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Striated muscle activator of Rho signalling (STARS) is a PGC-1α/oestrogen-related receptor-α target gene and is upregulated in human skeletal muscle after endurance exercise

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Non-technical summary Exercise improves the ability of skeletal muscle to metabolise fats and sugars. For these improvements to occur the muscle detects a signal caused by exercise, resulting in changes in genes and proteins that control metabolism. We show that endurance exercise increases the amount of a protein called striated muscle activator of Rho signalling (STARS) as well as several other proteins influenced by STARS. We also show that the amount of STARS can be increased by signals directed from proteins called peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC-1α) and oestrogen-related receptor-α (ERRα). We also observed that when we reduce the amount of STARS in muscle cells, we block the ability of PGC-1α/ERRα to increase a gene called carnitine palmitoyltransferase-1β (CPT-1β), which is important for fat metabolism. Our study has shown that the STARS pathway is regulated by endurance exercise. STARS may also play a role in fat metabolism in muscle.

Abstract The striated muscle activator of Rho signalling (STARS) is an actin-binding protein specifically expressed in cardiac, skeletal and smooth muscle. STARS has been suggested to provide an important link between the transduction of external stress signals to intracellular signalling pathways controlling genes involved in the maintenance of muscle function. The aims of this study were firstly, to establish if STARS, as well as members of its downstream signalling pathway, are upregulated following acute endurance cycling exercise; and secondly, to determine if STARS is a transcriptional target of peroxisome proliferator-activated receptor gamma co-activator 1-α (PGC-1α) and oestrogen-related receptor-α (ERRα). When measured 3 h post-exercise, STARS mRNA and protein levels as well as MRTF-A and serum response factor (SRF) nuclear protein content, were significantly increased by 140, 40, 40 and 40%, respectively. Known SRF target genes, carnitine palmitoyltransferase-1β (CPT-1β) and jun B proto-oncogene (JUNB), as well as the exercise-responsive genes PGC-1α mRNA and ERRα were increased by 2.3-, 1.8-, 4.5- and 2.7-fold, 3 h post-exercise. Infection of C2C12 myotubes with an adenovirus-expressing human PGC-1α resulted in a 3-fold increase in S*tars* mRNA, a response that was abolished following the suppression of endogenous ERRα. Over-expression of PGC-1α also increased *Cpt-1β, Cox4* and *Vegf* mRNA by 6.2-, 2.0- and 2.0-fold, respectively. Suppression of endogenous STARS reduced basal *Cpt-1β* levels by 8.2-fold and inhibited the PGC-1α-induced increase in *Cpt-1β* mRNA. Our results show for the first time that the STARS signalling pathway is upregulated in response to acute endurance exercise. Additionally, we show in C2C12 myotubes that the STARS gene is a PGC-1α/ERRα transcriptional target. Furthermore, our results suggest a novel role of STARS in the co-ordination of PGC-1α-induced upregulation of the fat oxidative gene, CPT-1β.

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Abbreviations Abra, actin-binding rho activating protein; CPT-1β, carnitine palmitoyltransferase 1β; COX4, cytochrome c oxidase subunit IV; DAPI, 4,6-diamidino-2-phenylindole; ERRα, oestrogen-related receptor-α; IGF-1, insulin-like growth factor-1; JUNB, jun B proto-oncogene; MRTF-A, myocardin-related transcription factor-A; ms1, myocyte stress-1; MHCI, myosin heavy chain I; MHCIIa, myosin heavy chain IIa; PGC-1α, peroxisome proliferator-activated receptor gamma co-activator-1α; SRF, serum response factor; STARS, striated muscle activator of rho signalling; VEGF, vascular endothelial growth factor.

Introduction

Skeletal muscle contraction is a catalyst for numerous physiological adaptations including enhanced substrate metabolism, growth and regeneration (Hawke, 2005; Hawley *et al.* 2006; Leger *et al.* 2006*a*; Russell, 2010). For these skeletal muscle adaptations to occur the extracellular signals must be detected, then transmitted to the cell (Schiaffino *et al.* 2007). These signals result in the intracellular activation of transcriptional co-activators and transcription factors (McGee *et al.* 2006) with a consequent increase in their target genes that encode proteins mediating metabolic, structural and contractile function of skeletal muscle (Hawley *et al.* 2006; Hood, 2009). Understanding the molecular targets and intracellular signals activated by exercise is important for identifying potential therapeutic targets to combat diseases characterized by perturbed skeletal muscle metabolism, growth and regeneration.

The striated muscle activator of Rho signalling (STARS), also known as myocyte stress-1 (ms1) (Mahadeva *et al.* 2002) and actin-binding rho activating protein (Abra) (Troidl *et al.* 2009), is an actin-binding protein specifically expressed in cardiac, skeletal and smooth muscle (Arai *et al.* 2002; Mahadeva *et al.* 2002; Troidl *et al.* 2009). STARS, in part in collaboration with RhoA, enhances the binding of free G-actin to F-actin filaments, resulting in enhanced or stabilized actin polymerization (Arai *et al.* 2002). As a consequence there is a reduction in free G-actin, which permits the nuclear translocation of the transcriptional co-activator myocardin-related transcription factor-A (MRTF-A) (Sotiropoulos *et al.* 1999). MRTF-A is a transcriptional co-activator of SRF (Miralles *et al.* 2003), a transcription factor known to regulate genes involved in muscle metabolism and growth (Charvet *et al.* 2006; Miano *et al.* 2007). *STARS* gene expression is increased in rat heart during pressure overload (Mahadeva *et al.* 2002) and its mRNA and proteins levels are increased in rabbit smooth muscle following fluid shear stress-induced arteriogenesis (Troidl *et al.* 2009). Forced over-expression of STARS causes cardiac hypertrophy in mice (Kuwahara *et al.* 2007) and increases the proliferation of porcine smooth muscle cells (Troidl *et al.* 2009). STARS mRNA, as well as several members of the STARS signalling pathway, are upregulated in hypertrophied human skeletal muscle following increased muscle loading caused by resistance exercise training and downregulated following reduced training (Lamon *et al.* 2009). STARS has therefore been suggested to provide an important link between the transduction of external stress to intracellular signalling pathways and the control of genes involved in the maintenance of muscle function (Arai *et al.* 2002; Troidl *et al.* 2009; Russell, 2010).

Moderate-intensity skeletal muscle contraction induced by endurance exercise has positive health implications for diseases such as diabetes, obesity and cardiovascular disease as well as maintaining skeletal function in the elderly (Booth *et al.* 2002; Ventura-Clapier *et al.* 2007; Koutroumpi *et al.* 2008). It has recently been observed that endurance exercise increases the phosphorylation of SRF (Rose *et al.* 2007), a key downstream target of the STARS signalling pathway (Arai *et al.* 2002; Kuwahara *et al.* 2005). Whether endurance exercise increases the levels of STARS, members of the STARS signalling pathway and/or downstream transcriptional targets involved in muscle metabolism and growth has not been investigated.

The STARS gene has recently been identified as a transcriptional target of MEF2 in cardiac muscle (Kuwahara *et al.* 2007) as well as MyoD in C2C12 myotubes (Ounzain *et al.* 2008). Analysis of the human STARS promoter reveals also an oestrogen-related receptor-α (ERRα) binding site, located 150 bp upstream of the transcriptional start site. ERRα, in combination with its transcriptional co-activator PGC-1α, is known to regulate genes involved in skeletal muscle function (Hock & Kralli, 2009). As both ERRα and PGC-1α are upregulated in human skeletal muscle following endurance exercise (Cartoni *et al.* 2005; Russell *et al.* 2005), it is possible that STARS is a target of the PGC-1α/ERRα transcriptional program.

Therefore the primary aim of the present study was to determine if STARS and members of its downstream signalling pathway, including MRTF-A and SRF, as well as several SRF target genes including CPT-1β, JUNB, α-actin and insulin-like growth factor-1 (IGF-1), are upregulated

following acute endurance exercise. A secondary aim was to determine if the STARS gene is regulated via PGC-1α/ERRα signalling.

Methods

Subjects

Nine healthy male subjects participated in the study: age 24 ± 5 years, weight 84 ± 10 kg, single leg $\dot{V}_{O_2max}$ 36.6 ± 3.8 ml kg^{-1} min^{-1}. The study was approved by the Deakin University Human Research Ethics Committee in accordance with the *Declaration of Helsinki* (2000). All participants gave their informed consent and agreed to muscle biopsies and physiological testing.

Preliminary testing

Subjects were familiarised with the electromagnetically braked cycle ergometer (Lode B.V, Groningen, The Netherlands) and single leg cycling on two separate occasions prior to a single leg cycling $\dot{V}_{O_2peak}$ test. The $\dot{V}_{O_2peak}$ test involved single leg cycling at three incremental work rates of 65, 80 and 95 W for 3 min followed by an increase of 15 W min^{-1} until exhaustion. Expired air was collected and analysed using zirconia cell O_2 and infrared CO_2 analysers (Applied Electrochemical Instrument, Pittsburgh, PA, USA) that were calibrated against 0.01% alpha-rated gases (Medgraphics, St Paul, MN, USA).

Experimental procedure

Subjects commenced the experimental trial 1 week after the $\dot{V}_{O_2peak}$ test and abstained from strenuous exercise during this time. For each testing session the subjects were required to report to the laboratory having fasted overnight, and abstained from caffeine and alcohol consumption for 24 h. On the first day of the trial a resting muscle sample (pre) was obtained from the non-exercising (NEx) leg to avoid the potential influence of a stress response from the biopsy needle. With the exercising (Ex) leg subjects then performed single leg cycling at 65% single leg $\dot{V}_{O_2peak}$ (95.9 ± 19.5 W) until exhaustion (1.2 ± 0.2 h), which was determined by the subjects' choice to cease cycling or by the subjects' inability to maintain the required cadence. Muscle samples were taken from the Ex leg at 3 and 24 h post-exercise.

Muscle biopsies

Skeletal muscle samples were obtained under local anaesthesia (1% Xylocaine) from the belly of the vastus lateralis muscle using a percutaneous needle biopsy technique (Bergstrom, 1962) modified to include suction (Evans *et al.* 1982). Following a small incision through the skin, muscle biopsies were taken using a Bergstrom needle, snap frozen and stored in liquid nitrogen until required for analysis.

RNA extraction

Total RNA was extracted from 5–20 mg skeletal muscle samples using Tri-Reagent Solution (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocol. First-strand cDNA was generated from 1 μg RNA in 20 μl reaction buffer using the AMV reverse transcriptase (RT) kit (Promega, Madison, WI, USA) and Random Hexomer primers (500 ng) (Promega) according to manufacturer's protocol.

Real-time quantitative PCR

Real-time PCR was carried out using either a 7500 Real-Time PCR system (Applied Biosystems, Forster City, CA, USA) or a Stratagene MX3000 PCR system (Agilent Technologies, Santa Clara, CA, USA) to measure mRNA levels of STARS, α-actin, IGF-1, PGC-1α, ERRα, CPT-1β, cytochrome c oxidase subunit IV (COX4) and JUNB. To compensate for variations in input RNA amounts and efficiency of the reverse transcription, data were normalized to cyclophilin mRNA levels when assaying muscle biopsies, and ribosomal protein 36B4 (also known as RPLPO) RNA levels when assaying C2C12 cells. PCR primers for the mouse genes are as follows; *Stars,* forward TCA AAC GCC CCT TGC TCT C, reverse CGT GTT CAT CGG CCC ACT; *Cpt-1β*, forward ATT CTG TGC GGC CCT TAT TGG AT, reverse TTT GCC TGG GAT GCC TGT AGT GT; *Cox4*, forward ACT ACC CCT TGC CTG ATGTG, reverse GCC CAC AAC TGT CTT CCA TT; *Vegf*, forward AAG CCA GCA CAT AGG AGA GAT GA, reverse TCT TTC TTT GGT CTG CAT TCA CA. Other primer sequences and PCR conditions for the amplified genes have been published previously (Puntschart *et al.* 1998; Russell *et al.* 2003*a*; Cartoni *et al.* 2005; Leger *et al.* 2006*b*; Lamon *et al.* 2009).

Protein extraction

Nuclear and cytosolic proteins were extracted from skeletal muscle using the NE-PER Nuclear and Cytoplasmic extraction kit (Pierce Biotechnology, Rockford, IL, USA) as performed previously by our group (Lamon *et al.* 2009). Exactly 2 μl of inhibitor cocktail, containing protease and phosphatase inhibitors (Sigma, St Louis, MO, USA) at a ratio of 1:1 per 20 mg of muscle sample was added before the extraction of both cytosolic and nuclear proteins. In brief, the muscle samples were homogenised in the

CER I solution provided in the NE-PER kit using a Polytron PT 1200C (Kinematica AG, Luzern, Switzerland). The following steps were performed in accordance with the manufacturer's protocol. For myotubes, total protein was extracted using 1× RIPA buffer (Millipore, North Ryde, NSW, Australia) with 1 μl ml^{-1} protease inhibitor cocktail (Sigma, Castle Hill, NSW, Australia) and 10 μl ml^{-1} Halt Phosphatase Inhibitor Single-Use Cocktail (Thermo Scientific, Rockford, IL, USA). Total protein content was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

Western blotting

Electrophoresis and protein transfer were performed using the XCell Surelock Novex Mini-Cell (Invitrogen, Carlsbad, CA, USA) system. Protein lysates were separated by SDS-PAGE using pre-cast NuPAGE Novex 4 to 12% Bis-Tris gels (Invitrogen) and transferred to a PVDF membrane (Millipore, Billerica, MA, USA). Membranes were then incubated at room temperature for 1 h in blocking buffer (0.1% Tween 20–Tris-buffered saline (TBST)) supplemented with 5% gelatin. After blocking, membranes were incubated overnight at 4°C with the following primary antibodies diluted 1:1000 in 5% gelatin–TBST: STARS (Institute of Medical and Veterinary Science, Adelaide, SA, Australia); SRF (Santa Cruz, sc-335; CA, USA); MRTF-A (Santa Cruz, sc-32909). Following washing the membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in 5% gelatin–TBST. After washing, the specific proteins were revealed using ECL-Plus (GE Healthcare, Buckinghamshire, UK) and exposed on a Kodak 2000 mm image station (Eastman Kodak, Rochester, NY, USA). Individual protein band optical densities were determined using ImageJ Software (National Institutes of Health, Bethesda, MA, USA). Tubulin (cytoplasmic fractions) and Lamin A (nuclear fractions) as well as Ponceau staining (0.5% Ponceau-S (w/v), 1% acetic acid (v/v)) were used to control for protein loading as published previously by our group (Lamon *et al.* 2009). A representative blot is shown in Supplementary Fig. S1. Validation of our STARS antibody using siRNA Knockdown of STARS and adenoviral over-expression of STARS is shown in Supplementary Fig. S2.

Immunofluorescence

Immunfluorescence experiments for identifying fibre-specific protein levels of STARS were performed as reported previously by our group (Russell *et al.* 2003*b*, 2004). The antibodies raised against STARS, MHC type I and MHC type IIa were diluted 1:200, 1:10 and 1:10, and visualized using a goat anti-rabbit (IgG) fluorescein isothiocyanate (FITC), donkey anti-mouse (IgM)-TRITC and a goat anti-mouse (IgGγ1)-Alexaflour 320, respectively. Negative controls, using only the secondary antibody, were also performed in parallel and on the same glass slide as the experimental samples. Sections were viewed and photographed using an Olympus IX70 fluorescent microsope and a Magna Fire camera with dedicated software. Due to freezing artifacts only 6 of the 9 subjects were analysed. Between 80 and 120 fibres were visualized per subject.

C2C12 myotubes

C2C12 myoblasts (ATCC) were plated in 12-well or 6-well tissue culture plates in complete DMEM (10% fetal bovine serum). As the cultures approached confluence (∼90% confluent), media was changed to differentiation medium (DMEM) supplemented with 2% horse serum. Differentiation media was replaced on days 2, 4 and 6.

Adenoviral infections

For siERRα experiments, myotubes were infected with multiplicity of infection of 200 (MOI 200) siERRα or vector control adenovirus on day 4 of differentiation. On day 6 of differentiation, myotubes were infected with an additional dose of MOI 200 siERRα (or vector control) and MOI 50 of PGC-1α (tagged with a FLAG epitope) or LacZ (control) expressing adenovirus. Twenty-four hours later, RNA or DNA was harvested for RT-qPCR gene expression analysis or ChIP analyses, respectively. For expression of a constitutively active ERRα (VP16-ERRα), day 6 myotubes were infected with MOI 50 of adenoviruses expressing LacZ (control) or VP16-ERRα (Schreiber *et al.* 2004). RNA was harvested 24 h later.

siRNA knockdown of STARS combined with PGC1α over-expression

STARS knockdown in C2C12 myotubes was achieved using 100 pmol of Stealth RNAi siRNA (target sequence: 5′-ACCAGAACGGTTGTCAGCAAGGCTT-3′; Invitrogen) over a 72 h period. Stealth RNAi^{TM} siRNA Negative Control Hi GC (Invitrogen) 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 Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, the C2C12 myotubes were infected with either a green fluorescent protein (GFP; control) or PGC-1α adenovirus at a MOI of 25 for a further 48 h.

Chromatin immunoprecipitation (ChIP)

ChIP was performed on adenovirus-transduced C2C12 myotubes as described previously (Cartoni *et al.* 2005). Briefly, C2C12 myotubes were crosslinked for 10 min at 37°C in 1% formaldehyde in PBS. After quenching, sonication to ∼500 bp fragments and pre-clearing by treatment with protein A/G sepharose, soluble chromatin was immnuoprecipitated with the following antibodies: anti-GFP (control), anti-FLAG (clone M2) for the detection of FLAG-tagged PGC-1α, or anti-ERRα (a generous gift of Dr V. Giguere (Sladek *et al.* 1997)). Genomic DNA (gDNA) from each immunoprecipitation was quantified by real-time PCR, using primers (TCTCCTCCAAGTGCCAGAGT and AGCTGTATTGAGGGCTACCG) to amplify an ERR response element (ERRE) 272 bp upstream of *Stars*, as well as primers specific to the ERR response element-containing region of the *Esrra* gene (positive control) and a region that lacks ERREs, distal to the *Esrra* promoter (negative control) (primers described in Villena *et al.* 2007). Data were normalized first against total genomic input and then expressed relative to levels of immunoprecipitated DNA from the ERRα negative control region (Villena *et al.* 2007). Values shown are the mean and standard deviation of three independent experimental replicates.

Statistics

Differences between time were determined using a one-way ANOVA. Experiments combining over-expression and knockdown of targets were analysed using a two-way ANOVA. The level of significance was set at $P < 0.05$. Where required, a *post hoc* analysis was

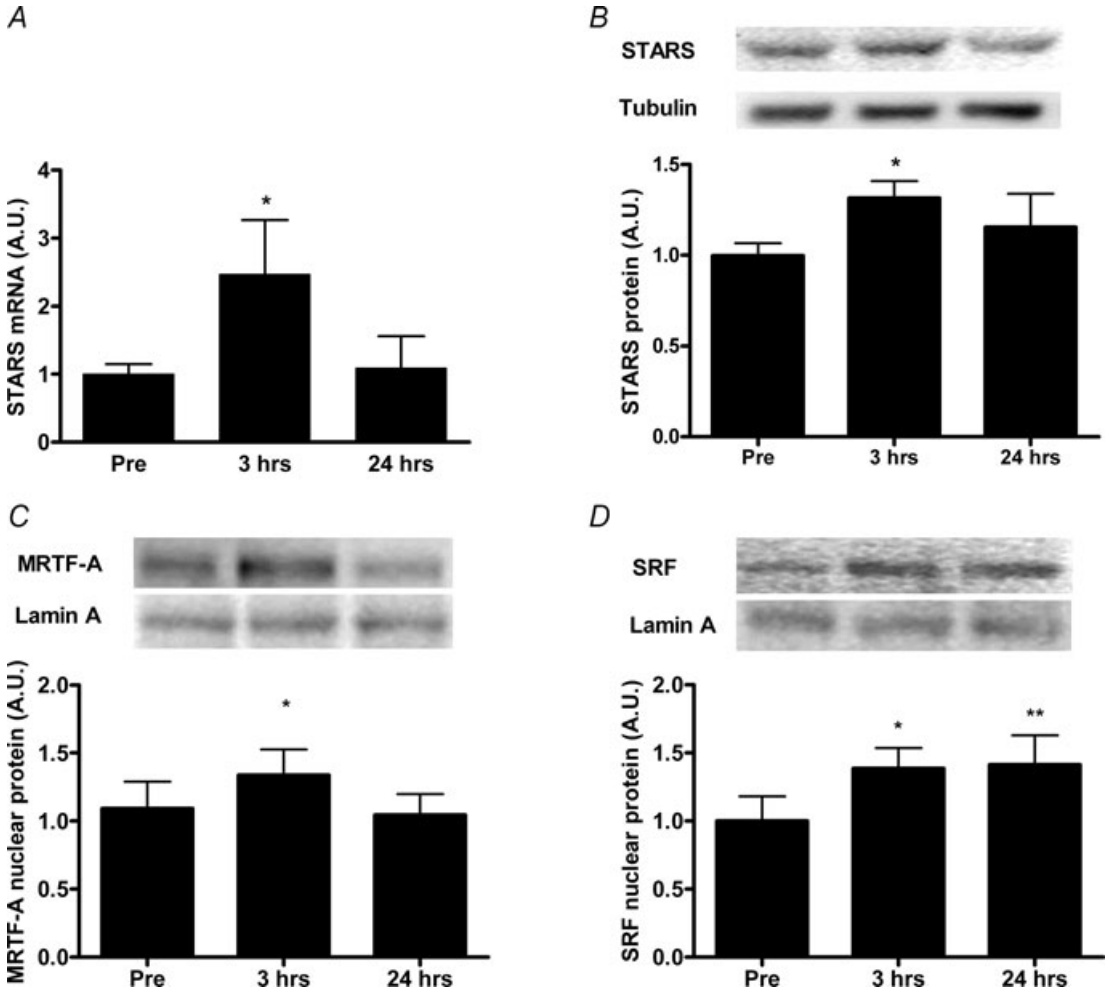

Figure 1. Effect of acute endurance exercise on STARS mRNA, STARS protein, MRTF-A and SRF nuclear protein
Effect of acute endurance exercise on the levels of: *A*, STARS mRNA; *B*, STARS protein; *C*, MRTF-A; and *D*, SRF nuclear protein. Pre, before exercise; 3 h, 3 h post-exercise; 24 h, 24 h post-exercise. $^{*}P < 0.05$, significantly different from the other groups, $^{**}P < 0.01$, significantly different from pre.

performed using either paired or unpaired *t* tests with a Bonferroni adjustment.

Results

STARS mRNA and protein levels were increased by 140% and 40%, respectively ($P < 0.05$), when measured 3 h post-exercise, with both returning to near-basal levels 24 h post-exercise (Fig. 1). MRTF-A nuclear protein content increased by 34% ($P < 0.05$) 3 h post-exercise and returned to basal lines levels 24 h post-exercise. SRF nuclear protein content also increased by 39% when measured 3 h post-exercise ($P < 0.05$); however, it remained elevated 24 h post-exercise ($P < 0.01$) (Fig. 1).

Levels of the SRF targets genes, CPT-1β and JUNB mRNA increased by 2.3- and 1.8-fold ($P < 0.05$), respectively, 3 h post-exercise and returned to baseline levels 24 h post-exercise (Fig. 2). No change in other SRF target genes, α-actin or IGF-1 mRNA was observed. PGC-1α mRNA and ERRα mRNA were also increased 4.5- and 2.7-fold, respectively 3 h post-exercise and returned to baseline levels 24 h post-exercise (Fig. 2), a response mirroring that of STARS, CPT-1β and JUNB mRNA. As PGC-1α is known to transcriptionally regulate CPT-1β (Moore *et al.* 2003), it was of interest to determine if STARS could also be regulated via a PGC-1α/ERRα

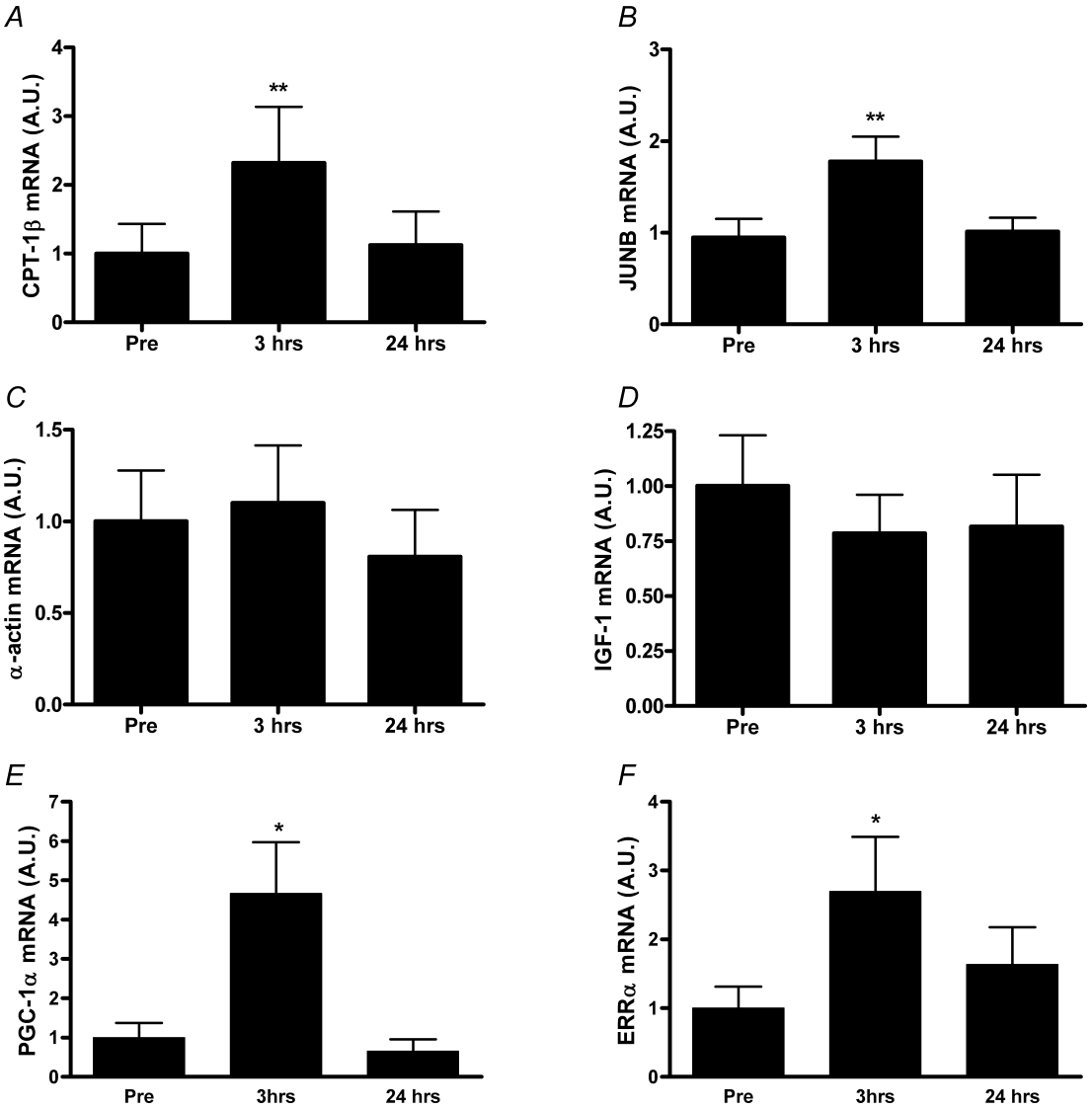

Figure 2. Effect of acute endurance exercise on CPT-1β, JUNB, α-actin, IGF-1, PGC-1α and ERRα mRNA levels
Effect of acute endurance exercise on: *A*, CPT-1β; *B*, JUNB; *C*, α-actin; *D*, IGF-1; *E*, PGC-1α; and *F*, ERRα mRNA levels. Pre, before exercise; 3 h, 3 h post-exercise; 24 h, 24 h post-exercise.. Significantly different from the other groups; $^{*}P < 0.05$; $^{**}P < 0.01$.

transcriptional program. Infection of C2C12 myotubes with an adenovirus-expressing human PGC-1α resulted in a 3-fold increase in *Stars* mRNA ($P < 0.01$). This was abolished when the myotubes were infected with PGC-1α as well as an adenovirus containing an siRNA sequence against ERRα. Additionally, expression of a constitutively active form of ERRα, VP16-ERRα, resulted in a 7-fold increase in *Stars* mRNA ($P < 0.01$) (Fig. 3).

To test if ERRα and PGC-1α act directly at the *Stars* gene to induce its expression, we asked whether ERRα and PGC-1α interact physically with the *Stars* promoter. Analysis of the *Stars* genomic sequence indicated a putative ERRα binding site matching the known 9 bp ERRE consensus 5′-TNAAGGTCA-3′ (Sladek *et al.* 1997) and was conserved across species (Fig. 4*A*). To determine binding of endogenous ERRα and exogenous adenovirally expressed PGC-1α at this region, we immunoprecipitated crosslinked chromatin with antibodies against either GFP (control antibody measuring background), FLAG-tagged PGC-1α or ERRα, and measured the enrichment of the *Stars* locus DNA in the immunoprecipitated material. Both PGC-1α and ERRα were detected at the *Stars* ERRE (Fig. 4*B*), as well as at the previously defined ERRE of the *Esrra* gene that was used as positive control (Fig. 4*C*). ERRα was detected at the *Stars* locus only when PGC-1α was expressed, i.e. when endogenous ERRα levels were induced (Cartoni *et al.* 2005). Knockdown of endogenous ERRα expression by siERRα ablated binding of PGC-1α to the *Stars* ERRE (Fig. 4*B*), demonstrating a critical role for ERRα in recruitment of the PGC-1α co-activator to the *Stars* promoter.

In human skeletal muscle we have previously shown that PGC-1α protein levels are greater in oxidative type I and oxidative-glycolytic type IIa fibres than in glycolytic type IIx fibres (Russell *et al.* 2003*a*). Using immunofluorescence staining we observed that STARS protein levels are also predominately expressed in oxidative type I and oxidative-glycolytic type IIa fibres when compared with glycolytic type IIx fibres (Fig. 5*A* and *B*). STARS was also expressed in the myonuclei as shown via its co-localization with the 4,6-diamidino-2-phenylindole (DAPI) stain (Fig. 5*C*–*E*). Due to limited nuclear protein extracts STARS nuclear protein content could only be measured in 6 of the 9 subjects. Following exercise, 5 of 6 subjects showed an increase in STARS nuclear protein levels. Although the increase was by 60% this did not reach statistical significance (Fig. 5*F*).

As PGC-1α is known to regulate genes involved in mitochondrial biogenesis, angiogenesis and oxidative phosphorylation, it was of interest to see if knockdown of STARS could ablate this response. In C2C12 myotubes PGC-1α over-expression resulted in a 2.0-fold increase in *Cox4* and *Vegf* mRNA, an effect not influenced by the knockdown of STARS. PGC-1α over-expression also increased *Cpt-1β* mRNA by 6.6-fold. However, the endogenous knockdown of STARS significantly reduced the basal levels of *Cpt-1β* mRNA levels by 8.2-fold and ablated the PGC-1α-induced increase in *Cpt-1β* mRNA (Fig. 6).

Discussion

Identifying the molecular targets and intracellular signals activated by endurance exercise will significantly enhance our understanding of how skeletal muscle adapts to increased contractile load and may identify potential

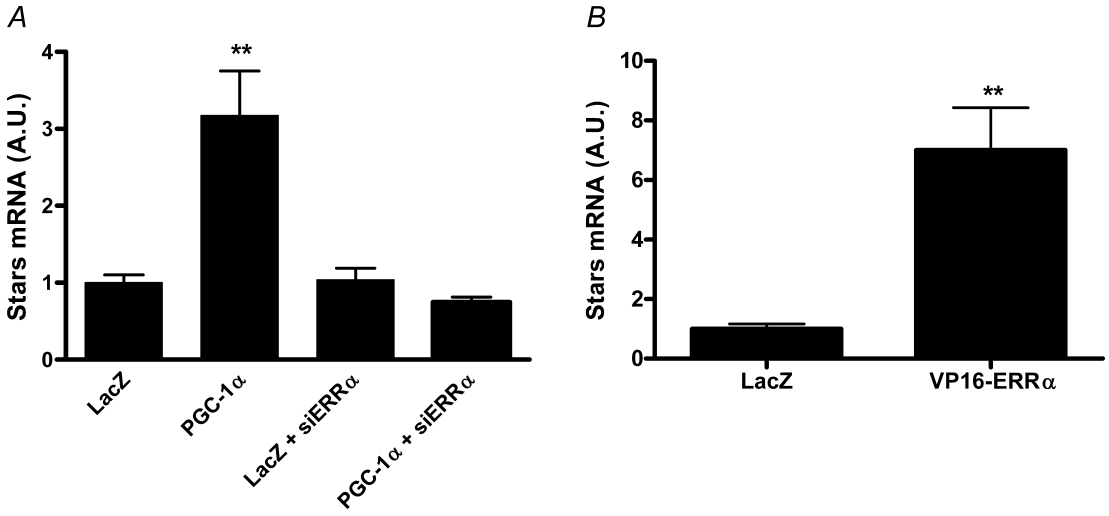

Figure 3. *Stars* is regulated via the PGC-1α/ERRα transcriptional program in C2C12 myotubes
A, *Stars* mRNA levels 24 h after expression of LacZ (control) or PGC-1α, in the absence or presence of siRNA against ERRα (siERRα). $N = 6$ per group (duplicate samples from 3 separate experiments). *B*, *Stars* mRNA levels 24 h after expression of LacZ (control) or the constitutively active VP16-ERRα. $N = 4$ per group. $^{**}P < 0.01$, significantly different from the other groups.

therapeutic targets to combat diseases characterized by perturbed skeletal muscle metabolism, growth and regeneration. The present study demonstrates several new and important findings, which are summarized in Fig. 7. Firstly, we show for the first time that an acute bout of single-leg endurance exercise is sufficient to induce an increase in STARS mRNA and protein levels; secondly, the response of STARS is paralleled by an increase in the nuclear protein content of several key members of the STARS signalling pathway, MRTF-A and SRF; thirdly, in C2C12 myotubes the STARS gene is a target of the PGC-1α/ERRα transcriptional complex; and finally, the presence STARS appears to be required for the PGC-1α regulation of the CPT-1β gene in C2C12 myotubes.

STARS, an actin-binding protein, is considered to communicate mechanical stress signals to downstream transcriptional regulators, such as MRTF-A and SRF, via its influence on actin dynamics (Arai *et al.* 2002; Kuwahara *et al.* 2005). SRF is known to regulate genes involved in muscle structure, function and growth such as α-actin, MHC, IGF and JUNB (Charvet *et al.* 2006; Lindecke *et al.* 2006; Miano *et al.* 2007) as well as fat metabolism, such as CPT-1β (Moore *et al.* 2001). Therefore, STARS may be an upstream regulator of signals controlling the expression of genes which encode proteins involved in muscle function, growth and metabolism. In support of this notion, forced over-expression of STARS *in vivo* (Kuwahara *et al.* 2007) and *in vitro* (Koekemoer *et al.* 2009) results in hypertrophy of cardiac tissue and protects against apoptosis. Additionally, we have recently shown that the STARS signalling pathway is increased in hypertrophied human skeletal muscle following resistance exercise (Lamon *et al.* 2009). In contrast, STARS and several members of its signalling pathway are reduced in skeletal muscle of sarcopenic mice (Sakuma *et al.* 2008) and after 1 day of limb immobilization in rats (Giger *et al.* 2009). However, after 20 days of limb immobilization in humans there were no observed changes in STARS

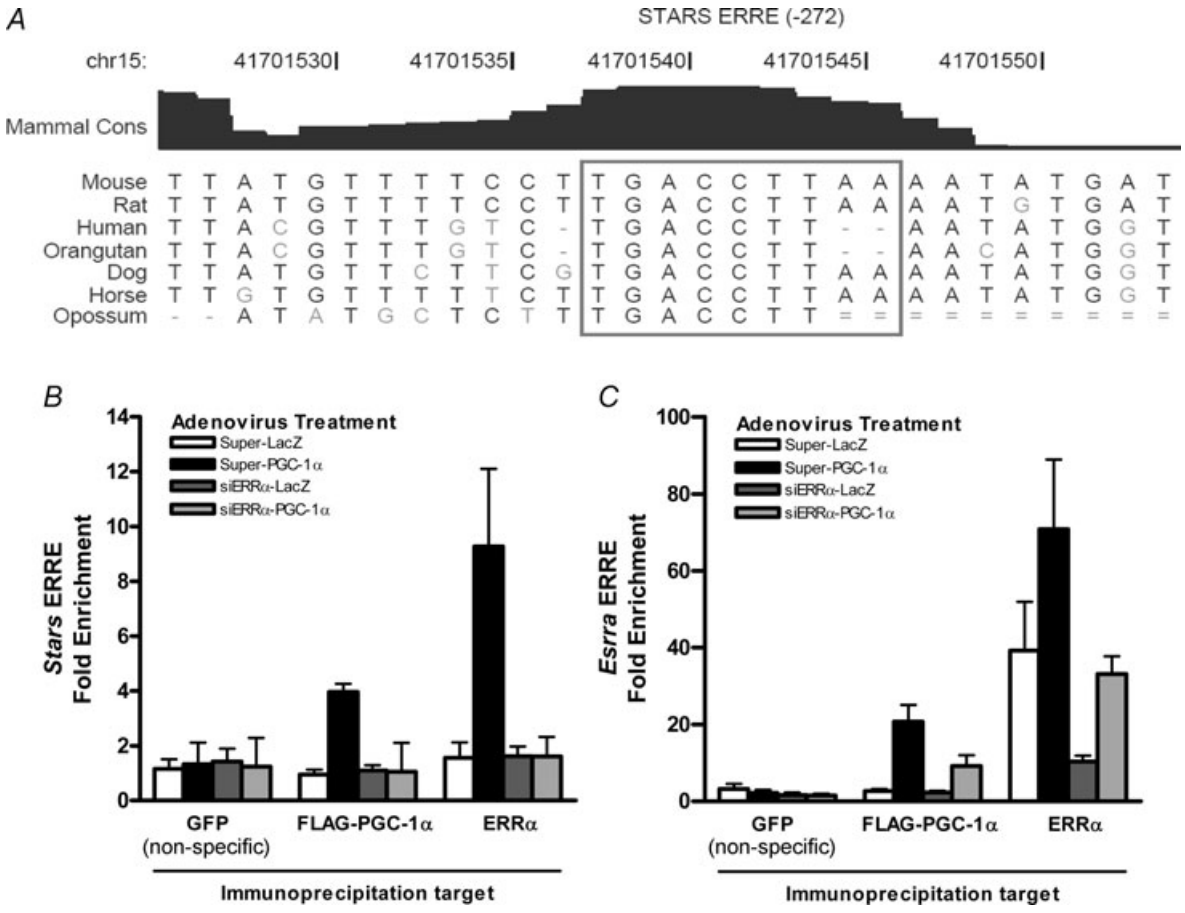

Figure 4. PGC-1α and ERRα bind an ERRE upstream of *Stars* in C2C12 myotubes
A, a University of California, Santa Cruz (UCSC) genome browser screenshot depicting the presence of an ERRE (at 272 bp 5′ of the *Stars* transcription site and its conservation across species. Occupancy of PGC-1α and ERRα at the *Stars* (*B*) and *Esrra* (positive control) (*C*) ERREs, as determined by ChIP assays in C2C12 myotubes expressing LacZ (control) or PGC-1α, in the absence (Super) or presence of siRNA for ERRα (siErrα). Data are expressed relative to a region devoid of ERREs and are the mean ± SD of triplicates. $^{**}P < 0.01$, significantly different from all treatments within the group.

(Sakuma *et al.* 2009). The results from the present study show that acute moderate-intensity exercise results not only in increased levels of STARS mRNA but also the STARS protein. Combined, these observations suggest that the regulation of STARS is rapid and transient which is a logical regulatory response for a protein believed to be responsible for the sensing and transmission of extracellular stress signals. It should be noted that this is the first measurement of endogenous STARS protein levels and shows, at least in the present experimental model, that the regulation of STARS mRNA is closely mirrored by the regulation of its protein.

In parallel with the increase in STARS, we show for the first time that both MRTF-A and its transcriptional target SRF have enhanced nuclear abundance following endurance exercise, a response consistent with enhanced STARS activity (Kuwahara *et al.* 2005). These results support our previous observation following high-intensity resistance training (Lamon *et al.* 2009). Along similar lines, endurance exercise training has also

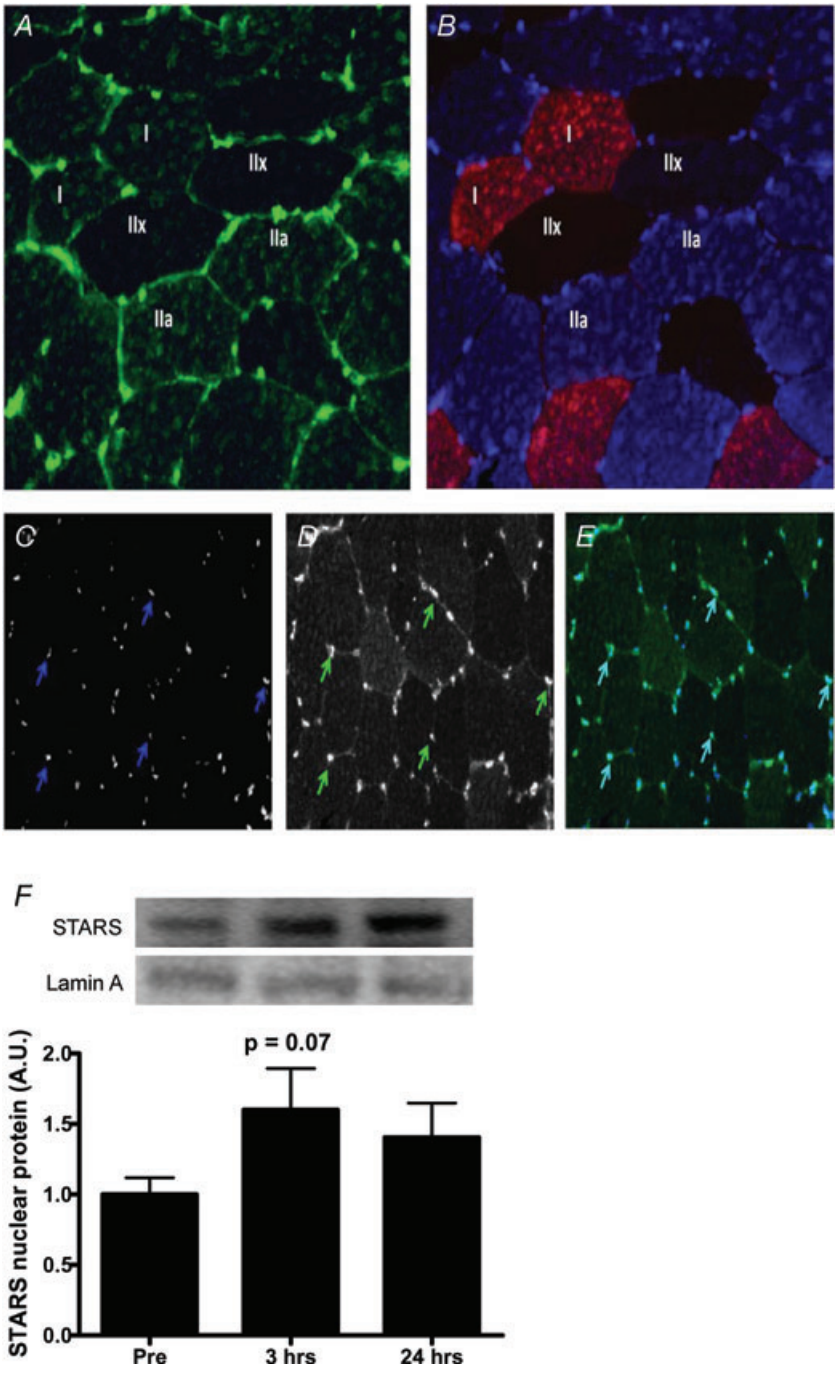

Figure 5. The STARS protein is more abundantly expressed in oxidative type I and oxidative-glycolytic type IIa fibres, than in glycolytic type IIx fibres and is also found in the nucleus
Immunofluorescence staining of: *A*, the STARS protein; and *B*, MHCI (red; oxidative type I), MHCIIa (blue; oxidative-glycolytic type IIa) and MHCIIx (not stained; glycolytic type IIx). The same fibres to the marks of I, IIa or IIx in figures *A* and *B*. *C*, DAPI stain; *D*, STARS stain; and *E*, their co-localization, respectively, is shown in the section. Arrows represent co-localization of the nuclei and STARS. *F*, Western blot showing the STARS protein levels in nuclear protein fraction before, 3 h after and 24 h post-exercise.

been shown to increase SRF activity, as shown by enhanced phosphorylation levels, in human skeletal muscle (Rose *et al.* 2007). In the present study, we also observed an increase in the SRF target genes CPT-1β, a regulator of long-chain fatty acid β-oxidation, and JUNB, a cell-cycle and growth regulator, but not the expression levels of α-actin and IGF-1, genes involved in structure and growth. While these results suggest that both moderate and intense exercise elicits stress signals that can be detected by STARS and transmitted downstream to its effector targets, such as MRTF-A and SRF, their transcriptional control might be dependent on the exercise intensity. For example, SRF is known to regulate over 200 genes, many of which are involved in muscle structure, function, growth and regeneration (Charvet *et al.* 2006; Miano *et al.* 2007) but not all are regulated at the same time. The selective regulation of certain SRF target genes might be attributed to their sensitivity to the stress signals initiated by the acute nature of the endurance exercise protocol.

While we show here that endurance exercise, and previously that resistance exercise (Lamon *et al.* 2009), regulates STARS transcription and translation, the exact stress signals initiating this are unknown. During exercise, numerous stress signals are initiated by changes in motor nerve activation and calcium concentrations mechanical and contractile stress, increased muscle blood flow and shear stress, hormonal and metabolic stress which are known to activate intracellular signalling pathways controlling skeletal muscle gene transcription and translation (Bassel-Duby & Olson, 2006; Koulmann & Bigard, 2006; Park *et al.* 2007; Favier *et al.* 2008; Russell, 2010). Identifying the primary stressor/s activated by endurance exercise which regulate STARS, and consequently its

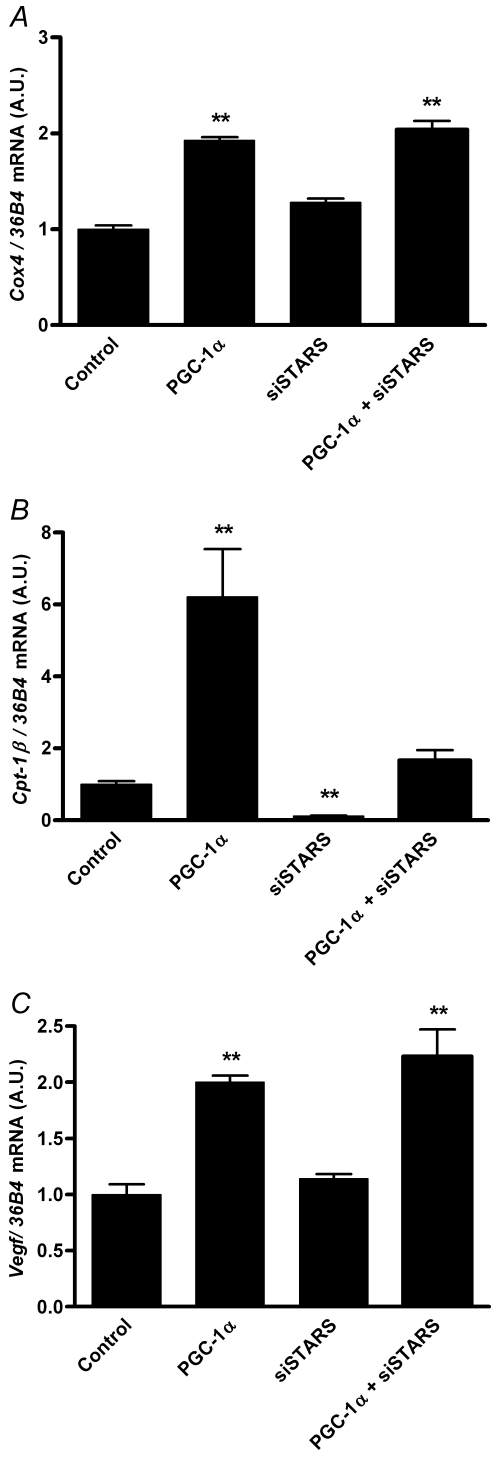

Figure 6. Regulation of: *A*, *Cox4*; *B*, *Cpt-1β*; and *C*, *Vegf* mRNA by PGC-1α with and without STARS knockdown in C2C12 myotubes
$N = 6$ per group. $^{**}P < 0.01$, significantly different from the other groups.

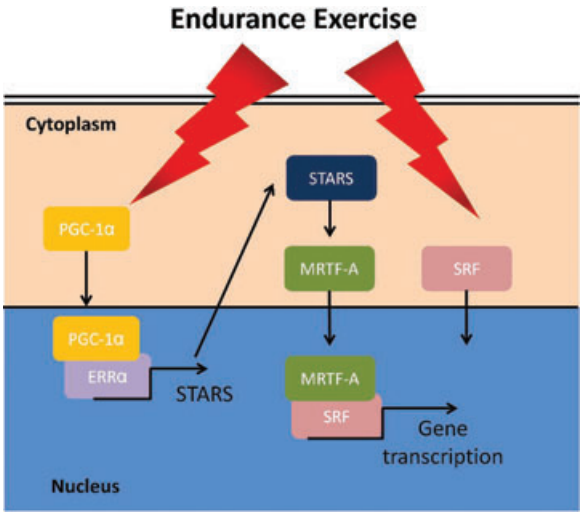

Figure 7. A diagram summarizing the main findings from the study
STARS is transcriptional target of PGC-1α/ERRα signalling. Acute endurance exercise increases STARS mRNA and protein levels as well as the nuclear translocation of the transcriptional co-activator MRTF-A and its target transcription factor SRF. Increased nuclear abundance of MRTF-A and SRF should increase the potential for SRF target transcription.

downstream signalling pathway, is beyond the scope of this study; however, this should be an area investigated in the future.

Our observation that STARS is increased in response to moderate-intensity endurance exercise suggests that the STARS signalling pathway may also play a role in skeletal muscle adaptations associated with endurance exercise such as substrate oxidation, capillary function and mitochondrial biogenesis, factors often perturbed in metabolic diseases (Schrauwen & Hesselink, 2004). Indeed SRF, a downstream target of STARS signalling, has also been shown to control the transcription of CPT-1β (Moore *et al.* 2001), a regulator of fat metabolism, as well as vascular endothelial growth factor (VEGF), a regulator of angiogenesis (Franco *et al.* 2008). Another commonalty between CPT-1β and VEGF is that they can also be regulated by the transcriptional co-activator PGC-1α (Moore *et al.* 2003; Zhang *et al.* 2011). PGC-1α is upregulated in human skeletal muscle in response to both moderate- and high-intensity endurance exercise (Russell *et al.* 2003*a*, 2005; Cartoni *et al.* 2005). PGC-1α co-activates several transcription factors that regulate genes involved in skeletal muscle substrate oxidation and mitochondrial biogenesis (Knutti & Kralli, 2001; Hock & Kralli, 2009). We identified that one of these transcription factors, ERRα, has a binding site on the STARS promoter. Therefore, we performed *in vitro* experiments using C2C12 myotubes whereby we manipulated PGC-1α and ERRα levels using adenoviral infection. Our results demonstrate that STARS is a transcriptional target of the PGC-1α/ERRα transcriptional program.

Previous work by our group has shown that in human skeletal muscle, PGC-1α protein levels are greater in oxidative type I and oxidative-glycolytic type IIa fibres than in glycolytic type IIx fibres (Russell *et al.* 2003*a*). In the present study, we also observed a similar fibre type expression for the STARS protein. In addition to this, we observed that the STARS protein was also localized to the nucleus, which supports observations made in transiently transfected COS cells (Arai *et al.* 2002) and in rat endothelial cells (Troidl *et al.* 2009). The PGC-1α regulation of STARS, their similar fibre type expression profile, as well as the localization of STARS in the nucleus, suggested to us that a PGC-1α/STARS transcriptional relationship may exist. Our observation that the knockdown of endogenous STARS reduces CPT-1β mRNA and attenuates the PGC-1α upregulation of CPT-1β mRNA supports this possibility. However, it remains to be determined whether STARS has the capacity to directly bind DNA or whether its downregulation impacts indirectly on transcriptional signalling pathways, potentially through SRF. These *in vitro* results, combined with our observation of increases in PGC-1α and ERRα mRNA following acute endurance exercise, suggest that a possible feed-forward loop involving SRF, PGC-1α, ERRα and STARS may exist during moderate-intensity muscle contraction which controls oxidative metabolism and muscle function. In contrast to the increase in these genes following exercise, skeletal muscle SRF, PGC-1α and STARS are all reduced in conditions such as ageing (Ling *et al.* 2004; Lahoute *et al.* 2008; Sakuma *et al.* 2008) and limb immobilization (Oishi *et al.* 2008; Giger *et al.* 2009), situations that present reduced skeletal muscle mass, function and attenuated metabolism. The possible existence of this novel transcriptional pathway requires further investigation.

In conclusion, we show for the first time that STARS mRNA and protein as well as MRTF-A and SRF nuclear content are increased following endurance exercise. Additionally, we have identified the STARS gene as a PGC-1α/ERRα transcriptional target and a gene that may be involved in the regulation of oxidative metabolism. Future investigations should focus on STARS and its downstream signalling pathway on the regulation of skeletal muscle oxidative function, potentially via its influence on actin polymerization.

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Author contributions

All *in vivo* experiments and *in vitro* experiments required for the antibody validation were performed at Deakin University while the *in vitro* experiments completed in Figs 5 and 6 were performed at the Scripps Research Institute. M.A.W. completed the analyses using of human muscle samples, validated the STARS antibody, performed the PGC-1/siSTARS experiments and assisted with the manuscript preparation; M.B.H. and B.C.H. completed the adenoviral over expression, siRNA and ChIP experiments and participated with the manuscript preparation; A.K. was responsible for designing the *in vitro* experiments and manuscript preparation; R.J.S. was responsible for designing the *in vivo* human exercise study and manuscript preparation; A.P.R. was responsible for management of the project, supervision of M.A.W., funding the project and manuscript preparation. All authors approved the final version of the manuscript.

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