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RESEARCH ARTICLE

Skeletal muscle LINE-1 ORF1 mRNA is higher in older humans but decreases with endurance exercise and is negatively associated with higher physical activity

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Roberson PA, Romero MA, Osburn SC, Mumford PW, Vann CG, Fox CD, McCullough DJ, Brown MD, Roberts MD. Skeletal muscle LINE-1 ORF1 mRNA is higher in older humans but decreases with endurance exercise and is negatively associated with higher physical activity. *J Appl Physiol* 127: 895–904, 2019. First published August 1, 2019; doi:10.1152/japplphysiol.00352.2019.—The long interspersed nuclear element-1 (L1) is a retrotransposon that constitutes 17% of the human genome and is associated with various diseases and aging. Estimates suggest that ~100 L1 copies are capable of copying and pasting into other regions of the genome. Herein, we examined if skeletal muscle L1 markers are affected by aging or an acute bout of cycling exercise in humans. Apparently healthy younger (23 ± 3 y, $n = 15$) and older participants (58 ± 8 y, $n = 15$) donated a vastus lateralis biopsy before 1 h of cycling exercise (PRE) at ~70% of heart rate reserve. Second (2 h) and third (8 h) postexercise muscle biopsies were also obtained. L1 DNA and mRNA expression were quantified using three primer sets [5′ untranslated region (UTR), L1.3, and ORF1]. 5′UTR and L1.3 DNA methylation as well as ORF1 protein expression were also quantified. PRE 5′UTR, ORF1, or L1.3 DNA were not different between age groups ($P > 0.05$). ORF1 mRNA was greater in older versus younger participants ($P = 0.014$), and cycling lowered this marker at 2 h versus PRE ($P = 0.027$). 5′UTR and L1.3 DNA methylation were higher in younger versus older participants ($P < 0.05$). Accelerometry data collected during a 2-wk period before the exercise bout indicated higher moderate-to-vigorous physical activity (MVPA) levels per day was associated with lower PRE ORF1 mRNA in all participants ($r = -0.398$, $P = 0.032$). In summary, skeletal muscle ORF1 mRNA is higher in older apparently healthy humans, which may be related to lower DNA methylation patterns. ORF1 mRNA is also reduced with endurance exercise and is negatively associated with higher daily MVPA levels.

NEW & NOTEWORTHY The long interspersed nuclear element-1 (L1) gene is highly abundant in the genome and encodes for an autonomous retrotransposon, which is capable of copying and pasting itself into other portions of the genome. This is the first study in humans to demonstrate that certain aspects of skeletal muscle L1 activity are altered with aging. Additionally, this is the first study in humans to demonstrate that L1 ORF1 mRNA levels decrease after a bout of endurance exercise, regardless of age.

aging; endurance exercise; LINE-1; methylation; retrotransposons

INTRODUCTION

Transposable elements are repetitive DNA sequences that exist in almost every organism tested and possess the ability to mobilize and change positions. DNA transposons have been suggested to play a role in human evolution but have not been active in the human genome for over 50 million years (24). Retrotransposons, however, are still active within the human genome, and the long interspersed nuclear element-1 (L1) is highly abundant with an estimated 500,000 copies existing in the genetic code (36). L1 is ~6.0 kilobases in length, contains a 5′ untranslated region (UTR) with an internal promoter, two open reading frames that encode for the ORF1 and ORF2 proteins, and a 3′ UTR containing a polyadenylation tail (34).

Retrotransposition is the process whereby the insertion of new L1 copies into the genome occurs, and this event requires numerous coordinated steps (26). First, L1 transcription is facilitated by RNA polymerase II in the nucleus. L1 mRNA is exported to the cytoplasm where it associates with ribosomes. Translated ORF1 and ORF2 proteins show cis preference to bind to L1 mRNA (39). The ORF1 protein is ~40 kDa and forms trimer complexes to facilitate L1 translocation back into the nucleus (29). The ORF2 protein possesses multiple domains and contains endonuclease and reverse transcriptase activities to aid in the retrotransposition of L1 (16). The resultant ribonucleoprotein particle is then capable of entering the nucleus whereby endonuclease activity of the ORF2 protein cleaves DNA and reverse transcriptase activity of the ORF2 protein catalyzes the integration and insertion of a new L1 copy into the genome (10). Integration of new L1 sequences appears to be random, and insertion of these sequences within protein-coding sequences can alter protein structure and function (31). Although there are numerous L1 copies in the genome, it is notable that only 80–100 copies are active and capable of retrotransposition (2).

There are various mechanisms that can act to inhibit the retrotransposition process, albeit increases in L1 promoter methylation is a well-known inhibitory mechanism of L1 transcription (7). White blood cell L1 hypomethylation appears to be related to aging (9), increased adiposity (28, 35), and various cancers (15). Furthermore, cardiac ischemia upregulates L1 transcription in rats, and when L1 transcripts were silenced by antisense oligonucleotides, infarct size was significantly lower compared with controls receiving a scrambled

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oligonucleotide (27). L1 mRNA has also been shown to be upregulated in synovial fluid samples obtained from patients experiencing rheumatoid arthritis (33). Although no investigations have examined if skeletal muscle L1 markers are differentially expressed between younger and older humans, De Cecco et al. (12) demonstrated that skeletal muscle L1 mRNA expression was 4-fold greater in 36-mo-old mice versus 5-mo-old mice, and L1 DNA content was ~80% greater in the former versus the latter group. Collectively, these data suggest that markers of increased L1 activity coincide with adverse health outcomes and, specific to skeletal muscle, increased L1 activity may be a hallmark of tissue aging.

It is generally recognized that physical activity and exercise improve overall health outcomes (4). An acute bout of endurance exercise has been shown to alter global methylation status in skeletal muscle (1), and chronic exercise activity has been shown to be associated with a higher L1 methylation percentage in white blood cells (28). Resistance training has been shown to reverse the transcriptomic profile associated with aging in skeletal muscle (30). Furthermore, our laboratory has shown that acute bouts of resistance exercise as well as chronic resistance training increase skeletal muscle L1 methylation and reduce L1 mRNA expression in humans (37). Given some similarities in metabolic demand and cellular signaling between endurance and resistance exercise (19), it is possible that endurance exercise may also alter L1 methylation and mRNA expression.

The aforementioned evidence suggests that skeletal muscle L1 expression and retrotransposition increases with aging, and an acute bout of endurance exercise may increase L1 methylation and decrease L1 mRNA expression. Higher levels of physical activity, regardless of age, may also accomplish a similar molecular phenotype. Therefore, the purpose of this study was to determine baseline differences in skeletal muscle L1 DNA, L1 mRNA, and L1 DNA methylation status between apparently healthy younger and older humans. Furthermore, we sought to determine if an acute bout of endurance exercise alters these markers and whether daily physical activity levels were associated with these markers.

MATERIALS AND METHODS

Participants. This study was approved by the Auburn University Institutional Review Board and conformed to the standards set by the latest revision of the Declaration of Helsinki (Institutional Review Board protocol no. 18–226 AR 1806). Participants read and provided a written informed consent form before participating in the study. Inclusion criteria for this study were as follows: *1*) participants could be a man or woman between the ages of 18–30 yr ("young") and 50–80 yr ("old"), *2*) participants had to be able to cycle for at least 60 min, and *3*) participants had to be apparently healthy and free of any known disease. Notably, men and women were recruited for this study. As a result, we cannot discredit the effect of sex-based hormones on the present study's findings; however, literature is lacking on this topic, and as a result, we did not find it necessary to exclude either sex. Physical activity and medical history questionnaires were filled out before participation to establish physical activity patterns, health status, and to identify potential risk factors that could be aggravated by exercise. Eligible participants that provided written informed consent then engaged in the study as described below.

Experimental design. Figure 1 presents an illustrative figure of the study design. During the initial visit participants provided written consent and then were screened using medical history and physical activity questionnaires as stated above. Thereafter, participants were split into two groups based on age and were given an Actigraph GT3X accelerometer (Actigraph, Pensacola, FL) to monitor physical activity throughout the study. Accelerometers were worn on the right hip and attached with an elastic strap. Participants were asked to wear the device as often as possible with the exception of sleeping and water-based activities, and they were asked to fill out an on-off log and record an off time if the accelerometer was taken off for more than 5 min. Cut points for each activity level were based on Freedson et al. (17). Wear time when participants were unable to exercise because of study design were excluded from analyses.

Participants arrived 1 to 7 days following the initial visit, 8 h fasted, not having consumed alcohol in 24 h, exercised in 48 h, nor ingested caffeine within 8 h. Maximum leg extensor torque was assessed using an isokinetic dynamometer on the right leg. During the same session, maximal aerobic power ($\dot{V}O_{2peak}$) testing occurred on a cycle ergometer to determine each participant's maximal aerobic power. Following at least 7 days, but no more than 14 days, the acute exercise bout occurred, which consisted of body composition testing, ultrasonography for vastus lateralis thickness, a resting vastus lateralis biopsy and blood draw (PRE), cycling for 60 min at 70% heart rate (HR) reserve (HRR), an immediate postexercise blood draw (POST), a 2-h postexercise muscle biopsy and blood draw (2 h), and an 8-h postexercise muscle biopsy and blood draw (8 h). Notably, blood draws were performed for other analyses not included in this study but are included in these methods to disclose all procedures being performed on participants. Each test is described in greater detail below.

Muscle function and $\dot{V}O_{2peak}$ testing. Participants first performed maximal isokinetic right leg extensions on an isokinetic dynamometer (System 4 Pro, BioDex Medical Systems, Shirley, NY) at 30° and 120°/s. Participants performed a standardized warm-up before exercise by completing 1 set of 4 repetitions at 30°/s at 80% maximal effort. Participants then performed two sets of four repetitions for each velocity. For each set, the first two repetitions served as a graded

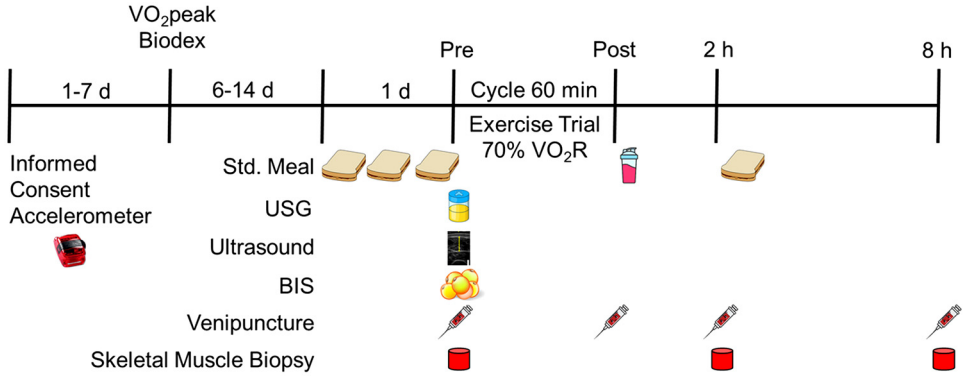

Fig. 1. Graphical illustration of experimental design. Detailed methods are described in-text. BIS, bioelectrical impedance spectroscopy; USG, urine-specific gravity; $\dot{V}O_{2peak}$, maximal aerobic power; $\dot{V}O_2R$, oxygen uptake reserve.

warm-up to maximal effort, whereas the following two repetitions were maximal effort. Sets were separated by 60 s of rest. The highest value for each velocity was determined as maximal torque.

Ten minutes following isokinetic dynamometer testing, $\dot{V}O_{2peak}$ and peak power (W_{max}) testing were performed on an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA). Participants performed a self-selected warm-up for 5 min and began the test at an intensity corresponding to what the subject perceived as comfortable for a 60-min cycling session. Intensity was increased by 30 W every 2 min until volitional fatigue or until cadence dropped below 50 revolutions/min. Participants then rested 10 min and performed a validation stage thereafter. The validation stage consisted of participants cycling to exhaustion at the wattage during the uncompleted stage they ceased during the preceding test. During the cycling test, indirect calorimetry (Parvomedics; Sandy, UT) was used to collect expired gases for oxygen uptake ($\dot{V}O_2$) and respiratory exchange ratio, and values were averaged in 30-s intervals. HR was measured using an HR monitor (Polar; Lake Success, NY), and following exercise, HRR for each participant was measured by subtracting resting, pre-exercise HR from maximal HR during the maximal exercise test. Participants indicated rating of perceived exertion (RPE) by pointing to a Borg RPE scale (6–20) at the end of each stage. A test was deemed as maximal effort if HR was within 10% of age-predicted max HR, RPE $\geq$17, respiratory exchange ratio $\geq$1.10, and a plateau in $\dot{V}O_2$ occurred ($<$250-mL increase in $\dot{V}O_2$ from the end of the original test and over the validation stage).

Standardized meals before the exercise trial. The exercise trial occurred 7–14 days following right leg extensor torque testing and $\dot{V}O_{2peak}$ testing. Given that calorie consumption affects skeletal muscle L1 markers in rodents (12), we posited that controlling participants' diets was a critical aspect of the study design. To control for dietary intakes, participants were provided standardized meals 24 h before their exercise trial. Each participant's basal metabolic rate was determined using the Harris-Benedict equation (21) and then multiplied by 1.5 as an activity coefficient to ensure adequate nourishment. Standardized meals were provided in the form of commercially prepared sandwiches (Uncrustables, Smucker's; Orrville, OH). Each sandwich provided 210 kcal, 9 g total fat, 28 g carbohydrates, 2 g fiber, and 6 g protein. Thus, 10 sandwiches provided 2,100 kcal, 90 g total fat (39% of total kcal), 280 g carbohydrates (50% of total kcal), and 60 g protein (11% of total kcal). If a participant's estimated basal metabolic rate ($\times$1.5 activity coefficient) was 2,100 kcal/day, then 10 sandwiches were provided, and the participant was instructed to evenly consume them over breakfast, lunch, and dinner 24 h before the exercise trial.

Exercise trial. Participants were instructed to report for the exercise trial between 0500 and 0800, 8 h fasted, not having consumed alcohol in 48 h, exercised in 72 h, nor ingested caffeine within 8 h. Testing began with assessment of hydration status measured through urine testing by an ATAGO 2392 handheld refractometer (Bellevue, WA). Participants were allowed to proceed with testing only if urine-specific gravity level was less than 1.030 g/mL; notably, all participants presented acceptable values below the cutoff. Following hydration assessment, muscle thickness was determined by ultrasound using a 3–12-MHz multifrequency linear phase array transducer (Logiq S7 R2 Expert, General Electric; Fairfield, CT). Briefly, the ultrasound probe was positioned in a transverse orientation on the vastus lateralis, halfway between the greater trochanter and lateral epicondyle, where a cross-sectional image was captured. Vastus lateralis thickness was measured using associated software by spanning the measurement tool from superficial to deep aponeuroses, and midthigh thickness was measured from the outer femur edge to superficial aponeurosis. Further details regarding test-retest reliability of this procedure have been previously published by our laboratory (23).

After the ultrasound testing, bioelectrical impedance spectroscopy was utilized to determine whole body lean soft tissue mass and fat mass (Imp SFB7, Impedimed; Pinkemba Queensland, Australia). Briefly, participants rested in a supine position on an athletic training table for 5 min, during which electrodes each were placed ~5 cm apart on the right hand and right foot according to manufacturer's instructions. Readings were conducted twice and averaged to obtain whole body lean soft tissue mass and fat mass. Further details regarding test-retest reliability of this procedure have been previously published by our laboratory (23).

After the body composition testing, participants were instructed to lie supine on another athletic training table in preparation for a venipuncture and a vastus lateralis skeletal muscle biopsy. Following 5 min of supine rest, venous blood samples were obtained from an antecubital vein. Blood samples were collected into a serum-separating tube and a mononuclear cell preparation tube with sodium citrate (BD Vacutainer; Franklin Lakes, NJ). Serum and mononuclear cell preparation tubes were spun at 1,800 *g* for 30 min, and supernatants were stored at -80°C for other analyses not presented herein. Following venipuncture, the outer aspect of each participant's upper right leg was prepared for a skeletal muscle biopsy as described in previous publications by our laboratory (23, 32). Briefly, a 3 $\times$ 3-in. area approximately halfway between the iliac crest and the patella was shaved, cleaned with alcohol, and cleansed with betadine. Lidocaine (1.0 mL) was injected to provide local anesthesia and given 5 min to enact effects. With the use of aseptic technique, a single-use, sterile no. 11 blade was used to make a 1-cm incision into the skin and muscle fascia. Tissue was then extracted using a 5-gage Bergstrom biopsy needle with suction. Tissue was blotted of blood, and any connective tissue was immediately removed. Participants were then dressed with a sterile butterfly bandage, sterile gauze, and an elastic bandage. Tissue distribution for future analyses and analysis procedure is later described.

After the skeletal muscle biopsy, participants were fitted with an HR monitor (Polar), mounted to an electronically-braked cycle ergometer (Velotron, RacerMate, Seattle, WA), and performed a 5 min warm-up at a self-selected pace. Wattage was then adjusted to achieve 70% $\dot{V}O_{2Reserve}$, which was measured by the corresponding HRR determined during $\dot{V}O_{2peak}$ testing, and participants cycled for 60 min. HR was continuously monitored throughout the cycling bout, and cycling resistance was adjusted accordingly to ensure 70% HRR was maintained. Drinking water was administered ad libitum throughout the trial.

Immediately following the 60-min cycling exercise trial, venipuncture was performed as described previously. Since participants had not consumed calories since the day prior, a standardized postexercise recovery beverage [~30 g carbohydrate via Maltodextrin (Dymatize Nutrition; Dallas, TX) and ~30 g protein (Six Star Pro Nutrition, Six Star Plant-Based Protein Smoothie; Blasdell, NY)] was provided to participants. Participants were monitored in the laboratory until 2 h following the cycling trial when a second muscle biopsy and third venipuncture was performed. Participants were then instructed to consume standardized meals within 1 h, which consisted of 25% of their basal metabolic rate in the form of commercially prepared sandwiches (Uncrustables) and could leave the facility. Participants were instructed to maintain low levels of physical activity (e.g., do not walk briskly or climb stairs and sit when possible) and consume no other calories aside from standardized meals. Eight hours following the cycling trial, a third muscle biopsy and fourth venipuncture were performed.

Skeletal muscle processing. Immediately following the procurement of muscle as previously stated, tissue was rapidly removed from the needles, teased away from blood, connective tissue, and fat, wrapped in prelabeled foils, and flash frozen in a cryogenic dewar containing liquid nitrogen. At the end of the day, tissue was placed in a -80°C freezer until study completion for batch processing. For protein analysis, tissue was pulverized on a liquid nitrogen-cooled ceramic mortar and pestle, and ~20 mg frozen tissue was placed in 1.7-mL microcentrifuge tubes containing 500 μL of ice-cold cell lysis buffer [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA,

1 mM EGTA, 1% Triton; cat. no. 9803, Cell Signaling; Danvers, MA] prestocked with protease and Tyr/Ser/Thr phosphatase inhibitors (2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin). Samples were then homogenized on ice by hand via micropestle manipulation, insoluble proteins were removed with centrifugation at 500 *g* for 5 min, and obtained sample lysates were stored at −80°C before Western blotting.

Frozen tissue was also used for DNA and RNA isolation (~30 mg for each assay). RNA-free DNA was prepared using the Qiagen DNeasy Blood and Tissue Kit following manufacturer's instructions (cat. no. 69504, Qiagen; Germantown, MD). DNA-free RNA was prepared using the Qiagen RNeasy Fibrous Tissue Kit following manufacturer's instructions (cat. no. 74704, Qiagen). After the RNA and DNA isolations, concentrations were quantified using a spectrophotometer (Nanodrop Lite, Thermo Fisher Scientific; Waltham, MA), quality was assessed for DNA and RNA using a 260/280 ratio, DNA samples were adjusted to 1 μg, and RNA was adjusted to 100 ng and reverse transcribed to cDNA using a commercially available kit (cat. no. 95048, Quanta Biosciences; Gaithersburg, MD) following the manufacturer's recommendations. Notably, all samples yielded 260/280 ratios above 1.80. DNA and cDNA were then subsequently frozen at −80°C until batch processing for L1 DNA and mRNA expression analyses described below.

Polymerase chain reaction. L1 DNA and mRNA expression were measured via real-time PCR with SYBR green chemistry (cat. no. 95053, Quanta Biosciences), and three primer sets were used to target the L1 gene. 5′ UTR primers amplified a portion of the L1 gene containing a CpG-rich promoter. L1.3 primers amplified 5′ UTR portions of the L1.3 and L1.4 gene sequences, which are full-length L1 sequences that possess the greatest ability to undergo retrotransposition (5, 13). ORF1 primers amplified a portion of the ORF1 coding sequence. Primer sequences are displayed in Table 1. Fold change scores for each L1 mRNA of interest were calculated as previously reported by our laboratory (37). Briefly, delta quantitation cycle (ΔCq) values were calculated using the geometric mean Cq of B2M, GAPDH, and PPIA minus the gene of interest Cq. $2^{\Delta Cq}$ values were then calculated, and gene of interest fold change values were obtained by dividing each individual $2^{\Delta Cq}$ value by the younger PRE $2^{\Delta Cq}$ group mean. For example, if the younger PRE group average $2^{\Delta Cq}$ was 100 and an individual's $2^{\Delta Cq}$ value was 200, then the fold change score of this individual was 2.0.

L1 promoter methylation. L1 promoter methylation analyses were performed from isolated DNA (described above) using a commercially available methylated DNA immunoprecipitation kit (cat. no. 117133, Abcam; Cambridge, MA). L1 5′ UTR and L1.3 primers listed above were utilized, given that both span CpG-rich areas in the 5′ UTR. Prior to methylated DNA immunoprecipitation being performed, 1.5 μg DNA was digested using MseI (cat. no. R0525, New England BioLabs; Ipswich, MA). Thereafter, total methylated DNA from a total of 1 μg input DNA was immunoprecipitated using an anti-5-methylcytosine antibody provided within the kit. Additionally, 500 ng of residual input DNA from each sample was used to normalize real-time PCR results. Fold change scores L1 5′ UTR and L1.3 methylation were calculated as previously reported by our laboratory (37). Briefly, $2^{\Delta Cq}$ values were calculated whereby ΔCq = input DNA Cq − methylated DNA Cq. Fold change values were then obtained by dividing each individual $2^{\Delta Cq}$ value by the younger PRE $2^{\Delta Cq}$ group mean.

Western blotting. Whole tissue sample lysates obtained through cell lysis buffer processing were batch process-assayed for total protein content using a BCA Protein Assay Kit (cat. no. 23225, Thermo Fisher Scientific). Lysates were then prepared for Western blotting using 4× Laemmli buffer at 1 μg/μL. Following sample preparation, 25 μL samples were loaded onto 4%–15% SDS-PAGE (cat. no. 5671085, Bio-Rad; Hercules, CA) and subjected to electrophoresis (150 V for 75 min) using premade 1× SDS-PAGE buffer (cat. no. 97061, VWR; Radnor, PA). Proteins were then transferred (200 mA for 2 h) to polyvinylidene difluoride membranes (cat. no. 1620177, Bio-Rad), Ponceau stained, and imaged to ensure equal protein loading between lanes. Membranes were then blocked for 60 min at room temperature with 5% nonfat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST; cat. no.J60764, Alfa Aesar; Haverhill, MA). Mouse anti-ORF1 (1:1,000; cat. no. MABC1152, EMD Millipore; Billerica, MA), rabbit anti-phospho (p) AMPKα (Thr172) (1:1,000; cat. no. 2535, Cell Signaling), and rabbit anti-AMPKα (1:1,000; cat. no. 2532, Cell Signaling) were incubated with membranes overnight (ORF1 and AMPK) or 72 h (p-AMPK) at 4°C in TBST with 5% BSA. The ORF1 antibody was successfully validated with HeLa cell lysate (data not shown); all antibodies yielded proteins that corresponded with predicted molecular weights. Additionally, the allocated antibodies have been validated elsewhere (22, 37). The following day, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:2,000, cat. no.7076, Cell Signaling) or anti-rabbit IgG (1:2,000, cat. no.7074, Cell Signaling) in TBST with 5% BSA at room temperature for 60 min. Membrane development was performed using an enhanced chemiluminescent reagent (Luminata Forte horseradish peroxidase substrate, cat. no. WBLUF0100, EMD Millipore), and band densitometry was performed using a gel documentation system and associated software (UVP; Upland, CA). Densitometry values for ORF1 were normalized to Ponceau densitometry values. Densitometry values for p-AMPK were normalized to AMPK densitometry values. Each participant's PRE, 2-h and 8-h samples were then normalized to the group mean of young PRE for each protein target.

Statistics. All statistics were run using SPSS v22.0 (IBM; Armonk, NY). Shapiro-Wilk testing was used to determine normality for all dependent variables. Levene's test for equality of variances was determined, and when appropriate, sphericity tests were performed. Baseline differences in dependent variables between age groups were determined using independent samples *t*-tests. Changes in dependent variables over time were tested using mixed factorial ANOVA tests, in which the within-subject factor was time (PRE, 2 h, and 8 h) and the between-subject factor was age group (younger or older). In the event that a significant effect or interaction was observed, pairwise comparisons or least-significant difference post hoc tests were utilized. Pearson correlations were performed between select variables. All data are presented as mean ± SD, and statistical significance was established at $P < 0.050$.

Table 1. *PCR primer sequences*

Gene	Forward (5′ → 3′)	Reverse (5′ → 3′)
L1 ORF1	TAAGGGCAGCCAGAGAGAAA	GCCTGGTGGTGACAAAATCT
L1 5′UTR	GAACAGCTCCGGTCTACAGC	CCGGCTGCTTTGTTTACCTA
L1.3	GAACAGCTCCGGTCTACAGC	CAATATTCGGGTGGGAGTGA
B2M	ATGAGTATGCCTGCCGTGTGA	GGCATCTTTCAAACCTCCATG
PPIA	CGATGTCTCAGAGCACGAAA	CCCACCTGTTTCTTCGACAT
PGC-1α	CAAGCCAAACCAACAACTTTATCTCT	CACACTTAAGGTGCGTTCAATAGTC
GAPDH	AACCTGCCAAATATGATGAC	TCATACCAGGAAATGAGCTT

L1, long interspersed nuclear element-1; UTR, untranslated region.

RESULTS

Participant characteristics. Participant characteristics are summarized in Table 2. Older participants were older than younger participants ($P < 0.001$). Interestingly, whole body lean soft tissue mass, fat mass, midthigh thickness, and vastus lateralis thickness were not different between older and younger participants ($P > 0.050$ for all metrics). Likewise, relative and absolute $\dot{V}O_{2peak}$ were not different between older and younger participants ($P > 0.050$ for both metrics). Resting and maximal HR were lower in older participants ($P < 0.001$). However, maximal cycling power (W_{max}) was not different between groups ($P = 0.768$). Exercise trial HR was higher in younger compared with older participants ($P < 0.001$), although percent of HRR ($P = 0.944$) and average power output during exercise ($P = 0.639$) were not different between groups. Leg extensor peak torque was greater in younger versus older participants at 30°/s ($P = 0.020$), but other BioDex metrics were not different between age groups.

Accelerometry metrics. Accelerometry data is presented in Table 3. Both groups wore accelerometers for 13 ± 4 days before the exercise trial ($P = 0.784$). The older group wore the accelerometer (min/day) more often than the younger group ($P = 0.045$). Moreover, older participants registered more time per day performing light activity relative to younger participants ($P = 0.007$). However, there were no differences between groups in time spent per day performing moderate activity ($P = 0.614$), vigorous activity ($P = 0.292$), or moderate-to-vigorous physical activity (MVPA) ($P = 0.431$).

Skeletal muscle L1 DNA and RNA expression. Baseline skeletal muscle L1 DNA expression is presented in Fig. 2. Neither 5′ UTR DNA expression ($P = 0.410$; Fig. 2*A*), L1.3 DNA expression ($P = 0.588$; Fig. 2*B*), nor ORF1 DNA expression ($P = 0.867$; Fig. 2*C*) were different between younger and older participants.

mRNA expression analysis was conducted in 15 younger participants and 13 older participants because of low RNA yield for 2 participants in the older group. L1 5′ UTR mRNA expression did not reveal a significant time effect ($P = 0.223$), group effect ($P = 0.366$), or interaction ($P = 0.261$) (Fig. 3*A*). L1 ORF1 mRNA expression demonstrated significant time ($P = 0.034$) and group effects ($P = 0.014$) but not a significant interaction ($P = 0.526$) (Fig. 3*B*). Post hoc analyses indicated that older participants expressed more L1 ORF1 mRNA than younger participants irrespective of exercise ($P = 0.014$), and cycling lowered L1 ORF1 mRNA expression 2 h postexercise regardless of age ($P = 0.027$). L1.3 mRNA expression (Fig. 3*C*) did not reveal a significant time effect ($P = 0.081$), group effect ($P = 0.290$), or interaction ($P = 0.708$). PGC-1α mRNA expression was assessed as a positive control, given that this gene is responsive to acute endurance exercise (Fig. 3*D*). PGC-1α mRNA demonstrated a significant interaction ($P = 0.010$) as well as a significant time effect ($P < 0.001$). Within both groups, PGC-1α mRNA expression increased from PRE to 2 h ($P < 0.001$) but then decreased from 2 h to 8 h ($P < 0.001$); however, PGC-1α mRNA expression was still elevated at 8 h compared with PRE for both groups ($P < 0.001$). Although a significant interaction was found, expression was not different between groups at each time point ($P > 0.050$). A representative agarose gel of single PCR products for each primer set from a random younger and older participant is depicted in Fig. 3*E*.

Skeletal muscle protein content. Protein expression of select targets was conducted in 15 younger participants and 14 older participants because of limited tissue from 1 older participant. ORF1 protein content violated the assumption of sphericity, and thus a Huynh-Feldt correction was utilized. ORF1 protein content did not reveal a significant time effect ($P = 0.805$), group effect ($P = 0.242$), or interaction ($P = 0.789$) (Fig. 4*A*). p-AMPK/AMPK also did not reveal a significant time effect ($P = 0.395$), group effect ($P = 0.183$), or interaction ($P = 0.305$) (Fig. 4*B*).

Skeletal muscle L1 methylation status. L1 methylation status was conducted in 15 young participants and 14 old participants because of limited tissue availability in 1 older participant. L1 5′ UTR DNA methylation did not reveal a significant time

Table 2. *Baseline characteristics between age groups*

	Younger	Older	*P* Value
Sample size	15	15	
Men/women	7/8	10/5	
Age, yr	23 ± 3	58 ± 8	**<0.001**
Body composition			
Height, cm	172 ± 9	172 ± 8	0.861
Mass, kg	71.9 ± 14.9	71.4 ± 8.7	0.913
WB LSTM, kg	58.27 ± 12.48	57.72 ± 7.47	0.886
WB fat mass, kg	13.25 ± 6.99	13.30 ± 4.51	0.979
Midthigh thickness, cm	5.10 ± 0.82	4.78 ± 0.76	0.283
VL thickness, cm	2.45 ± 0.52	2.22 ± 0.44	0.207
Aerobic measures			
$\dot{V}O_{2peak}$, mL·kg^{-1}·min^{-1}	38.0 ± 7.7	35.6 ± 7.3	0.394
$\dot{V}O_{2peak}$, L/min	2.70 ± 0.63	2.56 ± 0.66	0.552
Resting HR, beats/min	69 ± 13	58 ± 8	**0.005**
HRmax, beats/min	190 ± 9	165 ± 17	**<0.001**
ET HR, beats/min	156 ± 12	135 ± 13	**<0.001**
ET HRR, %	71.4 ± 4.4	71.5 ± 1.8	0.944
Max power, W	209 ± 46	204 ± 52	0.768
ET power, W	112 ± 30	107 ± 24	0.639
Strength measures			
120° extension, N·m	148.1 ± 42.2	124.0 ± 22.6	0.064
120° flexion, N·m	70.2 ± 15.9	67.6 ± 12.7	0.622
30° extension, N·m	196.1 ± 50.6	158.0 ± 31.7	**0.020**
30° flexion, N·m	92.0 ± 24.6	85.3 ± 14.6	0.374

Values are means ± SD. Bold-faced *P* values are significant ($P < 0.050$). ET, exercise trial; HR, heart rate; HRmax, maximal heart rate; HRR, heart rate reserve; LSTM, lean soft tissue mass; VL thickness, vastus lateralis thickness; $\dot{V}O_2$, volume of oxygen consumption; $\dot{V}O_{2peak}$, maximal aerobic power; WB, whole body.

Table 3. *Accelerometry measures between young and old participants*

	Younger	Older	*P* Value
Calendar days worn	13 ± 4	13 ± 4	0.784
Total time worn, min/day	721 ± 100	803 ± 112	**0.045**
Sedentary time, min/day	497 ± 91	524 ± 105	0.471
Light activity time, min/day	184 ± 57	244 ± 55	**0.007**
Moderate activity time, min/day	35 ± 12	32 ± 15	0.614
Vigorous activity time, min/day	5 ± 6	2 ± 5	0.292
MVPA/day, min/day	40 ± 15	35 ± 16	0.431
Steps taken, steps/h	600 ± 195	566 ± 169	0.784

Values are means ± SD. These data show accelerometry metrics between young and old. Light activity time is time spent <3 METs. Moderate activity time is time spent 3–6 METS. Vigorous activity time is time spent >6 METs. Bold-faced *P* values are significant ($P < 0.050$). MET, metabolic equivalent of task; MVPA, moderate-to-vigorous physical activity.

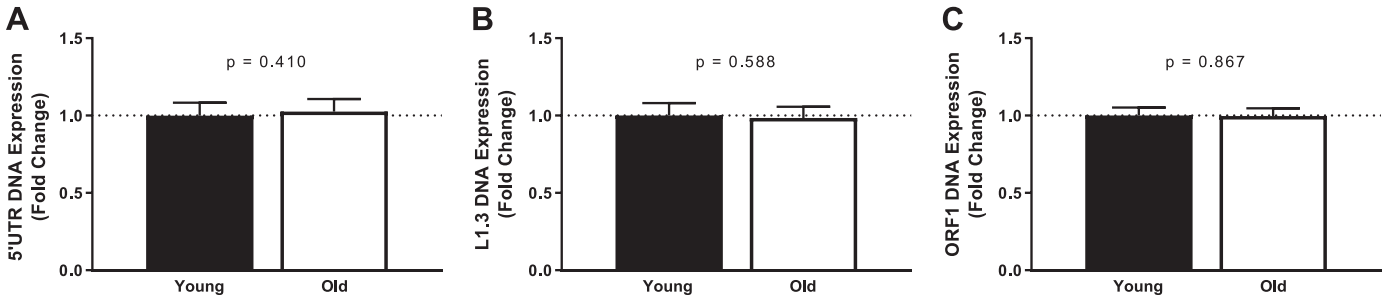

Fig. 2. Skeletal muscle L1 DNA expression at PRE between age groups. The expression of 5′UTR DNA (*A*), L1.3 DNA (*B*), and ORF1 DNA (*C*) between younger ($n = 15$) and older participants ($n = 15$) only at the PRE time point. Data are presented as fold change from the younger group's PRE mean average (±SD). UTR, untranslated region.

effect ($P = 0.226$) or interaction ($P = 0.502$); however, a group effect was evident ($P = 0.006$) whereby younger participants possessed higher values compared with older participants (Fig. 5*A*). L1.3 DNA methylation did not reveal a significant time effect ($P = 0.251$) or interaction ($P = 0.521$); however, again, a group effect was evident ($P = 0.002$) whereby younger participants possessed higher values compared with older participants (Fig. 5*B*).

Correlations. Select correlations are shown in Table 4. Age did not correlate with any DNA markers ($P > 0.050$), RNA markers ($P > 0.050$), or ORF1 protein ($P = 0.405$). Notably, age was correlated with 5′ UTR methylation ($r = -0.371$, $P = 0.048$), PRE ORF1 DNA was correlated with 5′ UTR methylation ($r = -0.532$, $P = 0.003$), and MVPA/day was correlated with PRE ORF1 RNA ($r = -0.398$, $P = 0.032$).

DISCUSSION

This is the first study to analyze skeletal muscle L1 markers in younger and older humans during resting conditions and following a bout of endurance exercise. The participants in this study were (on average) ~20 yr old in the younger group and 60 yr old in the older group. Despite this age gap, an interesting and unanticipated observation was that both groups were similarly matched in aerobic fitness, body composition, and daily physical activity levels. Strength decreases with aging (20), and this was evident in the current study through significant difference between groups in maximal knee extension torque at 30°/s. It is notable, however, that maximal knee extension torque does not seem to translate to functional metrics of endurance exercise performance (11). Thus, we speculate that

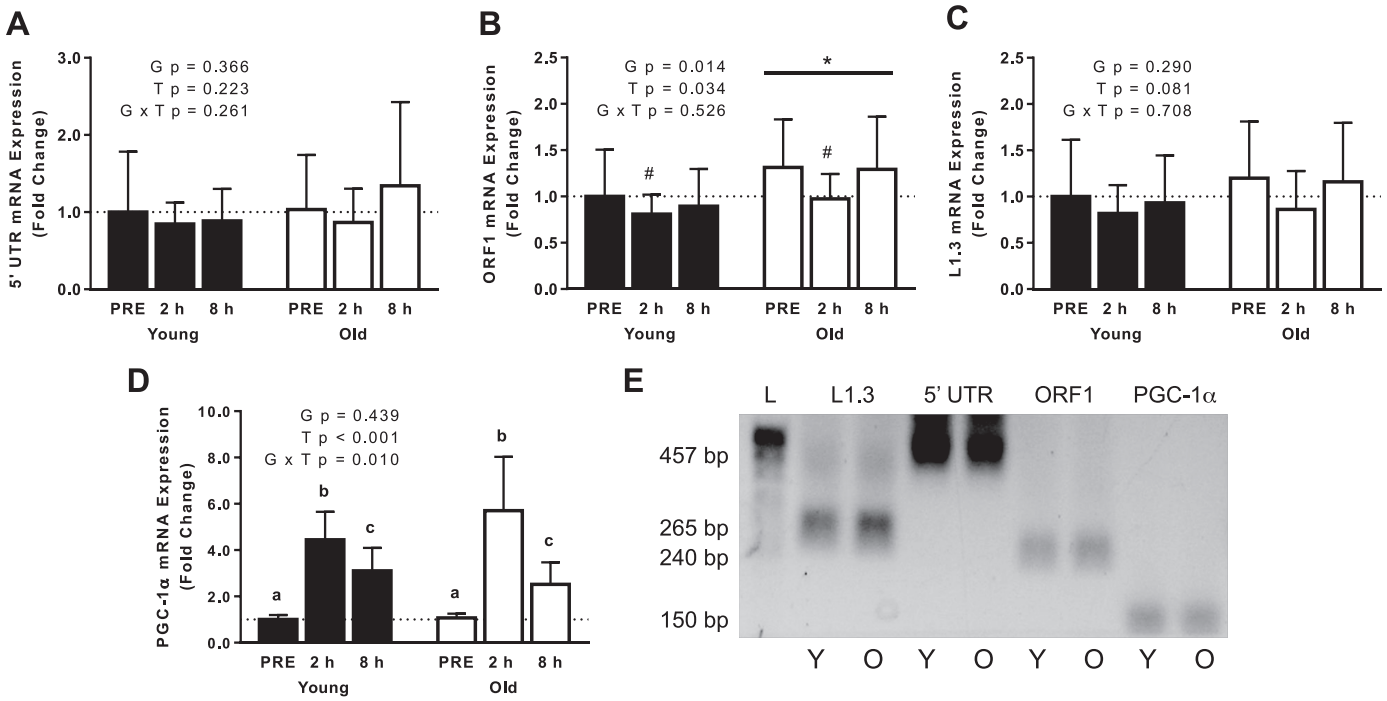

Fig. 3. Skeletal muscle mRNA expression between age groups. The expression of 5′UTR mRNA (*A*), ORF1 mRNA (*B*), L1.3 mRNA (*C*), and PGC-1α mRNA (*D*) between younger ($n = 15$) and older participants ($n = 13$). Notably, PGC-1α was assessed as a positive control, given the robust response the gene has to acute endurance exercise. *E*: representative agarose gel of single PCR products for each primer set from a random younger and older participant. Data are presented as fold change from the younger group's PRE mean average (±SD). "G" indicates group *P* value, "T" indicates time *P* value, and "G × T" indicates the group by time interaction *P* value. *Group differences whereby older participants were significantly higher than younger participants. #Time differences whereby 2 h was significantly lower than PRE and 8 h. Differing letters denote significant differences. UTR, untranslated region.

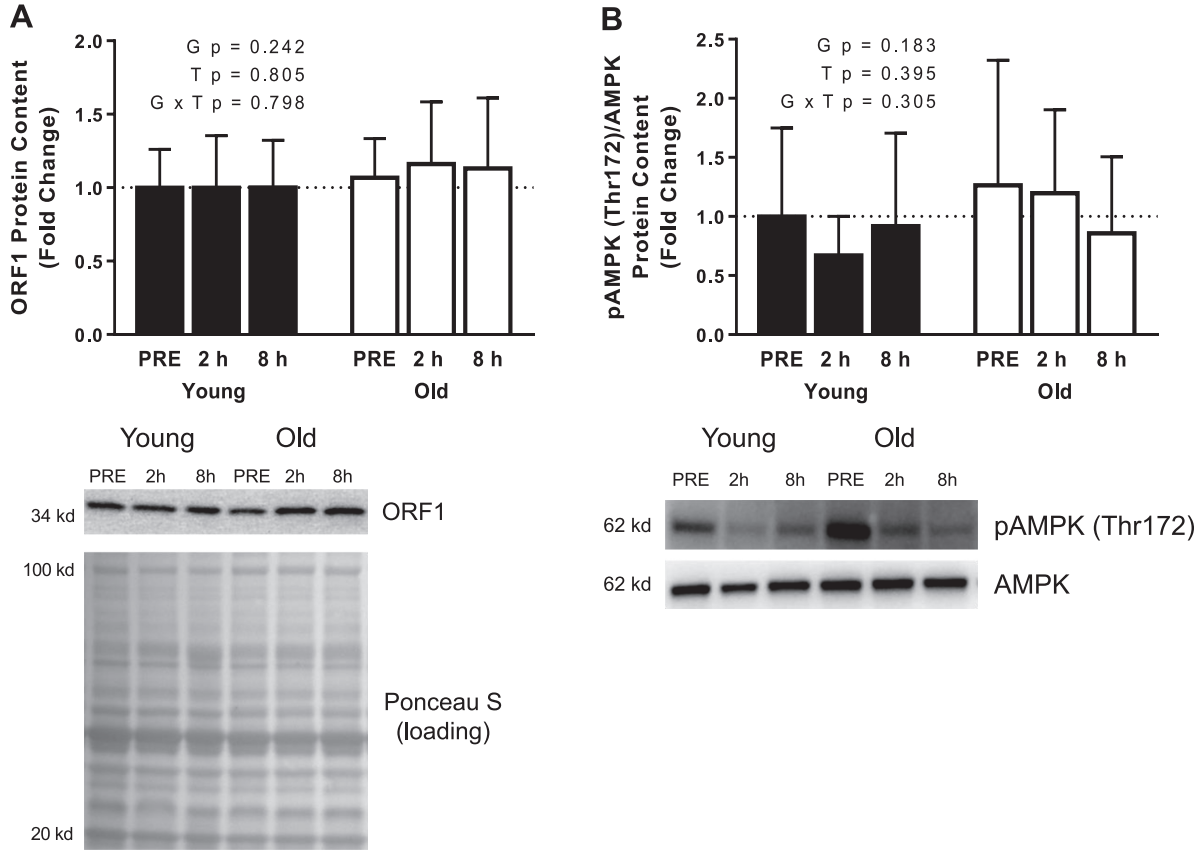

Fig. 4. Skeletal muscle protein content between age groups. ORF1 protein content (*A*) and p-AMPK/AMPK (*B*) between younger ($n = 15$) and older participants ($n = 14$). Western blot representative images are depicted below each figure. Data are presented as fold change from the younger group's PRE mean average (±SD). "G" indicates group *P* value, "T" indicates time *P* value, and "G × T" indicates group by time interaction *P* value.

the molecular differences discussed below relate mostly to aging and not body composition or aerobic fitness.

Skeletal muscle L1 DNA expression patterns were nearly identical between younger and older humans. As mentioned previously, De Cecco et al. (12) reported that skeletal muscle L1 DNA copy number was 80% greater in 36-mo-old mice compared with 5-mo-old mice, and the authors hypothesized that this phenomenon was likely due to increased L1 retrotransposition with aging. However, these authors did not find a difference in this metric between 5- and 24-mo-old mice. Given that the older participants' age was ~60 yr old, it is possible that these subjects were not old enough to observe increases in skeletal muscle L1 DNA. It has been suggested that 24-mo-old mice have a human age equivalent of ~70 yr, and 36-mo-old mice have a human age equivalent of ~95 yr (14). Assuming that these animal data translate, it would

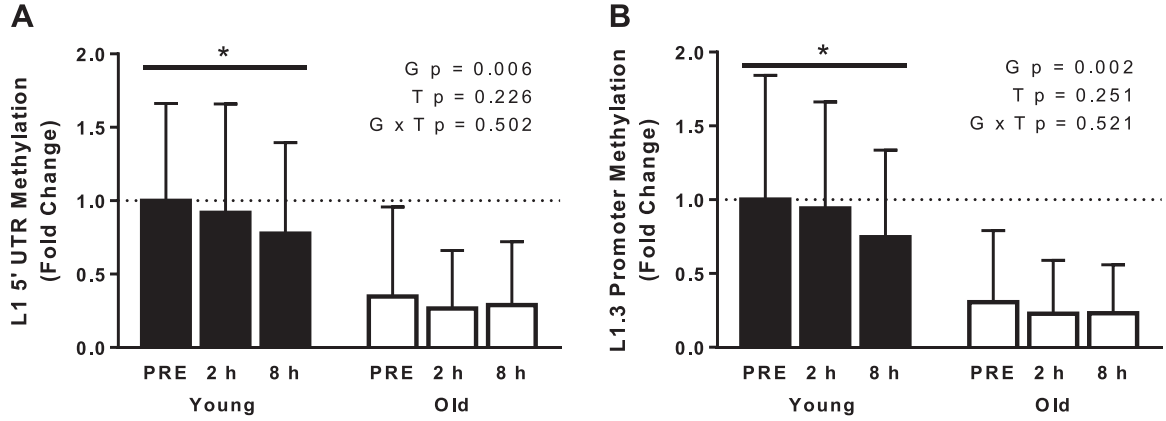

Fig. 5. Skeletal muscle L1 methylation between age groups. Methylation of 5′UTR (*A*) and L1.3 (*B*) between younger ($n = 15$) and older participants ($n = 14$). Data are presented as fold change from the younger group's PRE mean average (±SD). "G" indicates group *P* value, "T" indicates time *P* value, and "G × T" indicates group by time interaction *P* value. *Group differences whereby younger participants were significantly higher than older participants. UTR, untranslated region.

Table 4. *Select correlations between skeletal muscle L1 markers and participant characteristics*

Variable 1	Variable 2	*r*	*P* Value
Age	PRE ORF1 DNA	−0.116	0.542
Age	PRE ORF1 RNA	0.088	0.645
Age	PRE ORF1 Protein	0.161	0.405
Age	5′UTR Methylation	−0.371	**0.048**
ORF1 DNA	5′UTR Methylation	−0.532	**0.003**
PRE ORF1 RNA	MVPA/day	−0.398	**0.032**

These data are Pearson correlation coefficients between select dependent variables. Bold-faced *P* values are significant ($P < 0.050$). MVPA, moderate-to-vigorous physical activity; UTR, untranslated region.

suggest that humans would need to be very old for L1 DNA copy number differences to be found. It should also be noted that cellular turnover differences among various tissues appears to play a role in L1 DNA copy number, such that cell types that undergo rapid proliferation are susceptible to L1 insertions. In this regard, Billingsley et al. (3) demonstrated differences in L1 enrichment and total repetitive elements between skin cells and white blood cells. Since skeletal muscle fibers are postmitotic, it is possible that skeletal muscle tissue is resilient to de novo L1 insertions because of lack of cellular turnover. Finally, as mentioned above, fitness and daily physical activity levels were similar between age groups. This may have played a role as to why L1 DNA content was similar between age groups, and future studies using frailer and/or more sedentary older participants may be needed to observe age-related increases in skeletal muscle L1 DNA levels.

Although skeletal muscle L1 DNA expression was not different between younger and older participants, this is the first study to demonstrate that skeletal muscle L1 ORF1 mRNA expression is higher in older versus younger humans. This finding agrees with the rodent findings of De Cecco et al. (12) and is supported by the concomitant finding that L1 DNA methylation was lower in older individuals. As mentioned prior, blood L1 methylation negatively correlated with aging in 32 healthy men aged 21 to 57 yr old (9). Additionally, global methylation has also been found to be negatively correlated with aging in white blood cells from 76 healthy individuals aged 4 to 94 yr old (18), and because of the high abundance of L1 in the genome, L1 methylation has been used as a surrogate marker for global methylation. Hence, not only is skeletal muscle L1 hypomethylated with aging but also the genome as a whole may be hypomethylated because of defective DNA methylation mechanisms. This speculation is not far-fetched given that others have reported global methylation patterns in older human (68–89 yr old; $n = 24$) skeletal muscle are appreciably altered versus younger humans (18–27 yr old; $n = 24$) (41), and the mRNA expression of DNA methyltransferase enzymes are dysregulated in other cell types from older versus younger human fetal lung fibroblasts in which <30 population doubling was considered "young" and >45 population doubling was considered "senescent" (8). These parallel findings suggest decreased genomic integrity as humans age. Within the context of disease, L1 reinsertion acts primarily through insertional mutagenesis, which poses a threat to gene function given that the insertion of L1 within a gene could potentially alter the function of the gene and lead to disease. As genomic integrity parishes with age, the opportunity for L1 reinsertion becomes greater, given that decreased global methylation and increased L1 RNA expression occurs with aging. Therefore, future research is needed to determine if skeletal muscle L1 mRNA dysregulation with aging is indirectly due to age-related changes in DNA methylation mechanisms.

Regardless of age, moderate-intensity endurance exercise transiently decreased skeletal muscle L1 ORF1 mRNA expression. Furthermore, higher daily MVPA levels were negatively associated with ORF1 mRNA levels. Other groups have reported similar findings in that blood cell L1 hypermethylation is associated with increased physical activity (28, 40), and our current findings agree in principle with prior data from our laboratory, suggesting that skeletal muscle L1 mRNA expression is downregulated following acute and chronic exercise (in the form of resistance training) in college-aged men (37). It is interesting that both endurance and resistance exercise downregulate skeletal muscle L1 mRNA expression given the different molecular signaling associated with each exercise modality (19). Indeed, exercise did not alter L1 methylation patterns in the current study, suggesting that other mechanisms are likely involved with transient ORF1 mRNA decreases during endurance exercise.

Interestingly, the current study did not find a significant difference between groups or following exercise in either ORF1 or p-AMPK (Thr172)/total AMPK relative protein expression. The lack of difference in ORF1 protein expression with concomitant increases in mRNA expression in the older group suggests that ORF1 is posttranscriptionally regulated. Future studies should aim to determine if this regulation occurs in the cytoplasm or nucleus and if the regulation is related to translational machinery. We attempted to associate L1 RNA patterns to p-AMPK (Thr172)/total AMPK patterns given that: *1*) long-term caloric restriction, which is a well-known activator of skeletal muscle AMPK (6), has been shown to downregulate L1 mRNA in rodent skeletal muscle (12); and *2*) AMPK activation is central in facilitating endurance training adaptations. Additionally, we have preliminary data in C2C12 myotubes demonstrating that 3-h treatments with 2 μM and 4 μM of 5-aminoimidazole-4-carboxamide ribonucleotide significantly downregulates L1 mRNA relative to DMSO-treated myotubes (Romero et al., unpublished observations). However, no apparent relationships existed between p-AMPK levels and L1 mRNA levels in the current study. Another possible mechanism for exercise-mediated skeletal muscle L1 mRNA regulation may be through the Sirtuin (SIRT) proteins, which are histone deacetylase enzymes. In this regard, Sirt6 knockout mice exhibit greater L1 mRNA expression and DNA integration in isolated mouse embryonic fibroblasts, whereas Sirt6 overexpression represses these events (38). Furthermore, we recently observed that rats that had access to running wheels for 5 mo exhibit lower skeletal muscle L1 mRNA expression and elevated skeletal muscle SIRT activity compared with littermates who never engaged in wheel running (Romero et al., unpublished observations). Thus, regardless of species or modality of training, we posit that exercise in general is capable of downregulating markers of skeletal muscle L1 activity, and the mechanisms modulating L1 downregulation along with the physiological relevance of this phenomenon are ripe for further investigation.

Finally, it is notable that only ORF1 mRNA demonstrated significant age and exercise effects, whereas the other primer

sets (5′ UTR and L1.3) yielded numerically similar trends but no significant time or group effects. Although these data are difficult to reconcile, this phenomenon is likely due to the numerous iterations of L1 transcripts that are able to be transcribed due to 5′ end truncations and variable transcription initiation sites (25).

Limitations. Although skeletal muscle L1 DNA expression was similar between age groups, this could have been due to either: *1*) the older participants likely not being old, diseased, or sedentary enough to demonstrate an aging effect relative to the younger participants; or *2*) increased L1 DNA content through retrotransposition only appreciably occurs in rodents. Thus, future research examining skeletal muscle L1 DNA expression in very old humans (i.e., >90 yr old) or individuals that have musculoskeletal diseases is warranted. Additionally, we did not determine a mechanism as to how exercise affects skeletal muscle L1 mRNA expression. Although we speculate that this may be due to increased SIRT activity, future research is needed to clarify which mechanism reduces L1 mRNA expression in skeletal muscle following acute and chronic exercise. Finally, although not the scope of the current study, it remains to be determined how L1 mRNA expression affects skeletal muscle physiology. Indeed, this endeavor will require substantial efforts through the development of mechanistic mouse models and innovative assays to monitor which genes in skeletal muscle are potentially disrupted via de novo retrotransposition events.

Conclusions. Despite the aforementioned limitations, this is the first study to examine how aging and acute endurance exercise influence L1 markers in human skeletal muscle. These findings build upon past human and rodent literature reporting that skeletal muscle L1 markers are reduced with exercise and increased with aging. Future research is needed to determine how exercise affects mechanisms that regulate skeletal muscle L1 mRNA expression and, more importantly, how aberrant L1 activity affects skeletal muscle physiology.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.A.R. conceived and designed research; P.A.R., M.A.R., S.C.O., P.W.M., C.G.V., C.D.F., and M.D.R. performed experiments; P.A.R. analyzed data; P.A.R., M.A.R., P.W.M., C.G.V., D.J.M., M.D.B., and M.D.R. interpreted results of experiments; P.A.R. prepared figures; P.A.R. and M.D.R. drafted manuscript; P.A.R., M.A.R., S.C.O., P.W.M., C.G.V., C.D.F., D.J.M., M.D.B., and M.D.R. edited and revised manuscript; P.A.R., M.A.R., S.C.O., P.W.M., C.G.V., C.D.F., D.J.M., M.D.B., and M.D.R. approved final version of manuscript.

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