Reductive stress impairs myogenic differentiation

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Abstract

Myo-satellite cells regenerate and differentiate into skeletal muscle (SM) after acute or chronic injury. Changes in the redox milieu towards the oxidative arm at the wound site are known to compromise SM regeneration. Recently, we reported that abrogation of Nrf2/antioxidant signaling promotes oxidative stress and impairs SM regeneration in C57/B16 mice. Here, we investigated whether the activation of intracellular Nrf2 signaling favors antioxidant transcription and promotes myoblast differentiation. Satellite cell-like C2C12 myoblasts were treated with sulforaphane (SF; 1.0 & 5.0 μM) to activate Nrf2/antioxidant signaling during proliferation and differentiation (i.e. formation of myotubes/myofibers). SF-mediated Nrf2 activation resulted in increased expression of Nrf2-antioxidants (e.g. GCLC and G6PD) and augmented the production of reduced glutathione (GSH) leading to a reductive redox state. Surprisingly, this resulted in significant inhibition of myoblast differentiation, as observed from morphological changes and reduced expression of MyoD, Pax7, and Myh2, due to reductive stress (RS). Furthermore, supplementation of N-acetyl-cysteine (NAC) or GSH-ester or genetic knockdown of Keap1 (using siRNA) also resulted in RS-driven inhibition of differentiation. Interestingly, withdrawing Nrf2 activation rescued differentiation potential and formation of myotubes/myofibers from C2C12 myoblasts. Thus, abrogation of physiological ROS signaling through over-activation of Nrf2 (i.e. RS) and developing RS hampers differentiation of muscle satellite cells.

Keywords:
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Differentiation markers
 Reactive oxygen species (ROS)

1. Introduction

Skeletal muscle (SM) contributes to about 50% of the total body mass. SM generates force through the contractile movement of myofibers during physical activity, which are tightly regulated through bioenergetics, stress response, antioxidant defense systems, and redox metabolism [1–4]. Adult SM tissue regains regenerative capacity upon injury or damage [5–7]. Satellite cells undergo regeneration to form myofibers and maintain muscle mass through their activation following injury [6,8,9]. Myogenic differentiation is a multistep process consisting of the expression of various transcription factors such as paired-box (Pax3 and Pax7) [10–13] and canonic myogenic regulatory factors (MRFs; Myf-5, MyoD, myogenin and Mrf4) for the development [14,15]. The adult SM requires Pax7 and MyoD in the satellite stem cells to regulate cell lineage during SM regeneration [16–18]. SMs including muscle precursor cells are constantly exposed to a pro-oxidizing environment due to their high rate of oxygen consumption and metabolic activity [19,20]. During aging or chronic conditions such as diabetes mellitus and AIDS, loss of skeletal muscle mass and activity are coupled with decreased glutathione and increased oxidative stress (OS) [21–23]. In addition, sarcopenia, an age-related condition, characterized by a reduction in the size and number of muscle fibers is strongly associated with increased OS [24–26]. While OS impairs muscular strength and health, it is likely that the use of antioxidants would be beneficial. However, a pro-oxidative signaling is crucial for satellite cell activation at the wound site of a regenerating skeletal muscle [20,27–30]. Nonetheless, response to extreme changes in the redox conditions (i.e. oxidative vs. reductive stresses) during SM regeneration remains unexplored.

Here, we hypothesized that an excess reductive capacity (i.e. abundant intracellular antioxidants) may abrogate the obligatory pro-oxidant signals required for the activation of satellite cells (i.e.
proliferation and differentiation), thereby delaying or dampening the process of muscle regeneration. In this study, we investigated whether the activation of Nrf2 induces RS and impairs proliferation and differentiation of muscle stem cells, which are crucial for SM regeneration.

2. Material and methods

2.1. Cell culture, treatments and experiment design

C2C12 myoblasts were cultured in proliferation medium (DMEM containing 10% FBS). The cultured myoblasts (~80% confluency) were induced to myogenic differentiation by replacing proliferation medium with differentiation medium [31] (DMEM containing 2% horse serum). Myoblasts were subjected to pro-reductive environment or RS by dose-dependent treatment of sulforaphane (SF), N-acetyl cysteine (NAC) and glutathione reduced ethyl ester (GEE) or Keap1-knockdown using shRNA during proliferation (24 h) and differentiation phases (up to 5 days of differentiation). Following these treatments, the proliferating and differentiating myoblasts were analyzed for rate of differentiation (morphological, gene and protein markers) by qPCR [31] and immunoblotting [31], glutathioneredoxstateby enzyme kinetics (glutathione reductase recycling assay) [32], reactive oxygen species measurements (using Fluorescent probe) [33], and apoptosis using Annexin-V/propidium iodide [32,34] (FACS). Detailed methods are provided in the supplemental section.

2.2. Statistical analysis

Data are expressed as mean ± SEM. One-way ANOVA analysis was used to determine significant differences between control and SF/NAC/GEE treated groups, and Student T-test was used to compare the control and Keap1 knockdown cells. All the statistical comparisons were made between controls/undifferentiated cells to differentiated cells vs respective drug treated groups in each figure. p values smaller than 0.05 were considered statistically significant.

3. Results

3.1. Sulforaphane (SF) induces reductive stress (RS) and inhibits myoblast differentiation

Glutathione (GSH) is an essential non-protein antioxidant thiol involved in maintaining redox homeostasis by neutralizing reactive oxygen species and scavenging oxidized proteins [35,36]. Previous studies demonstrated SF could enhance GSH levels and result in an intracellular reductive state [37,38]. Here, we tested the effect of SF on the status of Nrf2-regulated antioxidant genes during the proliferation and differentiation phases of myoblast growth. Exposure of the cells with SF resulted in a dose dependent increase in glutathione (GSH) levels and redox ratio (GSH/GSSG) [Fig. 1A] along with an upregulation of antioxidant genes, suggestive of an intensification of reductive stress in C2C12 myoblasts.
Fig. 2. Reductive stress impairs myoblast differentiation. C2C12 myoblasts were cultured in proliferation medium to 70–80% confluence, subjected to differentiation with or without SF (1.0 & 5.0 μM). (A) Bright field microscopy images of C2C12 myoblast during proliferation and differentiation phase under SF treatment (day 1 & 5). (B) Q-PCR based relative gene expression of myoblast differentiation markers (Myod1 and Myogenin) during myoblast proliferation and differentiation (day 1 & 5). (C) Immunoblots for differentiation markers (Myogenin and Myh2) during myoblast proliferation and differentiation (day 1 & 5) phases. ***p < 0.001, n = 3–4.

3.2. A pro-oxidative milieu is a pre-requisite for myogenic differentiation

Since we observed diminished levels of antioxidants during basal differentiation of myoblasts, we next addressed whether SF-mediated increase in antioxidants resulted in decreased cellular ROS (than the basal/physiological settings), which may lead to an impaired differentiation. To this end, we determined the ROS levels by flow cytometric analysis of proliferating & differentiating myoblasts incubated with DCFDA, a ROS sensitive probe that fluoresce upon oxidation. During normal differentiation, increased fluorescence was observed as an indication of elevated ROS levels in C2C12 myoblast (Fig. S2). On day-1 of differentiation, ROS levels were increased significantly from basal levels and further increased on days 3 & 5 (Figs. S1A–B). This finding is consistent with our observation that antioxidant proteins are decreased during myoblast differentiation (Fig. 1C). While the levels of antioxidants were augmented by SF treatment (1, 3 and 5 μM), basal ROS levels (observed during proliferation) and elevated ROS levels (observed during normal differentiation) were significantly decreased (Fig. S1). Though treatment with a lower dose of SF (1.0 μM) showed no significant change in ROS levels, 5.0 μM of SF dramatically decreased the ROS levels, which was associated with poor differentiation (Fig. 2). These results suggest that a moderate generation of ROS is necessary to facilitate the myoblast differentiation. In addition, shifting the redox milieu towards the reductive arm by SF treatment appears to prevent the differentiation of myoblasts into myotubes (Fig. 2A–C). Furthermore, FACS using Annexin V/Propidium Iodide (PI) and immunoblotting analyses indicated no evidence for apoptosis in the SF-treated myoblasts that experienced poor to no differentiation (Fig. S2).

3.3. Direct augmentation of intracellular glutathione also hampers myoblast differentiation

We next determined whether direct augmentation of intracellular glutathione could result in RS and inhibit myoblast differentiation. To address this possibility, we governed the effects of N-acetyl cysteine (NAC), a precursor of glutathione synthesis [39], and glutathione ethyl ester (GEE), a cell-permeable derivative of glutathione [40]. Both NAC and GEE supplementations significantly increased total GSH levels and the GSH/GSSG ratio (Fig. 3A–B). Either NAC or GEE at the concentration of 1.0 mM increases the total GSH levels by ~3-fold and the GSH/GSSG ratio by 1.5 fold (P < 0.05; Fig. 3A–B). Under these conditions, both NAC and GEE moderately inhibited the myoblast differentiation. The higher doses of NAC (3 mM) or GEE (5.0 mM) significantly increased total GSH levels by ~7–8 fold and GSH/GSSG ratio by 2–3 fold (P < 0.01) which resulted in substantial inhibition of myotube formation (Fig. 3A–B). Immunoblot analysis of early and terminal differentiation markers, myogenin and Myh2, respectively, further corroborated with the inhibition of myoblast differentiation (Fig. 3A–B). These results suggest that reductive conditions established by NAC or GEE are sufficient to inhibit the myogenic differentiation process.

3.4. Silencing Keap1 activates the Nrf2/antioxidant signaling and retards myoblast differentiation

Keap1 is a negative regulator of Nrf2 activation [41–43]. Under basal conditions, Keap1 binds to Nrf2 and facilitates its proteasomal degradation [44]. Here, we silenced Keap1 by shRNA to inhibit proteasomal degradation of Nrf2 and induce Nrf2-dependent antioxidant signaling (Fig. 3C). The proliferating myoblasts were transfected with four different Keap1 shRNAs (1 to 4) and the protein levels of NQO1, a classical target of Nrf2, were measured to determine the type of shRNA that efficiently silences Keap1. Myoblasts expressing Keap1 shRNA-4 with highest Keap1 silencing showed impaired differentiation when compared to myoblasts transfected with mock shRNA (Fig. 3C). Rate of differentiation was semi-quantitatively analyzed by scoring on a scale of 1–10 (1 – Proliferating cells vs. 10 – fully differentiated myotubes) (Fig. 3C). Both early and late differentiation markers, myogenin and Myh2 protein levels were decreased in differentiating myoblasts expressing Keap1 shRNA-4 in relation to control shRNA (Fig. 3C). In addition, antioxidant proteins (GCLC, NQO1, and catalase) were also significantly increased in proliferating and differentiating myoblasts expressing Keap1 shRNA-4 (Fig. 3C). These results indicate that a genetic silencing of Keap1 and resultant activation of the Nrf2 antioxidant pathway impairs myoblast differentiation due to an enhanced reductive condition (i.e. RS). Of note, preventing RS through SF washout on day
3, efficiently rescued the differentiation of myoblasts (Fig. 4A-C).

4. Discussion

Nrf2/antioxidant signaling constitutes cellular defense systems and protects them during stress conditions. We previously demonstrated that an age-related loss of Nrf2 function results in oxidative stress (OS) and delays satellite cell activation and skeletal muscle (SM) regeneration [45]. In the present investigation, we attempted to attenuate OS-mediated impaired SM regeneration by pharmacologically/genetically activating Nrf2-antioxidant signaling. Unexpectedly, a pharmacological activation of Nrf2 by sulforaphane (SF) resulted in a gradual inhibition of myoblast differentiation. Importantly, the geneticsilencing of Keap1, a suppressor of Nrf2, promoted the Nrf2-dependent induction of antioxidants, which also resulted in significantly impaired myoblast differentiation. These results indicate that shifting the redox milieu toward the reductive arm (i.e. RS) could negatively influence the process of myogenic differentiation.

Our data demonstrate a marked decrease in expression of major antioxidant enzymes (GCLC, GLCM, G6PD, etc.) at both protein (Fig. 1C) and transcript levels (Fig. 1B) during the normal transition of myoblasts from proliferation to differentiation. Importantly, the genetic silencing of Keap1, a suppressor of Nrf2, promoted the Nrf2-dependent induction of antioxidants, which also resulted in significantly impaired myoblast differentiation. These results indicate that shifting the redox milieu towards the reductive arm (i.e. RS) could negatively influence the process of myogenic differentiation.

During RS, inhibition of myoblast differentiation was accompanied with a decrease of early (myogenin and Myod1) and terminal (Myh2) myogenic differentiation markers (Fig. 2). In contrast, age-related OS has been reported to impairsatellite cell activation causing inhibition of myogenic differentiation [45]. Age-related OS has been demonstrated to promote apoptosis of muscle and progenitor satellite cells leading to muscle loss [46,47]. Of note, RS partially induced C2C12 myoblast proliferation and did not induce apoptosis during either the proliferation or differentiation phase (Fig. S2). To our surprise, the myoblasts under RS were neither proliferating nor differentiating, suggesting that the RS engages a quiescent state (G1 phase of the cell cycle) in the myoblasts. Interestingly, preventing the RS by withdrawing SF restored the myogenic differentiation process. Although a G1 arrest is typically coupled to the differentiation process, an inhibition of the differentiation process was observed without apoptosis. Of note, besides G1 arrest, several factors and events such as activation of p38 MAPK kinase and histone deacetylase are essential to drive the differentiation process [48,49]. One or more of these events may be hindered by an extensive reductive redox (i.e. RS) condition in the myoblasts receiving chronic SF. These interesting questions are to be addressed in future studies.

Consistent with this notion, the SF wash-out experiments (Fig. 4) partially rescued the differentiation, indicating that the cells have been maintained in a functionally active and conditionally reversible state. Thus, the RS-induced cell cycle arrest could be related to cell senescence-associated cytostasis [50].
In summary, this study demonstrates that suppression of Nrf2-antioxidant signaling occurs during physiological myogenic differentiation that creates a pro-oxidative environment, which is essential for myoblast differentiation. Nonetheless, activation of Nrf2/antioxidant signaling establishes a reductive condition that causes RS, which impairs myogenic differentiation.

Declaration of competing interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101492.

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