Inhibition of Drp1-dependent mitochondrial division impairs myogenic differentiation

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Muscle precursor cells (or myoblasts) are adult muscle stem cells that have significant therapeutic potential to treat cardiovascular disease, muscle dystrophy, and severe skeletal muscle trauma (16, 38, 50). Upon activation, they recapitulate embryonic muscle development, which involves proliferation, cell cycle withdrawal, and subsequent differentiation to fuse into multinucleated syncytial myotubes (9, 41). During this process, mitochondria are dynamic organelles forming a tubular network that is continuously fusing and dividing to control their morphology and functions. Recent literature has shed new light on a potential link between the dynamic behavior of mitochondria and muscle development. In this study, we investigate the role of mitochondrial fission factor dynamin-related protein 1 (Drp1) in myogenic differentiation. We found that differentiation of C2C12 myoblasts induced by serum starvation was accompanied by a gradual increase in Drp1 protein expression (to ~350% up to 3 days) and a fast reduction of Drp1 phosphorylation at Ser-637 (to ~30%) resulting in translocation of Drp1 protein from the cytosol to mitochondria. During differentiation, treatment of myoblasts with mitochondrial division inhibitor (mdivi-1), a specific inhibitor of Drp1 GTPase activity, caused extensive formation of elongated mitochondria, which coincided with increased apoptosis evidenced by both enhanced caspase-3 activity and increased number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells. Furthermore, the mdivi-1-treated myotubes (day 3 in differentiation media) showed a reduction in mitochondrial DNA content, mitochondrial mass, and membrane potential in a dose-dependent manner indicating defects in mitochondrial biogenesis during myogenic differentiation. Most interestingly, mdivi-1 treatment significantly suppressed myotube formation in both C2C12 cells and primary myoblasts. Likewise, stable overexpression of a dominant negative mutant Drp1 (K38A) dramatically reduced myogenic differentiation. These data suggest that Drp1-dependent mitochondrial division is a necessary step for successful myogenic differentiation, and perturbation of mitochondrial dynamics hinders normal mitochondrial adaptations during muscle development. Therefore, in the present study, we report a novel physiological role of mitochondrial dynamics in myogenic differentiation.

myogenesis; mitochondrial dynamics; dynamin-related protein 1; mdivi-1

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tentially in the differentiation of muscle stem cells during muscle regeneration.

MATERIALS AND METHODS

Cell culture and differentiation. C2C12 mouse myoblasts (American Type Culture Collection) were grown in growth medium (GM) consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin-streptomycin at 37°C and 5% CO₂. Primary myoblasts were isolated from hindlimb muscles of 2-wk-old pups as we previously described (47). All mice were maintained and handled in accordance with the Institutional Animal Care and Use Committee at Temple University. Myogenecity determined by Pax-7 staining was over 90% of total number of cells in each culture (data not shown). Myoblast differentiation was initiated when they reached 90–95% confluency by incubating the cells in differentiation media (DM) consisting of DMEM supplemented with 2% horse serum (Invitrogen) and 1% penicillin-streptomycin. For some experiments, C2C12 myoblasts were maintained in DM supplemented with small molecule mitochondrial division inhibitor (mdivi-1, between 1 and 20 μM in DMSO, DMSO final concentration = 0.2%) and insulin (1 μg/ml) for the indicated period of time. IC₅₀ of mdivi-1 is ~10 μM. Control cells were treated with 0.2% DMSO in DM. For some experiments, DM was supplemented with ROS scavengers Mito-TEMPO (25 μM) (Enzo Life Sciences) and N-acetylcysteine (NAC) (500 μM) (Sigma-Aldrich) in the presence or absence of mdivi-1 (10 μM). During differentiation, medium was changed every 24 h.

Transfection. C2C12 cells were stably transfected with pcDNA3-Drp1-GFP or pcDNA3-Drp1(K38A) using FuGENE 6 (Promega) according to the manufacturer’s instruction. Selectively generated stable transfectants were maintained for more than 3 wk in G418 (200 μg/ml) containing medium. G418 was added to all media used for the following experiments.

Subcellular fractionation. For Drp1 translocation assay and creatine kinase (CK) activity assay, subcellular fractionation was performed using a protocol modified from Frezza et al. (19). Briefly, the cell pellet was resuspended in isolation buffer (320 mM sucrose, 1 NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, pH 7.4) and disrupted using a Dounce homogenizer. To remove precipitated nuclei, membranes, and non-disturbed large cell debris, the homogenized sample was initially centrifuged at 1,000 g for 10 min at 4°C. Then the supernatant was centrifuged at 10,000 g for 15 min at 4°C. Subsequently, the subfractionated mitochondrial pellet and cytosolic fractions were lysed in RIPA buffer and subjected to a Bradford assay for protein quantification.

Immunoblotting. Cells were washed three times with cold DPBS and lysed in RIPA buffer (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, pH 7.5). After precipitation of insoluble fraction of the RIPA samples by centrifugation (16,000 g for 15 min at 4°C), supernatants were collected and subjected to Bradford assay to quantify protein concentrations. For sample preparation for Drp1 translocation assay, please see Subcellular fractionation. The resulting protein samples underwent SDS-PAGE and were transferred to Immobilon-P membrane (Millipore). Subsequently, the membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) for 20 min at room temperature and incubated overnight with respective primary antibodies. Antibodies were purchased from the following sources: mouse monoclonal anti-Drp1 (BD Biosciences), rabbit polyclonal anti-phospho-Drp1 (Ser 637) (Cell signaling), mouse monoclonal α-tubulin (Sigma-Aldrich), mouse monoclonal anti-porin (Invitrogen), and rabbit polyclonal anti-caspase-3 (Cell signaling). The MF 20 monoclonal antibody developed by Dr. Donald A. Fischman (Cornell University Medical College, New York, NY) was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA. The membranes were then washed twice in TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h. After washing three times with TBST, membranes were then subjected to standard enhanced chemiluminescence (Thermo Fisher Scientific) method for visualization.

Mitochondrial staining. Mitochondrial morphology and activity were monitored by using MitoTracker Red CMXRos (Molecular Probes) (24). Briefly, at the indicated differentiation time points, cells were incubated with 200 nM of MitoTracker Red in prewarmed medium for 20 min in a 37°C cell culture incubator. After three washes in PBS, 3.7% paraformaldehyde solution in cell medium was applied to the cells for 20 min. The fluorescence intensity and mitochondrial morphology were analyzed and quantified using the Image J software (NIH).

Mitochondrial morphometric analyses. Morphometric analysis of mitochondria was performed using methods described previously (14, 32). Briefly, microscopic images were processed using Image J (NIH) to enhance brightness and contrast and subjected to convolutions to emphasize the edges of each mitochondrial particle. After the threshold was modified, individual mitochondrial particles were analyzed for circularity and major/minor axes. Form factor (FF: the reciprocal of circularity value) and aspect ratio (AR: major axis/minor axis of an ellipse equivalent to the object) were calculated, and a scatter plot of AR versus FF was generated for each image. Both parameters have a minimal value of 1 when it is a small perfect circle and the value increase as mitochondria become elongated. Specifically, AR is a measure of mitochondrial length, and FF represents both mitochondrial length and branching. Furthermore, mitochondrial morphology of each cell was categorized into either normal (tubular), elongated, or fragmented. Then the number of cells in each category was counted and summarized as a percentage of the total cell number. Approximately 200 cells were analyzed for each condition. The analyses were performed blinded in triplicate by at least two individuals.

Immunocytochemistry. For myosin heavy chain staining, myotubes were fixed in ice-cold methanol for 5 min and rehydrated in PBS. After being blocked for 30 min at room temperature, anti-sarcomeric myosin antibody MF-20 in 2% BSA-PBS was incubated for 2 h. Final concentration of 2 μg/ml of Alexa 488 secondary antibody was incubated for 1 h at room temperature. Cover slips were mounted on Prolong Gold with DAPI (Molecular Probes) and micrographs were captured using a fluorescence microscope (Axioimager, Zeiss). To quantify the densities of differentiated myotubes, the number of myonuclei was estimated by determining the average myonuclei number within a given field (56). MHC-positive-stained areas having a minimum number of three nuclei were considered to be differentiated myotubes.

TUNEL staining. Terminal deoxynucleotidyl transferase-mediated DUTP nick-end labeling (TUNEL) staining (Roche Applied Science) was performed to determine apoptotic nuclei during C2C12 differentiation with mdivi-1 treatment according to the manufacturer’s instructions. After 24 h of mdivi-1 treatment (0, 1, 10, or 20 μM) in DM, cells were fixed in 4% paraformaldehyde for 1 h. Cells were then permeabilized (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice. TUNEL reaction mixture was added to cells and incubated for 1 h at 37°C in a humidified incubator with occasional mixing. Cells were mounted in Prolong Gold with DAPI (Invitrogen) to label all nuclei. Stained nuclei were analyzed using a fluorescence microscope. Numbers of TUNEL-positive nuclei were counted in each condition and time point. DNase I-treated C2C12 cells were used as a positive control and those without terminal transferase enzyme were used as a negative control.

Creatine kinase activity assay. CK activity was measured in isolated cytosolic supernatants using EnzyChrom CK Assay Kit. For sample preparation for the CK assay, please see Subcellular fractionation. The assay was performed according to the manufacturer’s instructions (BioAssay Systems). The values were normalized to protein concentration measured by Bradford assay. The activities were
presented as unit values. One unit of CK activity is equal to the activity required to transfer 1 μmol of phosphate from phosphocreatine to ADP per minute at pH of 6.0.

*mtDNA content quantification.* Total genomic DNAs of C2C12s that had been treated in DM with indicated concentration of mdivi-1 were isolated by using the DNeasy kit (QIAGEN). Their mtDNA content were assessed by semiquantitative PCR. The relative ratio between mitochondrial DNA (ND II; NAHD dehydrogenase subunit 2) compared with nuclear DNA (18s rRNA) amount was calculated. Primer sequences were as follows: NDII, sense, 5′-CTTAGAGGGACAAGTGGCGTTC-3′; antisense, 5′-CTATCACCCT-3′; 18s rRNA, sense, 5′-CTGAGGATGTGCTGTCTGGGAA/CCTTTGCCTCCACTTCGGTC-3′; antisense, 5′-CGCTGAGCCACCTGCTGTTAG-3′.

*mtDNA isolation, cDNA synthesis, and semiquantitative PCR.* mRNAs were isolated using Dynabeads direct kit, and cDNA synthesis were performed on poly-DT magnetic beads by reverse transcription using superscript II (Invitrogen). mRNA expression levels for muscle differentiation markers were measured by semiquantitative PCR. Three housekeeping genes TIF1, HPRT1, and GAPDH were used as controls to ensure reliability of the assay. The primer sequences used are described in Table 1.

*Statistics.* The results are presented as mean ± SD (unless otherwise indicated) for a minimum of three independent experiments in triplicate. All comparisons were made to the control conditions (Sub-confluent or 0 μM mdivi-1) for each variable using either one-way ANOVA or a two-tailed t-test analysis depending on how many conditions were compared in each experiment. One-way ANOVA was followed by Tukey’s post hoc test. The significance level was set at P < 0.05.

**RESULTS**

*Increased Drp1 expression and translocation to mitochondria during C2C12 myotube differentiation.* To examine the importance of Drp1 during myogenic differentiation, we assessed Drp1 protein expression level in subconfluent myoblasts and differentiating myotubes. When C2C12 cells reached between 90 and 95% confluency, myotube differentiation was induced by replacing GM with DM as described above. The majority of myoblasts were differentiated and fused to form multinucleated syncytial myotubes indicated by myonuclei per field of ~60% within 3 days in DM (Fig. 1A). During the 3-day differentiation period, protein expression of Drp1 was significantly and gradually upregulated (to ~350% in 3 days) (Fig. 1B). This observation raised a question as to whether the increased cytosolic Drp1 translocated to mitochondria and participated in mitochondrial remodeling during muscle differentiation. Therefore, we next assessed the phosphorylation state at Ser-637 residue of Drp1. As shown in Fig. 1C, there was a fast reduction of Drp1 phosphorylation (to ~30%) which was initiated as early as 30 min after DM exposure. The dephosphorylation state of Drp1 was maintained during the entire experimental period of up to 3 days (data not shown). As the changes in phosphorylation state at Ser-637 are associated with mitochondrial localization of Drp1, we next examined the abundance of Drp1 protein in subfractionated cytosolic and mitochondrial portions in differentiating C2C12 cells. Expectedly, we found that the level of Drp1 protein in mitochondria fraction increased during C2C12 myotube differentiation, and there was no complementary reduction in the amount of cytosolic Drp1 (Fig. 1D, left). The mitochondrial-to-cytosolic Drp1 protein ratio peaked on the second day of myogenic differentiation (Fig. 1D, right). These data indicate that Drp1 protein expression is upregulated and rapidly translocalized to mitochondrial compartment during C2C12 myotube differentiation.

**Inhibition of mitochondrial division by mdivi-1.** We analyzed mitochondrial morphology at the single cell level by an objective systematic quantification method as described previously (14, 32). As shown in Fig. 2A, each C2C12 cell within a given micrograph field showed a distinct mitochondrial morphology that was confirmed by a morphometric analysis (Fig. 2A, right), and we categorized them into either normal (tubular)-, elongated- or fragmented-shaped mitochondria containing cells. A large number of cells (~70%) presented a tubular shape under the no treatment condition whereas the frequency of elongated mitochondria-containing cells was dramatically increased with mdivi-1 treatment in a dose-dependent manner (from 26.8% to 61.7%) (Fig. 2B).

**Drp1 inhibition and myotube differentiation.** To further assess a role of Drp1 in myogenic differentiation, we performed pharmacological inhibition studies using mdivi-1. We examined mRNA expression levels of key myogenic regulatory factors (MRFs) (i.e., MyoD and Myogenin) by semiquantitative PCR. MyoD is required for the formation, propagation, and survival of skeletal muscle myoblasts, and myogenin acts later in the program likely as differentiation factor (6). The mRNA expression levels of MyoD and Myogenin were significantly suppressed by mdivi-1 treatment in differentiating C2C12 cells (Fig. 3, A and B). Also, the muscle fiber-specific gene MHC I mRNA level was remarkably decreased in the presence of mdivi-1 (Fig. 3, A and B). We also measured CK activity in the differentiating myotubes. CK activity was significantly diminished in mdivi-1-treated differentiating myotubes compared with the control (Fig. 3C). Accordingly, immunostaining showed a dramatic decrease in total myosin expression levels in mdivi-1-treated myotubes derived from both C2C12 cells and primary myoblasts (day 3) (Fig. 4A, top). Analysis of the number of myonuclei per field indicated significantly fewer multinucleated myotube formations in mdivi-1-treated cells (Fig. 4A, bottom). As low as 1 μM concentration of mdivi-1 was sufficient to cause the comparatively slower myogenesis than nontreated cells, and 10 μM and 20 μM of mdivi-1 resulted in further reduction of myotube formation. Likewise, stable overexpression of a dominant negative mutant

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**Table 1. Primer sequences for semiquantitative PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequence (5′-3′) Sense/Antisense</th>
<th>Amplicon Length, bp</th>
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<tbody>
<tr>
<td>MyoD</td>
<td>CAGAAGTCTGTCCTAGATCCAGCCC/CTGTCCTGAAACCAGGAGGG/ACACATTGGGGGTAGGAACA</td>
<td>359</td>
</tr>
<tr>
<td>Myogenin</td>
<td>COTTTGAGAAGACAGATGATGGCTGGCTGAC/GAAATCAGGCCCAAGAGCCCGACC</td>
<td>300</td>
</tr>
<tr>
<td>MHC1</td>
<td>GAAGAAGAAAGCAATGGTGG/AGGTCATCTTTGGTGTACCTTTCOC</td>
<td>361</td>
</tr>
<tr>
<td>TIF1</td>
<td>CTTGATGAGTCCTGTGCTGGGA/ACCTTGGCCACTCAGTCCTGC</td>
<td>277</td>
</tr>
<tr>
<td>HPRT1</td>
<td>GCTTTTGAGGACTAGGGCAGATTCG/GCTGCGCTATAGGCTCATACTGTC</td>
<td>346</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AACCTTTGGCCTGTCGAGG/ACACATTGGGGGTAGGAACA</td>
<td>223</td>
</tr>
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Fig. 1. Dynamin-related protein 1 (Drp1) expression and translocation profile during C2C12 myotube differentiation. A: phase-contrast micrographs of undifferentiated subconfluent C2C12 myoblasts and differentiating myotubes (DM). Bar = 100 μm. B: protein expression of Drp1 in subconfluent myoblasts (SC) and differentiating myotubes. Total cell lysates were harvested and subjected to Western blot. The loading volume was normalized by the expression level of α-tubulin. N = 5 each condition. C: Drp1 dephosphorylation during early phase of C2C12 myogenesis. The phosphorylated-Drp1 (Ser-637) during differentiation was determined by Western blotting. The amount of phosphorylated-Drp1 was normalized by the amount of Drp1 protein. N = 3 each condition. D: translocalization of Drp1 from cytosol to mitochondria. Mitochondrial and cytosolic portions were fractionated from subconfluent myoblasts and differentiating myotubes as described in MATERIALS AND METHODS. Equal amounts of proteins were loaded across the samples (mitochondria: 10 μg, cytosol: 20 μg). Voltage-dependent anion channel (VDAC) and α-tubulin were used as markers for mitochondrial and cytosolic fractions, respectively. N = 3 each condition. All densitometric analyses values are shown as means ± SD. *P < 0.05 vs. SC; **P < 0.01 vs. SC.

Drp1-K38A decreased myotube differentiation compared with the control (Fig. 4B).

Inhibition of Drp1-dependent mitochondrial division and apoptosis. To assess whether inhibition of Drp1 increase cell death, we assessed the activated form of caspase-3, an executioner caspase. A 3.5-fold increase in caspase-3 activation was observed shortly after induction of myogenic differentiation (Fig. 5A). Under the Drp1-inhibited condition, increased caspase-3 activation was observed at 6 and 9 h of differentiation (Fig. 5B). Also, we observed a dramatic increase in the number of floating dead cells in mdivi-1-treated condition over the entire experimental period in a dose-dependent manner as shown in the phase-contrast micrographs (Fig. 5C). Furthermore, mdivi-1 significantly increased the number of TUNEL-positive cells (up to 10-fold) in a dose-dependent manner (Fig. 5D).

Inhibition of Drp1-dependent mitochondrial division and mitochondrial biogenesis. We first measured mtDNA copy number as a surrogate marker for mitochondrial content in differentiating myotubes that were cultured in mdivi-1 or DMSO supplemented DM for 3 days. There was a significant increase in mtDNA copy number in 0 μM of mdivi-1-treated myotubes compared with the subconfluent myoblasts. In addition, we observed a significant and dose-dependent reduction of mtDNA copy number in mdivi-1-treated myotubes compared with the subconfluent myoblasts. Interestingly, differentiation-mediated mitochondrial biogenesis was completely blocked by 20 μM of mdivi-1-treated myotubes (Fig. 6D). To confirm the observed difference in mitochondrial biogenesis, we stained cells with MitoTracker Red (Fig. 6A), a lipophilic cation dye that is sensitive to the mitochondrial membrane potential, and determined mitochondrial content and mitochondrial membrane potential by measuring percent red fluorescence-positive pixels (Fig. 6B) and fluorescence intensity (Fig. 6C) (51), respectively. Mdivi-1-treated differentiating C2C12 cells displayed significantly lower percentage of fluorescence-positive pixels. In addition, the MitoTracker Red fluorescence intensity was also significantly decreased (∼70% reduction) in the mdivi-1-treated myotubes (Fig. 6C).
ROS and myotube differentiation. Mitochondrial fission has been associated with elevated production of ROS (62). Therefore, we used a mitochondrial-targeted superoxide scavenger Mito-TEMPO and NAC, to determine a possible role of ROS in C2C12 myotube differentiation. As shown in Fig. 7, we observed that both Mito-TEMPO and NAC treatment impaired myotube formation. NAC treatment decreased myotube formation to a greater extent compared with Mito-TEMPO. Cotreatment of the antioxidants with mdivi-1 further decreased the myotube formation compared with their (H11006/M, for 1 h), and the ratio of each morphology was summarized as mean percentages ± SE. Over 200 cells were analyzed in each condition.

DISCUSSION

Although mitochondrial fusion/fission events have been consistently documented in muscle cells, the physiological importance of these processes in myogenesis remains unclear. In the present study, we report an essential role of Drp1, a master fission mediator, in myotube differentiation. We observed dephosphorylation of Drp1 (Ser-637) and consequent translocalization to mitochondria in the early phase of differentiation. Genetic and pharmacological inhibitions of Drp1 led to an impaired myotube formation that coincided with increased apoptosis and defects in mitochondrial biogenesis. These data suggest that mitochondrial dynamic events are crucial for myogenic differentiation through regulating mitochondrial remodeling.

Drp1 and apoptosis. The present study indicates that mitochondrial fission plays a critical role in myogenesis through several distinct pathways. First, we observed that a disruption of mitochondrial division led to an elevation of apoptotic potential indicated by the increases in active caspase-3.
Apoptosis concurrently occurs during myogenic differentiation through at least three different apoptotic pathways previously described (i.e., extrinsic death receptor, intrinsic mitochondrial, and ER stress-mediated) (44-45, 54, 59). In muscle progenitor cells, the dysregulation of apoptosis contributes to the low regeneration capability of aging skeletal muscle (12) and poor outcomes of stem cell-based therapy (22, 49). On the other hand, the mitochondrial fission process has been recognized as an important initial step to induce apoptosis through modulating mitochondrial outer membrane permeabilization and cytochrome c release (3, 60). Thus we initially hypothesized that inhibition of Drp1 would decrease apoptosis. Surprisingly, however, we observed that inhibition of Drp1-mediated mitochondrial fission led to increased apoptosis with only a transient reduction of cytochrome c release (data not shown). To this end, although a link between mitochondrial fission and apoptosis has been evidenced in some models, the concept of a direct relationship between these two processes has been recently challenged. For instance, apoptotic stimuli induce expression of pro-fission molecules and their mitochondrial translocalization, and suppression of Drp1 desensitizes cells to apoptotic insults (18, 48); however, in other cell culture systems, Drp1 depletion led to a higher rate of cytochrome c release and increased apoptosis of these cells (27).

**Drp1 and mitochondrial biogenesis.** We found that the imbalance between mitochondrial fission and fusion events hinders successful mitochondrial remodeling during myogenic differentiation (Fig. 6). During myogenic differentiation, mitochondria undergo extensive remodeling in terms of their size, content, and activity through a process referred to as mitochondrial biogenesis (33, 43). A number of studies demonstrated that the mitochondrial biogenesis is a necessary step in successful muscle development. For instance, enhancing (29, 42, 52) or suppressing (23, 55) mitochondrial biogenesis potently alters myogenic differentiation of myoblasts in culture and animal models. The formation of myotube was severely impaired in PGC-1α knockout model or mtDNA-depleted myoblasts (55). Moreover, previous studies showed that defects in mitochondrial fusion and fission processes were asso-

(cleaved), floating dead cells, and TUNEL-positive cells (Fig. 5). Apoptosis concurrently occurs during myogenic differentiation through at least three different apoptotic pathways previously described (i.e., extrinsic death receptor, intrinsic mitochondrial, and ER stress-mediated) (44-45, 54, 59). In muscle progenitor cells, the dysregulation of apoptosis contributes to the low regeneration capability of aging skeletal muscle (12) and poor outcomes of stem cell-based therapy (22, 49). On the other hand, the mitochondrial fission process has been recognized as an important initial step to induce apoptosis through modulating mitochondrial outer membrane permeabilization and cytochrome c release (3, 60). Thus we initially hypothesized that inhibition of Drp1 would decrease apoptosis. Surprisingly, however, we observed that inhibition of Drp1-mediated mitochondrial fission led to increased apoptosis with only a transient reduction of cytochrome c release (data not shown). To this end, although a link between mitochondrial fission and apoptosis has been evidenced in some models, the concept of a direct relationship between these two processes has been recently challenged. For instance, apoptotic stimuli induce expression of pro-fission molecules and their mitochondrial translocalization, and suppression of Drp1 desensitizes cells to apoptotic insults (18, 48); however, in other cell culture systems, Drp1 depletion led to a higher rate of cytochrome c release and increased apoptosis of these cells (27).
associated with failure of normal mitochondrial proliferation (15). Therefore, our data suggest that mitochondrial division protein Drp1 may participate in C2C12 differentiation through, at least in part, regulation of mitochondrial remodeling. Further studies are needed to identify the upstream mechanism of Drp1 activation during myogenic differentiation and the possible downstream function of Drp1-dependent mitochondrial division responsible for mitochondrial quality control during myogenic differentiation.

**Drp1 and mitochondrial ROS.** Mitochondrial fusion/fission dynamics are closely related to intracellular redox state. In particular, our data (61) as well as others (30) showed that inhibition of mitochondrial fission is associated with reduced ROS generation under certain stress conditions. Therefore, we hypothesized that the impaired myogenesis observed in mdivi-1 treatment condition is associated with limiting essential mitochondrial ROS. To test this hypothesis, we treated cells with NAC or Mito-TEMPO and determined whether these antioxidants caused similar myogenic dysregulation. As shown in Fig. 7, supplementation of these ROS scavengers in DM significantly reduced myotube formation, and the presence of mdivi-1 in each condition further decreased myogenesis. ROS, generally, is thought to be cytotoxic when excessive amount is produced and sustained. However, ROS at low concentrations has been shown to serve as an important intracellular signaling molecule mediating cellular responses. Malinska et al. (36) reported a significant increase in ROS production during myogenic differentiation, and the authors speculated that this might...
Fig. 5. Effect of inhibition of mitochondrial division on apoptosis during C2C12 differentiation. A: activation of caspase-3 in total cell lysates was examined during C2C12 myotube differentiation by Western blot with an antibody that detects both nonactivated and activated cleaved forms of caspase-3. Densitometric analyses values are shown as means ± SD; N = 3 each condition. *P < 0.05 vs. SC; **P < 0.01 vs. SC. B: caspase-3 activation in the presence or absence of mdivi-1 (10 μM) was examined at 3, 6, and 9 h of differentiation. Arrows indicate 17 kDa of cleaved caspase. N = 3 each condition. Densitometric analyses values are shown as means ± SD; N = 3 each condition. **P < 0.01 vs. 0 μM of mdivi-1. C: increased number of floating dead cells under mdivi-1 treatment conditions during myotube differentiation. DM supplemented with indicated concentration of mdivi-1 was replaced every 24 h. Phase-contrast micrographs were taken at day 1, 2, and 3 of differentiation. The bright rounded shapes represent floating cells. D: increased apoptosis determined by transferase-mediated dUTP nick-end labeling (TUNEL) staining in mdivi-1-treated differentiating myoblasts (day 1). TUNEL-positive nuclei are shown in green, and DAPI counterstainings are shown in blue. Representative fluorescence images are shown (bottom). Average percentages of TUNEL-positive cells were assessed in each condition (top left). TUNEL-positive percentages are shown as means ± SE; N = 3. **P < 0.01 vs. 0 μM of mdivi-1. Representative TUNEL-positive (DNase I treated) and -negative (without enzyme) stained C2C12 pictures are shown (top right). Bar = 100 μm.
be related to metabolic remodeling during myotube formation (36). Furthermore, it has been shown that both genetic and pharmacological inhibition of NADPH oxidase (53) or antioxidants (25) inhibit C2C12 muscle differentiation. Activation of the ROS-sensing transcription factor NF-κB and its downstream target (i.e., inhibitory nitric oxide synthase, iNOS) has been shown to be responsible for the positive regulatory effect of ROS in myogenic process (1, 31). There is, however, discrepancy regarding the exact role played by ROS in the differentiation program by altering cell cycle progression. As cell cycle withdrawal is linked to S-phase entry during cell cycle development (7, 28). Recently, mitochondrial fission/fusion dynamics have been linked to cell cycle progression such that antagonistic and balanced activities of fission/fusion proteins were critical for cell proliferation and normal embryonic development (7, 28). Thus the failure of mitochondrial biogenesis and enlargement of mitochondrial networks, which we observed here, may contribute to the switching off the normal differentiation program. Moreover, mitochondrial dynamics has been shown to be precisely coupled with cell cycle progression such that mitochondrial retrograde signaling pathways are potentially involved in myogenesis.

Studies have demonstrated that impairment of mitochondrial function and activity blocks myogenic differentiation. For instance, inhibition of a necessary metabolic shift from glycolysis to oxidative phosphorylation inhibits myogenesis (33, 43). Thus the failure of mitochondrial biogenesis and enlargement of mitochondrial networks, which we observed here, may contribute to the switching off the normal differentiation program. Moreover, mitochondrial dynamics has been shown to be precisely coupled with cell cycle progression such that antagonistic and balanced activities of fission/fusion proteins were critical for cell proliferation and normal embryonic development (7, 28). Recently, mitochondrial fission/fusion dynamics have been linked to S-phase entry during cell cycle progression (40). The levels of Drp1 are regulated in a cell cycle-dependent manner (26, 57), and cells with downregulated expression of Drp1 did not exit the cell cycle and failed to progress to differentiation (39). As cell cycle withdrawal is a prerequisite for muscle cell differentiation (20), disruption of mitochondrial dynamics may block initiation of a terminal differentiation program by altering cell cycle progression.

Interestingly, a recent study by De Palma et al. (13) reported that enhanced Drp1 activity and the resultant excessive mitochondrial fragmentation delayed myogenic differentiation, especially in the early phase of differentiation (3–12 h in DM). This study (13) elegantly demonstrated that, using a specific...
regulation of the key myogenic regulatory factors. These re-

binding to mitochondria, which was accompanied by down-

pathway increased Drp1 GTPase activity, translocation, and 

nitric oxide (NO) synthase inhibitor, inhibition of NO/cGMP 

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binding to mitochondria, which was accompanied by down- 

regulation of the key myogenic regulatory factors. These re-

Fig. 7. Reactive oxygen species (ROS) scavengers, Mito-TEMPO and NAC, impair myotube differentiation. C2C12 myoblasts were treated with DMSO (NT control), Mito-TEMPO (25 μM), or NAC (500 μM) in the presence or absence of mdivi-1 (10 μM) during myotube differentiation. MF20 immunocytochemistry staining was performed on day 3 of differentiation. Myotubes (MF-20 antibody) are shown in green, and nuclei (DAPI) are shown in blue. The densities of differentiated myotubes are presented as the number of myonuclei per field. Values are shown as means ± SE; N = 5. **p < 0.01 vs. NT (−) mdivi-1. ##p < 0.01 vs. (−) mdivi-1 of each counterpart. Bar = 100 μm.

Perspectives and Significance

In the present study, we report a novel physiological role of Drp1-dependent mitochondrial division in myoblasts survival and mitochondrial remodeling during myogenic differentiation. Further investigation is warranted to determine whether the Drp1 and mitochondrial fission process is a critical component in muscle regeneration in adult skeletal muscles. Furthermore, there seems to be a logical necessity of examining potential adverse effects of mdivi-1 treatment in vivo on muscle development and regeneration, particularly under pathological conditions such as severe muscle injuries.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: B.K., Y.Y., M.D.B., and J.-Y.P. conception and design of research; B.K. and J.-S.K. performed experiments; B.K. and Y.Y. analyzed data; B.K. and J.-Y.P. interpreted results of experiments; B.K. prepared figures; B.K. and J.-Y.P. drafted manuscript; Y.Y., M.C.S., M.D.B., and J.-Y.P. edited and revised manuscript; J.-Y.P. approved final version of manuscript.
REFERENCES


