Comparison of GH, IGF-I, and testosterone with mRNA of receptors and myostatin in skeletal muscle in older men

TAYLOR J. MARCELL,1 S. MITCHELL HARMAN,1 RANDALL J. URBAN,1
DANIEL D. METZ,1 BUEL D. RODGERS,2 AND MARC R. BLACKMAN2
1Intramural Research Program, National Institute on Aging, National Institutes of Health;
2Division of Endocrinology and Metabolism, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21224; and 3Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas 77555

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Marcell, Taylor J., S. Mitchell Harman, Randall J. Urban, Daniel D. Metz, Buel D. Rodgers, and Marc R. Blackman. Comparison of GH, IGF-I, and testosterone with mRNA of receptors and myostatin in skeletal muscle in older men. Am J Physiol Endocrinol Metab 281: E1159–E1164, 2001.—Growth hormone (GH), insulin-like growth factor I (IGF-I), and testosterone (T) are important mediators of muscle protein synthesis, and thus muscle mass, all of which decline with age. We hypothesized that circulating hormones would be related to the transcriptional levels of their respective receptors and that this expression would be negatively related to expression of the myostatin gene. We therefore determined content of mRNA transcripts (by RT-PCR) for GH receptor (GHR), IGF-I, androgen receptor (AR), and myostatin in skeletal muscle biopsy samples from 27 healthy men >65 yr of age. There were no significant relationships between age, lean body mass, or percent body fat and transcript levels of GHR, IGF-I, AR, or myostatin. Moreover, there were no significant correlations of serum GH, IGF-I, or T with their corresponding target mRNA levels (GHR, intramuscular IGF-I, or AR) in skeletal muscle. However, GHR was negatively correlated (r = −0.60, P = 0.001) with myostatin mRNA levels. The lack of apparent relationships of muscle transcripts with their respective ligands in healthy older adults suggests that age-related deficits in both GH and T may lead to an increase in myostatin expression and a disassociation in autocrine IGF-I effects on muscle protein synthesis, both of which could contribute to age-related sarcopenia.

androgens; reverse transcription-polymerase chain reaction; gene expression; elderly; protein metabolism

IN HEALTHY YOUNG ADULTS, under equilibrium conditions, skeletal muscle protein synthesis and degradation are a balanced, dynamic process with no net change occurring in skeletal muscle mass. During aging, however, muscle tissue is gradually lost, often resulting in diminished mass and strength, a condition referred to as sarcopenia, which contributes to frailty. This loss of protein results from a net imbalance between the rates of protein synthesis and degradation (22). Protein synthesis can be stimulated by various signals, including hormones, metabolic demand, and functional overload (10, 23). Protein breakdown is also under the influence of these same factors, which stimulate lysosomal and ubiquitin degradation processes. Insulin, growth hormone (GH), insulin-like growth factor I (IGF-I), and testosterone (T) are anabolic hormones, all of which increase muscle mass by stimulating protein synthesis and/or inhibiting protein breakdown, whereas cortisol is a potent stimulus to protein catabolism (6, 9, 22). Circulating levels of these various hormones are altered by the aging process, potentially contributing to sarcopenia (2, 5, 12, 27).

Although GH-induced IGF-I production in the liver is the major source of circulating IGF-I and mediates many GH metabolic effects, local IGF-I production within target tissues, under the influence of both GH and T (28), accounts for >50% of total IGF-I production and appears to be more important for stimulating muscle growth and repair (10, 14, 16). How changes in hormone levels with age affect the IGF-I pathway in skeletal muscle remains poorly understood.

Myostatin, a recently discovered member of the transforming growth factor (TGF)-β superfamily, is an autocrine factor that is a potent inhibitor of muscle development (20). Postnatally, myostatin is expressed in varying levels exclusively in skeletal muscle (4, 29) and preferentially in fast-type skeletal muscle fibers (4). Age-related loss of skeletal muscle is associated with a selective atrophy of the fast-type skeletal muscle fibers (17). Thus, whether the age-related decline in anabolic hormonal status allows a progressive increase in myostatin expression that would contribute to sarcopenia is an important question (15). However, by what mechanism hormones interact with myostatin remains unclear.

Therefore, in the current study, we measured plasma levels of GH, IGF-I, T, and cortisol and, using the reverse transcription-polymerase chain reaction (RT-PCR), determined gene expression of IGF-I and myostatin in skeletal muscle biopsy samples from healthy older men. B135, Phoenix, AZ 85018 (E-mail: marcell@kronosinstitute.org).

Address for reprint requests and other correspondence: T. J. Marcell, Kronos Longevity Research Institute, 4455 E. Camelback Rd., Ste. B135, Phoenix, AZ 85018 (E-mail: marcell@kronosinstitute.org).

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older men. Because circulating hormones can auto-
regulate their actions by effects on their own receptors
(3), we also determined mRNA levels for GH receptor
(GHR) and androgen receptor (AR). The present study
was designed to test the hypotheses that 1) circulating
anabolic hormone concentrations are significantly re-
lated to the transcriptional levels of their respective
receptors, 2) the expression of GHR, IGF-I, and AR is up-
or downregulated coordinately, and 3) the expres-
sion of anabolic hormone receptors is negatively re-
lated to expression of the myostatin gene.

SUBJECTS AND METHODS

Subjects. Twenty-seven relatively healthy, ambulatory,
community-dwelling older men (Table 1) were recruited by
mass-mailed advertisement. All subjects underwent a com-
prehensive screening assessment that included a physical
examination, routine laboratory profiles, and a graded tread-
mill exercise electrocardiogram. No subject had diabetes mel-
itus, coronary artery disease, or untreated thyroid disease.
Study participants were nonsmokers, consumed <2 oz. of
alcohol per day, and took no medications known to interfere
with the GH/IGF-I or sex steroid axes or any other outcome
measure. Because these subjects were to participate in an
intervention study examining the separate and interactive
effects of exogenously administered recombinant human GH
and/or T, subjects were included only if serum IGF-I values
were >1 SD below the mean for subjects aged 20–35 yr (i.e.,
<230 ng/ml) and if serum total testosterone levels were <470
ng/dl. Over 70% of the men screened for participation in this
study had circulating IGF-I levels below this exclusion range.
The study was approved by the combined Institutional Re-
view Board of the Johns Hopkins Bayview Medical Center
and Intramural Research Program, National Institute on
Aging (NIA), and each subject provided written, informed
consent.

Study protocol. All subjects were admitted to the General
Clinical Research Center at the Johns Hopkins Bayview
Medical Center, where their body weight and height were
recorded and subjects underwent an assessment of body
composition for determination of percent body fat and lean
body mass (LBM) via dual-energy X-ray absorptiometry
(DEXA; Lunar Radiation, DPX-L, Madison, WI). At 900,
an intravenous catheter was inserted into a forearm vein for
subsequent blood sampling, and heparinized (1,000 U/I) 0.9%
saline was infused slowly to prevent clotting. Blood was
sampled for GH at 20-min intervals from 0000 until the
following morning at 0800, at which time blood samples for
GH-I and testosterone were drawn in the fasted state.

Hormone assays. Serum GH and total serum T were me-
sured in duplicate in the Endocrine Research Laboratory of
the Intramural Research Program, NIA. The sensitivity of
the GH immunoradiometric assay was 0.05 ng/ml. Intra-
assay coefficients of variation (CVs) at mean GH concentra-
tions of 2.5, 6.4, and 10.9 ng/ml were 2.0, 1.7, and 1.3%,
respectively, and interassay CVs at mean GH levels of 2.4,
6.2, and 11.2 ng/ml were 3.6, 2.7, and 4.1%, respectively.
Total T was measured by RIA (Diagnostic Products, Los
Angeles, CA) with a sensitivity of 10 ng/dl. Intra-assay CVs
at mean T concentrations of 60, 300, 597, and 998 ng/dl were
11.2, 6.7, 1.5, and 3.1%, respectively, and interassay CVs at
mean T levels of 76, 299, 707, and 1,041 ng/dl were 5.9, 3.9,
3.2, and 4.8%, respectively. Cortisol was measured by RIA
(ICN Pharmaceuticals, Diagnostics Division, Costa Mesa,
CA) with a sensitivity of 0.25 mg/dl. Intra-assay CVs at mean
cortisol concentrations of 4.0, 12.5, and 25.6 mg/dl were 4.3,
6.8, and 10.5%, respectively, and interassay CVs at mean
cortisol levels of 3.9, 10.9, 13.0, 16.8, and 19.4 mg/dl were
4.5, 4.5, 4.5, 4.5, and 5.5%, respectively. Total serum IGF-I was measured by RIA
after acid-ethanol extraction at Endocrine Sciences (Calabasas
Hills, CA). Sensitivity of the IGF-I assay was 30 ng/ml,
and the intra- and interassay CVs were 5.9 and 7.3% at 289
ng/ml and 4.6 and 6.3% at 591 ng/ml.

Skeletal muscle mRNA measures by RT-PCR. Skeletal
muscle samples were obtained from the midbelly of the dom-
inant vastus lateralis muscle, midway between its origin and
insertion, by use of the Bergstrom needle biopsy technique
(11), in the morning after the overnight blood sampling.
Visible fat and connective tissue were excised on a chilled
plate, and samples were placed into preweighed vials
and quick-frozen in liquid nitrogen. For RNA extraction,
the muscle sample was first pulverized in liquid nitrogen with
a mortar and pestle, followed by homogenization for 30 s at
8,000 rpm. Total RNA was then extracted using RNA
STAT-60 (Tel-Test, Friendswood, TX) and quantified by
determining absorbance at 260 nm in duplicate, and the values
were averaged.

Complementary DNA (cDNA) was reverse transcribed
(RT) from 0.5 μg of total RNA using 1 μM random hexamers
and 100 U Superscript II reverse transcriptase (GIBCO-BRL,
Rockville, MD) in a final volume of 20 μl at 42°C for 50 min,
followed by 5 min of heat inactivation at 99°C, following
the manufacturer’s recommendation.

cDNA was amplified from the 20 μl of RT reaction mix
in the same tube in a final concentration of 1× PCR buffer
(Perkin-Elmer, Norwalk, CT; 25 mM Tris·HCl, 50 mM KCl),
1.5 mM MgCl₂, 1.0 mM deoxy-NTP, forward and reverse
primers (gene of interest, 10 pmol glyceraldehyde-3-phos-
phate dehydrogenase (GAPDH), 1.5 pmol), 5 U AmpliTaq
DNA polymerase (Perkin-Elmer), and 0.0225 μCi/μl deoxy-
[32P]CTP (Amersham, Arlington Heights, IL) in a final vol-
ume of 50 μl. The linear portion of the amplification curve
for each transcript was defined and then utilized to determine
the appropriate number of PCR amplification cycles in the
RT-PCR analyses. As an example, data for the AR cycle
titration are depicted in Fig. 1. Similar curves for GHR,
IGF-I, and myostatin were also generated independently
(data not shown). PCR for AR started with an initial dena-
turation at 94°C for 3 min, followed by 26 cycles of denatur-
aturation at 94°C for 35 s, annealing at 58°C for 45 s, and
extension at 72°C for 40 s, with a final extension cycle at 72°C
for 7 min. GHR transcripts were similarly amplified for 26
cycles, whereas IGF-I was amplified for 28 cycles and myo-
statin for 30 cycles.
duplicate samples were then electrophoresed, and optical density was determined from the $^{32}$P incorporation into the subsequent bands. The arbitrary value for optical density was then averaged between duplicate samples. Analysis of duplicate samples minimizes the potential variability in reverse transcription, PCR cycle efficiency, and gel loading variations. The intra-assay variance for the entire set of duplicate samples determined (GHR, IGF-I, AR, and myostatin) was $0.1 \pm 9.7\%$ (mean $\pm$ SD; $n = 206$ pairs). Therefore, the mRNA data are expressed as the average optical density (arbitrary units) of the duplicate samples measured. Because this study was designed to focus on relationships of circulating hormone concentrations with expression of hormone receptor genes, we did not normalize our molecular data to a “housekeeping” gene such as GAPDH, because changes in such a housekeeper could influence the relationships studied. Relationships between continuous variables were analyzed using simple linear regression analysis and were expressed as a correlation coefficient ($r$). Significance was set at $P \leq 0.05$.

RESULTS

Relationships of skeletal muscle transcript levels with age, body composition, and circulating hormones. Subject characteristics are described in Table 1. Mean total LBM ($51.6 \pm 0.8$ kg) and percent body fat ($30.0 \pm 0.8\%$), determined by DEXA, were consistent with basic good health and did not suggest frailty or morbid obesity. To determine whether muscle transcript levels were related to age, body composition (total weight, body mass index, LBM, %fat) or circulating hormone concentrations (GH, IGF-I, T, and cortisol), we performed a series of simple and multiple regression analyses. There were no significant relationships between age or any of the measured indexes of body composition and transcript levels of GHR, IGF-I, AR, or myostatin (data not shown). Moreover, there were no significant correlations of GH, circulating IGF-I, or T with their corresponding target mRNA levels (GHR, intramuscular IGF-I, or AR) in skeletal muscle (Table 3). Furthermore, no relationships were observed between serum GH, circulating IGF-I, T, or cortisol and skeletal muscle myostatin mRNA levels (Table 3).

**Table 2. Oligonucleotide primer sequences for GHR, intramuscular IGF-I, AR, and myostatin**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHR 5’</td>
<td>5’-AAC-GGA-TTG-ATC-CAG-ATC-TTC-TCA-AAG-3’</td>
<td>185 bp</td>
</tr>
<tr>
<td>GHR 3’</td>
<td>5’-ACT-CTG-TAC-AAG-TCT-GCT-GCT-GTC-3’</td>
<td>225 bp</td>
</tr>
<tr>
<td>IGF-I 5’</td>
<td>5’-AGT-CTT-CCA-ACC-CAA-TTA-ATT-TAA-GTC-3’</td>
<td>387 bp</td>
</tr>
<tr>
<td>IGF-I 3’</td>
<td>5’-GCG-ACA-TGC-CCA-AGA-CCC-AGA-AG-3’</td>
<td>255 bp</td>
</tr>
<tr>
<td>AR 5’</td>
<td>5’-ATT-TGC-ACC-TGC-CGG-CTT-CTT-CCT-GCT-3’</td>
<td>860 bp</td>
</tr>
<tr>
<td>AR 3’</td>
<td>5’-TAG-GGT-ATA-ACA-TGA-ATG-3’</td>
<td>387 bp</td>
</tr>
</tbody>
</table>

GHR, growth hormone receptor; AR, androgen receptor; MSTN, myostatin.
significant (NS; Table 3). However, we did detect a significant direct relationship between GHR and AR mRNA levels \( (r = 0.41; P = 0.04; \text{Fig. 2}) \), as well as a trend indicating an inverse relationship between AR and IGF-I mRNA levels \( (r = -0.37; P = 0.06; \text{Table 3}) \), indicating possible interactions between these anabolic pathways.

Muscle transcript levels of the negative regulator myostatin were inversely related to the anabolic GHR mRNA \( (r = -0.60; P = 0.001; \text{Fig. 2}) \) gene expression, and a negative trend was observed between myostatin and AR \( (r = -0.36; P = 0.07) \) mRNA, but myostatin was not significantly related to IGF-I \( (r = -0.16; P = \text{NS}) \) mRNA levels (Table 3).

### DISCUSSION

In the present study of healthy older men, we observed a significant negative relationship between skeletal muscle myostatin and GHR gene expression, as determined by mRNA levels. Myostatin is thought to be a negative regulator of skeletal muscle growth, because null mutations (31) and gene knockout experiments of myostatin (20) have resulted in significant increases in muscle mass. Myostatin functionally decreases protein synthesis by inhibiting cell proliferation and DNA synthesis by blocking satellite cell (myoblast) activity (25, 26). In contrast, GH may exert its stimulatory effects on muscle protein synthesis, in part, by activating satellite cells (14). Thus the inverse relationship between myostatin and GHR gene expression in the current study suggests that GH effects on muscle protein synthesis (14) may, in part, occur by inhibiting myostatin’s effects on satellite cell activation. Although the direct mechanism by which GH signaling could inhibit myostatin remains unclear, a GH-responsive element has been identified upstream of the myostatin promoter (18, 25). Thus GH could directly downregulate myostatin transcription. However, Kirk et al. (13) observed no change in the expression of myostatin levels associated with GH-induced muscle hypertrophy in regenerating rat skeletal muscle.

Alternatively, the GH-induced expression of IGF-I may indirectly inhibit myostatin expression. Muscle-derived IGF-I is associated with an upregulation of myogenin (7), a regulatory factor that binds to myocyte enhancer factor 2 (MEF2) start sites, and these start sites have been identified within the myostatin promoter (13). Therefore, GH/IGF-I could modulate myostatin expression by interacting with various muscle promoters in a competitive manner. However, we observed no relationship between the autocrine production of IGF-I and that of myostatin. Currently, we are investigating this potential hypothesis further using a cell culture model.

Taken together, these data suggest that GH excess may override myostatin’s inhibition of satellite cell activation rather than GH directly inhibiting myostatin promoter activity. Thus an increased expression of muscle myostatin levels with age (S. Bhasin, personal communication) may be due, in part, to the age-related

<table>
<thead>
<tr>
<th></th>
<th>GHR mRNA</th>
<th>IGF-I mRNA</th>
<th>AR mRNA</th>
<th>MSTN mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum GH, ng/ml</td>
<td>( r = -0.16 ) ( (&gt; 0.4) )</td>
<td>( r = -0.29 ) ( (&gt; 0.1) )</td>
<td>( r = -0.03 ) ( (&gt; 0.8) )</td>
<td>( r = -0.03 ) ( (&gt; 0.9) )</td>
</tr>
<tr>
<td>Serum IGF-I, ng/ml</td>
<td>( r = -0.20 ) ( (&gt; 0.3) )</td>
<td>( r = -0.07 ) ( (&gt; 0.7) )</td>
<td>( r = 0.19 ) ( (&gt; 0.3) )</td>
<td>( r = -0.07 ) ( (&gt; 0.7) )</td>
</tr>
<tr>
<td>Serum testosterone, ng/dl</td>
<td>( r = -0.28 ) ( (&gt; 0.1) )</td>
<td>( r = -0.15 ) ( (&gt; 0.4) )</td>
<td>( r = 0.26 ) ( (&gt; 0.1) )</td>
<td>( r = -0.11 ) ( (&gt; 0.5) )</td>
</tr>
<tr>
<td>Serum cortisol, mg/ml</td>
<td>( r = -0.08 ) ( (&gt; 0.7) )</td>
<td>( r = -0.31 ) ( (&gt; 0.1) )</td>
<td>( r = 0.03 ) ( (&gt; 0.9) )</td>
<td>( r = -0.17 ) ( (&gt; 0.4) )</td>
</tr>
<tr>
<td>GHR mRNA</td>
<td>( r = -0.16 ) ( (&gt; 0.4) )</td>
<td>( r = 0.41 ) ( (&gt; 0.04) )</td>
<td>( r = -0.60 ) ( (&gt; 0.001) )</td>
<td></td>
</tr>
<tr>
<td>IGF-I mRNA</td>
<td>( r = -0.37 ) ( (&gt; 0.06) )</td>
<td>( r = 0.16 ) ( (&gt; 0.4) )</td>
<td>( r = -0.36 ) ( (&gt; 0.07) )</td>
<td></td>
</tr>
<tr>
<td>AR mRNA</td>
<td>( r = -0.36 ) ( (&gt; 0.07) )</td>
<td>( r = -0.36 ) ( (&gt; 0.07) )</td>
<td>( r = -0.36 ) ( (&gt; 0.07) )</td>
<td></td>
</tr>
</tbody>
</table>

Values = Pearson correlation coefficients (significance).
decrease in endogenous GH and testosterone levels, contributing to age-related sarcopenia. Although the functional significance of increased myostatin expression has been well documented, the mechanism by which anabolic hormones could inhibit myostatin remains uncertain.

In keeping with the basic state of good health of these study participants, none of the healthy older men we studied would be considered frail or sedentary on the basis of published values for fitness (24) or body composition (8) for this age group. Nonetheless, aging is associated with a progressive loss of lean body mass and muscle strength (2, 21), and thus these men were relatively sarcopenic compared with published data for younger men (17). Additionally, overnight mean circulating GH, serum IGF-I, and T values were similar to levels previously observed in comparably aged men but were significantly reduced compared with values for young adults (5, 12, 19).

Both circulating GH and T are thought to elicit their effects on skeletal muscle protein synthesis by binding to their receptors and increasing muscle gene transcription, including that of IGF-I (1, 28). Several lines of evidence suggest that GH exerts effects on muscle protein synthesis via locally produced rather than circulating IGF-I (14). Despite these mechanisms, we could not detect relationships between circulating GH or T and mRNA levels for their receptors, nor were circulating GH and T related to intramuscular IGF-I gene expression, as we had hypothesized.

As noted earlier, all of the subjects studied were old and had relatively low hormone levels. Therefore, our findings may reflect the limited variability of measured GH and T within the group studied. Such relationships may be better observed in younger persons in whom a wider range of hormone values would be obtained. Another possibility is that the normal relationships of GH, IGF-I, and T to muscle gene activation are disrupted by the aging process itself. For example, in one study of six elderly subjects, skeletal muscle IGF-I mRNA levels were not increased after an acute GH injection (30).

On the basis of the data in the present study, we conclude that age-related deficits in both GH and T may lead to an increase in myostatin expression and a dissociation in autocrine IGF-I effects on skeletal muscle protein synthesis, both of which could contribute to age-related sarcopenia.

We thank Dr. Tom Wood [University of Texas Medical Branch (UTMB) Molecular Core Laboratory] for critical comments in optimizing the RT-PCR protocol utilized and in sequencing the DNA fragments, Dr. S. Bhasin and colleagues for supplying the primer sets for myostatin, Dr. J. Jayme for valuable assistance in obtaining the muscle biopsies, Carol St. Clair for performing the serum GH and testosterone assays, Tracey A. Roy for performing the DECA scans, and the nursing staff of the Johns Hopkins Bayview Medical Center’s General Clinical Research Center for expert assistance with patient studies.

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