Human Sarcopenia Reveals an Increase in SOCS-3 and Myostatin and a Reduced Efficiency of Akt Phosphorylation

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ABSTRACT

Age-related skeletal muscle sarcopenia is linked with increases in falls, fractures, and death and therefore has important socioeconomic consequences. The molecular mechanisms controlling age-related muscle loss in humans are not well understood, but are likely to involve multiple signaling pathways. This study investigated the regulation of several genes and proteins involved in the activation of key signaling pathways promoting muscle hypertrophy, including GH/STAT5, IGF-1/Akt/GSK-3β/4E-BP1, and muscle atrophy, including TNFα/ SOCS-3 and Akt/FKHR/atrogene, in muscle biopsies from 13 young (20 ± 0.2 years) and 16 older (70 ± 0.3 years) males. In the older males compared to the young subjects, muscle fiber cross-sectional area was reduced by 40–45% in the type II muscle fibers. TNFα and SOCS-3 were increased by 2.8 and 1.5 fold, respectively. Growth hormone receptor protein (GHR) and IGF-1 mRNA were decreased by 45%. Total Akt, but not phosphorylated Akt, was increased by 2.5 fold, which corresponded to a 30% reduction in the efficiency of Akt phosphorylation in the older subjects. Phosphorylated and total GSK-3β were increased by 1.5 and 1.8 fold, respectively, while 4E-BP1 levels were not changed. Nuclear FKHR and FKHRL1 were decreased by 73 and 50%, respectively, with no changes in their atrophy target genes, atrogin-1 and MuRF1. Myostatin mRNA and protein levels were significantly elevated by 2 and 1.4 fold. Human sarcopenia may be linked to a reduction in the activity or sensitivity of anabolic signaling proteins such as GHR, IGF-1, and Akt. TNFα, SOCS-3, and myostatin are potential candidates influencing this anabolic perturbation.

INTRODUCTION

Sarcopenia is the general term for a reduction in muscle quality and function due to aging. This can be seen by an increase in muscle atrophy, common in type II fibers,1 which is related to the reduction of maximal voluntary strength.2–4 The impairment of muscle development and function with age tends to accelerate after the age of 50 years5 and is linked with increases in falls and fractures, as well as a decreased capacity to recover from such injuries.6 Sarcopenia has an important socioeconomic consequence as falls are a major source

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of morbidity and mortality in the increasing population of the elderly. The molecular mechanisms controlling age-related skeletal muscle atrophy are not well understood and is likely to involve multiple cell signaling pathways controlling both protein synthesis and degradation. Growth hormone (GH) plays a key role in muscle development. Much of the anabolic effects of GH is mediated via insulin-like growth factor-1 (IGF-1). IGF-1 gene transcription is controlled by GH via a Janus kinase-2 (JAK2)/signal transducer and activator of transcription-5b (STAT5b) signaling pathway. In aging human skeletal muscle, a reduction in IGF mRNA has been observed and suggested to result from a reduced GH secretion or a reduced GH sensitivity. The mechanisms regulating GH and IGF levels in aged skeletal muscle are unknown. A possible mechanism may be the catabolic cytokine, tumor necrosis factor-α (TNFα), which is increased in aging skeletal muscle. TNFα and IGF-1 mRNA levels are increased and decreased, respectively, in mouse skeletal muscle following lipopolysaccharide injections, while the addition of TNFα directly to C2C12 mouse muscle cells also decreases IGF-1 mRNA. TNFα is known to regulate the transcription of suppressor of cytokine signaling-3 (SOCS-3), with the latter able to inhibit GH signaling to JAK2 and STAT5b. This could possibly lead to a reduction in IGF transcription. Whether SOCS-3 is increased and the activation of the JAK/STAT pathway decreased in aged human skeletal muscle is unknown.

A reduction in IGF-1 may also have a direct effect on muscle hypertrophy and atrophy signaling pathways as IGF-1 influences the activation of Akt (also known as protein kinase B [PKB]). IGF-1 regulates the Akt/mTOR and the Akt/GSK-3β signaling pathways, which are involved in the skeletal muscle hypertrophy process by activating downstream regulators of protein initiation and translation. In the elderly when compared to younger subjects, a reduction on the total protein content of several protein initiation and translation targets, such as mTOR, p70s6k, 4E-BP1, and eIF2B, all of which are either direct or indirect targets of Akt, have been observed. Additionally, the sensitivity and responsiveness of these proteins to stimulation via administration of essential amino acids are reduced in older subjects. These results demonstrate that human age-related sarcopenia is associated with a decrease in the content and sensitivity of several proteins downstream of Akt, which are implicated in catabolic processes. Inhibition of IGF-1 to phosphorylate, and activate, Akt removes the ability of Akt to phosphorylate, and inactivate, the forkhead family of transcription factors (FKHR). The inhibition of Akt phosphorylation of FKHR allows FKHR nuclear translocation where they have been shown, at least in rodent muscle, to transcribe two muscle-specific “atrogenes”—atrogin-1/MAFbx and muscle ring finger-1 (MuRF1), which are involved in the muscle atrophy process. A reduction in FKHR nuclear content in rodent muscle is associated with a reduction in atrogin-1/MAFbx and MuRF1. At present it is unknown if there is a perturbation in the activity of Akt /FKHR signaling in human sarcopenia.

Another interesting target, potentially making a link between GH, IGF, and Akt signaling, is myostatin. Myostatin, a member of the transforming growth factor-β family, inhibits muscle development. Myostatin mRNA in elderly skeletal muscle has been shown to be either increased or unchanged, when compared to younger skeletal muscle, while myostatin protein levels in elderly muscle have not been reported. Myostatin levels are inhibited by GH in human muscle, suggesting that a perturbation in GH levels, or GH activity, may result in increased myostatin levels. It has recently been reported that myostatin reduces the activity of Akt in both cardiomyocytes and in C2C12 cells where it also activates FKHR.

Findings in the literature have prompted us to hypothesize that human sarcopenia may be linked to increased levels of in TNFα and SOCS-3 causing perturbations in GH signaling and therefore increasing myostatin. Consequently, this would result in a reduced activation of Akt signaling and therefore an inhibition and activation, respectively, of the muscle hypertrophy and atrophy signaling cascades. The aims of the present study were to determine if age-related sarcopenia in humans was...
linked with perturbations in TNFα, SOCS-3, GH, STAT5, and IGF levels, as well decreases in the Akt/GSK/mTOR and increases in the Akt/FKHR/atrogene signaling pathways.

METHODS AND MATERIALS

Subjects

Thirteen healthy males (age, 20 ± 0.2 years; body weight, 73.3 ± 6.6 kg) and 16 older males (age, 70 ± 0.3 years; body weight, 78.0 ± 8.5 kg) participated in the present study. All subjects were healthy and active but were not trained. The research protocol was approved by the institutional ethics committee and informed consent was obtained from all participants.

Muscle biopsies

All subjects agreed to a skeletal muscle biopsy taken from the vastus lateralis muscles using a Bergstrom needle, as reported previously.33 All biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analyzed.

Fiber size

Identification of type I, IIa, and IIx muscle fiber types was performed using immunohistochemical staining and their cross-sectional areas were measured, as previously described.34

RNA extraction and real-time quantitative PCR

RNA was extracted from skeletal muscle using a commercially available preparation, peqGOLD Tri-Fast (Peqlab, Erlangen, Germany). Three μg of RNA was reverse transcribed to cDNA using Random Hexomer primers and a Stratascript enzyme (Stratagene, Amsterdam, The Netherlands) while quantitative PCR was performed using the MX3000p thermal cycler system, as published previously.35 PCR primer and probe sequences (Stratagene, La Jolla, CA) for atrogin-1, MuRF1, and ribosomal phosphoprotein P0 (RPLP0; 36B4) have been reported previously.35,36 Primers for TNFα, SOCS-3, IGF-1 and myostatin were forward, CG TCT CCT ACC AGA C; GTG GAG ACA GGG GCT TTT ATT TC, AGG TAT ACT GGA ATC CGA TCT CTG A and reverse, CC AAA GTA GAC CTG CCC AGA; CGG CAG CTG GGT GAC TTT; CTT GTT GTT TCC TGC ACT CCC TCT ACT; CAC TGT CTT CAC ATC AAT. GHR (MAB263) antibody from AbD Serotec (Oxford, United Kingdom). All antibodies were diluted 1:1000.

Statistical analysis

Unpaired t-tests were used to test for differences between the younger and older subjects. The level of significance was set at p < 0.05. Data are mean ± sem.

RESULTS

A representative immunohistological staining of a muscle cross section from a young and older subject is presented in Figure 1A. The differences in the cross-sectional area of type I, IIa, and IIx fiber types are shown in Figure 1B. In comparing the older with younger subjects, the cross-sectional area of the type I muscle fibers was 18% smaller, but this did not reach statistical significance. However, the cross-sectional area of type IIa and IIx muscle fiber was 40 and 45% smaller (p < 0.01 and p < 0.05, respec-
tively). There was no statistical difference in the percentage of type I, IIA, and IIX fiber types between groups.

In the skeletal muscle of the older compared with the younger subjects, TNFα, SOCS-3 mRNA, and SOCS-3 protein levels were increased by 2.8, 1.5, and 1.5 fold, respectively ($p < 0.05$). Growth hormone receptor (GHR) protein content was decreased by 45% ($p < 0.05$). There was no difference between STAT5 protein content; however, nuclear STAT5$^{Tyr694}$ protein levels tended to be increased by 1.4 fold in the muscle of the older subjects, but this did not reach statistical significance ($p = 0.056$) (Fig. 2).

There was a significant (45%; $p < 0.05$) reduction in IGF-1 mRNA in the older compared with the younger subjects. This led us to determine if this was paralleled with a reduction in IGF-1 protein content, as well as several targets downstream of IGF-1 known to be involved in muscle hypertrophy and atrophy. There was no significant difference in IGF-1 protein levels between the older and younger subjects. There was, however, a 2.5-fold increase ($p < 0.05$) in total Akt protein content in the older compared with the younger subjects. This did not result in an increase in the levels of phosphorylated Akt (Fig. 3). However, when determining the amount of phosphorylated active Akt, relative to the total amount of Akt, there was a 30% reduction ($p < 0.05$) in the older compared with the younger subjects.

GSK-3β and 4E-BP1 are downstream targets of Akt, and regulators of protein initiation and translation. In comparing the older with the younger subjects, there was a 1.8-fold increase ($p < 0.05$) in total GSK-3β protein content, which was paralleled by a 1.5-fold increase ($p < 0.01$) in phosphorylated GSK-3β protein levels. When the ratio between phosphorylated and
total GSK-3β protein levels was determined, there was no significant difference between groups. Total and phosphorylated 4E-BP1 protein levels were also not different between groups (Fig. 4).

The FKHR family of transcription factors are activated when Akt phosphorylation is reduced and can increase the transcription of atrogin-1 and MuRF1, two genes involved in muscle atrophy. Both FKHR (Foxo1) and FKHRL1 (Foxo3) were decreased by 73% (p < 0.01) and 50% (p < 0.05), respectively, in nuclear fractions from the older subjects, with no changes in the mRNA levels of atrogin-1 and MuRF1 (Fig. 5).

Myostatin, a negative regulator of muscle development and inhibitor of Akt activity, was also measured. Both myostatin mRNA and protein levels were significantly elevated by 2.0 (p < 0.05) and 1.4 fold (p < 0.01), respectively, in the muscle of the older subjects (Fig. 6).

For all mRNA analyses, the quantitative PCR measurements for the genes of interest were normalized to the housekeeping gene, ribosomal phosphoprotein PO (RPLPO; 36B4), which remained stable across groups. The values for the young and old subjects were 1.25 ± 0.11 and 1.35 ± 0.30 arbitrary units, respectively.

The Western blot images are of three representative young and old subjects.

**FIG. 2.** Differences in the mRNA levels of TNFα and SOCS-3 and protein levels of SOCS-3, growth hormone receptor (GHR), phosphorylated STAT5Tyr694, and total STAT5 in young and old subjects. The ratio of phosphorylated STAT5Tyr694 and total STAT5 is also shown. Significantly different from young subjects (*p < 0.05). The Western blot images are of three representative young and old subjects.
plasmic and nuclear proteins is provided in 
Supplementary Figure 2 (<www.liebertonline. 
com/rej>). An example of several complete 
Western blots is provided in Supplementary 
Figure 3 (<www.liebertonline.com/rej>).

**DISCUSSION**

Age-related sarcopenia is associated with in-
creases in falls and fractures, which have an 
important socioeconomic consequence as falls 
are a major source of morbidity and mortality 
in the increasing population of the elderly. The 
mechanisms contributing to skeletal muscle at-
rophy are not well understood and the molec-
ular signaling pathways influencing sarcopenia 
in humans have not been thoroughly inves-
tigated. Here we present several novel and 
important findings showing that age-related 
sarcopenia in older subjects compared with 
younger ones reveals an increase in SOCS-3

*FIG. 3. *Differences in the mRNA and protein levels of IGF-1 and protein levels of total Akt and phosphorylated AktSer473. The ratio of phosphorylated AktSer473 and total Akt is also shown. Significantly different from young sub-
jects (***p < 0.01; *p < 0.05). The Western blot images are 
of three representative young and old subjects.
and myostatin mRNA and protein levels. There were a reduced phosphorylation efficiency of the total Akt pool and an increase in phosphorylated GSK-3β. Furthermore there was a decrease in FKHR and FKHRL1 nuclear protein levels without changes in the mRNA levels of their atrogene targets, atrogin-1 and MuRF1.

The GH/IGF-1 axis is a key regulator of anabolism\textsuperscript{8,19} and has been shown to be perturbed in several models of muscle wasting, including

**FIG. 4.** Differences in the protein levels of phosphorylated GSK-3β\textsuperscript{Ser9} and 4E-BP1\textsuperscript{Thr37/46} total GSK-3β and 4E-BP1 in young and old subjects. The ratios of phosphorylated and total GSK-3β and phosphorylated and total 4E-BP1 are also shown. Significantly different from young subjects (**p < 0.01; *p < 0.05). The Western blot images are of three representative young and old subjects.
sepsis, trauma, burns, AIDS, cancer, and renal or liver failure,\textsuperscript{37–41} as well sarcopenia.\textsuperscript{42} In HEK 293 cells, GHR signaling can be blocked by SOCS-3 binding to the GHR,\textsuperscript{17} resulting in the inhibition of JAK2/STAT5 mediated signaling.\textsuperscript{18} Alternatively, SOCS-3 can also directly inhibit STAT5 activity.\textsuperscript{43,44} We therefore hypothesized that perturbations in TNF/\textit{H9251}/SOCS-3/GHR signaling cascade may play a key role in age-related human sarcopenia, via the inhibition of JAK/STAT transcription of IGF-1 mRNA. As observed previously,\textsuperscript{14} we found a substantial increase in TNF/\textit{H9251} mRNA in older subjects, which was associated with an increase in the TNF target gene SOCS-3 at both the mRNA and protein levels. This increase in SOCS-3 in human sarcopenia is similar to that recently observed in old versus young rats.\textsuperscript{45} We observed a tendency (\(p = 0.056\)) for an increase in the nuclear protein content of STAT5 in the older compared with the younger subjects. STAT5 can be activated via GHR signaling and inhibited by SOCS-3. In human sarcopenia, STAT5 may be activated, in a GHR-independent manner, in an attempt to offset a reduction in GHR sensitivity and reduce the amount of muscle loss. However, as the STAT5 target gene IGF-1 was downregulated, it appears that there may be a reduction in transcriptional activity of STAT5 or that other transcription factors may have a more important regulatory role for IGF-1 mRNA in elderly skeletal muscle. It has recently been observed that serum response factor (SRF) can also transcriptionally regulate IGF-1 mRNA in muscle.\textsuperscript{46} Whether SRF does play a role in human sarcopenia remains to be elucidated, and we are presently investigating this possibility. Combined, these results suggest that GHR sensitivity may be perturbed via a TNF/\textit{H9251}/SOCS-3 mechanism that reduces IGF-1 mRNA transcription.

Another key signaling pathway regulating the maintenance of human skeletal muscle...
mass involves the IGF-1 phosphorylation and activation of Akt, which initiates a series of signaling cascades promoting protein synthesis and, in parallel, inhibits the transcription of genes involved in protein degradation. Our observation of a decrease in IGF-1 mRNA led to the speculation that there would be a decrease in IGF and Akt protein levels in the older compared to the younger subjects. There was no significant decrease in IGF-1 protein, and surprisingly there was an increase in total Akt protein level. The increase in total Akt was not associated with a concomitant increase in phosphorylated Akt levels in the older subjects. This observation is novel and pertinent and indicates the failed attempt of a potential mechanism aimed at increasing protein synthesis through providing more Akt protein. However, the inability of the older skeletal muscle to phosphorylate more of the available Akt pool demonstrates a reduced efficiency of Akt phosphorylation. When expressed as the ratio phosphorylated Akt/total Akt, there was a 30% reduction in the older skeletal muscle. This ob-

![Graph showing differences in mRNA and protein levels of myostatin in young and old subjects.](image)

**Fig. 6.** Differences in the mRNA and protein levels of myostatin in young and old subjects. Significantly different from young subjects (*p < 0.01; †p < 0.05). The Western blot images are of three representative young and old subjects.

![Diagram summarizing the results.](image)

**Fig. 7.** Schematic summary of the results. Overview of the putative and proven signalling molecules involved in protein synthesis and protein degradation. The gene and proteins measured in the present study are shown in grey boxes. The results of the present experiments are indicated as follows: † increased; - unchanged; ‡ decreased in older vs. younger subjects. ▶ stimulation; ◄ inhibition.
ervation supports those recently made in older rat skeletal muscle and suggests a reduced efficiency in skeletal muscle Akt phosphorylation in elderly subjects.

Downstream targets of Akt involved in protein synthesis, 4E-BP1 and GSK-3β, were also measured. As the absolute amount of phosphorylated Akt was the same between the older and younger subjects, it would have been expected that phosphorylated 4E-BP1 and GSK-3β would also have been the same. As expected, no differences in the phosphorylation levels of 4E-BP1 were observed. However, there was an increase in the total protein content of GSK-3β, which, unlike Akt, was paralleled by an increase in the phosphorylation levels of GSK-3β in the older subjects. The increased pool of GSK-3β protein may be a result of increased protein translation or protein stability, aimed at providing the cell with a source to maintain protein synthesis. The mechanisms responsible for reducing the capacity to phosphorylate Akt appear not to influence GSK-3β phosphorylation, as increased levels of the latter were measured in the older subjects. These observations also suggest the existence of a mechanism that is able to phosphorylate GSK-3β, independently of Akt. A previous investigation has observed an Akt-independent increase in GSK-3β in mechanically stretched rodent muscle. In line with this is the recent suggestion that muscle protein synthesis rates may be increased in a futile attempt to maintain muscle mass; however increased levels of protein degradation is the determining factor during sarcopenia. Sarcopenia, unlike severe acute muscle-wasting disorders, such as ALS, is a gradual process. This gradual loss of muscle mass with age may be due to a number of attempted “survival” feedback mechanisms, including increases in the total pool of Akt and GSK-3β, as well as GSK-3β phosphorylation.

It has been demonstrated in rodents and myotubes that a reduced phosphorylation of Akt also results in an increase in the nuclear content of the FKHR and FKHR1 transcription factors, as well as an increase in the expression of their target genes, atrogin-1 and MuRF1, two muscle-specific E3-ligases involved in protein degradation. However, in the present study, both FKHR and FKHR1 nuclear protein levels were reduced in the skeletal muscle of older compared to the younger subjects, suggesting that factors other than Akt may be regulating human FKHR and FKHR1 activities. This is not without precedent as we and others have previously observed that human Akt and FKHR and FKHR1 do not always follow the same regulatory patterns. As FKHR and FKHR1 have been shown to regulate the atrogin-1 and MuRF1 genes, at least in rodents, this would suggest that the older subjects may have a reduction in the gene expression of these two atrophy genes. However, no differences in the mRNA levels of atrogin-1 and MuRF1 were observed between the two groups, which supports previous observations in aging human skeletal muscle. This suggests that other transcription factors may be involved in regulating human atrogin-1 and MuRF1.

A reduction in the efficiency to phosphorylate Akt, observed in the present study and by others in rodents, may be a crucial factor in controlling sarcopenia. Therefore identifying the factor(s) that may inhibit Akt phosphorylation is of importance. One such factor may be myostatin, which has recently been reported to reduce Akt phosphorylation in both cardiomyocytes and in mouse C2C12 cells. Myostatin mRNA, but not protein, levels have been measured and compared previously in older versus younger skeletal muscle, with investigations showing either increases in older subjects or no difference when compared to younger subjects. However, it was deemed relevant to measure the myostatin mRNA and protein levels in the present study and to determine if an association existed with Akt phosphorylation levels. Both myostatin mRNA and protein levels were increased in the older compared with younger subjects.

Although it is not within the scope of the present study to establish a cause and effect relationship between myostatin and Akt phosphorylation in human sarcopenia, these results, in combination with those from Morissette et al. and McFarlane et al., suggest that elevated myostatin levels may contribute to a reduced efficiency of Akt phosphorylation and human age-related muscle loss.

The present study has focused on the potential perturbation of key signaling proteins that may be involved in the reduction of muscle mass in age-related sarcopenia. However, it is
important to realize that there are other abnormalities occurring in aged skeletal muscle. Other common phenotypical traits include, but are not limited to, increased intramuscular triglyceride (IMTG) accumulation,\(^55,56\) insulin resistance,\(^57\) and a reduced myofiber regenerative potential.\(^58\) Although these factors were not measured in the present study, it is worth mentioning that several of the novel observations made in the present study may be relevant to these additional age-associated muscle alterations. For example, SOCS-3 is also implicated in triglyceride storage via its activation of sterol regulatory element binding protein (SREBP)-I\(^59\) and can also cause insulin resistance through downregulation of tyrosine phosphorylation of insulin receptor substrate (IRS) proteins.\(^60\) The reduced efficiency of Akt phosphorylation is also linked to increased IMTGs.\(^61\) The increased in skeletal muscle myostatin protein levels may be linked with myofiber necrosis, as myostatin levels have been shown to be elevated within necrotic, but not regenerating fibers.\(^62\) Additionally, increased levels of myostatin may contribute to reduced myofiber regenerative potential\(^58\) by inhibiting myoblast proliferation.\(^53\) Future investigations should be aimed at establishing if the alterations in signaling proteins observed in the present study are also implicated in other abnormalities occurring in aged skeletal muscle.

In summary, this is the first study to compare the regulation of several key signaling pathways known to control skeletal muscle hypertrophy, including GH/STAT5, IGF-1/Akt/GSK/4E-BP1, and skeletal muscle atrophy, including TNF\(\alpha\)/SOCS-3 and Akt/FKHR/atrogene, in muscle biopsies from young and old men (Fig. 7). It appears that human sarcopenia is associated with an increase in TNF\(\alpha\) and SOCS-3, which may result in a reduction of GHR levels or sensitivity. The significant increase in total Akt protein content, but not Akt phosphorylation in muscle from the older subjects, suggests an inefficiency in Akt activation and, by analogy, reduced protein synthesis. The observed increase in myostatin mRNA and protein levels in the older subjects, combined with recent observations in cardiac and rodent cells, suggest that myostatin is a prime candidate inhibiting Akt phosphorylation. Establishing if this is the case should be a priority for future investigations aimed at reducing human sarcopenia.

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Supplementary FIG 1. Western blot analysis showing the stability of α-tubulin in cytoplasmic fractions and Lamin A in nuclear fractions.

Supplementary FIG 2. A Ponceau stain of membranes with cytoplasmic or nuclear proteins demonstrating the accuracy of protein loading.
Supplementary FIG 3. Examples of two complete Western blots.
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