OXFORD

Human Molecular Genetics, 2017, Vol. 0, No. 0 1–10

doi: 10.1093/hmg/ddx173
Advance Access Publication Date: 3 May 2017
Original Article

ORIGINAL ARTICLE

Oxidative stress in Duchenne muscular dystrophy: focus on the NRF2 redox pathway

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Abstract

Oxidative stress is involved in the pathogenesis of Duchenne muscular dystrophy (DMD), an X-linked genetic disorder caused by mutations in the dystrophin gene and characterized by progressive, lethal muscle degeneration and chronic inflammation. In this study, we explored the expression and signaling pathway of a master player of the anti-oxidant and anti-inflammatory response, namely NF-E2-related Factor 2, in muscle biopsies of DMD patients. We classified DMD patients in two age groups (Class I, 0–2 years and Class II, 2–9 years), in order to evaluate the antioxidant pathway expression during the disease progression. We observed that altered enzymatic antioxidant responses, increased levels of oxidized glutathione and oxidative damage are differently modulated in the two age classes of patients and well correlate with the severity of pathology. Interestingly, we also observed a modulation of relevant markers of the inflammatory response, such as heme oxygenase 1 and Inteleukin-6 (IL-6), suggesting a link between oxidative stress and chronic inflammatory response. Of note, using a transgenic mouse model, we demonstrated that IL-6 overexpression parallels the antioxidant expression profile and the severity of dystrophic muscle observed in DMD patients. This study advances our understanding of the pathogenic mechanisms underlying DMD and defines the critical role of oxidative stress on muscle wasting with clear implications for disease pathogenesis and therapy in human.

Introduction

Increasing evidence shows that oxidative stress is involved in the pathogenesis of Duchenne muscular dystrophy (DMD), an X-linked genetic disorder caused by mutations in the dystrophin gene (1,2). Loss of the sarcolemmal integrity by the absence of the dystrophin protein, in both humans and mdx mouse model, leads to a cascade of pathological events that cause muscle fiber degeneration.

Altered enzymatic antioxidant responses, increased levels of oxidized glutathione and enhanced protein oxidation have been reported in mdx mice and in muscle biopsies of DMD patients (3–5). A role for oxidative stress in DMD is further supported by pre-clinical studies performed on mdx mice that report benefits

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Received: February 28, 2017. **Revised:** April 11, 2017. **Accepted:** April 26, 2017

(improved muscle pathology, decreased necrosis) for many antioxidant drugs (coenzyme Q_{10}, catalase, green tea extracts, resveratrol, N-acetylcysteine) (6–17), and on patients with DMD not using glucocorticoids (18–20). A double-blind randomized placebo-controlled phase 3 trial showed that the short-chain benzoquinone idebenone significantly reduced the loss of respiratory function in patients with DMD, suggesting this drug as a suitable treatment option to ameliorate a life-threatening complication of the disease (20). However, the efficacy of antioxidants in clinical trials has to be fully elucidated; thus monitoring glutathione systemic levels and understanding the precise sequence of events deriving from oxidative stress in DMD could be certainly useful.

Glutathione (γ-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular-weight thiol, and GSH/glutathione disulfide (GSSG) is the major redox couple in animal cells. The synthesis of GSH from glutamate, cysteine and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase and GSH synthetase. Compelling evidence shows that GSH synthesis is regulated primarily by γ-glutamylcysteine synthetase activity, cysteine availability and GSH feedback inhibition (21).

In order to shed light on the cascade of events triggered by oxidative stress, we analyzed the most important redox-activated pathway in muscle biopsies obtained from 11 children with DMD. This study moves from recent papers that recognize NF-E2-related Factor 2 (NRF2) as an important player in the anti-oxidant and anti-inflammatory response in dystrophin-deficient mice (22,23).

NRF2 is an inducible transcription factor that, in response to oxidative stress, regulates the expression of phase II enzyme genes by interacting with the antioxidant responsive element (ARE) sequence (24–26). Under normal condition, NRF2 is sequestered in cytoplasm and its activity is regulated by Kelch ECH associating protein 1 (KEAP1) through the complex KEAP1-NRF2, which promotes the ubiquitination and degradation of NRF2. Upon exposure to several stressors (environmental agents, endogenous inducers, drugs), NRF2 is released and translocates in the nucleus where its antioxidant functions occur (26–28).

Several genes are target for NRF2: those involved in glutathione synthesis, such as the glutamate-cysteine ligase (GCL), those implicated in the detoxification of xenobiotics, including the NAD(P)H quinone dehydrogenase 1 (NQO1) and the heme oxygenase 1 (HO-1), and the genes involved in the neutralization of reactive oxygen species (ROS) (Superoxide Dismutase, SOD) (24,25,29).

Given that previous studies have identified NADPH oxidase (NOX)-dependent ROS production and NRF2 activation as mainly responsible for the enhanced susceptibility to injury in dystrophin-deficient heart and skeletal muscle (30,31), the aim of this study was to explore the NRF2 expression and its signaling pathway in muscle biopsies of children with DMD. Moreover, in light of previous data showing a decrease in redox related genes in DMD, we also investigated HO-1, NQO-1 and SOD expression in DMD muscle biopsies. Importantly, since ROS levels appear progressively and temporally enhancing in dystrophic mouse models (32,33), we classified our patients in two age groups, in order to evaluate the antioxidant pathway expression during the disease progression. Finally, as pharmacological antioxidant approaches showed effects in reducing the pathogenic progression of the muscular dystrophies (34), we also wanted to assess the levels of two critical markers of systemic oxidative stress: the oxidized and reduced forms of glutathione (GSSG/GSH ratio) and the carbonyl content, indicative of the irreversible oxidative damage to proteins. Overall in this study, we focus on the redox signaling pathway of NRF2 in human DMD, attempting to address additional targeted pharmacological approaches.

Results

Analysis of local (muscle) oxidative stress in DMD

In order to correlate the oxidative stress response with the progression of the disease, we subdivided the DMD patients into two groups, namely Class I (0–2 years) ($n = 6$) and Class II (2–9 years) ($n = 5$) children, according to the age at the biopsy. Of note, none of the participants at the time of biopsy collection was under corticosteroid treatment.

NAPDH oxidase 2 (NOX2) has a central role in the pathology of DMD as source of ROS (35–37), thus we examined the expression of the NOX2 catalytic subunit (gp91-phox) on muscle biopsies of 11 patients. As reported in Fig. 1A, the qRT-polymerase chain reaction (PCR) analysis showed a significant increase of NOX2 gene expression in both groups of patients compared with age-matched controls (5.5-fold Class I, 5.7-fold Class II).

Moving from the evidence that NOX-dependent ROS has been shown to activate the NRF2 redox signaling pathway (38), we analyzed the expression of NRF2 and its target genes by qRT-PCR and western blotting. NRF2 gene expression was significantly increased in both age groups of patients, compared with controls (Fig. 1B), although the activation appeared less pronounced in patients of Class II (3.3-fold increase in Class I, 1.9 in Class II). Western blot analysis confirmed the significant increase of NRF2 protein amount in younger patients (66%) compared with age matched controls, whereas in Class II it fell to levels comparable to those of age-matched controls (Fig. 1C). To note, when compared Class II with Class I patients a consistent reduction of NRF2 protein levels was observed (Fig. 1C), suggesting an impairment of the antioxidant compensatory mechanism during the progression of pathology.

To examine if the NRF2 activation up-regulated phase II enzymes genes, we evaluated the expression of NQO1, the enzyme catalyzing the reduction of quinones to hydroquinones, Superoxide dismutase (MnSOD, CuZnSOD) that functions as scavenger of ROS, and HO-1, which is protective against early phases of inflammation. As reported in Fig. 2A, expression of these genes significantly increased in muscle of both classes of patients, especially NQO1 (6.2-fold increase in Class I and 1.7-fold in Class II) and HO-1 (6.9-fold in Class I and 5.5-fold in Class II). Furthermore, SOD1 and SOD2 mRNA significantly increased, but the increments were comparable in both classes compared with age-matched controls (SOD1, 2.7-fold in Class I, 2.3-fold in Class II; SOD2, 2.9 in Class I, 2.1 in Class II).

Western blot analysis confirmed the high protein levels of NQO1 in Class I (85% increase) and the slight increase in patients of Class II (Fig. 2B). Conversely HO-1, whose gene expression consistently increased in both classes, significantly decreased in Class I patients and remained at levels of controls in Class II DMD (Fig. 2C). These results are quite surprising, given the protective role of HO-1 against the immune-mediated tissue injury (39). Therefore, as it has been shown that HO-1 deficiency causes chronic inflammation (40,41), we measured the amount of Inteleukin-6 (IL-6), a key mediator of chronic inflammation (42), in muscle of DMD patients. As showed in Fig. 2D, the IL-6 mRNA expression was significantly increased in both

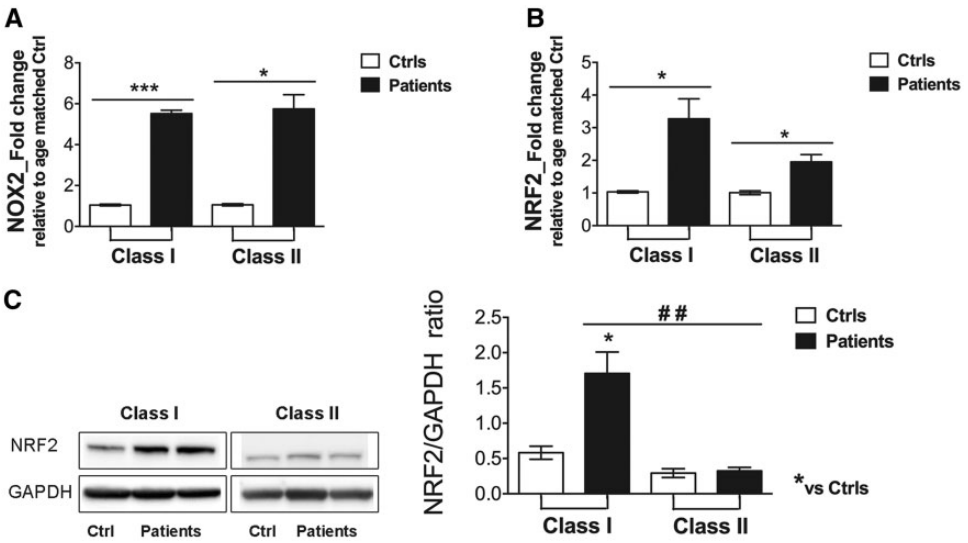

Figure 1. Redox status in muscle of DMD patients. (**A** and **B**) Real time PCR analysis performed on biopsies of DMD patients (n=11) and control subjects (Ctrls) for the expression of NOX2 (A) and NRF2 (B) mRNA. (**C**) Representative western blot (left panel) and densitometry (right panel) for NRF2 protein levels. Values represent mean±SEM. *$P<0.05$, *** $P<0.001$; ## $P<0.01$ by Student's two-tailed t test. Class I and II gels were simultaneously run under same experimental conditions.

groups of age, although more consistent in the children of Class I (6.3-fold in Class I; 2.1-fold in Class II).

These findings are in agreement with previous observations, in both humans and mice, where the up-regulation of inflammatory molecules, in particular IL-6, was reported in DMD and mdx muscles (43,44).

To support the link between oxidative stress and chronic inflammatory response mediated by IL-6, we analyzed relevant markers of oxidative stress in a recent generated animal model, namely mdx/IL6, in which increased levels of IL-6 exacerbate the dystrophic muscle phenotype, sustaining inflammatory response and repeated cycles of muscle degeneration and regeneration, leading to exhaustion of satellite cells (45). Of note, IL-6 overexpression paralleled the human antioxidant expression profile, upregulating several antioxidant genes, such as Nqo1, Ho-1 and Sod1, in mdx mice (Fig. 3A). Moreover, the expression of Nrf2 and Nox2 proteins was significantly increased in mdx/IL6 mice compared with mdx littermates, similarly to the expression pattern observed in human DMD patients (Fig. 3B-C).

Muscle glutathione status in DMD

As NRF2 modulates the antioxidant response mainly inducing the expression of the γ-GCL gene, the rate-limiting enzyme of the *de novo* glutathione synthesis, we evaluated if the activation of NRF2 may have an impact on the muscle glutathione status. As expected, a significant increase of mRNA GCL gene expression in both DMD age groups was obtained (3.9-fold in Class I, 4.2-fold in Class II, Fig. 4A). However, this gene induction was not followed by a corresponding increase of tissue GSH levels, whose local concentration was reduced in both classes, compared with controls, and it was 38% lower in Class I patients with respect to Class II (Fig. 4B).

Similarly to patients, we observed an increase of Gcl mRNA expression (2.1-fold, Fig. 4C) and a decrease of GSH levels (32%, Fig. 4D) in the muscles of 4-week old mdx/IL6 mice, compared with mdx littermates.

Collectively, these findings demonstrate a local response to oxidative stress by the activation of NRF2 redox pathway that, however, does not appear to fully compensate the muscle redox imbalance.

Analysis of systemic oxidative stress in DMD patients

In order to evaluate whether muscle oxidative imbalance was correlated to the systemic oxidative stress, we measured GSH levels in whole blood of DMD patients (Fig. 5). In agreement with the GSH decrement observed in muscle, a significant GSH decrease was found in blood of both Classes (Fig. 5A, 22% decrement in Class I, 21% in Class II). Accordingly, a consistent increase of GSSG was evidenced in both classes (Fig. 5B), leading to a significant imbalance of the GSSG/GSH ratio (Fig. 5C).

GSH is the co-factor of many glutathione-dependent enzymes, thus we also determined the activity of the glutathione peroxidase (GPx) enzyme in blood of patients (Fig. 5D). A significant decrease of GPx activity in both Class I (18%) and Class II (26%) patients was obtained, with respect to age-matched controls. In addition, a marked protein carbonylation was further detected in plasma of both classes of children with DMD (51% increase in Class I, 48.5% 1 in Class II, Fig. 5E), evidencing oxidative protein dysfunction in this disease. Indeed, protein carbonylation, caused by the insertion of carbonyl groups into amino acid residues, irreversibly modifies the protein structure, making proteins not functional and causing cell and tissue dysfunction (46,47). Carbonyls content is a hallmark of oxidative damage and its increase in plasma of our patients is strongly suggestive of systemic oxidative stress. Moreover, despite the well-known role of carbonylated proteins as redox markers, the carbonylation of specific muscle proteins could have an additional significance in DMD, by contributing to muscle dysfunction, and further exacerbating the pathological context.

Discussion

Because of their contractile activity and their high oxygen consumption and metabolic rate, skeletal muscles continuously produce moderate levels of ROS, which are buffered by multiple antioxidant systems to maintain redox homeostasis. NOX is

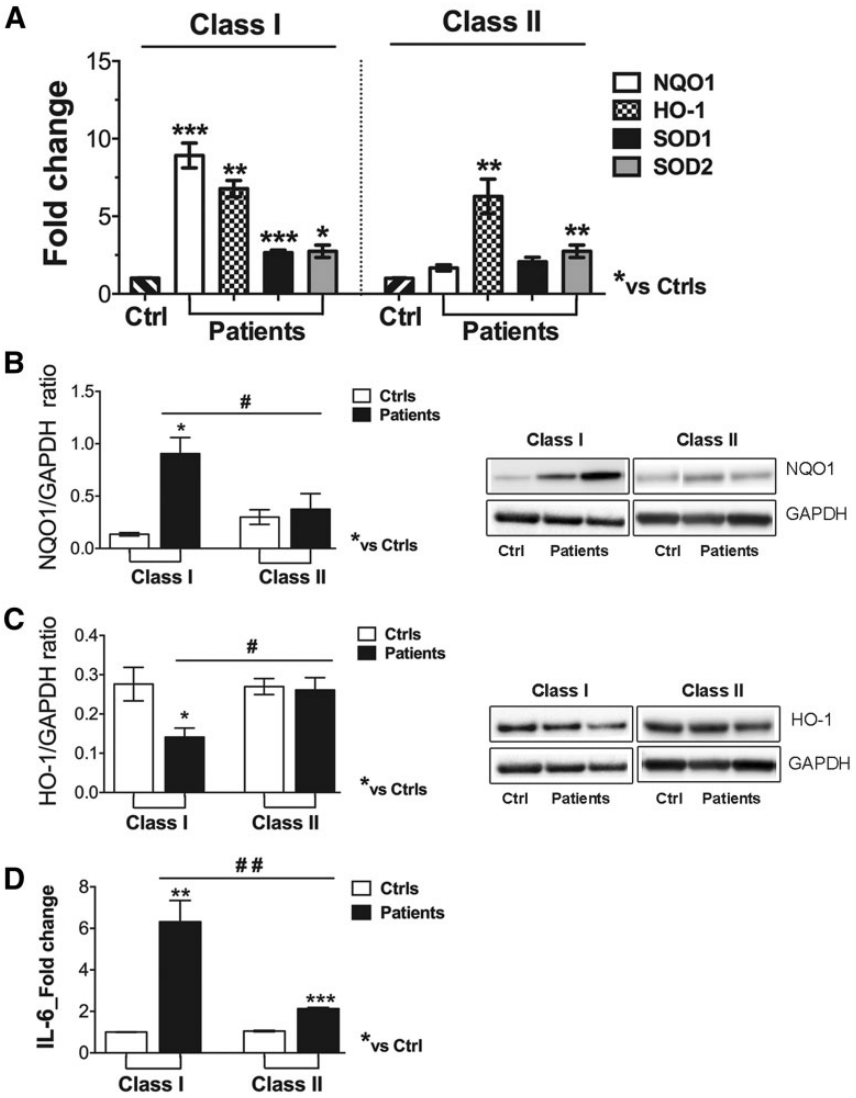

Figure 2. NRF2-dependent antioxidant response in muscle of DMD patients. (**A**) Real time PCR analysis of NRF2-downstream genes (NQO1, HO-1, SOD1 and SOD2) on biopsies of DMD patients (n =11). (**B** and **C**) Representative western blots (right panel) and densitometric analysis (left panel) of NQO1 (B) and HO-1 (C) protein levels. (**D**) Real time PCR analysis for the expression of IL-6 on muscles of DMD patients. Values represent mean±SEM. *P<0.05, **P<0.01, ***P<0.001 respect to controls; #P<0.05, ##P<0.01 compared with Class I. Class I and II gels were simultaneously run under same experimental conditions.

critical for the generation of ROS in muscle, and previous studies have demonstrated a dramatic enhancement of NOX2-dependent ROS production in dystrophin-deficient heart and skeletal muscle (30,32). To note, the pharmacological decrease of NOX2 expression significantly improved muscle function in the mdx mouse model protecting from tissue damage and inflammation (48). In this study, we analyzed NOX gene expression in muscle biopsies of 11 patients with DMD. Furthermore, as an age-related progressive increase of oxidative stress has been shown in dystrophic animals (32,33), we divided our patients into two age classes: the Class I (0–2 years) and Class II (2–9 years). According to the literature on mouse models, we found a progressive increase of NOX gene expression in muscles from both groups of patients, followed by a significant increase of NRF2 gene expression that was more evident in the Class I patients.

Unlike on mouse models, we found a similar increase of NOX gene expression in muscles from both groups of patients, followed by a significant increase of NRF2 gene expression that was more evident in the Class I patients. This is confirmed by NRF2 protein levels showing a consistent increase in the 0–2 years patients, whereas in Class II it fell to levels comparable to those of age-matched controls. Indeed, the increase of NRF2 gene expression, although occurring in all patients, failed to activate some genes encoding phase II detoxifying enzymes in older patients. In particular, NQO1, which is highly expressed in the Class I patients appears to slow down its antioxidant buffering activity in the children of Class II. NQO1 catalyzes the reduction of quinone to hydroquinone, thus preventing the toxic accumulation of quinone products that bind to cellular nucleophiles, including the reduced form of glutathione, causing cellular damage (49,50). Therefore, the decreased NQO1 expression in older patients may lead to accumulation of quinones, GSH depletion and contribute to induce further generation of ROS in DMD muscles.

Conflicting with the increased levels of NQO1, the amount of HO-1 protein appears down-regulated in the Class I patients

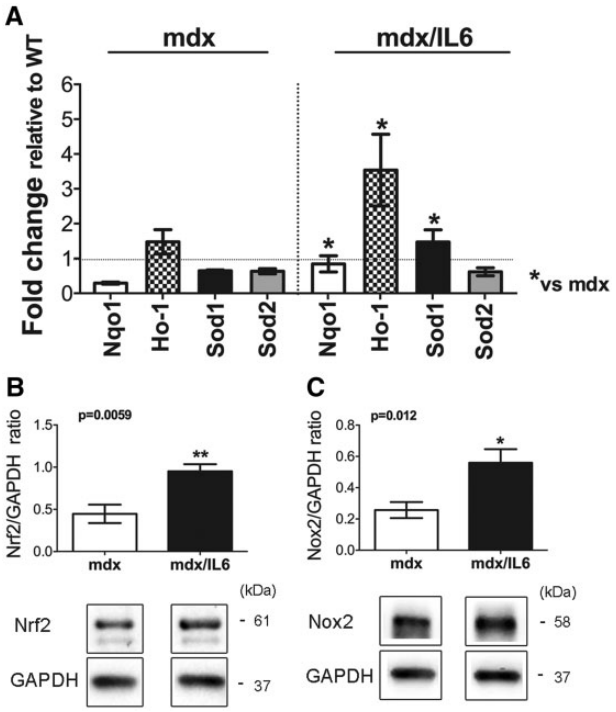

Figure 3. IL-6 overexpression parallels the human antioxidant expression profile in mdx mice. (**A**) Real time PCR analysis performed on diaphragm muscles from wild-type (WT), mdx and mdx/IL6 mice at 4 weeks of age, for the expression of Nqo1, Ho-1, Sod1 and Sod2. Values are reported as fold change in expression relative to calibrator (WT, horizontal dot-line) and represent mean ± SEM; $n = 4$–5 mice per group. *$P < 0.05$ compared with mdx mice (by ANOVA with Bonferroni correction). (**B** and **C**) Western blot analysis (bottom panel shows a representative image) for Nrf2 (B) and Nox2 (C) proteins, performed on diaphragm of 4-week-old mdx and mdx/IL6 mice. Values represent mean ± SEM; $n = 4$–6 per group. *P* value using Student's two-tailed *t* test. In (B and C), the lanes were run on the same gel but were not contiguous.

(46% decrease), with respect to Class II, and this decrease could explain abnormalities in inflammatory responses occurring early in DMD. HO-1, the rate-limiting enzyme of heme degradation, plays a pivotal role in the early phases of immune response and its impairment can determine the fate of downstream events in the dystrophic muscle (40). Its protective role in modulating the immune response has been demonstrated in HO-1 knockout mice, where high levels of pro-inflammatory cytokines [especially IL-1, IL-6 and tumor necrosis factor (TNF-α)] were observed (40). Our findings show a 50% reduction of HO-1 protein in Class I patients, with respect to controls, thus suggesting that the beneficial anti-inflammatory effect of the enzyme does not fully occur in the younger children. This partial HO-1 deficiency is associated with a consistent up-regulation of IL-6 in Class I patients (6-fold increase, while to 2-fold increase in Class II) and can predispose to an excessive inflammatory response or to delay the resolution of the early inflammation.

To support these evidences we made use of a novel dystrophic animal model we recently generated, namely the mdx/IL6 mice, which closely approximates the human disease and more faithfully recapitulates the disease progression in humans (45). Indeed, we revealed both an enhanced oxidative stress and a decrease of the antioxidant-compensatory mechanism during the progression of pathology in mdx/IL6 mice, paralleling human DMD.

This evidence indicates that chronic inflammatory response further exacerbates the dystrophic phenotype impinging the anti-oxidant defense. The existence of a molecular borderline between the 'presymptomatic' phase of the disease and the later stage of DMD suggests that a pharmacological approach, specifically acting in the presymptomatic phase of the pathology, should ameliorate the efficacy of later therapeutic interventions on DMD patients.

A combined impairment of HO-1 in younger children and of NQO1 in older patients may contribute to the early-onset of the inflammation and to the progressive increase of ROS production.

The temporal progression of muscle oxidation has been reported in dysferlin-deficient muscle and in aged mdx mice (32), where an amplified ROS signaling was also found (33,51). Therefore, an efficient and long-term active antioxidant system is critical to guarantee a prompt and stable response against the increasing ROS production in DMD.

The essential contribution of NRF2 in the antioxidant response derives also from its ability to modulate the expression of the rate-limiting enzyme GCL, which is responsible of the *de novo* synthesis of glutathione (26). Glutathione is the major tissue redox buffer and it is a good indicator of the cellular redox environment, as it promptly responds to redox fluctuations (52). Indeed, the cellular redox balance is warranted by equilibrated ratios among reduced (GSH) and oxidized (GSSG) glutathione forms. Under physiological conditions, GSH normally exceeds GSSG, but a slight increase of GSSG level is enough to dramatically alter GSSG/GSH ratio thus triggering oxidative stress (53). Therefore, the GSSG/GSH couple may be considered the most important redox control node in redox homeostasis (54).

In our patients, we observed a significant increase of GCL expression in both age classes, indicating a consistent effort of the muscle tissue in buffering the redox imbalance. However, as the GSH is synthesized, it is promptly converted to GSSG or used as a co-factor for the GPx enzyme, further perturbing the GSSG/GSH ratio. Nevertheless, this antioxidant effort is not enough to counteract oxidative stress in DMD, as demonstrated by the significant increase of plasma carbonyls in our patients. However, while the correlation between protein carbonylation and oxidative stress is widely recognized, more studies are needed to better understand the role of protein carbonylation in the etiology of DMD, also considering that a role for protein carbonyls has been reported in the activation of NRF2 signaling pathway (55).

Recently, NOX-mediated oxidative stress has been shown to reduce autophagy in the mdx mouse (56). Although we do not have a conclusive link between severity of pathology and autophagy impairment we observed a reduction in the microtubule-associated protein light chain (LC3) II/I levels in Class 2 DMD muscles, leading to tentatively hypothesize an impairment of autophagy in the disease (data not shown). Additional work will clarify the potential involvement of autophagy in DMD pathogenesis.

Overall, our study confirms a central role for NRF2 in the pathogenesis of DMD and, at the same time, shows a weakening of the antioxidant response along the disease progression.

Our work is consistent with a model (Fig. 6) in which the absence of dystrophin expression activates an anti-oxidant pathway to cope with the toxic effects induced by oxidative stress. Nevertheless, the decrease of HO-1 protein levels, concomitantly with an increase of IL-6 expression, impinge upon the activation of a compensatory mechanism and promote the establishment of a chronic inflammation, leading to exacerbation of the dystrophic phenotype.

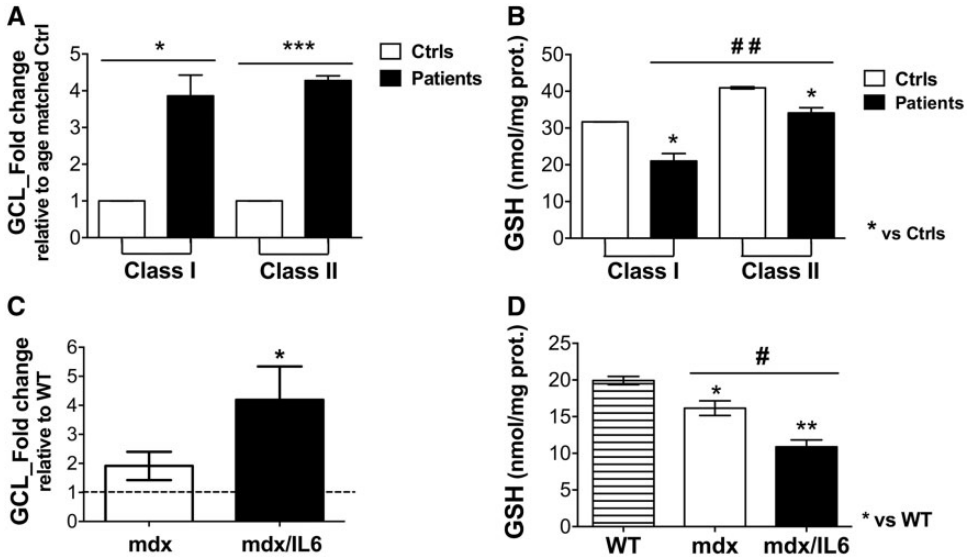

Figure 4. Muscle glutathione status in DMD. (A) The expression of GCL gene, the rate-limiting enzyme of the *de novo* glutathione synthesis, was analyzed by Real time PCR in DMD patients ($n = 11$). (B) The GSH muscle concentration in DMD patients ($n = 11$) was measured by the enzymatic re-cycling assay. Values represent mean ± SEM; $^{*}P < 0.05$, $^{***}P < 0.001$ respect to controls (Ctrls); $^{\#\#}P < 0.01$ compared with Class I (by Student's two-tailed *t* test). (C) Real time PCR analysis for the expression of GCL in diaphragm of 4-week-old WT, mdx, and mdx/IL6 mice. Values are expressed as the fold change relative to calibrator (WT, horizontal dot-line) and represent mean ± SEM; $n = 4$–12 mice per group. $^{*}P < 0.05$ compared with mdx (by ANOVA with Bonferroni correction). (D) Measurements of GSH in gastrocnemius of 4-week-old WT, mdx and mdx/IL6 mice. Values represent mean ± SEM; $n = 3$ per group. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{\#}P < 0.05$ by Student's two-tailed *t* test.

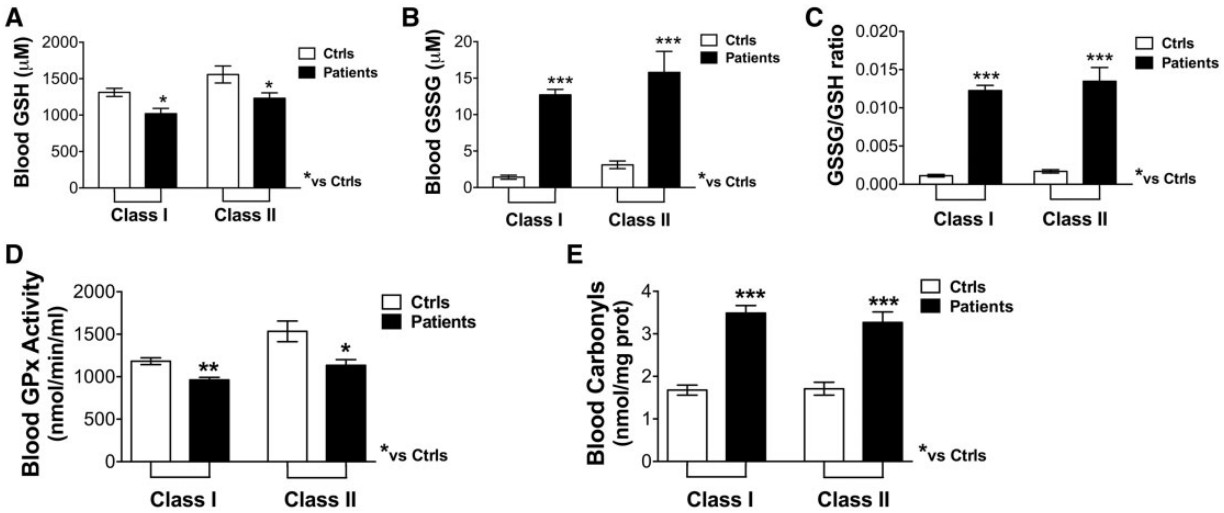

Figure 5. Systemic oxidative stress in DMD patients. GSH (A), GSSG (B), GSSG/GSH ratio (C), GPx activity (D) and protein carbolyls content (E) were measured in whole blood of patients, as described in Materials and Methods. Values represent mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.001$, respect to controls (by Student's two-tailed *t* test).

The encouraging element deriving from this and other studies is that oxidative stress is a drug-targetable condition, thus paving the way for the implementation of pharmacological trials using NRF2 inducers and redox drugs already clinically approved for other diseases (57,58).

Materials and Methods

Patients

This study was performed on 11 genetically confirmed DMD patients (mean age 6 years, age range 0–9 years) followed at the Unit of Neuromuscular Diseases at the Bambino Gesù Children's Hospital. In order to correlate the variability in the antioxidant response with the progression of the disease, our cohort of patients was subdivided into two groups, named Class I (0–2 years) ($n = 6$) and Class II (2–9 years) ($n = 5$) children, according to the age at the biopsy. Muscle biopsy samples were collected at the time of diagnosis. Control biopsies ($n = 3$ aged-matched for each group of patients) were from individuals in whom a neurological diseases was suspected, but did not show any sign of muscle pathology on histology and biochemical examinations. None of the participants at the time of biopsy or blood collection was under corticosteroid treatment. The local ethics committee approved the study and informed consent was obtained from all subjects.

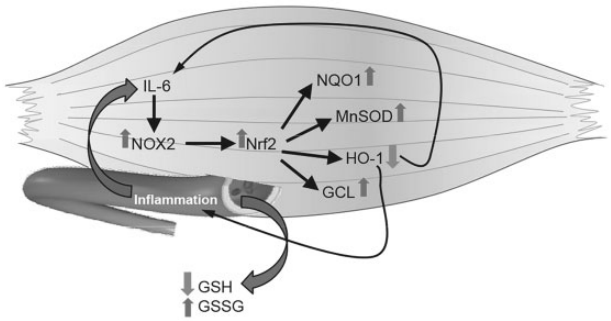

Figure 6. Scheme representing the relationship between the Nrf2-signaling pathway and inflammation in DMD. The dystrophin deficiency induces Nrf2 activation to counteract NOX2-triggered oxidative stress. Nevertheless, low levels of HO-1 together with high IL-6 concentrations weaken the efficacy of the antioxidant compensatory mechanism and promote the establishment of a chronic inflammation, which ultimately exacerbates the dystrophic phenotype.

Mice

Wild-type C57Bl/6J mice, mdx (Jackson Laboratories) and mdx/IL6 (45) mice of 4 weeks of age used in the current study were maintained according to the institutional guidelines of the animal facility of the unit of Histology and Medical Embryology. All animal experiments were approved by the ethics committee of Sapienza University of Rome-Unit of Histology and Medical Embryology and were performed in accordance with the current version of the Italian Law on the Protection of Animals.

Blood sample collection

Blood samples from 11 patients, obtained early after diagnosis during routine analysis performed before the beginning of steroid treatment, were collected into 5% ethylenediaminetetraacetic acid (EDTA) Vacutainer Tubes (Becton Dickinson, Rutherford, NY, USA) to evaluate GSH and antioxidant enzyme (GPx), or in Vacutainer Tubes prefilled with both EDTA and N-ethylmaleimide (NEM, 30 mM final concentration, Sigma-Aldrich, St. Louis, MO, USA) for GSSG determinations. Whole blood was centrifuged at 450*g* for 3 min, to separate plasma from erythrocytes. For carbonyl content analysis, plasma was rapidly removed by aspiration and stored at −80 °C until analysis.

Quantitative real-time PCR

Total mRNA from muscle biopsy of patients and three age-matched healthy controls was isolated using TRI Reagent (Sigma-Aldrich), according to the manufacturer's protocol. One microgram of each RNA samples was reverse transcribed with the SuperScript™ First-Strand Synthesis system and random hexamers as primers (Life Technologies, Carlsbad, CA, USA). The expression levels of NRF2, GCL, HO-1, SOD1 and 2, NQO1, IL-6 were measured by quantitative Real-Time PCR (qRT-PCR) in an ABI PRISM 7500 Sequence Detection System (Life Technologies, Carlsbad, CA, USA) using Power SYBR Green I dye chemistry. Data on the expression levels of NOX2 were obtained using a TaqMan™ Universal PCR Master Mix and the appropriate Assay-on-Demand™ Gene Expression Mix (Hs00166163 m1) (Life Technologies). Data were analyzed using the 2-DDCt method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as housekeeping gene and data are shown as fold change relative to control. Notably, the use of alternative housekeeping genes, such as TATA box binding protein (TBP) and glucuronidase beta genes did not modified the data obtained with GAPDH gene. Total mRNA from diaphragm muscle of wild-type, mdx and mdx/IL6 mice was extracted in TRI Reagent (Sigma-Aldrich) using Tyssue Lyser (Quiagen). One microgram of each RNA sample was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen). Quantitative Real-Time PCR was performed on an ABI PRISM 7500 SDS (Applied Biosystem) using specific premade TaqMan assays for Ho-1, Nqo1, Sod1, Sod2, gcl and hypoxanthine guanine phosphoribosyl transferase as housekeeping gene (Applied Biosystem). Data were analyzed using the 2-DDCt method and reported as mean fold change in gene expression relative to wild-type.

Western blot analysis

Muscle samples were homogenized and lysed on ice with RIPA buffer, including dithiothreitol (DTT) and protease inhibitors. For western blotting analysis, 40 μg of proteins were subjected to SDS PAGE on 4–12% denaturing gel and probed with the following antibodies: NRF2 (1:500, Abcam, Cambridge, UK), NQO1 (1:500, Novus Biologicals, Minneapolis, USA), HO-1 (1:2000, Abcam, Cambridge, UK) and GAPDH (1:15000, Sigma-Aldrich) as loading control. Immunoreactive bands were detected using the Lite Ablot Extend Long Lasting Chemiluminescent substrate (Euroclone, MI, Italy). Densitometric measurements were performed using ImageLab Software (BioRad, Hercules, CA, USA). Diaphragm muscles from 4-week-old mdx and mdx/IL6 mice were homogenized in lysis buffer [Tris–HCl, pH 7.5/20 mM, EDTA/2 mM, ethylene glycol tetraacetic acid/2 mM, sucrose/250 mM, DTT/5 mM, Triton-X/0.1%, phenylmethylsulfonyl fluoride (PMSF)/1 mM, sodium fluoride (NaF)/10 mM, sodium orthovanadate (SOV4)/0.2 mM, cocktail protease inhibitors/1× (Sigma-Aldrich)]. Western blotting analysis was performed using 70 μg of protein extracts and filters were blotted with antibodies against gp91phox (Nox2; 1:300, BD Transduction Laboratories), Nrf2 (1:1000, Santa Cruz Biotechnology) and GAPDH (1:1000, Santa Cruz Biotechnology). Signals derived from appropriate HRP-conjugated secondary antibodies (Bethyl Laboratories, Montgomery, TX, USA) were captured by Chemi DocTM XRS 2015 (Bio-Rad Laboratories, Hercules, CA, USA) and densitometric analysis was performed using Image Lab software (Version 5.2.1; © Bio-Rad Laboratories).

GSH and GSSG analyses

GSH and GSSG levels were detected in muscle and whole blood using the enzymatic re-cycling assay (59), with minor modifications. Briefly, samples were de-proteinized with 5% (w/v) sulphosalicylic acid (SSA, Sigma-Aldrich) and the glutathione content was determined after dilution of the acid-soluble fraction in Na-phosphate buffer containing EDTA (pH 7.5). To prevent an overestimation of GSSG due to oxidation of thiols during sample manipulation, blood samples were collected in tubes prefilled with 30 mM NEM. GSH and GSSG concentrations were measured with ThioStar® glutathione detection reagent (Arbor Assays, Michigan, USA), using respectively GSH and GSSG as standards (Sigma Chemicals, St. Louis, MO, USA). The fluorescence was measured using an EnSpire® Multimode Plate Reader (Perkin Elmer, Waltham, MA, USA).

GPx assay

GPx activity was measured in whole blood samples by spectrophotometric assay using the Glutathione Peroxidase Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA), according to the manufacturer's instructions. GPx activities were determined following NADPH oxidation at 340 nm and using cumene hydroperoxide as substrate.

Carbonylated protein

Plasma protein carbonyls were assayed according to the protocol provided by the manufacturer (Cayman Chemicals, MI, USA). Protein concentrations (mg/ml) were measured using Pierce BCA (Thermo Scientific). Carbonyl concentrations were expressed as nmol/mg protein.

Data analysis and statistics

Statistical analysis was performed using the GRAPHPAD/Prism 5.0 Software (SanDiego, CA, USA). Statistically significant differences between groups were analyzed using Student's *t*-test for normally distributed variables. All data are presented as mean ± standard deviation or as mean ± standard error of the mean (SEM). Statistical significance was defined as $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared with healthy controls and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ between classes. Statistical differences among animal groups were assessed using one-way analysis of variance (ANOVA) with a Bonferroni post-test or Student's *t*-test.

Acknowledgements

We thank F. De Benedetti for IL6 mice and scientific support.

Conflict of Interest statement. None declared.

Funding

This work was supported by Ricerca Corrente of the Italian Ministry of Health, Fondazione Roma, Telethon, and ASI.

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