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

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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). GH, however, 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 dissociation 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 muscle 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

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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 related 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 expression of anabolic hormone receptors is negatively related 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 comprehensive screening assessment that included a physical examination, routine laboratory profiles, and a graded treadmill exercise electrocardiogram. No subject had diabetes mellitus, 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 Review 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 1900, an intravenous catheter was inserted into a forearm vein for subsequent blood sampling, and heparinized (1,000 U/L) 0.9% saline was infused slowly to prevent clotting. Blood was sampled for GH at 20-min intervals from 2000 until the following morning at 0800, at which time blood samples for IGF-I and testosterone were drawn in the fasted state.

Hormone assays. Serum GH and total serum T were measured 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 concentrations 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, 33.3, 45.5, 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 dominant 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 glass 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 gyceraldehyde-3-phosph dehydrate 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 volume 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 denaturation at 94°C for 3 min, followed by 26 cycles of denaturation 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 myostatin for 30 cycles.

Table 1. Subject characteristics of the healthy older men

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Range</th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>72.7 ± 0.9</td>
<td>66–82</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>78.5 ± 1.7</td>
<td>58–92</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.7 ± 0.6</td>
<td>22–32</td>
</tr>
<tr>
<td>Lean body mass, kg⁺</td>
<td>51.6 ± 0.8</td>
<td>42–59</td>
</tr>
<tr>
<td>Body fat, %⁺</td>
<td>30.0 ± 0.8</td>
<td>22–38</td>
</tr>
<tr>
<td>GH, ng/ml</td>
<td>0.75 ± 0.10</td>
<td>0.3–2.4</td>
</tr>
<tr>
<td>IGF-I, ng/ml</td>
<td>126.0 ± 8.3</td>
<td>63–219</td>
</tr>
<tr>
<td>Testosterone, ng/dl</td>
<td>428.5 ± 19.1</td>
<td>252–627</td>
</tr>
<tr>
<td>Cortisol, mg/dl</td>
<td>6.6 ± 0.2</td>
<td>4.8–9.4</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 27 men. BMI, body mass index; GH, growth hormone; IGF-I, insulin-like growth factor I. Body composition determined by dual-energy X-ray absorptiometry. Mean 12-h (Q₁₀, min, 0800–2000) overnight value.
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 ± 9.7% (mean ± 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 ± 0.8 kg) and percent body fat (30.0 ± 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).

**Interrelationships among skeletal muscle transcript levels.** We observed no significant interrelationship between basal levels of skeletal muscle GHR and intramuscular IGF-1 gene expression ($r = -0.16$; $P = non-

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'-AAAG-GGA-TTG-ATC-CAG-ATC-TTC-TCA-AGG-3'</td>
<td>185 bp</td>
</tr>
<tr>
<td>GHR 3'</td>
<td>5'-ATC-GCT-TAG-AAG-TCT-GTC-GTC-3'</td>
<td></td>
</tr>
<tr>
<td>IGF-1 5'</td>
<td>5'-AGT-CTT-CCA-ACC-CAA-TTA-TTT-AAG-3'</td>
<td>387 bp</td>
</tr>
<tr>
<td>IGF-1 3'</td>
<td>5'-CCG-ACA-TGG-CCA-AGA-AGC-3'</td>
<td></td>
</tr>
<tr>
<td>AR 5'</td>
<td>5'-TTG-TCC-ACC-TGG-TCT-CTT-GTG-3'</td>
<td>225 bp</td>
</tr>
<tr>
<td>AR 3'</td>
<td>5'-TTG-ACC-TGG-TCT-CTT-GAT-3'</td>
<td></td>
</tr>
<tr>
<td>MSTN 5'</td>
<td>5'-ATG-ATG-CAT-CAA-3'</td>
<td>860 bp</td>
</tr>
<tr>
<td>MSTN 3'</td>
<td>5'-TAG-AGG-GTA-AAG-ACA-GCA-TG-3'</td>
<td></td>
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</tbody>
</table>

GHR, growth hormone receptor; AR, androgen receptor; MSTN, myostatin.

Fig. 1. Optimizing RT-PCR for androgen receptor (AR) by determining the linear phase of PCR amplification after a cycle titration. AR was expressed in duplicate between 20 and 40 cycles. Bottom: representative gel electrophoretic pattern for AR. Similar curves were determined for growth hormone receptor (GHR), insulin-like growth factor I (IGF-I), and myostatin (data not shown).
Table 3. Correlations between serum hormone levels and skeletal muscle gene transcripts

<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>$r = 0.41 (0.04)$</td>
<td>$r = -0.03 (&gt; 0.8)$</td>
<td>$r = -0.60 (0.001)$</td>
</tr>
<tr>
<td>IGF-I mRNA</td>
<td>$r = -0.31 (&gt; 0.1)$</td>
<td>$r = -0.37 (0.06)$</td>
<td>$r = 0.16 (&gt; 0.4)$</td>
<td>$r = -0.36 (0.07)$</td>
</tr>
<tr>
<td>AR mRNA</td>
<td>$r = -0.20 (&lt; 0.1)$</td>
<td>$r = 0.41 (0.04)$</td>
<td>$r = -0.03 (&gt; 0.8)$</td>
<td>$r = -0.60 (0.001)$</td>
</tr>
</tbody>
</table>

Values = Pearson correlation coefficients (significance).

significant (NS); Table 3]. However, we did detect a significant direct relationship between GHR and AR mRNA levels ($r = 0.41; P = 0.04$; Fig. 2), as well as a trend indicating an inverse relationship between AR and IGF-I mRNA levels ($r = -0.37; P = 0.06$; Table 3), indicating possible interactions between these anabolic pathways.

Muscle transcript levels of the negative regulator myostatin were inversely related to the anabolic GHR ($r = -0.60; P = 0.001$; 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 ($r = -0.16; P = NS$) 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
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 DEXA 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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