Uphill running improves rat Achilles tendon tissue mechanical properties and alters gene expression without inducing pathological changes

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Uphill running improves rat Achilles tendon tissue mechanical properties and alters gene expression without inducing pathological changes. J Appl Physiol 113: 827–836, 2012. First published July 12, 2012; doi:10.1152/japplphysiol.00401.2012.—Overuse Achilles tendinopathy is a common and challenging problem in sports medicine. Little is known about the etiology of this disorder, and the development of a good animal model for overuse tendinopathy is essential for advancing insight into the disease mechanisms. Our aim was to test a previously proposed rat model for Achilles tendon overuse. Ten adult male Sprague-Dawley rats ran on a treadmill with 10° incline, 1 h/day, 5 days/wk (17–20 m/min) for 12 wk and were compared with 12 control rats. Histological, mechanical, and gene-expression changes were measured on the Achilles tendons after the intervention, and local tendon glucose-uptake was measured before and after the intervention with positron emission tomography. No differences were detected between runners and controls in tissue histology or in glucose uptake, indicating that tendon pathology was not induced. Greater tendon tissue modulus (P < 0.005) and failure stress/body weight (P < 0.02) in runners compared with controls further supported that tendons successfully adapted to uphill running. Several genes of interest were regulated after 12 wk of running. Expression of collagen III and insulin-like growth factor I was increased, while collagen I was unchanged, and decreases were seen in noncollagen matrix components (fibromodulin and biglycan), matrix degrading enzymes, transforming growth factor-β1, and connective tissue growth factor. In conclusion, the tested model could not be validated as a model for Achilles tendinopathy, as the rats were able to adapt to 12 wk of uphill running without any signs of tendinopathy. Improved mechanical properties were observed, as well as changes in gene-expression that were distinctly different from what is seen in tendinopathy and in response to short-term tendon loading.

loading; exercise; tendinopathy; collagen
Surprisingly, our data show no detrimental effects of the uphill running regime on the tendon tissue. In fact, the Achilles tendon mechanical properties were enhanced as a result of the training, and changes in gene expression were clearly different from what is seen, both in tendinopathy and in response to short-term tendon loading.

METHODS

Animals

All rat experiments were approved by the Danish Animal Experiments Inspectorate (J.nr.2006/561–1124). The rats were housed in pairs and fed chow and water ad libitum. The animal room was maintained at 21°C with a 12:12-h light-dark cycle. From a group of 30 male Sprague-Dawley rats, 22 rats were selected by their ability and willingness to run on the treadmill. These 22 rats weighed 363 ± 12 g (means ± SE) and were randomly divided into groups of controls (n = 12) and 12 wk running (n = 10). For practical reasons, only 10 rats were included in the running group, since only 10 lanes were available on the treadmill. In the control group, we chose to include 12 rats in the case of dropouts in this group.

Running Protocol

The employed running protocol has been described previously by Glazebrook et al. (18), although it was slightly modified in the present study. The rats ran for 1 h on a treadmill with 10° incline for 5 days/wk in 12 wk. To promote running, a grid at the tail end of the treadmill provided a mild electric shock. Before the start of the intervention, 1 wk was focused on acclimatization to the running protocol, as shown in Table 1. The only modification relative to the protocol published previously (18) consisted of an increased running speed during the time course of the intervention, progressing up to 20 m/min instead of maintaining the speed at 17 m/min (Table 1).

Two rats were excluded from the running group because of unwillingness to run after 4 wk of training. Accordingly, all data are based on 8 runners and 12 controls.

PET/CT Image Acquisition and Processing

To evaluate whether the running protocol influenced the energy metabolism in Achilles tendon tissue at rest, PET, combined with CT (computerized tomography), was employed to detect glucose uptake in the tendinous tissue. The uptake of the glucose-analog 18F-fluorodeoxyglucose in Achilles tendons was obtained using PET/CT 3 days before the running regime was initiated and again 3 days after the last day of the training protocol. The control group was equally scanned twice with 12-wk intervals. PET/CT scans were carried out according to a modified protocol previously described (43). Before the PET/CT scans, the rats were anesthetized with a subcutaneous injection of Hypnorm/Dormicum (5 mg/0.625 ml/kg). 18F-fluorodeoxyglucose was injected as a bolus 21.3 ± 1.7 MBq in 0.5 ml saline through a tail vein catheter (BD Neoflon 24 GA) and allowed to accumulate for 60 min. The rats were placed in a prone position on the acquisition bed, and a 20-min static emission PET scan was performed with the two hindlimbs in the field of view, followed by a CT scan. During PET and CT scans, the rats’ temperature was maintained with a heating pad (M2M, Cleveland, OH) attached to the acquisition bed. PET data were acquired with a micro-PET Focus 220 (Siemens Medical Solutions, Malvern, PA). All list-mode data were sorted into three-dimensional (3D) sinograms using a span of 3 and a ring difference of 47 and reconstructed using Ordered Subset Expectation Maximization 2D. Images were attenuation corrected using the CT-based attenuation correction method and, in addition, corrected for dead time and decay time, and the system was calibrated to provide the quantification unity becquerel per milliliter. CT data were acquired with a MicroCAT II Tomograph (Siemens Medical Solutions). The x-ray tube was set at 40 kVp and had 0.5 mm plate of added aluminum filtration. Exposure time was 700 ms per projection, and the tube current was 500 μA. A total of 360 projections were used for a full 360° scan. Images were reconstructed using the Shepp-Logan algorithm. The uptake of 18F-fluorodeoxyglucose in the Achilles tendon was quantified (Bq/ml) using the image analysis software Inveon (Siemens Medical solutions). Regions of interest (ROIs) were manually outlined as 3D volumes on the CT images and included 1) the proximal one-third of the Achilles tendon, starting at the musculo-tendinous junction; 2) the one-third midportion of the Achilles tendon; and 3) the distal one-third limited by the insertion to the calcaneus. ROIs were drawn on both hindlimbs, and the values given as an average of two sides. During subsequent analysis, standardized uptake values in the individual ROIs were calculated by dividing the mean specific activity with injected dose and multiplying by the weight of the individual rat, assuming 1 cm³ equals 1 g.

CT images were also used to determine the tendon diameter at the proximal, mid, and distal level as an average of three measurements for each level.

Dissection

After the 12-wk PET/CT measurements (performed 3 days after the last running bout), the anesthetized rats were killed by decapitation, and immediately both Achilles tendons were isolated, and care was taken not to include any muscle tissue. The right Achilles tendon was divided into two equal parts in the longitudinal plane. One part was frozen in liquid nitrogen and stored for RNA analysis at −80°C. The other half was wrapped with PBS-soaked gauze and stored at −20°C until mechanical testing. The left Achilles tendon was embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and stored at −20°C for subsequent histological analyses.

Mechanical Testing

Instrumentation. Tendon mechanics were measured as described previously (19). In summary, a micro-tensile testing stage was used for the mechanical testing of the Achilles tendon bundle dissected from the rats. The stage (200 N tensile stage, Petri dish version, Deben UK, Suffolk, UK) consists of a load cell (1% accuracy), a specimen liquid chamber, and two specimen mounting plates driven by a computer-controlled motor fitted with a linear variable differential transducer that registers changes in mounting plate displacement. The stage with the sample mounted is placed directly under a stereomicroscope (SMZ1000, Nikon, Tokyo, Japan) with a C-mount lens (×0.38). The microscope is equipped with a 15-Hz digital camera (DFW7X700, Sony, Tokyo, Japan) with a 1.024 × 768 output signal format that allowed acquisition of detailed image data of the sample.
Mechanical testing procedures. The Achilles tendon bundles, with a length of ~10 mm, were processed for mechanical testing, as described previously (21). Briefly, each ~2.5 mm of end of the tendon bundle were allowed to air dry at room temperature, while the central region of the tendon bundle was wrapped in PBS-soaked gauze. Next, the dried tendon ends were glued to the uncoated aluminum specimen mounting plates of the mechanical rig with cyanoacrylate. The tendon bundle and mounting plates were then immersed in PBS solution in a Petri dish fitted in the mechanical rig. The tendon bundle was hydrated for 20 min before the failure tests were conducted at a strain rate of 2.0 mm/min. The initial specimen testing length (mounting plate-to-mounting plate distance) was ~5 mm. For all samples, the exact original length \( L_0 \) was determined once the tendon bundle was mounted in the testing apparatus. \( L_0 \) was measured at force onset by slowly straining the tendon bundle until a slight rise in force (\( \sim 0.03 \) N) was detected. A high-resolution stereomicroscopic image of the mounted tendon bundle was obtained in this position, and the exact diameter was measured from the acquired image. The measured \( L_0 \) was subsequently used for strain calculations, and the diameter was used for calculations of stress.

RNA Extraction

Achilles tendon tissue was homogenized in 1 ml of TriReagent (Molecular Research Center, Cincinnati, OH) containing five stainless steel balls of 2.3 mm in diameter (BioSpec Products, Bartlesville, OK), and one silicon-carbide sharp particle of 1 mm (BioSpec Products), by shaking in a FastPrep-24 instrument (MP Biomedicals, OH) and one silicon-carbide sharp particle of 1 mm (BioSpec Products) containing five stainless steel balls of 2.3 mm in diameter (BioSpec Products, Bartlesville, OK), and one silicon-carbide sharp particle of 1 mm (BioSpec Products), by shaking in a FastPrep-24 instrument (MP Biomedicals, OH) for 20 min before the failure tests were conducted at a strain rate of 2.0 mm/min. The initial specimen testing length (mounting plate-to-mounting plate distance) was ~5 mm. For all samples, the exact original length \( L_0 \) was determined once the tendon bundle was mounted in the testing apparatus. \( L_0 \) was measured at force onset by slowly straining the tendon bundle until a slight rise in force (\( \sim 0.03 \) N) was detected. A high-resolution stereomicroscopic image of the mounted tendon bundle was obtained in this position, and the exact diameter was measured from the acquired image. The measured \( L_0 \) was subsequently used for strain calculations, and the diameter was used for calculations of stress.

Table 2. Primers for PCR

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Primer sequences used for real-time RT-PCR. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RPLP0, large ribosomal protein P0; COL1A1, collagen type I alpha 1; COL3A1, collagen type III alpha 1; COL5A1, collagen type V alpha 1; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; TGF-B1, transforming growth factor-beta; CTGF, connective tissue growth factor; VEGFA, vascular endothelial growth factor A; IL-6, interleukin-6; IGF-I, insulin like growth factor-I.
riboosomal protein P0 (RPLP0) was chosen as internal control, as RPLP0 mRNA has been suggested to be constitutively expressed (15). To validate this assumption, another unrelated “constitutive” RNA, GAPDH mRNA, was measured, and RPLP0 was normalized to GAPDH and shown to be completely stable.

**Histology**

Serial sections of 10-μm thickness were cut longitudinally on a cryostat from the anterior, mid, and posterior regions of the Achilles tendons embedded in Tissue-Tek (Fig. 1). Sections from all three regions were stained with hematoxylin and eosin and with Alcian blue combined with picrosirius red (37).

For evaluation of the tissue histology, the longitudinal tendon sections were divided into a proximal, mid, and distal part (Fig. 1), and in each of these parts three separate random fields of 390 μm × 280 μm were analyzed. The average pathology score of these three fields was used for the statistical analyses. The histological analyses were all performed by the same experienced histologist, who was blinded to the identity of the slides.

The degree of tendon histopathology was evaluated according to a slightly modified Bonar scale, assessing tenocyte morphology, tenocyte proliferation, collagen organization, glycosaminoglycan (GAG) content, and vascularization, as described previously by Refs. 11 and 37. Scores from 0 to 3 were given in each of the five categories and summed for a total score.

**Statistics**

All data are presented as means ± SE, except for mRNA data, which were log-transformed before statistical analyses and are presented as geometric means ± back-transformed SE.

For statistical analyses of body weight, tendon diameter, and tendon metabolism (PET scan results), a two-way repeated-measures ANOVA on group × time was performed using SigmaPlot 11.0 (Systat Software, San Jose, CA). If a significant group × time interaction was found, individual differences between groups (within time points) and between time points (within groups) were tested with a post hoc test (Holm-Sidak). For tendon diameter and tendon metabolism, individual two-way repeated-measures ANOVAs were performed for the three different tendon regions (proximal, mid, and distal). For mRNA data and mechanical data, unpaired t-tests were performed to compare the end point results for controls vs. runners. Differences were considered significant when P < 0.05.

**RESULTS**

**Body Weight**

The rats of the running group steadily gained weight up to 10 wk of running, but they were significantly lighter than the control rats from 4 wk of running and throughout the rest of the intervention. At 12 wk, the running rats weighed 471 ± 7.5 g (means ± SE), while the controls rats weighed 573 ± 14 g (Fig. 2).

**Tendon Diameter**

Proximal, mid, and distal tendon diameter was measured, with use of a CT scan, to evaluate the effect of the training program on the gross morphology of the Achilles tendon. Tendon diameter did not appear to be affected by the running protocol, as no interaction was found between group and time in any of the tendon regions (Fig. 3). At the proximal level, however, the tendon diameter increased as a function of time (time, P < 0.004), presumably related to growth of the animals, and at the midlevel the running group in general had a greater tendon diameter (group, P < 0.02) (Fig. 3).

**Mechanical Properties**

Force-deformation measures, as well as force at tendon tissue failure, were measured to evaluate possible changes in mechanical properties in response to the running regime. Most importantly, the tendon modulus was considerably higher in the running group (283 ± 23 mPa) (mean ± SE) than in the
controls (186 ± 20 mPa) (Table 3 and Fig. 4). Furthermore, the stress at failure, when related to body weight, was significantly higher in the running group (0.086 ± 0.012 mPa/g) compared with the control group (0.051 ± 0.008 mPa/g) (Table 3).

**Histology**

The Achilles tendon tissue histology of the control and running rats was evaluated in nine different regions of the tendon (Fig. 1). No differences were found between the Bonar scores of the tendons in the two groups of rats in any of these regions (Fig. 5).

**Glucose Uptake in Achilles Tendon Tissue**

To evaluate metabolism in the tendon tissue of the control vs. running rats, PET scans were used to detect uptake of 18F-fluoro-deoxyglucose in the proximal, mid, and distal tendon areas (Fig. 5). No differences were found between the Bonar scores of the tendons in the two groups of rats in any of these regions (Fig. 5).

**Gene Expression**

Total RNA. Levels of total RNA were ~100 ng/mg tendon tissue, and there was no difference between controls and runners (98 ± 7.8 ng/mg in controls and 105 ± 9.8 ng/mg in runners, means ± SE).

**Target genes.** Several of the genes of interest (Table 2) were regulated in the Achilles tendon tissue as a result of the running protocol (Fig. 7).

### Table 3. Tendon mechanical properties

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<td>8</td>
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<tr>
<td>Strain, %</td>
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<td>23.6 ± 1.4</td>
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<tr>
<td>Failure stress, MPa</td>
<td>28.5 ± 4.3</td>
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<td>Failure stress/body weight, MPa/g</td>
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<td>0.086 ± 0.012*</td>
<td>0.02</td>
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<tr>
<td>Modulus, MPa</td>
<td>186 ± 20</td>
<td>283 ± 23**</td>
<td>0.005</td>
</tr>
<tr>
<td>Energy, mJ/mm³</td>
<td>18.9 ± 3.9</td>
<td>26.7 ± 4.4</td>
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</table>

Values are means ± SE; n, no. of rats; Achilles tendon mechanical properties in control rats and rats subjected to 12 wk of uphill treadmill running. Significant differences: *P < 0.05, **P < 0.01.

**Collagens and Related Growth Factors**

The measured collagen genes were differentially regulated in response to the running protocol, as expression of type I collagen (COL1A1) remained stable, while type III collagen (COL3A1) was upregulated and type V collagen (COL5A1) downregulated (Fig. 7A). Similarly, the expression of some collagen-stimulating growth factors were downregulated by the intervention, including transforming growth factor-β1 (TGF-β1) and connective tissue growth factor (CTGF), while both splice forms of insulin-like growth factor-I (Ea and Eb) were expressed at higher levels in the running group compared with controls (Fig. 7C).

**Noncollagen Matrix Components**

With regard to the mRNA expression of noncollagenous matrix proteins, these were all either unchanged or downregulated in tendon tissue of the runners compared with controls (Fig. 7B). The small proteoglycans, fibromodulin and biglycan, were decreased by approximately eightfold and approximately sixfold, respectively, and the glycoprotein, fibronectin, was downregulated by approximately fourfold (Fig. 7B). The expression levels of decorin, versican, aggrecan, and tenascin C were very similar in the two groups and showed a relatively low individual variation.

**Matrix Degrading Enzymes**

The regulation of the expression of MMPs gave no clear indication of either an increased or decreased potential for matrix breakdown, as MMP-2 was downregulated (4- to 5-fold), while MMP-3 appeared slightly upregulated, although not significantly (P = 0.07), and MMP-12 was unchanged (Fig. 7D). The expression of tissue inhibitors of metalloproteinases (TIMP) did not give any clear picture either, as TIMP-1 was slightly upregulated and TIMP-3 was decreased. Finally, a sixfold decrease in ADAMTS-5 (a disintegrin and metalloproteinase with thrombospondin motifs) expression was seen (P < 0.001), whereas ADAMTS-4 was unchanged.

MMP-1,-8, and -13, as well as ADAMTS-1 and substance P, were expressed at very low levels and cannot be reliably reported (data not shown).
Values are means ± SE, bars), pre and post 12 wk of running, at the prox, mid, and dist tendon level.

280

Tendon histology

Fig. 5. A: pathology assessment with the Bonar scale in nine different areas of the Achilles tendons from control rats (n = 12; open bars) and rats subjected to 12 wk of uphill running (n = 8; shaded bars). Values are means ± SE. AD, anterior distal; AM, anterior mid; AP, anterior proximal; MD, mid distal; MM, mid mid; MP, mid proximal; PD, posterior distal; PM, posterior mid; PP, posterior proximal. B: mid dist tendon cryosection from control rat at 12 wk (390 μm × 280 μm). C: mid dist tendon cryosection from running rat at 12 wk (390 μm × 280 μm).

DISCUSSION

The 12-wk uphill running model that was investigated in the present study has previously been shown to induce pathological changes in rat Achilles tendon, including increased cell density and disorganization of collagen matrix (18). By replicating this tendinopathy model, we aimed to confirm these findings and to further characterize the induced tissue changes by measuring mechanical properties, tissue metabolism, and gene expression. However, we found no indications of tissue pathology on the histological level and did not observe any changes in tissue metabolism. On the contrary, our results indicate that the rat Achilles tendon is able to adapt to the load induced with this model and is, in fact, mechanically strengthened. Importantly, it seems that the changes seen in gene expression in response to this long-term physiological loading regime are clearly different to what is seen in tendon pathology and to what is observed in response to short-term loading of tendons.

Achilles Tendinopathy Models in Rats

Our lack of knowledge regarding the etiology of tendinopathy in humans makes the development of good animal models an important goal. This would give the possibility to study all phases of the pathological development, as well as potential treatments in relation to the temporal progress of the disease. For many practical reasons, the rat is an obvious choice for development of an animal tendinopathy model, and several groups have investigated possible models for functional overuse of tendons in rats. Soslowsky et al. have developed a downhill running model that reliably induces histopathological changes in the supraspinatus tendon comparable to those of human tendinopathy (37, 44), and this model has also been shown to induce mechanical weakening of the supraspinatus tendon (44). However, the tendinopathy developed in this downhill running model is presumably related to the compression of the supraspinatus tendon, and, accordingly, it may not be a good model for other tendons, such as the Achilles tendon, which is very often affected by overuse in humans in association with tensile loading (32). A well-established rat model is still lacking for the Achilles tendon, although several attempts have been made to develop such a model (18, 25, 34). Recently, Glazebrook and coworkers (18) tested the effect of 12 wk of uphill treadmill running (1 h/day, 5 days/wk, 17 m/min) on rats and found histological changes in the Achilles tendon tissue comparable to those seen in human tendinopathy. In the present study, we copied this model, with the intention of using it for future studies on Achilles tendinopathy. To increase the likelihood of tendon overuse, the running speed was changed compared with the original study, as we progressively increased the speed from 17 to 20 m/min during the 12 wk of training (Table 1).

Tissue Histology

Although observations were made in nine distinct Achilles tendon regions (Fig. 1) and for a number of histological parameters, including cell morphology, cellularity, collagen organization, presence of ground substance, and vascularity, we could not detect any differences between Achilles tendon tissue of running and control rats (Fig. 5). Thus we cannot confirm the observations, made by Glazebrook and coworkers (18), of decreased collagen organization, as well as increased cell density in response to the uphill running protocol. It is difficult to explain this discrepancy, especially as our histology data represent nine different regions of the Achilles tendon, while the previous data represent only three regions, namely the proximal, mid, and distal levels of the midtendon (18). Therefore, any presence of histological change should, with all
probability, be detected in our analysis. In addition, the running speed was progressively increased in the present study, which should only increase the potential for development of tendinopathy. Of course it cannot be excluded that the progression from healthy adaptation to maladaptation is a fine balance, and that small, seemingly inconsequent, differences may be important. Furthermore, there are differences in the histological methods in relation to tissue embedding and to the methods for scoring histological changes. The present study used the sum of scores for tenocyte morphology, tenocyte proliferation, collagen organization, GAG content, and vascularization to evaluate tendon pathology in accordance with the modified Bonar scale (11, 37), while, in contrast, the previous study assessed nuclear number, collagen organization, and collagen staining intensity as individual parameters (18). Although it seems unlikely, it cannot be excluded that these differences in methodology may have affected the outcome. Based on the present data, however, it must be concluded that the uphill running model does not induce Achilles tendinopathy in rats.

During the course of data collection for the present study, one additional study has been published using the uphill running model (1). Here microregions of abnormal-appearing tenocytes were found in Achilles tendons of only running rats (not in controls). However, in that study, the degree of pathology was not quantified in a conventional manner (e.g., Bonar scale), as the study was focused on the use of second harmonic generation microscopy to evaluate the difference in organization of the collagen surrounding abnormal- and normal-appearing cells. In addition, the rats were considerably older, and, therefore, a direct comparison to the present study is not possible. Finally, a very recent study by Silva et al. (40) has evaluated the effect of uphill running also with 10° incline, but at a significantly higher speed (26.8 m/min), and found increases in cellularity, microtearing, and GAG content. This could indicate that higher levels of speed are needed for a reliable induction of histopathological changes.

**Tendon Mechanics**

No mechanical data for the Achilles tendon tissue were published in the previous reports using the uphill running model (1, 18). However, the previously mentioned rat model for supraspinatus overuse showed decreased failure stress and modulus, along with the histopathological changes (44), and in both humans and rats diseased Achilles tendons appear to have weakened mechanical properties (7, 34). Contrary to this, our mechanical testing showed an increase in failure stress per body weight, as well as increased modulus (Table 3 and Fig. 4). The increase in failure stress per body weight might be explained partly by the lower body weight of the rats in the running group (Fig. 2), although the failure stress per se was close to being significantly higher in the runners ($P = 0.12$). Importantly, the difference in modulus ($283 \pm 23$ MPa for the
runners compared with 186 ± 20 MPa for the controls) underlines that this long-term loading model substantially improves the material properties of the tendon tissue. In line with the histological data, this supports that the uphill running regime does not have adverse effects on the Achilles tendon tissue. In fact, the enhanced mechanical properties indicate that the tissue is well able to adapt to the increased load in this model. Several studies on humans confirm that tendon stiffness and modulus may increase in response to long-term loading, although this has mainly been shown in response to resistance type training (e.g., Refs. 5, 30, 39). In rats, very few studies have been published regarding alterations in tendon mechanical properties in response to functional long-term nonpathological loading. Legerlotz et al. (31) found no changes in mechanical properties of female rat Achilles tendons after either 12 wk of voluntary wheel running (10 km/day) or strength training, and, similarly, Huang et al. (25) showed no effect of 16 wk of downhill treadmill running on rat Achilles tendons. On the other hand, an early study showed a preventive effect of long-term swim training on the age-related deterioration of Achilles tendon mechanical properties (41). Accordingly, no clear picture of the adaptability of rat tendon mechanical properties exists. However, the present data indicate that long-term functional loading can lead to substantial changes in material properties of rat Achilles tendons.

Tendon Diameter

In agreement with the previous observations (18), no changes were found in the diameter of the Achilles tendons as a result of the running regime. However, we found an overall group effect at the midtendon level (Fig. 3), indicating that the running group had a generally greater tendon diameter than the controls, independent of the intervention. This difference must be presumed to be a random difference (at least at the pre-time point), since the rats were randomly selected from a group of rats that were all willing to run. The possible impact of this group difference should, of course, be considered, as the tendon diameter could potentially affect the resistance to development of tendinopathy. Meanwhile, none of the animals developed any consistent signs of tendinopathy, and absolutely no correlation was seen between the histopathological score and the tendon diameter (pre or post) (data not shown). Thus the difference between groups in midtendon diameter seems very unlikely to have had any significant impact on what conclusions can be made from this study. With regard to the mechanical data, the tendon diameter will presumably have some impact on the failure stress, and the higher tendon diameter at the midtendon level in the running group may contribute slightly to the difference seen in failure stress between the runners and controls (Table 3). However, since we also saw an increase in the tendon modulus, there is no doubt that the running regime did have a true effect on mechanical properties. In fact, a recent study indicates that the measured modulus is inversely related to the diameter of the tested specimen (30a), and thus the higher diameter in the running group may have led to an underestimation of modulus in this group.

Tendon Tissue Metabolism

In addition to histological and mechanical analyses, we tried to advance the examination of the Achilles tendons by measuring local tissue energy metabolism. This was done by detecting uptake of 18F-fluoro-deoxyglucose in the tendon tissue, with use of PET combined with CT scans. The rationale for trying to detect tendinopathy with use of 18F-fluoro-deoxyglucose-PET is that cell density and vascularity is often increased in tendinopathic tendons (8, 37), both of which would be expected to increase glucose uptake. In addition, it is possible to detect the increase in glucose uptake seen in response to acute exercise in rats and humans (9, 29, 43), and it seems likely that an increase in tendon metabolism, due to tendinopathy, would be within a similar range as the increase seen in response to exercise. We observed no difference between pre- and post-levels of glucose uptake at rest in the Achilles tendons of running rats, and also the glucose uptake was similar in controls and running rats at all time points (Fig. 6). These results indicate that the 12-wk training program did not alter resting tendon tissue metabolism and fit well with the histological data that indicated no alterations in vascularity and cell morphology or number.

Regulation of Gene Expression

No well-defined profile exists of the alterations in gene expression occurring in tendinopathy. There are, however, some common findings in studies that have investigated differences in mRNA expression in tendinopathic tendon tissue vs. healthy tissue. These include an increased expression of collagen I and III mRNA (12, 14, 26, 27), increased levels of MMP-2 mRNA (3, 12, 26, 27) and decreased levels of MMP-3 mRNA (12, 14, 26, 27, 28). Since we found unchanged type I collagen expression, decreased MMP-2 expression, and a trend toward an increased MMP-3 expression (P = 0.07), it seems clear that the gene expression profile of the rat Achilles tendons subjected to 12 wk of running does not fit well with that of a tendinopathic tendon. In further support of this, we found decreased levels of VEGF and proteoglycan mRNA (fibromodulin and biglycan), which are thought to be elevated with tendinopathy (4, 12, 13, 36, 38). These mRNA results serve to underline the more fundamental finding of unchanged tissue histology, which clearly indicates that the training model does not induce Achilles tendinopathy. The fact that we do find an increase in collagen type III mRNA and a depression of both TIMP-3 and ADAMTS-5, which has also been seen in tendon pathology (6, 28), might indicate that there is some degree of underlying pathology present that does not penetrate to a change in tissue histology. However, previous data suggest that an induction of type III collagen and downregulation of at least TIMP-3 is merely a part of tendon loading in general, and not necessarily connected to pathological changes (6, 23, 35).

Considering the lack of pathological changes on the histological level and the enhanced tissue mechanical properties, the present mRNA data can most likely be interpreted as long-term changes in gene expression related to a successful adaptation of tendon tissue to increased daily loading. Apparently, no human and very few animal studies have previously investigated how tendon tissue responds to extended periods of physiological training with regard to regulation of mRNA expression. One study found increased TIMP-1 mRNA and unchanged levels of collagen (I and III), TGF-β1, and CTGF mRNA in Achilles tendons in response to voluntary wheel running in female rats (31). A more
recent study showed elevated levels of TIMP-1 and IGF-IIEa, after 7 wk of jump training in male rat Achilles tendons, while collagen I and III were unchanged (33). Considering these findings, combined with the data of the present study, it seems that TIMP-1 and IGF-I expression may be consistently upregulated even after long periods of tendon loading. Similarly with short-term tendon loading in rats, levels of TIMP-1 mRNA and IGF-I mRNA (and protein) have been shown to increase (20, 23, 24, 35), and it may be speculated that the upregulation of these factors is initiated in the beginning of the training period and persists throughout the 12 wk of training. Although it is difficult to conclude on the significance of this, both IGF-I and TIMP-1 could play an important anabolic role in the maintenance of the loaded tendon tissue, since IGF-I induces collagen synthesis (2), and TIMP-1 inhibits MMP-mediated breakdown of collagen matrix (10). In general, however, the response to long-term loading does not equal that of short-term loading. For example, collagen I and III mRNA expression has consistently been shown to be substantially induced with different kinds of short-term loading (including treadmill running) in rat Achilles tendon, and TGF-β1 was also clearly upregulated with short-term strength training (23, 35, 42). Our data indicate that only collagen III remains upregulated after 12 wk of loading, whereas collagen I mRNA levels are equal to that of control tendons, and TGF-β1 is in fact downregulated, along with its proposed downstream mediator CTGF. Thus it may be proposed that an initial anabolic response, i.e., simultaneous increases in collagen I and III, IGF-IIEa, and -IEb, TGF-β1, and TIMP-1 is somewhat reduced when a steady state is reached, and that only increased levels of collagen III, IGF-I mRNA, and TIMP-1 is maintained at the 12-wk time point. This is, however, speculative, and studies of the tendon gene expression in the initial week should be done to confirm this.

Limitations

One of the most interesting findings of the present study is that, even after 12 wk of physiological loading, a gene expression response was still present. However, the study is limited by the fact that only the 12-wk time point is available for interpretation. It would have been interesting to include more rats in the study to enable comparison of gene expression and other parameters at several time points during the intervention. In addition, the initial sample size should perhaps have been greater to compensate for the exclusion of rats that refused to continue running for the entire 12 wk. Furthermore, the study would have been strengthened if tissue quantities had allowed for analyses of changes in protein expression, as well as levels of collagen cross-links.

With regard to the relevance for humans, recent observations suggest that human tendons may be far less responsive to loading, at least with regard to induction of changes in gene expression (22). Thus we cannot be certain of the relevance of the long-term changes seen in rat tendon mechanics and gene expression in relation to human tendon tissue.

Finally, in relation to the mechanical data, the present data only examined the effect of the intervention on tendon properties and not that of the entire muscle-tendon unit.

Conclusion

The uphill running model proposed earlier (18), consisting of 12 wk of daily, 1-h, 10° uphill running sessions, cannot be validated as an Achilles tendinopathy model. We found no changes in tendon histology when repeating this loading program, and the lack of pathological change was further supported by unchanged tendon metabolism. On the contrary, the tendon tissue mechanical properties were improved (increased modulus), indicating that the rat tendon tissue was able to successfully adapt to this level of increased loading. In support of this, alterations in gene expression were markedly different to what is observed in tendinopathy and also distinct from the changes in gene expression seen in tendon tissue after short-term loading.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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