Differential Anabolic Effects of Testosterone and Amino Acid Feeding in Older Men

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The interaction between testosterone and exogenous amino acids was studied in older men before and after 6 months of testosterone administration. Twelve healthy older male subjects were randomly assigned in double-blind fashion to receive either testosterone enanthate [T; n = 7; 68 ± 3 (±SE) yr] or placebo (n = 5; 67 ± 3 yr) for 6 months. Muscle protein kinetics were determined using stable isotope methodology, arterial-venous difference across leg muscle, and muscle biopsies. In addition, ubiquitin-proteasome activity was measured in muscle biopsies as an indicator of muscle protein breakdown. T improved fasting net protein balance, although it remained significantly negative. The improvement in net balance was due to a decrease in muscle protein breakdown, as protein synthesis was unchanged. Ubiquitin-proteasome activity was also decreased with T. Exogenous amino acids increased protein synthesis in both placebo and T groups, but to a lesser degree after 6 months of T treatment. These results indicate that prolonged T administration increases net protein balance in the fasted state, but no additive effect is demonstrated when combined with amino acid feedings. Taken together, however, these diverse stimulatory effects can increase lean body mass and muscle strength over time. (J Clin Endocrinol Metab 88: 358–362, 2003)
a 20-gauge polyethylene catheter was inserted into an antecubital vein to obtain baseline blood samples for measurement of background amino acid enrichment, indocyanine green concentration, and hormonal concentrations. Infusion studies were conducted as depicted in Fig. 1. A primed continuous infusion of the stable isotope tracers \([^{2}H_{3}]ketoisocaproic\) acid (priming dose, 4.8 \(\mu\)mol/kg; infusion rate, 0.15 \(\mu\)mol/kg/min) and \(^{15}N\)-[ring-\(^{2}H_{3}\)]phenylalanine (priming dose, 2 \(\mu\)mol/kg; infusion rate, 0.07 \(\mu\)mol/kg/min; Cambridge Isotope Laboratories, Andover, MA) was given throughout the 8-h study. Biopsies of the vastus lateralis were performed as previously described (15) after 2, 5, and 8 h of tracer infusion. Muscle protein net balance (NB) was determined by arterio-venous balance of phenylalanine across leg muscle by:

\[
NB = (C_a - C_v) \times BF
\]

where \(C_a\) and \(C_v\) are phenylalanine concentrations in the femoral artery and femoral vein, respectively, and BF is blood flow as measured by indocyanine green dilution. Appearance (Ra) and disappearance (Rd) from the plasma pool, an indicator of protein breakdown and synthesis, respectively, were calculated by:

\[
Ra = \frac{[E_aC_a - EvC_v]/E_a}\times BF
\]

and

\[
Rd = \frac{E_p1 - E_p2}{E_a}\times BF
\]

where \(E_a\) and \(E_v\) are the arterial and venous tracer enrichments, respectively. The fractional synthetic rate (FSR) of skeletal muscle was determined by the rate of \([^{2}H_{3}]ketoisocaproic\) acid enrichment, indocyanine green concentration, and hormonal concentrations. Appearance (Ra) and disappearance (Rd) of the intracellular pool as the precursor:

\[
FSR = \frac{[E_p1 - E_p2]/[E_a] \times 60}{100}
\]

where \(E_p1\) and \(E_p2\) are the enrichments of the protein-bound \([^{2}H_{3}]\)leucine (from transamination of \([^{2}H_{3}]ketoisocaproic\) acid) from the biopsies at 2 and 5 h of isotope infusion and from 5–8 h to determine the effects of exogenous amino acids. \(E_a\) represents the average intracellular \([^{2}H_{3}]\)leucine enrichment over the time of incorporation, and \(t\) is the time in minutes. The factors 60 and 100 are required to express FSR as percentage per hour.

After isotope infusions were started, 3Fr 8-cm polyethylene catheters (Cook, Inc., Bloomington, IN) were inserted into the femoral vein and femoral artery under local anesthesia. Both femoral catheters were used for blood sampling, and the femoral arterial catheter was also used for indocyanine green infusion for the determination of leg blood flow. A 2nd 20-gauge polyethylene catheter was placed in the contralateral femoral artery for the determination of blood flow, a continuous infusion (infusion rate, 0.5 mg/min) of indocyanine green was started 15 min before the sampling hour. Subsequent sampling was performed simultaneously from the femoral vein and the peripheral vein for approximately 30 min.

**Analysis of samples**

**Blood.** The blood concentration of unlabeled phenylalanine as well as the enrichment of \([^{2}H_{3}]\)leucine were simultaneously determined by gas chromatography-mass spectrometry (GCMS) using the internal standard approach and the tert-butyldimethylsilyl derivative as previously described (17). The isotopic enrichment of free amino acids in blood was determined on an HP model 5973 GCMS instrument (Hewlett-Packard Co., Palo Alto, CA) by electron impact ionization and selected ion monitoring (18).

**Muscle.** Tissue biopsies of the vastus lateralis were immediately rinsed with cold saline, blotted, and frozen in liquid nitrogen. Samples were then stored at \(-80^\circ\text{C}\) until processed. The tert-butyldimethylsilyl derivative was prepared for the intracellular free water as previously described (17) and was analyzed by GCMS (model 5989B, Hewlett-Packard Co.) using electron impact ionization. The protein-bound enrichment of leucine was analyzed as previously described (8, 19) by GCMS.

**Blood hormones.** Blood was drawn from the femoral vein at the beginning (\(-0708\) h) of each stable isotope study for the determination of total T concentrations. All hormonal analyses were determined by a double-antibody method with commercial RIAs (Diagnostic Products, Los Angeles, CA). The intraassay coefficient of variation for T was 5.0%, and the interassay coefficient of variation was 7.0%.

**Proteasome activity assay.** To determine muscle proteolytic activity, a portion of the muscle biopsy was analyzed for chymotrypsin-like peptidase activity according to the method described by Liu et al. (20). Briefly, muscle tissue was powdered in liquid nitrogen and sonicated in buffer (50 mM HEPES, 2 mM EDTA, 30 mM sodium pyrophosphate, 100 mM NaF, 150 mM NaCl, 2 mM NaVO_4, pH 7.2). Samples were then centrifuged for 50 min at 10,000 \(\times\) g at 4°C, and the supernatant was used for the assay. Seventy-five micrograms of the muscle lysate were used in 0.2 mM suc-llvy-amc (Affiniti Research Products Ltd., Mamhead Castle, UK) as substrate and were run in duplicate with and without adding lactacystin. After 1-h incubation at 37°C, proteasome assay activity was measured with a 96-well plate fluorometer by the amount of 7-amino-4-methylcoumarin liberated (excitation, 360 nm; emission, 460 nm) at 20, 40, and 60 min (values averaged and reported). This methodology yields a coefficient of variation of 5.8%. Final results were expressed as fluorescence units per microgram of protein per minute. The assay was run with and without lactacystin (Affiniti Research Products Ltd., final concentration, 10 \(\mu\)M), a specific inhibitor of proteasome peptidase activity. Lactacystin covalently modifies the N-terminal threonine of the \(\beta\)-subunits, which are involved in catalysis (21). Lactacystin does not inhibit other proteases, nor does it affect lysosomal proteolysis (20). Thus, the use of this inhibitor allows for the determination of ubiquitin-proteasome pathway activity. This pathway is primarily responsible for accelerated proteolysis in catabolic conditions (22).

**Data presentation and statistical analysis**

Data are presented as the mean \(\pm\) SEM. Comparison of baseline and 6-month measurements for each group were compared by two-way repeated measures ANOVA with Scheffe’s multiple comparison test. \(P < 0.05\) is considered significant.
Results

TE injections were adjusted by an independent clinician (every 2 wk) to maintain levels within the normal range (17–28 nmol/liter); thus, the serum T concentrations and doses of TE were variable from individual to individual, as described by Ferrando et al. (1). However, serum T concentrations were greater in the treatment group at all time points after baseline (baseline = month 0, i.e. beginning of the study; P < 0.05) (1). The average serum concentrations over the 6-month study period were 23.0 ± 1.1 nmol/liter for the T group and 11.3 ± 0.3 nmol/liter for the placebo group. Subject characteristics, clinical measures, and functional outcomes are presented in the report by Ferrando et al. (1); however, Table 1 depicts body mass and T changes in each group over the 6-mo study. Briefly, the placebo and T groups were similar in starting age and weight. T administration did not adversely affect clinical outcomes, although estradiol and hematocrit were significantly increased.

Lean body mass (Table 1) and muscle strength increased, and percent body fat decreased in the T group (1).

The change in the net balance of phenylalanine across leg muscle improved significantly during the fasted state after 6 months of T treatment (Fig. 2). Although the net balance of skeletal muscle protein was still significantly negative, T administration improved net balance by 18 ± 8 nmol phenylalanine/min-100 ml leg vs. –9 ± 10 nmol phenylalanine/min-100 ml in the placebo group (P < 0.05). The change in net balance during fasting accounts for the demonstrated changes in leg lean body mass. Converting these changes in phenylalanine net balance during the fasted state after 6 months to milligrams of lean body mass (assuming that protein is 4% phenylalanine and muscle is 73% phenylalanine and, in turn, milligrams of leg muscle protein balance during the fasted state after 6 months to milligrams of leg in the T group, whereas the placebo group demonstrated an increase of 61 ± 27 nmol phenylalanine/min-100 ml leg (P < 0.01). The change in the rate of disappearance of phenylalanine from the plasma (Rd) was not significantly different between groups (P > 0.05).

Net balance (Fig. 2) and FSR increased in response to amino acid infusion (P < 0.0001), although there was no additive effect of T. In the placebo group, FSR increased 3-fold with amino acids (from 0.067 ± 0.011% to 0.199 ± 0.033%/h) at baseline and again at 6 months (from 0.073 ± 0.019% to 0.224 ± 0.043%/h). The same increase was apparent in the T group at baseline (from 0.071 ± 0.02% to 0.213 ± 0.043%/h); however, at 6 months the increase was slightly more than 2-fold (from 0.073 ± 0.019% to 0.144 ± 0.033%/h). This increase in FSR due to amino acid infusion was not significantly different between groups (P = 0.4). The changes over time in net balance, FSR, Ra, and Rd with amino acid feeding are outlined in Table 2. There was a trend toward down-regulation of protein turnover with T treatment, as measured by Ra and Rd (P < 0.05), demonstrating that there is no additive effect of T and amino acids.

The proteasome peptidase activity decreased significantly after T. Figure 3 outlines activity with and without the lactacystin inhibitor. Although there were no changes without the inhibitor in either group, T reduced the inhibitable proteolytic activity at 6 months compared with baseline (0.068 ± 0.007 fluorescence units/µg protein/min to 0.043 ± 0.002; by t test, P < 0.02). The change between groups was also significant (by t test, P < 0.03), indicating that ubiquitin-proteasome pathway activity was decreased after 6 months of T treatment. These data corroborate our kinetic data, in that protein breakdown is decreased in the fasted state after 6 months of T treatment.

Discussion

Our data indicate that T and amino acid administration exert their anabolic effects on skeletal muscle via different mechanisms in older men. Long-term T treatment is anabolic to skeletal muscle by reducing protein breakdown in the fasted state, whereas amino acid infusion results in a stimulation of protein synthesis. Contrary to our hypothesis, there were no additive effects of T on amino acid administration. Acute amino acid infusion stimulated net protein synthesis in both groups, an effect that was greater than that of T. However, the effect of T on fasting muscle, when added over days and months, led to an increase in lean body mass and strength in older men (1).

This study also demonstrates the differences in the anabolic response of skeletal muscle in young and old men given T. In young men, acute T administration eliminated the neg-

TABLE 1. Subject body mass and T concentrations

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>T</th>
<th>Placebo</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variable</strong></td>
<td>Baseline</td>
<td>6 months</td>
<td>Baseline</td>
</tr>
<tr>
<td>Total mass (kg)</td>
<td>89.7 ± 5.0</td>
<td>90.3 ± 5.0</td>
<td>97.6 ± 6.6</td>
</tr>
<tr>
<td>Total lean mass (kg)</td>
<td>62.2 ± 3.6</td>
<td>66.4 ± 4.0</td>
<td>68.2 ± 2.9</td>
</tr>
<tr>
<td>Total leg lean mass (kg)</td>
<td>18.3 ± 1.2</td>
<td>19.9 ± 1.1</td>
<td>20.9 ± 1.3</td>
</tr>
<tr>
<td>Total T (nmol/liter)</td>
<td>12.4 ± 2.0</td>
<td>23.6 ± 3.7</td>
<td>9.8 ± 1.9</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. Mass measures are by DEXA and total T by RIA. P value is by two-way repeated measures ANOVA. NS, Not significant.
ative net balance of phenylalanine across the leg during the fasted state (6), whereas the net balance improved, but remained significantly negative, in these older men. In the young men, muscle anabolism was due to the increase in muscle protein synthesis, with a trend toward an increase in protein breakdown (6), whereas the effect in the older men was a decrease in protein breakdown, with no change in protein synthesis. The demonstrated decrease in proteasome activity supports the metabolic findings that changes in muscle protein breakdown occurred in response to T administration in these older men. The observation that approximately 75% of the chymotrypsin-like proteolytic activity is inhibitable by lactacystin suggests that the ubiquitin-proteasome pathway contributes significantly to the overall proteolytic activity in skeletal muscle in older men (20). Further, the reduced inhibition at 6 months suggests that this pathway is down-regulated with T administration.

The reason for these dissimilar mechanisms is unclear and may be related to T dosage, timing of T administration, and length of T administration. The young subjects were studied 5 d after a 200-mg dose was given, with T levels being well above the physiological range (6). In the present study a similar dose may have been given; however, our subjects were studied 2 wk after injection, when T concentrations were in the normal range (1). Hypogonadal men studied 1 wk after injection demonstrated an increase in muscle FSR (3). We have previously demonstrated that after 1 month of T administration, muscle FSR is increased (2). However, T concentrations were in the upper normal range in subjects studied 6 d after injection (2). The 1-month period in the present study was also coincident with higher blood T concentrations and study time post-treatment. We also cannot exclude the possibility that the anabolic response to T changes throughout prolonged administration.

The effect of amino acid infusion on skeletal muscle anabolism is acutely greater than that of T at the dosages tested.
Although T improved net balance in the fasted state, the improvement was modest, and net balance remained negative. Regardless of the treatment group, however, amino acids result in a positive net balance of phenylalanine and amino nitrogen (9). Amino acid uptake into muscle was related to a dramatic stimulation of muscle protein synthesis. Interestingly, these data reveal that the change in synthetic response to amino acids (as determined by Rd) is lowest in the T group. After 6 months of T treatment, the combination of T and amino acids increased the rate of phenylalanine disappearance from blood (Rd), but not to the degree demonstrated at non-T-supplemented time points. This may be related to the T-mediated increase in the synthetic efficiency of the muscle with amino acids (16) and the ability of T to stimulate reutilization of intracellular amino acids (6, 16). This finding is further supported by the trend for protein turnover (Ra and Rd) to decrease when T and amino acids are combined. This finding is not limited to older men. Sheffield-Moore et al. (16) studied young men in the fasted state and again after amino acid infusion. The researchers noted that before receiving T, muscle protein synthesis (FSR) increased by 94% with amino acid infusion, whereas after 5 d of T treatment, protein synthesis increased by only 53% after amino acid infusion (16). However, the combined effect of T and amino acids resulted in a significant increase in protein synthetic efficiency (16). Thus, for a given amount of intracellular amino acids, a greater portion is routed toward protein synthesis with T. Taken together, these data indicate that T may serve to limit skeletal muscle protein turnover when combined with amino acids.

Despite the apparent blunting of amino acid-stimulated muscle protein synthesis with T, the overall effect is one of anabolism. The anabolic effect of T in this older population is the reduction of muscle nitrogen loss in the fasted state, whereas amino acids stimulate protein synthesis. Although the present administration method of amino acids is neither physiologial nor practical, a bolus ingestion, similar to postmeal delivery of amino acids, also stimulates muscle nitrogen uptake (9). Our laboratory has previously demonstrated that elderly subjects respond to oral amino acids to the same degree as young subjects (10). The positive net balance across the muscle is short-lived after a bolus ingestion (9), but with the amelioration of muscle catabolism by T in the nonfed states, the net effect of multiple feedings and T is muscle anabolism over a 24-h period. Extrapolated over 6 months, these small changes equate to significant gains in LBM and muscle strength, even in the absence of additional exercise (1). In fact, the change in leg lean body mass could be entirely explained by the effects of T on fasting muscle (see Results).

In summary, prolonged T administration in older men promotes muscle anabolism by reducing protein breakdown in the fasted state, whereas amino acid administration results in an acute stimulation of muscle protein synthesis. T does not provide any additional stimulation of muscle anabolism when combined with amino acid supplementation and, in fact, limits protein turnover. The primary effects of T demonstrated in the fasted state combined with the stimulation of synthesis by amino acid infusion (feeding) result in net muscle anabolism over time.

Acknowledgments

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