Stra13 regulates oxidative stress mediated skeletal muscle degeneration

Cécile Vercherat¹, Teng-Kai Chung¹,³, Safak Yalcin², Neriman Gulbagci¹, Suma Gopinadhan³, Saghi Ghaffari¹,² and Reshma Taneja¹,³,⁴,*

¹Department of Developmental and Regenerative Biology and ²Department of Gene and Cell Medicine, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY, USA, ³Department of Physiology and ⁴NUS Graduate School of Integrative Sciences and Engineering, National University of Singapore, Singapore, Singapore, 117597

Received June 22, 2009; Revised August 3, 2009; Accepted August 10, 2009

Duchenne Muscular Dystrophy (DMD), caused by loss of dystrophin is characterized by progressive muscle cell necrosis. However, the mechanisms leading to muscle degeneration in DMD are poorly understood. Here, we demonstrate that Stra13 protects muscle cells from oxidative damage, and its absence leads to muscle necrosis in response to injury in Stra13-deficient mice. Interestingly, Stra13−/− mutants express elevated levels of TNFα, reduced levels of heme-oxygenase-1, and display apparent signs of oxidative stress prior to muscle death. Moreover, Stra13−/− muscle cells exhibit an increased sensitivity to pro-oxidants, and conversely, Stra13 overexpression provides resistance to oxidative damage. Consistently, treatment with antioxidant N-acetylcysteine ameliorates muscle necrosis in Stra13−/− mice. We also demonstrate that Stra13 expression is elevated in muscles from dystrophin-deficient (mdx) mice, and mdx/Stra13−/− double mutants exhibit an early onset of muscle degeneration. Our studies underscore the importance of oxidative stress-mediated muscle degeneration in muscular dystrophy, and reveal the contribution of Stra13 in maintenance of muscle integrity.

INTRODUCTION

Muscular dystrophies are a group of heterogeneous genetic disorders that lead to degeneration and wasting of skeletal muscles (1). Duchenne Muscular Dystrophy (DMD), caused by mutations in the dystrophin gene (2), is the most prevalent muscular dystrophy. DMD is characterized by progressive skeletal muscle degeneration and the subsequent replacement of muscles by fibrotic and fat tissue. Patients typically are wheelchair bound in their early teens, and die in the early twenties due to respiratory and cardiac failure. The mdx mouse lacking dystrophin (3) have been widely studied as a mouse model for DMD and for exploring therapeutic strategies. However, muscle dystrophy in mdx mice is quite mild compared with DMD patients. Muscle degeneration and necrosis in mdx mice occurs between 3 and 8 weeks of age, and subsides in limb muscles thereafter, with the exception of the diaphragm that displays progressive degenerative changes and fibrosis (4).

Dystrophin, a large cytoskeletal protein, associates with many membrane glycoproteins including dystroglycans, sarcoglycans and syntrophins to form the dystrophin–glycoprotein (DGC) complex that connects the cytoskeleton to the extracellular matrix (ECM). The absence of dystrophin disrupts the interactions of the DGC with the ECM and the cytoskeleton, and renders the sarcolemma susceptible to damage (5,6). Although the degeneration of muscles is apparent in both DMD patients and in mdx mice, the mechanisms by which loss of dystrophin leads to muscle degeneration are still unclear. Several hypotheses have been proposed which include an increased sensitivity of dystrophin-deficient muscles to contraction that leads to muscle damage and degeneration; an excessive calcium influx due to the loss of sarcolemmal integrity that results in the activation of proteases (calpains) leading to muscle death; free radical damage leading to muscle necrosis; vascular defects; and neural innervation defects (7–11). Many correlative studies have associated oxidative stress with muscle degeneration in DMD (12,13). These include observations that the muscle pathology seen in conditions of vitamin E deficiency, as well as in ischemia, which are attributed to an increase in free radical mediated injury, are similar to DMD (14,15). Moreover, lipid and protein oxidation that are hallmarks of oxidative

*To whom correspondence should be addressed. Tel: +1 6565163236; Fax: +1 65 67788161; Email:phsrt@nus.edu.sg

© The Author 2009. Published by Oxford University Press. All rights reserved.
For Permissions, please email: journals.permissions@oxfordjournals.org
injury are evident in dystrophic muscles (10). Consistent with a role for oxidative stress in muscle degeneration, mdx muscles were reported to express increased levels of the antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutases prior to their degeneration, indicative of a cellular response to oxidative injury (16). In addition, dystrophin-deficient muscles exhibited increased susceptibility to oxidative damage (17). However, despite such evidence, the role of oxidative damage as a key pathogenetic event in the degeneration of dystrophic muscles in DMD has not been clarified. In addition, the mechanisms by which oxidative stress is regulated in dystrophic muscles are largely unclear.

Stra13, a basic helix-loop-helix transcription factor, is expressed in many tissues and cell types (18). Several studies using gain of function and loss of function strategies have revealed its roles in cell cycle arrest (19,20), differentiation of a number of cell types (21,22) and in apoptosis (23–27). We have previously demonstrated that Stra13 is expressed in embryonic and adult skeletal muscles (21,28). Nevertheless skeletal muscle development occurs normally in Stra13−/− mice. Interestingly however, the ability of Stra13−/− mutants to regenerate in response to acute injury is compromised, and the mutant mice exhibit a dystrophy-like phenotype characterized by reduced membrane integrity, myotube necrosis and fibrosis. In this study, we have investigated the role of Stra13 in muscular dystrophy, and the mechanisms underlying myotube degeneration in Stra13−/− mutants. We demonstrate Stra13 deficiency leads to elevated TNFα expression, reduced heme-oxygenase-1 (HO-1) levels, and increased oxidative damage prior to the onset of muscle degeneration, likely due to production reactive oxygen species (ROS). Consequently, treatment of Stra13−/− mice with the anti-oxidant N-acetylcysteine (NAC) prevents muscle necrosis in response to injury. Consistent with these observations, Stra13 overexpression is sufficient to protect muscle cells from free radical mediated death, and up-regulates the expression of HO-1 that is cytoprotective against oxidative injury. We show that Stra13 expression is upregulated in mdx muscles, and mdx/Stra13−/− double mutants exhibit an early onset of muscle degeneration and progressive dystrophinopathy. Together, our studies provide evidence that Stra13 is essential for protecting muscle cells from oxidative damage mediated death, and underscores its role in muscular dystrophy.

RESULTS

Stra13 expression is elevated in dystrophic muscles

We have previously demonstrated that Stra13−/− mutants do not exhibit any alterations in skeletal muscle development, but their regenerative capacity is altered, and the mutant phenotype is characterized by late stage muscle necrosis and fibrosis (Fig. 1; 28). Given this distinct phenotype of Stra13−/− mutants that is reminiscent of mdx muscles where myotubes are formed but subsequently degenerate, we determined whether there were any changes in the membrane integrity of the mutant muscle. Wild-type (WT) and Stra13−/− muscles were subjected to freeze injury, and 9 days later, injected with Evans blue dye (EBD) that penetrates damaged and necrotic fibers, and is therefore used to visualize membrane damage (29). In contrast to WT mice, necrosis was evident in the mutant regenerating tissue (Fig. 1A; 28), along with clusters of EBD+ fibers (Fig. 1B), indicating reduced sarcolemmal integrity in the mutant muscle. Moreover, alizarin red staining, which identifies fibers with high intracellular calcium in dystrophic muscles (30), revealed increased calcium deposits in the mutant muscles compared with WT mice (Fig. 1C). Given the dystrophy-like phenotype of Stra13−/− mutants, we then examined whether Stra13 is involved in muscular dystrophy. To address this, we first examined various muscles from littermate WT and mdx mutants for Stra13 expression by quantitative real time RT–PCR (Q-PCR). Interestingly, Stra13 expression was significantly upregulated (P < 0.05) in several mdx muscles including quadriceps, tibialis anterior, gastrocnemius, soleus and diaphragm (Fig. 1D) at postnatal day 20 (P20). Stra13 expression was also elevated in mdx muscles analyzed from 6- to 9-month-old mice (data not shown). The increase in Stra13 expression in mdx muscles prior to the onset of necrosis suggested that the upregulation is not a consequence of muscle degeneration, but rather may play a compensatory role for the loss of dystrophin in dystrophic muscles.

mdx/Stra13−/− mutants display early degeneration of myofibers

To investigate the mechanisms leading to muscle degeneration in Stra13−/− mutants and examine its potential role in muscular dystrophy, we crossed Stra13−/− mice with mdx mutant mice. Double mutants lacking both dystrophin and Stra13 (mdx/Stra13−/−) were viable, and did not show any overt phenotypes at birth compared with littermate mdx mutants. Previous studies have shown that the onset of muscle necrosis occurs ~3 weeks after birth in mdx mice, prior to which the mutants do not show any histological changes in skeletal muscles. In contrast to WT mice and mdx mutants, the diaphragms of mdx/Stra13−/− mutants showed an early onset of muscle degeneration (Fig. 2A) as seen by EBD uptake at postnatal day 15 (P15). Quantification of the percentage of EBD+ fibers indicated that at P15, 7% myofibers were EBD+ in the double mutants, compared with 2% in mdx mice (P < 0.05). At P20 (Fig. 2B), 19% of EBD+ myofibers were present in mdx/Stra13−/− diaphragms versus 4% in mdx (P < 0.05). Small clusters of necrotic fibers in quadriceps muscles were evident histologically in mdx/Stra13−/− mutants at P20, at which point no necrosis was evident in mdx mutants (Fig. 2C). To confirm that these muscles were indeed damaged, we stained them with the membrane impermeable marker IgG (31). Positive intracellular fiber staining for IgG was seen in the necrotic fibers in mdx/Stra13−/− mutants (Fig. 2D), confirming the loss of membrane integrity. Moreover, immunostaining with anti-Mac3 antibody revealed the presence of activated macrophages surrounding the necrotic fibers, further confirming muscle degeneration in mdx/Stra13−/− mice (Fig. 2E).

mdx/Stra13−/− mutants develop increased dystrophic pathology upon aging

Histological analysis of littermate mdx and mdx/Stra13−/− mutants that were 6-month-old revealed increased dystrophic pathology. The double mutants expressed increased levels of calpain 1 and 3, which are markers of myofiber degeneration (Fig. 3A). Moreover, tibialis anterior muscles were subjected to freeze injury, and 9 days later, injected with Evans blue dye (EBD) that penetrates damaged and necrotic fibers, and is therefore used to visualize membrane damage (29). In contrast to WT mice, necrosis was evident in the mutant regenerating tissue (Fig. 1A; 28), along with clusters of EBD+ fibers (Fig. 1B), indicating reduced sarcolemmal integrity in the mutant muscle. Moreover, alizarin red staining, which identifies fibers with high intracellular calcium in dystrophic muscles (30), revealed increased calcium deposits in the mutant muscles compared with WT mice (Fig. 1C). Given the dystrophy-like phenotype of Stra13−/− mutants, we then examined whether Stra13 is involved in muscular dystrophy. To address this, we first examined various muscles from littermate WT and mdx mutants for Stra13 expression by quantitative real time RT–PCR (Q-PCR). Interestingly, Stra13 expression was significantly upregulated (P < 0.05) in several mdx muscles including quadriceps, tibialis anterior, gastrocnemius, soleus and diaphragm (Fig. 1D) at postnatal day 20 (P20). Stra13 expression was also elevated in mdx muscles analyzed from 6- to 9-month-old mice (data not shown). The increase in Stra13 expression in mdx muscles prior to the onset of necrosis suggested that the upregulation is not a consequence of muscle degeneration, but rather may play a compensatory role for the loss of dystrophin in dystrophic muscles.

mdx/Stra13−/− mutants display early degeneration of myofibers

To investigate the mechanisms leading to muscle degeneration in Stra13−/− mutants and examine its potential role in muscular dystrophy, we crossed Stra13−/− mice with mdx mutant mice. Double mutants lacking both dystrophin and Stra13 (mdx/Stra13−/−) were viable, and did not show any overt phenotypes at birth compared with littermate mdx mutants. Previous studies have shown that the onset of muscle necrosis occurs ~3 weeks after birth in mdx mice, prior to which the mutants do not show any histological changes in skeletal muscles. In contrast to WT mice and mdx mutants, the diaphragms of mdx/Stra13−/− mutants showed an early onset of muscle degeneration (Fig. 2A) as seen by EBD uptake at postnatal day 15 (P15). Quantification of the percentage of EBD+ fibers indicated that at P15, 7% myofibers were EBD+ in the double mutants, compared with 2% in mdx mice (P < 0.05). At P20 (Fig. 2B), 19% of EBD+ myofibers were present in mdx/Stra13−/− diaphragms versus 4% in mdx (P < 0.05). Small clusters of necrotic fibers in quadriceps muscles were evident histologically in mdx/Stra13−/− mutants at P20, at which point no necrosis was evident in mdx mutants (Fig. 2C). To confirm that these muscles were indeed damaged, we stained them with the membrane impermeable marker IgG (31). Positive intracellular fiber staining for IgG was seen in the necrotic fibers in mdx/Stra13−/− mutants (Fig. 2D), confirming the loss of membrane integrity. Moreover, immunostaining with anti-Mac3 antibody revealed the presence of activated macrophages surrounding the necrotic fibers, further confirming muscle degeneration in mdx/Stra13−/− mice (Fig. 2E).
Figure 1. Stra13−/− mutants exhibit dystrophic pathology in response to injury and Stra13 expression is elevated in mdx muscles. (A–C) Cross sections of quadriceps muscles from WT and Stra13−/− mice (n = 4) were analyzed by H&E staining (A); EBD uptake (B), and alizarin red staining (C). The mutant regenerating tissue showed increased degenerating fibers and calcium deposits which occur due to tissue necrosis. Scale bar: 100 μm. (D) Stra13 mRNA expression was analyzed by Q-PCR in various muscles including quadriceps (QUA), tibialis anterior (TA), gastrocnemius (GAS), soleus (SOL) and diaphragm (DIA) from littermate WT and mdx mice at P20. GAPDH transcripts were used as an internal control. Stra13 expression is increased significantly (*P < 0.05) in mdx muscles. Error bars indicate mean ± SD.
pathology in the double mutants (Fig. 3A). Various muscles including quadriceps, biceps, tibialis anterior, gastrocnemius and diaphragm in mdx/Stra13−/− mutants revealed signs of increased myopathy characterized by variation in fiber size, presence of regenerating and degenerating muscle fibers, increased connective tissue, and calcium deposits. Not all muscles were affected to the same extent in the double mutants, indicating heterogeneity in the pathology.
To examine whether the phenotype of *mdx/Stra13−/−* double mutants share the same phenotypic characteristics as *Stra13−/−* muscle during injury, we examined fibrosis, calcification and membrane integrity. Similar to *Stra13−/−* mutant regenerating tissue (Fig. 1A–C; 28), a significant increase (*P < 0.05) in collagen deposition was detectable by Masson’s Trichrome staining in quadriceps and diaphragm of *mdx/Stra13−/−* double mutants compared with control *mdx* mice (Fig. 3B and C). Moreover, *mdx/Stra13−/−* double mutants exhibited reduced membrane integrity as seen by increased intracellular IgG staining (Fig. 3D), and increased dystrophic calcification (Fig. 3E). Together, these data indicated that

**Figure 3.** Increased dystrophic pathology in *mdx/Stra13−/−* mutants. (A) Cross sections from quadriceps (QUA), biceps (BIC), tibialis anterior (TA), gastrocnemius (GAS) and diaphragm (DIA) of 6-month-old *mdx* and *mdx/Stra13−/−* animals were stained with H&E. Arrows indicate fibrosis in quadriceps, necrosis in biceps, regenerating myofibers with central nuclei in tibialis anterior, and calcium deposits in the diaphragm. (B) Cross sections from quadriceps and diaphragm of 6-month-old *mdx* and *mdx/Stra13−/−* animals were stained with Masson’s Trichrome, which revealed increased collagen deposition (blue staining) in *mdx/Stra13−/−* mutants. (C) To estimate the extent of fibrosis, the fibrotic area was measured relative to the total area using ImageJ in two randomly chosen fields on H&E stained sections from *mdx* and *mdx/Stra13−/−* mutants (*n = 3*). A significant increase (*P < 0.05) in the percentage of fibrosis was seen in the double mutants. (D) Quadriceps sections from *mdx* and *mdx/Stra13−/−* mutants were stained using anti-IgG antibody. Decreased sarcolemmal stability is evident in the double mutants as evidenced by increased intracellular IgG staining. (E) Alizarin red staining of cross sections from gastrocnemius muscles from 6-month-old animals revealed increased calcium deposits in *mdx/Stra13−/−* muscle. Scale bar: 100 μm.
similar degenerative changes occur in Stra13−/− mutants during injury-induced regeneration, as well as in mdx/Stra13−/− double mutants.

Regeneration is not altered in mdx/Stra13−/− mice
Regeneration of skeletal muscle relies on satellite cells. We have previously demonstrated that there is no alteration in the number of Pax7+ satellite cells in Stra13−/− mutants (28). To investigate whether mdx/Stra13−/− mutants exhibit changes in satellite cell numbers, quadriceps muscle sections from P20 animals were stained with anti-Pax7 antibody. No significant differences in the percentage of Pax7+ cells were seen between mdx and mdx/Stra13−/− mutants (Fig. 4A and B). Moreover, the regeneration index, calculated as the percentage of myofibers with central nuclei, was also unchanged in 6-month-old mdx/Stra13−/− mutants (Fig. 4C). Together, these results demonstrate that mdx/Stra13−/− muscles show no changes in the number of Pax7+ satellite cells and in the regeneration index, suggesting that increased degeneration, rather than defective regeneration, might underlie the increased pathology of mdx/Stra13−/− mutants.

Expression of structural proteins and fiber type composition is unchanged in mdx/Stra13−/− mutants
Several possibilities may account for the degeneration of muscle fibers in Stra13−/− mutants during regeneration, as well as the early onset of degeneration in mdx/Stra13−/− mutants. Stra13, like dystrophin, could be required for structural integrity of muscle, and its absence may lead to decreased sarcolemmal integrity and degeneration. Alternatively, since fast fibers are preferentially affected in DMD (32), a change in fiber type specification could underlie the increased muscle degeneration in Stra13−/− mutants. Lastly, Stra13 may be required for muscle survival.
Dystrophin deficiency affects the expression levels and/or localization of other components of the DGC complex, which can also cause muscular dystrophy. Typically, reduced levels and/or changes in distribution of sarcoglycans, dystroglycans, sarcospan, nNOS, syntrophin and dystrobrevin are seen in DMD (33). In contrast, utrophin, which shows functional similarity with dystrophin, is upregulated in mdx muscles, and compensates for the loss of dystrophin. Immunofluorescence analysis of dystrophin, α-dystroglycan and β-dystroglycan revealed similar expression and localization in mdx and mdx/Stra13−/− quadriceps muscles at P20 (Supplementary Material, Fig. S1A). Consistent with previous reports, the levels of utrophin were upregulated in mdx compared with WT mice. This over-expression was also seen in the mdx/Stra13−/− double mutants (Supplementary Material, Fig. S1A). Integrin α7β1, the principle receptor for laminin α2 in skeletal muscle represents an indispensable linkage between the muscle fiber and ECM that is independent of the DGC (34). No changes in α7 integrin or α2 laminin expression were apparent in mdx/Stra13−/− mutants, indicating that a reduction in α2 laminin or α7 integrin expression does not underline the early degeneration of muscles in mdx/Stra13−/− mutants. To investigate whether modifications in fiber type composition could underlie the degeneration of myofibers, frozen sections of quadriceps muscles from mdx and mdx/Stra13−/− mutants were stained with antibodies against slow and fast myosin isoforms and analyzed by immunofluorescence microscopy (Supplementary Material, Fig. S1B). A similar percentage of slow and fast fibers were present in mdx and mdx/Stra13−/− muscles, indicating that a switch in fiber type was not the underlying basis for muscle degeneration in the double mutants.

Altered oxidative stress and cytokine production lead to necrosis in Stra13−/− muscle

Oxidative stress has been implicated in the degeneration of dystrophic muscles (12,13). To examine whether increased oxidative stress underlies muscle necrosis, we analyzed Stra13−/− regenerating tissue, as well as mdx/Stra13−/− muscles, for hallmarks of oxidative damage. ROS cause the oxidation of amino acid residues on proteins, forming protein carbonyls. To directly examine oxidative damage, protein extracts from WT and Stra13−/− regenerating tissue at various time points after injury [6 h, day 2 (d2), and d10] were treated with 2,4-dinitrophenylhydrazine (DNPH) to derivatize carbonyl groups of proteins formed through protein oxidation, and immunoblotted with anti-DNP antibody. In contrast to WT regenerating tissue, Stra13−/− muscle exhibited higher levels of protein carboxylation several days (d2 and d10) after injury (Fig. 5A). Similarly, mdx/Stra13−/− muscles also exhibited increased protein oxidation compared with mdx muscles at P20 (Fig. 5B). Consistent with increased oxidative damage, the expression of the anti-oxidant enzymes peroxiredoxins 1, 2 and 3 were increased in Stra13−/− regenerating tissue at d5 and d10 after injury (Fig. 5C–E). The elevated expression of peroxiredoxins at d5 after injury indicates that a cellular response to oxidative damage occurs prior to the onset of necrosis, as myogenin+ and eMHC+ myotubes are present in mutant regenerating tissue at d5 after injury (Supplementary Material, Figs. S2 and 28). We then examined whether Stra13−/− muscle is indeed susceptible to undergoing necrosis due to increased oxidative stress. For this, we tested the response of WT and Stra13−/− primary myoblasts to exogenously added hydrogen peroxide (H2O2) (Fig. 5F). Interestingly, Stra13−/− cells exhibited significantly increased sensitivity to H2O2, confirming that Stra13 deficiency results in an increased response to oxidative stress mediated death. The pro-inflammatory cytokine TNFα that is expressed in regenerating muscle has been associated with muscle wasting and can lead to muscle necrosis through ROS-dependent pathways (35–37). Moreover, peroxiredoxins are associated with TNFα-mediated death (38). Because of the apparent signs of oxidative damage, increased peroxiredoxin expression, and overt muscle degeneration in Stra13−/− mutants, we analyzed TNFα expression in the freeze injury model of regeneration, as well as in mdx/Stra13−/− double mutants. Consistent with previous reports (39), TNFα levels were elevated in the early phases of the regenerative response in WT muscles, and then declined by d10 after injury. In Stra13−/− mutants, TNFα was expressed at much higher levels at 6 h and d2 after injury, and subsequently its levels declined to levels comparable with WT tissue (Fig. 5G). Similarly, TNFα was also expressed at higher levels in the mdx/Stra13−/− muscles compared with mdx mice at P15 and P20 (Fig. 5H) indicating that similar alterations in gene expression occur in the freeze injury model as well as in mdx/Stra13−/− mutants. TNFα is expressed by a number of cell types including macrophages that infiltrate the site of injury, and by skeletal muscle. To examine whether the increased TNFα expression in the mutant tissue is derived from skeletal muscle itself, we examined its expression in undifferentiated and differentiating WT and Stra13−/− primary myoblasts. Interestingly, TNFα levels were elevated at early phases during differentiation in the mutant cells, indicating that TNFα expression is increased, at least in part, in a cell autonomous manner during regeneration (data not shown). Previous studies have demonstrated that HO-1 plays a protective role in oxidative tissue injuries (40,41). HO-1 is up-regulated in response to TNFα, and blocks TNFα-mediated death (40–44). To examine the mechanisms leading to elevated oxidative stress in Stra13−/− regenerating muscle, we analyzed HO-1 expression by western blot analysis. Interestingly, in WT muscles, HO-1 expression was highly up-regulated during the regenerative process at d2, d5 and d10 after injury. In contrast, HO-1 was expressed at much lower levels in the mutant tissue at d2, d5 after injury, and declined to negligible levels at d10 (Fig. 5I). Given its critical role in protection from oxidative injury, these results suggest that the inability to up-regulate HO-1 levels may underlie increased susceptibility to oxidative damage in Stra13−/− regenerating tissue. Since our data suggested that increased oxidative damage occurs prior to the onset of muscle degeneration in Stra13−/− mutants, we then tested whether inhibition of oxidative stress with the anti-oxidant NAC modulates muscle necrosis and the regenerative response of Stra13−/− mice in vivo. Two-month-old WT and Stra13−/− mice were injected intraperitoneally with NAC, or with PBS as a control for one week prior to injury, and
the treatment continued until the mice were sacrificed and muscles analyzed histologically 10 days later. As previously reported, in the absence of NAC treatment, Str13−/− mutants exhibited a significant increase in necrotic myotubes (Fig. 6A), and calcium deposition (Fig. 6B) compared with WT mice in response to injury. Approximately 25% of the injured area in Str13−/− mutants (\(P < 0.001\)) contained necrotic fibers at d10 after injury as evidenced by H&E staining.
staining (Fig. 6C). Treatment with NAC did not alter muscle regeneration in WT mice. Strikingly however, administration of NAC rescued the degeneration of muscles in Stra13−/− mutants (Fig. 6A–C). Histological analysis by H&E staining and alizarin red staining revealed an obvious improvement in histopathology of Stra13−/− muscles upon NAC treatment (Fig. 6A and B). Moreover, the extent of necrosis within the injured area was significantly reduced (\( \*P < 0.05 \)) to ~6% upon NAC treatment in Stra13−/− mutants (Fig. 6C). Together these results strongly suggest that oxidative damage is the underlying reason for muscle necrosis and the inability of Stra13−/− mutants to effectively regenerate.

**Stra13 protects myoblasts from oxidative stress mediated death**

Since Stra13 mutant regenerating tissue exhibits signs of oxidative damage; we tested whether Stra13 is directly involved in the regulation of oxidative stress. We first analyzed whether Stra13 overexpression in muscles is sufficient to confer resistance to free radical mediated death. C2C12 cells were transduced with a retrovirus expressing Stra13 (pBabe-Stra13), or a control virus (pBabe). Transduced cells were treated with hydrogen peroxide, and the viability was measured by trypan blue exclusion assays. Strikingly, in contrast to control virus expressing cells that rapidly died in presence of the pro-oxidant, Stra13 expressing myoblasts were protected from H2O2 induced death (Fig. 7A and B). To further examine the mechanism by which Stra13 regulates oxidative stress, we examined HO-1 and TNFα mRNA levels in control and pBabe-Stra13 cells in the absence and presence of H2O2 treatment (Fig. 7C). Interestingly, Stra13 expression was sufficient to up-regulate endogenous HO-1 mRNA levels relative to vector control cells (Fig. 7C). Moreover, although TNFα expression was up-regulated in control cells within 1 h of H2O2 addition, its expression was reduced in Stra13 overexpressing cells. Together, these results confirm that Stra13 can function as an anti-oxidant, and confer protection to muscle cells from ROS mediated death at least in part by regulation of HO-1 and TNFα expression.

**DISCUSSION**

ROS are constantly generated in cells through normal metabolic pathways. A tight control of the generation and removal of free radicals is essential for cellular homeostasis. An excessive accumulation of ROS either due to increased production, or defective cellular responses that impair its removal, lead to oxidative stress. Elevated ROS levels result in modification of lipids, proteins, and DNA, and this free radical injury is associated with many disease states including DMD, neurodegeneration, diabetes, cancers and ageing (45,46). In the case of DMD, the absence of dystrophin leads to necrosis of skeletal muscles. However, given the progressive nature of the disease, it is likely that dystrophin deficiency alone is not sufficient for death of dystrophic muscles, but rather enhances the sensitivity of muscle to
stimuli that eventually lead to muscle degeneration (47). Although previous studies have suggested a correlation between oxidative stress and the degeneration of muscle fibers characteristically seen in DMD, the role of oxidative stress as a primary cause of muscle degeneration has not been established. Moreover, the effects of anti-oxidants in preventing the pathologic degenerative changes in dystrophic muscles in clinical trials as well as in mdx mice have not been promising likely due to the timing of administration of the anti-oxidants (12,13). Our current study demonstrates that oxidative damage is indeed a key event in muscle degeneration, and provides evidence for the existence of an

Figure 7. Stra13 protects myoblasts from oxidative stress. (A) C2C12 myoblasts were infected with a retrovirus expressing Stra13 (pBabe-Flag-Stra13) or an empty vector (pBabe) alone. Stra13 expression levels were analyzed by western blot using anti-Flag antibody (right panel). β-actin was used as loading control. Both control and pBabe-Stra13 cells were left untreated, or treated with H2O2 for 5 h and analyzed microscopically. (B) The viability of control and pBabe-Stra13 cells was measured by trypan blue exclusion assays. The data is representative of four independent experiments. (C) HO-1 and TNFα mRNA levels were analyzed in pBabe and pBabe-Stra13 cells in the absence and presence of H2O2 treatment for 1 h as indicated. The amount of RNA in each lane was normalized to 36B4 transcripts. The signal intensities were quantified (Kodak Imaging Software v 4.0.5) and are shown on the right.
oxidative stress response pathway in muscle cells that is regulated by Stra13. Our principle findings are that Stra13 provides critical protection to muscle cells from the deleterious effects of ROS, and the loss of such protective mechanisms underlies muscle degeneration in muscular dystrophy.

Several lines of evidence demonstrate that the primary trigger for muscle degeneration in Stra13−/− mutants is oxidative stress (1). Increased levels of TNFα, which can lead to ROS (35–37), are evident early on the mutant regenerating tissue (2). The elevated TNFα expression is followed by altered protein carbonylation in the mutant regenerating tissue demonstrating oxidative damage (3). A cellular response to oxidative damage is apparent by upregulation of peroxiredoxins at d5 after injury. It is worth highlighting that the hallmark of oxidative damage such as increased anti-oxidant enzyme expression and protein carbonylation occur prior to the onset of muscle necrosis in Stra13−/− mutants, indicating that such alterations are likely to be causal to degenerative process (4). HO-1, which facilitates cellular resistance to oxidative injury by blocking the cytotoxic effects of TNFαs and attenuating the production of ROS (41–43), is down regulated in Stra13−/− regenerating tissue. The timing of TNFα increase and HO-1 decrease is in agreement with overt signs of oxidative damage in the mutant tissue, which occurs prior to the onset of muscle degeneration. Nevertheless, our studies do not exclude changes independent of TNFα that may contribute to ROS production and oxidative damage in the mutant regenerating tissue (5). Stra13−/− myoblasts exhibit increased susceptibility to death following oxidative damage (6). Conversely, Stra13 expression protects myoblasts from oxygen radicals, and up-regulates endogenous HO-1 mRNA levels concomitant with reduced TNFα expression (7). Strikingly, muscle necrosis in Stra13−/− mutants can be prevented by treatment with the anti-oxidant NAC in vivo. We have previously demonstrated increased Notch signaling in Stra13−/− regenerating muscle, and its inhibition partially rescues muscle necrosis (28). It would be interesting to determine whether deregulated redox homeostasis is linked to altered Notch activity in the regenerating tissue in Stra13−/− mutants.

The distinctive dystrophy-like phenotype of Stra13−/− mice that is characterized by loss of sarcolemmal integrity, muscle necrosis, calcification and fibrosis, is reminiscent of mdx mutants. However, unlike mdx mice where the primary defect is loss of the structural protein dystrophin, no changes in either the expression or localization of additional structural proteins is evident in mdx/Stra13−/− mutants. In particular, no changes in utrophin, α7 integrin and α2 laminin, which are upregulated in the mdx mutants, and compensate for loss of dystrophin, were apparent. Nor were there any changes in fiber type specification that could account for the degeneration of muscle fibers. Moreover, since neither the percentage of Pax7+ satellite cells, nor the regeneration index is altered in mdx/Stra13−/− mice, our results point to a specific role for Stra13 in muscle degeneration rather than muscle regeneration. The molecular mechanisms by which Stra13 regulates oxidative stress remain to be investigated. Stra13 is expressed at low basal levels in myoblasts (28), and its overexpression is sufficient to down regulate TNFα expression and up-regulate HO-1 expression. In response to oxidative stress, it is possible that Stra13 expression/activity is modulated, that allow it to maintain reduced TNFα levels, as well as increase expression of cytoprotective enzymes such as HO-1. Together, these results suggest that Stra13 regulates muscle integrity by providing critical protection to muscle cells from oxidative damage. The combined loss of both structural and survival signals likely lead to an early onset of muscle degeneration in mdx/Stra13−/− mutants. Previous findings regarding the role of Stra13 in cell death have indicated that it can function both as a pro-death, and anti-apoptotic factor (23–27). These findings indicate that Stra13 may have complex context- and cell type-dependent roles. Post-transcriptional modifications or interaction with different protein partners may modulate Stra13 activity and enable it to perform seemingly opposing functions. Nevertheless, it is likely that our present findings have broader implications in various pathologies associated with increased oxidative stress such as cancers and ageing.

**MATERIALS AND METHODS**

**Mice**

Quadiceps muscles of littermate WT and Stra13−/− mice were subjected to freeze injury as described (28). For antioxidant treatment, 2-month-old mice (n = 3) were given daily NAC intraperitoneal injections (100 mg/kg) as described (48), or with PBS as a control for 1 week prior to freeze injury. After injury, treatment with NAC was continued until mice were sacrificed 10 days later. mdx/Stra13−/− animals were obtained by breeding Stra13−/− mice with C57BL/10ScSnJ-Dmdmdx/J mice (Jackson Laboratory, Bar Harbor, USA). All animal protocols followed institutional guidelines.

**EBD uptake**

Twenty-four hours before sacrifice, mice were injected intraperitoneally with 1% EBD (Sigma) in PBS (pH 7.5) as described (30). Muscles were sectioned and analyzed by fluorescence microscopy. The percentage of EBD+ myofibers to the total number of myofibers in the field was calculated by counting at least 300 myofibers. The data presented are an average from three mice of each genotype.

**Histological and immunohistochemical analysis**

For all analyses, at least three animals of each genotype were used. Muscles were collected and either paraffin-embedded, or immediately frozen in isopentane. Eight to ten micrometer sections were used for histological and immunohistochemical analysis. Masson’s Trichrome staining was used to detect collagen deposition according to the manufacturer’s protocol (Diagnostic Biosystems). To detect calcification, sections were stained with 2% alizarin red S. To calculate the regeneration index, cross-sections from the mid-belly of quadriceps muscles (n = 4) were stained with H&E. The ratio of myofibers with centrally located nuclei to the total myofibers in the field was counted. A total of 400 myofibers were counted in each field. To quantify fibrosis, two fields were randomly chosen on cross sections of HE stained quadriceps muscles from mdx and mdx/Stra13−/− mice (n = 3).
Non-muscle areas were measured using ImageJ (version 1.37v, National Institutes of Health) and plotted as the ratio of fibrotic area to the total area in the field. The extent of necrosis was quantified by determining the area covered by degenerated myotubes within the total injured area. For immunostainings, sections were blocked with 5% BSA-PBS and incubated with the primary antibody. Negative controls were performed by omitting the primary antibody. Primary antibodies used in this study are MANDYS8 (Sigma) for dystrophin; MANDAG2 (DSHB) for β-dystroglycan; MANCHO3 clone 8A4 (DSHB) for utrophin; α2 laminin (Sigma); M8421 for slow myosin (Sigma); MyHC A4.74 for fast myosin (DSHB); Mac3 (BD Pharmingen) and HO-1 (Stress-Gen). Antibodies against α-dystroglycan and α7 integrin were kind gifts from Stephen Kroger and Ulrike Mayer, respectively. Microscopy was performed using a microscope (Axioshot, Zeiss) with plan neofluar 10X/0.3 and 20X/0.5 objectives (Zeiss), and images were captured using a camera (2.3.0 Spot RT Color, Diagnostic Instruments) and Spot software (version 3.3.2, Diagnostic Instruments).

Oxyblot analysis

Proteins were extracted in Cell Lysis Buffer (Cell Signaling). Samples were concentrated with a 10K NMMWL-0.5 ml Ultrafree filter unit (Millipore). Fifteen microgram protein extracts were separated on 8% acrylamide gels, transferred onto PVDF membranes (Millipore) and carboxyl groups were detected using the OxyBlot kit (Chemicon International) as per the manufacturer’s protocol.

RT–PCR

Total RNA was extracted from muscles, or cells, using TRIZOL (Invitrogen). After treatment with RNase free DNeasy kit (Ambion), 10 ng of total RNA was incubated with 5 ng of oligo(dT) (Promega) at 70°C for 5 min, and reverse transcribed using AMV reverse transcriptase (Promega) for 90 min at 42°C. Quantitative real time PCR (Q-PCR) was done using the Quantitect SYBR Green PCR Kit (Qiagen). Ten microliter of cDNA was used as a template and PCR was performed in an ABI Prism 7900HT thermocycler (Applied Biosystems) in triplicates. Amplification steps were: 95°C 15 min [95°C 15 s, 55°C 30 s, 72°C 30 s] 40 cycles. cDNA from d2 regenerating muscle was used to generate standard curves. All results were compared using an unpaired t test, and P-values of <0.05 were considered to be statistically significant.

Statistical analysis

Values are reported as mean ± standard error (SE). For all analyses, at least three mice per genotype were used. Data were compared using an unpaired t test, and P-values of <0.05 were considered to be statistically significant.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We sincerely thank Hart Lidov for help with histological analysis of the double mutants. We are grateful to Stephen Kroger and Ulrike Mayer for generously providing antibodies and Gladys Tan for assistance with the experiments.

Conflict of Interest statement. None declared.

FUNDING

This work was supported in part by funds from the Muscular Dystrophy Association (#92922), and from the National University of Singapore (R.T.).

REFERENCES


