Knockdown of metallothionein 1 and 2 does not affect atrophy or oxidant activity in a novel in vitro model

Robert D. Hyldahl,1 Kevin S. O’Fallon,1 Lawrence M. Schwartz,2,3 and Priscilla M. Clarkson1

1Muscle Biology and Imaging Laboratory, Department of Kinesiology; and 2Department of Biology, University of Massachusetts, Amherst; and 3Pioneer Valley Life Sciences Institute, Springfield, Massachusetts

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Skeletal muscle atrophy is a significant health problem that results in decreased muscle size and function and has been associated with increases in oxidative stress. The molecular mechanisms that regulate muscle atrophy, however, are largely unknown. The metallothioneins (MT), a family of genes with antioxidant properties, have been found to be consistently upregulated during muscle atrophy, although their function during muscle atrophy is unknown. Therefore, we hypothesized that MT knockdown would result in greater oxidative stress and an enhanced atrophy response in C2C12 myotubes subjected to serum reduction (SR), a novel atrophy-inducing stimulus. Forty-eight hours before SR, myotubes were transfected with small interfering RNA (siRNA) sequences designed to decrease MT expression. Muscle atrophy and oxidative stress were then measured at baseline and for 72 h following SR. Muscle atrophy was quantified by immunocytochemistry and myotube diameter measurements. Oxidative stress was measured using the fluorescent probe 5-(and-6)-carboxy-2′,7′-dichloro-dihydrofluorescein. SR resulted in a significant increase in oxidative stress and a decrease in myotube size and protein content. However, there were no differences observed in the extent of muscle atrophy or oxidant activity following MT knockdown. We therefore conclude that the novel SR model results in a strong atrophy response and an increase in oxidant activity in cultured myotubes and that knockdown of MT does not affect that response.

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Skeletal muscle atrophy occurs from a myriad of distinct stimuli. Conditions generally resulting in atrophy include immobility, disuse, denervation, or as the consequences of certain chronic disorders such as sarcopenia, cancer, AIDS, and diabetes (25, 31, 36, 42, 46). Muscle atrophy is commonly characterized by a loss of balance between protein synthesis and degradation that ultimately results in a net loss of contractile proteins (11, 24). Atrophy results in decreased muscle fiber size, force production, and fatigue resistance, which in turn can lead to weakness, instability, and a decreased quality of life.

Muscle atrophy can be mediated by a number of distinct signal transduction pathways. As examples, disease-related atrophy seems primarily to be brought about by elevated levels of cytokines and/or glucocorticoids (3, 7), while disuse atrophy appears to be triggered by intracellular signaling events as a result of decreased muscular tension (13). Muscles that no longer bear weight or are immobilized for extended periods of time initiate pathways leading to protein loss, and some of these pathways have been well characterized (1, 13). However, potential upstream molecules regulating these processes are still poorly defined.

It has been extensively documented that expression of metallothioneins (MT) is significantly upregulated in muscle cells following an atrophy stimulus in both humans and animal models (17, 18, 42, 43). MTs are ubiquitously expressed proteins with a diversity of reported intracellular functions (4). In rodents, there are four known isoforms of MT (1–4), two of which (MT1 and 2) are expressed in skeletal muscle. In humans, at least 17 isoforms have been identified. Different isoforms of MT are distributed in a wide variety of tissue and for the most part appear to have redundant functions although some specialization has been noted (35). While it has been difficult to define a consensus biological function for MTs, it is clear that they play important roles in both binding of heavy metals and as endogenous antioxidants (5). More recently, their role as antioxidants and their ability to protect against oxidative damage in various tissues have been elucidated (15).

As part of our ongoing studies to define the molecular mechanisms that mediate skeletal muscle atrophy in humans, we have performed microarray analysis with mRNA isolated from muscle biopsies 48 h after knee joint immobilization, unilateral lower limb suspension, and spinal cord injury (30a, 42, 43). This unique set of data, collected at the same time point relative to the experimental manipulation or injury for three distinct conditions, has permitted a detailed comparative analysis to ascertain a possible common program for gene expression following disuse. One of the few gene clusters to be commonly increased following all three forms of disuse was the MTs. These observations are consistent with data from animal models, where a significant increase in MT mRNA has been measured following a diverse range of atrophy-inducing manipulations (14, 18, 31). Despite the preponderance of gene expression data indicating increased expression levels of MTs following an atrophy stimulus, the potential role(s) for MT in the muscle atrophy program have not been identified. Given their ability to scavenge free radicals and protect against oxidative injury (16), it has been suggested that increases in MT expression may be an initial response to offset increases in oxidative stress associated with a muscle atrophy stimulus.

In the present study, we use an in vitro model of muscle atrophy to knockdown MT expression to test the hypothesis that MTs are protective against atrophy and oxidant activity. We chose to take an in vitro approach because 1) it facilitated the use of siRNA methodology to transiently reduce MTs, thus overcoming potential confounding compensatory mechanisms that have been described in knockout mouse models of oxidative stress-related proteins (22, 33); and 2) it enabled the use of...
an intracellular probe to measure oxidant activity in living cells over time. To test our hypothesis, we have also sought to develop and characterize a novel in vitro model of muscle atrophy in C2C12 myotubes that does not require the treatment of cultures with exogenous factors not normally associated with disuse atrophy (13). Herein we describe a simple and reliable method for the induction of atrophy in cultured myotubes and subsequently apply this model to more clearly delineate the role of MTs during muscle atrophy.

MATERIALS AND METHODS

Cell culture to induce myotube atrophy. C2C12 myoblasts were obtained from the American Type Culture Collection (ATCC) and expanded and frozen down. Only early passage cells were used in these studies. For experiments, cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Atlanta Biologicals Norcross, GA), to create a growth medium. Following 96 h in DM, fused myotubes were reintroduced into the high serum GM for 48 h to produce a hypertrophic response. A group of cells were not transferred to GM and were maintained in DM to serve as a basal myotube control condition (DM control). Of the myotubes that were switched to GM for 48 h, the experimental cells (atrophy condition) were then switched back to DM for a maximum of 72 h to produce the desired atrophic response (serum reduction [SR]). A subset of the GM enhanced cells was kept as an additional control and remained in GM (GM control). Figure 1 provides a detailed schematic of the experimental design and associated controls for the induction of atrophy via the method that we term serum reduction (SR). Data were collected at baseline (0), 6, 12, 24, 48, and 72 h under the growth factor reduced and control conditions.

siRNA. All siRNA duplexes were obtained from IDT (Coralville, IA) and used at a final concentration of 10 nM for knockdown experiments. The sequences for the MT1 and MT2 siRNA duplexes were as follows: MT1, 5'-AGCGUCUUCUACAGUCCAGGUUCCCATCCC-3'; and MT2, 5'GUAAUAAGACCAUGUAGAAGCCUAG-3'. A standard nonsilencing, scrambled control siRNA duplex was obtained from IDT.

Transfections. Following 96 h of differentiation (the onset of GM reintroduction), myotubes were transfected in either 24-well plates, 6-well plates, or slide flasks with 10 nM MT1, MT2, or scrambled control siRNA nucleotides using lipofectamine RNAi max (Invitrogen, Carlsbad, CA). This time point was chosen so that MT1 and MT2 expression would be reduced at the onset of DM reintroduction (0 h). Both lipofectamine and the appropriate siRNA sequence(s) were first diluted individually in Opti-MEM I Reduced Serum medium (Invitrogen). The diluted lipofectamine and siRNA sequence(s) were then mixed and allowed to complex for 20 min at room temperature. Lipid/siRNA complexes were added to each well at a concentration of 10 nM in an appropriate volume for each plate size and incubated for 24 h. After 24 h, transfection medium was replaced with GM. A scrambled, nonsilencing sequence was transfected into parallel wells for all experiments. To determine transfection efficiency and siRNA stability, parallel cultures were also transfected with TYE-563 labeled double-stranded control sequences (IDT) under the same conditions. Myotubes were then visualized and photographed using a Nikon model TMS inverted fluorescence microscope and a SPOT Insight imaging color camera (Diagnostic Instruments, Sterling Heights, MI). Efficiency of knockdown was also determined on the RNA level with quantitative real-time PCR (qRT-PCR) and on the protein expression level using Western blot analysis.

Immunocytochemistry. To assess changes in myotube morphology, myoblasts were grown in chamber slide flasks and treated as above. At each time point, myotubes were fixed for 2 min in 2% paraformaldehyde at room temperature. The myotubes were then permeabilized for 3 min with Karsenti’s Lysis Buffer (0.5% Triton X-100, 80 mM PIPES, 1.0 mM MgSO4, 5.0 mM EGTA, pH 7.0), after which they were rinsed twice with PBST (PBS, 0.1% Tween-20). Myotubes were then incubated with the mouse monoclonal MF20 antibody against myosin heavy chain (Developmental Hybridoma Bank, Iowa City, IA) for 60 min in the dark at room temperature. After three washes with PBST, the myotubes were incubated with AlexaFluor 488 anti-mouse (Invitrogen) for 60 min in the dark. Myotubes were washed again three times in PBST and nuclei stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO). Samples were visualized and measured as described below.

Myotube morphology analysis. Myotubes were visualized and photographed with a 20× objective on a Nikon model TE-2000 inverted microscope and a SPOT Insight imaging color camera (Diagnostic Instruments). The diameters of the myotubes were quantified using Metamorph Imaging software (Molecular Devices, Downingtown, PA) at 12, 24, 48, and 72 h time points under all experimental and control conditions. Approximately 100 diameters were randomly selected from 12 representative microscope fields in each condition from three or four independent experiments. Three lines running perpendicular to the orientation of the fixed myotubes were drawn at evenly spaced intervals across each field using the Metamorph software. Diameter measurements were made in micrometers at all points.
where the line crossed a myosin heavy chain (MHC)-positive myotube.

Total RNA, DNA, and qRT-PCR. Plates were scraped and the myotubes separated from the residual mononucleated myoblasts by filtering through a 50-μm sieve. Verification of the separation was made via phase-contrast microscopy. Total RNA was extracted from myotubes at baseline (0), 6, 12, 24, and 48 h following SR using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA concentration was determined by optical density on the NanoDrop ND-100 spectrophotometer (NanoDrop Products, Wilmington, DE). Total RNA was reverse transcribed using a cloned murine leukemia virus reverse transcriptase (Fermentas, Glen Burnie, MD) according to the manufacturer’s instructions. The relative levels of MT1, MT2, arogin1, and MuRF1 mRNAs were determined by quantitative real-time PCR (qRT-PCR). ABgene Absolute qPCR SYBR Green Master Mix (ABgene, Surrey, UK) with ROX dye was used for all PCR protocols. qRT-PCR reactions were performed in 96-well plates with all cDNA samples from each time point for both experimental and control myotubes run in triplicate for each gene of interest. The average cycle threshold (Ct) value for triplicate samples was used for data analysis. Samples were run for 40 cycles of amplification on a MX3000p real-time PCR System (Stratagene, La Jolla, CA). At the end of each reaction, a melting curve analysis was run to ensure target specificity. Differences in gene expression were determined by the delta Ct relative quantification method. Values were normalized to the levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an internal reference gene whose expression did not change in response to the treatment (data not shown). Forward and reverse primers (IDT) for all genes of interest (see Table 1 for sequences) were designed using NCBI gene sequences with the Primer Express program v 2.0 (Applied Biosystems, CA). All primers were tested for efficiency via a standard curve and demonstrated efficiencies between 95 and 104%.

Western blotting and antibodies. C2C12 myotubes were isolated as above, and incubated in lysis buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 0.25% NP-40, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate (Sigma-Aldrich), which was then incubated with the primary antibody in 5% BSA in 1X PBST with rocking motion overnight at 4°C. After washing in PBS supplemented with 0.5% Tween-20, the membrane was incubated with a horseradish peroxidase (HRP)-labeled secondary antibody. Membranes were then treated with enhanced chemiluminescence (ECL) for 2 min, wrapped in plastic film, and exposed to Kodak film. Bands from each blot were quantitated via densitometry and normalized to the loading control using ImageJ software (NIH). The antibodies used include rabbit polyclonal anti-GAPDH (1:5,000; Abcam, Cambridge, MA); mouse anti-MHC monoclonal antibody MF20 (1:100, Developmental Hybridoma Bank, Iowa City, IA); rabbit polyclonal anti-phospho-Akt (ser473) (1:500, Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-metallothionein antibody (which recognizes all MT isoforms) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA); and anti-mouse and anti-rabbit HRP-conjugated secondary antibodies (1:1,000, Bio-Rad Laboratories, Hercules, CA).

Assessment of intracellular oxidant activity. A 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate, diacetate, acetyl ester (CM-H2DCFDA) (Invitrogen) assay was used to measure net intracellular oxidant activity and provided a nonspecific measure of oxidative stress. The chemically reduced parent compound (CM-H2DCFDA) is nonfluorescent until the acetate groups are removed by intracellular esterases. Oxidation by reactive oxygen and nitrogen species (RONS) occurs within the cell and forms fluorescent dichlorofluorescein (DCF). Fresh stock solutions of CM-H2DCFDA were reconstituted in cell culture grade DMSO (ATCC) and diluted in 1X PBS (≤0.01% vol/vol PBS) immediately before each experiment. Extracellular hydrolysis of CM-H2DCFDA was minimized using PBS as a loading buffer. After dye loading, a ~15-min recovery period was given to allow cellular esterases to hydrolyze the acetate groups on the dye that prevent oxidation. This process renders the dye responsive to oxidation by free radicals. All experiments were conducted in darkness to reduce inadvertent photo bleaching. Before experimental manipulations, myotubes cultured in sterile 96-well plates were washed once with PBS and incubated in loading buffer (PBS supplemented with 10 μM CM-H2DCFDA) for ~30 min at 37°C. Cells were then washed twice with PBS and incubated for ~15 min (recovery period) prior to returning myotubes to their corresponding experimental culture medium (serum reduced or control). DCF fluorescence was measured at 37°C on a fluorescence multimicroplate reader (FLUOstar Optima, BMG LABTECH) at excitation and emission wavelengths of 485 and 520 nm, respectively. Cells were maintained and measured at 37°C, and all experiments were conducted in a darkened laboratory to prevent spurious DCF photo-oxidation. Transit time between the incubator, tissue culture hood, and microplate reader were minimized at all times. Baseline DCF fluorescence was measured immediately before SR and at baseline (0), 6, 12, 24, and 48 h post-SR. To account for any artifactual DCF fluorescence (i.e., background signal) observed due to potential DCFH leakage from myotubes over time or unknown nonoxidant cellular processes related to atrophy, the raw data were processed by subtracting the mean fluorescence values of cell-free controls (i.e., cell free wells containing 10 μM DCFH in either DM or GM only) from the mean fluorescence values of each condition (i.e., myotube-rich wells loaded with 10 μM DCFH) at each time point. Net intracellular oxidant activity in the serum-reduced conditions was expressed as the percentage of DCF fluorescence in the control condition (high serum). The rate of change in intracellular oxidant production was calculated as the average slope of the normalized values between time points. To minimize the variability in DCF signal between replicates, the cell seeding density was standardized across all wells in each 96-well microplate and the DCF signal was measured in the same cells over time.

Statistical analysis. Data are presented as means ± SE. All qRT-PCR, morphology, Western blot, and DCF data were analyzed using a two-way ANOVA with factors for treatment and time. Where there was a significant effect, Tukey’s honest significant difference test was applied. All statistical analyses were computed using a SAS statistical software package. Significance was set a priori at P < 0.05.

Table 1. Forward and reverse primer sequences

<table>
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<tr>
<th>Gene Name</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Atrogin 1</td>
<td>5′-AGGCTCTGTAATATTGAGCTGGGA-3′</td>
<td>5′-TCCCAGTCGTTTGGACAGAAGA-3′</td>
</tr>
<tr>
<td>MuRF1</td>
<td>5′-TCGAGTGGGTTTGGACAGAGACAGA-3′</td>
<td>5′-AGGCTCTGTAATATTGAGCTGGGA-3′</td>
</tr>
<tr>
<td>MT 1</td>
<td>5′-ACACAGACTCAAGGCTGCTGAGT-3′</td>
<td>5′-TGAGGCTGCACCTTGACCTGAGAT-3′</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-GGAAATCTCCAGGTCACTCC-3′</td>
<td>5′-ACAGATTGAGGTTAAGAA-3′</td>
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</tbody>
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RESULTS

Development of an in vitro model for skeletal muscle hypertrophy and atrophy. To examine the role(s) of metallothioneins in muscle atrophy, we needed to develop an appropriate in vitro model. After several experimental paradigms, we selected the one outlined in Fig. 1. Briefly, we differentiated C2C12 myoblasts into myotubes by changing the cells from a growth factor rich-growth medium (GM) to a differentiation medium (DM) that was deficient in these factors. After 96 h in DM, myotubes were returned to GM for 48 h. Control cultures were either maintained in DM for the entirety of the protocol or remained in GM for the subsequent 72 h while test cultures (referred to as “SR” for serum reduced) were returned to DM for the same period of time.

We then tested the hypothesis that SR results in myotube atrophy as measured by myotube diameter and protein loss. Most importantly, myotube diameter was significantly different at all time points under the SR condition compared with baseline (0 h) levels ($P < 0.05$) (Fig. 2, A and B). Myotube diameter was expressed in micrometers, rather than as a percentage of control, to demonstrate that significant decreases in size were being driven by SR rather than an increase in size of the GM control myotubes. Myotube diameter was also found to be significantly decreased in SR cultures relative to the GM control cells at 24, 48, and 72 h ($P < 0.01$). DM control myotubes, which remained in DM (were not enhanced by GM) for the duration of the protocol, did not significantly change in size over time, indicating that changes in myotube size were not due to maturation of the myotubes, but rather, the serum stimulus. Further confirmation of morphological changes following SR was assessed by changes in the protein expression of the sarcomeric protein MHC. We found that 24, 48, and 72 h of SR was effective at reducing MHC over time compared with baseline (0 h) levels ($P < 0.01$) (Fig. 2, C and D). Also noted was a significant increase in MHC protein level over time in the GM control condition ($P < 0.01$) and significant differences between SR and GM control myotubes ($P < 0.01$). GAPDH was used as a loading control for the Western blots.

SR-mediated changes in protein synthesis/degradation pathway components. To further characterize the SR model of atrophy, we used Western blot and qRT-PCR analyses to monitor the expression of several key regulatory proteins following SR. Because there were no differences in size or protein content of DM control myotubes, we did not include that control in the subsequent experiments. Phosphorylation of the serine/threonine kinase protein kinase B (Akt) is associated with muscle cell growth (1). The levels of Akt phosphorylation at Ser473 significantly declined over time following transfer to

Fig. 2. Serum reduction (SR) reduces myotube diameter and myosin heavy chain (MHC) protein content. A: representative images from DM control, GM control, and SR myotubes at baseline (0), 12, 24, 48, and 72 h stained with an antibody against MHC. Scale bar equals 100 μm. B: myotube diameter of DM control, GM control, and SR myotubes at baseline (0), 12, 24, 48 and 72 h; data are expressed as mean diameter in micrometers ± SE from 4 independent experiments. *Significant difference from baseline (0 h) within the same condition ($P < 0.05$). *Significant difference from GM control ($P < 0.05$). 

C: representative Western blots of MHC and GAPDH (loading control) expression from DM control, GM control, and SR myotubes at baseline (0 h), 24, 48, and 72 h. D: MHC protein in SR treated myotubes from 4 independent experiments expressed in arbitrary units. *Significant difference from baseline (0 h) within the same condition ($P < 0.05$). #Significant difference from GM control ($P < 0.05$).
SR (Fig. 3. A and B). In contrast, transfer of myotubes into GM (GM control) resulted in Akt phosphorylation at 12 and 72 h. SR treatment also led to increased expression of the atrophy-associated ubiquitin E3 ligases atrogin1 and muscle ring finger protein 1 (MuRF1) at 48 h by 1.8- and 2.1-fold, respectively, when normalized to control cells (P < 0.05) (Fig. 3C).

**Metallothionein expression in SR and siRNA transfected myotubes.** To determine if MT is induced in our SR model similarly to other atrophy models, we measured endogenous MT1 and MT2 expression at various time points following SR. Expression of MT1 and MT2 mRNA increased by 2.3- and 2.6-fold, respectively, at 6 h post-SR compared with control myotubes (Fig. 4A). There were no other significant differences in expression of either gene at any other measured time point. Both genes also tended to follow a very similar pattern of expression.

We next designed siRNA sequences that would selectively target MT1 and MT2 transcripts. Following cotransfection of myotubes with these siRNAs 48 h before SR, we observed via qRT-PCR that there were 78% and 69% reductions in the basal levels of MT1 and MT2 mRNA, respectively, at the 0 h time point (beginning of the SR stimulus). At 24 h post-SR, there were 82% and 69% reductions for MT1 and MT2, respectively (Fig. 4B). Western blot analysis was used to verify that decreases in MT mRNA resulted in decreases in protein expression. At 48 h post-MT1 and MT2 siRNA cotransfection (0 h), there was a notable reduction in MT protein (Fig. 4C).

**Oxidant activity in SR and siRNA transfected myotubes.** One of the more well-characterized roles for MTs in cells is to quench RONS levels (15). To test our hypothesis that MT1 and MT2 function to reduce oxidative stress following an atrophic stimulus, myotubes were preloaded with DCF and then fluorescence intensity was measured (in the same cells) at 0, 6, 12, 24, and 48 h post-SR. SR resulted in a significant increase in oxidant activity over time compared with control myotubes (P < 0.01) (Fig. 5, A–C). The greatest changes in oxidant activity occurred at the early time points between 6 and 12 h post-SR (Fig. 5C) and maximal oxidant activity was observed at 24 and 48 h post-SR (Fig. 5, A and B). Knockdown of MT expression with siRNAs did not significantly reduce SR-induced changes in oxidant activity at any time point (Fig. 5, A–C).

**Myotube diameter in siRNA transfected myotubes.** To determine the effect of MT1 and MT2 knockdown on the development of muscle atrophy, we cotransfected myotubes with an siRNA sequence specific for MT1 and MT2 or a control, nonsilencing siRNA 48 h before SR. Myotube size was calcu-
lated by measuring myotube diameters in each condition. Representative images of siRNA, control siRNA, and nontreated (SR) myotubes following 48 h of SR are shown in Fig. 6A. Knockdown of MT1 and MT2 did not augment myotube atrophy as we had hypothesized. There was no difference in myotube diameter between any condition at 24 or 48 h post-SR (Fig. 6, A and B). Similarly, under the GM control condition (high serum) MT1 and MT2 knockdown had no effect on myotube size or morphology (data not shown).

DISCUSSION

As early as 1992, Kondo et al. (17) first observed changes in MT expression following an atrophy stimulus. Since then numerous reports in both animals and humans have shown increased MT gene expression in response to a muscle atrophy perturbation (14, 31, 36, 42, 43). Inquiries into the role that MTs play during atrophy, however, are recent. The prevailing hypothesis to explain rises in MT expression during atrophy has suggested that MTs may play a role in the oxidative stress response that accompanies muscle atrophy (30), thereby providing a source of protection from oxidative damage. This hypothesis is indeed plausible considering the reported antioxidant capacity of MTs in other tissues (16). Thus we sought to test this hypothesis by knocking down the expression of the muscle-expressed MT isoforms MT1 and MT2 before applying a novel atrophy stimulus in cultured myotubes. Contrary to our hypothesis, we found that MT1 and MT2 knockdown did not affect myotube atrophy or oxidant activity. However, we found that serum reduction (SR) induced a significant increase in oxidant activity within 6 h (∼200%), which remained elevated (relative to GM controls) for the duration of the experimental protocol. The increase in oxidant activity was inversely proportional to the subsequent decline in myotube diameter and MHC protein content at 24 and 48 h, demonstrating that SR induced a rapid increase in oxidant activity, followed by significant myotube atrophy. The temporal relationships between SR, increased oxidant activity, and myotube atrophy suggest that increased oxidant activity may initiate activation of proteolytic pathways (e.g., calpains, caspases, and the ubiquitin proteasome pathway) responsible for muscle catabolism. In this particular atrophy model, the mechanisms by which SR induced increased oxidant activity are currently unknown; however, the withdrawal of growth factors from the culture medium is likely the primary initial stimulus for increased oxidant activity and subsequent myotube atrophy.

It is well known that increased oxidant activity stimulates proteolytic pathways and leads to significant muscle atrophy.
For example, Li et al. (21) demonstrated that incubation of C2C12 myotubes with 100 μM H2O2 upregulated proteolytic gene expression of MuRF1, atrogin1/MAFbx, and E214k within 3–6 h of exposure, and increased the rate of ubiquitin conjugation to muscle proteins in whole cell extracts. Notably, the increase in ubiquitin conjugating activity occurred within 4 h of exposure to H2O2 and persisted for 24 h (21). More recently, McClung et al. (23) showed that exogenous treatment of C2C12 myotubes with H2O2 induced significant atrophy within 24 h. In that study, H2O2 treatment induced oxidative damage, calpain-1 protease activation, and diminished content of several sarcomeric proteins. Those studies revealed that increased oxidant activity stimulated muscle catabolic pathways within 6 h and induced significant muscle atrophy within 24 h of exposure. In our model, we used a different approach (SR) to induce myotube atrophy; nonetheless, we observed a significant increase in oxidant activity (within 6 h) that peaked, and remained elevated at 24–48 h. Moreover, elevated oxidant activity corresponded with diminished myotube diameter and MHC protein content (i.e., characteristics of atrophy) at 24 and 48 h. Collectively, those findings demonstrate that oxidant activity plays a key role in protein catabolism, regardless of whether atrophy is induced via serum reduction (as in the present study) or via exogenous H2O2 treatment [as previously reported (21, 23)].

Various sites and sources of RONS production have been identified in skeletal muscle that contribute to net intracellular oxidant activity (as observed in this study) leading to cellular damage and muscle atrophy. Superoxide (SO) is generated in the mitochondria at complex I and III (during state 4 respiration), the plasma membrane, and sarcoplasmic reticulum by NADPH oxidase complexes (8, 29). The enzymatic dismutation of SO gives rise to H2O2 in mitochondrial and cytoplasmic compartments, and to a lesser extent, forms the highly damaging hydroxyl radical. Nitric oxide (NO) is generated by neuronal nitric oxide synthase (nNOS) and can react with SO to form peroxynitrite, which is also highly damaging, although NOS-mediated oxidant formation does not appear to contribute to atrophy (29, 34). In the present study, the nonspecific RONS probe CM2-DCFDA was used to quantify net intracellular oxidant activity in living muscle cells and the contribution of individual RONS to oxidant activity and atrophy in this model is unknown. At present, it is unclear which individual RONS are the key players in oxidative damage and redox signaling during muscle atrophy, nor is it known which subcellular compartments (e.g., mitochondria, cytoplasm, nucleus, and sarcoplasmic reticulum) contribute to net intracellular oxidant activity and muscle catabolism (29).

The development of a novel in vitro model of muscle atrophy. Our experimental approach necessitated that we develop an in vitro model of muscle atrophy in C2C12 myotubes. Several models of muscle atrophy have been developed previously (20, 41), most of which have relied on either the treatment of cell cultures with exogenous glucocorticoids or cytokines. However, previous work has noted the unpredictability of the effect of these agents on muscle culture response. For example, TNF-α has been shown in vitro to reduce muscle protein content, promote muscle wasting, and inhibit myogenesis (10, 20). In contrast, a similar concentration of TNF-α has been demonstrated to have mitogenic effects by promoting myoblast proliferation and enhancing myoblast fusion and differentiation (19). Likewise, in our hands, both glucocorticoid and TNF-α treatment has resulted in varying effects on myotube size (unpublished observations). Therefore, it became important for us to develop and characterize a more reliable model of muscle atrophy in cultured myotubes.

Here we have shown that a SR protocol is a simple and reliable stimulus to achieve atrophy of myotubes and loss of contractile protein content. Furthermore, we have demonstrated this effect by the simple manipulation of standard cell culture medium, rather than the treatment of cultures with exogenous agents. In this, we believe that we have developed a model of considerable value for the future study of muscle atrophy. Although others have experimented with serum stimulation as a means to produce myotube hypertrophy (26), we are the first to use the serum-stimulated (hypertrophied) myotubes as a platform to then induce a muscle atrophy response via subsequent serum reduction.

We then extended these analyses to examine changes in key signaling pathways that are associated with muscle atrophy in vivo. It has been shown that changes in Akt phosphorylation can regulate the induction of atrophy by control of the forkhead box (FOXO) family of transcription factors, which control the expression of the ubiquitin E3 ligases atrogin1 and MuRF1 (32, 37). Thus we measured both Akt phosphorylation and expression of the genes atrogin1 and MuRF1 in response to SR. We observed decreases in Akt phosphorylation, which correlated with concomitant increases in atrogin1 and MuRF1 mRNA abundance at 48 h post-SR. However, it should be noted that myotube atrophy occurred primarily between 0 and 24 h post-SR during which there were no measurable changes in Akt phosphorylation or atrogin1 and MuRF1 expression. This could mean that these molecular changes were not associated with myotube atrophy in our model, or that our detection methods were insufficiently sensitive to detect the initial changes in mRNA and protein abundance. While the bulk of the literature support a role for decreases in Akt phosphorylation following unloading or immobilization in rodents (1, 2, 39), others have reported muscle atrophy following unloading with no associated changes in Akt phosphorylation or its downstream targets (44). Furthermore, unloading or disuse-related atrophy can be driven by changes in signaling pathways distinct to the Akt signaling pathway (9, 12, 29).

Muscle atrophy and oxidative stress following MT knockdown. We chose to test our hypothesis using siRNA directed at the two muscle-expressed MT isoforms. Immunoblot analysis confirmed that we were able to significantly reduce the amount of MT protein in our transfected cells. Because our antibody recognized multiple isoforms of MT, we also show via qRT-PCR specific reductions in our targets MT1 and MT2. Contrary to our hypothesis, targeted knockdown of the MT isoforms MT1 and MT2 did not affect myotube atrophy or alter the levels of intracellular cytosolic oxidant activity in our model. Our observations are consistent with and strengthen recent in vivo data published by DeRuisseau et al. (6), who also failed to detect either a decline in fiber cross-sectional area or oxidative injury in soleus muscles following spinal cord transection in MT1−/−/MT2−/− mice. Interestingly, they did report greater soleus muscle contractile impairment following spinal cord transection in these null mice. These observations, together with our data, suggest that MTs may not directly influence the muscle atrophy response. DeRuisseau et al. (6) speculate that
MTs are involved in the regulation of calcium handling during pathological conditions and thus may result in diminished contractile force. These changes in calcium homeostasis may result from the metal binding capacity of MT proteins. For example, regulation of zinc and other metals is important in modulating muscle force and work capacity in skeletal muscle (45) and likely is important in zinc finger transcription factor activity and thus overall gene expression under various stress stimuli.

There are a number of published reports supporting a role for MTs as protective antioxidants in various tissues (16, 27, 38, 40). For example, Sun et al. (40) were able to demonstrate reduced cardiac myopathy in response to an oxidative stress stimulus using a MT-overexpressing transgenic mouse model. There is, however, little evidence supporting this role in skeletal muscle. Penkowa et al. (28) have documented increased expression of MT1 and MT2 protein in response to acute exercise, a stimulus known to increase oxidative stress. They suggested that the upregulation of MTs represented a response to counter oxidative stress-induced damage to muscle fibers, but there were no data provided to substantiate this suggestion. More recently, there was no difference shown in lipid peroxidation, protein oxidation, or the expression of endogenous antioxidant enzymes in skeletal muscle of MT1 and MT2 deficient mice 7 days following spinal cord transection (6), suggesting that the antioxidant role of MTs is minimal during atrophy. Here we show, with the use of an in vitro intracellular oxidant probe, that Sr results in a significant increase (~3-fold relative to controls) in net intracellular oxidant activity, which is sustained for 48 h; yet the knockdown of MT1 and MT2 did not significantly alter that activity. Therefore, within the parameters of our in vitro model, we could not support the hypothesis that MTs play a significant role in the endogenous antioxidant response to a muscle atrophy stimulus. Although clearly more work needs to be done to define a protective antioxidant role for MTs in skeletal muscle, the accumulating evidence would suggest that, if at all, they perform a minor function in this capacity.

Conclusion. In conclusion, we have described a novel, nonpathological in vitro model for both muscle atrophy and hypertrophy. The model requires only the manipulation of standard culture medium and thus may be of considerable value for future mechanistic studies of muscle atrophy. Using siRNA knockdown methodology, we were unable to demonstrate a role for MTs 1 and 2 in either atrophy or intracellular oxidant activity within the parameters of our in vitro atrophy approach. These findings are corroborated by recent in vivo knockout mouse data; and together, they lend strong support to the notion that, despite the reports of widespread mRNA increases during atrophy, MTs may not play a direct functional role in the atrophy response.

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DISCLOSURES

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REFERENCES


