Mechanical and structural adaptations of tendon with aging

by

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Dissertation Abstract

Tendon provides a critical functional link between muscle and the skeleton. Thus, any change in tendon mechanical properties with aging may contribute to disability and loss of mobility of the elderly. Despite the importance of tendon to the function of the musculoskeletal system, the effects of age on tendon structure and function are incompletely understood. In addition, the responses of tendons in old animals to altered loading or biochemical environment and the mechanisms underlying the changes represent fundamental gaps in the field of musculoskeletal biology. Therefore, the goals of this dissertation were to (1) clarify how tendon structure and mechanics are altered with aging and (2) establish if age-associated changes can be delayed or reversed with exercise or the lifespan-extending drug Rapamycin. Using a novel technique of coupling regional mechanics and structural properties, we analyzed hindlimb tendons of adult (8-12 months) and old (28-30 months) mice. Tibialis anterior (TA) tendons stiffened with age, with the most pronounced increase in the region of the tendon nearest the muscle, where a 2-fold increase was observed. The mechanical changes in tendon were accompanied by suppressed collagen turnover and increased collagen crosslinking and calcification without changes in collagen fibril morphology. A similar degree of age-associated stiffening was observed for plantaris tendons, but 10 weeks of treadmill running, initiated at 27 months of age, essentially restored the adult

phenotype in terms of mechanics and collagen turnover. Age-associated calcification of Achilles tendons was also partially abrogated following treadmill running. In contrast to reports from adult mice, the tendon extracellular matrix (ECM) remodeling was achieved without increased cell density or activation of tendon developmental pathways. Chronic administration of Rapamycin also impacted tendon properties, preventing the age-related changes in mechanical properties of TA tendons. This preservation of adult levels of tendon stiffness in old Rapamycin treated mice was accompanied by higher cell density than untreated old mice without changes in collagen synthesis. These findings demonstrate that mechanical and structural properties of mouse hindlimb tendons are altered in old age, but tendon maintains the ability to remodel its ECM, potentially through multiple mechanisms.

Chapter I

Introduction

Background and Motivation

In humans, old age is associated with dramatic declines in musculoskeletal performance, including decreases in muscle strength and power (9, 42) and decreased bone mass (18). These age-associated modifications are well established as major contributors to impairments in mobility and elevated risk of musculoskeletal injury in old age (30). The effect of aging on tendon properties is less well established, but there is emerging evidence that the functional performance of tendon is also compromised in old age. In humans, old age is associated with elevated rates of tendon dysfunction, which can manifest as a wide range of pathological changes including structural degeneration, abnormal thickening, and calcification (56). At least one third of persons over the age of 70 suffer from some degree of tendon degeneration (41, 69, 82), although tendinopathies are frequently asymptomatic (75, 81), suggesting that the incidence is underestimated. Tendon degeneration increases the risk of full tendon rupture (24, 68), which is of great consequence in the elderly population where a minor slip, fall, or sudden movement may induce full tendon tearing (48).

Although tendon ruptures are painful and debilitating injuries (27), functional degeneration of tendon without frank rupture can adversely influence mobility and

quality of life of the person affected. Age-associated changes in tendon functional performance lead to decreased muscle power (64) and torque (37), which can negatively affect the magnitude and fidelity of force transfer to the skeleton (8). Additionally, the functional properties of limb tendons highly influence balance and therefore recovery from a slip or trip (28, 46), an ability which is compromised in old age (16, 23). Finally, surgical treatment of tendon disorders in older individuals is associated with a high rate of complications, and most patients do not return to their pre-injury activity level (44).

The high prevalence of tendon dysfunction in the elderly coupled with limited options for successful treatments highlight the need for greater knowledge of the mechanisms underlying the progression of the changes. Since the influence of aging on tendon functional properties has not been fully characterized, establishing how healthy tendon structural and mechanical properties are altered by aging is essential to provide a baseline from which the progression of age-associated changes can be determined. In addition, increased understanding of the ability of tendons from old mammals to adapt to changes in their external environment and the mechanisms underlying the changes is critical to facilitate the development of treatments aimed at delaying or preventing the condition.

Tendon Structure

Tendon extracellular matrix. Tendon is a complicated, hierarchical tissue that is primarily composed of collagen, which makes up 60-85% of the tissue's dry weight (14). This collagen is dominated by the fibril-forming type I collagen (COL1), but types II, III,

and V have been observed in small amounts, especially in disease states (30). The basic unit of fibrillar collagens is tropocollagen, which is a triple-helix peptide chain. Tropocollagen molecules cross-link into fibrils, which are further crosslinked into fascicles and, finally, whole tendons (Figure 1.1). Collagen fibril diameters vary wildly, typically ranging between 40 and 500 nm in diameter (77) and, at rest, have a distinct crimp configuration (54). Collagen fibrils are embedded in an amorphous extrafibrillar matrix consisting of proteoglycans (PGs), elastin, and water. PGs constitute 1-5% of tendon dry mass (59), and help regulate fibrillogenesis (13) and aid in resisting compressive forces (71).

Collagen turnover in developing and adult tendon. Collagen molecules are interconnected with crosslinks that impart stability and mechanical integrity to the fibrils. The mechanical integrity of collagen in developing and mature tendon is achieved through a balance between the deposition and crosslinking of young collagen, mediated by the enzyme lysyl oxidase (LOX), and collagen degradation via matrix metalloproteinase (MMP) collagenases. LOX binds to specific lysine or hydroxylysine residues on the ends of newly synthesized collagen molecules and converts these residues to aldehydes (63). These aldehydes then condense with hydroxylysine and other residues in neighboring molecules resulting in trivalent intermolecular crosslinks that stabilize collagen fibrils (4). MMPs cleave collagen fibrils, creating fragments that can be further degraded (74). Collagen fibrils are notably more resistant to degradation than lone collagen monomers and only those molecules on the surface of the fibril are

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accessible for enzymatic collagenolysis (49) contributing to the extremely slow turnover of collagen with a half-life estimated to be on the order of decades (39, 70).

Collagen turnover in tendon with aging. The exceptionally slow turnover of collagen allows for the accumulation of post-translational modifications in collagen, which are initiated by the spontaneous reaction of glucose with lysine residues on the fibrils (72). Subsequent oxidation of these modifications can form advanced glycation end-products (AGEs), which include the crosslinks N^{ε}–(carboxymethyl)lysine and pentosidine (65). These crosslinks accumulate in long-lived collagenous tissues such as tendon and have the effect of progressively increasing the stiffness of collagen fibrils and tendons (55, 58). Additionally, AGE crosslinks increase the resistance of collagen to enzymatic breakdown (11), further decreasing the turnover of collagen and encouraging the development of AGE crosslinks.

Cellular components. Tendon fibroblasts, typically referred to as tenocytes, constitute the main cellular component of tendons. These cells reside in between and in parallel with the collagen fibrils (29). With maturation and aging, cell density in tendon drops precipitously, and adult tendon cell density is much lower than in muscle or bone (25). Tenocytes are responsible for the maintenance and repair of tendon extracellular matrix.

Muscle Structure

Skeletal muscle is composed of multinucleated cells, called fibers, which are embedded in a collagen-rich extracellular matrix (ECM). The skeletal muscle ECM

makes up approximately 1% to 5% of the muscle mass (51) and is functionally continuous to tendon tissue, partially via the aponeurosis. The aponeurosis is essentially flattened tendon that extends from the tendon into the muscle belly. Muscle ECM is organized into three levels. The endomysium directly surrounds individual muscle fibers, the perimysium encloses bundles of muscle fibers, and the epimysium surrounds the entire muscle. Since the extracellular matrix of tendon and muscle are functionally linked, the extracellular matrix of muscle plays a vital role in the transmission of force produced by muscle fibers to the tendon (53, 67).

Tendon Function

The primary function of tendon is to transfer forces produced by the contractile elements of muscle to the skeleton, allowing for joint motion and everyday movement. Tendons act as springs that store and release elastic energy during normal locomotion (1, 5). As a tendon is stretched, it stores strain energy (5). During relaxation, the stored strain energy is converted into mechanical energy that is used to do useful work, such as reaccelerating the body during a step (5, 6, 62). This cyclical storage and release of energy reduces the work done by muscle fibers (60), and can lead to power outputs that exceed the power-producing capacity of muscles (61). Tendon serves this function without metabolic cost, unlike muscle that requires the consumption of metabolic energy to produce work. Tendons also serve to protect muscle fibers from damage that can occur when muscles are stretched during lengthening contractions (17) by limiting the amount of strain placed on muscle fibers (36). Given that sarcomeres can be highly

damaged during lengthening contractions (10), a compliant tendon protects muscle fibers from strain-induced injury, and allows muscle fibers to maintain an isometric length or even continue to shorten as the joint angle increases (15, 17, 32, 57, 60). During maximal isometric contractions, a more compliant tendon results in more shortening of muscle fibers than if the tendon were stiffer (34, 43, 45), causing the muscle to operate at lower sarcomere lengths. Thus, a more compliant tendon would lead to a reduction in muscle force output, especially for muscles that normally operate on the ascending limb of the muscle's force-length curve, as is the case in most human muscles (38). Additionally, the mechanical properties of tendon have an impact on how quickly muscle torque is developed (8, 76).

Mechanical Testing of Tendon

The mechanical properties of tendon are typically characterized with a uniaxial tensile test, in which the tissue is gripped on both ends and stretched to a predetermined length. Applied loads and tendon displacements are determined using real-time force measurements and imaging, respectively. In order to determine the intrinsic properties of the tendon tissue, load is normalized to tendon cross-sectional area and displacement normalized to original tendon length. The resulting stress and strain data are plotted in order to characterize the tendon's mechanical properties. Tendons demonstrate complicated mechanical behaviors that are both nonlinear and viscoelastic. When tendons are subjected to a uniaxial tensile test, their stress-strain behavior displays a characteristic shape consisting of a concave toe-region of low

stresses and high extensibility followed by a linear region of constant slope (Figure 1.2). The toe-region has been shown to correspond to the uncrimping of the collagen fibrils (19), while the linear region corresponds to gliding and stretching of the fibrils (52). By taking the slope of the linear portion of the stress-strain curve (i.e., the maximum tangent modulus), the intrinsic stiffness of the tendon tissue material can be determined. Tendons also show viscoelastic behavior, indicated by energy loss during a cyclical test (i.e., hysteresis).

Mechanical testing of tendon is complicated by difficulty in both mounting samples in the testing apparatus and accurately tracking strain of the sample. When tendons are severed from their bone and muscle attachments and mounted in testing grips, sample artifacts such as stress concentrations or collagen fibril slipping may be introduced that can affect the measured sample strains (20). Studies that keep the tendon unit intact for testing have primarily measured strain either on the central tendon region or by measuring grip-to-grip changes. Tendons actually possess regional variations in their mechanical properties, with a compliant region nearest the muscle that becomes stiffer towards the bone insertion (3). Strains measured in the central region differ from end-to-end strains, and neither central region nor end-to-end strain measurements reflect the complicated regional strain behavior of tendons (3). Also, if grip-to-grip movement is used to determine sample strain, the resulting values will be 2- to 3-fold larger than the true strain (80). These issues highlight the need to ensure that the tendon attachments to bone and muscle are kept intact for mechanical testing. It is also imperative that true local strains are measured during testing so that a complete

and accurate understanding of the regional mechanics of tendons can be obtained. Finally, may tendon mechanical studies have utilized tail tendons that do not experience muscle loads during locomotion. Since physical activity and loading patterns directly affect tendon mechanical properties (78, 79), load-bearing tendons are the most appropriate for studies of the etiology of tendon dysfunction that influences mobility.

Tendon Adaptation to Environmental Changes

Tendon is a dynamic tissue that is capable of adapting in both structure and function to changes in its environment. The most well characterized tendon adaptations occur in response to alterations in the habitual pattern of loading achieved through exercise. Endurance training increases tendon stiffness in a wide range of species, including rodents (22), pigs (78, 79) and humans (12). Resistance training also results in increased tendon stiffness in human patellar (31) and vastus lateralis (33) tendons. Exercise induces structural changes in adult tendon by promoting increased expression and concentration of type I collagen (35), resulting in net collagen deposition and an increased tendon diameter (7, 40, 78). These structural changes are accompanied by elevated cell proliferation following training (40).

Adult tendon is also capable of adapting to pharmacological intervention. In humans, exposure to steroids such as dexamethasone leads to decreased collagen synthesis in tendon (2). Additionally, the antibiotic class fluoroquinolones alters

collagen expression, most notably in older patients (84), and results in severe collagen fibril disruption and degeneration (83).

Tendon adaptations in adult animals have been widely studied, but there is a lack of data regarding the ability of tendon in old animals to adapt to similar changes in loading or pharmacological environment. Although there are reports of increased stiffness of patellar tendons in elderly subjects following resistance training (47, 57), as well as the attenuation of age-associated increases in rat tail tendon stiffness by lifelong treadmill running (3), the response of load-bearing tendons in old animals to treadmill running started late in life has not been previously explored. Furthermore, the effects of pharmacological interventions on tendon structure and function in old animals are even less well studied.

Dissertation Aims

The mechanisms underlying the increased incidence of tendon dysfunction with aging are unknown but are thought to be a result of changes in the functional properties of tendon brought about by alterations in underlying structure. This dissertation addresses fundamental gaps in the field of musculoskeletal biology through studies that reveal the age-related changes in tendon and muscle ECM structural and functional properties, and to establish if the changes in tendon can be reversed or prevented with exercise or pharmacological interventions. In this dissertation, we use mouse models of tendon aging. Given their similar anatomy, short lifespan, and ability to perform highly controlled and invasive procedures, the use of mice is an attractive and appropriate

alternative to human beings for these basic studies. In Chapter 2, we report data characterizing how the mechanical and structural properties of mouse tibialis anterior (TA) tendons are affected by aging. Results from the work described in Chapter 2 motivated the work described in Chapter 3, in which the impact of treadmill training on mouse hindlimb tendon mechanics and structure was determined. Chapter 4 explores the ability of treatment with the drug Rapamycin (RAPA) to protect TA tendons from age-associated degeneration. RAPA extends lifespan in mice (21) and influences collagen synthesis and MMP expression in cultured fibroblasts (50), but the effects of RAPA on tendon tissue *in vivo* remain unclear. Finally, since tendon and muscle ECM form a functional link, Chapter 5 examines if TA muscle ECM undergoes similar age-associated changes in structure as tendon.
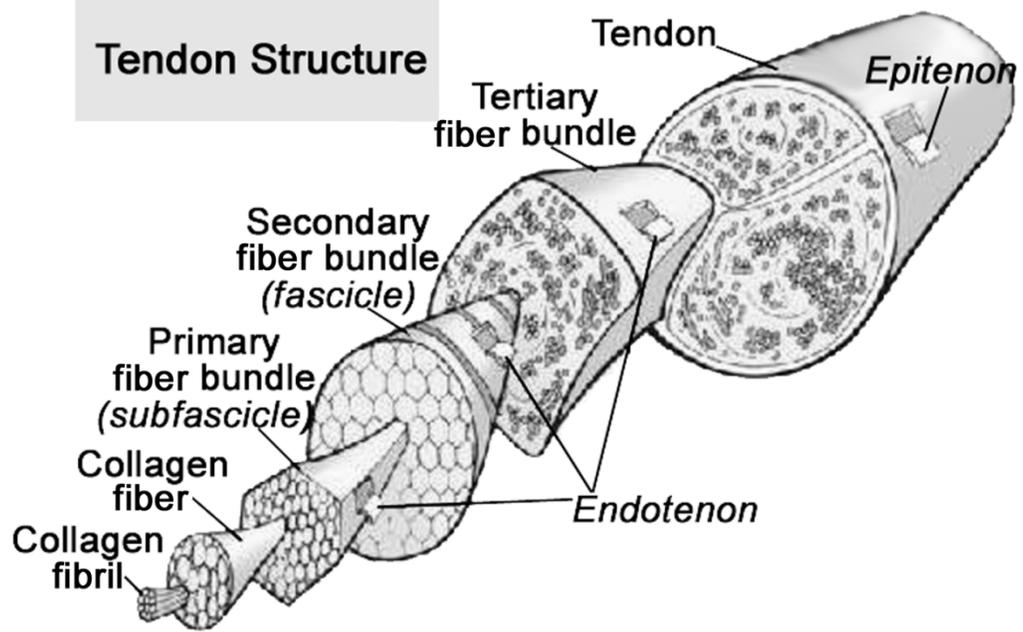
Figure 1.1. General structural hierarchy of tendon. From reference 66.
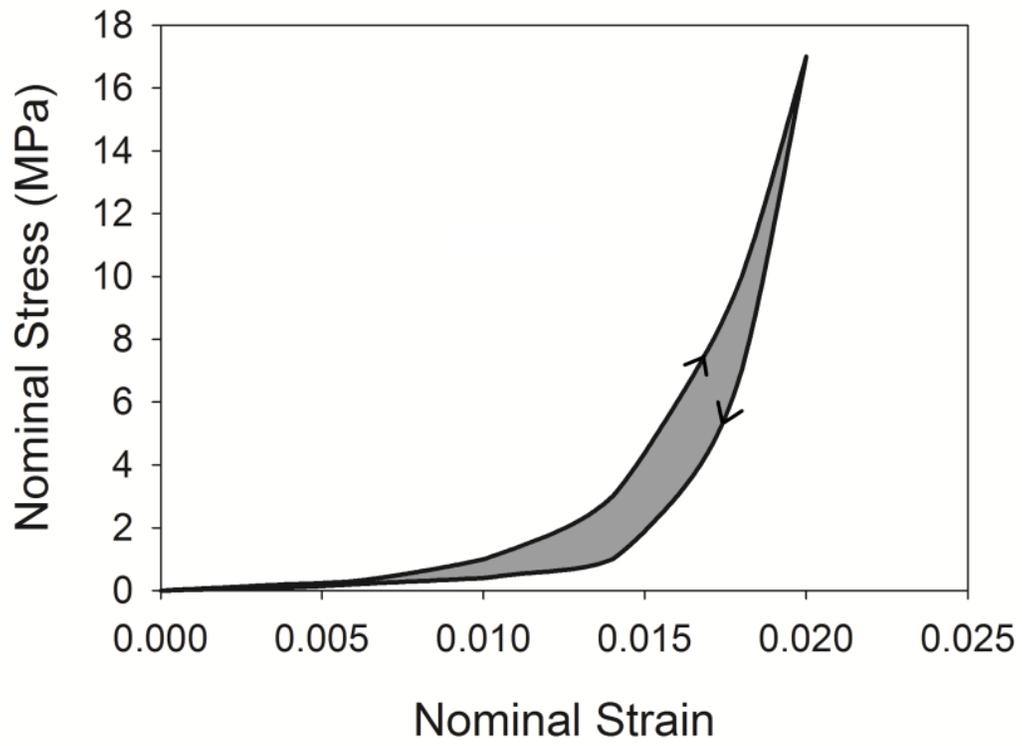
Figure 1.2. Representative stress-strain curve of tendon undergoing a single cyclical test.

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Chapter II

Regional stiffening with aging in tibialis anterior tendons of mice occurs independent of changes in collagen fibril morphology

Abstract

The elevated incidence of frailty and impaired mobility in the elderly arises at least in part due to structural and functional impairments in the musculoskeletal tissues. Although age-related changes in muscle and bone have been well established, tendon has not been thoroughly characterized. Our purpose was to determine the effect of aging on tendon mechanical properties and collagen fibril morphology. Regional mechanical properties and collagen fibril characteristics were determined along the length of tibialis anterior tendons from adult (8-12 months) and old (28-30 months) mice. Tangent modulus of all regions along the tendons increased in old age, but the increase was substantially greater in the proximal region adjacent to the muscle compared with the rest of the tendon. Overall end-to-end modulus at maximum tendon strain was 70% greater for tendons of old compared with adult mice and 50% greater at physiologically relevant levels of strain. Collagen fibril diameters and packing fractions were also quantified in the proximal and distal regions of the tendon. Despite the dramatic changes in tendon mechanical properties, collagen fibril morphology and packing fraction remained relatively constant in all tendon regions from adulthood to

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old age. Since tendon properties are influenced by the external loading environment, we also examined the effect of aging on TA muscle contractile properties. Maximum isometric force did not differ between the age groups. We conclude that tibialis anterior tendons stiffen in a region-dependent manner throughout the life span, but the changes in mechanical properties are not accompanied by corresponding changes in collagen fibril morphology or force-generating capacity of the TA muscle.

Introduction

Tendons provide a critical functional connection between muscles and bones, transferring force produced by the contractile elements of muscle to the skeleton, allowing for movement. Age-associated changes in tendon function are likely contributors to the high incidence of frailty and impaired mobility among the elderly (16, 21). At least a third of persons over the age of 70 present with some degree of tendon degeneration or tearing (28, 33), which is often asymptomatic and increases the risk of full tendon rupture (22). This increased risk is of great consequence in the elderly population where tendon rupture can result from a minor slip or fall (29).

The mechanisms underlying the impaired functional performance of tendon with old age are unknown, but may arise because of age-associated changes in the mechanical properties of tendon (8). Furthermore, tendon mechanical properties are governed by the characteristics of its underlying structural components, but data correlating mechanical properties with underlying structure in tendons of old animals are scant. Even attempts to develop structure-function relationships in tendons from

younger animals have been inconclusive. Collagen fibril diameter has been positively correlated with tendon stiffness (13), but others have failed to demonstrate such correlations (18, 25). These inconsistencies may arise due to the mechanical and structural heterogeneity of tendons. The tibialis anterior (TA) tendon of healthy adult rats has a compliant region near the muscle and then gradually becomes stiffer towards the bone insertion (1). Additionally, anterior fascicles from human patellar tendons are stronger and stiffer than posterior fascicles (18, 20). Collagen characteristics in tendon are region-dependent as well, with rabbit patellar tendons showing local variations in collagen fibril diameter and area fraction (36), and chicken gastrocnemius tendons displaying regional differences in collagen content (11).

The local dependency of tendon properties coupled with conflicting results in the literature and a lack of data on the effects of age have made it difficult to develop clear structure-function relationships that can be applied to aging tendon. Further complexities arise since the external loading environment also influences tendon mechanical properties (6, 23). Significant reductions in muscle force output with aging are well documented in many muscles (4, 5, 12), but the adaptive response of tendons to those age-associated changes remains unclear. Previous studies have demonstrated decreases in tendon stiffness following unloading due to prolonged bed rest (30) or spinal cord injury (27). Conversely, we have shown that decreased muscle activity associated with denervation results in tendon stiffening that is most pronounced in the region nearest the muscle (1). Therefore, the goals of this work were to examine how regional TA tendon mechanics are related to collagen fibril morphology and TA muscle

contractile properties in adult and old mice. Our working hypothesis is that decreased force generation with aging will lead to stiffening of the tendon with the region nearest the muscle most affected. We tested the hypotheses that (1) compared with adult mice, maximum isometric force of TA muscles of old mice will be lower and the stiffness of TA tendons will be greater due primarily to changes in the region of the tendon nearest the muscle, and (2) regional mechanical properties will correlate with collagen fibril diameter and packing fraction both within individual tendons and between age groups.

Methods

Animals. Twenty 8-12 month-old and eleven 28-30 month-old C57BL/6 male mice were obtained from Charles River Laboratories (Wilmington, MA). Body masses (mean ± SD) for the two age groups were 34.6 ± 2.5g and 32.9 ± 3.7g for the 8-12 month and 28-30 month old mice, respectively ($p > 0.4$). Mice were randomly assigned into groups for testing tendon mechanics, muscle contractile properties, or electron microscopy. In total, tendon mechanical properties were determined for ten tendons from adult mice and five tendons from old mice. TA muscle contractile properties were determined for eight adult muscles and six old muscles. Three tendons per age group were examined with electron microscopy. Animals were housed in a specific-pathogen-free barrier facility in the Unit for Laboratory Animal Medicine at the University of Michigan with food and water provided *ad libitum*. All procedures were approved by the University of Michigan Committee for the Use and Care of Animals.

Determination of tendon mechanical properties. Mice were anesthetized with an intraperitoneal injection of tribromoethanol, which was supplemented as necessary during the procedure. The TA tendon unit (TA muscle, TA tendon and 1st metatarsal) was removed from one hindlimb, and overlying connective tissue carefully removed. Samples were stored in sterile PBS at 4°C until testing, which was within 3 hours of tissue removal. The stress-strain response of each tendon was determined using a previously validated testing setup (24), with modified grips to accommodate the small size of the mouse TA tendon unit. The TA unit was placed in a room temperature PBS bath, and the TA muscle and 1st metatarsal were secured to custom grips so that the tendon was free-standing (Figure 2.1a). Tendon cross-sectional area (CSA) was determined by optically measuring the diameter at six equally-spaced points along the tendon at 0° and 90° rotation using a calibrated microscope eyepiece, and fitting the values to an ellipse (Table 2.1). 25µm polystyrene beads (IMT Laboratories, Irvine, CA) were brushed along the length of the tendon to serve as optical markers for strain measurements (Figure 2.1b). The tendon was stretched to a pre-load of ~0.02N, which served as the zero point. The sample was then subjected to a load-unload cycle of 10% grip-to-grip strain at a constant strain rate of 0.01/sec. Since preconditioning can shift the stress-strain response of collagenous tissue (20, 32), no preconditioning protocol was used.

Synchronized force and image recordings were obtained and compiled using LabVIEW (Austin, TX). Bead positions were tracked using MetaMorph software (Molecular Devices, Sunnyvale, FL), and nominal strains in the proximal (near the

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muscle), central and distal (near the bone) tendon regions were calculated as the change in separation between two beads in each region divided by their initial separation. Regional nominal stress was determined by dividing raw load data by the local CSA. End-to-end tendon strain was calculated using beads that were positioned one tendon diameter length proximal to the bone insertion and one diameter length distal to the muscle insertion (Figure 2.1a). This bead positioning avoided any strain concentrations created by the grips. End-to-end tendon stress was calculated using average tendon CSA. Stress-strain data were fit to a third-degree polynomial and maximum tangent modulus of each tendon region was calculated as the maximum slope of the curve. Generally, the polynomials fit the raw mechanics data very well. Fits were poorest in the distal tendon region (average $r^2=0.756$). Fits in the remaining regions were similar to each other (average $r^2 = 0.935$). Tangent modulus served as our measure of tendon stiffness.

TA muscle contractile properties. The distal tendon of the TA was exposed by an incision at the ankle of anesthetized mice. The tendon was cut several millimeters distal to the end of the muscle. The tendon was tied with 4.0 nylon suture as close to the muscle attachment as possible, and the tendon was folded back onto itself and tied again. The mouse was placed on a heated platform maintained at 37°C and the hind limb was secured by pinning the distal femur and clamping the foot to the platform. The tendon was tied securely to the lever arm of a servomotor (Model 305B, Aurora Scientific, Richmond Hill, ON, Canada). The tendon and exposed muscle were kept moist by periodic applications of isotonic saline. The TA muscle was stimulated with

0.2 ms pulses (Grass, model S88) via two needle electrodes that penetrated the skin on either side of the peroneal nerve near the knee. Stimulation voltage and subsequently muscle length (L_o) were adjusted for maximum isometric twitch force (P_t). While held at L_o, the muscle was stimulated at increasing frequencies, stepwise from 150 Hz by 50 Hz, until a maximum force (P_o) was reached, typically at 250 Hz. A one- to two-minute rest period was allowed between each tetanic contraction. Muscle length was measured with calipers, based on well-defined anatomical landmarks near the knee and the ankle. Optimum fiber length (L_f) was determined by multiplying L_o by the TA L_f/L_o ratio of 0.6 (7).

Transmission electron microscopy. One TA tendon was removed from three anesthetized mice in each age group. Immediately after excision, tendons were fixed in primary fixative (2.5% glutaraldehyde in 0.1 M Sorensen's buffer), stored at 4°C overnight and then post fixed (1.0% osmium tetroxide in 0.1 M Sorensen's buffer). Samples were then rinsed with Sorensen's buffer, dehydrated in ascending strengths of ethanol, cleared in propylene oxide and embedded in Epon according to standard procedures. Two transverse sections were taken from each tendon using a Reichert-Jung Ultracut E microtome, one ~1.5mm proximal to the bone insertion, and one ~1.5mm distal to the muscle insertion. Sections were stained with uranyl acetate and lead citrate, and viewed using a Philips CM 100 TEM at an accelerating voltage of 60kV. Ten random micrographs were obtained from each section (64,000X magnification, 2.97μm^2 each). The six tendons thus yielded a total of 120 images. The major and minor axes of all complete fibrils in each micrograph were measured using ImageJ software (NIH).

Fibril diameter was defined as the major axis for each fibril. Fibril area was calculated using each fibril's major and minor axes and fitting to an ellipse. Packing fraction was defined as the proportion of area in each micrograph occupied by collagen fibrils. Data from all ten images from each section were combined and considered representative of the fibril profile for that tendon region. All measurements were performed by a single, blinded investigator.

Statistics. Values are given as means ± SD. Statistical analyses were performed using SigmaPlot software (San Jose, CA). Differences in mean values for both tendon regional mechanical and collagen fibril data were determined using a two-way analysis of variance (ANOVA), with age and tendon region as independent factors. Differences in mean values for muscle contractility data were determined using a Student's t-test. Significance was set at $p < 0.05$.

Results

Tendon morphology. Within each age group, tendon CSA gradually increased from the distal bone insertion to the muscle, but there were no age-associated changes seen in regional tendon CSA (Table 2.1). Similarly, tendon length remained constant with age.

Regional stress-strain responses. Tendons from both adult (Figure 2.2a) and old (Figure 2.2b) mice show regional variations in their stress-strain responses. For both age groups, strains were smaller and stresses larger in the distal end compared with the other tendon regions. In addition, end-to-end tendon responses were different from any of the individual regional responses. In adult mice, the proximal region of tendons

displayed a large toe region that was sharply truncated for tendons of old mice. Maximum strain achieved during the single cyclical test also decreased with old age in the proximal tendon region (p = 0.020). Finally, for the end-to-end response of tendons of old mice, maximum strains were substantially decreased (p = 0.018) and toe regions diminished compared with those observed for tendons of adult mice.

Regional differences in tangent modulus. Maximum tangent modulus varied dramatically along the length of TA tendons displaying an increase of approximately four-fold between the proximal and distal regions for adult mice (Figure 2.3a). In all tendon regions, modulus increased significantly with old age, but the increase was not uniform; rather, it was substantially more pronounced in the proximal tendon region compared with the other regions. The non-uniformity of the changes with aging along the tendons resulted in a loss of heterogeneity in mechanical properties such that the range in modulus values between the proximal and distal ends was only ~2.5-fold for old mice. Tangent modulus determined from the overall end-to-end stress-strain responses was also greater for tendons of old compared with adult mice at both maximum tendon strain (799 ± 157 MPa vs. 1419 ± 91 MPa, p < 0.001) and at 1.5% tendon strain (377 ± 137 MPa vs. 798 ± 104 MPa, p < 0.001) (Figure 2.3b).

Collagen fibril distribution. Representative TEM images and tendon collagen fibril diameter distributions are shown in Figure 2.4. Regional dependency of fibril density was only observed in tendons from old mice, where the number of fibrils per square micrometer was greater in the distal region than in the proximal region (103 ± 21 fibrils/μm^2 vs. 49 ± 7 fibrils/μm^2, p = 0.030) (Table 2.2). In contrast to the fibril density,

fibril packing fraction, which measures how much of a given area is occupied by fibrils, was greater in the proximal region as compared to the distal region for tendons of both age groups (adult: 0.90 ± 0.03 vs. 0.79 ± 0.03, p = 0.046; old: 0.88 ± 0.03 vs. 0.76 ± 0.09, p = 0.022). No effect of age on packing fraction was observed (Table 2.2). Mean fibril diameter was similar across all age groups and tendon regions. Despite no difference between tendon regions nor between tendons of adult and old mice for mean fibril diameter, the proportion of small diameter fibrils (<60 nm) in tendons of old mice was greater in the distal tendon region (0.311 ± 0.079) compared with that for both the proximal region of the same tendons (0.211 ± 0.023, p = 0.033) and the distal region of tendons from adult mice (0.213 ± 0.065, p = 0.025) (Figure 2.5a). For both age groups, there was a greater proportion of large diameter fibrils (>160 nm) in the proximal region than in the distal region (adult: 0.281 ± 0.006 vs. 0.098 ± 0.057, p < 0.001; old: 0.294 ± 0.023 vs. 0.041 ± 0.039, p < 0.001), but no changes were seen with aging (Figure 2.5b).

TA muscle contractile properties. TA muscle mass decreased by ~30% between adulthood and old age (p = 0.003, Table 2.3). Despite the substantial TA muscle atrophy, maximum isometric force was not different between the age groups (Table 2.3). The maintenance of force generating capability with age was due in part to architectural changes within the muscle. A decrease in fiber length of ~15% was observed for muscles of old compared with adult mice (p = 0.014) that partially compensated for the decreased mass to maintain force levels.

Discussion

The major finding of the present study was that TA tendons of mice display regionally dependent changes with aging in mechanical properties. We showed that tangent modulus of TA tendons increased sharply for tendons of old compared with adult mice, with the most pronounced increase occurring in the proximal region of the tendon nearest the muscle. Also significant was the finding that the mechanical changes were not accompanied by major changes in collagen fibril morphology. Moreover, the maximum force generating capacity of TA muscles remained relatively constant throughout the age range studied. These results indicate that aging has a considerable effect on the mechanical characteristics of TA tendons that is independent of collagen morphology or skeletal muscle contractile force.

Data in the literature regarding the impact of aging on tendon mechanical properties are inconsistent. The modulus of rat tail tendons has been reported to both increase (34) and remain constant (17, 35) from young adulthood to old age. Additionally, some studies show that the modulus of human patellar tendons decreases with advancing age (2) but others failed to show such declines (8, 10, 14). The discrepancies likely arise in part from methodological differences in the procedures used for mechanical testing. When tendons are severed from their bone and muscle attachments and gripped directly, collagen fibril slippage and stress-strain concentrations can be introduced at the grip interfaces. These artifacts can result in measured mechanical properties that are underestimated by up to 50% (20, 37). Further disagreements in the literature may be due to difficulties in comparing results from *in*

vivo and *in vitro* experiments. *In vivo* testing requires that the tendon be loaded by the subject's own muscle contraction, a situation under which the rate of tendon loading cannot be easily controlled. Tendon is a viscoelastic material whose mechanics are dependent on the rate of loading (9, 31), and as such, any inconsistency in that variable has the opportunity to affect the measured mechanical properties. By keeping the muscle-tendon unit intact and controlling the rate of tendon loading, the testing method presented in this paper minimizes damage to the tendon and creates uniformity across age groups in parameters that are important for consistent mechanical properties to be determined in the tissue.

The present data corroborate the findings of Arruda and colleagues (1) that the TA tendon is functionally graded, with a compliant proximal region and a substantially stiffer region towards the bone insertion. The patellar tendon has also been shown to possess regional variations in mechanical properties (18, 20). Thus, tendons should be considered heterogeneous in mechanics studies, and the mid-tendon mechanical response typically will not represent the response of the tendon as a whole (present study, 1). Moreover, global mechanical measurements mask the complicated regional behavior of the tissue (1). This study expands upon the work of Arruda and colleagues (1) by establishing that the tangent modulus of TA tendons increases dramatically with advanced age. The observation that the increases were more pronounced in the proximal tendon region with age also demonstrates that the effects of aging cannot be assumed to be uniform across all tendon tissues. Furthermore, prominent increases in tangent modulus were observed at physiologically relevant strain levels. *In vivo* data

suggest that human tibialis anterior tendons experience strains between 0% and 2.5% during normal physiological movement (26). In the present study, tendons from old mice showed two-fold larger modulus when compared to younger animals within that strain range. Therefore, a complete characterization of changes in tendon mechanical properties with aging cannot be determined unless regional properties and various strain levels are examined.

Our data demonstrated that despite a significant age-associated increase in tangent modulus for all tendon regions, fibril morphology remained relatively constant from adulthood to old age. Furthermore, fibril packing fraction was significantly greater in the proximal tendon region than in the distal region, indicating that a larger volume of collagen did not lead to increased tendon stiffness. The lack of an association between fibril morphology and mechanical properties is contrary to studies that have established a positive correlation between tendon mechanical properties and collagen fibril diameter (13) and packing fraction (17). These correlations, however, were generally established in tendons from young animals. As such, the observed correlations may be coincident with additional changes associated with biological development and maturation. Furthermore, assuming that collagen fibrils can be considered as cylindrical rods, from a purely mechanical standpoint, the tangent modulus of a fibril should be independent of the fibril's diameter (15). The maximum tangent modulus of a collagen fibril is dependent on the properties of the fibril itself, and is independent of the amount of material present. In contrast, an increased diameter of the fibril will affect its bending stiffness (15), which may be of consequence

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at low strain levels where the mechanical response to the strain is associated with the uncrimping of collagen fibrils (19). A comprehensive study of low-strain tendon mechanical properties coupled with structural analysis would be required to address the hypothesis that fibril diameter influences crimp behavior.

Tendons are responsible for transferring the force produced by the muscle to the skeleton with high fidelity while avoiding musculoskeletal injury. Thus, muscle and its associated tendon must function in concert and the mechanical properties of one tissue will undoubtedly influence the mechanical response of the other. In fact, *in vivo* investigations have demonstrated that tendon stiffness positively correlates with muscle power in young adult humans (3). Despite this important combined functional role, the association between muscle force output and tendon mechanical properties in aged animals and humans is poorly understood. The significant reductions in mass and force output with aging are well documented in many muscles (4, 5, 12). Since tendon tissue clearly adapts to its loading environment (for reviews see 6, 23), decreased muscle force output has the potential to dramatically affect the behavior and properties of the tendon. A substantial effect of reduced loading by the muscle on tendon was demonstrated by the findings of Arruda and colleagues (1) of a dramatic increase in TA tendon modulus following a five week period of muscle denervation. Despite the dramatic age-associated increase in modulus seen in TA tendons in the present study, associated declines in TA muscle maximum force generation were not observed. The relative constancy of TA force generation across the age groups studied indicates that the increased modulus we observed in tendons from old animals was likely caused by

intrinsic age-related changes in the tendon tissue itself rather than an adaptation to alterations of muscle force generating capability.

Our study is not without limitations. Ideally, mechanical and structural properties would be determined from the same tendons. We performed our EM analysis on fresh tendons fixed immediately upon removal from the animal to minimize degradation of the tissue or artifacts in structure resulting from the mechanical testing. Consequently, we are assuming that the mechanical properties of the tendons on which we performed the structural analysis were similar to those measured on a separate group of tendons from mice of the same age and strain. Furthermore, although we examined EMs from random areas within tendon cross-sections, regional differences in collagen fibril distributions may exist and we cannot rule out the possibility that specific regions of some cross-section were inadvertently favored. In addition, our reported overall tendon strain values fall below the imposed strain level of 10%. This is primarily a result of the bead placement, which leaves a portion of the tendon unsampled. Measuring strain over the entire muscle-tendon unit length would alter our results most noticeably by increasing strain values in the compliant proximal region, accentuating the local variations in mechanical properties rather than eliminating them. Finally, it is important to acknowledge that the results of this investigation may not generalize to all tendons or all species. Specific tendons are uniquely suited for specific functions. The TA tendon is the primary dorsiflexor of the mouse hind limb and is therefore highly important for locomotion, but differences in loading environment of other tendons may affect how individual tendons respond to aging. Additionally, the TA muscle is

primarily composed of fast-twitch fibers, and as such, our results may not generalize to tendons with attached muscles of mixed or slow fiber types.

In conclusion, we demonstrated that the tangent modulus of TA tendons increases in a region-specific fashion throughout the life span, with the most pronounced increases occurring in the proximal tendon region. This increase was not accompanied by dramatic alterations in collagen fibril morphology, suggesting that fibril size is not a significant contributor to the changes in tendon mechanical properties with aging. We also showed that the force-generating capacity of TA muscles did not decrease significantly over the age range of the present study, providing evidence that age-related changes seen in TA tendon mechanics are not an adaptation to wasting and weakness of the TA muscle but are likely a result of intrinsic alterations in the tendon tissue itself. These results have important implications for understanding the natural aging process in tendons that may help elucidate the underlying causes behind the increasing incidence of tendon dysfunction in the elderly.

Acknowledgements

tendons of mice occurs independent of changes in collagen fibril morphology. J Appl

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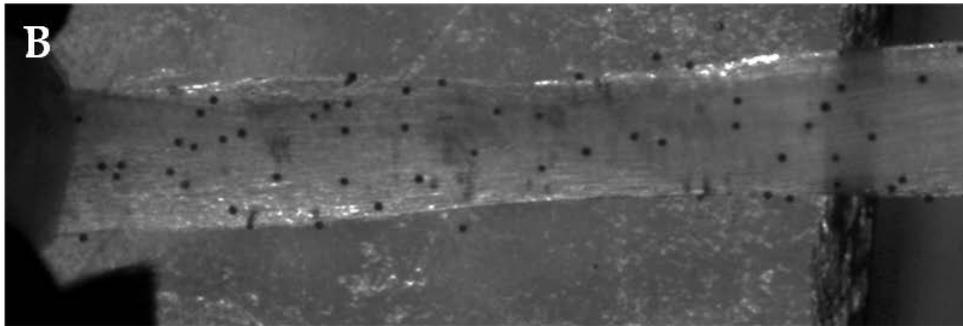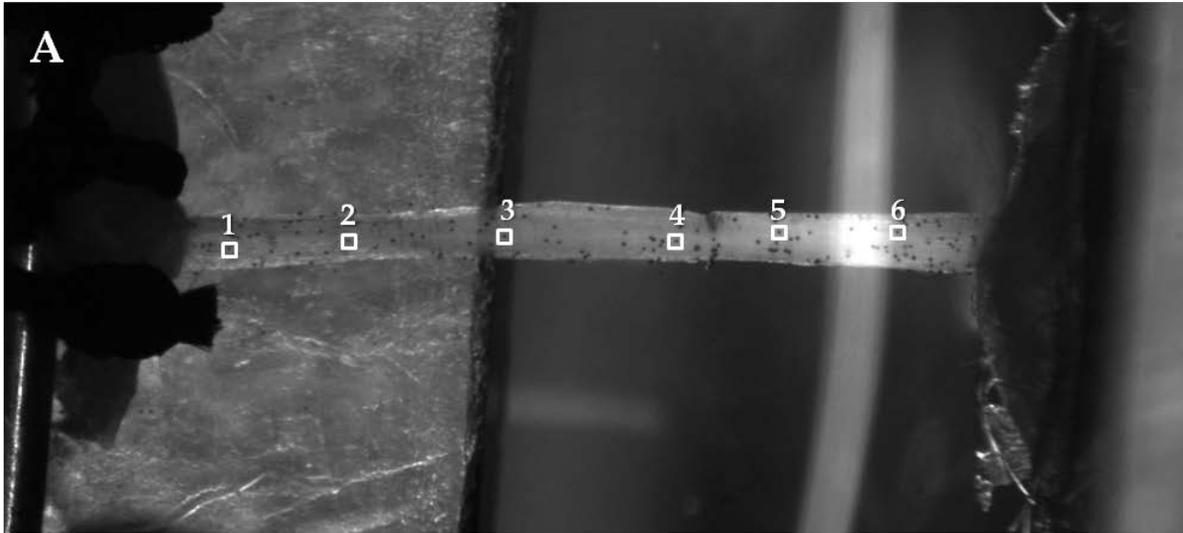
Figure 2.1. Mouse tibialis anterior (TA) muscle-tendon unit positioned in mechanical test fixture. A: Figure showing typical measurement regions along the tendon. Mechanical properties were determined at the distal (points 1 and 2), central (points 3 and 4) and proximal (points 5 and 6) tendon regions. Overall tendon response was also measured (points 1 and 6). Samples were loaded to 10% grip-to-grip strain at a rate of 0.01/second. B: Close-up of distal region of the tendon, showing the distribution of microspheres used to optically determine surface strains. Contrast has been increased in both images.
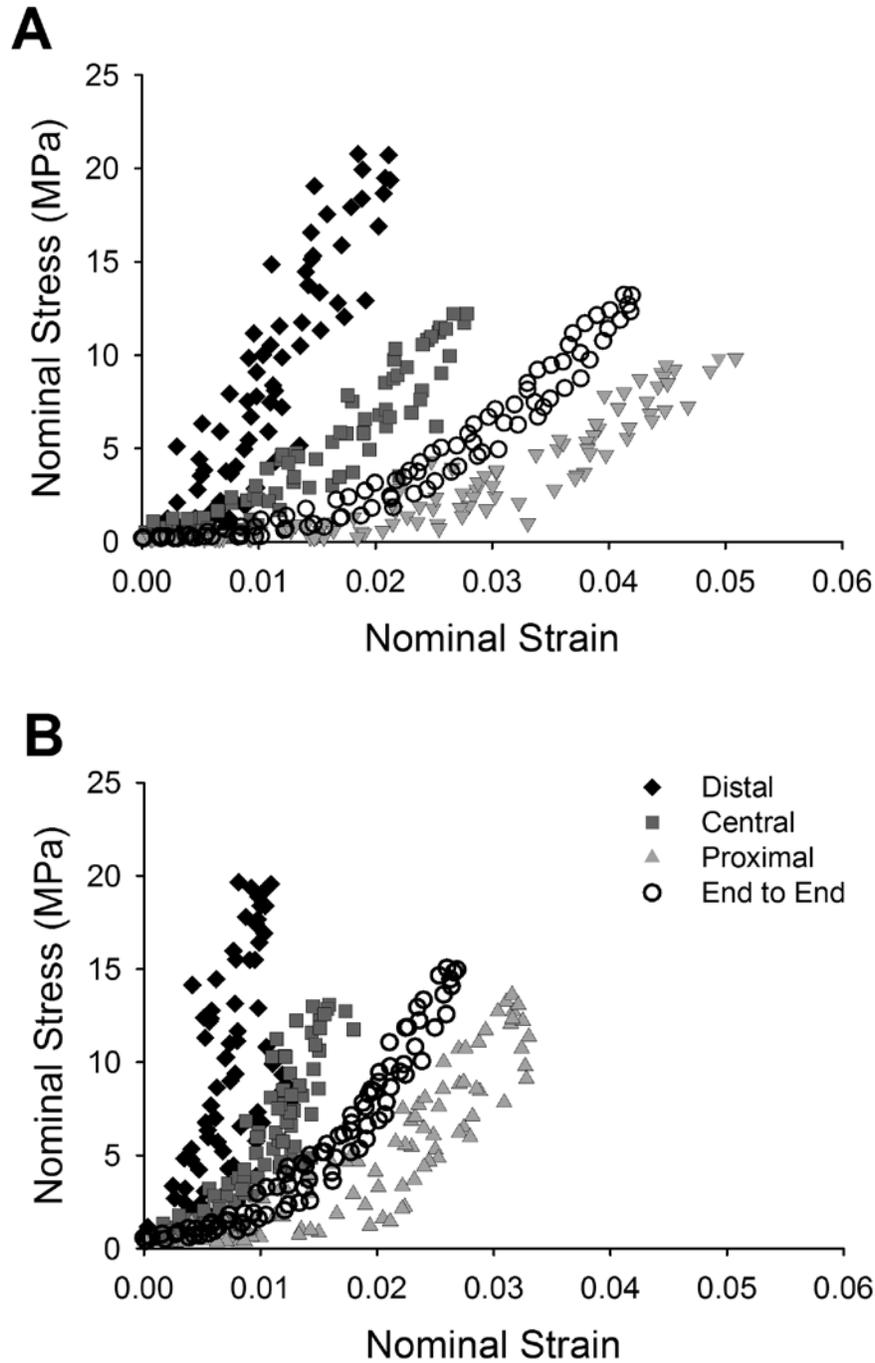
Figure 2.2. Representative stress-strain responses of TA tendons from adult (a) and old (b) mice undergoing a 10% grip-to-grip strain load-unload cycle. In both age groups tendon mechanical response is regionally dependent, with the distal region (diamonds) showing greater stresses and lower strains than the central (squares) and proximal (triangles) regions. Also, overall tendon response (circles) differs significantly from any of the regional responses. Maximum strains in the central and proximal tendon regions diminish in old age, leading to a stiffer tendon overall.
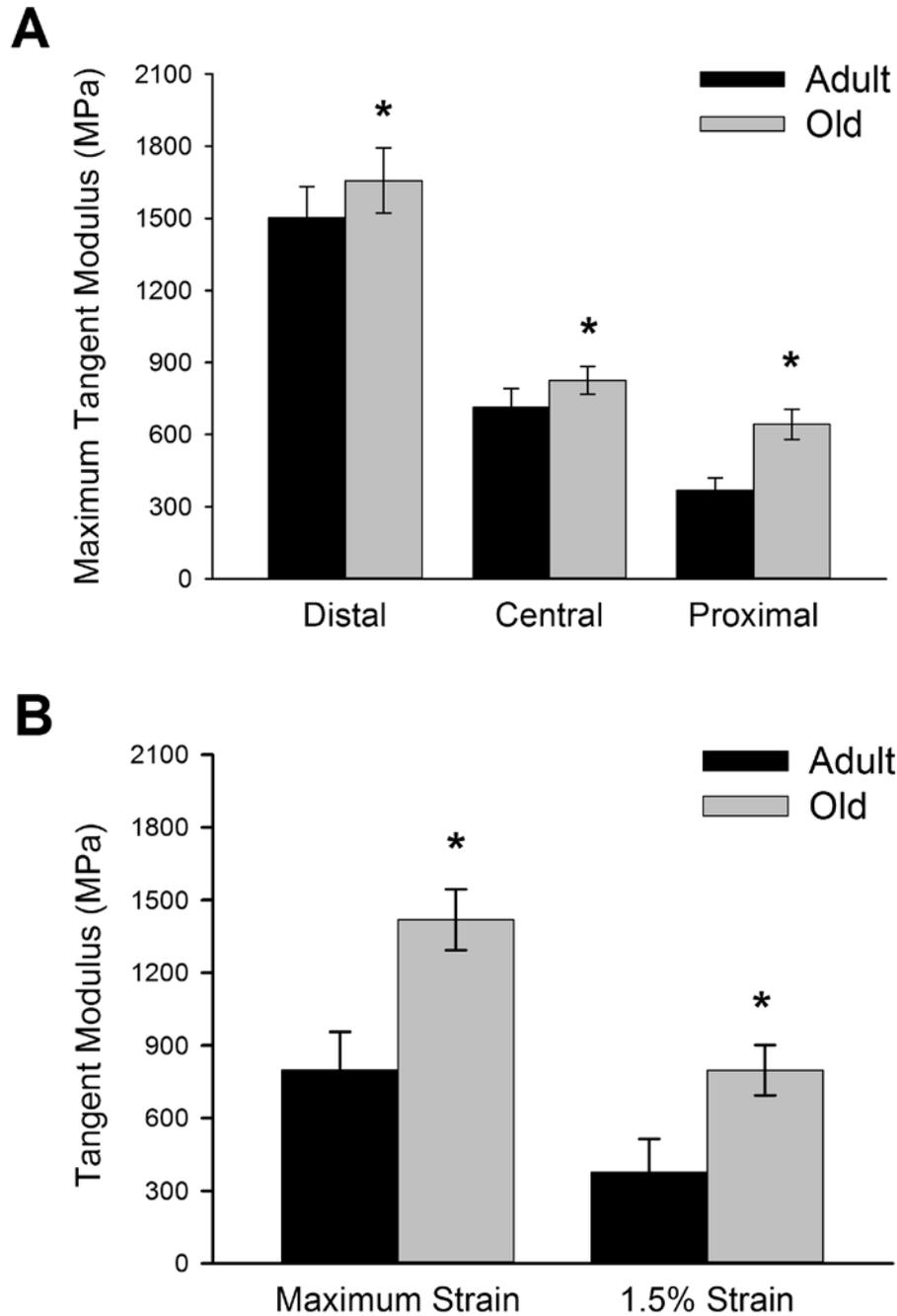
Figure 2.3. The effect of aging on the regional (a) and overall (b) tangent modulus of mouse TA tendons. In both age groups, maximum tangent modulus was greatest in the distal region and smallest in the proximal region of the tissue. Tendons from old mice showed increased modulus values among all tendon regions when compared to adult mice. End-to-end tangent modulus also increased with old age at maximum tendon strain and at 1.5% tendon strain. Data presented as means ± SD. *Indicates significantly different from adult age group ($p < 0.05$).
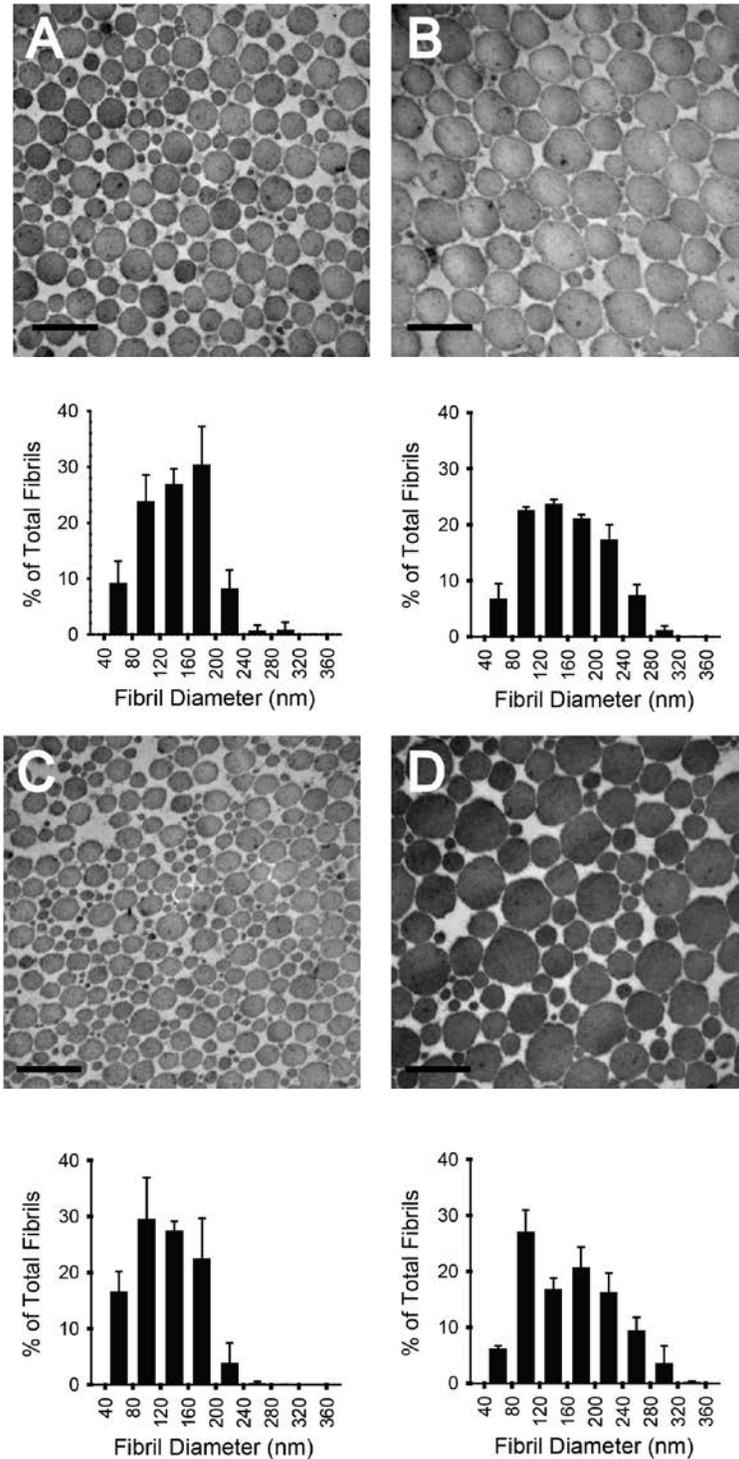
Figure 2.4. Representative electron micrographs and diameter distributions of collagen fibrils within the distal (left column) and proximal (right column) regions of TA tendons of adult (a and b) and old (c and d) mice. In both age groups, fibrils in the proximal region tended to have larger diameters than those in the distal region. Images taken at 64,000 x magnification. Scale bars = 300 nm.
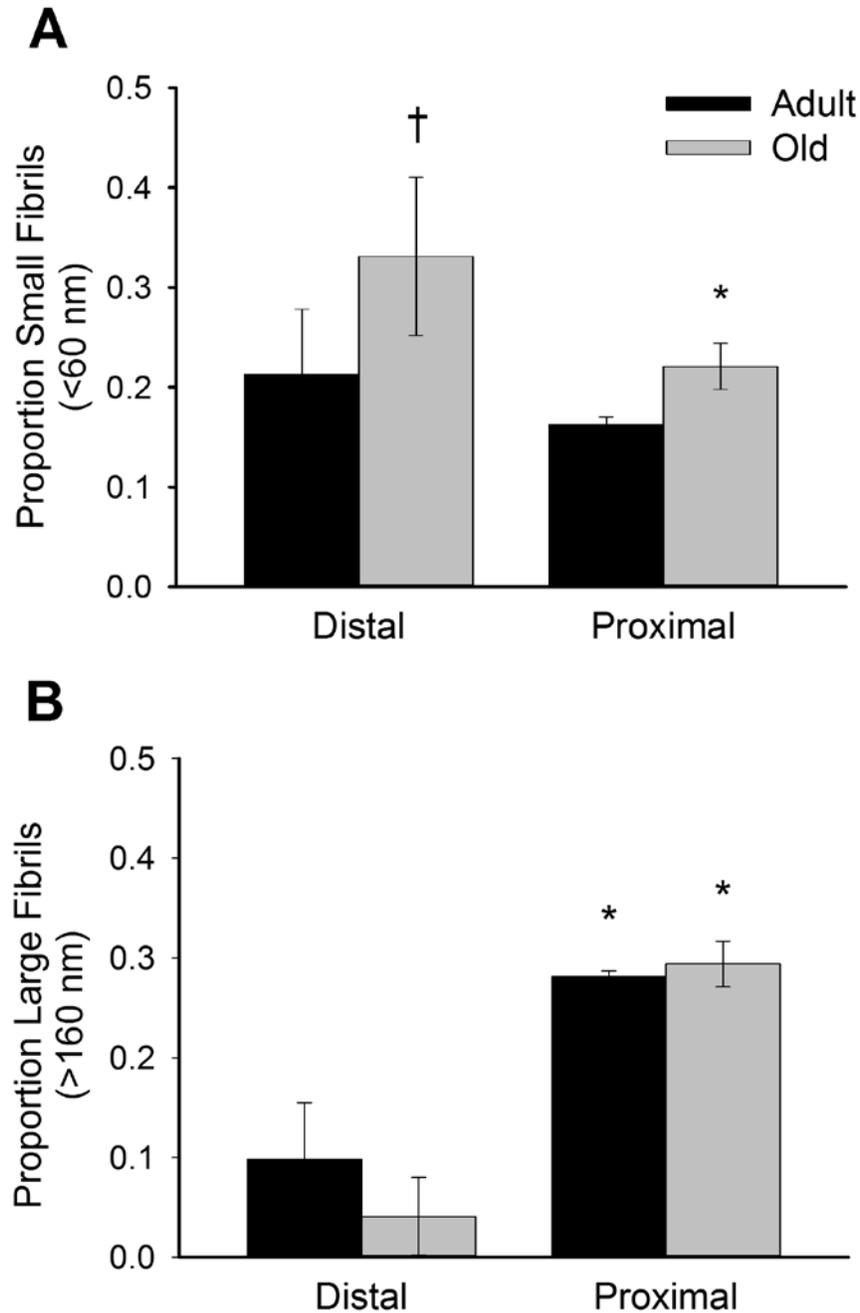
Figure 2.5. Proportion of small (a) and large (b) collagen fibrils in TA tendons. There was a significantly greater proportion of large fibrils in the proximal region than the in distal region for both age groups. The only age-related change seen was an increase in the proportion of small fibrils in the distal tendon region. Data presented as means ± SD. *Within age group, significantly different than distal region (p < 0.05). †Within tendon region, statistically different than adult age group (p < 0.05).

	Adult	**Old**
Length, mm	6.90 ± 0.19	6.94 ± 0.21
CA, mm^2		
Distal	0.048 ± 0.011	0.050 ± 0.011
Central	0.078 ± 0.008	0.084 ± 0.014
Proximal	0.099 ± 0.010	0.098 ± 0.010
Overall	0.074 ± 0.009	0.077 ± 0.009

Table 2.1. TA tendon morphological properties from adult and old BL6 mice. Values given as means ± SD; CSA, cross-sectional area; n = 10 (adult) and 5 (old); all properties were similar between age groups.

	Fibril Density (#/μm²)		Mean Fibril Diameter (nm)		Packing Fraction	
	Distal	Proximal	Distal	Proximal	Distal	Proximal
Adult	78 ± 20	53 ± 5	97.6 ± 49.3	80.4 ± 46.2	0.79 ± 0.03	0.90 ± 0.03[*]
Old	103 ± 21	49 ± 7[*]	105.6 ± 67.2	97.4 ± 74.2	0.76 ± 0.09	0.88 ± 0.03[*]

Table 2.2. Summary of TA tendon collagen fibril properties. Data presented as means ± SD. *Within age group, significantly different from distal region ($p < 0.05$).

	Adult	**Old**
Mass	56.7 ± 5.0	43.6 ± 7.6*
L_f, mm	9.36 ± 0.49	8.27 ± 0.74*
P_o, mN	1464 ± 236	1371 ± 238

Table 2.3. TA muscle morphological and contractile properties from adult and old BL6 mice. Values given as means ± SD. L_f, optimal fiber length; P_o, maximum isometric force; see methods for calculations; n = 8 (adult) and 6 (old). *Statistically different between age groups at p = 0.05.

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Chapter III

Ten weeks of treadmill running decreases stiffness and increases collagen turnover in tendons of old mice

Abstract

An increase in tendon stiffness in response to mechanical loading is well established in young animals. Given that tendons stiffen with aging, we aimed to determine the effect of increased loading on tendons of old animals. We subjected 28-month-old mice to ten weeks of uphill treadmill running; sedentary 8- and 28-month-old mice served as controls. Following training of old mice, plantaris tendon stiffness was reduced by approximately half, from the value for tendons of sedentary old mice to a level not different from that of adult animals. The restoration of "adult" mechanical properties was accompanied by a similar elimination of the age-related increase in advanced glycation end-product protein adducts. Elevated mRNA levels for collagen type 1, matrix-metalloproteinase-8, and lysyl oxidase following training suggest that collagen turnover was also increased. The dramatic mechanical and structural changes induced by treadmill training occurred independent of changes in cell density or tendon morphology. Finally, the extent of Achilles tendon calcification was significantly reduced following exercise, although restoration of adult levels was not achieved. These

results demonstrate that, in response to exercise, tendons from old animals are capable of replacing damaged and dysfunctional components of tendon extracellular matrix with tissue that is mechanically and structural similar to that of healthy adult tissue.

Introduction

Over one-third of persons over age 70 are estimated to experience some level of tendon degeneration or tearing (26). The high incidence of tendon dysfunction in the elderly may arise due to age-associated changes in tendon mechanical properties. We previously showed that mouse tibialis anterior tendon stiffens with aging (Chapter II). Given that the mechanical properties of tendon influence the force and power output of muscle (2), changes in tendon stiffness have the potential to severely affect range of motion and mobility (19). Tendon mechanical properties are determined by the underlying composition, which is primarily fibrillar type I collagen (COL1). Our previous observation (Chapter II) that age-associated tendon stiffening occurred in the absence of changes in collagen fibril morphology suggests that the material properties of the fibrils themselves are altered during aging. The mechanical integrity of collagen is achieved through a balance between the deposition and crosslinking of newly synthesized collagen and collagen degradation. The extremely slow rate of collagen turnover (27) allows for the accumulation of post-translational modifications including advanced glycation end-products (AGEs). AGEs form permanent molecular crosslinks that stiffen collagen fibrils (20) and may play an important role in compromising the

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mechanical integrity of tendon. Likewise, calcium deposition in tendon (18) can increase brittleness and further compromise the tissue.

Despite dramatic age-associated increases in tendon stiffness, there is a lack of knowledge regarding methods of delaying or preventing the changes. Increased tendon stiffness following exercise in adult mammals is well established (6, 14). Exercise in adult animals also induces structural changes in tendon by promoting increased expression of COL1 (11), and inducing hypertrophy and increased cellularity (16). Although there are reports that patellar tendon stiffness increases following resistance training in the elderly (21) and that lifelong treadmill running attenuates age-associated increases in rat tail tendon stiffness (28), the responses of load-bearing tendons from old mammals to treadmill running has not been adequately investigated. Our aim was to determine the impact of ten weeks of treadmill training on tendons from old mice. We hypothesized that treadmill training would attenuate age-associated changes in tendon mechanical properties by means of enhanced degradation of long-lived "aged" collagen and increased deposition of healthy "young" collagen.

Methods

Animals. Seventeen 28- to 30-mo-old (old) and five 8-mo-old (adult) C57BL/6 male mice were obtained from Charles River Laboratories (Wilmington, MA). Body masses were 34.6 ± 1.7 g and 32.4 ± 1.7 g (mean ± SD, $p < 0.01$) for the adult and old mice, respectively. Animals were housed in a specific-pathogen-free barrier facility in the Unit for Laboratory Animal Medicine at the University of Michigan, with food and

water provided *ad libitum*. All procedures were approved by the University Committee for the Use and Care of Animals.

Treadmill training. Eleven old mice (old trained) were randomly assigned to perform a 5 day/week, 10-week uphill treadmill running program. Following acclimation to the treadmill, mice were subjected to 30-minute running bouts of gradually increasing intensity, such that by the end of 10 weeks, mice were running at 13 m/min up a 6° incline. The remaining old mice (n = 6) (old sedentary) and all adult mice (n = 5) (adult) were restricted to normal cage activity and served as sedentary controls. After the ten weeks, body mass of the old sedentary controls increased (34.9 ± 1.2 g vs. 32.4 ± 1.7 g, p = 0.0020), whereas body mass of treadmill trained old mice did not change (32.6 ± 2.3 g vs. 32.4 ± 1.7 g, p = 0.80).

Tissue extraction. Two days after the final training bout, mice were anesthetized with an intraperitoneal injection of tribromoethanol (250 mg/kg). The plantaris tendon unit (plantaris muscle, plantaris tendon, and calcaneus) was removed from both hindlimbs, and tendons were stored in sterile PBS at 4°C for at most 4 hours before mechanical testing. Achilles and tibialis anterior (TA) tendons were also removed and snap frozen in liquid nitrogen, frozen in Tissue Tek for histology, or prepared for mRNA isolation.

Determination of tendon mechanical properties. In total, tendons from six adult, twelve old sedentary, and sixteen old treadmill-trained mice were mechanically tested. The stress-strain response of each plantaris tendon unit was determined as previously described (Chapter II). Briefly, the plantaris tendon unit was secured to custom grips in

a PBS bath, and 25µm polystyrene beads (IMT Laboratories, Irvine, CA) were brushed along the tendon to serve as optical markers. Cross-sectional area (CSA) was determined by measuring diameter at three points along the tendon at 0° and 90° rotation and fitting the values to an ellipse. Tendons were subjected to a load-unload cycle of 10% grip-to-grip strain at a constant strain rate of 1% per second. Synchronized force and image recordings were obtained using custom LabVIEW software (Austin, TX). The positions of beads in the proximal (near the muscle) and distal (near the bone) regions of the tendon were tracked with MetaMorph (Molecular Devices, Sunnyvale, FL), and strains were calculated as the change in separation between two beads in each region divided by their initial separation. Regional stress was determined by dividing loading data by the local CSA. Stress-strain data were fit to a third-degree polynomial, and maximum tangent modulus (ie, tendon stiffness) of each tendon region was calculated as the maximum slope of the curve.

Histology. Twelve micrometer cryosections were taken from the distal and proximal plantaris tendons from five tendons in each group. Separate sections from each tendon were stained with hematoxylin and eosin for visualization of cell nuclei and cytoplasm, or with the Herovici stain (7), which reveals "young" newly synthesized collagen as blue and "aged" long-lived collagen pink. Percent "young" collagen was quantified using ImageJ (NIH, Bethesda, MD).

AGE adduct content. Four TA tendons from each experimental group were finely homogenized and digested into peptides using a solution of 40 mg/mL proteinase K in

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PBS at 55ºC , and an enzyme-linked immunosorbent assay (ELISA) kit (Cell Biolabs, San Diego, CA) was used to determine the concentration of AGE protein adducts.

Gene expression. Total RNA was isolated by homogenizing three Achilles tendons from each group in Qiazol tissue lysis reagent (Qiagen, Valencia, CA). mRNA was isolated using a miRNeasy Mini Kit (Qiagen). Reverse transcription was performed using a QuantiTect Reverse Transcription kit (Qiagen), and cDNA was amplified in a CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA) using a QuantiTect SYBR Green I PCR Kit (Qiagen). Reactions were performed in duplicate. The specificity of amplification was verified with melting curve analysis. Primers for collagen I (COL1A1), matrix-metalloproteinase 8 (MMP8) and lysyl oxidase (LOX) were obtained from Qiagen. Custom primers for scleraxis (SCX; 15) and TGF-β (25) were obtained from IDT (Coralville, IA). Relative copy number for each sample was determined using the linear regression of efficiency method, which captures amplification efficiency for each reaction (23).

Tendon calcification. Frozen Achilles tendons were sectioned at 12μm in the central region and stained with 0.5% Alizarin Red [n =3 (adult,), 4 (old sedentary), and 4 (old trained)]. Percent area calcified was determined with ImageJ.

Statistics. Data are reported as means ± SD. Statistical analysis was performed using SigmaPlot (San Jose, CA). Differences in mean values for regional mechanical and histological data were determined using a one-way ANOVA. Significance was set at p < 0.05.

Results

Cell density and morphology. Plantaris CSA increased ~20% in the distal tendon region following treadmill training (Table 3.1) but did not change in the remainder of the tendon. Plantaris tendon length was similar among all three groups, and tendon fibroblast density remained unchanged with both aging and treadmill training (Figure 3.1).

Maximum tangent modulus. Plantaris tendon maximum tangent modulus increased with aging in both the distal and proximal regions (Figure 3.2). Although tangent modulus was not different between the proximal and distal regions, age-associated increases were more pronounced in the proximal region, for which modulus increased 2-fold compared with the distal region that showed a 70% increase in modulus. Following training, maximum tangent modulus decreased in both regions to levels similar to adult control values (Figure 3.2).

AGE adduct levels. AGE adduct concentration increased 60% with aging in TA tendons, whereas treadmill training decreased AGE adducts to a value not different from adult controls (Figure 3.3).

Gene expression. The effect of age and treadmill running on mRNA levels for key proteins associated with turnover of tendon extracellular matrix in Achilles tendons is shown in Figure 3.4. A 4.5-fold decrease in COL1A1 expression was seen with age, and expression of LOX, MMP8, and SCX was also severely attenuated, with decreases of 10-to-20-fold. With training, COL1A1 and MMP8 expression were restored to levels similar

to adult controls. TGF-β was also evaluated, but showed no effect of age or training (data not shown).

Deposition of new collagen. As demonstrated with Herovici polychrome stain, newly synthesized collagen made up a similar percentage of total collagen CSA in plantaris tendons from adult and old sedentary mice, and was independent of tendon region (Figure 3.5). Treadmill training induced the deposition of newly synthesized collagen as evidenced by an increase compared with age-matched sedentary controls in the area of brightly blue stained portions of tendon cross sections in both distal ($34.5 \pm 5.2\%$ vs. $14.9 \pm 8.2\%$, $p < 0.05$) and proximal (30.6 ± 4.2 % vs. 14.0 ± 7.8 %, $p < 0.05$) regions.

Area calcified. Alizarin Red stain revealed minimal calcification of adult Achilles tendons (Figure 3.6) that increased over 10-fold in old age. The extent of calcification was reduced by ~40% following training, although complete restoration to the characteristics of the tendons from the adult mice was not achieved.

Discussion

The major finding of the present study was that ten weeks of treadmill training restored the mechanical properties and collagen characteristics of hindlimb tendons of old mice to values similar to those of adult mice. Moreover, dramatic changes occurred in mechanical properties independent of substantial changes in cell density or tendon morphology. We also demonstrated that age-associated Achilles tendon calcification was significantly reduced following treadmill training. These findings confirm our

previous reports of dramatic age-associated increases in tendon stiffness (Chapter II), and demonstrate that even at advanced age, tendon is capable of adapting to an increased loading environment through altered structural and mechanical properties.

The reduction in plantaris tendon stiffness observed in the present study following treadmill training of old mice opposes the widely reported response of increased tendon stiffness in adult animals in response to exercise. For example, treadmill training increased the modulus of swine digital extensor (29) and rat Achilles tendons (6), and in humans, habitual badminton playing and fencing resulted in elevated patellar tendon stiffness (4). The effect of exercise on tendons of old animals has been poorly investigated, but there is evidence that the mechanical adaptation of tendon is highly influenced by the nature of the loading. Resistance training increases patellar tendon stiffness in elderly humans (21), whereas life-long treadmill training slowed the age-associated increase in rat tail tendon modulus (28). This study expands upon those results by demonstrating that treadmill training started late in life reduces modulus of tendons of old mice to levels similar to values of adult tendons.

Dissimilar mechanical responses to exercise between tendons in adult and old animals suggest a difference in the underlying molecular response to the loading. Mendias and colleagues recently demonstrated that tendons in adult mice adapt to treadmill training with increased cell proliferation (16), an adaptation that was not observed in the current study. In addition, treadmill training induced tendon hypertrophy in adult animals (16), but the decreases in tangent modulus in both the distal and proximal tendon regions in the present study occurred with only modest

hypertrophy in the distal region alone. As such, mechanical adaptations seen in the current study arise from changes in extracellular matrix composition and/or structure rather than an increased volume of the tissue. Additionally, the present study demonstrated that cell proliferation is not required for mechanical adaptations, and that tendon fibroblasts retain the ability to translate external stresses into measurable tissue remodeling in old animals.

Tendon mechanics are determined by the composition of the underlying components, the most abundant of which is fibrillar collagen. LOX and MMPs are responsible for maintenance of the mechanical integrity of collagen fibrils through crosslinking of newly synthesized collagen and collagen degradation, respectively. The observation in the present study of severe attenuation in COL1A1 expression in tendons of old mice is consistent with previous findings in rat tendon (10). Coupled with sharp age-associated declines in LOX and MMP8 expression, the current study provides evidence that collagen turnover in tendon is severely limited in old animals. The long half-life of tendon collagen (27) makes it highly susceptible to the accumulation of AGE crosslinks, the levels of which are highly correlated with age (3). AGE crosslinks inhibit collagen synthesis and MMP activity (5) and stiffen collagen fibrils (20). In tendons from adult animals, treadmill training triggers an increase in both COL1A1 expression (11, 16) and MMP activity (9), and the present study demonstrated similarly enhanced expression in tendons of old animals following exercise. We also observed decreased concentration of AGE adducts and increased deposition of newly synthesized collagen following treadmill training in old mice. Thus, increased loading of the hindlimbs of old

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mice through uphill treadmill running initiated pathways responsible for degrading AGE crosslinks and elevating MMP expression in Achilles tendon, allowing for the deposition of newly synthesized collagen into the tissue.

SCX is a transcription factor that appears to play an important role in tendon adaptation to loading (16) by inducing fibroblast proliferation and directing collagen expression (12). SCX is expressed at low levels in tendons of adult mice (15) and is essentially absent in old age (present study). In adult mice, adaptive changes in tendons in response to exercise were associated with increased SCX expression (16), whereas the present study showed no increase in SCX expression in tendon following exercise in old mice. Additionally, expression of TGF-β, which induces the expression of SCX (13), remained unchanged with aging and was unaffected by treadmill training in old animals. The lack of an effect of exercise on TGF-β expression in tendons of old mice observed in the present study differs from increases in TGF-β observed following training in adult animals (6). These data thus suggest that in old animals, tendon molecular responses to increased loading shift to SCX- and TGF-β-independent mechanisms to induce adaptive changes.

This study also demonstrated a significant reduction of Achilles tendon calcification following treadmill running. Spontaneous, age-associated tendon calcification in humans is associated with pain (22) and tears (8), and serves to stiffen the tissue (24). The pathogenesis of tendon calcification is largely unclear, but given that calcified deposits can be spontaneously resorbed (17), chronic tendon calcification with aging may be related to a disruption of the mechanisms underlying normal calcium

resorption. A recent study showed that treadmill training removed chondroid deposits and restored tendon mechanical properties in an adult murine Achilles tendinopathy model (1). This previous finding in the tendinopathy model coupled with the present finding of reduced calcification in old mice with treadmill running provides evidence that treadmill training activates pathways responsible for the identification and removal of tendon pathological changes.

Our study is not without limitations. Although the Achilles tendon acts as the primary hindlimb dorsiflexor during normal locomotion, we used the plantaris tendon to measure the mechanical adaptation in mice following treadmill training. The Achilles tendon has a low length-to-diameter ratio and has collagen fibrils that are twisted along the longitudinal axis of the tissue, which increase the probability of artifacts during mechanical testing. The plantaris tendon, with its long length and longitudinally oriented collagen fibrils, is therefore better suited for mechanical testing. Based on its anatomical location and function, we are assuming that the plantaris tendon is also loaded during uphill running. Given the dramatic mechanical alterations seen in the plantaris tendon following the training, we are confident that the use of the plantaris tendon gives a proper indication of tendon adaptation to loading in old mice, although we cannot rule out the possibility that the adaptations in the plantaris are not directly due to increased loading. Given that the present study examined tendon mechanical and structural properties at one time point following exercise, the time course of changes in structural and mechanical properties is unknown. Also, the small size of mouse plantaris tendons required that analysis of HyPro and AGE adduct

concentration be conducted in the larger TA tendon. Therefore, the signals resulting in biochemical changes in the TA tendon following training may not be the same that resulted in the reduction in plantaris tendon stiffness. Additionally, sensitivity limitations to the qPCR protocol precluded regional gene expression from being obtained and also required that the analysis be conducted on the Achilles tendon which provides more tissue for analysis. Finally, given that this 10-week intervention represented close to 10% of the mean lifespan of this strain of mice, it is important to recognize that these findings may not be observed in shorter-term investigations. Nonetheless, these data provide important information regarding the ability of tendon in old animals to respond structurally and mechanically to increased loading.

In summary, we demonstrated that 10 weeks of treadmill training decreased maximum tangent modulus and increased collagen turnover in hindlimb tendons of old mice, independent of changes in cell density or tendon morphology. We also demonstrated a significant reduction of Achilles tendon calcification and AGE protein adducts in tendons of old mice following training. This study has provided evidence that the ability of tendon to adapt to changes in loading environment is sustained into old age. Tendons of old animals retain the ability to replace components of the extracellular matrix with tissue that is mechanically and structurally more similar to that of adult tendon. These results have important implications for understanding the response of tendon in old animals to external loading, which may help facilitate development of methods to reduce or prevent age-associated changes in tendon function.
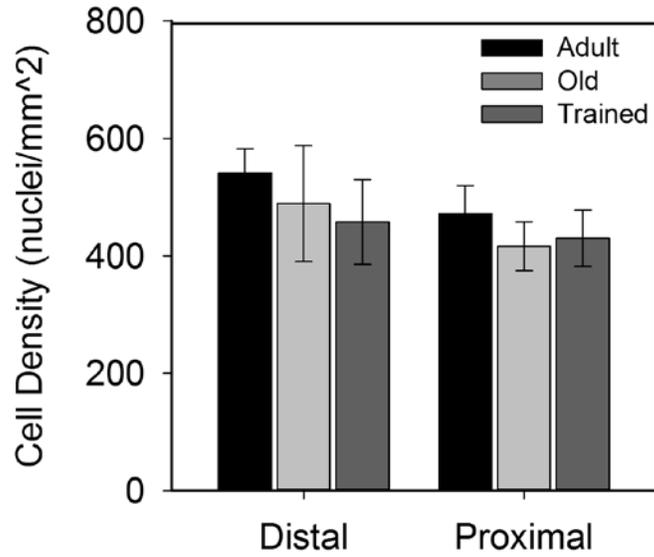
Acknowledgements

Figure 3.1. Fibroblast density in mouse plantaris tendons from adult (light gray bars), old untrained (gray bars), and old trained (dark gray bars) mice. No differences were seen between any of the groups, nor were regional differences present. Data presented as means ± SD.
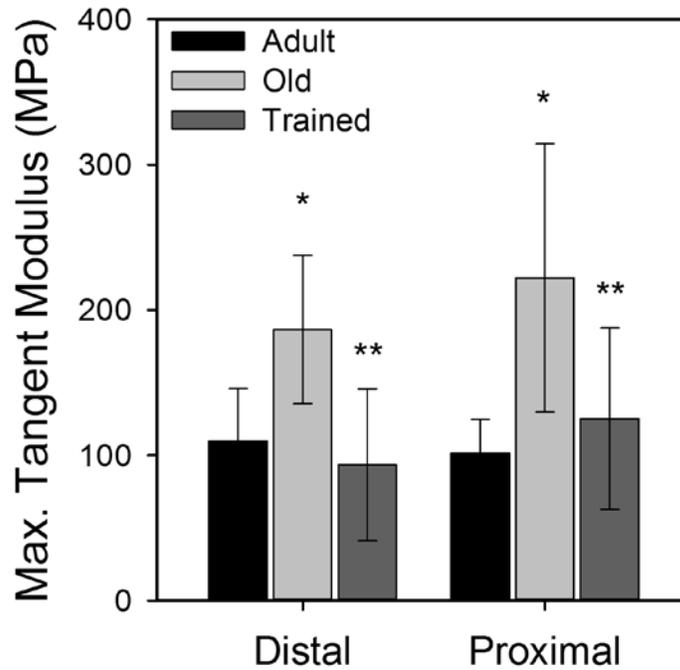
Figure 3.2. The effect of aging and treadmill training on the maximum tangent modulus of plantaris tendons from adult (light gray bars), old untrained (gray bars), and old trained (dark gray bars) mice. Compared with tendons from adult mice, tendons from old mice showed increase modulus that decreased to adult levels following training (p < 0.05). Data presented as means ± SD. *Statistically different from adult group, **statistically different from old group (p < 0.05).
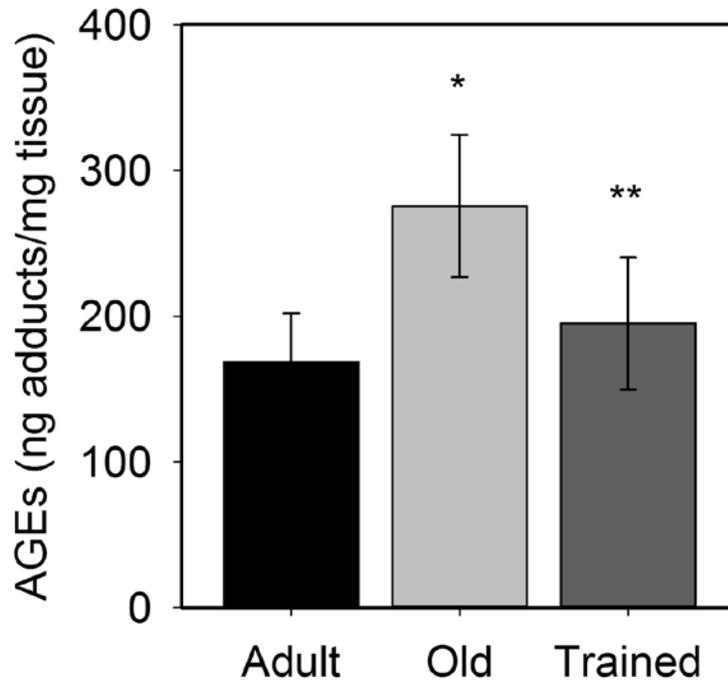
Figure 3.3. The effect of treadmill training on AGE adduct concentration in tibialis anterior tendons from adult (light gray bars), old untrained (gray bars), and old trained (dark gray bars) mice. In tendons from old mice, AGE adduct levels were significantly increased compared to adult tendons ($p < 0.05$), but decreased to adult levels following treadmill training ($p > 0.05$). Data presented as means ± SD. *Statistically different from adult group, **statistically different from old group ($p < 0.05$).
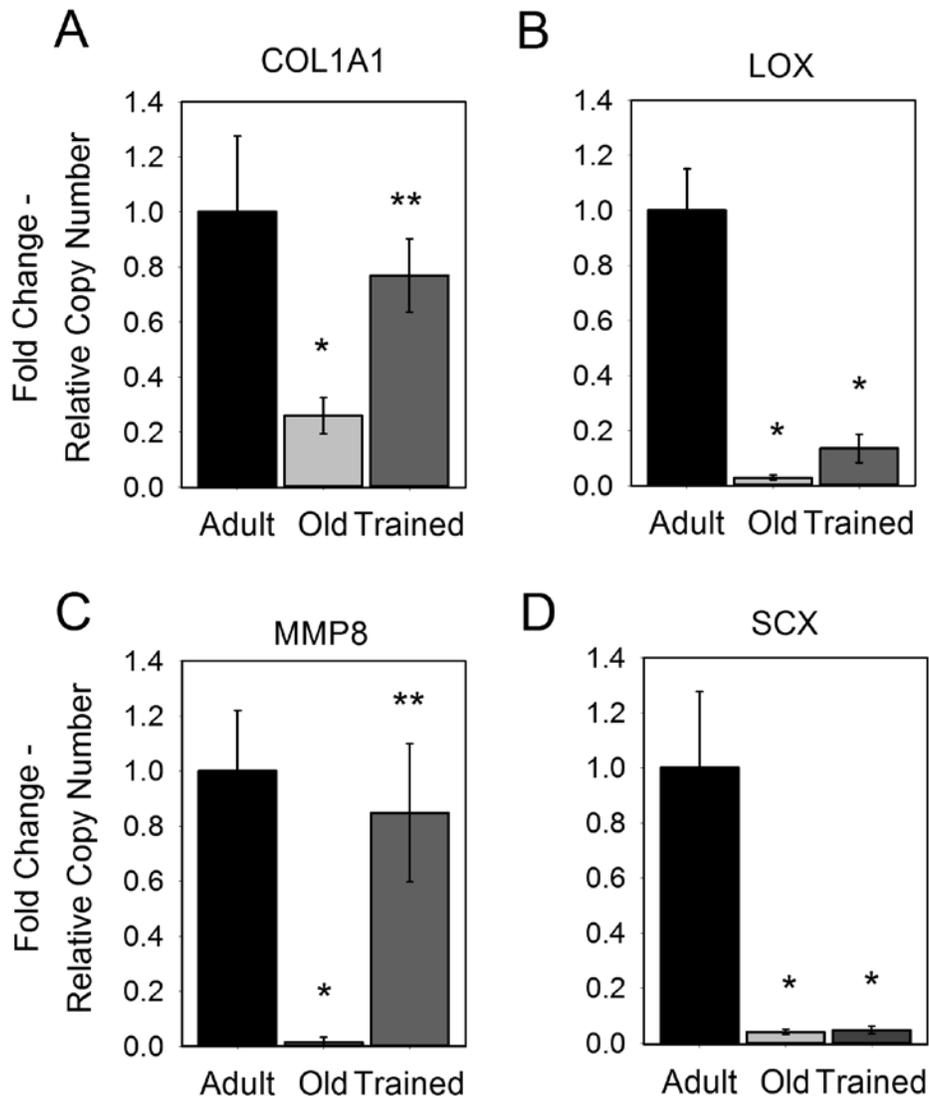
Figure 3.4. The effect of treadmill training on mouse Achilles tendon gene expression. Old mice showed decreased expression of COL1A1 (a), LOX (b), MMP8 (c) and SCX (d) compared to adult controls. Increased expression of COL1A1 and MMP8 was seen following training. TGF-β expression remained constant among all groups (data not shown). Data presented as means ± SD and are expressed relative to adult levels. *Statistically different from adult group, **statistically different from old group ($p < 0.05$).
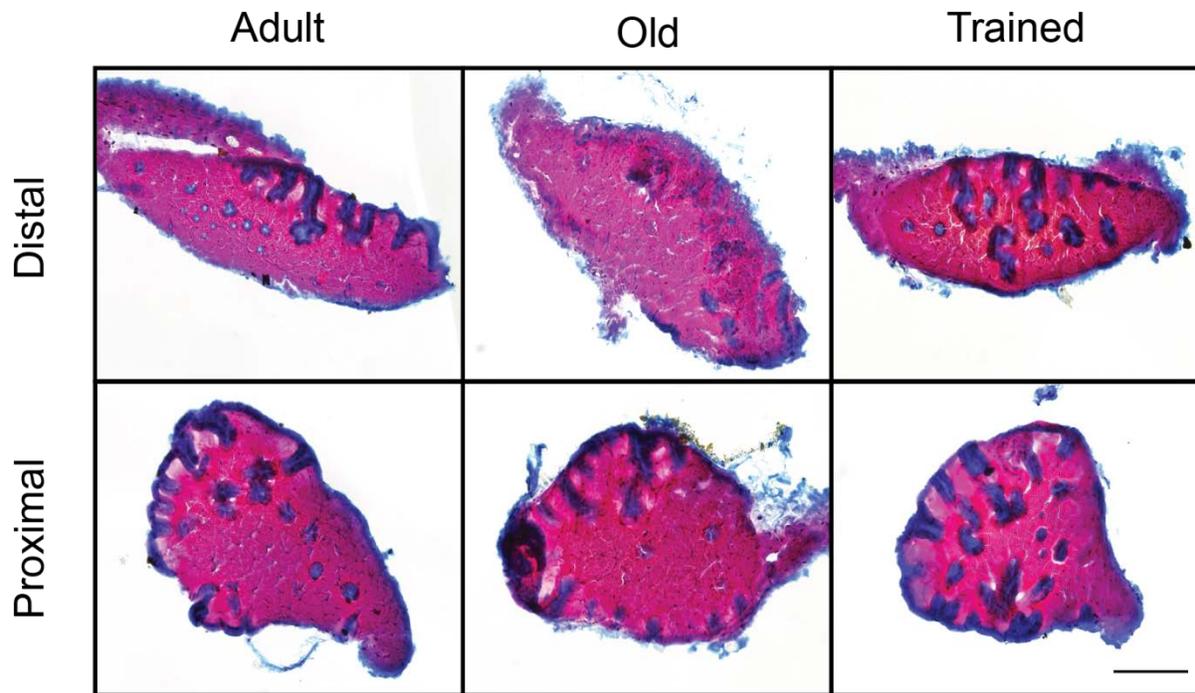
Figure 3.5. Herovici polychrome stain revealed deposits of newly synthesized collagen (blue) in mouse plantaris tendon cross-sections. Compared to sedentary old mice, tendons from old trained mice showed a greater percentage of newly synthesized collagen in the distal and proximal regions ($p < 0.05$). Scale bar = 250 μm.
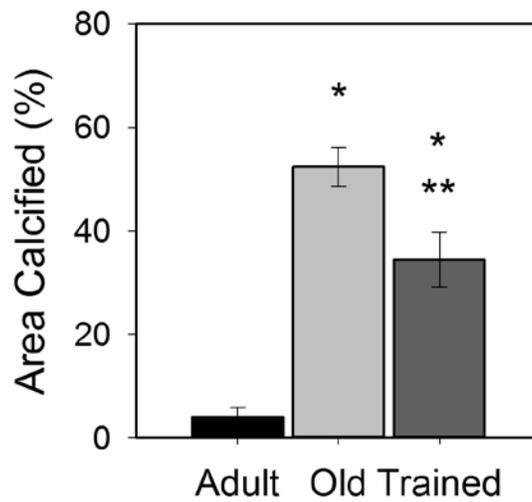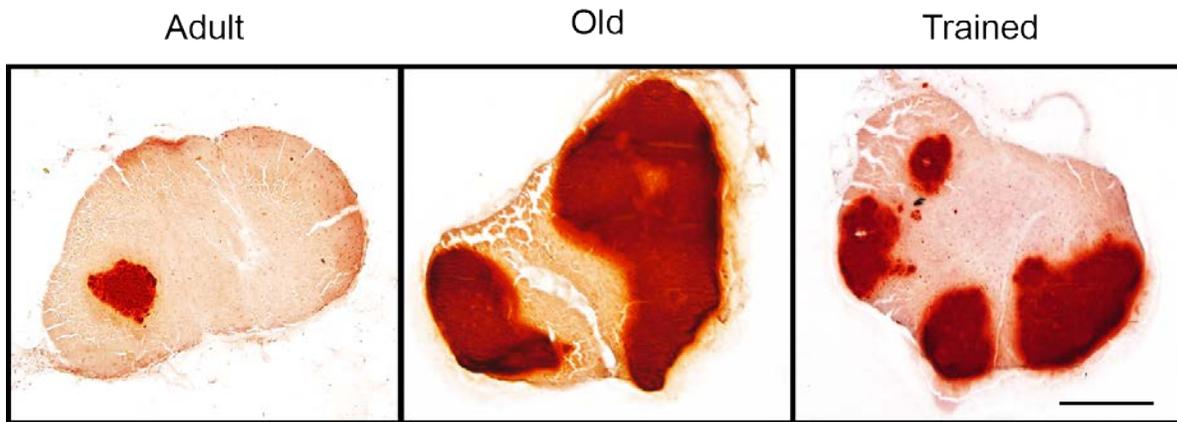
Figure 3.6. Area calcified in Achilles tendons from adult sedentary, old sedentary, and old treadmill-trained mice, as revealed with Alizarin Red staining. Tendons from old mice were extensively calcified, whereas tendons from young mice showed minimal calcification. Treadmill training reduced calcification by 34%. Scale bar = 250 μm. Data presented as means ± SD. *Statistically different from adult group, **statistically different from old group (p < 0.05).

	Age Range (months)	Body Mass (g)	Tendon Length (mm)	Distal Tendon CSA (mm^2)	Proximal Tendon CSA (mm^2)
Adult (n=6)	8	34.6 ± 1.7	4.87 ± 0.42	0.103 ± 0.025	0.086 ± 0.010
Old (n=6)	28-30	34.9 ± 1.2	4.93 ± 0.18	0.093 ± 0.015[a]	0.088 ± 0.011
Trained (n = 11)	28-30	32.6 ± 2.3	5.14 ± 0.23	0.113 ± 0.018[a,b]	0.091 ± 0.018[b]

Table 3.1. Adult and old mouse characteristics and plantaris tendon morphological properties. Values given as means ± SD. Different letters denote values that are significantly different (p < 0.05).

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Chapter IV

Rapamycin attenuates age-associated changes in tendon mechanical properties and collagen characteristics

Abstract

The drug Rapamycin (RAPA) extends mouse lifespan by 14%, but the extent to which RAPA prevents age-associated changes in specific tissues remains unclear. Given that stiffness increases and collagen turnover decreases in tendon with aging, our aim was to determine the effect of long-term RAPA treatment on the mechanical and structural properties of tendons from old mice. We determined mechanical and structural characteristics of tendons from 22-month-old UM-HET3 female mice fed RAPA throughout life (old RAPA), and 22-month-old (old) and 4-month-old (adult) controls fed a standard diet. Stiffness was 2-fold higher for tendons of old compared with adult mice, but administration of RAPA maintained stiffness for tendons of old RAPA mice at a value not different from that of the adults. Collagen turnover was enhanced in tendons of old RAPA fed mice compared with tendons of age matched controls and total hydroxyproline content trended toward decreased levels in RAPA fed compared with control old mice. Calcification of Achilles tendons from old mice, which was accompanied by elevated expression of chondrocyte and osteoblast markers, was decreased in old RAPA mice. These results suggest that long-term administration of

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RAPA slows the molecular pathways responsible for aging of tendon extracellular matrix, resulting in tissue that is structurally and mechanically more similar to tendons from adult mice.

Introduction

Tendon serves to transfer forces produced by the contractile elements of muscle to the skeleton, ideally with high fidelity while minimizing the risk of musculoskeletal injury. In old age, tendon's ability to perform this important role is compromised, and the incidence of tendon dysfunction and tendon injury is highly elevated past the seventh decade (15, 22). Age-associated increases in tendon dysfunction may be linked causally to alterations in the mechanical properties of tendons with aging (15). We have previously shown that mouse tendons stiffen in a region-dependent manner with aging, with the most pronounced changes occurring in the region of the tendon nearest the muscle (Chapter II). Changes in tendon stiffness can influence the effectiveness of force transfer from the muscle contractile elements to the skeleton (2) and may contribute to the impaired mobility and increased injury risk in the elderly (17).

The mechanical properties of tendons are determined by the underlying composition, which is primarily fibrillar type I collagen (COL1). In tendons of young, healthy individuals, the balance between the deposition of new collagen and collagen degradation via matrix metalloproteinase (MMP) collagenases helps to maintain the mechanical integrity of the collagen fibrils. With aging, however, collagen turnover is severely impaired (14), allowing for accumulation of post-translation modifications to

collagen that result in inter- and intramolecular cross-links that increase collagen fibril stiffness and compromise the structural and mechanical integrity of tendon (18). Spontaneous calcification of tendon also occurs with age (Chapter III, 9), which increases the brittleness of the tissue (21).

Mammalian target of rapamycin (mTOR) is an important protein complex whose numerous functions include the regulation of collagen and other extracellular matrix proteins at the translational level (5). The drug Rapamycin (RAPA) is a potent suppressor of mTOR activity that, when administered throughout life, results in robust lifespan extension in both male and female genetically heterogeneous mice (7). We previously showed that RAPA can delay the age-associated increase in tendon stiffness in the proximal region of tibialis anterior (TA) tendons (24), but the effects of RAPA on whole tendon mechanical properties and the mechanisms underlying the effects have not been explored. This study thus aimed to compare the mechanical and structural characteristics of tendons of old mice fed a diet containing RAPA with those of age-matched untreated mice. We tested the specific hypothesis that treatment of mice with RAPA, starting at nine months of age, would slow the progression of age-associated changes in tendon mechanical properties by maintaining collagen turnover into old age at levels observed in tendons from adult animals.

Methods

Animals. The mice used in this investigation were genetically heterogeneous UM-HET3 females, produced by a cross between (BALB/cByJ x C57BL/6J)F1 mothers and

(C3H/HeJ x DBA/2J)F1 fathers. All procedures were approved by the University of Michigan Committee for the Use and Care of Animals. Animals were housed in a specific-pathogen-free barrier facility in the Unit for Laboratory Animal Medicine at the University of Michigan, with food and water provided ad libitum. Weanlings were fed a diet of standard chow until 270 days of age, when a subset of the mice was switched to a diet that included encapsulated RAPA. Mice on the RAPA diet were administrated the drug at a concentration of 4.7, 14, or 44 ppm (7). Absorption of RAPA was confirmed by measuring the concentration of RAPA in serum, which increased in proportion to the RAPA dose (24). RAPA fed mice (RAPA mice) were euthanized at 22-months of age. Four- (adult mice) and 22-month-old (old) UM-HET3 mice fed standard chow served as controls. Body masses (mean ± SD) for adult and old mice were 23.5 ± 0.2 g and 37.8 ± 6.2 g, respectively. Body mass for the old RAPA mice was 42.6 ± 9.0 g and did not differ from the old control group but was significantly greater than in the adult group.

Tissue extraction. Tibialis anterior (TA) muscle-tendon units (TA muscle, TA tendon, and 1st metatarsal) were carefully dissected from one hindlimb and overlying connective tissue carefully removed. Samples were stored in sterile PBS at 4°C until testing, which was within 3 hours of tissue removal. The contralateral TA tendon and both Achilles tendons were also extracted and immediately coated with Tissue Tek and frozen in liquid nitrogen-cooled isopentane or prepared for mRNA isolation.

Determination of tendon mechanical properties. TA tendon mechanical properties were determined in six tendons from adult mice, nine tendons from old mice, and

sixteen tendons from old mice fed the various RAPA diets. The stress-strain response of each TA tendon was determined as described previously (Chapter II). Briefly, the TA muscle-tendon unit was placed in a room temperature PBS bath, and the TA muscle and 1st metatarsal were secured to custom grips so that the tendon was free-standing. 25µm polystyrene beads (IMT Laboratories, Irvine, CA) were brushed along the tendon to serve as optical strain markers. Cross-sectional area (CSA) was optically measured using a calibrated microscope eyepiece at three points along the tendons at 0° and 90° rotation and fitting the values to an ellipse. The tendons were stretched to a preload of ~0.03N, which ensured that there was no slack in the tendon prior to testing. The tendons were then subjected to a single load-unload cycle of 10% grip-to-grip strain at a constant strain rate of 0.01/second.

Synchronized force and image recordings were obtained using LabVIEW (Austin, TX). Bead positions were tracked with MetaMorph (Molecular Devices, Sunnyvale, FL), and nominal strains in the proximal (near the muscle) and distal (near the bone) tendon regions were calculated as the change in separation between two beads in each region divided by their initial separation. Regional stress was determined by dividing raw loading data by the local CSA. Overall tendon strain was calculated using beads that were positioned one tendon diameter length proximal to the bone insertion and one tendon diameter length distal to the muscle insertion. Overall tendon stress was calculated using average tendon CSA. Stress-strain data were fit to a third-degree polynomial, and maximum tangent modulus of each tendon region was

calculated as the maximum slope of the curve. Tangent modulus served as our measure of tendon stiffness.

Stiffness in all tendon regions as well as end-to-end values were independent of RAPA dose (data not shown). Thus, mechanics data from mice fed each of the three concentrations of RAPA were pooled for analysis of mechanical properties. Histological and gene expression data were examined only in tendons from mice fed the intermediate dose of 14ppm RAPA.

Determination of hydroxyproline concentration. Hydroxyproline content was determined in four TA tendons in each group using the method of Woessner (26). Briefly, tendons were dried at 110°C for 1 hr, weighed immediately, and then digested overnight at 100°C in 6N hydrochloric acid. The next day the samples were neutralized with sodium hydroxide. Chloramine T was added, and the tubes were incubated at room temperature for 20 min, after which the chloramines T was inactivated with perchloric acid. Ehrlich's solution was added and the tubes incubated at 60°C for 20 min, cooled, and absorbance measured at 560 nm. Hydroxyproline concentration was determined by normalizing total hydroxyproline content to tendon dry mass

Herovici staining. To distinguish between newly synthesized collagen from long-lived collagen, 12µm cyrosections were taken from the distal and proximal regions of TA tendons [n = 3 (adult,), 4 (old), and 5 (RAPA)]. Sections were stained with the Herovici stain (8), which reveals "young" newly synthesized collagen as blue and "aged" long-lived collagen pink. Percent "young" collagen was quantified using ImageJ (NIH, Bethesda, MD).

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Gene expression. Expression of collagen I (COL1A1) and matrix metalloproteinase-8 (MMP8) was determined in TA tendons [n = 4 (old), 4 (RAPA)], and expression of SOX-9 (SOX9), osterix (Sp7), osteocalcin (Bglap) and hypoxia-inducible factor 1-alpha (Hif1a) was determined in Achilles tendons [n = 5 (adult), 4 (old), 4 (RAPA)]. Total RNA was isolated by homogenizing tendons in Qiazol tissue lysis reagent (Qiagen, Valencia, CA). mRNA was isolated using a miRNeasy Mini Kit (Qiagen) and treated with DNAse I (Qiagen). Reverse transcription was performed using a QuantiTect Reverse Transcription kit (Qiagen), and cDNA was amplified in a CFX96 real-time thermal cycler (Bio-Rad, Hercules, CA) using a QuantiTect SYBR Green I PCR Kit (Qiagen). Reactions were performed in duplicate. The specificity of amplification was verified with melting curve analysis. Relative copy number of each sample was determined using the linear regression of efficiency method, which captures amplification efficiency for each individual reaction (20). All primers were obtained from Qiagen.

Tendon calcification. Four frozen Achilles tendons from each group were sectioned at 12µm the central tendon region and stained with 0.5% Alizarin Red to visualize calcium deposits. Percent area calcified was determined optically using ImageJ.

Statistics. Values are reported as means ± SD. Statistical analysis was performed using SigmaPlot software (San Jose, CA). Differences in mean values for regional mechanical and histological data were determined using two-way ANOVA, with treatment group and tendon region as independent factors. Differences in gene

expression and tendon calcification were determined using a one-way ANOVA with treatment group as the independent factor. Significance was set at $p < 0.05$.

Results

Tendon morphology and cell density. Within each age group, TA tendon CSA was smallest in the distal tendon region, but no age-related or RAPA-induced changes were seen for CSA in any region of the tendon (Table 4.1). Likewise, tendon length remained constant amongst all experimental groups. Fibroblast density decreased with age in both the distal and proximal regions of the tendon, while the loss of cellularity in the proximal region of the tendon was prevented in the old RAPA fed mice (Figure 4.1).

Changes in tendon mechanical properties. Maximum tangent modulus of TA tendons from all groups is shown in Figure 4.2 Maximum tangent modulus of the proximal tendon region increased 2-fold with age in the control group (adult: 226 ± 77 MPa, old: 507 ± 128 MPa), but no increase in modulus was observed in old RAPA mice as compared to age-matched controls (RAPA: 297 ± 110 MPa) such that the proximal region modulus was not different for tendons of adult and old RAPA mice. The overall end-to-end tendon modulus showed the same behavior (adult: 475 ± 148 MPa, old: 666 ± 137 MPa, RAPA: 512 ± 143 MPa).

Collagen fibril distribution. Collagen fibril morphology data for TA tendons from all groups are shown in Figure 4.3. Mean fibril diameter was similar across all groups and tendon regions (data not shown), and no effect of age was seen in any of the parameters examined. In the RAPA group, collagen fibril packing fraction was greater in the proximal region as compared to the distal region, whereas no regional difference

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was seen in either the adult or the old age groups (Figure 4.3a). In all groups, fibril density (Figure 4. 3b) was greater in the distal region compared to the proximal region.

Collagen turnover. The effect RAPA on mRNA levels for key proteins associated with turnover of tendon extracellular matrix is shown in Figure 4.4. Analysis of gene expression showed a 40% lower COL1A1 expression in tendons from RAPA fed mice compared with age-matched controls. MMP8, which was expressed in extremely low levels in old control tendons, was highly elevated in tendons from old RAPA mice. Total hydroxyproline content in TA tendons was unchanged with aging, but hydroxyproline content in tendons of old RAPA mice trended towards decreased levels compared to old controls ($p = 0.07$; Figure 4.5).

Tendon calcification. Alizarin Red staining revealed minimal calcification of adult Achilles tendons ($1.4 \pm 1.6\%$ calcified) that increased 10-fold with age ($15.2 \pm 4.1\%$) (Figure 4.6). In contrast, tendons of old RAPA mice did not exhibit the increased calcification observed in their age-matched counterparts, with calcification levels for tendons of old RAPA mice not different from those observed for tendons from adult mice. Given that fibroblasts from calcified avian tendon show chondrogenic and osteogenic phenotypes (1), we also examined the effect of RAPA on mRNA levels for proteins important for bone (Sp7 and Bglap) and cartilage (SOX9) formation (Figure 4.7). Expression of all three markers was minimal in tendons from adult mice, and highly elevated in tendons from old mice. Tendons from old RAPA mice showed 2-fold lower levels of Sox9 expression compared to age-matched controls, and expression of Sp7 and Bglap was almost undetectable in tendons from old RAPA mice. Given that

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hypoxia has been suggested as a driving factor in tendon calcification (12), we also measured expression of Hif1a, but no difference in expression was seen in any of the groups.

Discussion

A major finding of the present study was that age-associated changes in tendon mechanics previously observed at 28-30 months of age (Chapter II) occurred by 22 months of age, and that mechanical changes first manifested in the proximal tendon region. In addition, the previous findings were in the inbred strain of C57BL/6 mice, whereas the present study was performed on genetically heterogenous UM-HET3 mice, providing support that these changes are not specific to a single mouse strain. Finally, the current study demonstrated changes in tendon mechanical properties with aging in female mice, whereas our previous study was of male mice. We also demonstrate that in the proximal TA tendon region, long-term administration of RAPA delayed age-associated increases in stiffness while maintaining cell density and enhancing the turnover of collagen within the tissue. Moreover, treatment with RAPA reduced the extent of age-associated spontaneous calcification of the Achilles tendons. Overall, RAPA appeared to slow the cellular and molecular processes responsible for aging of tendon extracellular matrix, resulting in tendons in the old RAPA mice that approached the structural and functional characteristics of tendons from young adult control mice.

The ability of RAPA to extend lifespan has been extensively investigated, with increased longevity seen in response to treatment with RAPA in yeast (13), flies (10),

and mice (7). Recent studies have also attempted to elucidate the potential of RAPA as a therapeutic agent for various soft tissue pathologies. Administration of RAPA has been shown to reduce fatty infiltration in supraspinatus muscle six weeks after complete transaction of the attached tendon in adult rats (11). In addition to the impact of RAPA in the tendon injury model (11), in TSK/+ mice, which exhibit excessively tight skin and the accumulation of extracellular matrix proteins, one month of RAPA treatment significantly reduced skin fibrosis (26). Finally, in adult mice, a reduction of surgically-induced osteoarthritis severity was seen following ten weeks of RAPA (3). We recently demonstrated that RAPA also prevented or delayed many forms of age-related changes in tissues, including degenerative changes in the heart and liver, and the development of lesions in the adrenal gland and endometrium (24).

Our observation that administration of RAPA slowed or prevented the age-associated increase in tendon stiffness indicates that the underlying structure and/or composition is altered by RAPA. The tendon is composed primarily of fibrillar type I collagen. Collagen has an extremely slow turnover rate (23), allowing for the accumulation of post-translation modifications that can compromise the structural and mechanical integrity of collagen rich tissues, like tendons. These modifications include glucose-mediated permanent crosslinks such as pentosidine and carboxymethyllysine, both of which increase in concentration in a linear fashion with aging (6). The presence of these permanent crosslinks also reduces collagen turnover (4), consistent with reduced expression with aging of type I collagen (Chapter III, 14) and MMP-8 (Chapter III). Reports that mTOR is an important regulator of the expression of extracellular

matrix proteins (5) support the hypothesis that suppression of mTOR with RAPA may act to alter the expression profile of proteins important for collagen turnover in aging, including MMPs. Indeed, COL1 expression and protein levels were decreased and MMP-1 expression increased in cultured fibroblasts following treatment with RAPA (5, 16). The present investigation demonstrated similar changes in tendon tissue, with a 2-fold decrease in COL1A1 and a 110-fold increase in MMP8 expression in tendons of old RAPA mice compared with age-matched controls. RAPA also prevented the age-associated decline in cell density and enhanced the packing fraction of collagen fibrils in the proximal tendon region. Therefore, these data suggest that RAPA contributes to the preservation of pathways responsible for the maintenance of extracellular matrix mechanical integrity through 22 months of age, likely due to the preservation of cell density within the tissue and allowing for maintenance of collagen turnover.

The observation in the present study of age-associated calcification of Achilles tendons of genetically heterogeneous UM-HET3 mice expands on our previous observation of extensively calcified tendons in 28-month old C57BL/6 mice (Chapter III), and demonstrates the presence of tendon calcification at an earlier age. Although the mechanisms underlying the progression of age-associated calcification of tendon are largely unclear, previous reports have suggested that fibroblasts from calcified avian tendons show chondrogenic and osteogenic phenotypes (1). Consistent with this possibility is the present observation of highly elevated expression of markers for chondrocytes (Sox9), osteoblasts (Sp7) and bone formation (Bglap) in calcified tendons from old mice compared with adult mice, which showed minimal expression of all

three markers. Additionally, in tendons from old mice fed RAPA, expression of all three genes was maintained to levels equal to those from adult tendons. Hypoxia has also been suggested as a driving factor in tendon calcification (12); however, we saw no difference in Hif1a expression in tendons from any group. In the present study, tendons from old mice exhibited high levels of calcification, suggesting that age-associated tendon calcification involves chondrogenic and/or osteogenic processes but not hypoxia.

Our study is not without limitations. The mice used in this study were part of a larger investigation that required animal sacrifice at 22 months of age. The use of this age group precluded direct comparison between these data and previous data on 28-month-old TA tendons in genetically heterogeneous mice (Chapters II and III). However, given the observed age-associated mechanical and structural changes in the tendons, we are confident that the reported data adequately describe the effect of RAPA on aged tendon mechanical and structural properties. In addition, the small size and low mRNA yields from mouse TA and Achilles tendons precluded regional gene expression data from being obtained.

In summary, we demonstrated that long-term administration of RAPA delays the age-associated increase in tendon stiffness, as evidenced by the maintenance of adult levels of stiffness through at least 22 months of age for the RAPA treated mice, in contrast to tendons of age-matched untreated mice that showed increased tendon stiffness. The protective effects appear to be mediated through the maintenance of fibroblast density and collagen turnover. Additionally, we provide evidence that age-

associated tendon calcification is driven by chondrogenic and osteogenic processes that are inhibited by RAPA. This is in agreement with previous reports that Sp7 and Bglap expression are dependent on mTOR signaling in mouse bone marrow stromal cells (20). This study has provided evidence that the mechanisms underlying the aging of tendon tissue are dependent at least in part by the actions of mTOR, and that inhibition of this pathway results in delayed aging of the tissue through the maintenance of structural and mechanical integrity of the tissue from adulthood to old age.
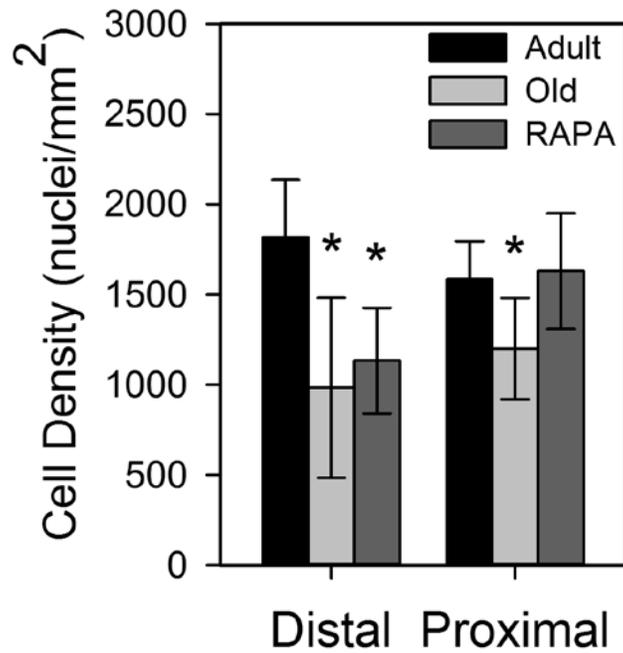
Acknowledgements

Figure 4.1. Density of tenocytes in plantaris tendons from adult, old, and RAPA-fed-old mice. There was an age-associated decrease cell density in both the distal and proximal tendon regions. Following RAPA treatment, distal region cell density remained depressed, but was maintained at adult values in the proximal region. Data are presented as means ± SD. *Significantly different from adult.
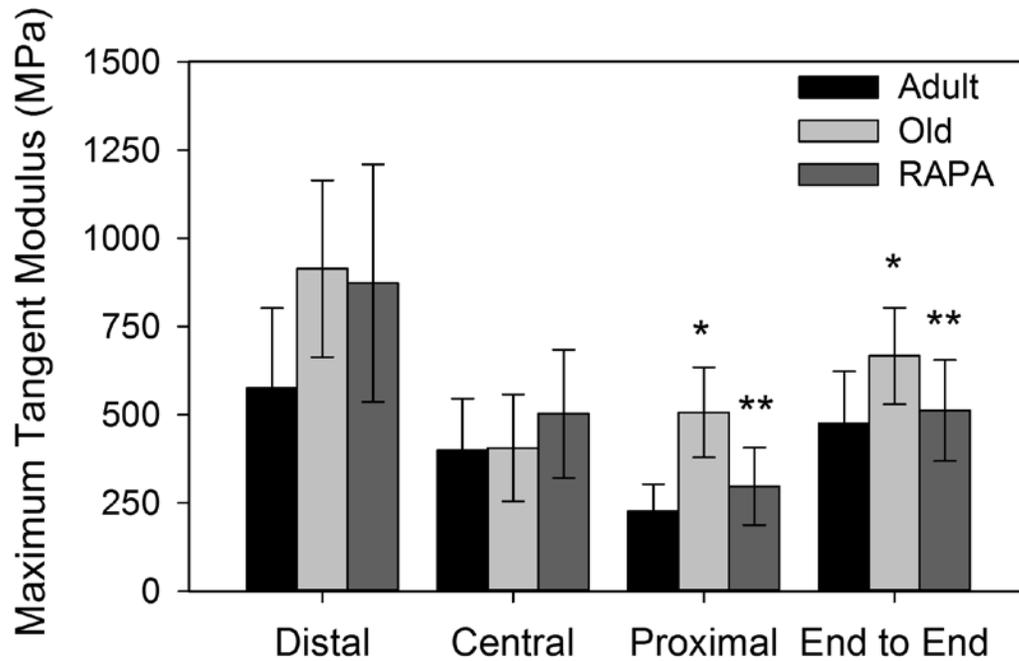
Figure 4.2. Effect of aging and RAPA on the tangent modulus of mouse TA plantaris tendons. Modulus increased with aging in the proximal tendon region and overall tendon response, but was reduced to adult control levels in age-matched RAPA mice ($p < 0.05$). Data are means ± SD. *Significantly different from adult, **significantly different from old.
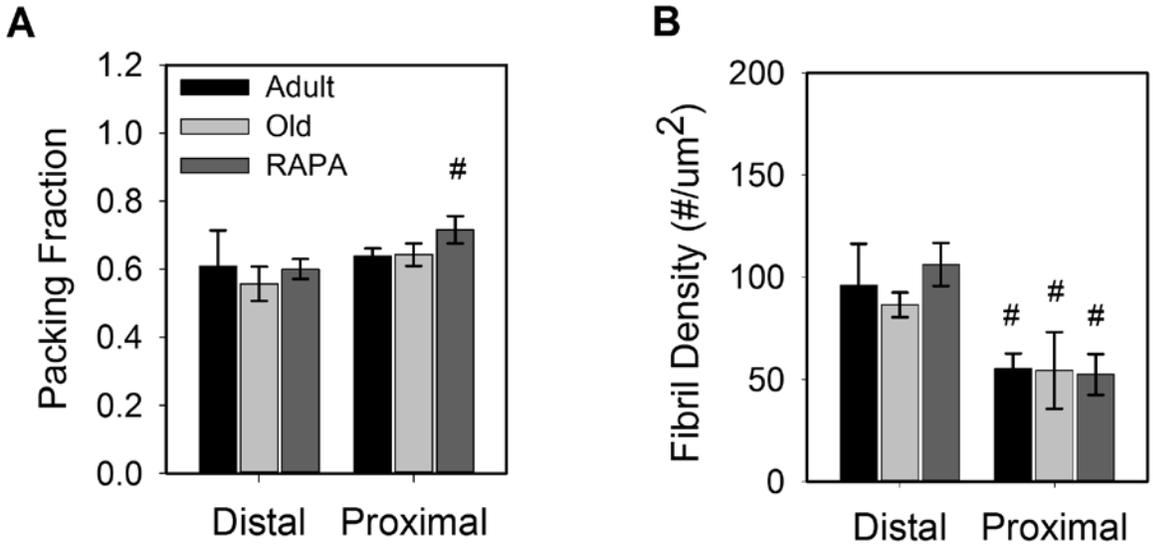
Figure 4.3. Collagen fibril morphology in TA tendons from adult, old, and old RAPA-fed mice. Mean fibril diameter was similar across all groups and tendon regions (data not shown). No effect of age was seen in packing fraction (a), or fibril density (b). Regional dependency of fibril packing fraction was only present in the RAPA-fed mice, where packing fraction was greater in the proximal region compared to the distal region. Fibril density showed regional dependency across all groups, with fibril density being greatest in the distal region. Data presented as means ± SD. #Within group, significantly different from distal region ($p < 0.05$).
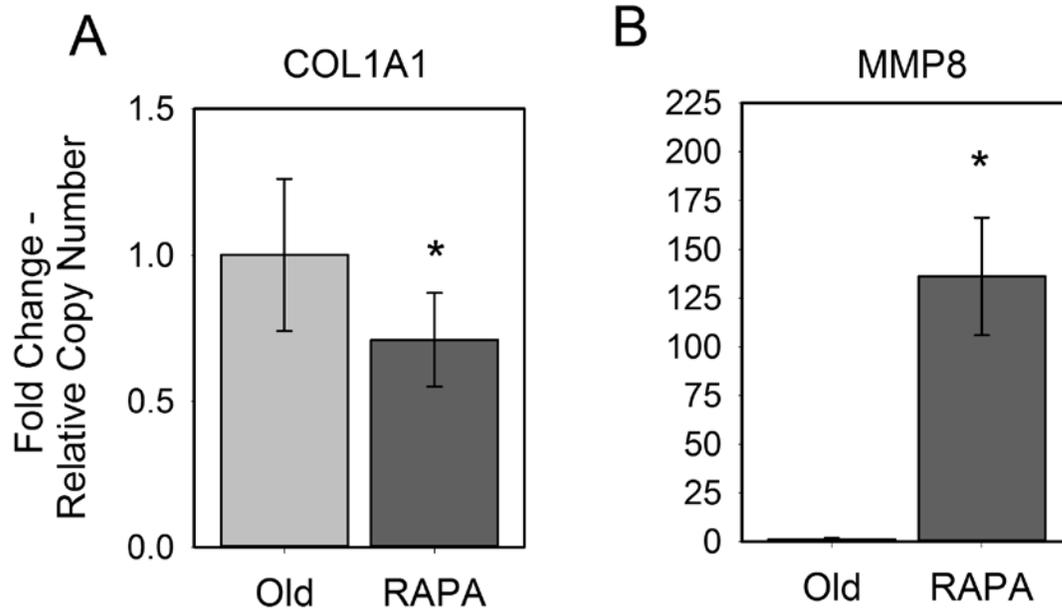
Figure 4.4. Expression of COL1A1 (a) and MMP8 (b) in TA tendons from old mice fed a standard diet, and old mice fed RAPA. COL1A1 expression decreased by 48% in tendons from RAPA fed mice compared to age-matched controls. MMP8 was expressed in extremely low levels in old mice, and was highly elevated in tendons from RAPA-fed mice. Data presented as means ± SD and are expressed relative to values from the old group. * Significantly different from old.
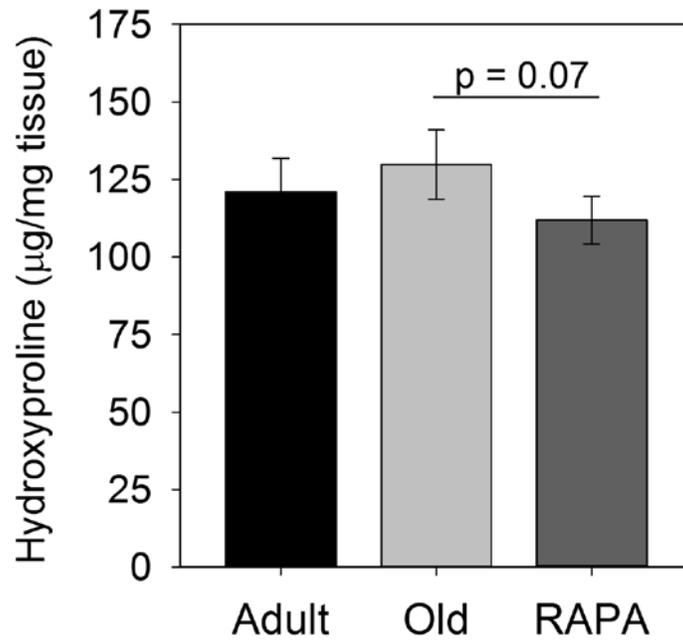
Figure 4.5. Hydroxyproline content in mouse TA tendons. No age-associated changes in hydroxyproline concentration were seen, although tendons from old RAPA fed mice trended towards decreased hydroxyproline content compared to age-matched controls ($p = 0.07$). Data are means ± SD.

Figure 4.6. Area calcified in mouse Achilles tendons, as revealed with Alizarin Red staining. Tendons from old mice were extensively calcified, whereas tendons from young mice showed minimal calcification. RAPA fed mice exhibited minimal tendon calcification equal to that from tendons of adult mice. Scale bar = 250 μm.
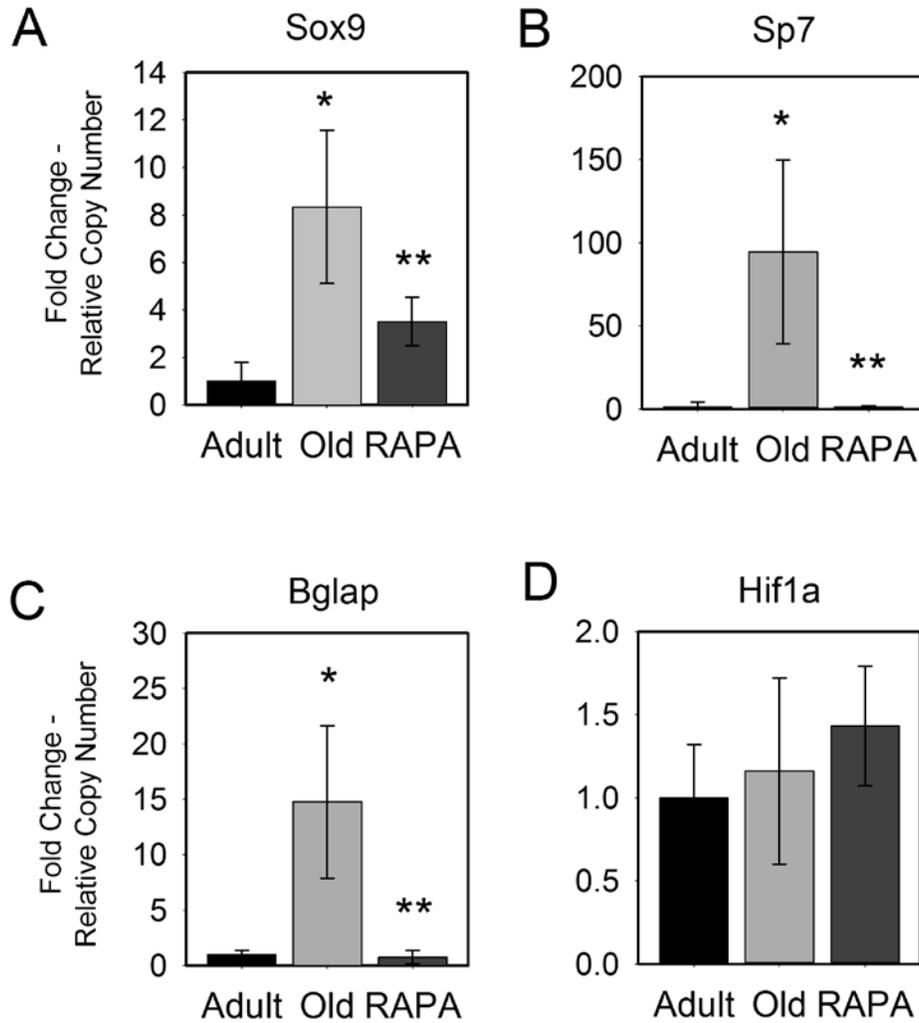
Figure 4.7. The effect of RAPA on mouse Achilles tendon gene expression of proteins important in bone and cartilage formation. Expression of the chondrocyte marker Sox9 (a), the osteoblast marker Sp7 (b) and the bone formation marker Bglap (c) was low in tendons from adult mice, but expression of all three markers was highly elevated in tendons from old mice. Administration of RAPA maintained expression of all three genes to levels equal to adult controls. Expression of the hypoxia marker Hif1a was no different among any of the groups (d). Data presented as means ± SD and are expressed relative to values from the adult group. *Significantly different from adult, **significantly different from old ($p < 0.05$).

	Adult (n=6)	Old (n=9)	RAPA (n=16)
Length, mm	6.5 ± 0.2	6.6 ± 0.2	6.7 ± 0.2
CSA, mm^2			
Distal	0.087 ± 0.008	0.082 ± 0.010	0.082 ± 0.012
Central	0.136 ± 0.014*	0.132 ± 0.013*	0.133 ± 0.012*
Proximal	0.118 ± 0.011*	0.112 ± 0.009*	0.116 ± 0.016*
Overall	0.113 ± 0.007	0.108 ± 0.007	0.110 ± 0.013

Table 4.1. TA tendon morphological properties from adult, old, and RAPA-fed UM-HET3 female mice. Values are means ± SD. CSA, cross-sectional area. *Amongst age groups, significantly different from distal region. No age-associated changes in regional CSA were seen.

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Chapter V

Collagen concentration and advanced glycation end-product crosslinking are elevated with age in tibialis anterior muscles of old mice

Abstract

Aging is associated with increased stiffness of both tendon and the extracellular matrix (ECM) of muscle, but the molecular basis for the increase in ECM stiffness is still being elucidated. We have shown that age-related tendon stiffening is associated with increased concentration of advanced-glycation end-product (AGE) crosslinking and decreased collagen turnover in the tissue. Given the observed changes in tendon ECM collagen with aging, we hypothesize that collagen in the ECM of muscle shows similar age related modifications that contribute to its increased stiffness. ECM collagen characteristics were determined from tibialis anterior muscles of adult (8- to 12-mo-old) and old (28- to 30-mo-old) male mice. With aging, muscle ECM hydroxyproline content increased 2-fold and advanced glycation end-product crosslinking increased 3-fold. In contrast, collagen fibril orientation and total ECM area were not different between muscles from adult and old mice. These findings present evidence that the age-associated increase in muscle ECM modulus is likely driven by an accumulation of densely packed extensively crosslinked collagen.

Introduction

The extracellular matrix (ECM) of muscle plays a vital role in the transmission of force produced by muscle fibers to the tendon (19, 21) and is thought to influence the risk of contraction-induced muscle fiber injury (15). To perform this function effectively, the ECM must remain intact during large strains associated with lengthening and shortening of muscle (18). The integrity of the ECM is largely determined by the mechanical properties of the tissue. Therefore, changes in ECM mechanical properties have the potential to influence both force transmission to the skeleton and susceptibility to contraction-induced muscle damage (4).

Aging is associated with declines in skeletal muscle force generation (6) and increased stiffness of both tendon (Chapter II) and muscle ECM (12). The decreased muscle force is due to a combination of reduced fiber size and number along with qualitative changes within the remaining fibers that reduce force generating capacity (8), but the precise molecular contributors to increased muscle stiffness with age remain unclear. ECM mechanical properties are determined by the underlying composition, a major component of which is fibrillar collagen. The slow turnover of type I collagen allows for post-translational modifications of collagen molecules including the accumulation of advanced glycation end-products (AGEs). AGEs can form permanent molecular crosslinks that stiffen collagen fibrils (20). Age-related tendon stiffening is associated with decreased collagen turnover in the tissue (Chapter III, 14) and increased AGE crosslinking (Chapter III).

Given that muscle ECM represents a functional extension of the tendon (13), changes similar to those observed for tendon may also play an important role in altering the mechanical characteristics of muscle ECM with age. Therefore, in this study we aimed to determine the influence of aging on the collagen characteristics of the muscle ECM. We hypothesized that the ECM of TA muscles from old mice would have greater AGE crosslinking and collagen content compared to muscles from adult mice.

Methods

Animals. Male C57BL/6 mice 8-12 (adult, n=10) and 28-29 (old, n=9) months of age were obtained from Charles River Laboratories (Wilmington, MA) and housed under specific-pathogen-free conditions with food and water provided *ad libidum*. All experimental procedures were approved by the University of Michigan Committee for the Use and Care of Animals.

Tissue extraction. Mice were anesthetized with an intraperitoneal injection of tribromoethanol (250 mg/kg), and the tibialis anterior (TA) muscles were removed. The muscles were divided equally between snap freezing at -80°C for biochemical analysis and frozen in Tissue Tek for histology. Immediately following removal of the muscles, mice were euthanized with an overdose of anesthetic followed by administration of a bilateral thoracotomy.

Immunohistochemical analysis. Three TA muscles from each age group were sectioned at a thickness of 10 μm in a cryostat. Sections were permeabilized with 0.2% Triton X-100 and incubated with wheat germ agglutinin conjugated to AlexaFluor 488

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(WGA, Invitrogen) to identify the ECM. Slides were mounted in ProLong Gold (Invitrogen) and imaged using a Zeiss Apotome fluorescence microscope with an 8 megapixel camera. Five random images were taken at 20x for each sample, and the total number of green pixels (ie, WGA-stained ECM tissue) was determined with a custom MATLAB program. Total ECM area was determined by dividing the number of green pixels by the total number of pixels in the image.

Picrosirius red staining was performed on 10 μm thick frozen sections (5 per age group) by incubating in 0.2% phosphomolybdic acid for 2 minutes, 0.1% picrosirius red for 90 minutes, and 0.01 N HCl for 2 minutes followed by drying and mounting in Permount. All samples were imaged using an Olympus BX51 microscope with an 8 megapixel camera under linearly polarized light. In muscle cross-sections, picrosirius red-stained collagen appears red if the fibrils are oriented at large angles to the muscle fibers, and yellow and green if the fibrils are oriented more parallel the muscle fibers (2). A custom MATLAB program was used to determine the number of pixels of each color present in each image. The distribution of red, yellow, and green pixels was determined by dividing the number of pixels of each individual color by the total number of colored pixels in each image.

AGE adduct concentration. The outer epimysium layer of connective tissue was carefully removed from four TA muscles in each age group, which were then finely homogenized and digested into peptides using a solution of 40 mg/mL proteinase K in PBS at 55°C. The concentration of AGE adducts was determined as previously described (Chapter III, 10).

Hydroxyproline content. Given that hydroxyproline is a major component of collagen, a hydroxyproline assay was performed as described previously (Chapter III, 16). Briefly, four flash-frozen TA muscles from adult mice and five flash-frozen TA muscles from old mice were thawed and the outer connective tissue layer carefully removed. The muscles were then dried, digested using 6.0N hydrochloric acid overnight at 110ºC, and the hydroxyproline content was determined using a colorimetric assay (24).

Statistics. Results are presented as mean ± standard deviation. Statistical analysis was performed using JMP (SAS Institute, Inc.). Differences in mean values for ECM collagen characteristics were determined with a Student's t-test, with significance set at $p < 0.05$.

Results

Morphology of muscle ECM. Representative images of cross-sections of TA muscles from adult and old mice immunostained with WGA and DAPI for visualization of total ECM content and cell nuclei, respectively, are shown in Figure 5.1. Total ECM area remained unchanged with age (adult: 18.4 ± 1.7% vs. old: 18.8 ± 1.8%, $p = 0.79$), indicating an absence of significant age-associated hypertrophy of ECM components.

Collagen fibril orientation. Figures 5.2a and 5.2b show representative cross-sections of muscles from adult and old mice, respectively, stained with picrosirus red and imaged under linearly polarized light. The total number of colored pixels in each image increased approximately 2-fold with aging, from 8.2 ± 3.7% area colored to 17.1 ± 6.0%

area colored (Figure 5.2c, p = 0.030), suggesting an age-associated increase in total collagen content in the muscle cross-sections. When analyzed as a percent of all colored pixels, the distribution of red, yellow, and green pixels remained constant with aging, indicating no age-associated change in collagen orientation (Figure 5.2d).

Hydroxyproline content and collagen crosslinking. Hydroxyproline content in muscles from old mice more than doubled compared with muscles from adult mice (1.13 ± 0.38 vs. 0.49 ± 0.30 μm/mg wet muscle mass, p = 0.024, Figure 5.3a). The concentration of AGE adducts in TA muscles also increased by more than 2X with aging from 690 ± 251 to 1,490 ± 620 pg/mg muscle (p = 0.042, Figure 5.3b).

Discussion

The main finding of the present study is that hydroxyproline concentration and collagen crosslinking in muscle ECM are increased with aging in TA muscles independent of significant hypertrophy of muscle ECM or changes in collagen fibril orientation. These findings allow the conclusion that the observed age-related increase in muscle ECM modulus (12) arise largely from changes in the material properties of the ECM tissue and not due to changes in collagen organization.

Muscle stiffness has been shown to increase in old age for both humans (5) and rodents (9, 17), although the molecular basis for the increase is unclear. ECM mechanical properties are determined by the composition of the underlying components, the most abundant of which is fibrillar collagen. Collagen has an exceptionally long half-life, which makes it highly susceptible to the accumulation of

glucose-mediated permanent intermolecular AGE crosslinks (23) that stiffen the collagen fibrils (20). The findings in the present study that the increase in ECM modulus with age (12) is accompanied by increased hydroxyproline content and levels of AGE protein adducts are consistent with previous reports that collagen concentration (1, 9) and collagen crosslinking (11, 22) increase in muscle with aging.

We also examined muscle cross-sections stained with picrosirius red, which gives an indication of the anisotropy of collagen fibrils when viewed under polarized light. Collagen fibrils that are aligned parallel with the muscle axis appear green, whereas fibrils that are at a greater angle relative to the muscle fiber appear yellow and red (2). Given that the load applied to a collagen fibril is determined by the cosine of the angle between the muscle axis and the fibril axis, fibrils that are more aligned with the muscle bear a greater proportion of the applied load, effectively increasing ECM stiffness (2). The lack of any changes with aging in the distribution of the three colors indicates that collagen fibril orientation is unchanged with aging in TA muscles of mice. The similarity in collagen fibril orientation between age groups coupled with the finding of no significant hypertrophy of the ECM with aging suggest that age-related changes in ECM mechanical properties result from changes in the intrinsic material properties of the ECM.

Muscle ECM and tendon form a functional link that serves to transfer force from muscle fibers to the skeleton. Consequently, the similarities between the age-associated changes in muscle ECM structural properties observed in the present study with those changes previously seen for tendon with aging may not be surprising. Tendon stiffness

increases two-fold with aging (Chapter II), a change that is likely driven by increased collagen content and AGE crosslinking in old age (Chapter III). Given that muscle ECM is directly continuous with tendon tissue, changes in the structural and mechanical properties of both tissues have the opportunity to influence the functional performance of the muscle-tendon unit. The precise influence of these changes on functional performance, whether protective or harmful, is still unclear. Increased stiffness of the muscle-tendon unit may indeed facilitate more effective force transmission from muscle contractile elements to the bone (4); however, such changes may also impair balance stability in the elderly (3) and decrease range of motion. During lengthening contractions, sarcomere lengths are stabilized by the transmission of force from the muscle fibers to the ECM (21), and changes in ECM mechanical properties may contribute to the observed impairment of transmission of force seen in old age (19). Increased stiffness of muscle ECM may also serve the protective role of limiting the amount of stretch a fiber undergoes during a lengthening muscle contraction. As such, future investigations are warranted to determine the precise influence of age-associated increases in ECM stiffness on functional performance of the muscle-tendon unit.

In summary, we demonstrated that the observed increase in muscle ECM modulus with aging is accompanied by an increase in hydroxyproline content and levels of AGE protein adducts. Coupled with the findings that the ECM fractional area and collagen orientation are unchanged with age, we conclude that the ECM of old muscles is more densely packed with highly crosslinked collagen than the ECM of adult muscles.
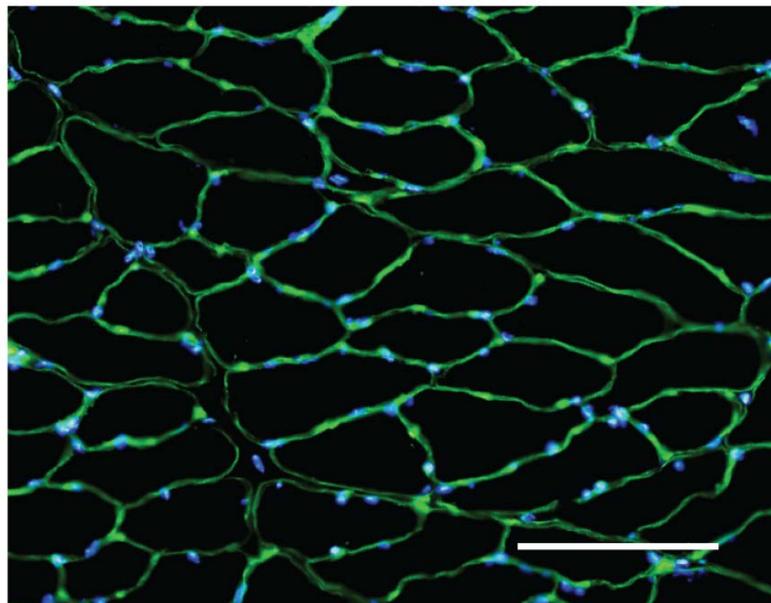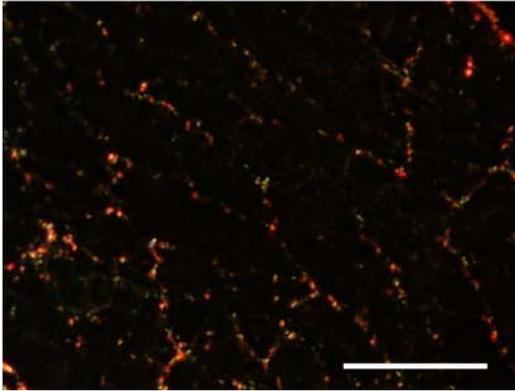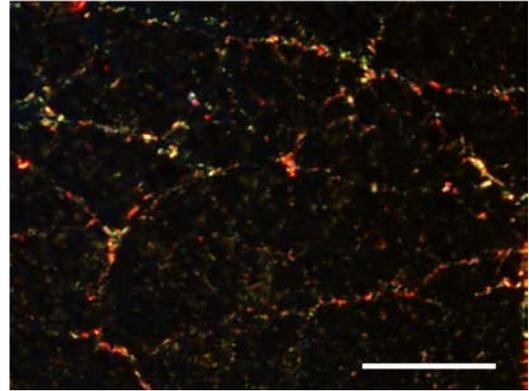
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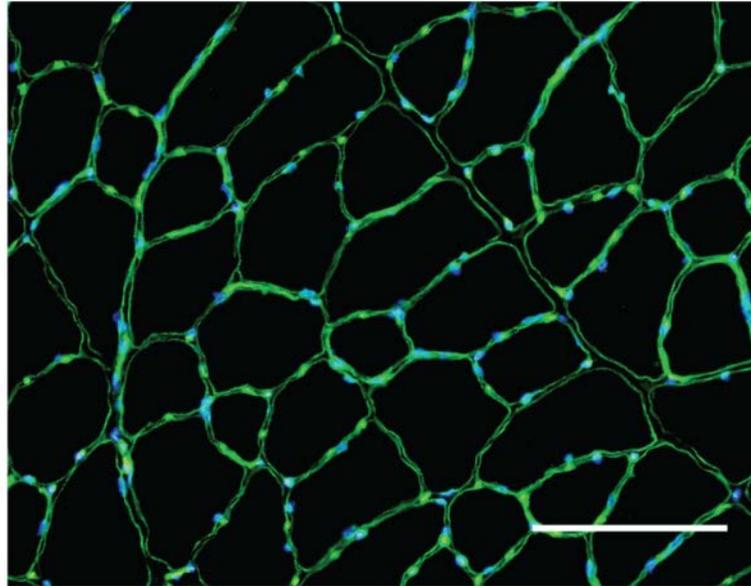
Figure 5.1. TA muscle cross-sections from adult (a) and old (b) mice immunostained with WGA and DAPI for visualization of extracellular matrix (ECM) and cell nuclei, respectively. Total ECM area remained unchanged with age (p = 0.79). Scale bars = 75 μm.
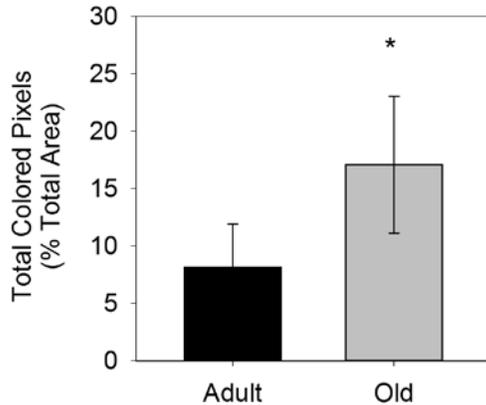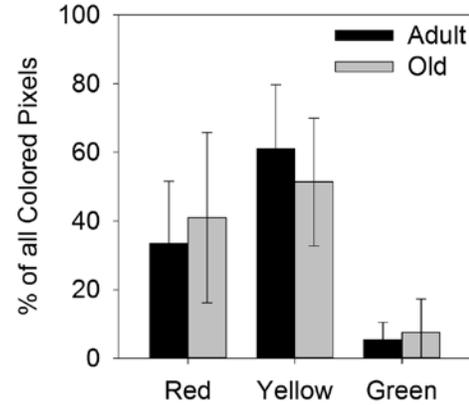
Figure 5.2. Cross-sections of TA muscles from adult (a) and old (b) mice stained with picrosirius red and viewed under linearly polarized light. Red staining indicates collagen fibrils that are oriented at a large angle with respect to the muscle fiber, and yellow and green staining indicates collagen fibrils that are more parallel to the fibrils. The total number of colored pixels increased with aging, suggesting an age-associated increase in total collagen content in the muscle cross-sections (c). When analyzed as a percent of all colored pixels, the distribution of red, yellow, and green pixels remained constant with aging, indicating no age-associated change in collagen orientation (d). *Indicates significantly different from adult group (p < 0.05). Scale bars = 200 μm.
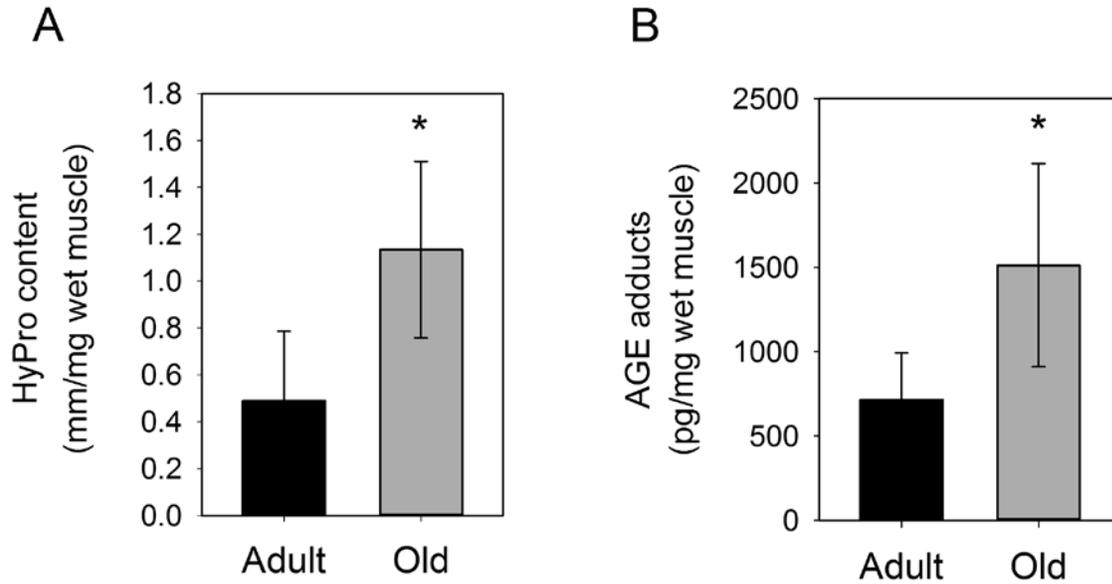
Figure 5.3. Hydroxyproline (a) and AGE adduct (b) concentration in whole TA muscles from adult and old mice. Muscles from old mice had increased hydroxyproline content and AGE adduct concentration as compared to muscles from adult animals. *Indicates significantly different from adult group (p < 0.05). Data presented as means ± SD.

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Chapter VI

Primary conclusions and recommendations for future work

This dissertation addresses three important knowledge gaps pertaining to aging of tendon and muscle ECM: (1) how are regional tendon structural and mechanical properties altered in aging? (2) is the impact of aging on muscle ECM similar to that observed for tendon? and (3) are tendons from old mice capable of adapting to changes in their external environment brought about by exercise or pharmacological intervention? To address these issues, we used a novel technique of coupling regional mechanics with regional structural properties in tendon. The primary conclusions of this dissertation are briefly summarized below.

1. TA tendons of mice stiffened significantly from 8 to 28 months of age, with the most pronounced changes occurring in the proximal tendon region. The dramatic changes in mechanical properties occurred independent of changes in collagen fibril morphology or force generating capacity of the TA muscle (Chapter 2).

2. Following 10-weeks of uphill treadmill training, hindlimb tendons from 28-month old mice had reduced stiffness and increased collagen turnover

compared to sedentary age-matched controls. Additionally, exercise reduced the number of AGE crosslinks in TA tendons and attenuated the extent of calcification in Achilles tendons from old mice independent of changes in cell density (Chapter 3).

3. The administration of RAPA, which extends lifespan in mice, delayed the age-associated stiffening of the proximal region of TA tendons through 22 months of age in genetically heterogeneous UM-HET3 mice. RAPA also increased fibroblast density and enhanced expression of MMPs while slowing the progression of Achilles tendon calcification through 22 months of age (Chapter 4).

4. The extracellular matrix of TA muscles from 28-month old mice had higher levels of AGE crosslinks and elevated collagen content compared to muscles from 8-month old adult mice, whereas collagen fibril orientation was unchanged with aging (Chapter 5).

The studies described in this thesis demonstrate that ECM of tendons from old animals is highly capable of remodeling. Our data also offers compelling evidence that multiple mechanisms may produce similar alterations in tendon functional properties. We showed that treadmill training (Chapter III) and RAPA treatment (Chapter IV) both resulted in decreases in tendon stiffness, but the effect of each intervention on cell density and collagen expression was unique. Based on these distinct responses, we hypothesize that the molecular mechanisms influencing the aging and remodeling of

tendon tissue are highly influenced, but not necessarily dependent, on the actions of mTOR. To test this hypothesis, the following experiments are proposed. First, by measuring the activity of mTOR along with collagen characteristics and tendon stiffness from adulthood to old age, a baseline measure of how mTOR activity correlates with tendon structural and functional properties in aging can be obtained. Given our observation of tendon remodeling following long-term RAPA administration (Chapter IV), the ability of acute RAPA treatment in both adult and old mice to induce similar remodeling on a shorter time scale would also be insightful. Additionally, a combined approach of RAPA administration with treadmill training in both adult and old mice will provide important information regarding the ability of tendon to remodel with impaired mTOR function. Finally, enhanced cell proliferation is commonly associated with treadmill training in adult animals (3), but the lack of an effect of treadmill training on cell density in tendons from old mice (Chapter III) suggests that enhanced proliferation is not required for training-induced functional adaptation. Therefore, it will be important to elucidate the ability of fibroblasts from all age groups to sense changes in their external environment. This can be accomplished in part by examining the age-related activities of network collagens, which aid in fibroblasts' ability to sense their external environments, and MMP-2 and MMP-9, which degrade network collagens (1). Examining these characteristics with aging and in both RAPA-fed and treadmill-trained mice will provide important insights into how each intervention affects the ability of tendon fibroblasts to sense and respond to changes in their external environment. Collectively, the above experiments will help elucidate the ability of

tendon ECM to remodel via pathways that are independent of mTOR throughout the lifespan of the mouse.

Our studies also suggest the underlying response of tendons from old animals to treadmill training is separate and unique to that seen in adult animals: tendons from adult animals stiffen following training, whereas tendons from old animals lose stiffness. We hypothesize that the reduction in tendon stiffness following treadmill training in old mice is driven by a reduction of AGE-crosslinks, whereas the increase in stiffness in tendons from adult animals is driven by an increase in LOX-derived crosslinks. A direct comparison of the training-induced molecular changes in tendons from the two age groups would therefore help to test our hypothesis. Specifically, the progression of changes in tendon tissue following 2, 4, 6, and 8 weeks of training should be determined in adult and old mice. Elucidation of the time course of fibrillar and network collagen deposition and degradation via MMPs would clarify how each age group responds to the training. Moreover, despite the restoration of adult levels for tendon stiffness, decreased AGE crosslinking, and increased collagen turnover, the extent of the positive adaptations to exercise may be limited in old animals. The lack of any increase with treadmill running in tendon cross-sectional area, fibroblast density, or scleraxis expression in old animals begs the question of whether longer duration exercise would induce tendon hypertrophy or may actually result in an overuse phenotype. Thus, a longer term intervention would be also informative. Finally, special attention should be given to the time course of training-induced changes in both LOX-derived and non-enzymatically derived crosslinks in both of the age groups. Given that

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increases in tendon stiffness in old age are correlated to the crosslinking of collagen (Chapters III) and not to collagen fibril morphology (Chapter II), it is likely that the increase in tendon stiffness following training in adults is due to increased crosslinking of collagen fibrils. Therefore, it would be beneficial to determine how crosslinking of collagen fibrils in both age groups is affected by the training protocol. The use of highly sensitive methods like Raman microscopy (2) would allow the determination of detailed crosslinking characteristics not achieved in the present data.

Overall, the studies described in this thesis provide important data clarifying the aging process in tendon tissue and elucidating the capacity of ECM of tendon from old mammals to adapt to changes in its external environment. Such information will be important in the development of treatments aimed at maintaining the functional performance of tendon through old age.

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