Collagen, cross-linking, and advanced glycation end products in aging human skeletal muscle

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Haus JM, Carrithers JA, Trappe SW, Trappe TA. Collagen, cross-linking, and advanced glycation end products in aging human skeletal muscle. J Appl Physiol 103: 2068–2076, 2007. First published September 27, 2007; doi:10.1152/japplphysiol.00670.2007.—We examined intramuscular endomysial collagen, cross-linking, and advanced glycation end products, as well as the general and contractile protein concentration of 20 young (25 ± 3 yr) and 22 old (78 ± 6 yr, range: 70–93 yr) sedentary men and women to better understand the underlying basis of changes in skeletal muscle mass and function that occur with aging. The old individuals had an impaired ability (increased time) (P < 0.05) to climb stairs (80%), rise from a chair (56%), and walk (44%), as well as lower (P < 0.05) quadriiceps muscle volume (−29%), muscle strength (−35%), muscle power (−48%), and strength (−17%) and power (−33%) normalized to muscle size. Vastus lateralis muscle biopsies revealed that intramuscular endomysial collagen (young: 9.6 ± 1.1, old: 10.2 ± 1.2 μg/mg muscle wet wt) and collagen cross-linking (hydroxylsylpyridinoline) (young: 395 ± 65, old: 351 ± 45 mmol hydroxylsylpyridinoline/mol collagen) were unchanged (P > 0.05) with aging. The advanced glycation end product, pentosidine, was increased (P < 0.05) by ~200% (young: 5.2 ± 1.3, old: 15.9 ± 4.5 mmol pentosidine/mol collagen) with aging. While myofibrillar protein concentration was lower (−5%, P < 0.05), the concentration of the main contractile proteins myosin and actin were unchanged (P > 0.05) with aging. These data suggest that the synthesis and degradation of proteins responsible for the generation (myosin and actin) and transfer (collagen and pyridinoline cross-links) of muscle force are tightly regulated in aging muscle. Glycation-related cross-linking of intramuscular connective tissue may contribute to altered muscle force transmission and muscle function with healthy aging.

hydroxyproline; hydroxylsylpyridinoline; pentosidine; myosin; actin; sarcopenia

A critical component in the transfer of force from the contractile units of the muscle out to the tendon and subsequent bone is the connective tissue scaffold that surrounds individual muscle fibers, muscle bundles, and the whole muscle (20, 23, 37). This skeletal muscle connective tissue network is primarily composed of collagen fibers and biochemical linkages within and between these fibers, which provide strength and stability. Numerous data from animals have shown that collagen concentration of intact skeletal muscle increases with aging (1, 12, 17, 24, 25, 34, 55), and a mature structural characteristic of collagen known as pyridinium cross-linking has also been shown to increase in aged animal muscle (17, 18, 36, 40, 55). The pyridinium cross-links are enzymatically added to collagen fibrils upon collagen maturity and replace an immature cross-link molecule. These mature pyridinium cross-links provide strength to the collagen molecule by linking the collagen fibrils in the characteristic one-third staggered arrangement.

Separate from the enzymatically derived, mature cross-links are an additional form of biochemical linkage found on connective tissue known as advanced glycation end products (AGEs). Accumulation of these compounds is the result of prolonged exposure to monosaccharides, in which a spontaneous nonenzymatic bond is formed between a reducing sugar and a protein residue. AGEs have been linked to disease and aging processes (2, 10, 35, 38, 41) and have been demonstrated to strongly correlate to increases in plasma glucose concentrations and chronological age. Evidence from humans and animals demonstrates the formation of these compounds in vascular tissues and lens proteins with diabetes and has shown a progressive accumulation in skin and cartilage with aging.

Skeletal muscle studied in aging animals demonstrates increases in collagen concentration, hydroxylsylpyridinoline (HP) cross-linking, and AGEs, all of which have been associated with increased muscle stiffness and reduced whole muscle function (1, 12, 17, 26, 52). Increases in tissue stiffness have been suggested to play a role in reduced whole muscle function with aging. However, almost no data exist from humans regarding intramuscular connective tissue changes with aging (3) or the extent of collagen cross-linking or the development of AGEs in healthy human skeletal muscle (14, 45). In aging humans, muscle strength and power are lost to a greater extent than the loss of muscle mass (i.e., whole muscle-specific force and specific power), suggesting that factors other than the loss of muscle mass are contributing to the decline in whole muscle function (47). We have shown that single-muscle fiber strength and power normalized to fiber size [i.e., myosin heavy chain (MHC) I and IIa myofiber-specific force and specific power] are not compromised with aging (47). These findings lead to the possibility that the transfer of force and power from the single fiber out to the whole muscle and subsequent tendon and bone may be impaired in aging skeletal muscle.

The intent of the present investigation was to expand on our previous findings (47) of the role of single-muscle fiber function in the decline of whole muscle function in aging through measurements of the intramuscular connective tissue network. Specifically, we measured the concentration of collagen (endomysium), collagen cross-linking (HP), and AGEs (pentosidine) in skeletal muscle biopsy samples taken from a well-characterized group of young and old sedentary men.
women and men. We hypothesized that the concentrations of endomyrial collagen, HP cross-links, and pentosidine would be elevated in old individuals compared with young. An additional aim was to measure the concentration of the general muscle protein fractions (mixed, sarcoplasmic, and myofibrillar) commonly studied in metabolic investigations of sarcopenia (5, 19, 42, 50, 51, 54), as well as the concentration of the two main contractile proteins that are responsible for force generation in skeletal muscle, myosin and actin. These measurements extend our laboratory’s previous investigation from a subset of the 42 individuals studied for the present investigation (47).

METHODS

Subjects

Twenty young (10 men, 10 women) and 22 old (10 men, 12 women) individuals were included in this investigation (Table 1) following a physical exam, which included blood and urine analyses, electrocardiogram (older subjects), and an interview to document their life history of physical activity. Subjects were excluded if they had any acute or chronic illness; cardiac, pulmonary, liver, or kidney abnormalities; uncontrolled hypertension; insulin- or noninsulin-dependent diabetes; abnormal blood or urine chemistries; arthritis; a history of neuromuscular problems; or if they smoked tobacco. Women taking oral contraceptives or hormone replacement therapy were included. It was our intent to only include life-long sedentary healthy older and younger individuals; therefore, we excluded individuals who, by self-report, had ever completed any formal exercise programs or physical activity outside of their activities of daily living. Following approval by the Institutional Review Board (IRB), all procedures, risks, and benefits associated with the experimental testing were explained to the subjects before they signed a consent form adhering to the guidelines of the IRB of the participating institutions. The study was conducted in accordance with the Declaration of Helsinki.

Experimental Design

Subjects who qualified for the study completed six different experimental sessions over a 2- to 3-wk period, described in detail below. The first session involved magnetic resonance imaging (MRI) for thigh muscle size. The second session involved the measurement of body composition via whole body air-displacement plethysmography (Life Measurement Instruments, Concord, CA) and a muscle biopsy of the vastus lateralis (VL). The remaining four visits involved the measurement of functional ability and muscle function. The first two of these four visits were used for familiarization to the tests, whereas the third was used for data analysis (i.e., the data presented here), and the fourth for the determination of the reliability of these measurements. Each of these four visits was separated by at least 2 days. The muscle biopsy and the measurements of muscle size and strength were completed on the dominant leg. Dominance was determined by asking the subject which leg he or she would use to kick a ball.

MRI

Following 1 h of supine rest to control for the influence of postural-related fluid shifts on muscle size (8), MRI were obtained for each subject, as our laboratory has previously described (48). Subjects were supine, and their heels were fixed on a nonmetallic support to control joint and scan angle and to minimize compression of the legs against each other and the MRI gantry. Imaging was completed in a 1.5-T GE Signa scanner (General Electric, Milwaukee, WI) to determine the volume and cross-sectional area (CSA) of the total quadriceps femoris, rectus femoris (RF), VL, vastus intermedius (VI), and vastus medialis (VM). Following an initial scout scan, interleaved transaxial images of 1 cm thick (repetition time/echo time = 2,000/9.0 ms, field of view 48 cm, 256 X 256 matrix) were taken from the top of the greater trochanter of the femur to the articular surface of the tibia. Magnetic resonance images were transferred electronically from the scanner to a personal computer (Macintosh Power PC) and analyzed with National Institutes of Health Image software (version 1.60) using manual planimetry. Analyses of the magnetic resonance images began with the first proximal slice not containing gluteal muscle and continued distally to the last slice containing RF. The average CSA (cm2) was taken as the average of all of the analyzed slices for an individual muscle and determined for the RF, VL, VI, and VM and summed for the total quadriceps femoris. Muscle volume (cm3) was calculated by multiplying the CSA of each individual muscle by the slice thickness (1 cm) for all analyzed images of the RF, VL, VI, and VM, and summed for the total quadriceps femoris. All measurements were made by the same investigator.

Muscle Biopsy

Each subject underwent a muscle biopsy (9) of the VL for the measurement of intramuscular connective tissue, general and contractile proteins, and MHC distribution. Following the biopsy, excess blood, visible fat, and connective tissue were removed, and portions of the muscle to be used for the aforementioned analyses were immediately frozen and stored in liquid nitrogen (−190°C) until analysis. An additional portion of the muscle was used to study the single-muscle fiber contractile characteristics of a subset (n = 24) of the subjects, and the results of these experiments are presented elsewhere (47).

Intramuscular Connective Tissue Quantification

Collagen concentration. Skeletal muscle collagen concentration was determined via measurement of the collagen-specific amino acid, hydroxyproline (HYP). The concentration of HYP was quantified by HPLC and fluorometric detection (1100 Series, Agilent Technologies, Wilmington, DE) via the precolumn derivatization method described by Hutson et al. (21) with modifications for human skeletal muscle. Muscle samples (~10 mg) were weighed at −35°C on a precision microbalance (AD-2Z Autobalance, Perkin-Elmer, Wellesley, MA) and hydrolyzed in 1 ml of 6 M HCl at 110°C for 30 h. In addition, a sarcosine internal standard (Sigma no. S7672, St. Louis, MO) prepared in water was added to each vial. The hydrolyzates were allowed to cool to room temperature and were neutralized with 6 M NaOH. HYP standards (Sigma no. 56250) of 1, 10, 25, 50, 75, 100, and 125 μM were prepared along with the 2 mM sarcosine internal standard and were used to generate a standard curve.

Derivatization was performed according to the procedures described by Hutson et al. (21), and separation was achieved through an XTerra RP 18, 5 μm, 250 mm × 4.6 mm column (Waters, Milford, MA) using an isocratic mobile phase of 65% acetic acid (3% glacial

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young</th>
<th>Old</th>
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<tbody>
<tr>
<td>n</td>
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<td>22</td>
</tr>
<tr>
<td>Age, yr</td>
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<td>78±1 (70–93)*</td>
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<td>167±2*</td>
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<td>Weight, kg</td>
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<td>Body fat, %</td>
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</table>

Values are means ± SE; n, no. of subjects. The Young group contained 10 men and 10 women. The Old group contained 10 men and 12 women. *P < 0.05 from young.
acetic acid, sodium acetate buffered to pH 4.3/35% acetonitrile (ACN) at a 1.0 ml/min flow rate. Peaks were monitored at 260/316 nm (excitation/emission) with a gain of 8 and integrated with chromatography software (ChemStation, Agilent Technologies) (Fig. 1). HYP signals were normalized to the internal standard for each injection, and HYP concentration was determined from standard curves of HYP (correlation coefficient of 0.983). All samples and standards were run in triplicate with a mean coefficient of variation of 0.32 and 0.69%, respectively. Collagen concentration was calculated from the HYP concentration, assuming collagen weighs 7.5 times the measured HYP weight and the molecular weight of collagen is 300,000 (13, 14). Samples were normalized to muscle wet weight, and data are expressed as micrograms of collagen per milligram wet weight of muscle.

**Collagen cross-link concentration.** The extent of collagen cross-linking was determined by measuring the amount of the pyridinium cross-link HP through HPLC, as previously described (6), with modifications for human skeletal muscle. Muscle samples (~10 mg) were weighed at −35°C and immediately placed into screw cap vials containing 1 ml of 6 M HCl, after which the samples were hydrolyzed at 110°C for 20 h. A 500-µl aliquot of the hydrolyzate was then added to a sample cocktail consisting of a 4:1:1 ratio of butanol-cellulose slurry-acetic acid, containing acetylated pyridinoline (Quidel no. 8006, San Diego, CA) as an internal standard.

Samples for collagen cross-links were extracted through CFI cellulose partition chromatography, as described previously (43). The cross-links were eluted with water and evaporated to dryness in a Savant Speedvac sample concentrator. Samples were reconstituted in 0.5% heptafluorobutyric acid (HFBA) in 10% ACN and then filtered (0.22 µM nylon filter) through centrifuge spin columns (Costar Spin X) at 10,000 g for 10 min to remove residual cellulose particles.

HP standards (Quidel no. 8004) of 5, 10, 25, 50, 75, and 100 pmol were prepared in 0.5% HFBA/10% ACN buffer with acetylated pyridinoline added as an internal standard. Standards and samples were then injected into the HPLC (Agilent Technologies) and eluted, as previously described with modifications (6). The column (XTerra RP 18, 5 µm, 250 mm × 4.6 mm, Waters) was equilibrated with 0.13% HFBA in 22% methanol, and cross-links were eluted with a 1.0 ml/min flow rate from 0 to 30 min. A washing step followed immediately using 0.1% HFBA in 75% ACN for 10 min. Fluorescence of HP was monitored at 295/395 nm (excitation/emission) and integrated with chromatography software (ChemStation, Agilent Technologies) (Fig. 1).

Standard and sample signals were normalized to the internal standard signal, and sample HP recovery was then calculated as the percentage of sample internal standard signal relative to the mean internal standard signal obtained from the known HP standard curve. Mean recovery of skeletal muscle HP was ~80% after CFI purification. The concentration of skeletal muscle HP was determined from the standard curve and adjusted for recovery. Cross-link concentration of each tissue sample was expressed as millimoles of HP per mole of collagen, assuming the molecular weight of collagen is 300,000 (7).

**AGE concentration.** The AGE, pentosidine, was measured in human skeletal muscle through HPLC and fluorometric detection (Agilent Technologies). Muscle samples (~10 mg) were weighed at −35°C and immediately placed into screw cap vials containing 1 ml of 6 M HCl. Samples were then hydrolyzed at 110°C for 20 h. Following hydrolysis, samples were evaporated to dryness in a Savant Speedvac sample concentrator followed by reconstitution with 0.5% HFBA/10% ACN buffer containing 300 pmol of pyridoxine internal standard (Sigma no. P5669). After reconstitution, the samples were filtered (0.22 µM nylon filter) through centrifuge spin columns (Costar Spin X) at 10,000 g for 10 min. Recovery of internal standard after spin column filtration was tested and found to be 100%. Pentosidine standards of 0.05, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, and 5 pmol were prepared from a known concentration of purified pentosidine obtained from the International Malliard Reaction Society (Vincent Monnier, CWRU, Cleveland, OH) in the same buffer as the samples (0.5% HFBA/10% ACN), which included 300 pmol of pyridoxine.

The column (XTerra RP 18, 5 µm, 250 mm × 4.6 mm, Waters) was equilibrated with 0.05% HFBA in water, and the standards and samples were subsequently injected into the HPLC (Agilent Technologies). Pyridoxine was eluted at ~10 min with an inverse gradient of HFBA (0.05–0%; 0–20 min) and methanol (0–2.5%; 0–20 min) at 1.0 ml/min flow rate. Pentosidine was eluted at ~22 min with 2.5% methanol from 20–30 min. The elution gradients were followed by a washing step of 100% ACN. Fluorescence was monitored at 295/395 at a gain of 12 from 0–17 min for detection of pyridoxine and 328/378 at a gain of 17–25 min for the detection of pentosidine (Fig. 1). Peaks were integrated with chromatography software (ChemStation, Agilent Technologies), and standard and sample signals were reduced with and without normalization to pyridoxine internal standard. Normalization had no effect on the concentrations of pentosidine compared with raw signal when calculated from the standard curve. Skeletal muscle pentosidine concentra-
tion was expressed as millimoles per mole of collagen, assuming the molecular weight of collagen is 300,000 (7).

General Protein Quantification: Mixed, Sarcoplasmic, and Myofibrillar Muscle Protein Concentration

For each subject, a piece of muscle weighing ~10 mg was divided and weighed on a precision microbalance (Cahn 35, Orion Research, Beverly, MA) at ~35°C. Each sample was homogenized in 40 volumes of cold homogenizing buffer (250 mM sucrose, 100 mM potassium chloride, 20 mM imidazole, and 5 mM EDTA; pH 6.8) in a ground-glass homogenizer (Radnoti Glass Technology, Monrovia, CA) (11). Samples were then centrifuged at 20,000 × g for 30 min at 4°C. The supernatant was taken as the sarcoplasmic protein fraction, and the pellet was resuspended in 40 volumes of cold homogenizing buffer and taken as the myofibrillar protein fraction (22, 44).

Aliquots of the homogenate (total mixed protein), sarcoplasmic, and myofibrillar protein fractions were measured for protein concentration using the bicinchoninic acid assay (Sigma), with bovine serum albumin used as the protein standard. The amount of protein in each of the three fractions was normalized to muscle wet weight and expressed as micrograms protein per milligram wet weight of muscle.

As collagen is contained in the mixed protein and myofibrillar protein (i.e., collagen pellets following centrifugation) (3, 32, 33), follow-up bicinchoninic acid protein assays with pure collagen (Sigma no. C9791) were completed to confirm that collagen contributes to the overall absorbance of the muscle measurements (Fig. 2). These measurements were completed because it has been suggested that collagen does not contribute to some assays of mixed protein in skeletal muscle (39, 53).

Contractile Protein Quantification: Myosin and Actin Concentration

Myosin (MHC) and actin concentrations were determined by quantitative gel electrophoresis, as previously described (22, 49). Aliquots of the myofibrillar protein fraction were diluted with SDS buffer [2% SDS, 125 mM Tris·HCl (pH 6.8), 12.5% glycerol, 5% 2-mercaptoethanol, 0.005% bromophenol blue] and heated at 60°C for 4 min. Myofibrillar protein (800 ng) was separated by SDS-PAGE (28). MHC was resolved with a 4% stacking gel and 10% separating gel. Actin was resolved with a 4% stacking gel and a 6–12% gradient separating gel that were allowed to polymerize overnight. For MHC and actin, electrophoresis was performed at a constant current of 20 mA per gel in the stacking gel, and 25 mA per gel in the separating gel with a Tris-glycine electrode buffer at 4°C (Hoeffer SE 600, Amersham Pharmacia Biotech, Piscataway, NJ).

The separating gels were silver stained (15) and digitally photographed (ChemiImager 5500, Alpha Innotech, San Leandro, CA), and densitometry was completed using National Institutes of Health Image software (version 1.60) (47). Each gel was loaded with five standards of either MHC or actin (Sigma nos. M7659, A2522), a molecular weight standard (Pierce, Rockford, IL), and subject samples. All standards and samples were loaded in duplicate, and each gel contained samples from young and old men and women. An average of the duplicate densities was taken to represent each standard and sample. All measurements were made in blinded fashion by the same investigator. Unknown sample amounts of MHC and actin were determined from regression analysis of the standard curves on each gel. Correlation coefficients were 0.98–1.00 for both MHC and actin. The amount of myosin or actin was normalized to muscle wet weight and expressed as micrograms per milligram wet weight of muscle.

MHC Isoform Distribution

MHC isoform composition of each of the myofibrillar protein samples quantified for myosin and actin protein concentrations was determined in triplicate from a SDS buffer-diluted aliquot of the myofibrillar protein fraction heated to 100°C for 5 min. MHC isoforms were separated with SDS-PAGE with a 3.5% stacking gel and 5% separating gel. Electrophoresis was performed at 150 V for ~15 h with a Tris-glycine electrode buffer at 4°C (Hoeffer SE 600, Amersham Pharmacia Biotech, Piscataway, NJ). The separating gels were silver stained (15) and digitally photographed (ChemlImager 5500, Alpha Innotech, San Leandro, CA), and densitometry was completed to determine the percent contribution of each isoform of the total (100%). The average density of each isoform from the three lanes loaded was taken as the MHC distribution for that sample.

Functional Ability

The ability to perform activities of daily living was determined with three functional tests: walk, chair stand, and stair climb. Each of the functional tests was completed three times during each session, with the average of the three tests recorded. Timing of the functional tests was accomplished using retro-reflective sensors (Banner Engineering, Minneapolis, MN) interfaced to a digital timer and display (Veeder-Root, Danaher Controls, Gurnee, IL). The sensors were placed such that the forehead (chair stand or walk tests) or foot (stair climb test) would break the light beams to start and stop timing.

Walk test. Subjects walked (i.e., one foot in contact with the ground at all times) a distance of 6.1 m as quickly as possible. Subjects started three steps behind the first photo eye and continued through the second photo eye. The coefficient of variation for the walk test was 3.8 ± 0.5%, with no group, age, or sex differences.

Chair stand test. Subjects stood from a seated position in a metal chair as fast as possible without support (i.e., via chair or thongs). The coefficient of variation for the chair stand was 6.9 ± 0.9%, with no group, age, or sex differences.

Stair climb test. Subjects started two steps before the first stair and climbed a flight of 10 stairs (3.3-m vertical distance) as quickly as possible without skipping a stair. The coefficient of variation for the stair climb was 3.6 ± 0.5%, with no group, age, or sex differences.

Muscle Function

Following the functional ability measurements, subjects completed light stretching of the legs and a 5-min warm-up on a bicycle ergometer (Monark Exercise AB). Muscle function testing was per-
formed on a Cybex Norm dynamometer (Lumenex, Ronkonkoma, NY) and associated equipment fabricated by the investigators. Measurements were made of in vivo maximal shortening velocity ($V_{\text{max}}$), as well as static and dynamic muscle strength and power of the quadriceps femoris muscle. Adequate rest periods separated all of the tests within each testing session.

$V_{\text{max}}$. $V_{\text{max}}$ was determined by having subjects extend their lower leg from a metal bar placed behind their heel to establish a starting position at 90° of knee flexion through a 90° arc and into a heavily padded bar as fast as possible. Fiber-optic sensors (Banner Engineering, Minneapolis, MN) interfaced with a computer timer (LabVIEW, National Instruments, Austin, TX) were positioned at 60° and 30° of knee flexion to measure the time to move through the 30°. Subjects were allowed three warm-up attempts followed by three timed leg extensions, with the fastest time of the three recorded. The coefficient of variation was 3.5 ± 0.5%, with no group, age, or sex differences.

Static and dynamic force and power. Following completion of the $V_{\text{max}}$ measurements, maximal isometric force ($P_o$) and concentric muscle forces were determined. $P_o$ was measured at 60° of knee flexion, and subjects were allowed one warm-up trial followed by two maximal contractions, with the greater value taken as $P_o$. A 1-min rest period separated the two maximal contractions. Coefficient of variation for the measurement of $P_o$ averaged 6.2%, with no age, group, or sex difference.

Maximal isokinetic concentric force was determined at 60, 120, 180, 240, 300, 360, 420, 480, and 500°/s. At each velocity, the subjects were allowed three warm-up attempts, immediately followed by three maximal concentric contractions, with the highest force attained for each velocity used for analysis. A 2-min rest period was given between velocities. Some subjects were not able to produce force at the higher velocities, and/or their measured $V_{\text{max}}$ was lower than some of the higher measurement velocities. Coefficient of variation for all concentric velocities, 60–500°/s, averaged 5.3, 3.9, 4.2,
Table 2. Functional ability and muscle characteristics

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<tr>
<td>Stair climb</td>
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<td>Power, W</td>
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<td>Chair rise time, s</td>
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<td>Walk time, s</td>
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Values are means ± SE; n, no. of subjects. Vₘₐₓ, maximal shortening velocity; MHC, myosin heavy chain. *P < 0.05 from young.

5.5, 5.5, 6.4, 5.8, 7.6, and 8.2%, respectively, with no age, group, or sex difference.

Peak power was calculated for each subject by curve fitting the data of power (force × velocity) vs. the force produced at each velocity, represented as a percentage of P₀ (%P₀). Data to develop the power curve was used, if the measurement velocity was below the measured Vₘₐₓ for that subject and force production at two consecutive velocities differed by >4 N·m.

Statistics

A two-way (age and sex) ANOVA was used to compare all variables. No significant interactions were detected. Therefore, post hoc comparisons were not completed, and the data were collapsed and presented as young and old. Significance was accepted at P < 0.05. Data are presented as means ± SE.

RESULTS

Intramuscular Connective Tissue

Collagen concentration (young: 9.6 ± 1.1; old: 10.2 ± 1.2 μg/mg muscle wet wt) and pyridinoline cross-links (young: 395 ± 65; old: 351 ± 45 mmol HP/mol collagen) were unchanged (P > 0.05) with aging, whereas AGEs were increased (P < 0.05) by ~200% (young: 5.2 ± 1.3; old: 15.9 ± 4.5 mmol pentosidine/mol collagen) with aging (Fig. 3).

General and Contractile Proteins

All three general skeletal muscle protein pools were reduced (P < 0.05) with aging (Fig. 4). Mixed protein concentration was reduced by 4% (young: 160 ± 3; old: 154 ± 1 μg/mg muscle wet wt), myofibrillar by 5% (young: 103 ± 2; old: 98 ± 2 μg/mg muscle wet wt), and sarcoplasmic by 8% (young: 64 ± 1; old: 59 ± 1 μg/mg muscle wet wt). The two main contractile proteins, myosin (young: 46 ± 3; old: 54 ± 4 μg/mg muscle wet wt) and actin (young: 21 ± 1; old: 17 ± 1 μg/mg muscle wet wt), were not changed (P > 0.05) with aging. The proportion of MHC I in the muscle increased (P < 0.05) with aging, whereas MHC IIa and IIx were reduced (P < 0.05) (Table 2).

Whole Muscle Characteristics

Quadriceps muscle volume was reduced (P < 0.05) by 29% with aging (Table 2). Quadriceps muscle CSA followed this same trend and was reduced (P < 0.05) by 23% (young: 62.9 ± 2.9; old: 48.7 ± 2.3 cm²) (Fig. 5). P₀ of the quadriceps was reduced (P < 0.05) by 35% (young: 218 ± 13; old: 141 ± 11 N·m), and P₀ normalized to muscle size was reduced (P < 0.05) by 17% (young: 3.46 ± 0.14; old: 2.85 ± 0.11 N·m·cm⁻²) (Fig. 5). Similarly, peak power of the quadriceps was reduced (P < 0.05) by 48% (young: 464 ± 36; old: 243 ± 23 W), and peak power normalized to muscle CSA was reduced (P < 0.05) by 33% (young: 7.25 ± 0.33; old: 4.85 ± 0.29 W/cm²) (Fig. 5). Stair climb, chair rise, and walk time were increased by 45–80%, and stair climbing power was reduced by 40% (Table 2) with aging. Quadriceps Vₘₐₓ was reduced by 23% (Table 2).

Fig. 5. Comparison between young and old skeletal muscle functional characteristics. CSA, cross-sectional area; P₀, peak isometric force; P₀/CSA, peak isometric force normalized to muscle CSA; peak power/CSA, peak power normalized to muscle CSA. *P < 0.05 from young.
DISCUSSION

The results of the present study show that endomysial collagen concentration and enzymatically mediated collagen cross-linking are tightly regulated with aging, as evidenced by the similar concentrations in young and old individuals. However, nonenzymatically regulated AGE cross-linking is significantly increased in muscle from healthy, sedentary elderly individuals. These results suggest that the formation of AGEs over the lifespan of an individual may contribute to increased muscle connective tissue protein stiffness and thus contribute to impaired muscle function in the elderly.

Contrary to our hypotheses, aging had no effect on the intramuscular collagen concentration. In addition, there was no sex-specific influence on intramuscular collagen levels. Several recent studies have shown the collagen fraction of skeletal muscle is much more dynamic than previously thought (3, 32, 33), and the synthesis rate of intramuscular collagen is elevated in older men (3). Based on these findings and the collagen concentration data from the present study, collagen breakdown would also have to be elevated to a similar degree to prevent the accretion of collagen within the muscle. The discrepancy between the present study and previous animal findings may be related to the specific portions of the muscle connective tissue studied. Data obtained from the muscle biopsy technique mainly reflect the endomysial collagen layers and not the peri- or epimysial layers of connective tissue, which have a different composition of collagen types and roles in the transfer of mechanical forces (20, 23, 37). This is contrary to the animal data, which utilizes excised whole muscles for the analysis of connective tissue. Thus the potential that biochemical changes are occurring in the outer connective tissue layers with aging cannot be excluded. Finally, it should also be considered that, whereas intramuscular collagen concentration did not change with aging, it is possible that the type or isoforms of collagen changed. Aging animal muscle has been shown to alter the collagen isoforms, either by increasing type IV collagen (27) or decreasing type III collagen (16). In these cases, the collagen may be changing morphology to accommodate a functional need.

To our knowledge, the present investigation is the first to examine the effects of aging on human skeletal muscle collagen cross-linking. Limited reports exist from postmortem analysis of human skeletal muscle that shows the primary mature intramuscular pyridinium cross-link is HP, and lysylpyridinoline is found only in trace amounts (14, 45). As a result, our focus in healthy adult skeletal muscle was limited to that of the abundant, mature HP species. The existing literature of collagen cross-linking in aging animals has focused mainly on HP and demonstrates that aging results in a significant increase in HP, with concomitant increases in muscle stiffness (17, 18). The lack of change seen in aging human muscle may indicate that the normal turnover of skeletal muscle collagen is robust enough to also turn over mature endomysial connective tissue cross-links, and these cross-links may not contribute to the reduced muscle function that occurs with aging.

In contrast to HP collagen cross-linking, the formation of AGE cross-links is not enzymatically regulated, but dictated by the presence of a reducing sugar, the appropriate protein side chain, and oxygen (4). The differences seen in AGE concentration between young and old in the present investigation are most likely related to the temporal component of aging. That is, the more time that protein residues have to come into contact with glucose, the greater the chance that AGEs will form. It should be noted that all of the subjects demonstrated normoglycemia and were absent of overt disease, thus the increased AGEs noted in the old are reflective of factors other than altered glucose metabolism, such as that seen in diabetic individuals. The accumulation of AGEs in collagenous tissues has been shown to negatively affect function, such as stiffening of the blood vessel walls and kidney structures (4, 35). It is likely that the 200% increase in pentosidine seen in the older individuals of the present study influenced tissue stiffness and the passive viscoelastic properties of the muscle and thus contributed to declines in muscle function. In this context, it should be noted that pentosidine is commonly used as a surrogate for the many other AGE cross-links (4), and the large increase seen herein is reflective of the accumulation of all possible AGEs. The accumulation of AGEs in the skeletal muscle of the aging women and men in the present study may be due to the lack of a regular and robust tissue turnover stimulus, such as exercise (19, 31, 33). These concepts warrant further study of AGE formation in aging human skeletal muscle and the influence of both acute and chronic exercise.

As the concentrations of mixed, myofibrillar, and sarcoplasmic proteins found in skeletal muscle are a result of the net turnover (i.e., the sum of protein synthesis and degradation) (5, 46, 50, 51, 54) and were reduced in the old men and women, there appears to be a net imbalance between these two processes in aging skeletal muscle. In addition, the reduction in the myofibrillar protein concentration, in light of the maintenance of myosin, actin, and collagen concentrations (Fig. 5), suggests that other proteins in the myofibrillar apparatus [i.e., titin, nebulin, C protein, M protein (37)] are disproportionately lost with aging. Collectively, the current and previous (47) data collected on the individuals in this study suggest a primary contributor to sarcopenia is the loss of MHC II fiber number (30) (Table 2). To this end, MHC IIa muscle fibers produce five to six times the normalized power as a MHC I fiber (∼9 vs. −1.5 W/l) (47). Thus an aging individual could afford to lose five to six MHC I fibers for every one MHC IIa fiber lost. Having a higher proportion of MHC I would lend itself to a slower contracting whole muscle, which is supported by the 23% slower in vivo maximal contraction velocity in the older subjects. Of course, we cannot rule out the role of a change in the neural control of muscle with aging (29).

This study and the data reported here are the first to comprehensively examine the intramuscular connective tissue network in aging men and women. These data suggest that, despite large changes in muscle mass, the concentrations of the two main contractile proteins, myosin and actin, the protein responsible for the transfer of force out to the whole muscle, collagen, and the enzymatically regulated cross-linking of collagen are tightly regulated in aging human skeletal muscle. It does appear that nonenzymatic addition of AGEs to the intramuscular connective tissue network may play a role in the reduction of muscle and physical function with aging.
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