et al. (19) have discovered that SFN reduces inflammatory responses of cardiopulmonary bypass. Furthermore, our previous study shows that SFN also ameliorates inflammation in mdx mice, but we mainly investigated its antioxidative role (20). Thus, despite its proven chemopreventive and anti-inflammatory efficacy in other diseases, the potential effects and mechanisms of SFN in inflammation of dystrophic muscles have not been well evaluated. We assumed that SFN-induced activation of Nrf2 has a favorable role in ameliorating inflammation of mdx mice via inhibition of the NF-κB signaling pathway.

Our previous study demonstrated that SFN improved muscle function and attenuated muscular pathology via its powerfully antioxidative efficacy (20). In the present study, we examined the anti-inflammatory effects of SFN on muscular dystrophy using the mdx mouse as the model of DMD. SFN-mediated activation of Nrf2 and its downstream phase II enzyme HO-1 was confirmed by Western blot, quantitative real time (qRT)-PCR, and immunohistochemistry. The anti-inflammatory effect of SFN in dystrophic muscles was assessed by immune cell infiltration assay and Western blotting, qRT-PCR, and immunofluorescence assay of inflammatory cytokine CD45 and proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) in the skeletal muscles. In addition, SFN also inhibited the expression of NF-κB(p65), p-1κB kinase (IKK)-α, and p-1κB-β and increased expression of 1κB-α in mdx mice in an Nrf2-dependent manner. Our results demonstrated that SFN may be used as a promising anti-inflammatory drug for DMD via activation of Nrf2-induced inhibition of the NF-κB signaling pathway.

**Experimental Procedures**

**Reagents and Antibodies**—DL-Sulforaphane (1-isothiocyanato-4-(methylsulfinyl)butane), trolox, DMSO, isopentane, and mammalian protease inhibitor mixture were all purchased from Sigma-Aldrich. 3’-Diaminobenzidine and 4’,-6-diamidino-2-phenylindole (DAPI) were from Vector Biologicals (Hamilton, Ontario, Canada). OCT (optimum cutting temperature compound) was from Sakura Finetek (Torrance, CA). TRizol reagent was purchased from Invitrogen. PVDF Western blotting membrane was from Bio-Rad. Anti-Nrf2, anti-NF-κB(p65), anti-CYD45, and anti-GADPH were from Affinity Biologicals (Hamilton, Ontario, Canada). Anti-HO-1, anti-p-IκB-α, anti-IκB-α, anti-p-IκB-β, anti-IκB-β, anti-p-1κB-α, and anti-TNF-α were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

**Animals**—C57BL/10ScSn-Dmdmdx/NJU mice were obtained from Nanjing Biomedical Research Institute of Nanjing University and bred in our facility. Twelve 4-week-old male mdx mice and six 4-week-old C57/Black 10 mice were housed in a controlled environment with a 12:12-h light-dark cycle at 22 °C and 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/Black10 mice (BL10), mdx control mice (mdx), SFN-treated mdx mice (SFN).

4-Week-old male mdx mice in SFN group were treated with SFN by gavage (2 mg/kg of body weight/day) solubilized in 0.5 ml of DMSO and then diluted in 49.5 ml of corn oil (0.1 mg/ml) for 4 weeks. At the same time, mice of mdx and BL10 groups were treated with corn oil (containing 1% DMSO) with the same doses. The 2 mg/kg of body weight dose was a little higher than that previously reported by Souza et al. (21) (they used 1 mg/kg of body weight by gavage). Moreover, de Souza et al. (22) used 0.1–0.5 mg/kg of body weight in an SFN gavage feeding protocol. Because DMD is a more serious disease than the two in above studies, we chose a higher SFN dose in our study.

**Sample Collection**—After 4-week gavage (8 weeks old), all mice were anesthetized with chloral hydrate (400 mg/kg of body weight) and then killed by cervical dislocation. TA, GAS, and TB muscles were removed, frozen in liquid nitrogen, and stored at −80 °C for further assays. In this study, we used TA muscle as a representative muscle for making muscle homogenates and the following Western blotting and qRT-PCR on account of the fact that the status of TA was usually treated as an indicator of DMD status in mdx mice.

**Western Blot Analysis**—Muscles lysates were prepared as described before (23). Protein concentrations were determined by the BCA protein assay. Muscle lysates were run on 10% SDS-polyacrylamide gels (50 μg/lane), and proteins were transferred...
TABLE 1

| Primer sequences for qRT-PCR | Gene      | Sequence (5’→3’) |
|------------------------------|-----------|-----------------|
| Mouse GAPDH                  | Forward   | CCACTTCTCGTTGAGGT |
|                             | Reverse   | GCGGACATTTCCATGAGCT |
| Mouse Nrf2                   | Forward   | CCACTTCTCGTTGAGGT |
|                             | Reverse   | GCGGACATTTCCATGAGCT |
| Mouse HO-1                   | Forward   | AAGCAGGAACTGCCAAGTCG |
|                             | Reverse   | AGTATGGGCTGTTGCTGGGCC |
| Mouse NF-κB(p65)             | Forward   | GCTGCTCTTCCGGGAGAGG |
|                             | Reverse   | GCTGCTCTTCCGGGAGAGG |
| Mouse IL-6                   | Forward   | TGGACTGAGGACACCACCA |
|                             | Reverse   | TGGACTGAGGACACCACCA |
| Mouse TNF-α                  | Forward   | CTGGAGAAGAACAGATAG |
|                             | Reverse   | CTGGAGAAGAACAGATAG |
| Mouse Bcl2                   | Forward   | GAGAAGTCTGCTGCTCTG |
|                             | Reverse   | GAGAAGTCTGCTGCTCTG |
| Mouse c-Myc                  | Forward   | GACCAGGAGAATTGCCATTG |
|                             | Reverse   | GACCAGGAGAATTGCCATTG |
| Mouse COX-2                  | Forward   | TGGACATTTGCCGACAGT |
|                             | Reverse   | TGGACATTTGCCGACAGT |


to poly(vinylidene fluoride) (PVDF) membranes by semidry electroblotting (1.5 mA/cm²). PVDF membranes were then incubated in blocking buffer (Tris-buffered saline (TBS) supplemented with 0.05% (v/v) Tween 20 (TBST)) containing 5% (w/v) 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-p-IKK-α (1:1,000), anti-IKK-α (1:1,000), anti-p-IKK-β (1:1,000), anti-IKK-β (1:1,000), anti-κB-α (1:1,000), anti-κB-α (1:1,000), anti-HO-1 (1:500), anti-CD45 (1:1,000), anti-TNF-α (1:500), anti-NF-κB(p65) (1:500), and anti-GADPH (1:5,000) as internal normalizers in TBST containing 5% (w/v) skimmed milk powder and goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5,000) at room temperature for 2 h. Sections were then incubated in blocking buffer and exposed to ECL Advance reagent (GE Healthcare) overnight at 4 °C on a three-dimensional rocking table. Then the membranes were washed three times for 10 min in TBST and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:12,000 dilution) 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) for 2 min as described in the manufacturer’s protocol. Then membranes were exposed to Hyperfilm-ECL (GE Healthcare) for 2–5 min and visualized using a Fluor S Multimager and Quantity One 4.1 (Bio-Rad). 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.

qRT-PCR Analyses—Total RNA was extracted using TRizol reagent according to the manufacturer’s instructions from muscles (TA). 2 μg of total RNA was reverse transcribed using the First Strand cDNA synthesis kit (Takara, Tokyo, Japan) according to the manufacturer’s protocol. qRT-PCR was carried out using Power SYBR Green PCR Master Mix with the StepOnePlus real time PCR system (Applied Biosystems, Warrington, UK). Gene-specific primer pairs used in qRT-PCR analysis are outlined in Table 1. All assays, including no-template controls, were done in triplicates. 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. Statistical analysis was performed on the -fold change.

Histological and Morphometric Analyses—Samples of skeletal muscle tissues (TA, TB, and GAS) were collected, then embedded with OCT (optimum cutting temperature compound), and fixed in precooled isopentane with liquid nitrogen. Serial cross-sections (10 μm thick) from 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 (PBS; pH 7.4) and stained with hematoxylin and eosin (H&E) as described by Nakaso et al. (24) with some modifications. They were finally observed under a light microscope (Olympus 600 autobiochemical analyzer, Tokyo, Japan). Using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) was used to record images and perform 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 v17 specifically testing the frequency with the χ² test.

Evans Blue Dye Staining—Evans blue dye injections were performed as described previously (20). The presence of Evans blue dye in myofibers was observed under a fluorescence microscope (Olympus 600 autobiochemical analyzer), and the intensity level was determined using Image-Pro Plus software by converting images to eight-bit grayscale and determining the total and average gray intensities taken as a measure of Evans blue dye fluorescence in the entire area of each 20× cross-section. Evans blue dye-positive areas were semi-quantified and represented as the percentage of total muscle area. Non-injected 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 non-injected 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.

Immunohistochemistry/Immunofluorescence—Immunohistochemistry and immunofluorescence of the muscle tissues (TA, TB, and GAS) were performed as described previously (25). 10-μm muscle sections were incubated with commercial rabbit polyclonal antibodies against Nrf2, HO-1, NF-κB(p65), and CD45 at 1:100 dilution overnight at 4 °C. For immunohistochemistry, sections were conjugated with horseradish peroxidase (HRP) antibody (1:500 dilution) at room temperature for 2 h and then covered by 3,3′-diaminobenzidine, nuclei were counterstained in hematoxylin, and slides were mounted with Vectorsheld mounting medium (Vector Laboratories). For immunofluorescence (NF-κB(p65) and CD45), sections were
visualized using a secondary antibody coupled to a fluorescent marker, Cy3, at room temperature for 2 h. Then slides were mounted using Vectashield with DAPI for detection of nuclei. Subsequently, all fields were observed under light (immunohistochemistry) and fluorescence (immunofluorescence) microscopes (Olympus 600 autobiochemical analyzer) at 20× magnification. Control experiments without primary antibody demonstrated that the signals observed were specific. Nrf2-, HO-1-, and CD45-positive areas were semiquantified and represented as the percentage of total muscle area.

**Statistics**—All values are reported as means ± S.E. of six animals. To check whether a difference was statistically significant, given the small sample sizes and the independence of samples, we adopted one-way ANOVA, which was statistically informative despite the small number of subjects in each group. 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). Values of $p < 0.05$ were considered as statistically significant.

**Results**

**SFN Improved the Dystrophic Features in mdx Mice**—Before sacrificing the mice, we measured the body weight of mice in the three groups, and then the muscle mass was weighed. Results indicated that mdx mice showed a lighter body weight than the BL10 control mice, but after treating mdx mice with SFN, the body weights of mdx mice were significantly increased and normalized to the normal controls (Table 2). To evaluate the potential of SFN in ameliorating hypertrophy of mdx mice, we isolated and weighed the mass of GAS, TA, and TB muscles and calculated the muscle mass to body weight ratio (Table 2). Results showed that mdx control mice showed a much heavier GAS than the BL10 controls ($n = 6$, $p < 0.05$), but SFN treatment significantly decreased the weights of GAS (~30%) in mdx mice, reaching normal levels ($n = 6$, $p < 0.05$) (Table 2). The TA and TB muscles were all lighter in mdx control mice than those in the BL10 mice ($n = 6$, $p < 0.05$), but when mdx mice were treated with SFN, the muscle mass increased for TA and TB muscles compared with mdx control counterparts and normalized to that in BL10 control mice ($n = 6$, $p < 0.05$) (Table 2). These results demonstrate that SFN treatment increases the body weight and skeletal muscle mass and improves gastrocnemius hypertrophy in mdx mice.

### TABLE 2

| BL10 control mice | mdx control mice | SFN-treated mdx mice |
|-------------------|------------------|----------------------|
| Body weight (g)   | 20.8 ± 1.6       | 17.3 ± 1.1$^a$       | 19.6 ± 0.9$^a$ |
| Gastrocnemius weight (mg) | 172.4 ± 8.6 | 275.0 ± 10.3$^a$ | 193.8 ± 49.6$^a$ |
| Tibialis anterior (mg) | 1257.4 ± 4.2 | 924.4 ± 35.6$^a$ | 1278.5 ± 97.6$^a$ |
| Triceps brachii (mg) | 163.2 ± 7.3 | 131.4 ± 51.1$^a$ | 159.9 ± 82.6$^a$ |
| GAS/body weight (mg/g) | 8.3 ± 0.1 | 5.3 ± 0.1$^a$ | 9.9 ± 0.1$^a$ |
| TA/body weight (mg/g) | 6.0 ± 0.1 | 7.6 ± 0.1$^a$ | 6.5 ± 0.1$^a$ |
| TB/body weight (mg/g) | 7.8 ± 0.1 | 6.0 ± 0.1$^a$ | 8.2 ± 0.1$^a$ |

$^a$ $p < 0.05$ versus BL10 mice.  
$^b$ $p < 0.05$ versus mdx control mice.

**SFN Activated Nrf2/HO-1 Signaling Pathway in mdx Mice**—To examine the positive effect of SFN on Nrf2 activation and Nrf2-mediated up-regulation of phase II enzyme HO-1, we examined the protein and gene expression of Nrf2 and its downstream phase II detoxifying enzyme HO-1. Western blot and qRT-PCR analyses confirmed that SFN successfully increased the protein (~1.9-fold) (Fig. 1A) and gene (~3-fold) (Fig. 1C) expression of Nrf2. Moreover, immunohistochemistry of Nrf2 in GAS, TB, and TA muscles (Fig. 1E) demonstrated that compared with the expression of Nrf2 in mdx counterparts, there were 2.7-, 2.8-, and 2.6-fold increases (Fig. 1F), respectively, in that of SFN-treated mdx mice as well. Thus, SFN successfully activated the expression of Nrf2 in skeletal muscles. We also determined the expression of Nrf2 and the downstream phase II detoxifying enzyme HO-1 by Western blotting, qRT-PCR, and immunohistochemistry. Results showed that protein (Western blot; Fig. 1A) and gene (qRT-PCR; Fig. 1D) expression of HO-1 was up-regulated ~2.2- and ~2.7-fold, respectively, by SFN treatment in comparison with mdx control mice. Furthermore, immunohistochemistry of HO-1 in GAS, TB, and TA muscles showed ~8.4-, ~4.2-, and ~3.6-fold increases (Fig. 1, G and H) in SFN-treated mdx mice compared with mdx counterparts. These results strongly indicate that SFN successfully activated the Nrf2/HO-1 signaling pathway in mdx mice. In addition, we also found that SFN successfully activated Nrf2/HO-1 signaling in BL10 mice (Fig. 2, A–C).

**SFN Enhanced Sarcolemmal Integrity of mdx Mice**—Evans blue dye 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 (26). Accordingly, we used Evans blue dye penetration into the muscle fiber cytoplasm as an index of sarcolemmal disruption resulting from inflammation induced by DMD. Our results showed that mdx mice exhibited much higher levels of Evans blue staining in TA, TB, and GAS muscles than the normal BL10 controls, whereas SFN treatment significantly decreased the levels of Evans blue staining in TB, TA, and GAS muscles by ~80, 76, and 62, respectively (Fig. 3, A and B), compared with mdx control counterparts. This finding demonstrates that SFN enhances the sarcolemmal integrity of mdx mice.

**SFN-induced Nrf2 Activation Inhibited NF-κB Signaling Pathway**—Activation of the NF-κB signaling pathway has been shown to be central to the pathophysiology of the muscular inflammatory response in patients with DMD (27, 28). To investigate the mechanism of SFN-induced Nrf2 activation in amelioration of muscular inflammation, we examined the protein expression of NF-κB(p65), p-IKK-α/IKK-α, p-IKK-β/IKK-β, and p-1κB-α/1κB-α as well as gene expression of NF-κB(p65), IL-1β, and IL-6. Results showed that mdx mice have a higher expression of NF-κB(p65), p-IKK-α, p-IKK-β, IL-1β, and IL-6 than BL10 control mice, whereas SFN significantly decreased the high levels of NF-κB(p65), p-IKK-β, p-IKK-α, IL-1β, and IL-6 in an Nrf2-dependent manner (Fig. 4, A, B, and D) compared with mdx counterparts. Moreover, SFN also inhibited nuclear translocation of NF-κB(p65) in mdx mice as indicated by immunofluorescence of NF-κB(p65) (Fig. 4C). In addition, SFN also reversed the low levels of 1κB-α in mdx muscles.
mice, normalizing them to normal levels (Fig. 4A). Moreover, we evaluated the correlation between NF-κB(p65) mRNA and Nrf2 expression in TA muscles of SFN-treated mdx mice.

Results demonstrated that expression of NF-κB(p65) mRNA and Nrf2 showed a significant inverse correlation as calculated by Pearson correlation (r = -0.7136, p = 0.0019) (Fig. 4E).

Furthermore, our results showed that SFN inhibited the protein expression of p-IKK-α, p-IKK-β, and NF-κB(p65), whereas 1κB-α levels were increased in BL10 mice (Fig. 5, A and B). In addition, we also examined the effect of SFN-induced Nrf2 on NF-κB-mediated antiapoptotic genes c-Myc, Bcl2, and COX-2. Results demonstrated that SFN did not affect expression of these antiapoptotic genes significantly (Fig. 4F). These results indicate that SFN-induced Nrf2 activation has a negatively regulatory role in the NF-κB signaling pathway.

SFN Had Anti-inflammatory Effects in Skeletal Muscle of mdx Mice—GAS, TB, and TA muscles were analyzed for signs of inflammation. An additional hallmark of dystrophic muscle is the presence of fibers with centralized nuclei, reflecting continuous muscle degeneration and regeneration (29). Our results of H&E staining showed that mdx mice exhibited many split fibers, and the percentage of centralized nuclei normalized to the number of muscle fibers was significantly higher in mdx muscles (n = 6; 12 images were taken per mouse) in comparison with BL10 control mice (Fig. 6, A and B). Moreover, the total area of inflammation in mdx mice and the frequency of inflammatory incidents of immune cell infiltration (n = 6; 12 images were taken per mouse) were also higher than in BL10 control mice (Fig. 6, A and C). Following treatment with SFN, mdx mice displayed a decrease in the number of centralized nuclei observed (Fig. 6, A and B) and a decrease in
FIGURE 3. Reduction in the levels of Evans blue staining in skeletal muscles from SFN-treated mdx mice. Cross-sectional views of Evans blue dye-positive regions of GAS, TB, and TA muscles from mice intravenously injected demonstrate levels of dye infiltration. Assessment of Evans blue dye staining in damaged regions of SFN-treated mdx GAS, TB, and TA muscles revealed ~80, 76, and 62% reductions, respectively, in comparison with age-matched mdx control counterparts. *, p < 0.05 versus BL10 mice; #, p < 0.05 versus mdx control mice. n = 6 for each group. Means ± S.E. (error bars) are shown. Statistical analysis was conducted using one-way ANOVA. Scale bar, 100 μm. Magnification, 20×.

FIGURE 2. SFN activated Nrf2/HO-1 signaling pathway in normal BL10 mice. 4-week-old male C57/Black 10 mice were separately treated with SFN (2 mg/kg of body weight by gavage), and 4-week-old C57/Black 10 mice were treated with corn oil in the same dose for 4 weeks. Then mice were sacrificed, and related muscles were collected. A, Western blot of Nrf2 and HO-1 protein in TA muscles. B, statistical analysis of Western blot of Nrf2 and HO-1 protein. C, qRT-PCR of Nrf2 and HO-1 genes in TA muscles. Data are the means ± S.E. (error bars) of three independent experiments. *, p < 0.05 versus BL10 mice. n = 6 for each group. Statistical analysis was conducted using Student’s t test.
the total area of inflamed muscles in comparison with mdx control mice (Fig. 6, A and C). CD45, which can detect all lymphocytes and is a specific marker of inflammatory cell infiltration, is frequently used as an indicator of inflammation (25). To further determine whether Nrf2 (activated by SFN)-mediated inhibition of NF-κB signaling pathway could result in an attenuation of the inflammation in dystrophic muscle, we also examined the protein expression of CD45 in dystrophic mouse TA muscles from BL10 and mdx mice with Western blotting and found that protein expression of CD45 was much higher (2.4-fold) in mdx mice.

FIGURE 4. SFN-induced Nrf2 activation inhibited NF-κB signaling pathway. 4-week-old male mdx mice were treated with corn oil or SFN (2 mg/kg of body weight/day), 4-week-old male BL10 mice were treated with corn oil for 4 weeks, and then skeletal muscle was harvested (TA) for related assays. A, Western blot of Nrf2, p-IKKα, p-IKKβ, IKKα, IKKβ, NF-κB(p65), p-IκBα, and IκBα in TA muscles. B, statistical analysis of Western blot of Nrf2, p-IKKα, p-IKKβ, NF-κB(p65), p-IκBα, and IκBα protein. C, immunofluorescence (IF) of NF-κB(p65) in TA muscles. The arrows indicate the nucleus location of NF-κB. D, qRT-PCR of NF-κB(p65), IL-6, and IL-1β genes in TA muscles. E, expression patterns of NF-κB(p65) with Nrf2 in TA muscles (r² = 0.5092, p = 0.0019, Spearman correlation). F, qRT-PCR of c-Myc, Bcl2, and COX-2 genes in TA muscles. Data are the means ± S.E. (error bars) of three independent experiments. n = 6 for each group. *, p < 0.05 versus BL10 mice; #, p < 0.05 versus mdx control mice. Statistical analysis was conducted using one-way ANOVA.
stem cell delivery (30); 2) genetic/molecular treatments based on exon skipping after RNA delivery (31), overexpression of insulin-like growth factor or AKT (32, 33), and activation of compensatory processes such as myostatin blockade (34); and 3) pharmacological treatments, which led to identification of glucocorticoids as the only approved pharmaceutical drug used to treat human patients with DMD. However, recent research shows that glucocorticoids only exert a small improvement in muscle function but have large numbers of undesirable side effects (7). Thus, it is imperative to find a safer and more effective succedaneum. In our previous study, we showed that SFN has a strong antioxidative efficacy and remarkably improves muscle function and muscle pathology in mdx mice through activation of Nrf2 with few secondary actions (20), indicating that Nrf2 activation by SFN may be useful in treatment of muscular dystrophy. In the present study, we further investigated the role of SFN-induced activation of Nrf2 in attenuating muscle inflammation (main reason for muscle pathology) in mdx mice and confirmed that its anti-inflammatory role is attributed to Nrf2-mediated inhibition of the NF-κB signaling pathway.

The lack of an effective cure for DMD may be due to multiple reasons such as the large size of the dystrophin gene, the multitude of mutations that cause the disease, and the complexity of successful and specific gene delivery to the affected tissues. This necessitates the need for alternative therapies to reduce the severity of the disease until a cure can be found. Currently, chronic treatment with glucocorticoids (prednisone and deflazacort) is the standard care for DMD; however, glucocorticoids are well known to induce muscle atrophy pathways via FOXO1 that may stunt the growth of pediatric patients and suppress the immune system, which plays an important role in myofiber repair cycles. Clinical improvement in patients with DMD treated with...
glucocorticoids may be the sum balance of beneficial anti-inflammatory effects and deleterious pathways. Both in vitro and in vivo data are consistent with this model. Heier et al. (35) have found that the net balance of prednisolone treatment increases muscle strength to normal, but at the same time, it stunts the growth of mice, resulting in lower maximal strength, and increases muscle damage in mdx mice. And Bauer et al. (36) have also found that prednisolone increases cardiac fibrosis in mdx mice. Furthermore, plenty of research shows that increased inflammation is widely distributed in dystrophin-deficient muscles (37–40) and that inflammation in young mdx mice exacerbates muscle damage and subsequently decreases muscle function (41). Thus, the attenuation of inflammation may be a potential therapeutic mechanism for the treatment of DMD.

In this study, we firstly checked the effect of SFN on inflammation in the dystrophic mdx mouse model. Our experimental results showed that mdx skeletal muscle sections exhibited strong inflammation with elevations in intracellular myophagocytosis and extracellular fibrotic infiltration, consistent with previous studies (42). However, the total immune cell infiltration in mdx mice was significantly reduced by 4 weeks of SFN treatment. SFN also improved morphology of dystrophic muscle as indicated by the reduction of nucleus centralization and small sized myocytes in mdx mice, which demonstrated that the necrosis/regeneration disorders of mdx muscles were partly restored by SFN treatment. The positive role of SFN on the morphological features of mdx mice is similar to the results of another report, which uses different paradigms and correlates muscle function and pathology in dystrophic muscle (43). Evans blue dye staining also showed a favorable role for SFN in attenuating muscular sarcolemmal integrity of mdx mice, indicating that SFN may attenuate the inflammation-induced muscle membrane damage of DMD. In addition, many negative side effects such as bone loss, heart problems, and diabetes seen with corticosteroid treatment (4) have not been reported with SFN treatment. Furthermore, SFN treatment released the growth retardation of mdx mice and attenuated gastrocnemius hypertrophy without any evidence of immunotoxicity in mdx.
mice, indicating that SFN treatment had no side effects like those of glucocorticoids.

Activation of the NF-κB signaling pathway has long been considered as the core to the pathophysiology of the muscular inflammatory response in patients with DMD, and NF-κB can be activated by lesion-induced oxidative stress and cytokines (mostly TNF-α) (28). The functional importance of NF-κB in inflammation is based on its ability to regulate the promoters of multiple inflammatory genes (45). Our experimental results showed that NF-κB(p65), p-IKK-α, and p-IKK-β are activated and that IkB-α is decreased in mdx mice, whereas SFN treatment significantly decreased expression levels of NF-κB(p65), p-IKK-α, and p-IKK-β and increased the expression level of IkB-α in an Nrf2-dependent manner. In addition, we also discovered that SFN inhibited nuclear translocation of NF-κB(p65) in mdx mice. Collectively, these results demonstrated that SFN-mediated activation of Nrf2 has a strong role in the inhibition of NF-κB signaling. It has been reported that long term treatment with the IKK-α inhibitor curcumin, another antioxidant, also reduces nuclear NF-κB activation in the mdx costal diaphragm (46). Compared with curcumin, SFN exerts a more powerful role in inhibiting the NF-κB signaling pathway. TNF-α is a major initiator of inflammation, is released early after an inflammatory stimulus, and plays an important role in the recruitment of inflammatory and immune cells to the injured site via activation of the NF-κB signaling pathway (28). In our study, SFN significantly ameliorated the gene and protein expression of TNF-α, which is up-regulated in mdx mice, and attenuated infiltration of immune and inflammatory cells in mdx mice. The inflammatory cytokines CD45, IL-1β,
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and IL-6 are widely distributed in dystrophic muscles and are regulated by activation of the NF-κB signaling pathway (47–49). Our results also demonstrated that the protein and gene expression of these inflammatory cytokines in skeletal muscles of mdx mice was remarkably decreased by SFN treatment. Despite increasing proinflammatory cytokines, NF-κB also mediates the transcription of several antiapoptotic genes, including c-Myc, Bcl2, p53, p21, c-FLIP, c-IAP-1, c-IAP-2, XIAP, IEX-1L, COX-2, TRAF-1, and TRAF-2 (50). However, in our present study, we determined the influence of SFN-induced Nrf2 on the antiapoptotic gene expression of c-Myc, Bcl2, and COX-2. The results demonstrated that SFN did not significantly affect the expression of these antiapoptotic genes, and this may be attributed to the fact that young mice (8 weeks old in our experiment) are in a fast growth stage, and apoptosis is not the major factor at this stage. Further study on old mice may clarify this question. These results indicate that SFN ameliorates muscle inflammation via Nrf2-mediated inhibition of the NF-κB signaling pathway in mdx mice.

Nrf2, a member of the cap ‘n’ collar family of basic leucine zipper transcription factors, is known to play an important role in the regulation of antioxidant responses, and recent studies have demonstrated that Nrf2 plays a broad role in modulating acute inflammatory response. Jin et al. (51, 52) revealed that Nrf2 plays an important role in protecting traumatic brain injury-induced secondary brain injury by regulating inflammatory cytokines and attenuating the pulmonary inflammatory response and NF-κB activation after traumatic brain injury. Mao et al. (44) also found that disruption of Nrf2 enhances the up-regulation of NF-κB activity, TNF-α, and MMP-9 after spinal cord injury in mice. Consistent with their results, our results showed that SFN-mediated activation of Nrf2 and its downstream phase II antioxidative enzyme HO-1 attenuated inflammation-induced muscle damage via inhibition of the NF-κB signaling pathway.

In summary, our experimental results show that SFN-induced activation of Nrf2 significantly improved the hallmarks of muscular dystrophy. This may be due to the reduction of inflammation in dystrophic muscles as indicated by reduced immune cell infiltration and decreased expression of inflammatory cytokine CD45 and proinflammatory cytokines TNF-α, IL-1β, and IL-6 expression, and this effect may be mediated by the inhibition of NF-κB signaling pathway, which indicate that SFN has a protective role in muscle inflammation in dystrophin-deficient mdx mice via Nrf2-mediated inhibition of the NF-κB signaling pathway. Therefore, Nrf2-mediated reduction of inflammation may be useful in amelioration of DMD pathology.

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