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# Morphological and Functional Characteristics of Skeletal Muscle Fibers From Hormone-replaced and Nonreplaced Postmenopausal Women

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We tested the hypothesis that cross-bridge mechanisms of contraction differed in early postmenopausal women who did or did not receive hormone replacement therapy (HRT). Vastus lateralis biopsies were obtained from 17 postmenopausal women (49–57 years old), 8 of whom were on HRT for the previous  $24 \pm 5$  months and 9 of whom were never on HRT. Electrophoresis and enzyme histochemistry revealed that fiber myosin heavy chain (MHC) isoform distribution, the cross-sectional area (CSA) of slow and fast fibers, and the relative CSA occupied by each, were similar for HRT and non-HRT groups. Single permeabilized fibers containing type IIa MHC had greater  $\text{Ca}^{2+}$ -activated peak specific force, unloaded shortening velocity, and peak power than fibers containing type I MHC, but in all cases the values for HRT and non-HRT groups were similar. In this cross-sectional study, we found no evidence that  $\text{Ca}^{2+}$ -activated fiber function, MHC isoform distribution, or relative CSA occupied by slow and fast fibers differed between HRT and non-HRT groups.

**H**UMAN muscular strength peaks during the third decade of life, remains relatively stable over the next three decades, and then begins to decline, falling to 60–70% of its peak level by the seventh to eighth decades of life (1,2). This decline in voluntary strength has important health-related consequences, ranging from the inability to perform activities of daily living, such as rising from a chair (3), to an increased risk for falling (4). The main peripheral contributors to these age-related losses in neuromuscular function are muscle atrophy and a reduction in the intrinsic ability of muscles to produce force (1,5,6). The latter is a consequence of impaired excitation–contraction coupling, and alterations in cross-bridge mechanisms of contraction (7–10).

Data from cross-sectional studies suggest that the age-related decline in voluntary strength occurs at an earlier age in women than in men (11,12). For instance, beginning in the 45- to 55-year-old age group, the specific strength (strength–muscle cross-sectional area) of the adductor pollicis was found to be substantially lower for female subjects than for male subjects (11). Further analysis of this age group revealed that the force deficit was confined to those women not on hormone replacement therapy (HRT), because women on HRT had strength comparable with their male counterparts. These observations lead to the hypothesis that a causal relationship exists between estrogen and muscular performance in postmenopausal women (11).

This hypothesis is supported by the finding that HRT treatments prevent an age-related reduction in knee extensor strength (13) and improve knee extensor and adductor

pollicis strength of postmenopausal women (14,15). Nevertheless, the hypothesis remains controversial, because several other longitudinal studies have found little support for the role of HRT in maintaining or improving handgrip strength, knee extensor strength, or leg extension power in postmenopausal women (16,17).

Previous HRT studies have evaluated muscle performance by assessing maximal voluntary contractions. Muscle performance measured under these conditions is a function of central (neural) and peripheral (muscular) factors. In an extensive review of the literature, Gandevia (18) concluded that “no muscle appears blessed with truly optimal drive during maximal isometric efforts.” To our knowledge, only one HRT study has directly addressed this issue of incomplete muscle activation (13), although other studies have assumed maximal activation (11,14) on the basis of results from their previous research (19).

Physiological cross-sectional area (CSA), fiber type composition, and the contractile properties of individual muscle fibers all contribute to the peripheral or mechanical properties of a muscle. In this context, it is noteworthy that the studies showing the greatest effects of HRT are those that have examined the function of the adductor pollicis (11,14), whereas those showing relatively small but statistically significant benefits with HRT (13,15), or no benefits at all (16,17), have been conducted on the knee extensors. The adductor pollicis has a relatively consistent fiber type composition (20) with a generally parallel fiber architecture (19). This latter characteristic means that the anatomical CSA of the muscle, which can be reliably

measured (19), is equivalent to its physiological CSA. In contrast, the muscles comprising the knee extensors generally have a mixed fiber type composition (21) and have a complex pennate architecture (22). This latter feature makes it difficult to properly normalize force measurements between subjects.

Our working hypothesis is that discrepancies between previous HRT studies may be attributed to the difficulty in normalizing or interpreting measurements of voluntary strength. Therefore, in this study we have taken an alternative approach to evaluate muscle function. Using adenosine triphosphatase (ATPase) histochemistry, single-fiber gel electrophoresis, and an *in vitro* single-cell functional preparation, we have assessed the CSA, fiber type distribution, and  $\text{Ca}^{2+}$ -activated contractile properties of muscle fibers obtained from two groups of early postmenopausal women: those on HRT since menopause and those never receiving HRT. This approach eliminates many of the uncertainties associated with measurements of voluntary neuromuscular strength. This methodology also allows us to test the hypothesis put forward by Phillips and coworkers (11) that the site of action of HRT is at the level of the actomyosin cross bridges.

## METHODS

### *Subjects*

Seventeen healthy, postmenopausal women volunteered to participate in this study. These individuals were a subset of subjects participating in a larger, longitudinal study that examined bone mineral density after menopause. Subjects were selected from a pool of volunteers who responded to newspaper advertisements or who had been referred to the study by their physicians. The study was approved by the Institutional Review Board at Oregon State University. All subjects provided written consent after being fully informed of the nature of the study.

Follicle-stimulating hormone levels were  $\geq 20$  mIU/ml for all subjects (confirmed by each subject's physician). Nine of the women had never taken HRT (non-HRT group:  $22 \pm 6$  months since being diagnosed as postmenopausal). The other 8 subjects had been taking HRT since, or shortly before, they were diagnosed as postmenopausal (HRT group:  $20 \pm 3$  months since being diagnosed as postmenopausal;  $24 \pm 5$  months on HRT). HRT consisted of conjugated estrogens (0.625 mg/d).

### *Body Composition*

Body composition was determined by dual-energy x-ray absorptiometry (QDR-4500 Elite A, Hologic, Inc., Waltham, MA) as previously described (23). Scans were analyzed by using Hologic software version 9.80 D (Hologic).

### *Voluntary Knee Extension Strength*

Peak torque of the left knee extensors was evaluated by isokinetic dynamometry (KIN-COM 500H, ChatteX Corp., Hixson, TN). All tests were conducted at a speed of 30 degrees per second and were gravity corrected. After a demonstration of procedures, each subject was positioned

to isolate the muscle group being tested and instructed to perform 10–12 trials at an intensity well below maximum. After this warm-up, subjects performed between three and five maximal efforts in order to determine peak force and torque. Each maximal effort was separated by approximately 60 seconds of rest.

### *Physical Activity*

Current physical activity levels were assessed by using a questionnaire administered during a laboratory visit (24). The questionnaire asked subjects to recall the duration and intensity of their typical weekly recreational, leisure, and household activities for the 6 months leading up to the study.

### *Muscle Biopsy*

A muscle sample of the left vastus lateralis was obtained by using the percutaneous needle biopsy technique. One portion of the sample was immediately placed in cold dissection solution (for composition, see the paragraphs that follow), where it was divided into small bundles of fibers for use in the functional experiments. These bundles were stored in skinning solution (composition is given in a later subsection) at 4°C for 24 hours before they were transferred to fresh skinning solution and stored at  $-20^{\circ}\text{C}$  for up to 3 weeks. Another portion of the biopsy sample was aligned vertically in tissue mounting medium and frozen in isopentane that was cooled to its freezing point with liquid nitrogen. This sample was stored in an airtight cryotube at  $-80^{\circ}\text{C}$  for later histochemical analysis.

### *Enzyme Histochemistry and Morphology*

The muscle samples were sectioned on a cryostat (model CM1800; Leica Microsystems, Nussloch, Germany) at  $-20^{\circ}\text{C}$ . Sections that were 8–10  $\mu\text{m}$  thick were picked up on cover slips, air dried, and stained for myofibrillar ATPase. Sections were stained at pH 9.4 after both alkaline (pH 10) and two different acid (pH 4.35 and 4.65) pre-incubations. Stained sections were mounted on glass slides and photos of the sections were taken at 100 $\times$ . Photographs were converted into digital files with a flatbed scanner. Image analysis software (Scion Image, Scion Corp., Frederick, MD) was used to determine the CSA of fibers that were free of artifacts, had distinct cell borders, and had no tendency toward a longitudinal cut.

### *Functional Studies*

The composition of the solutions used for the functional studies was determined by an iterative computer program (25) using stability constants adjusted for ionic strength, pH, and temperature (26). All solutions contained 7.0mM EGTA [ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid], 20.0mM imidazole, 1mM free  $\text{Mg}^{2+}$ , 4mM ATP (adenosine-5'-triphosphate), 14.5mM creatine phosphate, and 15 U  $\text{ml}^{-1}$  creatine kinase. The free  $\text{Ca}^{2+}$  concentration of the relaxing and activating solutions was adjusted to pCa 9.0 and pCa 4.5, where pCa is  $-\log[\text{Ca}^{2+}]$ , respectively, with a 100mM  $\text{CaCl}_2$  standard solution (Calcium Molarity Standard, Corning Inc., Corning, NY). In both solutions, pH was adjusted to 7.0 by using KOH

(potassium hydroxide), and sufficient KCl (potassium chloride) was added to bring the total ionic strength to 180mM.

The dissection solution consisted of relaxing solution and a protease inhibitor cocktail prepared according to the manufacturer (Complete Mini EDTA-Free Protease Inhibitor Tablets, Boehringer Mannheim, Indianapolis, IN). The skinning solution was made by mixing equal volumes of dissection solution and glycerol.

On the day of an experiment, a muscle bundle was transferred from skinning to relaxing solution. Muscle fiber segments were isolated by holding one corner of the bundle with forceps and inserting fine forceps (Dumont #5) into the opposite end of the bundle in order to grasp and gently remove a fiber segment. The single fiber segment was mounted between an isometric force transducer (Model 400, Aurora Scientific, Aurora, Ontario) and a servo-controlled motor (Model 308B, Aurora Scientific) as previously described (27). Outputs from the transducer and motor were amplified before being digitized (5 kHz) and interfaced to a personal computer by means of a data-acquisition board (Model AT-MIO-16E, National Instruments, Austin, TX). Data collection and analysis were carried out by using a computer program written in our laboratory (LabView, National Instruments, Austin, TX).

The mounted fiber was suspended in one of several glass-bottomed chambers milled into a stainless-steel dip plate. Depression and translation of the plate enabled the investigator to rapidly move the fiber segment from chamber to chamber. The plate was mounted on an inverted microscope (Olympus IX-70, Olympus America Inc., Melville, NY), where sarcomere length was adjusted to 2.5  $\mu\text{m}$  (measured by a calibrated eyepiece micrometer) by using three-axis micromanipulators attached to the transducer and motor. Fiber length (FL) was measured by translating the fiber across the microscope's field of view, using a digital micrometer attached to the mechanical stage of the microscope. Fiber CSA was determined by measuring the fiber width while the mounted fiber was briefly suspended in air (<5 seconds) and calculating the CSA by assuming that the fiber forms a cylinder in air (27,28). Three CSA measurements were made along the length of the fiber (with the fiber returned to the relaxing solution between measurements) and the mean was taken as fiber CSA. Temperature was monitored by a thermocouple that was inserted into the solution bathing the fiber. Solution temperature was maintained at 15°C throughout the experiments.

Unloaded shortening velocity ( $V_o$ ) was determined by using the slack test method (Figure 1A). Fibers were also subjected to a series of isotonic contractions in order to construct force-velocity-power relationships (Figure 1B). For both slack tests and isotonic contractions, total shortening never exceeded 20% of FL. Peak  $\text{Ca}^{2+}$ -activated force was determined as the difference in the maximum force produced during  $\text{Ca}^{2+}$  activation and the force baseline attained while the fiber was slack. Following the physiological measurements, the fiber was removed from the instruments, dissolved in 30  $\mu\text{l}$  of sodium dodecyl sulfate (SDS) sample buffer, and stored at -80°C.

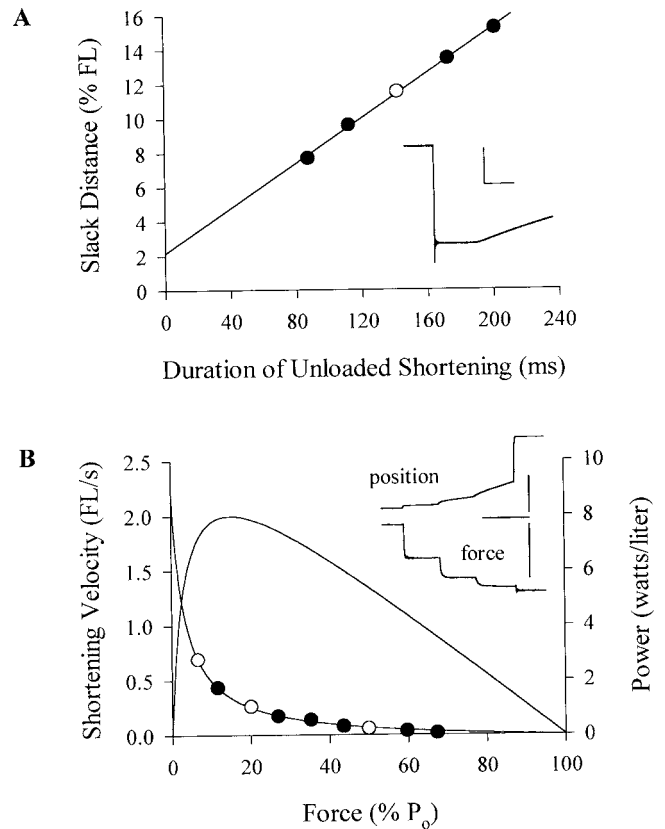


Figure 1. Examples of the slack test (A) and force-velocity test (B) methodology. A. The inset illustrates a force record obtained during a single slack step. Note that the slack step caused force to drop to baseline. The fiber shortened under no load until there was a rapid redevelopment of force (horizontal calibration bar, 100 ms; vertical calibration bar, 0.20 mN). In the main panel, the duration of unloaded shortening has been plotted against the slack step distance for this contraction ( $\circ$ ) along with four other slack steps ( $\bullet$ ). The plot was fit by a least-squares regression line ( $R^2 = .998$ ). The slope of the relationship, normalized to FL, defines  $V_o$  (0.65 FL/s). Later, gel electrophoresis indicated that the fiber in this example contained the type I MHC isoform. B. The inset illustrates one series of three isotonic contractions. After the fiber attained peak force, the servomotor subjected the fiber to three periods of isotonic shortening, followed by a slack step to zero the force transducer. The position record shows corresponding changes in motor position. Force and shortening velocity (slope of the position record) were measured over the second half of each step (horizontal calibration bars, 100  $\mu\text{s}$  and 0.20 mN). In the main panel, data from this contraction cycle ( $\circ$ ) and two other cycles ( $\bullet$ ) have been plotted and fit ( $R^2 = .998$ ) by the Hill equation (44). Parameters describing this particular relationship were  $V_{\max} = 2.22$  FL/s,  $a/P_o = 0.033$ , and  $P_o = 0.68$  mN (157 kN/m<sup>2</sup>). The force-power relationship illustrated in the figure was calculated from these parameters (45). Gel electrophoresis indicated that this fiber contained type IIa MHC isoform. FL = fiber length; MHC = myosin heavy chain.

#### Determination of Fiber Myosin Heavy Chain Isoform Content

Gel electrophoresis was used to determine the myosin heavy chain (MHC) isoform content of all of the fibers subjected to functional assessment. In addition, ~50 additional fiber segments per subject were isolated from the skinned muscle bundles (using the same technique described herein for the functional experiments) and stored in SDS buffer for MHC isoform analysis.

Gel electrophoresis and silver staining were carried out

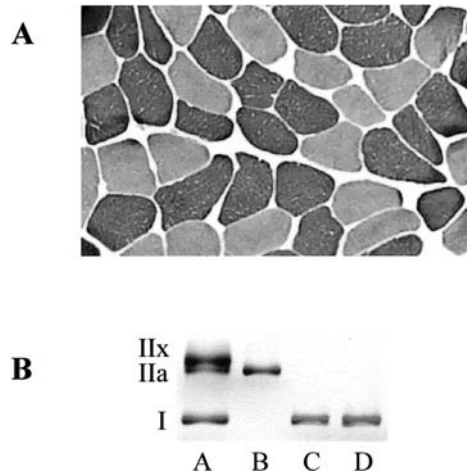


Figure 2. Examples of enzyme histochemistry and gel electrophoresis. **A**, Photograph of muscle section stained for myofibrillar adenosine triphosphatase at pH 10.0. Type I fibers are light in color; type II fibers are dark. **B**, Portion of a polyacrylamide gel used to determine fiber myosin heavy chain isoform content. Lane A was loaded with a human myosin standard. Note the separation of the three myosin heavy chain isoforms present in human skeletal muscle. Fiber segments from three separate experiments were run in lanes B, C, and D.

as previously described (27). A human myosin standard, prepared from an extract of human vastus lateralis muscle, was run on one or more lanes of each gel. This standard was used to identify the isoform(s) present in each fiber segment, using the order of migration of human adult MHC isoforms (29).

#### Statistical Analysis

Descriptive characteristics of the two groups were compared by using a one-way analysis of variance (ANOVA). An analysis of covariance was used to compare knee extension strength while left leg lean mass and total body lean mass were controlled for. The histochemical and single-fiber MHC isoform data were analyzed with a two-way ANOVA (main effects of HRT treatment and fiber type). Fiber functional data were analyzed by using an ANOVA (main effects of MHC isoform content and HRT treatment with subjects nested within hormone treatment). Statistical significance was accepted at  $p < .05$ . All data are presented as mean  $\pm$  SE.

## RESULTS

#### Characteristics of the Subjects

The 9 non-HRT and 8 HRT subjects were similar ( $p > .05$ ) in age ( $52.9 \pm 0.8$  and  $52.3 \pm 0.7$  years, respectively),

height ( $167 \pm 3$  and  $165 \pm 3$  cm), total body mass ( $66.3 \pm 3.8$  and  $67.4 \pm 5.3$  kg), and lean body mass ( $44.7 \pm 1.9$  and  $43.6 \pm 2.6$  kg). The non-HRT and HRT groups had similar levels of physical activity ( $54 \pm 11$  and  $43 \pm 7$  MET-h/wk, respectively, where 1 MET is 1 multiple of the resting metabolic rate). Voluntary knee extension strength (non-HRT,  $163 \pm 10$  N m; HRT,  $146 \pm 11$  N m) did not differ between groups in absolute terms or when adjusted for individual lean body mass or left leg lean mass (analysis of covariance).

#### Histochemistry

An example of a muscle cross section, stained for myofibrillar ATPase, is illustrated in Figure 2A. Three distinct fiber types could be distinguished on some, but not all, sections. Therefore, fibers were classified as type I or II on the basis of their histochemical staining characteristics, and no attempt was made to subclassify the type II fibers. We were unable to analyze samples from 2 subjects (both from the HRT group) because of an insufficient number of fibers that were free of artifact.

On average, 38% of the fibers were classified as type I, and these fibers were significantly larger in CSA than the type II fibers (Table 1). However, despite their smaller fiber CSA, the greater relative occurrence of fast fibers meant that a significantly greater percentage of the total sample area was composed of this fiber type. These relationships for fiber type distribution, fiber CSA, and relative fiber CSA were all independent of HRT status.

#### Fiber MHC Content

A total of 1110 single fibers were subjected to gel electrophoresis. A gel illustrating MHC isoform identification in single-fiber segments is presented in Figure 2B. There were no intergroup differences in the relative number of fibers expressing each particular MHC isoform or combinations of isoforms (Table 2). On average,  $46 \pm 1\%$ ,  $35 \pm 1\%$ , and  $5 \pm 1\%$  of the fibers expressed type I, IIa, or IIx MHC, respectively. The remaining fibers expressed various combinations of isoforms, including types I and IIa ( $5 \pm 1\%$ ) and types IIa and IIx ( $8 \pm 1\%$ ).

#### Functional Properties of Single Fibers

Approximately 90% of the fibers isolated for functional analysis contained either type I or IIa MHC. The average CSA, absolute force (millinewtons), and specific force (force per fiber CSA, in kilonewtons per square meter) of these fibers are presented in Table 3. Fibers expressing type

Table 1. Histochemical Analysis of Muscle Biopsy Samples From HRT and Non-HRT Groups

Group	Fiber Type (%)		Fiber Area ( $\mu\text{m}^2$ )		Fiber Area (% Total Area)	
	Type I	Type II	Type I	Type II	Type I	Type II
Non-HRT	$38 \pm 3$	$62 \pm 3$	$3560 \pm 222$	$2648 \pm 174$	$45 \pm 3$	$55 \pm 3$
HRT	$38 \pm 3$	$62 \pm 3$	$3603 \pm 396$	$2850 \pm 309$	$43 \pm 3$	$57 \pm 3$
Mean	$38 \pm 2$	$62 \pm 2^*$	$3577 \pm 198$	$2729 \pm 157^*$	$44 \pm 2$	$56 \pm 2^*$

Notes: Non-HRT = postmenopausal women not receiving hormone replacement therapy; HRT = postmenopausal women receiving hormone replacement therapy. Values are means  $\pm$  SE for 9 non-HRT and 6 HRT subjects. An average of  $106 \pm$  fibers were analyzed per subject.

\*This indicates a significant difference from type I fibers ( $p < .05$ ).

Table 2. MHC Isoform Content of Vastus Lateralis Muscle Fibers From HRT and Non-HRT Groups

Group	MHC Isoform						
	I	I/IIa	IIa	IIa/IIx	IIx	I/IIx	I/IIa/IIx
Non-HRT	46 ± 4	4 ± 2	35 ± 4	8 ± 2	6 ± 2	<1	1 ± 1
HRT	46 ± 4	5 ± 2	35 ± 4	9 ± 3	3 ± 1	0	2 ± 1

Notes: MHC = myosin heavy chain; non-HRT = postmenopausal women not receiving hormone replacement therapy; HRT = postmenopausal women receiving hormone replacement therapy. Values are average ± SE percents of fibers from 9 non-HRT (589 total fibers) and 8 HRT subjects (521 total fibers).

I MHC were significantly larger in CSA than the fibers expressing the IIa isoform. However, fibers expressing type IIa MHC produced greater specific force than fibers containing the type I isoform, and as a result, absolute peak  $Ca^{2+}$ -activated force was similar for type I and IIa fibers. There was no significant HRT effect or HRT by MHC interaction for any of these variables, indicating that the relationships among fiber MHC isoform content and CSA, absolute force, and specific force were similar for the HRT and non-HRT groups.

There was a significant main effect of MHC isoform expression on fiber  $V_o$  (determined from slack test measurements) with fibers expressing the type IIa isoform shortening ~4.5-fold faster than fibers containing the type I isoform (Table 4). This relationship did not differ between fibers from the non-HRT and the HRT groups. Consistent with the  $V_o$  results, analysis of force-velocity relationships indicated that fiber  $V_{max}$  (extrapolated velocity axis intercept of the force-velocity relationship) was significantly faster in fibers expressing type IIa MHC (non-HRT,  $1.98 \pm 0.11$  FL/s,  $n = 35$ ; HRT,  $1.93 \pm 0.12$  FL/s,  $n = 28$ ) than in fibers expressing type I MHC (non-HRT,  $0.69 \pm 0.02$  FL/s,  $n = 48$ ; HRT,  $0.69 \pm 0.04$  FL/s,  $n = 43$ ). The curvature of the force-velocity relationship, as indicated by the parameter  $a/P_o$ , was also greater for type IIa fibers (non-HRT,  $0.047 \pm 0.003$  FL/s; HRT,  $0.050 \pm 0.004$  FL/s) than for type I fibers (non-HRT,  $0.027 \pm 0.001$  FL/s; HRT,  $0.029 \pm 0.002$  FL/s). However, for both  $V_{max}$  and  $a/P_o$ , no significant differences were observed between fibers obtained from the non-HRT versus the HRT group for either fiber type.

Fiber peak power, expressed either in absolute terms (micronewtons per FL per second) or normalized to fiber volume (watts/liter), was greater in fibers expressing type IIa MHC than in those expressing the type I isoform (Table 5). There were no differences in peak power of either slow or fast fibers as a result of HRT status.

## DISCUSSION

To our knowledge, the present study is the first to examine cellular properties of muscle fibers from early postmenopausal women who differ in their HRT status. Consistent with previous studies conducted on female subjects, we found that slow and fast vastus lateralis fibers differed in several characteristic ways. For instance, slow fibers were, on average, larger in CSA than fast fibers. However, because slow fibers were less prevalent than fast fibers, slow fibers occupied less relative CSA of the vastus

Table 3. CSA and Peak  $Ca^{2+}$ -Activated Force of Skinned Fiber Segments From HRT and Non-HRT Groups

Variable/Group	MHC Isoform	
	I	IIa
CSA ( $\mu m^2$ )		
Non-HRT	5297 ± 193 (64)	4150 ± 231 (41)
HRT	5005 ± 226 (63)	4187 ± 232 (36)
Mean	5153 ± 148	4167 ± 163*
Peak force (mN)		
Non-HRT	0.61 ± 0.02	0.59 ± 0.03
HRT	0.57 ± 0.02	0.61 ± 0.03
Mean	0.59 ± 0.02	0.60 ± 0.02
Specific force (kN/m <sup>2</sup> )		
Non-HRT	117 ± 2	147 ± 4
HRT	116 ± 2	149 ± 3
Mean	116 ± 2	148 ± 3*

Notes: MHC = myosin heavy chain; CSA = cross-sectional area; non-HRT = postmenopausal women receiving no estrogen therapy; HRT = postmenopausal women receiving estrogen replacement therapy. Values are means ± SE with number of fibers indicated in parentheses. Fibers were obtained from 9 non-HRT and 8 HRT subjects.

\*This indicates a significant main effect of MHC ( $p < .05$ ).

lateralis sample than fast fibers. These results are in agreement with previous studies that have examined vastus lateralis fibers from younger female subjects (30,31). In addition, differences in the functional properties of  $Ca^{2+}$ -activated slow and fast fibers, including the lower specific force, unloaded shortening velocity, and peak power of the former, are consistent with previous data for young and middle-aged male (27,32-34) and young female (10,35) subjects.

The novel finding of this study is that these morphological and functional properties, which were specific to fiber type, were independent of HRT status. Fiber CSA, fiber type distribution, relative fiber type CSA, and  $Ca^{2+}$ -activated functional properties of slow and fast fibers did not differ between the early postmenopausal women who received HRT and those who did not.

Phillips and colleagues (11) have proposed that HRT acts at the level of the cross bridge to improve skeletal muscle performance. There are several mechanisms by which HRT could potentially improve muscle performance under this hypothesis. First, HRT could increase the number of cross bridges in parallel by inducing muscle fiber hypertrophy. This mechanism would be consistent with the results of Sipilä and coworkers (15), who reported 3% and 7% increases in quadriceps lean tissue CSA after 6 and 12 months of HRT, respectively. However, using two independent methods of analysis (skinned fibers and enzyme histochemistry), we found no evidence that slow or fast muscle fibers from the HRT subjects were larger in CSA than corresponding fibers from their non-HRT peers.

Alternatively, HRT could improve muscular performance by altering the quality of the muscle tissue. This mechanism is consistent with cross-sectional and longitudinal studies showing greater specific force of the adductor pollicis of women on HRT (11,14). Because we observed that fast muscle fibers produced greater specific force than slow fibers, a shift in fiber type distribution, or a change in the relative area occupied by either fiber type, might be

expected to increase the overall specific force of the muscle. However, fiber type distribution, the relative MHC isoform content of individual fibers, and the relative area occupied by either slow or fast fibers did not differ between HRT and non-HRT groups.

Finally, HRT could affect the specific force of individual muscle fibers. In support of this mechanism, Wattanapernpool and Reiser (36) found that slow soleus fibers obtained from young rats, ovariectomized at 8–10 weeks of age and then studied either 10 or 14 weeks later, produced significantly less specific force than fibers from sham-operated control animals. However, in the present study, no intergroup differences were observed in the average specific force of fibers containing type I or IIa MHC. These fibers accounted for ~80% of the total fibers isolated from the biopsy samples (Table 2). Only a few fibers containing types I/IIa ( $n = 5$ ), IIa/IIx ( $n = 13$ ), and IIx ( $n = 3$ ) were isolated for functional analysis, and the small sample sizes preclude statistical analysis. However, assuming these fibers make up 20% of total fibers (Table 2) and that all other factors are equal, we find 1.75- to 2-fold intergroup differences in the specific force of the I/IIa, IIa/IIx, and IIx fibers would be required to account for the 15–20% greater muscle specific force previously reported for women on HRT (11,14). A difference of this magnitude seems unreasonable, making it unlikely that the greater muscle specific force of HRT subjects can be attributed to differences in the specific force of individual muscle fibers.

Significant increases in vertical jump height following 6 and 12 months of HRT (15) suggest that HRT may improve muscle power. We therefore evaluated the ability of  $\text{Ca}^{2+}$ -activated fibers to shorten and to produce power. In agreement with previous work (27,33,34), both  $V_o$  and peak power were highly dependent on MHC isoform content. However, no intergroup differences were observed for either variable.

The primary finding of this study is the consistency in fiber type distribution, fiber CSA, and  $\text{Ca}^{2+}$ -activated fiber function between non-HRT and HRT subjects. Although these results do not eliminate an influence of HRT on muscle performance, they do suggest that any potential site of action is unlikely to be at the level of the cross bridge per se. This conclusion must be interpreted in light of the limitations of the present study. Because we used a cross-sectional design, individual subject variation in fiber characteristics may have obscured an effect of HRT. Although this is certainly a possibility, it is noteworthy that the specific force,  $V_o$ , and peak power–volume of the fibers obtained from both the HRT and non-HRT groups are in very good agreement with data obtained from 20- to 30-year-old male subjects recently studied in our laboratory (27). This suggests that the present middle-aged subjects had not experienced the reductions in muscle fiber specific force and  $V_o$  that have been observed in older populations (9,10). Although not directly tested, the present data suggest that cross-bridge mechanisms of contraction are not altered during the initial years of menopause. If this is the case, then it is difficult to envision how HRT could augment the contractility of  $\text{Ca}^{2+}$ -activated fibers that appear to have normal function.

Table 4. Unloaded Shortening Velocity of Skinned Fiber Segments From HRT and Non-HRT Groups

Group	MHC Isoform	
	I	IIa
Non-HRT	0.61 ± 0.02 (54)	2.80 ± 0.12 (39)
HRT	0.58 ± 0.01 (53)	2.67 ± 0.14 (29)
Mean	0.59 ± 0.01	2.75 ± 0.09*

Notes: HRT = postmenopausal women receiving hormone replacement therapy; non-HRT = postmenopausal women not receiving hormone replacement therapy; MHC = myosin heavy chain. Values are means ± SE with number of fibers indicated in parentheses. Fibers were obtained from 9 non-HRT and 8 HRT subjects.

\*This indicates a significant main effect of MHC ( $p < .05$ ).

On the basis of this argument, it is conceivable that the women in this study were too young to experience an effect of HRT on muscle fiber morphology or function. Alternatively, the duration of HRT treatment may have been too brief to induce a treatment effect. However, the age of the subjects and the duration of HRT treatment are in good agreement with the studies conducted by Greeves and coworkers (13) and Sipilä and coworkers (15), both of which reported beneficial effects of HRT on voluntary knee extension strength. It could also be argued that because no intergroup differences in knee extension strength were observed, our subjects were somehow different than those examined in these previous studies. However, as detailed at the beginning of this paper, there are limitations in using voluntary strength measurements as an index of muscular function. Because our goal was to investigate cellular mechanisms responsible for muscle contraction, the fact that knee extension strength did not differ between our groups has little bearing on our rationale for conducting this study. Because of the complexity of the intact neuromuscular system, changes in neuromuscular strength or performance are not always accompanied by alterations in the functional properties of single muscle fibers (37). Conversely, muscle fiber atrophy and contractile dysfunction (38,39), as well as reductions in electrically stimulated muscle torque (40), have been observed to occur without corresponding changes in the voluntary strength of the same subjects (40).

The single-cell experiments described here are labor and time intensive and must be completed while the fibers remain viable (within the 3–4 weeks immediately following the biopsy). This imposes limitations on sample size, and it is possible that this reduced our ability to detect differences between groups. We have investigated this possibility by computing effect size. For all physiological variables in Tables 3–5, effect sizes related to the HRT treatment were  $\leq 0.007$ . In contrast, effect size for comparisons based on MHC isoform content were 0.109, 0.005, and 0.408 for fiber CSA, absolute force, and specific force, respectively, and 0.854, 0.831, and 0.902 for fiber  $V_o$ , absolute power, and normalized power, respectively. On the basis of Cohen's criteria (41), effect sizes of 0.20–0.50 are considered small differences and those  $> 0.80$  are considered to be large. Because the effect sizes observed here for the HRT treatment are negligible, increasing the sample size would have little influence on meaningful differences between non-

Table 5. Peak Power of Skinned Fiber Segments From HRT and Non-HRT Groups

Group	MHC	
	I	Iia
Micronewtons per FL per second		
Non-HRT	7.73 ± 0.42 (48)	33.3 ± 1.5 (35)
HRT	7.42 ± 0.43 (43)	33.5 ± 1.8 (28)
Mean	7.58 ± 0.30	33.4 ± 1.2*
Watts/liter		
Non-HRT	1.50 ± 0.05	8.21 ± 0.36
HRT	1.52 ± 0.05	8.60 ± 0.31
Mean	1.51 ± 0.03	8.38 ± 0.24*

Notes: HRT = postmenopausal women receiving hormone replacement therapy; non-HRT = postmenopausal women not receiving hormone replacement therapy; FL = Fiber length; MHC = myosin heavy chain. Values are means ± SE with number of fibers indicated in parentheses. Fibers were obtained from 9 non-HRT and 8 HRT subjects.

\*This indicates a significant main effect of MHC ( $p < .05$ ).

HRT and HRT groups. Our conclusions regarding the HRT treatment cannot be easily dismissed as an artifact of sample size.

In this study, we examined only one aspect of muscle cell function under very controlled conditions. Because our preparation bypassed processes involved in normal cell activation and relaxation, one cannot rule out a potential effect of HRT on mechanisms of action potential propagation or excitation–contraction coupling. Indeed, faster relaxation times have been observed in several hindlimb muscles obtained from ovariectomized rats (42), suggesting an influence of estrogen on sarcoplasmic reticulum function. It has been suggested by Phillips and colleagues (11) that HRT may alter the distribution of low- and high-force cross bridges by modifying their sensitivity to intracellular metabolites such as inorganic phosphate ( $P_i$ ) or hydrogen ion ( $H^+$ ). Alternatively, HRT could alter the concentrations of these metabolites. Because the concentrations of  $P_i$  and  $H^+$  in slow and fast muscle fibers of postmenopausal women are not known, their potential effects on fiber contractility could not be tested in the present study. To reiterate, the present results do not eliminate an influence of HRT on those aspects of muscle contraction, including both neural and peripheral mechanisms, that were not examined in this study.

Finally, we performed histochemistry and functional measurements on fibers from the same muscle samples, and we wish to draw attention to two methodological issues pertinent to this type of dual analysis. The first issue concerns the large differences in fiber CSA one can observe between our histochemical results and our skinned fiber analysis. This apparent discrepancy can be attributed to the 20% increase in fiber diameter, or 44% increase in fiber CSA, that occurs during the chemical skinning process (43). Once the skinned fiber CSAs are adjusted for this swelling, the histochemical and skinned fiber results are in good agreement. Second, a lower percentage of fibers were classified as type I by enzyme histochemistry compared with the electrophoretic analysis of fiber MHC isoforms. Note that the histochemical fiber type analysis is based on the numerical occurrence of fibers in a sample. In contrast,

the isolation of fibers used in the MHC isoform analysis is based on the relative CSA occupied by each particular type of fiber in the sample (because fibers are isolated by randomly inserting forceps into the ends of the muscle bundles). Thus, the single-fiber MHC isoform content results are in much closer agreement with the histochemical relative fiber CSA results versus the histochemical fiber type percentages.

### Conclusions

In summary, we found no compelling evidence that fiber type distribution (on the basis of enzyme histochemistry and MHC isoform analysis), absolute fiber CSA, relative fiber CSA (CSA per fiber type), or the  $Ca^{2+}$ -activated functional properties (absolute force, specific force, shortening velocity, and peak power) of vastus lateralis muscle fibers differed between early postmenopausal women who had received HRT for 2 years versus those who had never received HRT. These results suggest that the morphological and functional characteristics examined in this study are insensitive to HRT during the initial years of menopause.

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