Increase in tendon protein synthesis in response to insulin-like growth factor-I is preserved in elderly men

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The aging tendon is associated with marked structural and biochemical changes. A reduced expression, alignment, and content of collagen, as well as altered fibrillar structure, have all been shown in old age (5, 7, 12). Furthermore, the function of the tissue seems to be impaired with an increased incidence of tendon overuse injuries and ruptures with aging (16). The physiological mechanisms behind these age-related changes leading to deterioration of tendon tissue are not known. However, animal studies and in vitro models suggest that a decrease in tenocyte number (7, 13, 22) and a cellular anabolic resistance (18, 19) could be important factors in tendon aging. Furthermore, human data show an age-dependent decrease in levels of some of the circulating humoral factors that have an anabolic effect on tendon tissue, and this reduction of stimulants could play a role for tendon aging (9, 17, 21). Two of these humoral factors, which appear to have an impact on tendon homeostasis, are growth hormone (GH) and insulin-like growth factor-I (IGF-I), which are both known to be important stimulators of collagen synthesis (1, 6, 8). Doessing et al. showed that systemic GH can stimulate the tendon collagen synthesis rate in healthy young men (6). Recently, Hansen et al. demonstrated that IGF-I directly injected in adult human patellar tendons (in subjects 55–70 yr of age) acutely increased tendon collagen synthesis rate (8). Based on these studies, it seems certain that IGF-I functions as an anabolic stimulus in human tendons. The activity of the GH/IGF-I axis as well as the GH and IGF-I serum concentrations are markedly decreased with aging (2, 3, 14). Furthermore, animal and in vitro studies show that the fibroblast response to IGF-I is blunted with increasing age (4, 15). Therefore, the age-dependent decrease in circulating IGF-I amplified by the decreased responsiveness of fibroblasts could be key elements in the phenotype of the aging tendon. However, the tendon anabolic response to IGF-I has never been investigated directly with focus on human aging.

In this study, we wanted to address the question of tendon responsiveness to IGF-I with human aging. We investigated the effect of IGF-I injections on protein synthesis in the patellar tendons in old and young men in vivo. Based on the earlier mentioned in vitro and animal studies, we hypothesized that the old group would be less responsive to IGF-I with regard to tendon protein synthesis compared with the young.

**MATERIALS AND METHODS**

**Participants.** Eleven young (20–30 yr of age) and 11 elderly (66–75 yr of age) men participated in the study. All participants were healthy (based on a health examination including a medical interview, physical examination, and routine blood samples), had a body mass index (BMI) between 19 and 26, and were moderately physically active (0–5 h of physical activity/wk). Participants were excluded from the study if they had any systemic diseases, history of patellar tendon problems, or previously had any patellar tendon biopsies taken. The study was approved by the Ethical Committee of the Capital Region of Denmark (H-1-2011-010), and it was conducted in accordance with the Helsinki Declaration.

**Baseline measurements.** To determine circulating IGF-I levels, a fasting blood sample was obtained between 0800 and 0900 in the morning, several days before the experiment, to control for the known effect of feeding and circadian rhythm on circulating IGF-I concentration (10, 11). Because we, at the experimental days, treated all participants with an IGF-I injection in one patellar tendon, we also wanted to measure if spillover from the IGF-I injections could be detected in the circulation. Therefore, an additional fasting blood sample was obtained in the morning 17 h after the first IGF-I injection and analyzed for serum IGF-I. All blood samples were obtained from the antecubital vein and left for a minimum of 30 min at room temperature. Whole blood was centrifuged (3,200 g, 15 min, 4°C), and serum was stored at −80°C until further analyses. Total serum IGF-I was measured with ELISA (RMEE20; Biovendor) following the
manufacturer’s manual. We verified that the assay was able to detect the correct amounts of exogenously added IGF-I in serum by adding the IGF-I compound, which was used for the injections (mecasermin, Incrlex; Ipsen), to human serum samples and analyzing these with the RMLE20 ELISA. All subjects had their lean body mass (LBM) measured in a dual-energy X-ray absorptiometer scanner (Lunar DPX-IQ; GE Healthcare, Chalfont St. Giles, UK) before experiments, which was used to calculate the exact amounts of tracer infused during the fractional synthesis rate (FSR) protocol (please see Experimental days).

Experimental days. An overview of the experimental days is given in Fig. 1. On day 1, participants came into the lab and had the first IGF-I and saline injections made 24 h before tendon biopsy collection. Dominant and nondominant legs were randomized to receive either 1 mg of recombinant human IGF-I (10 mg/ml, 0.1 ml, mecasermin, Incrlex; Ipsen) or placebo (0.1 ml isotonic saline) in a double-blinded manner. Injections were given in the midproximal part of the tendon. Live ultrasound images were used to guide the needle and to confirm the position of the injection bolus (Fig. 2). The injections were followed by 30 min of supine rest, and thereafter the participants were sent home and instructed not to perform any strenuous work or exercise. The next morning the participants arrived in the lab after an overnight fast. After obtaining a blood sample, they were given a standardized meal [32.2 KJ/kg LBM consisting of 20 energy (E)% fat, 23 E% protein, and 57 E% carbohydrates]. Hereafter, the IGF-I/placebo injections were repeated in each patellar tendon, which corresponded to 18 h after the first injections and 6 h before tendon biopsy collection (Fig. 1). The tracer infusion was then started allowing the determination of the FSR of tendon protein. Tendon protein FSR was measured according to the direct-incorporation, precursor-product approach over a 6-h period by applying the flood-primed continuous tracer infusion protocol of \([ring\text{-}13\text{C}_6\text{]}\text{phenylalanine}\) (Cambridge Isotope Laboratories, Andover, MA) (20). Before infusion the tracers were mixed in sterile 9 g/l saline and sterilized through a disposable 0.20-μm filter (Sartorius, Hanover, Germany). The flood-prime, containing 965 μmol \([ring\text{-}13\text{C}_6]\text{phenylalanine}\) (≥99 atoms%) and 8,000 μmol unlabeled l-phenylalanine, was given intravenously over a 2-min period, and the experimental days (from time 0 in Fig. 1. Overview of the experimental days. The timeline illustrates the two experimental days (from time 0 to 24 h). During this period, injections and tracer infusion were given, and blood samples and tendon biopsies were obtained at the time points marked. IGF-I, insulin-like growth factor-I. FSR of tendon protein. Tendon protein FSR was measured according to the direct-incorporation, precursor-product approach over a 6-h period by applying the flood-primed continuous tracer infusion protocol of \([ring\text{-}13\text{C}_6]\text{phenylalanine}\) (Cambridge Isotope Laboratories, Andover, MA) (20). Before infusion the tracers were mixed in sterile 9 g/l saline and sterilized through a disposable 0.20-μm filter (Sartorius, Hanover, Germany). The flood-prime, containing 965 μmol \([ring\text{-}13\text{C}_6]\text{phenylalanine}\) (≥99 atoms%) and 8,000 μmol unlabeled l-phenylalanine, was given intravenously over a 2-min period, and the continuous infusion (8 μmol \([ring\text{-}13\text{C}_6]\text{phenylalanine-LBM}^{-1}\text{h}^{-1}\)) was started thereafter. During the infusion, blood samples were drawn from an antecubital catheter (time points 10, 30, 60, 120, 240, and 360 min after infusion start). After the 6-h infusion one tendon biopsy was obtained from each patellar tendon following the procedure described below.

Tendon biopsy procedures. Tendon biopsies were taken from the midproximal part of the patellar tendon. The overlying skin was anesthetized with 2 ml lidocaine (1%), and the area was sterilized. A 5-mm proximal-distal incision was made exactly through the point of the needle punch from the injections, and the tendon biopsy was obtained with a 14-G automatic needle (Bard Magnum Biopsy Instrument; CR Bard, Covington, GA). The biopsies weighed 5–10 mg after visible blood and fat had been removed. The tendon tissue obtained with this method normally consists of two visually distinguishable fractions when viewed in a light microscope: one as a white, shiny densely packed fraction, and another one as a matte, more loosely packed fraction. We normally analyze these two parts together, but in this study we chose to subdivide each biopsy into the more densely and the more loosely packed tissue with the purpose of detecting potential differences in tendon FSR between these fractions. Samples were stored at −80°C until further analyses.

Calculation of tendon protein FSR. Tracer analyses and FSR calculations were performed following the protocol previously described (20). Plasma phenylalanine tracer enrichments were analyzed by gas chromatography-mass spectrometry (GC, Trace GC 2000 series, MS, Automass Multi; Thermo Quest Finnigan, Paris, France), as its t-butylidemethylsilyl derivative using a capillary column (CP-SIL 8, CB low Bleed, 30 m × 0.32 mm, coating 0.25 μm, Chrompack; Varian, Palo Alto, CA), and measuring on the fragment [M + 336 m/z] and using the \([M + 6]\) abundance to calculate the tracer-to-tracee ratio (TTR). The enrichment was calculated by subtracting the background TTR from all samples obtained during the infusion. Tendon biopsies were homogenized in buffer (0.15 M NaCl, 0.1% Triton X-100, 0.02 M Tris-HCl, and 5 mM EDTA, pH 7.4), left for 3 h, and centrifuged (1,600 g, 20 min, 4°C). The pellet was added to a high-salt solution (0.7 M KCl), homogenized one time, left overnight at 4°C, and the next day centrifuged (1,600 g, 20 min, 4°C). The pellet was washed one time in 70% ethanol and hydrolyzed overnight in 6 M HCl at 110°C. The liberated amino acids were then purified over cation exchange resin columns and derivatized as their N-acetyl-n-propyl (NAP) esters. The phenylalanine \(^{13}\text{C}\) abundance was determined by gas chromatography-combustion-isotope ratio mass spectrometry (Delta Plus XL; Thermo Finnigan, Bremen, Germany) using a CP-Sil 19 CB column, 60 m × 0.32 mm, coating 0.25 μm (ChromPack; Varian) to isolate the NAP-derivatized phenylalanine compound. The tendon protein FSR was calculated according to the precursor-product method: FSR (%/h) = \(Δ\text{E}_{\text{product}}/Δ\text{time} = \text{E}_{\text{product}}/Δ\text{time}\), where \(\text{E}_{\text{product}}\) is the difference in the tracer enrichment between the tendon protein samples obtained after the tracer infusion and the baseline protein (determined from plasma proteins in the background blood sample), \(\text{E}_{\text{precursor}}\) is the weighted average of tracer enrichment measured in plasma throughout the tracer infusion period, and \(Δ\text{time}\) is the incorporation time between tracer priming and the tendon biopsies measured in hours.

Statistics. Baseline measurements were compared between groups with unpaired t-tests. Serum IGF-I was analyzed with two-way repeated-measures (RM) ANOVA and Bonferroni post hoc test with age and effect of IGF-I injections as variables (old/young and before/after IGF-I injections). Tendon protein FSR was measured in four tendon protein fractions from each participant (1 biopsy from each leg each divided in loose and dense tissue fractions), making a total of 88 samples. Out of these 88 we had 10 missing samples because of insufficient specimen size (1 loose sample, young group; 3 dense samples, young group; and 6 dense samples, old group). These 10
missing samples originated from 9 participants, and we chose to exclude the 8 contralateral samples (7 dense samples and 1 loose).

Next a two-way RM ANOVA, with a Bonferroni post hoc test was used to analyze for FSR differences between tendon fractions with tendon fraction and treatment as variables (loose/dense and IGF-I/placebo). Because no differences in protein FSR were found between loose and dense tendon tissue, the mean value of these two was used in further analyses (when both were available, otherwise the single value was used). FSR values that deviated more than two SD from the mean were excluded as outliers and not included in the statistical analyses (2 placebo and 2 IGF-I samples from the young group and 1 placebo and 1 IGF-I sample from the old group). Finally, FSR differences with age and treatment were analyzed with a two-way RM ANOVA and Bonferroni post hoc test (old/young and IGF-I/placebo).

RESULTS

The characteristics of participants are listed in Table 1. The old group had a slightly (but significantly) higher BMI compared with the young group, but all individuals were within a BMI range of 19 and 26 kg/m², and no significant differences were found in LBM between the groups.

Serum IGF-I. The old group had a significantly lower serum IGF-I at baseline compared with the young group (165 ± 17 and 281 ± 27 ng/ml, respectively, Fig. 3). Serum IGF-I concentrations were affected by patellar IGF-I injections irrespective of age. We found a rise in systemic IGF-I concentrations after the first intratendinous injection in both the old and young groups (17 h postinjection, 21% increase in the old group and 20% in the young). Also at this time point the old group had significantly lower serum IGF-I levels compared with the young group (Fig. 3).

![Fig. 3. Serum IGF-I concentration. Blood samples were obtained in the morning after an overnight fast from 11 young men (20–30 yr of age) and 11 old men (66–75 yr of age). Baseline measurements were done several days before experimental days (young n = 11; old n = 11), and blood samples for “post-IGF-I” values were taken 17 h after the first IGF-I injection (young n = 9; old n = 10). Serum IGF-I in the old group is significantly lower compared with the young group at both time points (a), and there is a significant increase in serum IGF-I from baseline to post-IGF-I in both groups (b). Data are shown as means ± SE.](http://jap.physiology.org/)

Tendon protein FSR. Despite the obvious morphological differences between the two fractions in the obtained specimens, the tendon protein FSR was not statistically different between dense and loose tendon fractions, and we therefore used the mean values in the subsequent comparison between the young and old groups (see Statistics). Tendon protein FSR was significantly higher in the tendons injected with IGF-I (old group: 0.018 ± 0.015, young group: 0.016 ± 0.009%/h) compared with placebo (old group: 0.008 ± 0.008, young group: 0.009 ± 0.006%/h, P < 0.01), but there was no detectable difference in the response to IGF-I between the young and old groups (Fig. 4).

Fig. 4. Patellar tendon protein fractional synthesis rate (FSR). Shown is tendon protein FSR in young (20–30 yr of age, n = 10) and old (66–75 yr of age, n = 10) men in response to IGF-I injections. FSR is measured with the stable isotope technique. Tendon protein FSR is increased with IGF-I regardless of age (*). Data are shown as means ± SE.

DISCUSSION

The main finding in this study was that both the young and the old group had an increased patellar tendon protein synthesis rate in response to the intratendinously given IGF-I. In contrast to our hypothesis, we did not find any anabolic resistance to IGF-I in the aged tendons, and we therefore conclude that the ability of the tendon fibroblasts to synthesize protein in response to IGF-I stimulation is preserved with aging in humans. The finding that IGF-I stimulates tendon protein synthesis is consistent with existing human data on the effect of GH on tendon collagen (14, 22). Furthermore, it fits with a study in middle-aged men where IGF-I stimulated collagen synthesis (8). Thus, the existing studies show a stimulating effect of GH and IGF-I on tendon protein synthesis in humans at different ages, but the present study is, to our knowledge, the first to directly compare the response to IGF-I in young and elderly human tendon in vivo. This age-related response to IGF-I has, however, been studied in bone in an animal model and in vitro on chondrocytes. The effect of IGF-I on both collagen synthesis in mouse bone tissue and on proteoglycan synthesis in chondrocytes in vitro was blunted with age (4, 15). These data, in combination with animal and cell culture studies studying other growth factors than IGF-I (18, 19), point toward a decreased responsiveness of aged fibroblasts to growth factors, which is in contrast to our present results. However, a direct comparison between these studies may not be appropriate because of differences in species, tissue types, growth factor dosages, and growth factor types. We have in the present study tried as good as possible to address the question whether old and young individuals respond differently in collagen synthesis to administration of IGF-I, and that was not the case in vivo.

Table 1. Participants characteristics

<table>
<thead>
<tr>
<th></th>
<th>Young Group (n = 11)</th>
<th>Old Group (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>23 (20–30)</td>
<td>70 (66–75)*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21 (19–23)</td>
<td>23 (20–26)*</td>
</tr>
<tr>
<td>LBM, kg</td>
<td>60 (52–69)</td>
<td>55 (47–69)</td>
</tr>
</tbody>
</table>

Data are listed as means (range); n, no. of subjects. BMI, body mass index; LBM, lean body mass. *Significant difference compared with young group.
The discussion becomes more complex when we look at the tendon as an integrated part of the human body. As expected, we found a 40% lower circulating concentration of IGF-I in the old group. This means that fibroblasts in an old tendon are exposed to less IGF-I from the circulation than in a young tendon. Because we found that the old tenocytes respond with the same magnitude as young to directly injected IGF-I, it could be speculated that the low systemic levels of IGF-I in the elderly are in fact a limiting factor for maintaining tendon protein synthesis. However, we did not find any differences in protein synthesis rate at baseline between young and old tendons. The reason for this finding could be caused by a couple of factors. If the collagen content is in fact lower in older tendons (5), we would expect the synthesis rate to be suppressed with aging; however, it is plausible that a potential age-related decrease in synthesis rate would be too small for the stable isotope technique to detect it. Alternatively, the degradation rate of the tendon proteins can be increased, which unfortunately is hard to measure and thus investigate. Therefore, with the existing methodologies to study slow turnover protein synthesis and degradation rates, it is unfortunately not possible to determine the possible age effect on the baseline turnover rates in tendon. Furthermore, we found a systemic effect of the IGF-I injections in both groups, and therefore we cannot be sure that the placebo legs represent the true rate of tendon protein synthesis at baseline. Nevertheless, the moderate increase in serum IGF-I of ~20% was similar in all participants, and we are therefore certain that it did not influence the comparison of the age groups. The only possible effect would be that we overestimate the baseline level and underestimate the stimulus of IGF-I. The magnitude of the increase in tendon protein synthesis rate due to IGF-I in our study was around 0.008%/h, which is comparable to both the GH study by Doessing et al., who found values around 0.02%/h (6), and to the IGF-I study by Hansen et al. who found values around 0.01%/h (8). As a final comment, a dose-response investigation of the ability of IGF-I to stimulate tendon protein synthesis would be of utmost interest, if the methodologies would be adequate to allow small differences to be detected.

In the present study, we measured the rate of tracer incorporation into the two visually distinguishable fractions of a tendon tissue sample. We found no difference in tendon protein synthesis rate between the two fractions (the loose and dense tendon tissue). We are uncertain what these two fractions represent anatomically in the tendon, since our biopsy technique does not allow us to observe which part of the tendon we are sampling from, and does not tell us how the specimen was orientated in vivo. Furthermore, the apparently loose part of the tendon tissue biopsy could be a result of a tear and stretch of the dense tendon tissue, since we have seen that transition during handling of the tissue. Based on the present results we conclude that these visually distinguishable fractions are comparable with respect to protein synthesis rate and responsiveness to IGF-I.

In conclusion, we found that IGF-I stimulated patellar tendon protein synthesis to an equal magnitude in young and old men, which implies that the responsiveness to IGF-I of the human tendon is preserved with aging. Because we also found a markedly lower circulating concentration of IGF-I in the old group, it is plausible that low IGF-I in elderly can be a limiting factor for tendon protein synthesis. In a clinical perspective, growth factor therapy of tendon injuries might be as beneficial in old as in young age.

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GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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