This is the published version:

Trenerry, Marissa, Carey, Kate, Ward, Alister, Farnfield, Michelle M. and Cameron-Smith, David 2008, Exercise-induced activation of STAT3 signaling is increased with age, *Rejuvenation research*, vol. 11, no. 4, pp. 717-724.

Available from Deakin Research Online:

http://hdl.handle.net/10536/DRO/DU:30017521

Reproduced with the kind permissions of the copyright owner.

Copyright : 2008, Mary Ann Liebert Publishers
Exercise-Induced Activation of STAT3 Signaling Is Increased with Age

Marissa K. Trenerry,¹ Kate A. Carey,¹ Alister C. Ward,² Michelle M. Farnfield,¹ and David Cameron-Smith¹

Abstract

Activation of the transcription factor signal transducers and activators of transcription (STAT) 3 is common to many inflammatory cytokines and growth factors, with recent evidence of involvement in skeletal muscle regeneration. The purpose of this study was to determine whether STAT3 signaling activation is regulated differentially, at rest and following intense resistance exercise, in aged human skeletal muscle. Skeletal muscle biopsies were harvested from healthy younger (n = 11, 20.4 ± 0.8 years) and older men (n = 10, 67.4 ± 1.3 years) under resting conditions and 2 h after the completion of resistance exercise. No differences were evident at rest, whereas the phosphorylation of STAT3 was significantly increased in old (23-fold) compared to young (5-fold) subjects after exercise. This correlated with significantly higher induction of the STAT3 target genes including; interleukin-6 (IL-6), JUNB, c-MYC, and suppressor of cytokine signaling (SOCS) 3 mRNA in older subjects following exercise. Despite increased SOCS3 mRNA, cellular protein abundance was suppressed. SOCS3 protein is an important negative regulator of STAT3 activation and cytokine signaling. Thus, in aged human muscle, elevated responsiveness of the STAT3 signaling pathway and suppressed SOCS3 protein are evident following resistance exercise. These data suggest that enhanced STAT3 signaling responsiveness to proinflammatory factors may impact on mechanisms of muscle repair and regeneration.

Introduction

AGING IS ASSOCIATED WITH THE LOSS OF skeletal muscle mass and strength, which is a major contributing factor to frailty, falls, loss of independence, and, in severe cases, mortality.¹² Skeletal muscles of aged individuals have greater susceptibility to muscle loss during periods of unloading and disuse, while demonstrating a reduced regenerative response.³ This extends also to resistance exercise training, where the hypertrophic capacity of skeletal muscle is attenuated, but not completely lost.⁴ Indeed, training programs for older adults have been shown to promote marked increases in myofiber size,⁵ whereas improvements in functional capacity have been observed following resistance exercise training in even very elderly (~96 yrs) individuals.⁶

The cellular mechanisms for the loss of regenerative capacity in old age are not well understood. Recently we described the activation of the signal transducer and activator of transcription 3 (STAT3) signaling pathway in human skeletal muscle following an acute bout of resistance exercise.⁷ The activation of the Janus kinase (JAK)/STAT signaling cascade has been recognized as one of the key events in the signal transduction of numerous stimuli and is involved in the regulation of many different biological processes, including cellular proliferation, differentiation, programmed cell death, the immune response, and inflammation.⁸–¹² In the heart, STAT signaling is a major mediator of cardiac protection following inflammation and other stress-induced damage in cardiomyocytes,¹³ where it is essential in the prevention of cardiac failure¹⁴ and regulates myocyte elongation.¹⁵ In skeletal muscle, STAT3 regulates antiapoptotic signaling¹⁶ and recently has been suggested to be a key player in muscle regeneration through the activation of satellite cell proliferation following damage.¹⁷,¹⁸ STAT1 has also been implicated in regeneration, yet it is thought that STAT3 is required to mediate this proliferative response.¹⁷

The possible importance of STAT3 in skeletal muscle regeneration is underscored by its activation by numerous cytokines (including interleukin [IL] -6, IL-10, and leukemia inhibitory factor [LIF]), and growth factors (including insulin-like growth factor-1 [IGF-1], hepatocyte growth factor [HGF], epidermal growth factor [EGF], and basic fibroblast growth factor [bFGF]),¹⁹–²² many of which have been implicated in myogenic regulation.²³–²⁷ The activation and phosphorylation of this protein elicits dimerization and translocation to the nucleus where it participates in the reg-

¹School of Exercise and Nutrition Science, Deakin University, Victoria, Australia.
²School of Medicine, Deakin University, Geelong Victoria, Australia.
ulation of many target genes. Gene targets of STAT3 with potential roles in the regulation of skeletal muscle regeneration include those encoding the anti-apoptotic genes Bcl-2 and Bcl-XL, cell cycle regulators c-MYC and cyclinD1, the angiogenic factor VEGF (vascular endothelial growth factor), and the immediate early response genes c-fos and junB. The diverse nature of STAT signaling necessitates tight regulation through the activation of negative regulatory molecules to ensure appropriate and specific responses to each stimulus. Suppressor of cytokine signaling (SOCS) proteins are known inhibitors of STAT signaling in a variety of tissues. Of the eight family members characterized, SOCS3 has been identified as a mediator of IL-6- and LIF-induced signaling, suggesting it has a direct influence on STAT3 activation and induction of myoblast differentiation.

Aging is associated with higher levels of circulating inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and IL-6, which have been suggested to play major roles in the functional decline of older individuals. The importance of STAT3 activation in the transduction of inflammatory signals is well recognized; however, the regulation of this pathway in the skeletal muscle of older individuals has not yet been investigated. Given the likely importance of the STAT signaling pathway in the regulation of skeletal muscle adaptation and remodeling as well as its central role in the transduction of inflammatory signals, we sought to investigate the impact of age on STAT3 signaling.

Therefore, the purpose of this investigation was to determine whether age-altered STAT3 activation and the expression of its downstream target genes, including that encoding the negative regulator SOCS3 in skeletal muscle following acute resistance exercise. Increased STAT3 signaling was observed in aged skeletal muscle both at rest and following acute resistance exercise, although the changes in SOCS protein were less pronounced.

Methods
Study design: resistance exercise training

Subjects. Eleven healthy young men (age 20.4 ± 0.8; height 183.9 ± 1.6; mass, 73.9 ± 2.5; eccentric force, 245.3 ± 11.0) and 10 healthy older men (age 67.4 ± 1.3; height 174.3 ± 2.1; mass 83.4 ± 3.5; eccentric force, 201.2 ± 17.2) who had not participated in regular resistance exercise within a year prior to commencing the study were recruited. All subjects were informed of the nature and risks of the study before their written consent was obtained. A medical questionnaire was used to exclude subjects with a diagnosed condition or illness that would endanger them during strenuous exercise. A complete medical screen was undertaken in the older subjects, including a 12-lead electrocardiogram (ECG) exercise stress test to detect any underlying heart conditions prior to the inclusion in the trial. This study was approved by the Deakin University Human Research Ethics Committee.

Acute bout of resistance exercise. Familiarization and acute exercise regimes were completed as described previously. Briefly, subjects completed a familiarization session on the Cybex NORM dynamometer (Cybex International Inc., UK) 4 days before the trial and for the 24 h preceding. On the days of the trial, subjects abstained from alcohol, caffeine, tobacco, and additional exercise and reported to the laboratory in a fasted state for a resting muscle biopsy. The subjects then completed three sets of eight repetitions of maximal single-legged knee extension exercise on the Cybex NORM Dynamometer with a 2 min rest between each set. Each repetition involved concentric and eccentric contraction of the knee extensors. Subjects were instructed to contract as hard as possible and were verbally encouraged throughout each set. Subjects were required to remain resting in the laboratory for a further 2 h, when an additional muscle sample was collected.

Muscle biopsy procedure. The vastus lateralis muscle of the dominant leg was sampled by percutaneous needle biopsy technique modified to include suction. Excised muscle tissue from each biopsy was immediately frozen and stored in liquid nitrogen for subsequent analysis. Serial biopsy sites were located approximately 2 cm apart.

Protein extraction and western blot analysis

Tissue samples were prepared and separated as described previously. The proteins were transferred onto a nitrocellulose (STAT antibodies) or polyvinylidene fluoride (PVDF) membrane (SOCS3 and α-tubulin antibodies). Membranes were blocked in 5% (wt/vol) bovine serum albumin (BSA) in Tris-buffered saline with 0.1% (vol/vol) Tween 20 (TBST) for 2 h at room temperature (nitrocellulose), or with methanol for 2 min and left to air dry (PVDF). pSTAT3 (Tyrosine 705), total STAT3 (Cell Signaling Technology Inc, Danvers, MA), and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies diluted in 5% BSA/TBST, and SOCS3 (H103) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 5% skim milk in phosphate-buffered saline (PBS) with 0.1% (vol/vol) Tween 20 (PBST), were applied and incubated overnight at 4°C. Membranes were subsequently washed with TBST and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-rabbit (Chemicon, Melbourne, Australia) and anti-mouse (Quantum Scientific, Melbourne, Australia) secondary antibodies in respective blocking buffers before being washed again. Proteins were detected by enhanced chemiluminescence (Western Lighting Chemiluminescence Reagent Plus, Perkin-Elmer, Boston, MA). The density of the bands was quantified using Kodak Imaging software, Kodak ID 3.5 (Perkin Elmer Life Sciences, Boston MA).

RNA extraction and real-time-PCR

Total cellular RNA was extracted as previously described. The efficacy of cyclophilin as endogenous controls was examined using the equation 2−ΔΔCt. It was considered an appropriate endogenous control for this study because no changes in the expression of this gene were observed (data not shown). Primers were designed using Primer Express software package version 3.0 (Applied Biosystems) from gene sequences obtained from GenBank (see Table 1 for details).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4.1 (GraphPad Software, San Diego, CA). Means were com-
pared using two-way analysis of variance (ANOVA) and any significant differences analyzed using a Bonferroni post hoc test. Data were presented as mean ± standard error of the mean (SEM). A probability level of <0.05 was adopted throughout to determine statistical significance unless otherwise stated.

Results

STAT3 phosphorylation following acute resistance exercise increases with age

The effect of exercise on STAT3 phosphorylation (pSTAT3) at tyrosine 705 (Tyr705), was examined in both young and old males, following an acute bout of resistance exercise. pSTAT3 was significantly increased in both young and old subjects 2 h post exercise (Fig. 1A). However, quantification relative to total STAT3 (tSTAT3) protein, which was unaltered by age, revealed that the increase in pSTAT3 seen in old subjects (23-fold) was significantly greater than that observed in young subjects (5-fold) (Fig. 1B).

Genes associated with STAT3 signaling show increased gene expression following acute resistance exercise

The enhanced pSTAT3 prompted us to examine the effects on the expression of a gene encoding a known inducer of STAT3 in skeletal muscle, IL-6, as well as a series of known STAT3 target genes. The levels of IL-6 mRNA were significantly increased following exercise in the muscle of older men (156-fold increase, \( p < 0.05 \)), in comparison to a much smaller change in the muscle of younger men, which failed to reach significance (Fig. 2A). Furthermore, whereas there was a trend for increased expression of genes downstream of STAT3 signaling in younger men, none of the values reached significance, in contrast to older men who showed significant increases in JUNB (16-fold increase, \( p < 0.01 \)) and c-MYC (44-fold increase, \( p < 0.05 \)) but not VEGF and c-FOS (Fig. 2B–E).

SOCS3 protein and mRNA expression increases following acute resistance exercise

Because SOCS3 is a powerful negative regulator of STAT3, we sought to confirm the effects on SOCS3 mRNA and protein expression. Acute exercise elevated levels of a STAT3 regulator, SOCS3 protein, in both young and old subjects (Fig. 3A). In each case, the change was significant \( (p < 0.05) \); however, the younger men showed a much greater increase (18-fold) than the older men (6-fold) (Fig. 3B). The mRNA level of SOCS3 was significantly increased following exercise in the muscle of older men (16-fold, \( p < 0.05 \)), whereas no change was observed in younger muscle (Fig. 3C).

Discussion

The regenerative and hypertrophic capacity of skeletal muscle in older individuals is impaired relative to that of younger adults. Identification of cellular mechanisms that contribute to this diminished growth are significant for the establishment of strategies to maximize the benefit of resistance exercise, in addition to counteracting the atrophy of aging. This study has identified that the activation of STAT3

### Table 1. Primer Sequences

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Forward primer (5′–3′)</th>
<th>Reverse primer (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophilin</td>
<td>NM_021130</td>
<td>CATCTGCACCTGGCAAGACTGA</td>
<td>TTTATCGGCTTCTTTCGTCG</td>
</tr>
<tr>
<td>c-FOS</td>
<td>NM_002522</td>
<td>CGACCCCTTGTGACTCTTCT</td>
<td>GGGACGCGGTCGTCTCAGA</td>
</tr>
<tr>
<td>JUNB</td>
<td>NM_002229</td>
<td>GCACTAAAATGGAACAGCCCTT</td>
<td>GCCTGGAGTTCCAGAGTTT</td>
</tr>
<tr>
<td>c-MYC</td>
<td>NM_002467</td>
<td>CTCTCCACACATCAGCACAAA</td>
<td>TCTTGGCAGCAGGATAGTCCT</td>
</tr>
<tr>
<td>IL-6</td>
<td>NM_000600</td>
<td>GTGACATCCCTAGGCCATCTT</td>
<td>GTGACCTTTCGTGCTTTCAC</td>
</tr>
<tr>
<td>VEGF</td>
<td>AY041581</td>
<td>GCGCAAGAAATTCGCCGTATA</td>
<td>GCTTTCTCCCGCTCTGAGCAA</td>
</tr>
<tr>
<td>SOCS3</td>
<td>NM_003955</td>
<td>GACCCACGACTTTCCTCA</td>
<td>CTGGATCGCCAGGTTTTCG</td>
</tr>
</tbody>
</table>

Primer sequences were designed using Primer Express version 3.0 software (Applied Biosystems) and using sequences accessed through GenBank and checked for specificity using a nucleotide–nucleotide BLAST searches.

VEGF = Vascular endothelial growth factor; SOCS3 = suppressor of cytokine signaling 3.

![FIG. 1.](image-url) Activation of STAT3 in young and old human skeletal muscle following acute resistance exercise. (A) Representative western blots of protein extracted from samples taken at rest and 2 h post exercise, probed with anti-phospho-STAT3 (Tyr705). Phosphorylation of STAT3 increased significantly 2 h following an acute bout of exercise. (B) The graph shows arbitrary units of pSTAT3 normalized to tSTAT3 representing the mean of 11 young and 10 old individuals ± the standard error of the mean (SEM). (*) Significantly different from resting values \( (p < 0.05) \); (ψ) significantly different between age groups \( (p < 0.05) \).
signaling is heightened in the skeletal muscle of older men following an acute bout of resistance exercise. Under resting conditions, STAT3 phosphorylation at Tyr705 is undetectable in both young and older individuals. Two hours following a single bout of intense exercise, there is a marked activation of STAT3 in all individuals. What is most evident is that, whereas young muscle increased phosphorylated STAT3 by 5-fold following exercise, a 23-fold increase was observed in the muscle of older men. Consistent with this altered pattern of STAT3 activity in older muscle was a concomitant increase in the expression of several STAT3 target genes as well as an increase in expression of a negative regulator of STAT3 signaling, the SOCS3 protein. Therefore, these data describe altered activity of a key signaling pathway in skeletal muscle of aged individuals and may suggest novel molecular mechanisms contributing to the age-related decline of muscle mass and function.

The enhanced activation of STAT3 following a single bout of exercise in the older subjects is, in part, consistent with the limited number of previous studies, which are predominantly conducted in rodents. At both 24 and 48 h following a single bout of resistance exercise, increased Tyr705 STAT3 is evident in aged rats. However, following hindlimb immobilization, the failed regeneration of older rats did not correlate with alterations in STAT3 phosphorylation. Given the plethora of cytokines and growth factors that can potentially activate STAT3, the increased response measured in the older subjects may be the net result of differing pathways. One possible mechanism may be the rapid increase in the synthesis of IL-6, a myokine generated within the musculature, which is implicated in muscle remodeling and regulation. In aging, circulating IL-6 correlates with decline in muscle function. Differences in exercise type and intensity, as well as the timing of measurements, may have contributed to these discrepancies.

STAT3 dimerization and transcriptional activation are important in the regulation of many genes. To gauge whether the increased activation of STAT3 in older muscle corresponded with increased expression of downstream STAT3 target genes, we measured the expression of VEGF, c-FOS,
that there may be molecular cross-talk between other pathways in response to exercise. For example, the mitogen-activated protein kinase (MAPK) signaling cascade is activated by exercise, and is also known to serine phosphorylate STAT3 indirectly, which may lead to similar concomitant signals of exercise responsive genes such as c-FOS, although we saw no evidence of STAT3 serine phosphorylation (data not shown).

Central to the regulation of STAT3 signal activity is the SOCS family of proteins. Consistent with its role as a negative regulator of STAT3 signaling, augmented SOCS protein expression was observed in concert with increased STAT3 phosphorylation in older skeletal muscle. In younger muscle, higher levels of SOCS protein were evident in parallel with a slight increase in STAT3 phosphorylation. Cytokine-induced SOCS protein expression is regulated via the activation of STAT proteins and thus modulates cytokine action through a classical feedback loop. Therefore, the physiological role of SOCS proteins is most likely to prevent excessive cytokine signaling within the cell. Such negative feedback appeared to be occurring within young skeletal muscle following exercise. However, this was not the case in older muscle. Despite greater STAT3 phosphorylation and concomitant higher levels of SOCS3 mRNA, SOCS protein expression, while increased, was not as prominent as expected. These results show for the first time in humans that the regulation of cytokine signaling via the STAT3 pathway is altered in the skeletal muscle of older individuals, following an acute bout of resistance exercise.

Consistent with the results in the present study, higher levels of STAT3 protein in addition to high SOCS3 mRNA expression have been observed in the muscle of older rats following resistance exercise, whereas the absence of SOCS3 has previously been correlated with prolonged IL-6-induced STAT3 activation. Several possibilities exist that would explain these findings. First, the translation of SOCS proteins may be impaired in older muscle. This may be due to a reduction in the overall rate of protein translation, which has been observed in aged subjects, or this may be due to a more specific inhibition of SOCS3 protein translation by some unknown factor; indeed, potent translational control has been reported for SOCS proteins. Second, there is a precedent for control of SOCS3 in a manner such as via the phosphorylation of SOCS3 at two tyrosine residues in the conserved SOCS box that can destabilize SOCS3 and activate proteasome-mediated SOCS3 degradation, which may be higher in older muscle. Finally, the translational response may simply be delayed response in older subjects. Muscle samples were collected 2 h following exercise. Therefore, it remains a possibility that the peak increases in the signaling response and transcriptional activation of SOCS3 occurred at a later time point in the older subjects. Further studies are required to elucidate these relationships fully and to acquire a greater understanding of the effects of delayed SOCS3 protein translation. Although there appears to be a positive response of STAT3 in aged muscle, there are a myriad of signaling proteins and other biological factors that may contribute to the reduced regenerative capacity seen with aging, such as impaired hormonal secretion and regulation, decreased mitochondrial function, and an increased level of circulating reactive oxygen species (ROS).
The present study examined the activation and regulation of a major cytokine signaling pathway, the STAT3 kinase pathway, in human skeletal muscle. In summary, these data demonstrate that STAT3 signaling following acute resistance exercise is heightened in the skeletal muscle of older individuals. This occurs together with induction of the important negative regulator of cytokine signaling, SOCS3. Although the significance of this altered signaling in skeletal muscle remains to be determined, we suggest that the impaired regulation of cytokine signaling associated with delayed SOCS3 transcription may contribute to the diminished regenerative capacity and decline in skeletal muscle function seen with age.

Acknowledgments

We thank Dr. Andrew Garnham, Deakin University, for his excellent medical assistance and the research subjects for their invaluable participation in this research.

References

29. Matsui T, Kinoshita T, Hirano T, Yokota T, Miyajima A. STAT3 down-regulates the expression of cyclin D.


Address reprint requests to:
David Cameron-Smith
School of Exercise and Nutrition Sciences
Deakin University
221 Burwood Highway
Burwood, Victoria 3125
Australia

E-mail: david.cameron-smith@deakin.edu.au

Received: October 31, 2007
Accepted: April 11, 2008