Tendon collagen synthesis at rest and after exercise in women

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There is evidence that women sustain more exercise-related musculoskeletal injuries than men (7, 13). Sex differences in injury rates are apparent in some connective tissues such as ligaments. In some activities the incidence of anterior cruciate ligament (ACL) injuries in women is up to six times that in men (8). These injuries make up a large proportion of the $850 million spent annually in the United States on surgical repair (9). Although current evidence does not support a sex-related difference in tendon injury rates (18), the patellar tendon does represent a relatively easily accessible collagen-rich tissue for the study of connective tissue. In connective tissue that is load bearing (e.g., tendon or ligament), underlying reasons for sex-specific differences in propensity for injury, rates of healing, and connective tissue specificity have not been identified.

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541


Collagen protein is ubiquitous in connective tissue, and there is reason to believe that female sex hormones may alter collagen protein synthetic response. Ligament and tendon (11, 19), bone (17), and endometrium (25) all contain estrogen receptors responsive to female sex hormones. In rat tail tendon, collagen concentration decreases after treatment with estrogen (6). Furthermore, in fibroblasts cultured from rabbit ACLs, Liu et al. (19) demonstrated a dose-dependent inhibition of collagen synthesis with increasing estrogen concentration. Conversely, type I collagen mRNA is reportedly increased by estrogen in porcine ligament fibroblasts (17). However, on balance it seems that estrogen may have an inhibiting effect on collagen synthesis on noncutaneous mammalian extracellular matrix, and we hypothesized that this is true in human tendon as well.

Recently, our laboratory developed and applied methods for the direct measurements of collagen synthesis in human tissues and used them to show that in men an acute bout of exercise increased collagen synthesis markedly in quadriceps muscle and patellar tendon between 6 and 72 h after exercise (22). In women, rates of skeletal muscle myofibrillar protein and intramuscular connective tissue synthesis and responses to exercise were similar to those observed in men (22), without influence of menstrual phase (21). However, presently it is unknown to what extent blood estrogen and progesterone availability influences collagen synthesis in human tendon at rest and after exercise. We hypothesized that women, like men, would increase tendon collagen synthesis after a bout of strenuous exercise but that during the later luteal phase (LP), when estrogen and progesterone availability are increased, collagen synthesis in the tendon would be less than in women during the early follicular phase (FP). To test this hypothesis, we determined tendon collagen synthesis rates in women during the FP and the LP of the ovarian cycle at rest and after an acute bout of exercise known to result in an increased tendon collagen synthesis in men.

METHODS

Subjects

Sixteen young healthy women with normal menstrual cycles were recruited to the study (Table 1). These subjects were described in an earlier paper (21). All were nonsmoking, nulliparous, not currently taking oral contraceptives or other medications, and free of anatomic and metabolic disorders as judged by history and routine medical examination. The subjects gave informed consent to a protocol ad-

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ergometer. The workload for each subject’s subsequent 1-h exercise.

Menstrual Cycle Determination

Committee of Copenhagen and Frederiksberg Communities. Ran-

Table 1. Subject characteristics for subjects in the follicular
and luteal phases

<table>
<thead>
<tr>
<th></th>
<th>Follicular Phase (n = 8)</th>
<th>Luteal Phase (n = 7)</th>
<th>Men</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>26 ± 2</td>
<td>26 ± 4</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171 ± 5</td>
<td>173 ± 8</td>
<td>186 ± 9†</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>63 ± 8</td>
<td>60 ± 7</td>
<td>76 ± 8†</td>
</tr>
<tr>
<td>Body Fat, %</td>
<td>28 ± 7</td>
<td>28 ± 8</td>
<td>14 ± 7†</td>
</tr>
<tr>
<td>Fat-free mass, kg</td>
<td>45 ± 4</td>
<td>43 ± 4</td>
<td>65 ± 4‡</td>
</tr>
<tr>
<td>Workload, W</td>
<td>47 ± 9</td>
<td>45 ± 5</td>
<td>60 ± 9†</td>
</tr>
<tr>
<td>Workload, % maximum</td>
<td>65 ± 6</td>
<td>68 ± 5</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>Estrogen, nmol/l</td>
<td>0.21 ± 0.10</td>
<td>0.42 ± 0.20*</td>
<td></td>
</tr>
<tr>
<td>Progesterone, nmol/l</td>
<td>1.3 ± 0.3</td>
<td>47.3 ± 17.6*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of subjects. Estrogen and progesterone values represent the mean concentration during the 3 blood samples collected on the exercise day, 1 and 3 days after exercise. *Significantly different from follicular phase, P < 0.05, †Significantly different between men and all women combined, P < 0.05.

Measurement of Tendon Collagen Synthetic Rates

Measurement of rates of tendon collagen synthesis was performed according to our laboratory’s previously validated techniques (1–3, 21, 22). Briefly, on arrival, a 10-mg skin biopsy was obtained under local anesthetic from the posterior hip to be used for background enrichment of collagen. Subsequently, two cannulas were inserted into veins on opposite forearms for bolus tracer infusion and blood sampling from which a blood sample was obtained for measurement of background isotope enrichment. Next, a flooding dose of [15N]proline (flooding dose = 1,200 mg labeled to 2,000 mg unlabeled) was administered over 3 min. The tracer obtained from Cambridge Isotope Labs (Woburn, MA) was chemically pure (>99 atoms % in 15N) and certified to be sterile and without pyrogens. The tracer was dissolved on the morning of the infusion in 0.9% NaCl using a sterile technique and then passed through a 0.20-μm filter (Bie and Bernstein, Rødovre, Denmark). Blood samples were drawn at 10, 20, 30, 45, 60, 75, 90, and 120 min after the bolus injection for determination of labeling (tracer-to-tracee ratio) of the imino acid in plasma. At 90 min with ultrasound guidance, a small incision was made under local anesthetic (lidocaine 1%) in the region of the patellar tendon on both legs. Immediately following the blood draw at 120 min, a 10-mg sample of the patellar tendon was obtained from the resting and control legs. Tendon samples were taken with a 16-gauge Monopity biopsy instrument (Bard, Covington, GA) under ultrasound guidance. Confirmation of the sampling of tendon tissue by this technique has been previously published (22).

Free imino acid labeling. Plasma proline was prepared as previously described and analyzed as its r-butyldimethylsilylation derivative by gas-chromatography mass-spectrometry (1, 22, 27).

Extraction of collagen from tendon and skin. Details regarding this procedure have been given elsewhere (2, 22). Briefly, skin (10 mg) and tendon (10 mg) were ground in liquid nitrogen to a fine powder and hand-homogenized in a buffer containing 0.15 M NaCl, 0.1% Triton X-100, and 0.02 M Tris–HCl, pH 7.4, and then centrifuged at 1,600 g at 4°C for 20 min to pellet the collagen, which was then subjected to differential salt extraction. During the extraction of collagen from the tendon samples, some samples were lost because of insufficient concentration of sample.

Gas chromatography-combustion-isotope ratio mass spectrometry. Isolated collagen was hydrolyzed in a slurry of 0.05 M HCl-Dowex 50W×8-200 (Sigma, Poole, UK) (500 μl) at 110°C overnight, and the liberated free imino acids were eluted using 1 M HCl. The imino acid was derivatized as its N-acetyl-n-propyl (NAP) ester (20). The NAP imino acids were analyzed by capillary Gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; Delta-plus XL, Thermo Finnigan, Hemel Hempstead, UK); separation was achieved on a 25-m CP-SIL 19CB column (Chrompack). A proline standard was prepared as its NAP derivatives and analyzed by GC-C-IRMS.

Calculations The rate of tendon collagen synthesis was calculated using a standard equation (24). Thus protein synthesis [fractional synthesis rate (FSR); %/h] is the time (h) of tracer incorporation over time of the precursor for protein synthesis (taken as venous plasma proline labeling), and t is the time (h) of tracer incorporation (2 h). It has previously been demonstrated that, in a number of tissues, the values of plasma and tissue-free proline labeling are indistinguishable (1, 3). Therefore, venous plasma proline was taken to represent rate of each 30-min period, with a double dose prime at initiation of feeding. Basal metabolic rate was estimated from the subjects fat-free mass (FFM) determined by the skinfold technique (4, 5). Therefore, subjects were fed according to FFM.
the labeling of prolyl-tRNA in tenocytes for the calculation of tendon collagen synthetic rates.

Microdialysis

Local tissue fluid concentration of NH₂-terminal propeptide of human procollagen type I (PINP) was sampled by tissue microdialysis. Sterilized (ethylene oxide sterilization), high-molecular mass cutoff (3,000 kDa, membrane length 30 mm, ID 0.50 mm) fibers were inserted under ultrasound guidance as previously described (14) in the peritendinous spaces of the rested and exercised patella tendons 72 h after the exercise bout. The microdialysis catheters were perfused via a high-precision syringe pump (CMA 100, Carnegie Medicine, Solna, Sweden) at a rate of 2 μl/min with a Ringer-acetate solution and [3H]human type IV collagen. Dialysis fluid was collected in the third and fourth hour after insertion. The microdialysis relative recovery was estimated by the loss of [3H]-labeled human type IV collagen as calculated by the internal reference method (26) and exceeded 74%. Dialysate fluid was analyzed for PINP by the use of a sandwich ELISA utilizing purified α1-chain-specific rabbit antibodies (Teisner B, Department of Medical Microbiology, University of Odense, Denmark) (23).

Comparison of Results With Those of Men

Our laboratory previously published data collected in eight male subjects under the same conditions (22). Both the men and women underwent the same 4-day experimental trial, had the same investigative procedures, and were fed over the same time course and according to FFM. Furthermore, the studies were separated in the calendar year by 6 mo. Thus the data from the men are reported here for comparison.

Statistics

Results are presented as means ± SD. The effects of sexual dimorphism and menstrual cycle phases on rest and exercise values were compared by a t-test. The level of significance was set at P < 0.05 with subsequent adjustment for repeated comparisons by the Bonferroni method.

RESULTS

Workload

The absolute (FP = 47.1 ± 9.4 W; LP = 44.6 ± 5.3 W) and relative workloads (FP = 64.6 ± 6.3; LP = 68.4 ± 5% Wmax) were not significantly different between groups (Table 1).

Ovarian Hormones

Plasma estrogen and progesterone concentrations were significantly higher (P < 0.05) in LP than in FP during the experimental period based on three blood samples collected on the exercise day and 1 and 3 days after exercise (average estrogen: FP = 0.21 ± 0.10 nmol/l, LP = 0.42 ± 0.20 nmol/l; progesterone: FP = 1.3 ± 0.3 nmol/l, LP = 47.3 ± 17.6 nmol/l) (Table 1).

Directly Measured Tendon FSR

There were no significant differences between menstrual cycle phases for tendon collagen FSR at rest [FP = 0.025 ± 0.003%/h (n = 5); LP = 0.026 ± 0.001%/h (n = 3)] or 72 h after exercise [FP = 0.027 ± 0.006%/h (n = 6); LP = 0.028 ± 0.004%/h (n = 7)] (data not shown). When all data from the women irrespective of menstrual phase were combined and compared with those from men, there was a significant difference (P < 0.05) between women and men at rest (women = 0.025 ± 0.002%/h; men = 0.045 ± 0.007%/h) and 72 h after exercise (women = 0.027 ± 0.005%/h; men = 0.058 ± 0.008%/h) (Fig. 1). Furthermore, combined data for all women failed to demonstrate an increase in collagen FSR at 72 h after exercise compared with resting values (Fig. 1).

Indirect Measure of Tendon Collagen FSR

The concentration of PINP in the dialysate showed a significant exercise-induced increase in tendon collagen synthesis (P < 0.05, all women combined). However, no significant difference between phases was observed (Fig. 2). At rest, PINP
was higher in men compared with women \( (P < 0.05) \), but it was not different 72 h after exercise.

**DISCUSSION**

The results from the present study combined with our laboratory’s previous data (22) show that at rest and 72 h after exercise, tendon collagen FSR values are lower in women than in men. These results demonstrate a sex-based difference, which is compatible with the notion that estrogen and/or progesterone may alter the exercise-induced increase in collagen synthesis after an acute bout of exercise.

Although short-duration variations in plasma estrogen (and progesterone) may have limited effect on structural metabolism, it is possible that long-term exposure to such cyclic variability in ovarian hormones could set upper limits on the adaptive increases expected to be seen in collagen FSR with exercise. Indeed, at rest, tendon collagen FSR values in women were \( \sim 55\% \) of the rate in men. Furthermore, 72 h after exercise, the collagen FSR in women was only 47\% that of men. As far as we are aware, this is the first study to compare rates of tendon synthesis between women and men indirectly or directly. Lower collagen FSR in tendons of women than in those of men may be consistent with recent reports of smaller tendons in women than men (10, 12).

The question remains as to why collagen tissue in skeletal muscle is apparently not sensitive to sex hormones (21), whereas tendon is (present study). When considering skeletal muscle energy metabolism, it seems that the effects of feeding or exercise are the primary determinants of fuel preference with sex hormones secondary (28). An analogous hierarchy could be proposed for structural protein metabolism. In this case, mechanical loading and feeding (at least in noncollagen muscle) are likely to be primary determinants of protein synthesis, whereas female sex hormones might be secondary. The similarity between muscle collagen FSR values 24 h after exercise in men and women (21) may be due to a similar response to mechanical loading, whereas the differences in tendon collagen FSR values 72 h after exercise (present study) occur at a time when the effects of mechanical loading are diminished and those of sex hormones could be more pronounced. A study by Lee et al. (16) offers some support for this idea. When cultured porcine ACL fibroblasts were grown in an estrogen-free environment, mechanical stimulation increased type I collagen mRNA. However, the increase in type I collagen mRNA in response to loading was decreased at increasing estrogen concentrations (16). Therefore, it is possible that at sufficient concentrations (exceeding those in the present study), estrogen could directly modulate responses of the fibroblast to mechanical loading. It is also likely that the differences in type and density of estrogen receptors of the different tissues (6, 17, 29) could modulate the degree to which each of the tissues respond to loading.

Finally, one must consider the possibility that the mechanical forces transmitted from muscle to tendon may not be the same in women and men. Hashemi et al. (12) examined mechanical properties of patellar tendons collected postmortem from cadavers of women and men and concluded that there is no evidence of differences in the mechanical properties of tendons from women and men because the mean length of male tendons were longer than female tendons but the mass densities

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Fig. 2. NH2-terminal propeptide of human procollagen type I concentrations ([PINP]) measured at rest and after exercise. Top: individual data for the follicular phase \( (P = 0.08) \). Middle: individual data for the luteal phase (LP; \( P = 0.11 \)). Bottom: mean (SD) PINP concentration at rest and after exercise when all paired data are included \( (n = 14) \). *Significantly higher PINP concentration after exercise compared with resting values, \( P < 0.05 \). #Significantly higher than women, \( P < 0.05 \).
(tissue mass per cm$^3$) were not different. Nevertheless, the tendons from women had a much more variable ultimate stress for a given mass density than men (12). Although mass density could be ultimately responsible for determining mechanical properties, it should be recognized that differences in mass density and size of the tendon structures could be seen as a property of sex or changes in net deposition of tissue. Therefore, a decrease in tendon size and collagen FSR may be an important consideration when harvesting tendon from women for ACL reconstruction and the prospects of regaining proper tendon function.

Finally, one limitation in the present study is that tendon collagen FSR values were only measured directly by the isotope technique before and 72 h after exercise. We chose 72 h because the only exercise data available at the time indicated increased collagen synthesis in the tendon at this time point in men when measured indirectly (15). However, subsequent studies in men suggest that the exercise-induced changes in PINP in the local tissue fluid collected from the peritendinous space in front of the tendon are delayed compared with the actual change in fractional tendon collagen synthesis in vivo (15, 22). We now know that tendon collagen FSR values in men peak at 24 h after exercise (22) and therefore cannot conclude that women do not respond to exercise at an earlier time point. The fact that microdialysis–measured increases in PINP concentrations were measured 72 h after exercise lends evidence to the fact that female tendon collagen synthesis did increase at an earlier time point. It is therefore important to note that tendon collagen synthesis in women likely increases after a bout of exercise, but the response is both diminished in magnitude and in time compared with men.

In conclusion, tendon collagen FSR values seem to be diminished at both rest and following exercise in women compared with men. These results are compatible with the notion that estrogen may modulate the synthetic responses of the fibroblast to mechanical loading and may contribute to a lower rate of tissue repair after exercise.

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