Striated muscle activator of Rho signaling is required for myotube survival but does not influence basal protein synthesis or degradation

Marita A. Wallace and Aaron P. Russell
Centre for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin University, Burwood, Victoria, Australia

Submitted 24 December 2012; accepted in final form 22 May 2013

Wallace MA, Russell AP. Striated muscle activator of Rho signaling is required for myotube survival but does not influence basal protein synthesis or degradation. Am J Physiol Cell Physiol 305: C414–C426, 2013. First published May 29, 2013; doi:10.1152/ajpcell.00421.2012.—Skeletal muscle mass is regulated by sensing and transmitting extracellular mechanical stress signals to intracellular signaling pathways controlling protein synthesis and degradation. Striated muscle activator of Rho signaling (STARS) is a muscle-specific actin-binding protein that is sensitive to extracellular stress signals. STARS stimulates actin polymerization and influences serum response factor (SRF) and peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α transcription of genes involved in muscle growth, structure, and contraction. The role of STARS in skeletal muscle cells is not well understood. This study investigated whether STARS influenced C2C12 myotube growth by regulating protein synthesis and degradation. The influence of STARS on Pgc-1α, Srf, and Errα mRNA levels, as well as several of their downstream targets involved in muscle cell growth, contraction, and metabolism, was also investigated. STARS overexpression increased actin polymerization, with no effect on protein synthesis, protein degradation, or Akt phosphorylation. STARS overexpression increased Pgc-1α, Srf, Ckm2, Cpt-1β, and Mhc1 mRNA. STARS knockdown reduced actin polymerization and increased cell death and dead cell protease activity. It also increased markers of inflammation (Casp1, Il-1β, and Mep-1), regeneration (Socs3 and Myh5), and fast myosin isoforms (Mhc2a and Mhc2x). We show for the first time in muscle cells that STARS overexpression increases actin polymerization and shifts the muscle cell to a more oxidative phenotype. The suppression of STARS causes cell death and increases markers of necrosis, inflammation, and regeneration. As STARS levels are suppressed in clinical models associated with increased necrosis and inflammation, such as aging and limb immobilization, rescuing STARS maybe a future therapeutic strategy to maintain skeletal muscle function and attenuate contraction-induced muscle damage.

STARS; sarcomere; exercise; disuse; skeletal muscle

SKELETAL MUSCLE GROWTH is a process tightly controlled by signaling pathways regulating protein synthesis and protein degradation (14, 15, 45). When protein synthesis is greater than protein degradation, there is a net increase in protein accretion and skeletal muscle hypertrophy, a response influenced by increased mechanical loading, such as resistance exercise (23, 30, 61). When protein degradation occurs faster than protein synthesis, there is a net loss of muscle protein, resulting in skeletal muscle atrophy, a response observed following muscle disuse and unloading and in numerous chronic diseases (14, 15, 45). The activation of signaling pathways controlling muscle cell protein synthesis and degradation is dependent on the detection and transmission of extracellular stress signals (11). Once activated, intracellular signaling cascades stimulate transcription factors that influence skeletal muscle remodeling through changes in gene expression (4). Molecular targets that are sensitive to extracellular stress signals for the control of skeletal muscle hypertrophy and atrophy may present as potential therapeutic targets to combat muscle-wasting diseases and disorders.

The striated muscle activator of Rho signaling (STARS, also known as Abra and ms1) is a muscle-specific actin-binding protein (2, 31, 39, 58). STARS is sensitive to extracellular stress signals and, when activated, localizes to the sarcomere and stimulates actin polymerization (2). This has been shown to increase the nuclear translocation of the serum response factor (SRF) transcriptional coactivator myocardin-related transcription factor A (MRTF-A) (2) and then to enhance SRF activity (2, 24). SRF can transcribe genes that encode for proteins involved in muscle growth, structure, and contraction, such as IGF-I, actin, and myosin (6, 33). Combined overexpression of STARS and MRTF-A in rat primary cardiomyocytes increases phosphorylation of Akt (51), a key kinase known to positively regulate muscle growth and attenuate muscle wasting (14). We previously demonstrated that STARS is a transcriptional target of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1α/estrogen-related receptor (ERR)-α signaling (60), a pathway regulating genes involved in muscle protein translation, structure, and contraction, as well as substrate metabolism and angiogenesis (21, 43, 48). STARS knockdown inhibits transcription of the PGC-1α/ERRα target gene carnitine palmitoyltransferase-1B (Cpt-1β) (60). These observations suggest that STARS may be required for effective PGC-1α/ERRα transcriptional regulation.

Few studies have investigated the ability of STARS to directly regulate muscle size. Forced overexpression of STARS results in hypertrophy of H2c9 rat cardiomyocytes (22). Additionally, controlled overexpression of STARS in mouse cardiac tissue results in an exaggerated hypertrophic response, but only when it is combined with pressure overload (25). An increase in STARS gene expression is associated with skeletal muscle hypertrophy in humans after 8 wk of resistance training (26). With respect to conditions of muscle atrophy, STARS gene expression is reduced in skeletal muscle of aging mice and pigs (39, 46), after atrypathy-inducing detraining in humans (26), and after hindlimb suspension in rats (13), but not humans (47).

Together, these observations suggest that STARS may play a direct role in regulating skeletal muscle growth; however, this is yet to be investigated. Therefore, the primary aim of the present study was to determine whether STARS directly influences muscle cell growth by regulating C2C12 myotube protein synthesis and degradation, as well as Akt and p70 S6 kinase (p70S6K) phosphorylation. A secondary aim was to
determine if STARS regulates SRF and PGC-1α/ERKα target genes.

METHODS

Cell culture. For all experiments, C2C12 mouse myoblasts (American Type Culture Collection, Manassas, VA) were plated in six-well tissue culture plates in complete DMEM (10% FBS; Life Technologies, Melbourne, Australia) at a density of 1.5 × 10⁴. As the cultures approached confluence (~90% confluent), medium was changed to differentiation medium (DMEM) supplemented with 2% horse serum (Life Technologies), which was replaced every 48 h. For protein synthesis and degradation experiments, myoblasts were plated and allowed to differentiate as described above. Treatment and control media were adjusted to pH 7.4 before they were applied to the cells.

Adenoviral infection. Adenoviral constructs containing mouse STARS (Adv-STARS) or the control gene LacZ (Adv-LacZ) were generous gifts from Eric N. Olson (University of Texas Southwestern Medical Center). On day 4 of differentiation, C2C12 myotubes were infected with an adenovirus expressing STARS (Adv-STARS) at a multiplicity of infection of 100 or a vector control adenovirus (Adv-LacZ). After 48 h of infection, protein synthesis, protein degradation, and immunofluorescence analysis of actin polymerization were performed. RNA and protein were also harvested for RT-PCR gene expression and Western blot analysis.

Small interfering RNA transfection. STARS knockdown in C2C12 myotubes was achieved using 100 pmol of Stealth RNAi small interfering RNA (siRNA, target sequence 5′-TCCAGTCCCCAGAAGCTCAATCCCTT-3′; Life Technologies) over a 72-h period, as published by our group previously (60). Stealth RNAi siRNA Negative Control Hi GC (Life Technologies) was used as the control siRNA, which contains the same GC content as the target sequence. siRNA transfection of C2C12 myotubes was performed using 5 μl of Lipofectamine 2000 (Life Technologies).

Protein synthesis. Protein synthesis was determined by measurement of [³H]tyrosine (GE Healthcare, Sydney, NSW, Australia) incorporation into the myotubes (modified from Ref. 41). After adenoviral infection, C2C12 myotubes were treated for 24 h with or without 10 μM dexamethasone (Dex; Sigma-Aldrich, St. Louis, MO) to induce catabolic stress or 100 nM insulin (Ins) to induce anabolic stress. Also during this 24-h period, 1 mM L-tyrosine for 2 h (reading A). During this 24-h period, 1 mM L-tyrosine (Sigma-Aldrich) were added to the myotubes. The use of excess nonradioactive tyrosine in the medium gives an accurate indication of protein synthesis rates without alterations of the free intracellular tyrosine pool (59). Then myotubes were washed twice with cold PBS, and 1 ml of cold 10% trichloroacetic acid (TCA; Sigma-Aldrich) was added to each well. After they were scraped, the myotubes remained on ice for 1 h for precipitation of the protein and then centrifuged at 20,000 × g for 10 min. The supernatant was removed, and the precipitates were dissolved in 0.1 M NaOH with 1% Triton X-100 (Sigma-Aldrich) overnight at room temperature. Then 400 μl of this sample were mixed with 4 ml of Ultima Gold scintillation liquid (PerkinElmer, Boston, MA), and radioactivity was measured using a Wallac 1420 multishift scintillation counter (PerkinElmer) and expressed as counts per minute. Protein synthesis counts were normalized to total genomic DNA (41) in the myotubes. Genomic DNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen, Melbourne, Australia) according to the manufacturer's instructions. DNA was quantified using a spectrometer (model 2000, NanoDrop Products, Wilmington, DE).

Protein degradation. Protein degradation was determined by measurement of [³H]tyrosine release from the myotubes into the medium. After adenoviral infection, C2C12 myotubes were washed in serum-free high-glucose DMEM and then labeled with 2 μCi/ml [³H]tyrosine for 24 h. The myotubes were washed again in serum-free high-glucose DMEM and then incubated in 2 mM l-tyrosine for 2 h. After they were washed again, the myotubes were incubated in serum-free DMEM with 2 mM unlabeled tyrosine with or without 10 μM Dex or 100 nM Ins for 24 h for measurement of protein degradation under basal, catabolic, and anabolic conditions. Thereafter, 450 μl of the medium were added to 50 μl of TCA and left to precipitate on ice for 1 h. After centrifugation at 13,000 rpm for 5 min, 400 μl of the supernatant were added to 4 ml of Ultima Gold scintillation liquid, and TCA-soluble radioactivity was measured on the Wallac 1409 DSA liquid scintillation counter (reading A). Remaining medium from this sample was removed, and the pellet was solubilized in 500 μl of 0.5 M NaOH-0.1% Triton X-100 for 2 h at 37°C. Subsequently, 400 μl of this medium were added to 4 ml of scintillation liquid, and TCA-insoluble radioactivity was measured (reading B). The remaining medium from the culture plates was removed, the myotubes were washed twice with cold PBS, and the sample was incubated in 500 μl of 0.5 M NaOH-0.1% Triton X-100 for 2 h at 37°C. Another 500 μl of 10% TCA were added to the sample, which was incubated overnight at 4°C to precipitate the protein in the myotubes. On the following day, 400 μl were added to 4 ml of scintillation liquid, and myotube radioactivity was measured (reading C). Proteolytic rate was defined as TCA-soluble radioactivity (reading A), divided by the sum of TCA-soluble radioactivity (reading A), TCA-insoluble radioactivity (reading B), and myotube radioactivity (reading C), and then multiplied by 100. All radioactivity measurements are expressed as counts per minute.

Myotube size and actin polymerization. C2C12 myotubes were prepared on 35-mm μ-Dishes (ibidi, Munich, Germany) for quantification of myotube size and immunofluorescence analysis of F-actin expression using phalloidin staining (36). Cells were fixed for 10 min with 4.0% paraformaldehyde-PBS (Sigma-Aldrich), permeabilized with 0.1% Triton X-100-PBS for 5 min, and then blocked in 1.0% BSA-PBS for 1 h at room temperature. Antibody staining required incubation of myotubes at room temperature for 20 min with 5 μl Alexa Fluor 546-phalloidin (Life Technologies) to label F-actin, 9 μg/ml Alexa Fluor 488-DNase I (Life Technologies) to label G-actin, and 0.1 μg/ml 4,6-diamidino-2-phenylindole (Sigma-Aldrich) to label nuclei in 1% BSA-PBS. All images were obtained using the Fluoview FV10-W microscope (Olympus, Melbourne, Australia). For quantification of myotube size, diameter, and length were measured using Olympus Fluoview software. The amount of red fluorescent staining following the incubation with the phalloidin antibody was measured using ImageJ (National Institutes of Health, Bethesda, MD). An average of 10 myotubes were analyzed from 10 different fields of view for each treatment.

Cytotoxicity assay. To determine the cytotoxic effect of STARS knockdown in C2C12 myotubes, the Cytotoxicity assay (Promega, Madison, WI) was used according to the manufacturer's instructions to measure the relative number of dead cells per well. Briefly, 100 μl of the bis-AAF-R110 substrate were mixed with 10 ml of assay buffer, and 100 μl of this reagent were added to 100 μl of cell medium in each well of a 96-well plate. The plate was briefly subjected to orbital shaking and then incubated for 30 min at 37°C. Resulting fluorescence was measured using a multimode microplate reader (Synergy 2, BioTek, Winooski, VT) with filters set at 485 nm for excitation and 520 nm for emission. Serum-starved myotubes were used as the positive control and empty wells (no cells) as the negative control.

Apoptotic DNA laddering analysis. At 72 h after transfection with the siRNA sequences, DNA was extracted from the myotubes with use of an apoptotic DNA ladder kit according to the manufacturer's protocol. DNA samples were run on 1% (wt/vol) agarose-Tris-acetate-EDTA gel, and electrophoresis was performed in 1× Tris-acetate-EDTA buffer at 100 V for an appropriate time. DNA samples were run alongside a 1 Kb Plus DNA ladder and the apoptotic positive control (lyophilized apoptotic U937 cells). Gel images were obtained using the Gel Logic 112 imaging system (Kodak Scientific Imaging Systems, Rochester, NY) and a UV transilluminator (Vilber Lourmat, Marne la Vallée, France).
RNA extraction. Total RNA was extracted using TRI Reagent solution (Ambion, Austin, TX) according to the manufacturer’s protocol. First-strand cDNA was generated from 1–2 μg of RNA in 20 μl of reaction buffer using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. Before dilution of CDNA, 1 μl of RNase H (Life Technologies) was added to each sample, and the sample was incubated at 37°C for 30 min. Then cDNA was diluted to 5 ng/μl in nuclease-free water and stored at −20°C until further analysis.

Real-time quantitative PCR. Real-time PCR was carried out using the Stratagene MX3000 PCR system (Agilent Technologies, Santa Clara, CA) to measure mRNA levels of genes of interest. To compensate for variations in input RNA amounts and efficiency of the reverse transcription, data were normalized to ribosomal protein 36B4 (also known as RPLPO) mRNA levels or single-stranded DNA (ssDNA) concentration, which was quantified using the Quant-iT OliGreen ssDNA Assay Kit (Molecular Probes, Eugene, OR). PCR primers for the genes are provided in Table 1.

Protein extraction. Total protein was extracted using 1× RIPA buffer (Millipore, Billerica, MA) or SRF (catalog no. sc-335, Santa Cruz Biotechnology), PGC-1α/H9251, Cpt-1α/H9251, Ckmt2/NM_009807.2, Casp1α/H9251/NM_007475.5, Primers used for PCR analysis.

C416 STARS IS REQUIRED FOR MYOTUBE SURVIVAL

Tnf Stars Myh8 Mrtf-a Mhc2x NM_030679.1 NM_010855.2
Mhc2b Mhc2a NM_080728.2 NM_0139545.2
Mhc1 Mhc2a NM_0080728.2 NM_001039545.2
Mhc2b Mhc2a NM_010855.2 NM_0080728.2
Mhc1 Mhc2a NM_0080728.2 NM_001039545.2
Mhc2b Mhc2a NM_010855.2 NM_0080728.2
Mhc1 Mhc2b NM_010855.2 NM_0080728.2
Mhc2a Mhc2b NM_0080728.2 NM_001039545.2

Table 1. Primers used for PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>36B4</td>
<td>NM_007457.5</td>
<td>TTTGTCGAGGAGACAAATGGG</td>
<td>AGTCCCTGTTTTGACAGC</td>
</tr>
<tr>
<td>α-actin</td>
<td>NM_009606.2</td>
<td>GAGGTCGCTATTTCTCCTG</td>
<td>CTTTGAATGGGAGACAC</td>
</tr>
<tr>
<td>α-actinin2</td>
<td>NM_032689.4</td>
<td>CTGGTCCTCGACAAACAGCA</td>
<td>TTGACGATGGTTCTC</td>
</tr>
<tr>
<td>Casp1α</td>
<td>NM_009807.2</td>
<td>AAGGGACAAAGTTTAGGAAGAAGAA</td>
<td>GAGCGATGACAGGATTTGATTA TATCAT</td>
</tr>
<tr>
<td>Ckmt2α</td>
<td>NM_007953.1</td>
<td>CCTGCTCTCGTCTACGTCAG</td>
<td>CTTGAGCAGACAGGATCT</td>
</tr>
<tr>
<td>Erα</td>
<td>NM_010512.4</td>
<td>GGGGCTCAGAGAAAGAATC</td>
<td>TACAAGGTTTGAGAGGAC</td>
</tr>
<tr>
<td>Erβ</td>
<td>NM_008361.3</td>
<td>TCTGGCAAACCTTCTGTTG</td>
<td>CCAAGTACTGAGGAGGAC</td>
</tr>
<tr>
<td>Mrp-1</td>
<td>NM_011333.3</td>
<td>ACCCTGAGGTCGATGACCGA</td>
<td>TGGAGCCCCATAAGGACCGA</td>
</tr>
<tr>
<td>Mhc1α</td>
<td>NM_0080728.2</td>
<td>GACCCGATGTACTGAGCGAA</td>
<td>GAAGTTGACGAGGAGGAC</td>
</tr>
<tr>
<td>Mhc2aα</td>
<td>NM_0030679.1</td>
<td>GGACTGCGCTGATGACCGA</td>
<td>CAGGATGATGACAGGAGG</td>
</tr>
<tr>
<td>Myh8α</td>
<td>NM_153049.2</td>
<td>CACATTCTGGAAGAGACCGTG</td>
<td>AGATCTGCTCTGAGGAGG</td>
</tr>
<tr>
<td>Pgc-1α</td>
<td>NM_008904.2</td>
<td>TCAAGGGCCCTTTGAGTGC</td>
<td>GTGAGAGGTGTGGTCTT</td>
</tr>
<tr>
<td>Srf</td>
<td>NM_01392.2</td>
<td>AGACGCTTCACAGACTGAAG</td>
<td>AGGAGCTTCAGAGGAGGAG</td>
</tr>
<tr>
<td>Stars</td>
<td>NM_175456.4</td>
<td>TCAAGGGCCCTTTGAGTGC</td>
<td>GTGAGAGGTGTGGTCTT</td>
</tr>
<tr>
<td>Tnfa</td>
<td>NM_013693.2</td>
<td>CCCAGAAGGGAAATTGAGGGA</td>
<td>GCTGTGAGGAGGAGGAGGAG</td>
</tr>
</tbody>
</table>

RESULTS

STARS was overexpressed in C2C12 myotubes by transduction with an adenovirus expressing mouse STARS; Adv-LacZ was used as the infection control. This resulted in a 13- and 2.9-fold increase in STARS mRNA (Fig. 1A) and STARS protein (Fig. 1B) levels, respectively. STARS overexpression resulted in no change in C2C12 myotube length or diameter compared with LacZ control (Fig. 1, C, E, and F). To determine whether STARS overexpression regulated actin polymerization, F-actin expression was measured by phalloidin staining. A 60% increase in actin polymerization was observed in C2C12 myotubes overexpressing STARS (Fig. 1, D and G).

To investigate the effect of STARS overexpression on protein synthesis and degradation in C2C12 myotubes, we measured the incorporation and release, respectively, of [3H]tyrosine. STARS overexpression did not alter protein synthesis (Fig. 2A) or protein degradation (Fig. 2B) levels under basal conditions.
conditions in C2C12 myotubes. Dex treatment decreased protein synthesis by 18%, and Ins treatment increased protein synthesis by 34%. The combination of STARS with Dex or Ins did not influence protein synthesis levels. Dex treatment increased protein degradation by 17%, while Ins treatment decreased protein degradation by 23%. Again, the combination of STARS with Dex or Ins did not affect protein degradation levels. No differences were observed in total protein content (Fig. 2C) or phosphorylation of Akt (Fig. 2D) or p70S6K (Fig. 2E).

Fig. 1. Striated muscle activator of Rho signaling (STARS) overexpression in C2C12 myotubes increases actin polymerization. A and B: Stars mRNA and STARS protein levels in C2C12 myotubes at 48 h after infection with control adenovirus [Adv-LacZ (LacZ)] or adenovirus expressing STARS [Adv-STARS (STARS)]. Values are expressed in arbitrary units (AU, n = 6 per group). *P < 0.05 vs. LacZ. C and D: phase-contrast images of cells (C) and F-actin staining (D) using phalloidin in C2C12 myotubes 48 h after infection with Adv-LacZ or Adv-STARS. E and F: myotube diameter and length measured using Olympus Fluoview software. G: quantification of actin polymerization by measurement of red fluorescent staining following incubation with phalloidin antibody using ImageJ. An average of 10 fibers were analyzed from 10 different fields of view for each treatment group. *P < 0.05 vs. LacZ.
The STARS gene is a PGC-1α/ERRα and SRF transcriptional target. As gene expression of PGC-1α, ERRα, and SRF can be autoregulated by their own downstream gene targets, it was of interest to investigate whether a feedback loop existed between STARS and its transcriptional regulators. STARS overexpression increased Pgc-1α and Srf mRNA levels by 34% and 35%, respectively (Fig. 3, A and B). Despite this increase in mRNA levels, there was no change in PGC-1α or SRF protein levels following STARS overexpression (Fig. 3, C and D). Several PGC-1α target genes, including Cpt-1, creatine kinase mitochondrial 2 (Ckmt2), and Mhc1, were increased by 105%, 45%, and 31%, respectively (Fig. 4, A–C).

The mRNA levels of Errα and several downstream SRF target genes, including α-actin, Igf-1, and Mhc2a, as well as other myosin isoforms, such as Mhc2b and Mhc2x, remained unchanged (data not shown). As several oxidative metabolism genes were upregulated with STARS overexpression, it was of interest to investigate if STARS enhances typical markers of oxidative metabolism, such as citrate synthase activity and COX IV protein levels. At 48 h after STARS overexpression, there was no change in citrate synthase activity (Fig. 4, D) or COX IV protein levels (Fig. 4, E).

Knockdown of STARS in C2C12 myotubes by siRNA transfection resulted in an 80% and 47% decrease in Stars mRNA levels of Errα and several downstream SRF target genes, including α-actin, Igf-1, and Mhc2a, as well as other myosin isoforms, such as Mhc2b and Mhc2x, remained unchanged (data not shown). As several oxidative metabolism genes were upregulated with STARS overexpression, it was of interest to investigate if STARS enhances typical markers of oxidative metabolism, such as citrate synthase activity and COX IV protein levels. At 48 h after STARS overexpression, there was no change in citrate synthase activity (Fig. 4, D) or COX IV protein levels (Fig. 4, E).

Knockdown of STARS in C2C12 myotubes by siRNA transfection resulted in an 80% and 47% decrease in Stars mRNA levels.
mRNA (Fig. 5A) and STARS protein (Fig. 5B) levels, respectively. Several visual observations were noted following the knockdown of STARS. 1) There was cell debris floating in the wells of myotubes with reduced levels of STARS, which was not observed in the wells of the siCON myotubes, indicating that this observation was not an artifact of the transfection conditions. After the debris was removed and the cells were gently washed, a reduction in the number of myotubes was observed following STARS knockdown (Fig. 5C). 2) There was a 42% reduction in actin polymerization (Fig. 5, rows in Fig. 5, and membrane, an indication of increased cell death and damage (arrows). 3) There was an increased presence of myotubes that appeared to be rounding up and/or displaying blebs on the cell membrane, an indication of increased cell death and damage (arrows in Fig. 5, C–E).

To confirm these visual observations, dead cell protease activity was measured. A 13% increase in dead cell protease activity (Fig. 6A) and an 18% decrease in total protein content (Fig. 6B) were measured following STARS knockdown. It was of interest to establish if STARS knockdown regulated myotube protein synthesis and degradation. However, because of the increase in cell death following STARS knockdown, the accuracy of protein synthesis and degradation measurement in vitro was compromised. For example, the protein degradation assay involves prelabeling of proteins with a radioactive amino acid ([14C]tyrosine) prior to the treatment of interest. Protein degradation levels are calculated by dividing TCA-soluble radioactivity by the radioactivity in prelabeled proteins. As cell death would result in loss of prelabeled proteins, the calculation of protein degradation levels is inaccurate. The presence of cell membrane blebbing is an indication of necrotic and apoptotic cell death. As STARS knockdown increases cell death and membrane blebbing, typical apoptotic markers were measured to determine whether cell death occurred via apoptotic pathways. While apoptosis increases DNA fragmentation, no differences were observed between siSTARS- and siCON-transfected myotubes (Fig. 7A). Additionally, no differences were seen between cleaved caspase-3 (CASP3) and cleaved PARP protein levels following STARS knockdown (Fig. 7B).

Necrotic death results in membrane disruption, release of cellular contents into the extracellular environment, and a strong inflammatory response in surrounding cells. To determine if cell death was due to necrosis, gene expression of necrotic and inflammation markers was measured. STARS knockdown increased caspase-1 (CASP1; Fig. 8A), IL-1β (IL-1β, Fig. 8B), and monocyte chemoattractant protein 1 (Mcp-1, Fig. 8C) mRNA levels by 2.3-, 10.7-, and 1.5-fold, respectively. No differences were observed in tumor necrosis factor-α (Tnfα) mRNA levels (Fig. 8D). Markers of myotube regeneration were also measured to investigate whether the increase in inflammation following STARS knockdown was associated with myotube regeneration. Suppressor of cytokine signaling 3 (Socs3, Fig. 8E) and myosin heavy chain 8 (Myh8, Fig. 8F) mRNA levels increased by 90% and 61% following STARS knockdown.

STARS knockdown did not alter PGC-1α and SRF mRNA and protein expression (data not shown). However, the levels of the PGC-1α target genes Cpt-1β (Fig. 9A) and Ckmt2 (Fig. 9B) were reduced by 47% and 71%, respectively, and the level of Mhc1 expression was reduced by 29% (Fig. 9C). Despite this decrease in the mRNA levels of genes involved in oxidative metabolism, there was no change in citrate synthase activity (Fig. 9D) or COX IV protein levels (Fig. 9E). STARS knockdown resulted in a 37% increase in the SRF target gene α-actin (Fig. 10A) and a 2.2-fold increase in the sarcomeric z disk-associated protein α-actinin2 (Fig. 10B), as well as 7.8- and 1.7-fold increases in the fast-twitch myosin genes Mhc2a and Mhc2x (Fig. 10, C and D), respectively. No difference in Mhc2b mRNA levels was observed (data not shown).
DISCUSSION

Gene expression of STARS, a muscle-specific protein, increases in models of hypertrophy and declines in conditions of muscle atrophy in skeletal muscle (13, 26, 39, 42, 46). STARS stimulates an intracellular signaling pathway known to regulate muscle growth, suggesting that STARS may be involved in the regulation of skeletal muscle mass. However, it is yet to be established whether the increase or decrease in STARS gene expression is a cause or a consequence of muscle growth or muscle wasting, respectively. The present study demonstrates several novel and important findings. 1) Overexpression of STARS increases actin polymerization; however, this did not affect protein synthesis, protein degradation, or Akt phosphorylation. 2) Overexpression of STARS increased the mRNA levels of Pgc-1α and Srf, as well as several PGC-1α/ERRα target genes, such as Ckm2 and Cpt-1β, and the slow myosin isoform Mhc1. 3) Knockdown of STARS decreased actin polymerization and increased cell death, dead cell protease activity, markers of inflammation, including Casp1, Il-1β, and Mcp-1, and markers of regeneration, such as Socs3 and the
embryonic myosin Myh8. 4) STARS knockdown moved the myotubes toward a faster contractile phenotype, as indicated by a reduction of Mhc1 and an increase in Mhc2a and Mhc2x. There was also an increase in α-actin and α-actinin2.

STARS is an actin-binding protein that localizes to the sarcomere (2) and increases actin polymerization, MRTF-A nuclear translocation, and SRF reporter activity (2, 24). SRF transcriptionally regulates genes involved in muscle structure, function, and growth (6, 33), as well as oxidative metabolism (35). Overexpression of STARS in rat cardiomyocytes is associated with increased expression of several SRF target genes and muscle cell hypertrophy (22). Therefore, STARS may be an important upstream mediator of muscle growth and function. The results from the present study show that overexpression of STARS in differentiated mouse C2C12 myotubes also increases actin polymerization. However, this did not alter myotube diameter and length, protein synthesis and degradation, or Akt and p70S6K phosphorylation. Forced overexpression of STARS in the heart amplifies the hypertrophic response, but only after the addition of a mechanical loading stress (25), suggesting that overexpression of STARS alone is not sufficient to cause a hypertrophic response in cardiac muscle. However, an elevated level of STARS in cardiac muscle increases the sensitivity of the cell to extracellular mechanical stress signals, resulting in enhanced cardiac growth. Whether STARS overexpression in skeletal muscle also increases the sensitivity of the muscle to mechanical stress-induced protein synthesis and muscle growth is not known and will be the focus of future investigations. In addition to extracellular stress-induced growth, muscle is also sensitive to intracellular anabolic and catabolic stress signals, such as Ins and Dex, respectively. Elevated levels of STARS did not influence muscle cell protein synthesis or degradation in the presence of Ins or Dex. This suggests that STARS overexpression does not increase the sensitivity of the cell to intracellular catabolic or anabolic stress signals. STARS overexpression in rat primary cardiomyocytes only increases phosphorylated Akt levels in the presence of overexpressed MRTF-A (51). These observations further support the notion that STARS expression alone does not directly influence muscle hypertrophy signaling pathways in C2C12 muscle cells. Therefore, the observation that STARS is upregulated in hypertrophied skeletal muscle (26, 42) is most likely a consequence, and not a cause, of skeletal muscle hypertrophy.

STARS is a transcriptional target of PGC-1α/ERRα signaling (60), and it can also regulate its own transcription via a feed-forward mechanism requiring SRF binding to the serum response element on the STARS promoter (7). In the present study, it was observed for the first time that STARS overexpression increases Pgc-1α and Srf mRNA levels; however, protein expression of Pgc-1α and SRF remained unchanged. This discordance in changes in mRNA and protein levels at the time points measured may be due to posttranslational events controlling protein translation and stability (16). STARS overexpression also increases mRNA levels of several PGC-1α/ERRα target genes, such as Ckm1 (3, 17) and Cpt-1β (20, 34). Although STARS increased Srf gene expression, there was no change in SRF target genes involved in muscle growth, such as Igf-1, or contractile function, such as α-actin and Mhc2b. These observations suggest that a forced upregulation of STARS, at least under basal, nonmechanically stressed conditions, has a greater impact on transcriptional pathways involved in muscle oxidative metabolism, rather than growth and contractile function. An increase in PGC-1α expression has been correlated with markers of oxidative metabolism, such as COX IV expression and citrate synthase activity, in rat cardiac and skeletal muscle (12). However, in the present study the increased mRNA levels of Pgc-1α and several of its transcriptional targets following STARS overexpression did not alter citrate synthase activity or COX IV protein expression in C2C12 myotubes. These differences may be a reflection of in vivo vs. in vitro models.

STARS expression is reduced in conditions associated with reduced physical activity, mechanical loading, and muscle atrophy, such as aging, detraining, and hindlimb suspension (13, 39, 46), conditions associated with increased inflammation, necrosis, apoptosis, and loss of muscle mass (1, 8, 19, 27). In the present study, knockdown of STARS in myotubes resulted in an increase in cell death, dead cell protease activity, cellular debris, and membrane blebbing. There was also a decrease in the number of myotubes and total protein content. Membrane blebbing, loss of membrane integrity, and inflammation are typical signs of necrotic death (10). Membrane blebbing, DNA fragmentation, and cleavage of PARP following caspase-3 activation are also signs of cells undergoing apoptotic death (10). STARS knockdown in C2C12 myotubes increased the mRNA expression of several proteins involved in inflammation, such as Casp1, Il-1β, and Mcp-1; however, no
change in DNA fragmentation or protein levels of cleaved CASP3 and PARP was observed. Caspase-1, a key inflammatory enzyme, cleaves and activates inflammatory cytokines such as IL-1β (9). Caspase-1 and IL-1β are part of the inflammasome pathway and have been associated with inflammation following muscle damage and necrotic death (44). Caspase-1 is also more highly expressed in necrotic cell lines (37). Therefore, the cell death following STARS knockdown is

Fig. 7. STARS knockdown does not increase apoptotic cell death. A: DNA fragmentation of extracts from C2C12 myotubes 72 h after transfection with siCON or siSTARS. Lane 1, DNA ladder; lane 2, apoptotic positive control; lane 3, siCON; lane 4, siSTARS. B: cleaved caspase-3 (CASP3) and poly(ADP-ribose)polymerase (PARP) protein levels 72 h after transfection with siCON or siSTARS (n = 6 per group).

Fig. 8. STARS knockdown increases gene expression of inflammation and regeneration markers. A–F: caspase-1 (CASP1), IL-1β (IL-1β), monocyte chemoattractant protein 1 (Mcp-1), tumor necrosis factor-α (Tnfα), suppressor of cytokine signaling 3 (Socs3), and myosin heavy chain 8 (Myh8) mRNA levels 72 h after transfection with siCON or siSTARS (n = 6 per group). *P < 0.05 vs. siCON.
more than likely caused by necrosis, rather than apoptosis. A hallmark of necrotic death is the loss of membrane integrity (10). As STARS binds and stabilizes the sarcomere and the actin cytoskeleton (2), an increase in necrosis following STARS knockdown would potentially result in a loss of membrane structural integrity. This may have direct clinical relevance, as STARS is decreased in skeletal muscle from older animals (39, 46) and susceptibility to contraction-induced damage is increased, partly due to compromised sarcomeric structure (29). Rescuing STARS in the elderly may be a therapeutic strategy to reduce contraction-induced muscle damage and maintain muscle mass and function.

Inflammation plays an essential role in skeletal muscle regeneration following damage or injury (56). In the present study an increase in gene expression of the perinatal myosin heavy chain isoform Myh8, as well as Socs3, a cytokine-inducible negative regulator of cytokine signaling, was observed following STARS knockdown. Increased gene expression of Myh8 and Socs3 has been observed in several models of muscle regeneration, such as exercise, cardiotoxin treatment, hindlimb ischemia, and Duchenne muscular dystrophy (38, 52, 57). In addition, expression of MCP-1 is also essential for effective inflammatory response and muscle regeneration following injury (50). These findings suggest that STARS knockdown activates signals promoting myotube death and damage, as well as signals for muscle regeneration.

STARS knockdown altered the myosin heavy chain isoform profile: there was a reduction in the slow oxidative Mhc1
isofrom and an increase in the fast-oxidative Mhc2a and fast-glycolytic Mhc2x isofroms, as well as a reduction in Cpt-1β and Ckmt2. In combination, these changes in gene expression suggest a shift from a slow-oxidative to a fast-glycolytic muscle fiber phenotype. A slow-to-fast isofrom conversion is observed in humans and rodents following skeletal muscle unloading (40, 54, 55), models that also present decreases in STARS (13, 26). In addition, this switch in skeletal muscle myosin heavy chain isofrom expression is also observed in patients with chronic heart failure, a condition characterized by systemic inflammation (62). It is unknown if changes in the myosin heavy chain isofrom are a direct effect of reduced STARS or an indirect effect of the increased inflammatory response in the presence of reduced levels of STARS. Despite the reduction in mRNA expression of several oxidative metabolism genes, STARS knockdown did not alter the basal activity of citrate synthase or protein expression of COX IV.

STARS knockdown in C2C12 myotubes and primary neonatal rat ventricular myocytes decreases SRF activity (24). In the present study, STARS knockdown decreased actin polymerization, a cellular process required to increase SRF activity by STARS (2). However, this result was associated with an increased gene expression of the SRF target gene α-actin. This increase in α-actin may be due to the SOCS3 activation of SRF. It has previously been shown that SOCS3 stimulates SRF transcriptional activity and directly increases α-actin gene expression in C2C12 myotubes (52). Therefore, a potential loss of SRF transcriptional activity following STARS knockdown in muscle cells may be compensated by the upregulation of other proteins, such as SOCS3, in an attempt to maintain SRF target gene transcription and muscle contractile function. Also, in the current study, STARS knockdown upregulated α-actinin-2, a protein that plays an important structural role by cross-linking actin at the z-disc of the sarcomere (28). α-Acetin-2 interacts with a variety of proteins involved in skeletal muscle structure, contraction, mechanosensing, and intracellular signaling pathways involved in muscle hypertrophy (28) and, therefore, like STARS, is seen as an important link between muscle function and muscle adaptation. As STARS and α-actinin-2 seem to have similar localization, binding characteristics, and potential functions in skeletal muscle, it is plausible that the increase in α-actinin-2 expression following STARS knockdown is an attempt to maintain muscle structure and function.

While the present study demonstrates several novel and important findings, there are limitations to these observations. The C2C12 mouse myotubes used in the present study display similarities to skeletal muscle, such as the expression of metabolic, myogenic, and sarcomeric proteins, and similarities in their response to anabolic and catabolic compounds (5, 32, 49). C2C12 myotubes can spontaneously contract; however, they lack neural innervation and the contractile synchronicity of multiple muscle fibers (18). Therefore, the effect of STARS as a regulator of protein synthesis was investigated under basal conditions and following intracellular anabolic and catabolic stress. Investigation of the impact of STARS overexpression in skeletal muscle in vivo will be required to determine its role in mechanically induced skeletal muscle growth.

In conclusion, we show for the first time in C2C12 myotubes that STARS overexpression increases actin polymerization without a parallel increase in muscle cell growth. STARS increases Pgc-1α and Srf mRNA levels and shifts the muscle cell to a more oxidative phenotype. The suppression of STARS results in cell death and increases markers of necrosis, inflammation, and regeneration. As STARS levels are suppressed in clinical models associated with increased necrosis and inflammation, such as aging and limb immobilization, rescuing STARS may be a future therapeutic strategy to maintain skeletal muscle function and attenuate contraction-induced muscle damage.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.A.W. and A.P.R. are responsible for conception and design of the research; M.A.W. performed the experiments; M.A.W. and A.P.R. analyzed the data; M.A.W. and A.P.R. interpreted the results of the experiments; M.A.W. and A.P.R. prepared the figures; M.A.W. and A.P.R. drafted the manuscript; M.A.W. and A.P.R. edited and revised the manuscript; M.A.W. and A.P.R. approved the final version of the manuscript.

REFERENCES

C425


