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HIGHLIGHTED TOPIC | *Regulation of Protein Metabolism in Exercise and Recovery*

Effect of estrogen on tendon collagen synthesis, tendon structural characteristics, and biomechanical properties in postmenopausal women

Mette Hansen,[1] Mads Kongsgaard,[1] Lars Holm,[1] Dorthe Skovgaard,[1] S. Peter Magnusson,[1] Klaus Qvortrup,[3] Jytte O. Larsen,[4] Per Aagaard,[5] Morten Dahl,[1] Annette Serup,[1] Jan Frystyk,[2] Allan Flyvbjerg,[2] Henning Langberg,[1] and Michael Kjaer[1]

[1]*Institute of Sports Medicine, Bispebjerg Hospital, and Faculty of Health Sciences, University of Copenhagen, Copenhagen;* [2]*The Medical Research Laboratories, Clinical Institute and Medical Department M (Diabetes and Endocrinology), Aarhus University Hospital, Aarhus;* [3]*Department of Medical Anatomy, Faculty of Health Sciences and* [4]*Department of Neuroscience and Pharmacology, University of Copenhagen, Copenhagen, Denmark; and* [5]*Institute of Sports Science and Clinical Biomechanics, University of Southern Denmark, Odense, Denmark*

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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* 106: 1385–1393, 2009. First published October 16, 2009; doi:10.1152/japplphysiol.90935.2008.—The knowledge about the effect of estradiol on tendon connective tissue is limited. Therefore, we studied the influence of estradiol on tendon synthesis, structure, and biomechanical properties in postmenopausal women. Nonusers (control, $n = 10$) or habitual users of oral estradiol replacement therapy (ERT, $n = 10$) were studied at rest and in response to one-legged resistance exercise. Synthesis of tendon collagen was determined by stable isotope incorporation [fractional synthesis rate (FSR)] and microdialysis technique (NH_2-terminal propeptide of type I collagen synthesis). Tendon area and fibril characteristics were determined by MRI and transmission electron microscopy, whereas tendon biomechanical properties were measured during isometric maximal voluntary contraction by ultrasound recording. Tendon FSR was markedly higher in ERT users ($P < 0.001$), whereas no group difference was seen in tendon NH_2-terminal propeptide of type I collagen synthesis ($P = 0.32$). In ERT users, positive correlations between serum estradiol (s-estradiol) and tendon synthesis were observed, whereas change in tendon synthesis from rest to exercise was negatively correlated to s-estradiol. Tendon area, fibril density, fibril volume fraction, and fibril mean area did not differ between groups. However, the percentage of medium-sized fibrils was higher in ERT users ($P < 0.05$), whereas the percentage of large fibrils tended to be greater in control ($P = 0.10$). A lower Young's modulus (GPa/%) was found in ERT users ($P < 0.05$). In conclusion, estradiol administration was associated with higher tendon FSR and a higher relative number of smaller fibrils. Whereas this indicates stimulated collagen turnover in the resting state, collagen responses to exercise were negatively associated with s-estradiol. These results indicate a pivotal role for estradiol in maintaining homeostasis of female connective tissue.

connective tissue; tendon fibrils; insulin-like growth factor-I; extracellular matrix; bone

CROSS-SECTIONAL FINDINGS INDICATE that sex hormones influence tendon biomechanical properties (36), extracellular matrix adaptability in response to mechanical loading (11, 21, 36, 41, 59), and the risk of sustaining soft tissue injuries (11, 24, 25).

Estrogen receptors have been localized in ligaments (32, 33), and tendons express transcripts for estrogen receptors (23). Nevertheless, the effect of estrogen on tendon and ligament turnover is not clarified. Thus an inhibiting effect (34, 60), no effect (51), and a stimulating effect (32) on collagen synthesis and fibroblast proliferation in vitro have been observed in anterior cruciate ligament (ACL) tissue samples. These contrasting findings are probably related to the variation between animal species and the applied methods. This underlines the importance of performing human in vivo studies to elucidate the effect of estrogen on human collagen metabolism.

Collagen fibrils are the basic force-transmitting unit of tendons and, therefore, influence the mechanical properties of tendons (47). Case reports suggest that tendons with a higher proportion of larger collagen fibrils have greater tensile strength (38). Also, a positive relationship between fibril diameter and tendon stiffness has been demonstrated (7). However, the precise contribution of fibrillar diameter and composition to the mechanical properties and strength of tendinous tissue remains to be firmly established. The effect of estrogen on tendon fibril characteristic is not known.

Type I collagen (CTX-I) is the main protein component in bone and tendon tissue. Bone mass density (BMD) decreases by age, especially after menopause, whereas tendon cross-sectional area (CSA) is greater in postmenopausal women compared with young women (35). In postmenopausal women, estrogen replacement therapy (ERT) preserves bone mass (58), and the presence of estrogen, estrogen receptors, and mechanical loading has synergistic positive effects on bone mass (9, 55). The effect of combined ERT and physical training on tendons is elusive. A smaller tendon diameter has been observed in postmenopausal female golf players who used hormone replacement therapy compared with controls (11). Furthermore, an inhibition of CTX-I expression has been demonstrated when estradiol and tensile loading of ACL fibroblasts

Address for reprint requests and other correspondence: M. Hansen; Institute of Sports Medicine, Copenhagen, Bldg. 8, Bispebjerg Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark (e-mail: kontakt@mettehansen.nu).

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were combined, despite the fact that both stimuli upregulated the expression of CTX-I when applied separately (32). These findings suggest a differential effect of estrogen in bone and tendons and estrogen in combination with mechanical loading. The effect may either be direct or an indirect effect of estrogen on other growth factors and cytokines known to influence collagen turnover, such as insulin-like growth factor-I (IGF-I) (2, 6, 8, 43, 56).

The primary aim of the present study was to examine the effect of an enhanced serum concentration of estradiol on tendon collagen synthesis at rest and in response to exercise. In addition, we investigated the effects of long-term use of ERT on tendon CSA, fibril characteristics, and tendon biomechanical properties. Finally, BMD and markers for bone turnover were measured to study any differential effects of estrogen administration on bone and tendon collagen metabolism, respectively.

MATERIAL AND METHODS

Design

A cross-sectional design was used to compare the influence of high- vs. low-estradiol levels on tendon tissue by recruiting postmenopausal women who were nonusers or users of ERT. The effect of exercise on tendon collagen synthesis was measured 24 h after a one-legged exercise. The contralateral leg represented tendon collagen synthesis at rest.

Subjects

The participants were 20 healthy, postmenopausal women, who were nonsmokers and absent of metabolic disorders and orthopedic tendon injuries, as judged by history and routine medical examination. The subjects gave informed consent to the protocol, which was approved by the Ethics Committee of Copenhagen and Frederiksberg municipalities (KF-01–032/04). Ten women were long-term users of ERT [17.4 ± 2.6 treatment years (mean ± SD), range 6–30 yr]. The subjects were daily supplemented with 2 mg oral 17β-estradiol (n = 6 Oestradiol; n = 2 Oestrofem; n = 1 Femanest, or n = 1 Pygynon). All subjects had started the use of ERT following hysterectomy. In addition to ERT, one subject was supplemented with Trisekvens, which contains a further 1–2 mg of 17β-estradiol each day besides synthetic progestogens (1 mg norethisteronacetat) 10 out of 28 days in a pill cycle. The latter subject was excluded from the analysis for differences between groups. Additionally, we included 10 postmenopausal women (control) who had not been menstruating for the last 7.2 ± 1.4 yr (range 2–15 yr). One control subject had used ERT for a maximum of 3 mo 7 yr ago. Otherwise, none of controls had used ERT. However, six of the controls had used oral contraceptives before the menopause. To make the difference in estrogens (endogenous secreted and synthetic ethinyl estradiol) between groups as large as possible during the period in which samples were obtained, women who used ERT were tested in the hours after the last pill ingestion. The two groups were otherwise comparable based on age, height, weight, body mass index, and body composition quantified by dual-energy X-ray absorptiometry (Table 1).

Screening

All participants visited the laboratory 1–3 wk before study start to receive detailed information about the experiment, a health examination, and blood samples, including hemoglobin, serum (s)-ferritin, s-transferrin, leucocytes, s-thyroid stimulating hormone, plasma (p)-creatinin, p-cholesterol, p-C-reactive protein, p-aminostransferase, p-aspartate aminotransferase, p-alkaline phosphatase, and p-albumin. All results were within the normal range. Training status of the groups was compared in three different ways. *1*) Daily physical activity level (PAL) determined by use of a validated questionnaire was assessed (1) (Table 1). *2*) Estimated maximal oxygen uptake ($\dot{V}O_{2max}$) per kilogram body weight was estimated by a 10-min two-step submaximal bike test (3). The workload was increased once after 6 min, and the heart rate registered when it was stable. Two of the subjects in the ERT group were not tested due to influenza the week before. *3*) One-legged five repetitions maximum (RM) was measured in a Technogym leg extension R.O.M machine (for details, see Ref. 14). PAL and dynamic muscle strength were not significant different between the groups, whereas estimated $\dot{V}O_{2max}$ was significantly higher in control vs. ERT users.

Table 1. *Subject characteristics*

	Control	ERT Users
n	10	10
Age, yr	60±4	61±4
Weight, kg	64±4	64±9
Height, cm	163±6	162±5
BMI, kg/m^2	24.4±2.4	24.3±3.2
Body fat, %	34±5	36±7
LBM, kg	43±4	40±5
Estimated $\dot{V}O_{2max}$, l/min	2.2±0.4	1.8±0.4†
Estimated $\dot{V}O_{2max}$, l·min^{-1}·kg^{-1}	34±6	27.4±6*
PAL	1.8±0.2	1.7±0.3
Estimated 5 RM	31±9	27±9

Values are mean ± SD; n, no. of subjects. ERT, estrogen replacement therapy; BMI, body mass index (weight height^{-2}); LBM, lean body mass; estimated $\dot{V}O_{2max}$, maximal oxygen uptake estimated by a submaximal bike test; PAL, physical activity level (daily estimated energy expenditure/estimated basal metabolic rate) (1, 44); RM, repetition maximum during one-legged dynamic strength test (kg). *$P < 0.05$ and †$P = 0.06$: control vs. ERT users.

Experimental Protocol and Methods

At *day 1*, the subjects performed 10 sets of 10 repetitions of one-legged knee extension at 10 RM. A new set was started every third minute. Subject fasted overnight (12 h) and arrived at the laboratory at 8 AM the following day. The subjects were instructed to avoid strenuous physical activity for at least 2 days before and during the experimental days. Tendon collagen synthesis was measured by microdialysis technique and by infusion of a flooding dose of stable isotope labeled amino acids followed by patellar tendon biopsies bilaterally.

Microdialysis. Fluid collected from the interstitial fluid surrounding the patellar tendon by using a microdialysis technique was analyzed for a marker of CTX-I synthesis, the amino terminal propeptide of CTX-I (PINP) (31). The remaining dialysate was used for analyses of IGF-I and IGF-binding proteins (IGFBPs) (1–4). After previous preparation of incision sites with local anesthetic (lidocaine 1%), ethylene oxide sterilized catheters with high molecular mass cut-off (3,000 kDa, membrane length 30 mm, inner diameter 0.50 mm) were inserted under ultrasound guidance in the peritendinous spaces of patellar tendons, as previously described (31). The inflow tube of the microdialysis catheter was connected to a high-precision syringe pump with infusion rate of 2 μl/min. The catheters were perfused with a Ringer-acetate solution mixed with a small amount of radioactive labeled glucose (D-[3-^{3}H]glucose in aqueous solution steri-pack, Perkin Elmer Life and Analytical Science, Boston, MA; Net 331A, lot no. 3559-801). The total amount of radioactivity that the patients received was <0.001 mSv. For comparison, the normal radioactivity dose that a person in Denmark receives yearly is 3 mSv. A sample vial was placed at the end of the outflow tube, and, after 30-min perfusion of the catheter, dialysate was collected in three 1-h periods and stored at −80°C until the analyses were performed. The relative recovery (RR)

over the membrane was determined for each dialysate sample (49). The dialysate (3 μl) was pipetted into a counting vial in duplicates and mixed with 3 ml scintillation fluid (Ultima Gold, Perkin Elmer, Boston, MA), and the samples were counted in a β-counter. The mean relative recoveries for the microdialysis catheters did not differ between groups (means ± SE; control 36 ± 3%, ERT users: 49 ± 4%, $P = 0.16$).

Tendon collagen fractional synthesis rate. Measurement of tendon collagen fractional synthesis rate (FSR) was performed according to previously applied approaches (5, 42). Briefly, a flooding dose of proline (3.75 g total; 0.75 g labeled L-[1-^{13}C]proline; >99 atoms% [^{13}C]proline, Cambridge Isotope Laboratories, Andover, MA), 3.0 g unlabeled [^{12}C]proline (AppliChem, Darmstadt, Germany), was dissolved in 0.9% NaCl using a sterile technique and intravenously infused over 3 min. After flooding, blood samples were drawn at 10- to 60-min intervals to determine the area under the [^{13}C]proline enrichment curve in plasma measured as tracer-to-tracee ratio. Two hours after the isotope flood (24 h postexercise), tendon biopsies were taken from the patella tendon (~10 mg) of each leg after previously preparing incision sites with local anesthetic (lidocaine 1%). Tendon biopsies were obtained by using a 16-G Monopty biopsy instrument (Bard, Covington, GA) under ultrasound guidance. Biopsies were cleared of external adipose tissue and blood, frozen in liquid nitrogen, and stored at −80°C for subsequent analysis.

PLASMA PROLINE ENRICHMENT. Plasma proline was prepared as previously described and analyzed as its *t*-butyldimethylsilyl derivative by gas chromatography-mass spectrometry (Trace GC 2000 series, MS, Automass Multi, Thermo Quest Finnigan, Paris, France) (4, 50) using a CP-SIL 8, CB low bleed, 30 m * 0.32 mm, 0.25-μm column (Chromepack, Varian, Palo Alto, CA).

TENDON PREPARATION. Details regarding this procedure have been given elsewhere (5, 42). Briefly, tendon (5–10 mg) biopsies were homogenized in buffer (0.15 M NaCl, 0.1% Triton X-100, and 0.02 M Tris·HCl, 5 mM EDTA, pH 7.4) and hydrolyzed in 6 M HCl at 110°C overnight. Then amino acids were extracted through disposable columns using acidic cation exchange resin (Dowex AG-50W, Bio-Rad, Sundbyberg, Sweden). The amino acids were derivatized as their *N*-acetyl-*n*-propyl esters (40), and the enrichment of the proline peak was determined by gas chromatograph combustion isotope ratio mass spectrometry (Delta Plus XL, Thermo Finnigan, Bremen, Germany) using a column CP-Sil 19 CB 60 m × 0.32 mm, coating 0.25 μm (ChromPack).

CALCULATIONS. Plasma [^{13}C]proline enrichment was assumed to represent the enrichment of the true precursor pool, prolyl-tRNA (4). Thus tendon collagen FSR (%/h) = $\Delta E_t/E_p \times 1/t \times 100\%$, where ΔE_t is the change in enrichment of proline in the individual tendon samples compared with the tendon sample with the lowest enrichments in the whole group, E_p is the average enrichment of the precursor (plasma [^{13}C]proline) determined as the area under the time-plasma enrichment curve (Fig. 1), and t is the time (h) of tracer incorporation.

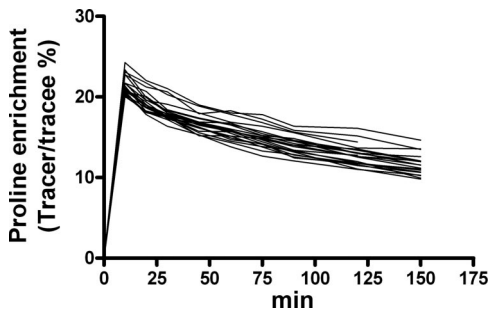


Fig. 1. Plasma enrichment of [^{13}C]proline after flooding dose of [1-^{13}C]proline (tracer-to-tracee ratio).

Transmission electron microscopy. In case of remaining tendon tissue after FSR analysis, a small specimen was used for transmission electron microscopy (TEM) (control specimen: $n = 9$, and ERT specimen: $n = 6$). The biopsy specimens for TEM were fixed in a 2% glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.2) and stored at 4°C until subsequent analyses. The procedure for TEM and the following measurements of collagen fibril diameter have previously been described in details (38).

STEREOLOGY. A simple, random sample of 10 digitized electron microscopy images was obtained from each biopsy cross section (Fig. 2). The stereological analyses of collagen fibrils were carried out on a computer monitor onto which the digitized electron microscopy image was merged with a graphic representation of the stereological test systems (C.A.S.T.-grid software, The International Stereology Center at Olympus). Each TEM image was examined with 16 uniformly positioned points and 16 uniformly positioned unbiased counting frames (19), each of area 0.0426 μm^2, in a fixed position relative to the image. The counting frames covered 15% of the area of the TEM images. On average, 175 ± 13 fibrils (range 118–253) were analyzed per biopsy cross section in the control specimens, and 189 ± 11 from each of the ERT specimens (range 157–235). All measurements were performed in a blinded fashion.

Patellar tendon CSA and length. Patellar tendon CSA and length were determined with magnetic resonance imaging (MRI) on the day before the biopsy procedure, as previously described (29). Patellar tendon CSA was measured *1*) just distal to the patellar insertion; *2*) just proximal to the tibia insertion; and *3*) midway between the two sites (29). The patellar tendon CSA and patellar tendon length were manually outlined using the software program WEB 1000 (AGFA). The mean value of three measurements of the same image was used for analysis. The MRI assessment investigator was blinded with regard to subject grouping. The average intraindividual coefficient of variation (CV%) was 4%, whereas the interindividual CV% was 11%. Tendon CSA was normalized to body mass to the power of 2/3 (39).

Patellar tendon mechanical properties. Measurements of patellar tendon biomechanical properties were obtained on a separate day before the experimental day. Details and reliability of this method have been reported previously (22). Briefly, subjects were tested following a 10-min warm-up on a stationary bike. Subjects performed four to five ramped isometric maximal voluntary contractions over a period of 10 s. A 2-min rest separated each ramp, and all measurements were performed on both legs. Synchronized values of patellar tendon elongation [change in length (ΔL)] (obtained from ultrasound recordings) and patellar tendon forces [change in force (ΔF)] were sampled during the ramp contractions. Tendon forces were calculated by dividing measured knee extension moments by moment arms estimated from individual femur lengths (57). Subsequently, all trials were analyzed to a greatest common patellar tendon force for all subjects (2,443 N) to take into account small differences in individual peak knee extension moment. Force-deformation curves were fitted to a second- or third-order polynomial fit that, in all cases, exceeded $R^2 = 0.95$. Tendon strain ($\Delta L/L_0$), tendon stiffness ($\Delta F/\Delta L$), and Young's modulus (Δstress/Δstrain) were calculated in the final 20% of the force-deformation and stress-strain curves, respectively (22). Analysis was performed in an investigator-blinded fashion. The proximal part of the tendon was used for the calculation of tendon stress. The proximal tendon CSA was used as this region had the smallest CSA and was, therefore, subjected to the highest stress values.

Blood, dialysate, and urine analysis. All blood samples were taken from an antecubital vein. Estradiol was analyzed by chemiluminescent competitive immunoassay (Immulite 2500) (NPU 1972, estradiol; Diagnostic Product, Los Angeles, CA), while testosterone was analyzed by liquid chromatography-mass spectrometry using an atmospheric pressure chemical ionization interface (CV% <15%)(21). s-IGF-I and dialysate IGF-I were determined by time-resolved immunofluorometric assay after acid-ethanol extraction, as previously described (17). All samples were measured in the same assay run

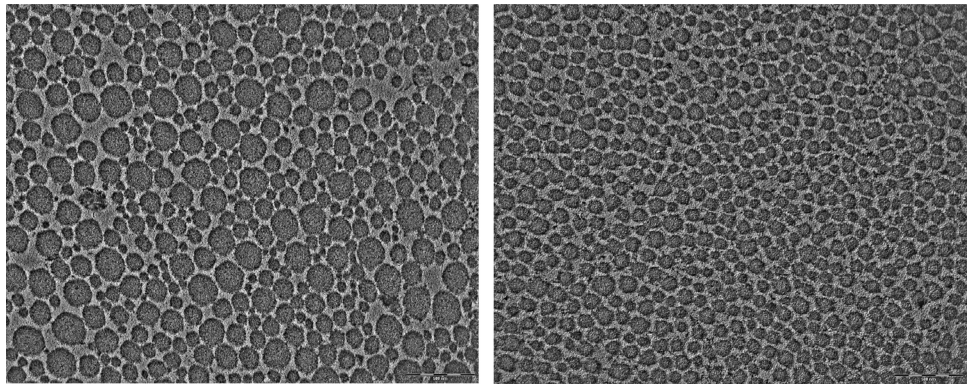


Fig. 2. Transmission electron microscopy of collagen fibrils from patella tendon with varying fibril diameter. *Left*: control subjects. *Right*: estrogen replacement therapy (ERT) user.

(intra-assay CV% <5%). s-IGFBP-1 was determined by an in-house radioimmunoassay with modifications (30) (CV% within and between assay averaged <5 and <16%, respectively). s-IGFBP-3 was measured by commercially available immunoradiometric assay (BioSource Europe, Nivelles, Belgium) (CV% within and between assay: <5 and <10%, respectively). Tendon dialysates were analyzed for IGFBP-1 to -4 by Western ligand blotting, as previously described (16). Serum was analyzed for PINP for indirect quantification of collagen synthesis in bone (s-PINP). Tendon dialysate was similarly analyzed for PINP for indirect quantification of local synthesis of CTX-I in tendon. The analysis for PINP was performed using a sandwich ELISA utilizing purified α_1-chain specific rabbit antibodies (donated by B. Teisner, Department of Medical Microbiology, University of Southern Denmark, Odense, Denmark) (26). The within- (double determination) and between-assay CVs were, on average, 2.2 and 4.9%, respectively. Urine samples were analyzed for CTX-I, as a marker for degradation of CTX-I in bone, by an enzyme immunosorbent assay (Urine CrossLaps ELISA, Nordic Bioscience Diagnostics a/s). Within- and between-assay CVs were 3.6 ± 2.3 and 2.7 ± 2.0%, respectively. Urine-creatinine was analyzed by a two-point kinetic slide method, with a Vitros 5.1 FS (670 nm).

Statistics and Data Analyses

Two-way repeated-measures ANOVA (one-factor repetition) was used to test for difference between groups in tendon FSR and tendon PINP and to elucidate if the response to exercise was influenced by the administration of estrogen. If results showed $P \leq 0.05$, post hoc analyses were performed using pairwise multiple-comparison procedures (Holm-Sidak method). Student's unpaired *t*-tests were used to test for differences between groups in the other parameters. For test of differences in the distribution of fibrils between groups, the fibrils were divided into three intervals: <60 nm, 60–90 nm, and >90 nm. The intervals chosen were based on earlier published results (54). The individual values for the biomechanical test parameters were analyzed at the same individual common force and the same common force for all of the subjects (29). Linear regression analyses between several of the measured parameters were performed between the main parameters [s-estradiol, tendon synthesis (PINP, FSR)]. The baseline characteristics of the subject groups are presented as means ± SD, whereas the results are presented as means ± SE. The level of significance was set at $P < 0.05$. The statistical analyses were performed using the statistical software package Prism version 4.01 (GraphPad, San Diego, CA, 2004).

RESULTS

Sex Hormones

Estradiol was below the detection level in 8 of 10 controls (<10 nmol/l). In the remaining two controls, s-estradiol was 0.11 and 0.21 nmol/l, respectively. In contrast, s-estradiol was above detection level in all women using ERT. Mean s-estradiol in ERT users was 0.39 ± 0.04 nmol/l (range 0.18–0.60 nmol/l). Estradiol was 1.3 nmol/l in the woman using a double dose. s-Testosterone was not significant different between groups (control: 0.87 ± 0.10 nmol/l; ERT users: 0.72 ± 0.08 nmol/l, $P = 0.30$).

Tendon Synthesis (Stable Isotope: FSR)

At rest, tendon collagen FSR was 47% higher in ERT users compared with control ($P < 0.01$) (Fig. 3). In the exercise leg tendon collagen FSR was 86% higher in ERT users compared with control ($P < 0.001$). When analyzing data from the ERT users, a positive association between s-estradiol and tendon FSR was observed at rest ($r^2 = 0.41$, $P = 0.06$) and after exercise ($r^2 = 0.80$, $P < 0.001$). Two-way repeated-measures ANOVA showed that no effect of exercise ($P = 0.33$) or interaction between sex hormonal levels and exercise ($P = 0.13$) was observed in the 24-h post-exercise biopsy.

Tendon Synthesis (Microdialysis: PINP)

The marker for tendon collagen synthesis (PINP) was not different between ERT users and control ($P = 0.32$) (Fig. 3). Neither was a significant response to exercise observed ($P = 0.21$, interaction $P = 0.59$). In ERT users, s-estradiol was positively correlated to tendon synthesis at rest ($r^2 = 0.47$, $P < 0.05$) (Fig. 3), and the subject receiving a double dose of estradiol strengthened this association ($r^2 = 0.68$, $P < 0.01$) (data not shown). When including the subject receiving a double dose of estradiol, a negative correlation between s-estradiol and the response to exercise was observed (ΔPINP, $r^2 = 0.75$, $P < 0.01$).

Tendon Fibril Characteristics

Tendon fibril characteristics were analyzed in a subgroup of subjects (control $n = 9$, ERT users $n = 6$). No significant difference in fibril density ($P = 0.32$), volume fraction ($P = 0.26$), or mean fibril size ($P = 0.53$) was observed between the two groups (Fig. 4). Nevertheless, in ERT users, a greater percentage of the fibrils had a diameter within the interval of 60–90 nm compared with controls ($P < 0.05$) (Fig. 4). In contrast, controls had a greater percentage of large fibrils (>90 nm in fibril diameter), although this difference only tended significance ($P = 0.10$). No significant group difference was observed with regard to percentage of small-sized (0–30 nm in fibril diameter) fibrils ($P = 0.32$).

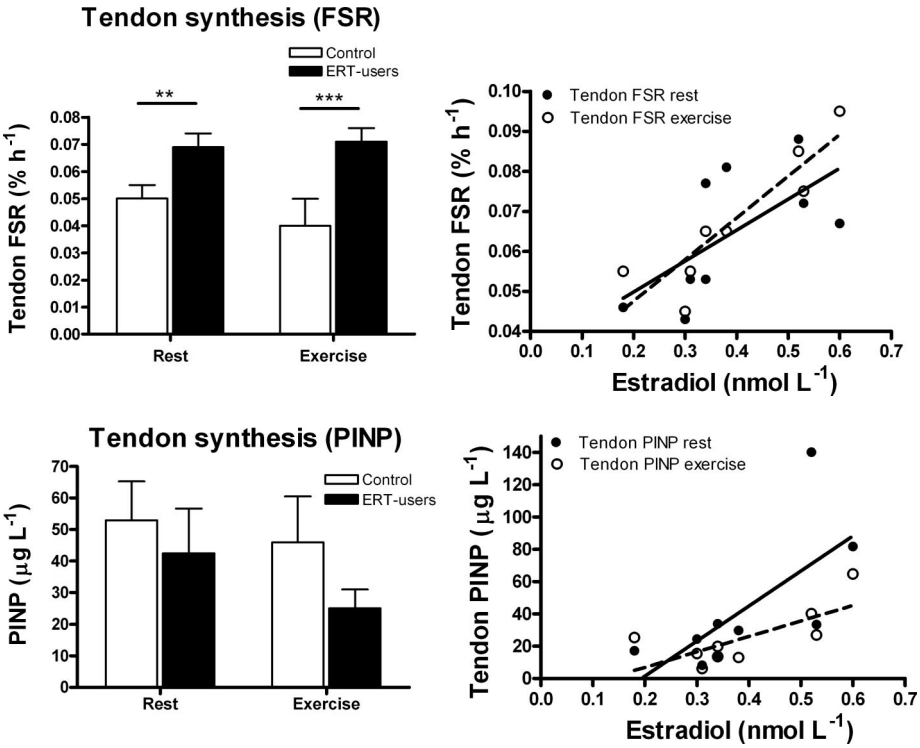
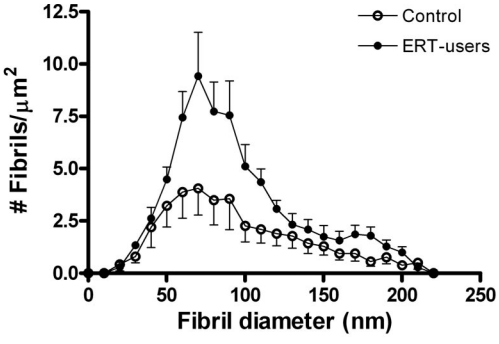


Fig. 3. *Top left*: patellar tendon collagen fractional synthesis rates (FSR) at rest and 24 h after exercise in postmenopausal women who used ERT and postmenopausal women who did not use ERT (control). $^{**}P < 0.01$ and $^{**}P < 0.001$, unpaired *t*-test, control vs. ERT users. *Top right*: relationship between tendon FSR and serum (s)-estradiol in ERT users at rest ($r^2 = 0.41$, $P = 0.06$) and postexercise ($r^2 = 0.80$, $P < 0.001$). *Bottom left*: NH_2-terminal propeptide of type I collagen (PINP) as a marker for tendon synthesis. *Bottom right*: relationship between tendon synthesis (PINP) and s-estradiol in ERT users at rest ($r^2 = 0.47$, $P < 0.05$) and postexercise ($r^2 = 0.52$, $P < 0.05$). Values are means ± SE.

Tendon CSA

ERT did not influence tendon CSA significantly at either the distal (control 84 ± 7 mm^2; ERT users 87 ± 4 mm^2, $P = 0.41$), the mid- (control 82 ± 7 mm^2; ERT users 82 ± 3 mm^2, $P = 0.98$), or the proximal level (control 73 ± 6 mm^2; ERT users 80 ± 4 mm^2, $P = 0.77$) of the patellar tendon. However, compared with young, healthy women (24 ± 3 yr), the proximal part of the patellar tendon CSA was greater in elderly ERT users, both in absolute values ($P < 0.01$) and after adjustment for weight ($P < 0.01$) (Fig. 5). Similarly, tendon CSA tended to be greater at the proximal level in elderly controls compared with young ($P < 0.10$). At the distal level of the tendon, no significant difference related to age was observed (Fig. 5).



	Control		ERT-users	
Density(#/mym2)	61 ±	4	68 ±	4
Volume fraction (%)	57 ±	2	62 ±	3
Mean area (nm^2)	8503 ±	562	7943 ±	614
% fibrils, dia 0-60 nm	27 ±	2	24 ±	2
% fibrils, dia 61-90 nm	30 ±	2	50 ±	8 *
% fibrils, dia > 90 nm	43 ±	2	26 ±	8 (*)

Fig. 4. Absolute collagen fibril diameter distribution in patellar tendon biopsies from postmenopausal women who use ERT ($n = 6$) and do not use ERT (control) ($n = 9$). The table shows data for fibril density (no. fibrils/μm^2), mean fibril diameter (dia) (nm^2), fibril volume (fibril area/total area), and the relative (%) distribution within each groups of fibrils with a fibril diameter within the intervals <60, 60–90 nm, and >90 nm. Values are means ± SE. $^{*}P < 0.05$ and (*)$P = 0.10$, unpaired *t*-test, control vs. ERT users.

Tendon Biomechanical Properties

The maximal isometric strength (peak moment) was not significant different between groups (control 118 ± 6 N·m, ERT users 108 ± 9 N·m, $P = 0.37$). However, the calculated maximal tendon force was higher in control compared with ERT users (4,145 ± 203 vs. 3,296 ± 204 N, $P < 0.05$) (Table 2). Young's modulus was significantly greater in controls compared with ERT users (Table 2). No other differences in tendon biomechanical properties were observed between groups (tendon stress $P = 0.16$, strain $P = 0.91$, stiffness $P = 0.55$, and peak moment $P = 0.24$) (Table 2).

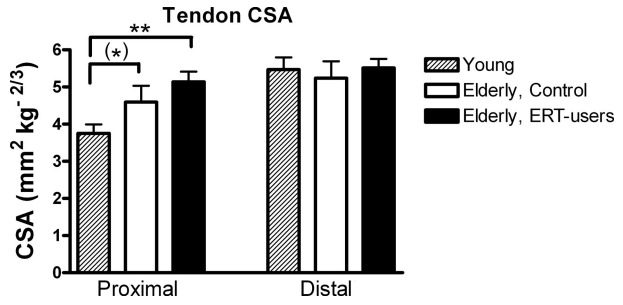


Fig. 5. Patellar tendon cross-sectional area (CSA) just distal to the patellar insertion (proximal) and just proximal to the tibia insertion (distal) in postmenopausal women who used ERT (ERT users) or did not use ERT (control), and in young eumenorrheic women. Values are means ± SE. $^{**}P < 0.01$ and (*) $P = 0.10$, unpaired *t*-test, control vs. ERT users.

Table 2. *Biomechanical tendon properties*

	Control	ERT Users
n	9	8
Stress, MPa	37±3	30±1
Strain, %	5.4±0.5	5.3±0.3
Stiffness, N/mm	3,094±248	2,821±339
Young's modulus, GPa/%	1.8±0.2	1.3±0.1*
Peak moment, N·m	118±6	108±8
Maximum force tendon, N	4,145±203	3,296±204*

Values are means ± SE; *n*, no. of subjects. The values are calculated based on the same common force (common force 2,443 N) to adjust for individual differences in muscle strength. Description of the test parameters is given in MATERIALS AND METHODS. *$P < 0.05$: control vs. ERT users.

BMD and Markers for Bone Turnover

BMD was higher in ERT users than in control when calculated as the average for whole body ($P < 0.01$), spine ($P < 0.01$), or legs ($P < 0.05$). Pelvis BMD was not significantly different between groups ($P = 0.13$) (Table 3). s-PINP, a marker for synthesis of CTX-I in bone, was lower in ERT users compared with control ($P < 0.01$). When s-estradiol in ERT users was compared with the corresponding s-PINP, a tendency toward a negative association was observed ($r^2 = 0.40$, $P = 0.07$). The marker for bone degradation, urine-CTX-I, was not significantly different between groups after adjustment for urine concentration ($P = 0.19$) (Table 3).

IGF-I and IGFBPs

IGF-I was significantly lower in ERT users than in control, both in serum ($P < 0.05$) and in the interstitial fluid surrounding the patellar tendon ($P < 0.01$) (Table 4). In contrast, no significant difference in IGFBPs in serum was observed (s-IGFBP-1 $P = 0.15$; IGFBP-3 $P = 0.14$). Furthermore, IGFBPs in the interstitial tendon fluid did not differ significantly between groups, although IGFBP-3 tended to be significant ($P = 0.06$) (Table 4).

DISCUSSION

The present study shows that ERT has a marked effect on tendon collagen metabolism. A higher tendon FSR was observed in ERT users compared with control. This might be related to the differences in the fibril structural distribution and the lower relative stiffness during maximal isometric voluntary contraction seen in ERT users compared with control.

Table 3. *BMD and markers for bone turnover in ERT users and controls*

	Controls	ERT Users	*P* Value
n	10	9	
Bone mass density			
Total BMD, g/cm^2	1.08±0.02	1.18±0.02	<0.01
Spine BMD, g/cm^2	0.96±0.03	1.16±0.05	<0.01
Pelvis BMD, g/cm^2	1.01±0.03	1.08±0.04	=0.13
Leg BMD, g/cm^2	1.13±0.06	1.22±0.03	<0.05
Bone turnover markers			
s-PINP, ng/ml	98±10	56±5	<0.05
Urine CTX-I, mg/mmol creatinine	216±55	136±14	=0.19

Values are mean ± SE; *n*, no. of subjects. Total BMD, mean whole body bone mass density; s-PINP, serum concentrations of NH_2-terminal propeptide of type I collagen (marker for type I collagen synthesis); urine-CTX-I, urine concentrations of c-terminal telopeptides of type I collagen (marker for type I collagen degradation). *P* values: control vs. ERT users.

Table 4. *IGF and IGFBP-1 to -4 in serum and tendon dialysate*

	n	Control	ERT Users
Serum			
IGF-I, μg/l	10/9	134±13	95±7†
IGFBP-1, μg/l	10/9	58±7	70±9
IGFBP-3, μg/l	10/9	3,562±98	3,419±106
Tendon dialysate			
IGF-I, μg/l	9/9	8.9±1.7	3.9±0.7*
IGFBP-1, AU/mm^2	8/8	117±31	131±26
IGFBP-2, AU/mm^2	8/8	258±62	187±24
IGFBP-3, AU/mm^2	8/8	2,338±667	1,092±330‡
IGFBP-4, AU/mm^2	8/8	145±56	153±53

Values are means ± SE; *n*, no. of subjects for control/ERT users. IGF-I, insulin-like growth factor I; IGFBP, IGF-binding protein; AU, arbitrary units (pixel intensity). *$P < 0.01$, †$P < 0.05$, and ‡$P = 0.06$: control vs. ERT users.

Tendon Synthesis

To our knowledge, the effect of ERT on tendon FSR has not been measured before, but the present results are in concert with observations showing a stimulating effect of estradiol on the expression of CTX-I in porcine ACL (32) and in pelvic connective tissue in human and rhesus macaques (10, 15). The stimulating effect of estradiol on tendon collagen synthesis in the resting state was further supported by a positive correlation between s-estradiol and tendon collagen synthesis.

In contrast to tendon FSR, no significant difference between groups was observed for tendon PINP. PINP is a marker for the synthesis of newly synthesized soluble collagen molecules into the pool of free collagen, which is not necessarily built into the final tendon collagen structure, whereas tendon FSR corresponds to the synthesis rate of both soluble, but probably primarily insoluble, mature collagen. Therefore, the observed differences between the isotope data and microdialysis data in the present study may partly be explained by ERT especially having a marked stimulating effect on the incorporation of immature collagen molecules into the fibrils. The stimulating effect of estradiol on tendon collagen synthesis (PINP) may be counteracted by the indirect effect of ERT administration, which may explain no difference between groups in tendon PINP. Although speculative, such an explanation could be supported by the fact that IGF-I was reduced in ERT users, whereas IGFBPs were unchanged, which indicates a lower IGF-I bioavailability, as observed by others (6, 8, 27). It is well documented, at least in young animals, that IGF-I exerts anabolic effects on tendon fibroblasts by increasing collagen synthesis in a dose-dependent manner (2, 43, 45, 56). In support of this, a lower tendon and muscle collagen FSR, and at the same time a markedly lower bioavailability of IGF-I within these tissues, have been observed in young oral contraceptive users compared with control (21). Moreover, when analyzing the synthesis data in ERT users separately, a positive correlation between tendon FSR and tendon PINP was observed ($r^2 = 0.43$; $P = 0.05$). This indicates that the differential effect of ERT on tendon synthesis when comparing the groups by the two present methods is probably related to indicted effects of ERT and is not only a methodological issue.

Exercise did not result in any significant response of tendon FSR in either group. This is supported by earlier findings, which showed no difference in tendon FSR 24 or 72 h after an acute bout of strenuous exercise in young women (21a, 41). A type II error cannot be excluded, but the results do not indicate a marked stimulating effect of exercise on tendon synthesis (FSR) in postmenopausal women. In contrast, tendon FSR has been observed to be enhanced above resting values 6 h postexercise, followed by a peak 24-h postexercise in men (42). Taken together with the present results, this may indicate a sex-specific difference in the response to exercise in tendon FSR.

In accordance with tendon FSR results, tendon PINP did not change in response to exercise. However, when focusing on the data from the ERT users only, a negative correlation was observed between s-estradiol and the response to exercise in tendon PINP, suggesting a negative interaction between estradiol and mechanical loading on CTX-I synthesis, as has been previously shown in vitro in porcine ACL preparations (32). In support of this, a negative correlation between s-estradiol and the response to strenuous exercise has been observed in young eumenorrheic women ($r^2 = 0.72$, $P < 0.05$) (41). Furthermore, an increase in tendon PINP in response to exercise has been observed in young, eumenorrheic women exposed to a low concentration of estradiol, whereas high exposure to synthetic estradiol by administration of oral contraceptives seemed to counteract the stimulating effect of exercise on tendon synthesis (21). Therefore, the response to exercise in tendon synthesis (PINP) seems to be negatively influenced by the presence of a high s-estradiol concentration (endogenous secreted and/or exogenous administrated estradiol). In this study, tendon FSR did not appear to be affected by exercise in postmenopausal women (Fig. 3); therefore, it cannot be concluded whether a small change in PINP postexercise would have had any implications for the force-transmitting collagen fibril units and tissue biomechanical properties when adapting to an increased mechanical loading during repetitive physical trainings bouts.

Tendon CSA and Fibril Characteristics

Tendon CSA did not differ between control and ERT users, even though tendon FSR was significantly higher in ERT users. Differences in tendon FSR without any significant change in tendon CSA have been observed before, when young users and nonusers of oral contraceptives were compared (21). A type II error may cause this, since the group sizes in both studies were rather small. An alternative explanation for the similar tendon CSA between groups could be that ERT also increases tendon collagen breakdown. This possibility is supported by findings in other types of collagen-rich tissues, where estrogen administration was linked to indications of a higher tendon collagen turnover and a more immature tendon structure (15, 26). Nevertheless, fibril characteristics showed no significant differences in mean fibril area or density between the two groups in the present study. However, interestingly, a more detailed analysis of the fibril size distribution showed a significantly larger relative contribution of medium-sized fibrils in ERT users (%total numbers of fibrils), whereas control tended to have a larger relative contribution of larger fibrils (%total numbers of fibrils). A larger fibril diameter in subjects characterized by low estradiol exposure is in accordance with animal findings (20).

Training and loading influence tendon size and biomechanical properties (29, 37). $\dot{V}O_{2max}$ and maximal tendon force were higher in control compared with ERT users, but no association was observed between either the mean fibril area, fibril volume fraction, or fibril density, when these parameters were pooled for all subjects and correlated to either $\dot{V}O_{2max}$, muscle strength, or PAL. Similarly, body composition (weight, lean body mass, body mass index) did not appear to be related to fibril characteristics. This observation supports the idea that the morphological difference between groups may be induced by ERT administration.

Tendon proximal CSA was greater in postmenopausal women compared with our laboratory's earlier findings in young women (21). This observation of an age-related increase in tendon CSA is in line with cross-sectional data showing a greater Achilles tendon CSA in postmenopausal women compared with young women (35). Furthermore, in the present study, ~25% of the fibrils in the specimen from the patellar tendon had a small diameter (between 0 and 60 nm). This is in contrast to young subjects (27 yr, range 19–40 yr), in whom ~73% of the fibrils had a diameter <60 nm (54). Taken together, these findings suggest an altered balance between collagen synthesis and breakdown with aging in women.

Biomechanical Properties

A lower relative tendon stiffness (Young's modulus) was observed in ERT users compared with controls. Based on the present knowledge about the relation between tendon structure and biomechanical properties (7, 37), this novel finding corresponds very well with *1*) the observed enhanced tendon synthesis in ERT users, indicating a higher collagen turnover; *2*) the fibril characteristics showing a nonsignificant higher fibril density and a lower mean fibril size; and *3*) the relative higher numbers of smaller fibrils in ERT users compared with controls. In addition, our observations provide a further clarification of earlier findings, revealing lower maximal load at tissue rupture in ACL from ovariectomized rabbits, which had been exposed to high estrogen levels for 1 mo compared with controls (52).

Bone Turnover and BMD

Bone collagen synthesis appears to increase during aging, indicated by markers for bone formation (18). Nevertheless, new findings suggest that the ability to process newly synthesized collagen to fully cross-linked mature bone collagen structure might be reduced in postmenopausal women (53). Taken together with an enhanced bone resorption in postmenopausal women, this may explain the well-known, age-dependent reduction in BMD (as an indicator for a reduction in bone mass). In the present study, use of ERT was associated with a lower concentration of a marker for bone collagen (PINP) synthesis compared with controls. This has been shown by others (58). The higher BMD in ERT users in the present study might be explained by an increase in the synthesis of mature bone collagen tissue, even though ERT seems to reduce the synthesis of new collagen molecules (indicated by lower s-PINP). An alternative explanation is that ERT reduces osteoclast activity and thereby bone collagen breakdown rate (12). However, the marker for bone collagen breakdown was not significantly reduced in ERT users in the present study compared with control.

The influence of ERT on collagen metabolism may differ between collagen-rich tissues. Results indicate that tendon collagen content is higher in elderly women compared with young women (21). This is in contrast to the well-documented, age-dependent decrease in bone mass. Furthermore, in the present study, a positive association between s-estradiol and tendon synthesis (peritendionous PINP) was observed, whereas a tendency toward a negative association between s-estradiol and the marker for bone collagen synthesis (s-PINP) was observed. The mechanism behind this discrepancy between tissues might be related to differences in type and distribution of estrogen receptors and/or other growth factors important for the signaling cascades in the two types of tissues.

Methodological Considerations

PINP was used as a marker of collagen synthesis in bone and in tendons. PINP, as the NH_2-terminal extension peptide, is cleaved from procollagen by specific proteinases in a one-to-one manner, which thereby enables the remaining collagen to aggregate and form fibrils (28). As CTX-I mainly resides in bone, s-PINP mainly reflects the synthesis of collagen in bone. However, by placing microdialysis catheters in the peritendinous space of the patella tendon, it is possible to measure local PINP, assumed to primarily represent synthesis of collagen within the patellar tendon tissue. This is supported by findings showing marked changes in PINP in the peritendinous space of the Achilles tendon and only minor changes in blood in response to mechanical loading (31). Another source, which could contribute to PINP in the dialysate, is the skin (dermis). CTX-I is the predominant type of collagen in skin (13). However, validation of the microdialysis technique demonstrated that the observed change in CTX-I propeptides does not originate from skin (46).

A drawback of microdialysis is that the exchange rate has to be determined over the semipermeable membrane and needs subsequent adjustment for the recovery value to calculate true interstitial values. Radioactive labeled glucose (0.180 kDa) was used as a marker for RR of PINP (35 kDa) and IGF-I (7.6 kDa), as no radioactive-labeled PINP was commercially available. The RR was high (42%, range 20–81%) and not significantly different between groups. When calculating interstitial concentrations, it was assumed that the relative loss of tracer over the membrane was similar to RR of the metabolite of interest. We cannot exclude an underestimation of the exact interstitial concentration of the metabolites, but the error in the calculation can be expected to be the same between subject groups and between the legs (rest and exercise leg).

Conclusion

ERT and thus elevation of estradiol levels seem to influence tendon morphology and biomechanical properties in postmenopausal women compared with women exposed to low-estradiol concentration. In contrast to the inhibiting effect of ERT on bone collagen synthesis, the present results showed a markedly higher tendon FSR in ERT users, which was related to the enhanced estradiol concentration in a dose-dependent manner. This observation did not have any significant influence on tendon size. However, it was linked to the presence of smaller fibrils and a higher fibril density, which supports the fact that ERT increases tendon collagen turnover. Moreover, the relative stiffness during mechanical loading was lower in ERT users, and this could be related to a relatively higher proportion of immature cross links and thereby a potential reduced tendon strength in women with higher levels of estradiol.

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