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RESEARCH ARTICLE

Tendon collagen synthesis declines with immobilization in elderly humans: no effect of anti-inflammatory medication

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[1]*Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery M, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark;* [2]*Institute of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; and* [3]*Department of Physical Therapy, Musculoskeletal Rehabilitation Research Unit, Bispebjerg Hospital, Denmark*

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Dideriksen K, Boesen AP, Reitelseder S, Couppé C, Svensson R, Schjerling P, Magnusson SP, Holm L, Kjaer M. Tendon collagen synthesis declines with immobilization in elderly humans: no effect of anti-inflammatory medication. *J Appl Physiol* 122: 273–282, 2017. First published December 8, 2016; doi:10.1152/japplphysiol.00809.2015.—Nonsteroidal anti-inflammatory drugs (NSAIDs) are used as pain killers during periods of unloading caused by traumatic occurrences or diseases. However, it is unknown how tendon protein turnover and mechanical properties respond to unloading and subsequent reloading in elderly humans, and whether NSAID treatment would affect the tendon adaptations during such periods. Thus we studied human patellar tendon protein synthesis and mechanical properties during immobilization and subsequent rehabilitating resistance training and the influence of NSAIDs upon these parameters. Nineteen men (range 60–80 yr) were randomly assigned to NSAIDs (ibuprofen 1,200 mg/day; Ibu) or placebo (Plc). One lower limb was immobilized in a cast for 2 wk and retrained for 6 wk. Tendon collagen protein synthesis, mechanical properties, size, expression of genes related to collagen turnover and remodeling, and signal intensity (from magnetic resonance imaging) were investigated. Tendon collagen synthesis decreased ($P < 0.001$), whereas tendon mechanical properties and size were generally unchanged with immobilization, and NSAIDs did not influence this. Matrix metalloproteinase-2 mRNA tended to increase ($P < 0.1$) after immobilization in both groups, whereas scleraxis mRNA decreased with inactivity in the Plc group only ($P < 0.05$). In elderly human tendons, collagen protein synthesis decreased after 2 wk of immobilization, whereas tendon stiffness and modulus were only marginally reduced, and NSAIDs had no influence upon this. This indicates an importance of mechanical loading for maintenance of tendon collagen turnover. However, reduced collagen production induced by short-term unloading may only marginally affect tendon mechanical properties in elderly individuals.

NEW & NOTEWORTHY In elderly humans, 2 wk of inactivity reduces tendon collagen protein synthesis, while tendon stiffness and modulus are only marginally reduced, and NSAID treatment does not affect this. This indicates that mechanical loading is important for maintenance of tendon collagen turnover and that changes in collagen turnover induced by short-term immobilization may only have minor impact on the internal structures that are essential for mechanical properties in elderly tendons.

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tendon unloading; tendon reloading; tendon mechanical properties; ibuprofen; scleraxis

THE RECOVERY FROM ACCIDENTAL INJURIES or acute diseases most often includes a period of unloading or even immobilization in otherwise healthy humans. Periods of unloading decrease both the quantity and quality of human skeletal muscle tissue dramatically, and especially in younger individuals the effect of unloading on human tendon connective tissue morphology and mechanical properties has been studied fairly extensively (4, 6, 25, 28, 37). From these studies, it seems that immobilization leads to loss of tendon mechanical properties, whereas tendon size appears to be unaffected by short-term immobilization. This is supported by animal studies as well (1, 33). However, an increased Achilles tendon cross-sectional area (CSA) was reported after 4 wk of unloading in young humans (25), a finding that was most pronounced in the distal tendon part and was explained to be related to increased tendon water content during immobilization (25). Moreover, only a very few studies have investigated tendon mechanical properties during immobilization in elderly humans, and these have found either decreased (16) or unchanged (7) stiffness and modulus after 2 wk of lower limb immobilization. Thus it is not clear whether the mechanical properties of tendon in the elderly respond to immobilization in a similar fashion as in younger counterparts.

The synthesis rate of human tendon collagen protein after periods of immobilization has been sparsely investigated (5), and only in tendons of young individuals. In the study by de Boer et al., the patellar tendon collagen fractional synthetic rate (FSR) was measured by use of stable isotope infusions and found to decrease by >80% after 21 days of unloading (5). This is supported by findings of decreased tendon collagen synthesis (39) and decreased type III collagen gene expression (19) in unloaded rat tendons. However, Christensen et al. used an indirect technique and reported an unchanged concentration of peritendinous collagen synthesis markers at the human Achilles tendon surface, indicating that the collagen production was unaffected by unloading in young individuals (12, 13). More recently, unchanged type I and III collagen gene expression has been found in unloaded patellar tendons of both young (6) and elderly (7) humans as well as in rat tendons (3, 22). Our knowledge of tendon adaptations to unloading is emerging, but

mostly based on studies in young tissue. Hence, more studies are required, especially in elderly individuals.

Generally speaking, tendons can respond to acute exercise (34) and adapt structurally to habitual mechanical loading (15, 27) in young individuals. However, tendon hypertrophy has not been observed after periods of resistance training in elderly individuals (9, 38). Moreover, tendon mechanical properties did not increase after 12 wk of resistance training in elderly subjects (9), which could indicate some resistance to the anabolic exercise stimuli in elderly tendons.

Because of their analgesic and antiswelling effect, nonsteroidal anti-inflammatory drugs (NSAIDs) are frequently used in the treatment of pain and swelling related to tendon overload and injury and to accelerate the subsequent recovery process, although NSAIDs do not seem to improve tendon healing (26). Furthermore, NSAIDs can be used as pain killers during periods of unloading that are caused by injuries other than tendon overload or injury, such as fractures of the lower extremities. It has been demonstrated that ibuprofen does not affect collagen type I and III expression in healthy resting rat tendons (43). Moreover, unloaded rat tendons may display signs of increased inflammatory activity (23, 44). However, it remains unknown whether NSAIDs affect tendon adaptation to periods of unloading. Furthermore, it is poorly understood how NSAIDs affect tendon adaptation to mechanical loading. It has been shown that NSAID ingestion reduces peritendinous blood flow during exercise (29) and collagen production (measured indirectly at the patellar tendon surface) after exercise in young individuals (11). In relation to this, the anabolic exercise response in tendons could be related to increased inflammatory activity (2, 11, 30), which may be inhibited by NSAIDs (11). However, others have not been able to show an effect of ibuprofen on tendon collagen FSR response to acute exercise (36) or on adaptations in tendon size or mechanical properties after 12 wk of resistance training in elderly individuals (9). Until now, no studies have investigated whether ingestion of NSAIDs influences tendon adaptation to periods of rehabilitation training.

We therefore decided to investigate tendon adaptations to periods of unloading and rehabilitation resistance training (retraining), with and without NSAID treatment, in elderly individuals. The primary outcome variable of the present study was tendon collagen synthesis, and secondary outcomes were tendon mechanical properties and tendon CSA. It was hypothesized that tendon collagen protein synthesis and mechanical properties would decrease with unloading in elderly individuals and that NSAID would have no influence on this.

METHODS

Study Design

Subjects. Nineteen healthy men (60–80 yr; age ± SD: 69 ± 7 yr) with a body mass index between 20 and 30 kg/m^2 participated in this study. All subjects underwent physical examination and were interviewed before inclusion to assess medical history, locomotive limitations of the hips and knees, physical activity level, and dietary/smoking habits. The included subjects were generally healthy (no cancer or metabolic, cardiac, or neurological diseases), nonsmokers, and moderately active and had not taken part in any form of strenuous endurance or resistance training before trial participation. Moreover, subjects did not have locomotive limitations of their lower extremities. Finally, the subjects were instructed not to take any kind of analgesic medication at least 2 wk before the beginning of the study. The study (H-1-2010-007) was approved by the local Ethics Committee of Region Copenhagen in accordance with Helsinki Declaration II. All subjects were informed of the risks associated with the study and gave their written informed consent.

Intervention protocol. The overall study design consisted of 2 wk of randomized, unilateral limb immobilization followed by 6 wk of supervised unilateral strength training (Fig. 1), which has been described previously (6, 7). Five of the 11 subjects in the placebo group of the present study were included in a previous study (7). Only the subject characteristics and raw biomechanical data from these five subjects are reused and integrated in the placebo group mean in the present study, whereas all other presented results from these five subjects have not been reported previously. Moreover, the present study was a part of another experiment (17) that investigated the influence of ibuprofen on skeletal muscle adaptations to immobilization and retraining.

Protein supplementation. To ensure that all subjects were given sufficient amounts of protein and essential amino acids to optimize their nutritional conditions (20), all subjects were provided 20 g of whey protein supplement (Lacprodan, Arla Foods Ingredients Group, Viby J, Denmark) twice daily throughout the study period. Subjects consumed the first drink in the laboratory 2 days before immobilization and were instructed to consume a drink after breakfast and a drink after lunch each day and always after exercise on retraining days. Besides the protein supplementation, subjects were encouraged to maintain their normal diets during the study period.

Limb immobilization. The immobilization procedure has been described in detail previously (6, 7, 41) and proven to impair the mechanical properties of the patellar tendon in immobilization studies involving young (6) and elderly (16) individuals . Immobilization was accomplished by a lightweight fiber cast applied from just above the malleoli to just below the groin. Notably, in contrast to earlier studies using a cast position at 30° of knee joint flexion (16, 41), the cast was positioned at 50° of knee joint flexion to further minimize walking ability (7). Furthermore, the subjects were carefully instructed to perform all activities on crutches and to abstain from ground contact and from doing isometric contractions of the quadriceps muscle. During the immobilization period, the subjects were contacted every

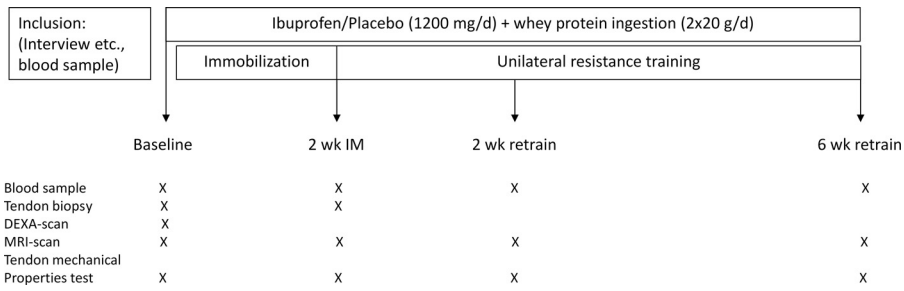

Fig. 1. Study intervention protocol. Nineteen elderly men completed 2 wk of immobilization and 6 wk of retraining, with either ibuprofen (n = 8) or placebo (n = 11) administration (2 × 600 mg/day) and whey protein ingestion (2 × 20 g/day). Measurements were performed at baseline, after 2 wk of immobilization (2 wk IM), after 2 wk of retraining (2 wk retrain), and after 6 wk of retraining (6 wk retrain).

second day by mail or telephone and were carefully instructed to contract the muscles around the ankle joint (venous pump exercises) several times a day to prevent the potential formation of deep venous thrombosis (DVT). Furthermore, to reduce the potential risk of DVT, all subjects were given acetylsalicylic acid tablets (75 mg/day) during the immobilization period. Even though acetylsalicylic acid is an NSAID, the anti-inflammatory effect of such a low dose was most likely very minor.

After postimmobilization biopsy collection, subjects were encouraged to start bearing weight on the immobilization leg for ~20 min before testing of tendon mechanical properties to minimize deficits due to knee joint stiffness. Additionally, the subjects did 5 min of warm-up on a stationary bike to ensure appropriate preconditioning of the joint and muscle-tendon unit before testing.

Unilateral retraining. After removal of the cast, the subjects received rehabilitation training for 6 wk, three sessions per week (all subjects completed a total of 18 training sessions). The retraining program consisted of supervised unilateral strength training and aimed to restore tendon mechanical properties to baseline levels. After 5 min of warm-up on a stationary bike, the subjects performed knee extension and leg press in training machines (Technogym, Gambettola, Italy). The training intensity was 3 or 4 sets × 12 repetitions [15 repetitions maximum (RM)] in *week 1*; 4 sets × 10 repetitions (12 RM) in *weeks 2–4*, and 4 sets × 8 repetitions (10 RM) in *weeks 5–6*. The training load was adjusted on a weekly basis by the use of 5 RM tests.

Randomization and ibuprofen treatment. The placebo group consisted of two groups of separately randomized, double-blinded, and placebo-controlled subjects. The first group of five subjects were included in a previous study (7) and received placebo injections in a double-blinded fashion (placebo or growth hormone). The remaining six subjects in the placebo group received placebo tablets in a double-blinded fashion (placebo or ibuprofen). In total, 11 subjects were included, and randomized according to body mass index, in the placebo group (Plc, $n = 11$) and 8 subjects were included in the ibuprofen group (Ibu, $n = 8$). The subjects were continuously randomized by the envelope method, and all subjects completed the study intervention during the same period of time. The subjects were included and randomized by the principal investigators. The persons who conducted the scans, analyzed tendon dimensions and signal intensity, measured the mechanical properties, and performed the tendon protein synthesis and gene expression measurements were all blinded with respect to the allocation of subjects.

Ibuprofen treatment started 2 days before immobilization (immediately after the baseline isotope infusion trial) to ensure that the effect of ibuprofen was obtained at the beginning of the immobilization period. Placebo tablets were visually identical to the ibuprofen tablets, and all subjects received the same number of tablets every second week and were instructed to take their daily tablets (2 × 600 mg/day) at the same time every morning and evening together with a meal. To check compliance, ibuprofen levels were traced in blood samples after completion of the intervention period. Additionally, to ensure normal liver and kidney function, blood samples were analyzed for creatinine, alkaline phosphatase, and cholesterol before, during, and after the intervention period. Subjects were instructed to report any discomfort that appeared throughout the intervention period and would be excluded from the study if blood parameters were negatively affected or if the patients experienced any signs of severe side effects from the medication or study intervention. Furthermore, subjects were not allowed to consume any cyclooxygenase-inhibiting drugs besides the tablets provided during the study period.

Measurements

Patella tendon CSA and mechanical properties were measured at baseline, immediately after 2 wk of immobilization, after 2 wk of retraining, and after 6 wk of retraining (Fig. 1). Blood samples were collected at these time points as well. Moreover, lean body mass (LBM) was determined by dual-energy X-ray absorptiometry scans at baseline (for calculation of tracer infusion rate). Finally, tendon biopsies were taken at baseline (from the nonimmobilized leg) and after immobilization for measurement of tendon collagen FSR and gene expression. Because of the repeated-biopsy effect on tendon tissue (18), no biopsies were taken during the retraining period. Before and after the immobilization period, magnetic resonance imaging (MRI) scans were performed before tendon biopsy sampling, whereas biomechanical tests were performed shortly (within 2 h) after biopsy sampling to minimize confounding effects from biopsies on MRI images and from biomechanical testing on tendon collagen synthesis and gene expression. Additionally, measurements of tendon CSA and mechanical properties were always performed ~48 h after the last training session during the retraining period.

Tracer infusion protocol. Trials to measure the fractional protein synthesis rate of tendon collagen were conducted before and immediately after removal of the cast from the immobilized leg. Subjects were instructed to refrain from alcohol and strenuous physical activity and to follow their normal eating pattern 72 h before the experimental trial. Furthermore, no intake of caffeine was allowed 24 h before the trial. Subjects arrived at the laboratory by car or public transportation after an overnight fast. Subjects were placed in a supine position, a catheter was inserted into the antecubital veins on both forearms, and a background blood sample was taken. On the trial before immobilization, a flood priming bolus of L-[*ring*-$^{13}C_6$]phenylalanine [1,485 mg of phenylalanine with a tracer-to-tracee ratio (TTR) corresponding to 12%] was given over 1–2 min and followed by a continuous infusion of tracer (1.2825 mg·kg $LBM^{-1}\cdot h^{-1}$), aiming at 12% TTR arterial enrichment (24). Throughout the trial (after 15, 30, 150, 170, 190, and 210 min of infusion), venous blood samples were collected into EDTA tubes that were cooled on ice for 10 min, followed by centrifugation (10 min at 3,970 *g* at 4°C). Plasma was stored at −80°C for measurement of phenylalanine tracer enrichment (TTR). After 210 min of infusion, a tendon biopsy was obtained from the patellar tendon of the nonimmobilized leg (a position between the mid and proximal parts) with a disposable 14-gauge needle (Bard Magnum Biopsy Instrument, C.R. Bard, Covington, KY). After sterilization, the sample site was prepared with local anesthetic (lidocaine, 1%). The total wet weights of the tendon biopsy samples were 8–10 mg. The samples were cleared of external adipose tissue and blood and divided into two portions for assessment of tendon collagen protein synthesis and gene expression, respectively, before they were frozen in liquid nitrogen and stored at −80°C. The infusion trial was repeated on the day of cast removal, with the tendon biopsy taken from the immobilized leg (the contralateral leg compared with the preimmobilization trial), and an alternative phenylalanine tracer, L-[^{15}N]phenylalanine, was infused because of enrichment of [^{13}C]phenylalanine tracer from the preimmobilization trial. The pre- and postinfusion trials were carried out at the same time in the morning to avoid effects of circadian variations on the biochemical parameters.

Tendon dimensions. Each subject was scanned at the same 1.5-T MRI scanner throughout the study period (Philips Intera, Eindhoven, The Netherlands or General Electric, Sigma Horizon, Milwaukee, WI). At each time point, subjects were scanned in the same supine position with a dedicated knee coil as well as an axial and sagittal T1w turbo spin echo sequence (TE: 17; TR: 500; matrix: 512 × 512; FOV: 150 mm; slice thickness: 3 mm). The knee was slightly bent to ensure removal of slack in the patellar tendon. The axial slices of the patellar tendon were positioned orthogonal to the length in the sagittal plane covering the distal patellar pole to the tibial insertion. A plastic tube containing 1.0% $CuSO_4$ was placed in the field of view for normalization of MRI contrast (8, 9). After normalization of the MRI images, tendon CSA and length were analyzed manually with the imaging software Osirix 2.7.5 (Osirix Imaging Medical, Geneva, Switzerland). Tendon length was measured as the distance from the most dorsal insertion part at the patella apex to the dorsal insertion on the tibia.

Tendon CSA was measured at the slice just proximal to the tibia insertion (distal part), at the axial slice just distal to the patellar insertion (proximal part), and midway between these two sites (medial part) (15, 27). Finally, the MRI signal intensity [mean gray value (MGV)] within the manually outlined area (tendon CSA) on the normalized MRI images was recorded and interpreted as a measure of tendon tissue material composition (8, 9). The same experienced and blinded investigator measured tendon length as well as CSA and signal intensity at the distal, medial, and proximal parts of the patellar tendon. Each image was analyzed three times, and the average of these measurements was used. The intraobserver coefficient of variation between consecutive measurements was 1.1% at the distal tendon part, 2.4% at the midtendon part, and 3.1% at the proximal tendon part.

Mechanical tendon properties. Subjects refrained from strenuous exercise 48 h before the measurements and performed a 5-min warm-up on a stationary bike to ensure a standardized preconditioning of the tendon before testing. As previously described in detail (7, 15), subjects were seated in a custom-made rigid chair with both hips and knees flexed to 90°. A leg cuff, connected to a strain gauge (Noraxon) through a rigid steel rod, was mounted on the leg just above the medial malleolus. A Hitachi EUB-6500 ultrasound scanner (Hitachi Medical, Tokyo, Japan) equipped with a 10-MHz, 100-mm-long, linear array B-mode transducer (Hitachi, model EUP-L53L) was secured to the skin above the patellar tendon in the sagittal plane. The ultrasound probe was placed so that the distal patella, the entire patellar tendon, and the proximal tibia were visible throughout the performed isometric ramp contractions.

The subjects performed four or five, unilateral, isometric knee extensor contractions, by applying gradually increasing force until a maximum was reached over a 10-s period, during which patellar tendon displacement and knee extension force were synchronously measured. Custom-made, frame-by-frame tracking software was used to assess the tendon deformation from the ultrasound videos. The accuracy and reproducibility of this tracking software have been assessed previously (31). The external tibia moment arm was measured (from the leg cuff to the lateral epicondyle of the knee) to calculate the knee extensor moment. The force applied to the patellar tendon was calculated by dividing the measured knee extensor moment by the internal patellar tendon moment arm, which was estimated from individually measured femur lengths (45). Tendon deformation was defined as the change in distance between the patellar apex and tibia (15, 27). Tendon stress was calculated by dividing tendon force with the average tendon CSA (mean of proximal, middle, and distal CSA from MRI). Tendon strain was calculated as tendon deformation normalized to tendon length (from MRI). Polynomial functions (2nd order) were fitted to each single force-deformation curve. Tendon stiffness and Young's modulus were calculated at the final 10% of the force-deformation and stress-strain curves, respectively. To ensure that the same region of the tendon mechanical response was analyzed at all time points, regardless of possible changes in strength, the mechanical behavior was analyzed at a fixed force: The highest common tendon force was determined for each subject across the time points, and all the raw data were cut off at this value before analysis.

Tendon collagen FSR measurement. Phenylalanine tracer analyses and FSR calculations were performed according to the protocol previously described (24, 35). Plasma phenylalanine ^{13}C and ^{15}N enrichments were measured by gas chromatography-mass spectrometry (GC-MS/MS, TSQ Quantum; Thermo Scientific, San Jose, CA) as *t*-butyldimethylsilyl derivatives, using a capillary column (CP-Sil 8 CB capillary column, 30 m × 0.32-mm ID, coating 0.25 μm) (Chrompack; Varian, Palo Alto, CA).

Connective tissue collagen protein was isolated from tendon biopsies, which were homogenized for 5 × 15 s in 1 ml of homogenization buffer (0.02 M Tris, pH 7.4, 0.15 M NaCl, 2 mM EDTA, and 0.05% Triton X-100), left for 2 h, and spun (1,600 *g*, 20 min, 4°C). One milliliter of high-salt solution (0.7 M KCl) was added to the pellet, which was homogenized and left overnight at 4°C. After a spin (1,600 *g*, 20 min, 4°C), 1 ml of KCl was added to the pellet, which was homogenized and left for 2 h. After spinning (1,600 *g*, 20 min, 4°C), the pellet was washed once in 70% ethanol and hydrolyzed overnight in 1 ml of 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 NAP-derivatized phenylalanine compounds were analyzed with a CP-Sil 19 CB capillary column (60 m × 0.25-mm ID, coating 0.25 μm) (AgilentJ&W) on a gas chromatograph-combustion-isotope ratio mass spectrometer (Delta Plus XL; Thermo Finnigan, Bremen, Germany) to isolate and measure ^{13}C and ^{15}N abundance in tissue protein samples. During ^{15}N analyses, CO was trapped in the column (by use of liquid nitrogen) to avoid CO influence on $^{14}N_2$ (mass 28).

Tendon protein FSR was calculated according to the precursor-product method: FSR (%/h) = $\Delta E_{product}/(E_{precursor} \times \Delta_{time})$, where $E_{product}$ is the difference in tracer enrichment between plasma proteins obtained from background blood samples and tendon samples obtained after the tracer infusions, $E_{precursor}$ is the weighted average of tracer enrichment measured in plasma throughout the tracer infusion period, and Δ_{time} is the time in hours of tracer exposure between priming and tendon biopsies.

We chose to determine tendon protein FSR before and after immobilization by use of a common background enrichment, which was blood protein enrichment. However, we measured the [^{15}N]phenylalanine background enrichment also in the tendon proteins derived from the first biopsy (where the ^{13}C-labeled phenylalanine tracer was used), to verify that the blood protein was useful as a valid surrogate background measure in elderly subjects, who never had been exposed to [^{15}N]phenylalanine infusions. The ^{15}N background delta value ($\delta = [r_{sample} - 1] \times 1{,}000$) enrichments did not differ ($P > 0.05$, 2-tailed, unpaired *t*-test) between plasma proteins (5.956 ± 0.609) and tendon proteins (4.626 ± 0.762).

Tendon mRNA measurements. Tendon RNA was extracted as described in Reference 7. Briefly, frozen tendon tissue was homogenized and phase-separated to precipitate RNA. RNA concentrations were determined by RiboGreen assay (Molecular Probes, Eugene, OR).

The amount of mRNA was measured with reverse-transcription real-time PCR, as previously described in detail (18). Briefly, total RNA (30 ng from each tendon sample) was converted into cDNA. cDNA was amplified in a SYBR Green PCR reaction and monitored in real time with the MX3005P real-time PCR machine (Stratagene). The threshold cycle values were related to standard curves (from PCR products) to determine the relative difference between the unknown samples, accounting for the PCR efficiency. The specificity of the PCR products was confirmed by dissociation curve analysis after amplification. The mRNA expression targets of type I collagen (COL1A1), type III collagen (COL3A1), insulin-like growth factor-1 (IGF-I)Ea, IGF-IEc, matrix metalloproteinase (MMP)-2, MMP-9, decorin, tenascin-C, scleraxis, lysyl oxidase (LOX), and RPLP0 were measured. As previously described and discussed (7), RPLP0 was used for normalization. The mRNA expression data are presented as the relative change from baseline values. Primer sequences are given in References 7 and 18, except for tenascin-C (CAACCATCACTGCCAAGTTCACAA;GGGGGTCGCCAGGTAAGGAG) and scleraxis (CAGCCCAAACAGATCTGCACCTT;CTGTCTTTCTGTCGCGGTCCTT).

Statistics

Data were analyzed with two-way (drug and time) repeated-measures analysis of variance (ANOVA). Significant effects were further analyzed by Student–Newman–Keuls post hoc test. Tendon mRNA data were log transformed before the statistical analysis and presented as geometric means ± back-transformed SE. Tendon CSA was analyzed as relative changes from baseline. Because of technical prob-

Table 1. *Patellar tendon mechanical properties at baseline, after 2 wk of immobilization, after 2 wk of retraining, and after 6 wk of retraining*

	Ibu				Plc			
	Baseline	2 wk IM	2 wk retrain	6 wk retrain	Baseline	2 wk IM	2 wk retrain	6 wk retrain
Deformation, mm†	2.5 ± 0.4	2.6 ± 0.2	2.8 ± 0.3	2.4 ± 0.2	1.8 ± 0.3	1.8 ± 0.3	1.9 ± 0.2	2.0 ± 0.2
Stiffness, N/mm	2,921 ± 403	2,504 ± 81	2,496 ± 209	2,733 ± 234	3,008 ± 641	2,589 ± 285	2,468 ± 276	2,469 ± 374
Stress, MPa	24 ± 3	24 ± 3	24 ± 2	25 ± 2	23 ± 3	23 ± 3	23 ± 4	23 ± 3
Strain, %‡	4.8 ± 0.7	5.0 ± 0.5	5.2 ± 0.4	4.7 ± 0.4	3.7 ± 0.7	3.7 ± 0.6	3.7 ± 0.4	4.0 ± 0.5
Modulus, GPa*	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.4 ± 0.3	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2

Data are means ± SE. Data were analyzed with a 2-way repeated-measures ANOVA. Biomechanical data were obtained from 9 and 8 subjects in Plc and Ibu groups, respectively. 2 wk IM, after 2 wk of immobilization; 2 wk retrain, after 2 wk of retraining; 6 wk retrain, after 6 wk of retraining. †Overall group effect ($P < 0.05$) for tendon deformation. ‡Overall group effect tendency ($P < 0.1$) for tendon strain. *Overall time effect tendency ($P < 0.1$) for tendon modulus.

lems, biomechanical data were obtained from nine (of 11) subjects in the Plc group and eight (of 8) subjects in the Ibu group, whereas tendon CSA could not be obtained for one subject in the Plc group. Furthermore, tendon collagen synthesis was measured in six subjects from each group, whereas tendon gene expression was measured in six and eight subjects in the Plc and Ibu groups, respectively. Because of upgrading of the MRI scanner during the study period, tendon signal intensity was only comparable in five and six subjects in Plc and Ibu groups, respectively. Data are presented as means ± SE. Differences were considered significant when $P < 0.05$. P values < 0.1 are also shown in figures. Statistical analyses were performed with SigmaPlot v. 12.3 (Systat Software, San Jose, CA and GraphPad Prism v. 6.0 (GraphPad Software, San Diego, CA).

RESULTS

Subject Characteristics

There was no difference in age, height, weight, or body mass index between groups. At baseline, after immobilization, after 2 wk of retraining, and after 6 wk of retraining, body weight (kg) was 78 ± 3, 79 ± 3, 80 ± 3, and 78 ± 3 in the Plc group and 84 ± 3, 84 ± 4, 84 ± 4, and 85 ± 3 in the Ibu group, respectively. An interaction effect ($P < 0.05$) was observed for body weight. Post hoc tests indicated that body weight increased after immobilization and 2 wk of retraining ($P < 0.05$) and returned to baseline levels after 6 wk of retraining in the Plc group, whereas it tended to be higher ($P < 0.1$) after 6 wk of retraining compared with baseline and 2 wk of retraining in the Ibu group.

Participants completed the immobilization period and all retraining sessions without reporting any clinical problems. There was no difference in training load or intensity between the Ibu and Plc groups (data not shown). Regarding ibuprofen administration and protein intake, subjects reported full compliance. This was supported by measurements of blood ibuprofen concentration, which indicated that all subjects in the Ibu group took their medication regularly (data not shown).

Tendon Mechanical Properties

The tendon mechanical properties investigated at maximum common force for each individual are shown in Table 1. Tendon stiffness did not change over time or between groups ($P > 0.05$). The 95% confidence intervals (CIs) for group differences in tendon stiffness were −820 to 993 at baseline, −786 to 957 after immobilization, −899 to 843 after 2 wk of retraining, and −1135 to 607 after 6 wk of retraining. For Young's modulus, a tendency ($P < 0.1$) toward an overall time effect, but no group effect ($P > 0.05$), was observed. The 95% CIs for tendon modulus group differences were −0.20 to 0.58 at baseline, −0.27 to 0.47 after immobilization, −0.22 to 0.52 after 2 wk of retraining, and −0.34 to 0.39 after 6 wk of retraining. Even though the mechanical properties did not differ between groups at baseline ($P > 0.05$), a group effect ($P < 0.05$) was found for tendon deformation and a tendency to a group effect ($P < 0.1$) was observed for tendon strain, which both were higher in the Ibu group compared with the Plc group. These findings may likely relate to a higher average level of force production (higher common force level), which was 3,205 ± 258 N and 2,672 ± 289 N in the Ibu and Plc groups, respectively. However, no time or interaction effects ($P > 0.05$) were observed for tendon deformation and strain.

Tendon Magnetic Resonance Imaging Signal Intensity

Tendon signal intensity is shown in Table 2. At the distal part of the patellar tendon, an overall time effect ($P < 0.05$) was observed. Post hoc tests indicated that the distal tendon signal intensity decreased after immobilization ($P < 0.01$) and returned to baseline levels after 2 wk of retraining. At the

Table 2. *Patellar tendon signal intensity at baseline, after 2 wk of immobilization, after 2 wk of retraining, and after 6 wk of retraining*

	Ibu				Plc			
	Baseline	2 wk IM	2 wk retrain	6 wk retrain	Baseline	2 wk IM	2 wk retrain	6 wk retrain
Distal (MGV)	24 ± 4	19 ± 5*	21 ± 5	21 ± 7	26 ± 11	14 ± 5*	19 ± 9	21 ± 7
Mid (MGV)	25 ± 6	21 ± 7	21 ± 6	21 ± 8	23 ± 5	16 ± 8	18 ± 6	24 ± 9
Proximal (MGV)	38 ± 8	24 ± 7*	28 ± 9*	30 ± 10*	38 ± 12	21 ± 12*	26 ± 10*	30 ± 9*

Data are means ± SE. Data were analyzed with a 2-way repeated-measures ANOVA. 2 wk IM, after 2 wk of immobilization; 2 wk retrain, after 2 wk of retraining; 6 wk retrain, after 6 wk of retraining. MGV, mean gray value. Overall time effect ($P < 0.05$). *Significantly lower than at baseline ($P < 0.05$). Tendon signal intensity was comparable in 5 and 6 subjects in Plc and Ibu groups, respectively.

middle part of the tendon, the signal intensity did not change ($P > 0.05$) throughout the study period. At the proximal part of the tendon, an overall time effect ($P < 0.005$) was observed. Post hoc tests indicated that the proximal signal intensity decreased after immobilization ($P < 0.05$) and remained decreased throughout the retraining period ($P < 0.05$).

Tendon Collagen Synthesis

Tendon collagen FSR is shown in Fig. 2. At baseline, tendon collagen FSR was 0.037 ± 0.003%/h in the Plc group and 0.032 ± 0.003%/h in the Ibu group. After immobilization, tendon collagen FSR was 0.007 ± 0.001%/h in the Plc group and 0.006 ± 0.002%/h in the Ibu group. A time effect ($P < 0.001$), but no group effect ($P > 0.05$), was observed. Furthermore, no difference appeared between the decreases in FSR from baseline to after immobilization (0.030 ± 0.004%/h and 0.026 ± 0.003%/h in the Plc and Ibu groups, respectively) ($P > 0.05$, 2-tailed, unpaired *t*-test). The 95% CIs for group differences in tendon collagen FSR were −0.003 to 0.012 at baseline and −0.007 to 0.008 after immobilization.

Tendon Gene Expression

Tendon gene expression is illustrated in Fig. 3. A tendency to a time effect ($P < 0.1$) was observed after immobilization for MMP-2. Moreover, group and interaction effects ($P < 0.05$) were found for scleraxis. Post hoc tests revealed that scleraxis expression decreased after immobilization ($P < 0.05$) in the Plc group, whereas it was unchanged in the Ibu group, resulting in a group difference after immobilization. All other gene expression targets did not change over time or between groups ($P > 0.05$).

Tendon Dimensions

Tendon CSA and length (Fig. 4) did not differ between groups at baseline ($P > 0.05$), and the values for each individual were set to 100% at baseline. For the distal part of the patellar tendon (1.20 ± 0.06 cm^2 and 1.29 ± 0.07 cm^2 in the Plc and Ibu groups, respectively, at baseline), no differences over time ($P = 0.1$) or between groups ($P > 0.05$) were observed. The 95% CIs for group differences in distal CSA were −0.19 to 0.01 at baseline, −0.09 to 0.11 after immobilization, −0.19 to 0.02 after 2 wk of retraining, and −0.20 to 0.00 after 6 wk of retraining. For the middle part of the patellar tendon (1.24 ± 0.05 cm^2 and 1.27 ± 0.07 cm^2 in the Plc and Ibu groups, respectively, at baseline), no differences over time or between groups were observed ($P > 0.05$). The 95% CIs for midtendon CSA group differences were −0.10 to 0.04 at baseline, −0.08 to 0.06 after immobilization, −0.12 to 0.03 after 2 wk of retraining, and −0.12 to 0.03 after 6 wk of retraining. For the proximal part of the patellar tendon (1.37 ± 0.07 cm^2 and 1.44 ± 0.09 cm^2 in the Plc and Ibu groups, respectively, at baseline), no time or group differences were observed ($P > 0.05$). The 95% CI for group differences were −0.17 to 0.02 at baseline, −0.16 to 0.03 after immobilization, −0.18 to 0.03 after 2 wk of retraining, and −0.14 to 0.06 after 6 wk of retraining. Finally, no changes in tendon length (Fig. 4*D*) or mean CSA (data not shown) were observed over time or between groups ($P > 0.05$).

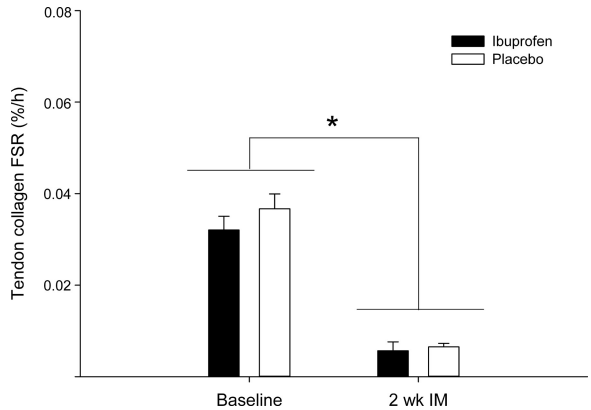

Fig. 2. Patellar tendon collagen fractional synthetic rate (FSR) at baseline and after 2 wk of immobilization (2 wk IM). Data were analyzed with a 2-way repeated-measures ANOVA: *Overall time effect ($P < 0.001$). Tendon collagen synthesis was measured in 6 subjects from each group. Data are means ± SE.

DISCUSSION

The main findings were that in elderly humans tendon collagen protein synthesis decreased after 2 wk of immobilization, whereas tendon stiffness and modulus were marginally reduced, and NSAIDs had no influence upon this. This indicates that mechanical loading is important for maintenance of tendon collagen turnover. Moreover, changes in collagen turnover induced by short-term immobilization may only have minor impact on the internal structures that are essential for mechanical properties in elderly tendons.

In line with previous findings in tendons of young humans (6) and rats (3, 22), unloading did not affect type I and III collagen gene expression in the present study (Fig. 3, *A* and *B*). Interestingly, the present observations of a maintained collagen expression alongside a decreased collagen protein synthesis after immobilization indicate that immobilization may reduce the translational activity of tendon collagen protein formation. Moreover, the expression of MMP-2 has been found to increase after immobilization in young humans (6), which is in accordance with the tendency observed in the present study (Fig. 3*E*). Although only assessed at the gene expression level, the increase in MMP-2 further indicates that even short-term withdrawal of mechanical loading may result in a slight negative regulation of collagen turnover in human tendons. Additionally, the signal intensity (Table 2), which may reflect the material composition of the tendon tissue (8), did decrease at the proximal part of the patellar tendon (close to where biopsies were taken) during immobilization in both groups. Together with the decreased collagen protein synthesis and the tendency to an increased gene expression of MMP-2, this indicates that the internal structure of the tendon may have been affected by immobilization. However, since tendon CSA was unchanged after immobilization, it is possible that a minor loss of tendon collagen content could have been counterbalanced by an increased interstitial water content of the tendon tissue. Importantly though, such possible change in the tendon internal structure only marginally influenced the tendon mechanical properties.

Even though Young's modulus displayed a slight change over time, no significant impairments in tendon mechanical properties were observed (Table 1), which was contrary to our

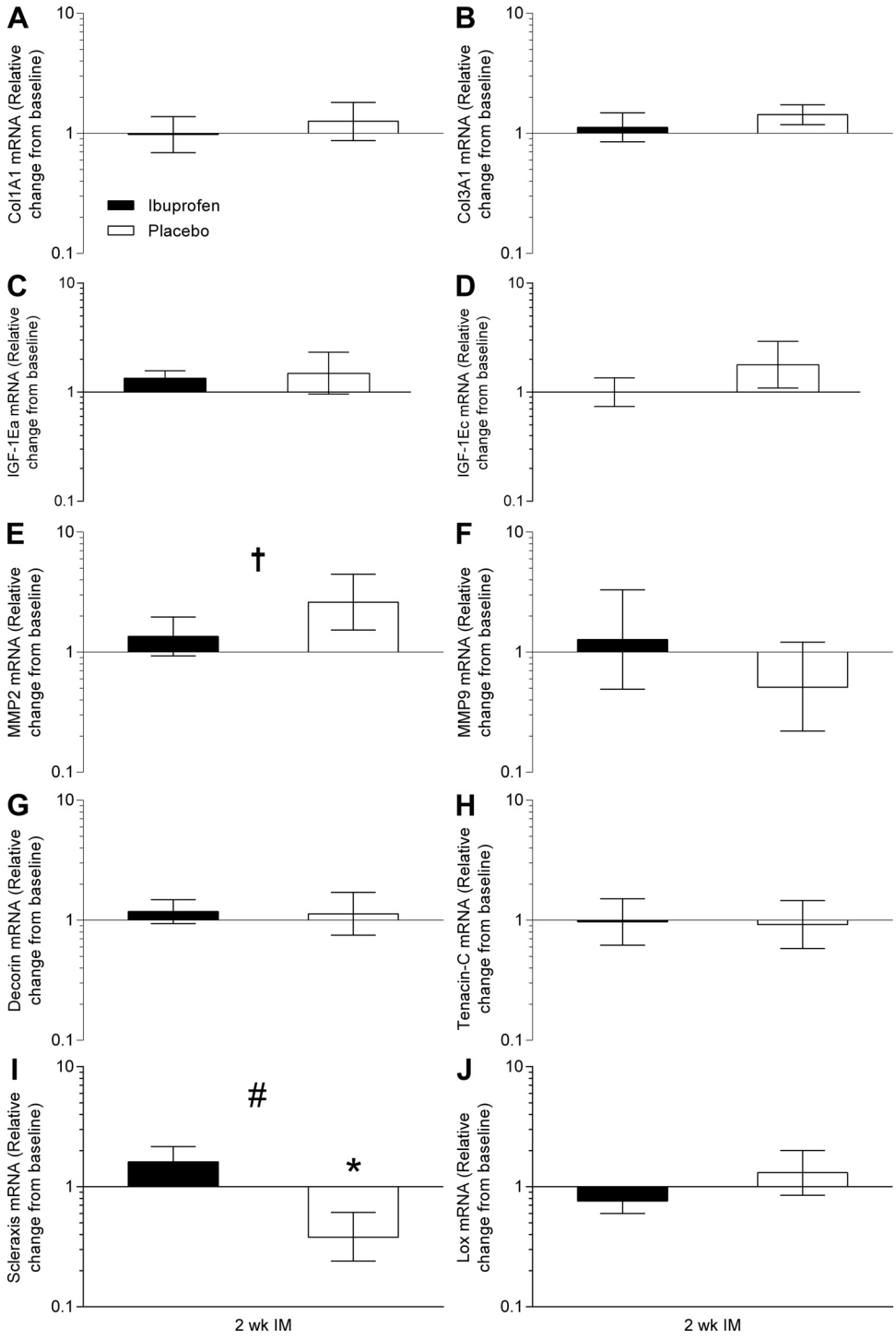

Fig. 3. Relative changes in tendon gene expression after 2 wk of immobilization (2 wk IM): Col1A1 (*A*), Col3A1 (*B*), IGF-1Ea (*C*), IGF-1Ec (*D*), MMP-2 (*E*), MMP-9 (*F*), decorin (*G*), tenascin-C (*H*), scleraxis (*I*), and lox (*J*). Data were analyzed with a 2-way repeated-measures ANOVA. For scleraxis, an overall interaction effect ($P < 0.05$) was observed: *Significant decrease ($P < 0.05$) from baseline in Plc, #Significant difference ($P < 0.001$) between groups. †Overall time effect tendency ($P < 0.1$) for MMP-2. Tendon gene expression was measured in 6 and 8 subjects in Plc and Ibu groups, respectively. Data are geometric means ± back-transformed SE.

hypothesis. Previously, tendon stiffness and modulus were found to decrease in elderly men completing an immobilization protocol similar to the present one (16). While the values for stiffness and modulus reported by Couppé et al. (16) are similar to those obtained in the present study (Table 1), the data variation is somewhat higher in the present study, which may have limited our ability to detect significant changes in tendon stiffness and modulus. In the present study, the knee joint was positioned in ~50° knee flexion during immobilization, whereas ~30° knee flexion was used previously (16). Although rather small, this difference may have resulted in a higher degree of passive tension in the patellar tendon in the present study compared with the previous study (16). Since it has been shown that loss of mechanical properties in unloaded rabbit patellar tendons depends on the degree of passive tension (32), a higher degree of passive tension may have protected the tendon mechanical properties to some extent, or at least caused the higher data variation in the present study compared with the previous study (16). Another part of the explanation for the unchanged mechanical properties after short-term unloading

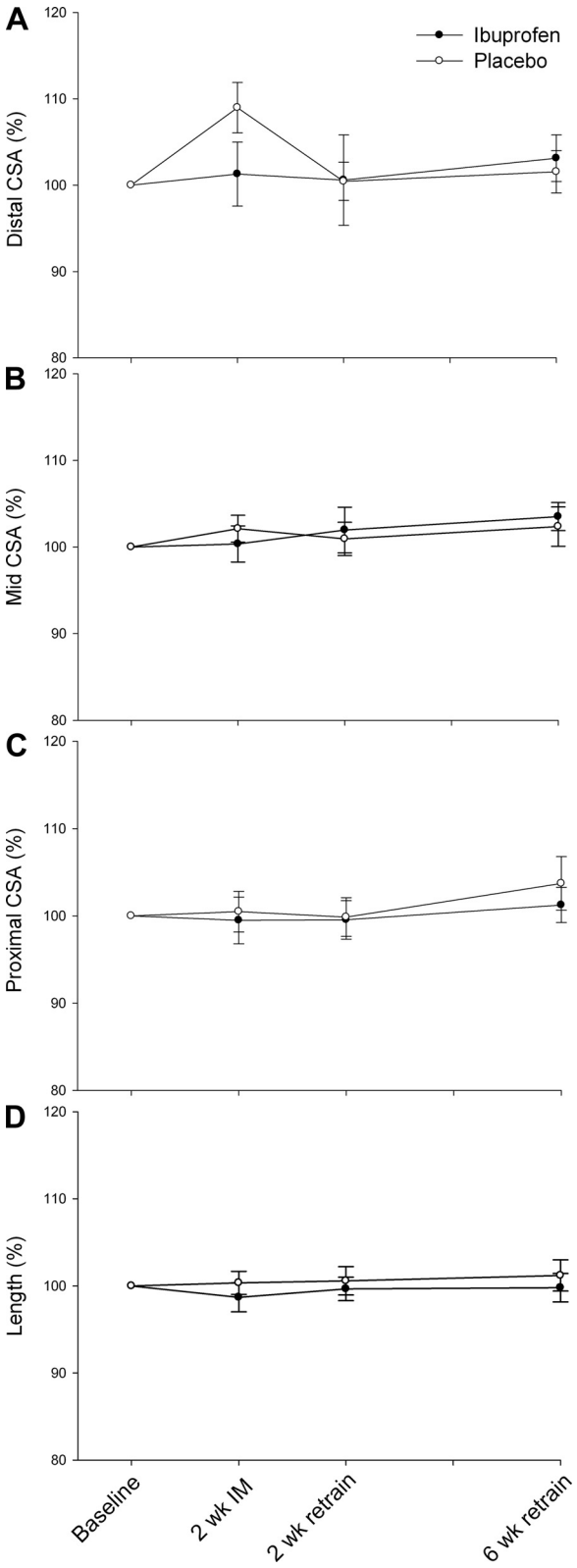

Fig. 4. Relative changes in patellar tendon cross sectional area (CSA) and length after 2 wk of immobilization (2 wk IM), after 2 wk of retraining (2 wk retrain), and after 6 wk of retraining (6 wk retrain): distal tendon CSA (*A*), midtendon CSA (*B*), proximal tendon CSA (*C*), and tendon length (*D*). Data were analyzed with a 2-way repeated-measures ANOVA, and no time or group differences were observed ($P > 0.05$). Tendon CSA data were obtained from 10 and 8 subjects in Plc and Ibu groups, respectively. Data are means ± SE.

could be that elderly tendons have a high degree of nonenzymatically and enzymatically derived cross-links, which are thought to be important for tendon stiffness (14). In young individuals, a decrease in tendon stiffness has been repeatedly demonstrated after periods of unloading (4, 6, 25, 28, 37). Therefore, the higher degree of both nonenzymatically and enzymatically derived cross-links in elderly compared with young tendons (14) could result in a higher degree of tendon stiffness preservation during periods of unloading in elderly individuals than in young individuals.

Even though no group or time differences were found for patellar tendon CSA, the distal tendon CSA did increase somewhat after immobilization in the Plc group (overall time effect: $P = 0.1$). Previously, tendon swelling has been reported at the distal part of human Achilles tendons after 4 wk of unloading (25). As suggested by Kinugasa et al., the increased tendon size most likely represents a greater relative content of interstitial water (25). In the present study, the distal CSA returned to baseline levels after 2 wk of retraining in the Plc group, which indicates that tendon swelling was transient and most likely neutralized shortly after reloading. To some extent, this is in line with the decreased signal intensity at the distal tendon part after immobilization and the return of this to baseline after 2 wk of retraining (Table 2). Notably, the distal patellar tendon CSA was unchanged after immobilization in the Ibu group. It has been demonstrated that acetaminophen reduces tendon water and cross-linking content in rat Achilles tendons (10). However, since ibuprofen and acetaminophen may have different effects on peripheral tissues (42), the picture is not clear, and further clarification of the effect of NSAIDs on tendon tissue hydration is needed. Moreover, the present findings indicate that a slight increase in tendon tissue hydration during periods of immobilization seems to be transitory and of minor importance for tendon function.

Tendon hypertrophy has previously been reported to occur at the distal and proximal parts of the patellar tendon during periods of resistance training in young individuals (15, 27, 40). However, tendon CSA did not increase during retraining, which is in accordance with our recent finding that—at least for the Plc group—the tendon collagen fibril size and density did not change throughout the study period (7). In accordance with this, an unchanged patellar tendon CSA has been reported after 12–14 wk of resistance training in elderly individuals (9, 38). Collectively, these findings indicate some degree of anabolic resistance to periods of training in elderly tendons. In line with this, it should be noted that tendon stiffness and modulus did not increase during retraining (Table 1), which is in accordance with the findings in elderly humans after 12 wk of resistance training (9).

As a final methodological comment, the magnitude of decrease in patellar tendon collagen FSR during immobilization was equal between groups (Fig. 2). Even though the decrease is in accordance with earlier findings based on the stable isotope technique in young individuals (5), other studies have indicated that the collagen production at the Achilles tendon surface is unaffected by unloading (12, 13). These different results may be due to the anatomically different locations of the tendons used for measurement. Alternatively, the use of microdialysis (12, 13), which is more indirect than the stable isotope technique, could result in diverging findings, as previously observed (21, 34). Nevertheless, it cannot be fully

excluded that the different labeling of the tracers used before and after immobilization may have influenced the FSR values, as the order of tracer administration was not randomized. However, we have no indications of any skewed tendency when comparing the tracer results. Moreover, the only other study that has investigated the change in human patellar tendon collagen FSR during immobilization (5) found a similar magnitude of decrease (~80%) in tendon FSR with different tracers ([1-^{13}C] and [^{15}N]proline) than those used in the present study, which indicates that the decrease in tendon FSR is, at least for the major part, due to decreased mechanical loading.

Conclusions and Perspectives

In conclusion, the present study demonstrated that in elderly tendons collagen protein synthesis decreased with short-term immobilization, whereas stiffness and modulus were only marginally reduced, and NSAID treatment had no influence upon this. This indicates an importance of mechanical loading for maintenance of tendon collagen turnover. However, reduced collagen production induced by short-term immobilization may only marginally influence the mechanical properties of elderly tendons. Furthermore, the present results indicate that NSAIDs potentially can be used as analgesic treatment during periods of unloading without negative mechanical consequences for healthy tendon adaptation in elderly individuals.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

K.D., A.P.B., and L.H. performed experiments; K.D., S.R., R.B.S., P.S., and L.H. analyzed data; K.D., S.R., C.C., R.B.S., P.S., S.P.M., L.H., and M.K. interpreted results of experiments; K.D. prepared figures; K.D. drafted manuscript; K.D., A.P.B., S.R., C.C., R.B.S., P.S., S.P.M., L.H., and M.K. edited and revised manuscript; K.D., A.P.B., S.R., C.C., R.B.S., P.S., S.P.M., L.H., and M.K. approved final version of manuscript; M.K. conceived and designed research.

REFERENCES

1. **Almeida-Silveira MI, Lambertz D, Pérot C, Goubel F.** Changes in stiffness induced by hindlimb suspension in rat Achilles tendon. *Eur J Appl Physiol* 81: 252–257, 2000. doi:10.1007/s004210050039.
2. **Andersen MB, Pingel J, Kjær M, Langberg H.** Interleukin-6: a growth factor stimulating collagen synthesis in human tendon. *J Appl Physiol (1985)* 110: 1549–1554, 2011. doi:10.1152/japplphysiol.00037.2010.
3. **Arruda EM, Mundy K, Calve S, Baar K.** Denervation does not change the ratio of collagen I and collagen III mRNA in the extracellular matrix of muscle. *Am J Physiol Regul Integr Comp Physiol* 292: R983–R987, 2007. doi:10.1152/ajpregu.00483.2006.
4. **De Boer MD, Maganaris CN, Seynnes OR, Rennie MJ, Narici MV.** Time course of muscular, neural and tendinous adaptations to 23 day unilateral lower-limb suspension in young men. *J Physiol* 583: 1079–1091, 2007. doi:10.1113/jphysiol.2007.135392.
5. **De Boer MD, Selby A, Atherton P, Smith K, Seynnes OR, Maganaris CN, Maffulli N, Movin T, Narici MV, Rennie MJ.** The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse. *J Physiol* 585: 241–251, 2007. doi:10.1113/jphysiol.2007.142828.
6. **Boesen AP, Dideriksen K, Couppé C, Magnusson SP, Schjerling P, Boesen M, Kjaer M, Langberg H.** Tendon and skeletal muscle matrix gene expression and functional responses to immobilisation and rehabilitation in young males: effect of growth hormone administration. *J Physiol* 591: 6039–6052, 2013. doi:10.1113/jphysiol.2013.261263.
7. **Boesen AP, Dideriksen K, Couppé C, Magnusson SP, Schjerling P, Boesen M, Aagaard P, Kjaer M, Langberg H.** Effect of growth hormone on aging connective tissue in muscle and tendon: gene expression, morphology, and function following immobilization and rehabilitation. *J Appl Physiol (1985)* 116: 192–203, 2014. doi:10.1152/japplphysiol.01077.2013.
8. **Carroll CC, Dickinson JM, Haus JM, Lee GA, Hollon CJ, Aagaard P, Magnusson SP, Trappe TA.** Influence of aging on the in vivo properties of human patellar tendon. *J Appl Physiol (1985)* 105: 1907–1915, 2008. doi:10.1152/japplphysiol.00059.2008.
9. **Carroll CC, Dickinson JM, LeMoine JK, Haus JM, Weinheimer EM, Hollon CJ, Aagaard P, Magnusson SP, Trappe TA.** Influence of acetaminophen and ibuprofen on in vivo patellar tendon adaptations to knee extensor resistance exercise in older adults. *J Appl Physiol (1985)* 111: 508–515, 2011. doi:10.1152/japplphysiol.01348.2010.
10. **Carroll CC, Whitt JA, Peterson A, Gump BS, Tedeschi J, Broderick TL.** Influence of acetaminophen consumption and exercise on Achilles tendon structural properties in male Wistar rats. *Am J Physiol Regul Integr Comp Physiol* 302: R990–R995, 2012. doi:10.1152/ajpregu.00659.2011.
11. **Christensen B, Dandanell S, Kjaer M, Langberg H.** Effect of anti-inflammatory medication on the running-induced rise in patella tendon collagen synthesis in humans. *J Appl Physiol (1985)* 110: 137–141, 2011. doi:10.1152/japplphysiol.00942.2010.
12. **Christensen B, Dyrberg E, Aagaard P, Enehjelm S, Krogsgaard M, Kjaer M, Langberg H.** Effects of long-term immobilization and recovery on human triceps surae and collagen turnover in the Achilles tendon in patients with healing ankle fracture. *J Appl Physiol (1985)* 105: 420–426, 2008. doi:10.1152/japplphysiol.00201.2008.
13. **Christensen B, Dyrberg E, Aagaard P, Kjaer M, Langberg H.** Short-term immobilization and recovery affect skeletal muscle but not collagen tissue turnover in humans. *J Appl Physiol (1985)* 105: 1845–1851, 2008. doi:10.1152/japplphysiol.90445.2008.
14. **Couppé C, Hansen P, Kongsgaard M, Kovanen V, Suetta C, Aagaard P, Kjaer M, Magnusson SP.** Mechanical properties and collagen cross-linking of the patellar tendon in old and young men. *J Appl Physiol (1985)* 107: 880–886, 2009. doi:10.1152/japplphysiol.00291.2009.
15. **Couppé C, Kongsgaard M, Aagaard P, Hansen P, Bojsen-Moller J, Kjaer M, Magnusson SP.** Habitual loading results in tendon hypertrophy and increased stiffness of the human patellar tendon. *J Appl Physiol (1985)* 105: 805–810, 2008. doi:10.1152/japplphysiol.90361.2008.
16. **Couppé C, Suetta C, Kongsgaard M, Justesen L, Hvid LG, Aagaard P, Kjær M, Magnusson SP.** The effects of immobilization on the mechanical properties of the patellar tendon in younger and older men. *Clin Biomech (Bristol, Avon)* 27: 949–954, 2012. doi:10.1016/j.clinbiomech.2012.06.003.
17. **Dideriksen K, Boesen AP, Kristiansen JF, Magnusson SP, Schjerling P, Holm L, Kjaer M.** Skeletal muscle adaptation to immobilization and subsequent retraining in elderly men: No effect of anti-inflammatory medication. *Exp Gerontol* 82: 8–18, 2016. doi:10.1016/j.exger.2016.05.009.
18. **Doessing S, Heinemeier KM, Holm L, Mackey AL, Schjerling P, Rennie M, Smith K, Reitelseder S, Kappelgaard AM, Rasmussen MH, Flyvbjerg A, Kjaer M.** Growth hormone stimulates the collagen synthesis in human tendon and skeletal muscle without affecting myofibrillar protein synthesis. *J Physiol* 588: 341–351, 2010. doi:10.1113/jphysiol.2009.179325.
19. **Eliasson P, Andersson T, Aspenberg P.** Rat Achilles tendon healing: mechanical loading and gene expression. *J Appl Physiol (1985)* 107: 399–407, 2009. doi:10.1152/japplphysiol.91563.2008.
20. **Farup J, Rahbek SK, Vendelbo MH, Matzon A, Hindhede J, Bejder A, Ringgard S, Vissing K.** Whey protein hydrolysate augments tendon and muscle hypertrophy independent of resistance exercise contraction

mode. *Scand J Med Sci Sports* 24: 788–798, 2014. doi:10.1111/sms.12083.

21. **Hansen M, Kongsgaard M, Holm L, Skovgaard D, Magnusson SP, Qvortrup K, Larsen JO, Aagaard P, Dahl M, Serup A, Frystyk J, Flyvbjerg A, Langberg H, Kjaer M.** Effect of estrogen on tendon collagen synthesis, tendon structural characteristics, and biomechanical properties in postmenopausal women. *J Appl Physiol (1985)* 106: 1385–1393, 2009. doi:10.1152/japplphysiol.90935.2008.
22. **Heinemeier KM, Olesen JL, Haddad F, Schjerling P, Baldwin KM, Kjaer M.** Effect of unloading followed by reloading on expression of collagen and related growth factors in rat tendon and muscle. *J Appl Physiol (1985)* 106: 178–186, 2009. doi:10.1152/japplphysiol.91092.2008.
23. **Hirose T, Nakazato K, Song H, Ishii N.** TGF-beta1 and TNF-alpha are involved in the transcription of type I collagen alpha2 gene in soleus muscle atrophied by mechanical unloading. *J Appl Physiol (1985)* 104: 170–177, 2008. doi:10.1152/japplphysiol.00463.2006.
24. **Holm L, Reitelseder S, Dideriksen K, Nielsen RH, Bülow J, Kjaer M.** The single-biopsy approach in determining protein synthesis in human slow-turning-over tissue: use of flood-primed, continuous infusion of amino acid tracers. *Am J Physiol Endocrinol Metab* 306: E1330–E1339, 2014. doi:10.1152/ajpendo.00084.2014.
25. **Kinugasa R, Hodgson JA, Edgerton VR, Shin DD, Sinha S.** Reduction in tendon elasticity from unloading is unrelated to its hypertrophy. *J Appl Physiol (1985)* 109: 870–877, 2010. doi:10.1152/japplphysiol.00384.2010.
26. **Kjaer M, Bayer ML, Eliasson P, Heinemeier KM.** What is the impact of inflammation on the critical interplay between mechanical signaling and biochemical changes in tendon matrix? *J Appl Physiol (1985)* 115: 879–883, 2013. doi:10.1152/japplphysiol.00120.2013.
27. **Kongsgaard M, Reitelseder S, Pedersen TG, Holm L, Aagaard P, Kjaer M, Magnusson SP.** Region specific patellar tendon hypertrophy in humans following resistance training. *Acta Physiol (Oxf)* 191: 111–121, 2007. doi:10.1111/j.1748-1716.2007.01714.x.
28. **Kubo K, Akima H, Ushiyama J, Tabata I, Fukuoka H, Kanehisa H, Fukunaga T.** Effects of 20 days of bed rest on the viscoelastic properties of tendon structures in lower limb muscles. *Br J Sports Med* 38: 324–330, 2004. doi:10.1136/bjsm.2003.005595.
29. **Langberg H, Boushel R, Skovgaard D, Risum N, Kjaer M.** Cyclo-oxygenase-2 mediated prostaglandin release regulates blood flow in connective tissue during mechanical loading in humans. *J Physiol* 551: 683–689, 2003. doi:10.1113/jphysiol.2003.046094.
30. **Langberg H, Olesen JL, Gemmer C, Kjaer M.** Substantial elevation of interleukin-6 concentration in peritendinous tissue, in contrast to muscle, following prolonged exercise in humans. *J Physiol* 542: 985–990, 2002. doi:10.1113/jphysiol.2002.019141.
31. **Magnusson SP, Hansen P, Aagaard P, Brønd J, Dyhre-Poulsen P, Bojsen-Moller J, Kjaer M.** Differential strain patterns of the human gastrocnemius aponeurosis and free tendon, in vivo. *Acta Physiol Scand* 177: 185–195, 2003. doi:10.1046/j.1365-201X.2003.01048.x.
32. **Majima T, Yasuda K, Fujii T, Yamamoto N, Hayashi K, Kaneda K.** Biomechanical effects of stress shielding of the rabbit patellar tendon depend on the degree of stress reduction. *J Orthop Res* 14: 377–383, 1996. doi:10.1002/jor.1100140306.
33. **Matsumoto F, Trudel G, Uhthoff HK, Backman DS.** Mechanical effects of immobilization on the Achilles' tendon. *Arch Phys Med Rehabil* 84: 662–667, 2003. doi:10.1016/S0003-9993(02)04834-7.
34. **Miller BF, Olesen JL, Hansen M, Døssing S, Crameri RM, Welling RJ, Langberg H, Flyvbjerg A, Kjaer M, Babraj JA, Smith K, Rennie MJ.** Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol* 567: 1021–1033, 2005. doi:10.1113/jphysiol.2005.093690.
35. **Nielsen RH, Holm L, Malmgaard-Clausen NM, Reitelseder S, Heinemeier KM, Kjaer M.** Increase in tendon protein synthesis in response to insulin-like growth factor-I is preserved in elderly men. *J Appl Physiol (1985)* 116: 42–46, 2014. doi:10.1152/japplphysiol.01084.2013.
36. **Petersen SG, Miller BF, Hansen M, Kjaer M, Holm L.** Exercise and NSAIDs: effect on muscle protein synthesis in patients with knee osteoarthritis. *Med Sci Sports Exerc* 43: 425–431, 2011. doi:10.1249/MSS.0b013e3181f27375.
37. **Reeves ND, Maganaris CN, Ferretti G, Narici MV.** Influence of 90-day simulated microgravity on human tendon mechanical properties and the effect of resistive countermeasures. *J Appl Physiol (1985)* 98: 2278–2286, 2005. doi:10.1152/japplphysiol.01266.2004.
38. **Reeves ND, Maganaris CN, Narici MV.** Effect of strength training on human patella tendon mechanical properties of older individuals. *J Physiol* 548: 971–981, 2003. doi:10.1113/jphysiol.2002.035576.
39. **Savolainen J, Myllylä V, Myllylä R, Vihko V, Väänänen K, Takala TES.** Effects of denervation and immobilization on collagen synthesis in rat skeletal muscle and tendon. *Am J Physiol Regul Integr Comp Physiol* 254: R897–R902, 1988.
40. **Seynnes OR, Erskine RM, Maganaris CN, Longo S, Simoneau EM, Grosset JF, Narici MV.** Training-induced changes in structural and mechanical properties of the patellar tendon are related to muscle hypertrophy but not to strength gains. *J Appl Physiol (1985)* 107: 523–530, 2009. doi:10.1152/japplphysiol.00213.2009.
41. **Suetta C, Hvid LG, Justesen L, Christensen U, Neergaard K, Simonsen L, Ortenblad N, Magnusson SP, Kjaer M, Aagaard P.** Effects of aging on human skeletal muscle after immobilization and retraining. *J Appl Physiol (1985)* 107: 1172–1180, 2009. doi:10.1152/japplphysiol.00290.2009.
42. **Trappe TA, Liu SZ.** Effects of prostaglandins and COX-inhibiting drugs on skeletal muscle adaptations to exercise. *J Appl Physiol (1985)* 115: 909–919, 2013. doi:10.1152/japplphysiol.00061.2013.
43. **Tsai W-C, Hsu C-C, Chang H-N, Lin Y-C, Lin M-S, Pang J-HS.** Ibuprofen upregulates expressions of matrix metalloproteinase-1, -8, -9, and -13 without affecting expressions of types I and III collagen in tendon cells. *J Orthop Res* 28: 487–491, 2010.
44. **Uchida H, Tohyama H, Nagashima K, Ohba Y, Matsumoto H, Toyama Y, Yasuda K.** Stress deprivation simultaneously induces overexpression of interleukin-1beta, tumor necrosis factor-alpha, and transforming growth factor-beta in fibroblasts and mechanical deterioration of the tissue in the patellar tendon. *J Biomech* 38: 791–798, 2005. doi:10.1016/j.jbiomech.2004.05.009.
45. **Visser JJ, Hoogkamer JE, Bobbert MF, Huijing PA.** Length and moment arm of human leg muscles as a function of knee and hip-joint angles. *Eur J Appl Physiol Occup Physiol* 61: 453–460, 1990. doi:10.1007/BF00236067.