Contractile function and myonuclear organization in single fibers from monozygotic female twins discordant for hormone replacement therapy

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Abbreviations

HRT          Hormone Replacement Therapy
MZ twins    Monozygous twins
ECM          Extra cellular matrix
MND          Myonuclear Domain
Abstract

Ageing is associated with a decline in muscle mass and strength leading to increased physical dependency in old age. Post-menopausal women experience a greater decline than men of similar age due to a dramatic decrease in sex hormones production. We recruited six monozygous female twin pairs (55 – 59 years old) discordant for postmenopausal hormone replacement therapy (HRT use = 7.8 ± 4.3 years) to investigate the association of HRT with the cytoplasmic domain supported by individual myonuclei (myonuclear domain size, MND) together with specific force at the single fiber level. MyHC isoform content of the fibers was determined using silver-stained SDS-PAGE. HRT use was associated with a significantly smaller (~27%; p < 0.05) mean MND size in muscle fibers expressing the type I but not the IIa MyHC isoform. An increase in specific force was recorded in the HRT user group both in muscle fibers expressing type I (~27%; p < 0.05) and type IIa (~23%; p < 0.05) MyHC isoforms. These positive effects on specific force were fiber-type dependent, i.e., in fast-twitch muscle fibers the increased specific force was primarily caused by an increased force per cross-bridge while slow-twitch fibers relied on both an increase in both number and force per cross-bridge. HRT use had no effect on fiber cross-sectional area (CSA), velocity of unloaded shortening (V_o) and relative proportion of MyHC isoforms. In conclusion, HRT has significant positive effects on both regulation of muscle contraction and myonuclei organization in menopausal women, but the response is fiber-type specific.
Sarcopenia is the aging-related progressive change in skeletal muscle quantity and quality leading to decline in strength and mobility (Frontera et al. 2000). The changes at the whole muscle level reflect aging-related changes in structure and function at the motor unit, muscle cell and motor protein levels, resulting in a decline in muscle mass, force generating capacity and contractile speed (Larsson et al. 1997; D'Antona et al. 2003; Yu et al. 2007). These changes are attributed to a complex interface of many factors ultimately leading to a progressively dependent life style in old age (Ryall et al. 2008).

Current theories attribute an altered endocrine activity as an important contributor to the aging-related muscle dysfunction. In this context, the most dramatic event in women is the menopause, resulting in an additional 15% loss in muscle mass (Phillips et al. 1993) making old women more vulnerable to fall and fall-related injuries (Frontera et al. 1991). Hence, hormone replacement therapy has been extensively used to partially counteract the deleterious effects on muscles (Skelton et al. 1999; Sipila et al. 2001). However, the beneficial impacts of HRT on muscle function are still in debate, mainly due to experimental limitations, such as genetic and life style differences among HRT users and non-users (Onambele-Pearson 2009). To overcome this limitation, two recent studies have been performed on postmenopausal monozygotic (MZ) sisters discordant for HRT (Ronkainen et al. 2009; Finni et al. 2011). In these studies, HRT users had an improved walking speed, jumping height and in vivo force production compared with non-user sisters. Similarly, a recent meta-analysis reported a 5% increase in muscle strength with HRT use in post menopausal women compared with age matched controls (Greising et al. 2009).

The cellular and molecular mechanisms underlying the in vivo improvements in response to HRT treatment remain unclear. It has been speculated, on one hand, that estrogen improves muscle directly by affecting actomyosin interactions (Phillips et al. 1993; Moran et al. 2006; Lowe et al. 2010). On the other hand, estrogen has been suggested to boost satellite cells activation, attenuating exercise-induced muscle damage and creating a pro-anabolic environment in muscles of post-menopausal women (Enns et al. 2008; Dieli-Conwright et al. 2009b; Dieli-Conwright et al. 2009a). Recent studies using HRT discordant MZ twin pairs have observed a positive anti-catabolic effect and an improved regulatory action on the cytoskeleton and extracellular matrix (ECM) in response to HRT treatment, leading to better muscle quality (Ronkainen et al. 2010). While some or all of the above mechanisms may influence skeletal muscle function, little is known about the action of estrogen on the regulation of muscle contraction at the cell and motor protein levels in humans. An investigation of the
actomyosin interactions at the cell and motor protein levels is forwarded as a relevant experimental model to improve our understanding of the mechanisms underlying the effects of HRT and in the identification of potential future pharmacological intervention strategies aiming at improving muscle function in post-menopausal women. Further, myonuclear organization is affected by aging and we have recently shown that myonuclear domain (MND) size is linked to specific force and the quantity of the molecular motor protein myosin in skeletal muscles fibers (Cristea et al. 2010; Qaisar et al. 2011).

It is hypothesized that the altered in vivo muscle function related to HRT treatment observed in MZ twin pairs (Ronkainen et al. 2009; Finni et al. 2011) is caused by the combined effect of an altered actomyosin interaction, contractile protein expression and myonuclear organization. This study aims at unraveling the effects of HRT treatment by studying a subset of the unique group of HRT discordant post-menopausal twin pairs described above by studying the force generation capacity, contractile speed and the 3D myonuclear organization in single muscle fiber segments.
RESULTS

Physical and Life style characteristics

Physical and life style characteristics of the participants have been described in detail elsewhere (Ronkainen et al. 2009). In summary, there were no differences in the physical activity, smoking behavior, alcohol use or daily energy intake between the HRT discordant postmenopausal twins. Further, weights, BMI, waist or hip circumference or body fat percentage did not differ between the twin sisters.

Single fiber cross-sectional area and MyHC isoform expression

The CSA of individual muscle fibers was measured in a total of 326 fiber segments from HRT non-users (n= 162) and users (n = 164) at a fixed sarcomere length assuming an elliptical circumference (Table 1). Statistical analysis was restricted to fibers expressing the type I (n = 176) and type IIa MyHC isoforms (n = 89) because of the scarcity and unequal distribution across subjects of fibers expressing the type IIx MyHC isoform (n = 5), co-expressing type I and type IIa (n = 29) or type IIa and IIx (n=27) MyHC isoforms. No statistically significant difference was found in the CSA of fibers expressing type I or type IIa MyHC isoform between twin pairs, resulting in a similar type IIa/I fiber area ratio between twin sisters. In young adults, type II fibers are typically larger than type I fibers independent of gender demonstrating that the preferential type II fiber atrophy reported in old age in skinned muscle fibers (Larsson et al. 1997; Cristea et al. 2010) or from enzyme-histochemically stained sections (Larsson 1978) becomes manifest already at 50-59 years of age.

The proportion MyHC isoforms expressed in dissected single muscle fibers used in contractile measurements and in muscle biopsy cross-sections are presented in Table 2. In short, no statistically significant difference was found in the relative proportion of different MyHC isoforms between twin sisters, neither in the analyzed muscle fibers nor at the muscle biopsy level.

Phenotypical Observations

Individual myonuclei typically had a rounded or elliptical appearance and both shapes were frequently observed in the same fiber segments irrespective of MyHC isoform and HRT status (Fig. 1). The longitudinal axis of elliptical nuclei was parallel with the longitudinal axis of muscle fiber in most, but not all, myonuclei. Deviations from the common rounded or elliptical shapes were rare, but a small number of nuclei were observed with “notches”. In accordance with our previous observations (Cristea
fibers expressing the type I MyHC isoforms frequently presented with groove like structures with long chains of aggregated nuclei (Fig. 1A, B), leading to an increased MND size variability. This type of spatial reorganization of myonuclei was typically observed in fibers expressing the type I MyHC isoform while it was relatively scarce in fibers expressing the type IIa MyHC isoform where myonuclei showed a more ordered organization (Fig. 1C, D). These observations were independent of HRT status.

Internal nuclei were rare, but a small number was observed in muscle fibers expressing the type I MyHC isoform in the HRT non-user (3 out of 31 fiber segments) and users (3 out of 29 fiber segments). Further, internal nuclei were infrequent in type I/IIa (1 of 7, in HRT user only) and in type IIa fibers (2 of 26, in non-users only), but they were not observed in fibers expressing the type IIx MyHC or co-expressing type IIa and IIx isoforms. When present, internal nuclei constituted 2-12% of all nuclei in the fiber segment.

**Nuclei number per unit length and MND size**

The MNDs of at the terminal part of the fiber segment may extend outside the fiber segment and give rise to erroneously small MND sizes therefore half of the terminal nuclei were randomly included in the analysis and half were excluded. Only the fibers expressing type I (n = 61) and type IIa (n = 36) MyHC isoforms were considered for statistical analysis because of the small number of fibers expressing other MyHC isoforms or a combination of MyHC isoforms. No significant difference was found in myonuclear number per unit length in muscle fibers expressing type I or type IIa MyHC isoforms between the HRT user and non-user groups (Fig. 2B).

In muscle fibers expressing the type I MyHC isoform, myonuclear domain size was 27% smaller (p<0.05) in HRT users than their non-user counterparts (Fig. 2A), due to the combined effect of small trends, not statistically significant, towards both smaller fibers and extra myonuclei in the HRT users. In fibers expressing the type IIa MyHC isoform, MND size did not differ significantly between HRT users and non-users.

**Contractile Properties**

A total of 216 fibers expressing type I (n = 129) and type IIa (n = 83) MyHC isoforms met the strict criteria for acceptance and were included in the analysis of contractile properties (Table. 1). Fibers expressing the type IIx MyHC isoform (n = 5), co-expressing type I and type IIa (n = 20) or type IIa and IIx (n = 21) MyHC isoforms were omitted from the statistical analysis due to paucity of these fiber types. Maximum force normalized to muscle fiber cross-sectional area (specific force) was higher in
the HRT user group both in muscle fibers expressing the type I MyHC isoforms ~ 27% (p<0.05) and type IIa MyHC isoform ~23% (p<0.05) (Fig. 3). The increased specific force may accordingly reflect changes in the regulation of muscle contraction, i.e., an increase in the number of strongly attached cross-bridges in series or force produced by each cross-bridge (Regnier et al. 2004). Stiffness recordings (E₀) represent a good index of the number of strongly attached cross-bridges in series. In muscle fibers expressing the type I MyHC isoform, a slightly higher stiffness (~13%) in the HRT group was not statistically significant (Fig. 3A) and it is accordingly unlikely that an increase in the total number of cross-bridges is the major source underlying the 27% higher specific force suggesting that the force per cross-bridge is higher in type I fibers from HRT users. In fibers expressing type IIa MyHC isoform, on the other hand, the ~17% increase in stiffness (p < 0.05) suggests that an increased number of cross-bridges contribute significantly to ~23% higher specific force among HRT users (Fig. 3B). Maximum velocity of unloaded shortening did not differ between users and non-users independent on MyHC isoform expression.
DISCUSSION

The results from this study favor a beneficial effect of hormone replacement therapy on skeletal muscle in post-menopausal women and the major findings from this study are as follows: (i) HRT preserves the specific force without affecting fiber CSA, (ii) stiffness values are a good reflector of change in specific force in fibers expressing type IIa MyHC isoform but not in type I fibers. (iii) smaller MND size is reported in fibers expressing type I MyHC isoform in response to hormone use while MND size in type IIa fibers remain unaffected. (iv) no significant change was observed in the velocity of unloaded shortening ($V_0$) and myonuclei number with HRT use.

Fiber CSA and MyHC isoform expression

The higher specific tension in muscle from the HRT user could be secondary to a myosin isoform switching towards a faster phenotype or an increase in the relative area of muscle fibers expressing the fast myosin isoform. Since human muscle fibers expressing fast myosin isoforms generate higher specific forces than fibers expressing the slow MyHC isoform (Medler 2002; Korhonen et al. 2006; Yu et al. 2007). This in part is due to a higher force-generating capacity of the human fast MyHC isoform (Li & Larsson 2010). However, analyses of MyHC isoform expression in single muscle fibers and from muscle cross-sections using sensitive SDS-PAGE and single muscle fibers CSA measurements does not show any significant differences in fiber CSA or any change in the proportion of MyHC isoforms between the HRT discordant twin pairs (Table 1 and 2). These observations are in accordance with a previous publication comparing hormone replacement with non-replacement post-menopausal women (Widrick et al. 2003).

HRT affects myosin function to preserve single fiber force generating capacity

The two prime determinants of specific force are the fraction of strongly attached cross-bridges and the force produced by individual cross bridges. The results from this study suggest that both factors contribute to the higher specific force in HRT users, but the relative contribution appears to be MyHC dependent.

In muscle fibers expressing the β/slow MyHC isoform, stiffness recordings suggest that only ~50% of the higher specific force in the HRT user is attributed to an increased fraction of strongly attached cross-bridges (Fig. 3A), indicating that the force/cross-bridge account for the remaining increase in specific force. In fibers expressing type IIa MyHC isoform, on the other hand, specific force is in good agreement with stiffness measurements demonstrating that the lower specific force in the non-user is primarily due to a smaller fraction of strongly attached cross-bridges (Fig. 3B). This
conforms with previous hypotheses (Phillips et al. 1993) and experimental results using EPR spectroscopy in ovariectomized rats treated with estrogen (Moran et al. 2007). Thus, the contractile recordings indicate a fiber type specific effect of the HRT treatment in the post-menopausal women where the positive effects are mainly due to a quantitative effect in fibers expressing the fast myosin isoform while the effect is both quantitative and qualitative in fibers expressing the slow myosin isoform. The higher metabolic rate, mitochondrial density and formation of reactive oxidative species in slow versus fast muscle fibers suggest that posttranslational protein modifications is a significant source underlying the qualitative changes in the force generation capacity of the type I fibers in post-menopausal women (McArdle et al. 2002).

The concentration of estrogen receptors is higher in slow- than fast-twitch fibers (Saartok 1984; Meeuwsen et al. 2000; Lemoine et al. 2002) and estrogen has anti-oxidant properties (Persky et al. 2000). The increased post-translational myosin modification by free radicals is one of the mechanisms leading to aging-related contractile dysfunction (Lowe et al. 2001; Lowe et al. 2004). HRT may accordingly reduce the impaired myosin function in post-menopausal women more efficiently in type I fibers by a specific protection against post-translational modifications.

Maximum velocity of unloaded shortening velocity did not differ between the HRT discordant twin pairs irrespective MyHC isoforms expression. This indicates that the greater in vivo muscle power reported in the HRT using twin pair (Ronkainen et al. 2009) was primarily due to the higher specific force.

**HRT reduces myonuclear domain size in slow-twitch fibers but not in fast-twitch fibers**

In accordance with our recent findings in old women (Cristea et al. 2010) average MND size did not differ between type I and type IIa fiber types in the non-user group (Fig. 2). This is probably related to the age-related preferential type IIa fiber atrophy (Tomonaga 1977; Larsson et al. 1978; Larsson 1982) since we previously found no change in myonuclei count per unit fiber length with aging (Cristea et al. 2010). In the HRT user group, MND size was significantly smaller in the muscle fibers expressing the type I MyHC isoform (Fig. 2A), but it did not differ between users and non-users in fibers expressing type IIa MyHC isoform (Fig. 2B). A possible explanation for this discrepancy is the observation that the aging-related oxidative stress has a more profound effects on slow-twitch fibers (McArdle et al. 2002) in part due to a decreased production of HSP70 (Broome et al. 2006). Slow-twitch fibers are also transcriptionally more active than fast-twitch fibers (Habets et al. 1999). Oxidative damage reduces transcriptional capacity leading to a reduced specific force and a need for smaller myonuclear domains. It is therefore possible that due to a higher concentration of its receptors in slow-twitch
fibers, estrogen not only arrests the age-related oxidative damage but also reduces myonuclear domain size to restore force generating capacity in aging fibers. This also optimizes transport distances for cellular proteins and synthetic capacity of the aging myonucleus.

In the fibers expressing the type IIa MyHC isoform, on the other hand, HRT seems to have no effect on MND size. We have recently reported an age-related decline in CSA and MND size in type IIa fibers (Cristea et al. 2010) and it is assumed that HRT usage impacts on existing nuclei to optimize their transcriptional and translational efficiency to restore functional capacity without a need for smaller domains or additional myonuclei.

Conclusion

There is growing interest in exploring the effects of HRT on skeletal muscle and the possible mechanisms by which it can assert its influence on muscle mass and strength. Results from our study indicate that HRT has significant positive effects on ability of single muscle fibers to generate more force without a change in size. This effect is obtained by modulation and direct influence on actin-myosin interactions and the number of such interactions as well as altered myonuclear organization. These effects are fiber-type dependent and the force per actin-myosin interaction plays a stronger role in fast- than slow-twitch fibers, with slow-twitch fibers relying on both the number and force per cross-bridge. These findings open a venue for future pharmacological interventions aiming at enhancing muscle mass and function in old age.
MATERIALS & METHODS

Study Design and Subjects

This study is part of a larger study, ‘Sarcopenia – Skeletal Muscle Adaptation to Postmenopausal Hypogonadism and Effects of Hormone Replacement Therapy and Physical Activity in older Women: a Genetic and Molecular Biological Study on Estrogen-related Pathways’ (SAWES). The study design, subject recruitment and exclusion criteria has been described previously (Ronkainen et al. 2009). Briefly, after screening and confirmation for monozygosity by multiple genetic markers, a total of six MZ twin pairs, clearly postmenopausal and discordant for HRT, were chosen. The mean age of the 12 subjects was 56.6 ± 1.3 years (range 55-59 years). Estradiol and progesterone were the effective agents given as pills in three HRT users while estradiol alone in another three subjects. The mean duration of HRT use was 7.8 ± 4.3 years (range 4-16 years).

Muscle biopsies

Bergstrom needles were used to obtain biopsies from the right vastus lateralis muscle with the understanding and consent of the subjects. The biopsy specimens typically contained segments of 200-800 muscle fibers and weighed 50-120 mg. Specimens were placed in relaxing solution at 4 °C, and bundles of ~50 fibers were carefully dissected free and then tied with surgical silk to glass capillary tubes at slightly stretched lengths. The muscle fiber bundles were chemically skinned for 24 h in relaxing solution 50% (v/v) glycerol at 4 °C, cryoprotected (Frontera & Larsson 1997) and subsequently stored at -180 °C before use. The relaxing solution contained (in mM): 4 MgATP, 1 free Mg2+, 20 imidazole, 7 EGTA, 14.5 creatine phosphate and sufficient KCl to adjust the ionic strength to 180. The pH was adjusted to 7.0. The free Ca2+ concentration, expressed as pCa (-log[Ca2+]), was 10⁻⁹ M. Apparent stability constant for Ca2+-EGTA was corrected for temperature and ionic strength (Fabiato & Fabiato 1979).

Single fiber contractile recordings

The experimental procedure has been described in detail elsewhere (Larsson & Moss 1993). Briefly, membrane permeabilized muscle fibers were used with an average segment length of 1.60 ± 0.20 mm (mean ± SD, range 1.00–2.00 mm) exposed to the solution between the connectors of the force transducer and servomotor. The sarcomere length (SL) of the single-fiber segment was set to 2.77 ± 0.05 μm (range 2.71–2.85 μm) by adjusting the overall segment length. Fiber CSA was
calculated from the width and depth, assuming an elliptical circumference. Specific tension (ST) was calculated as maximum tension ($P_o$) normalized to CSA, and was corrected for the 20% swelling that is known to occur during skinning (Moss 1979).

Maximum unloaded shortening velocity ($V_o$) was measured by the slack test procedure (Edman 1979). Fibers were activated at pCa 4.5 and, once steady state tension was reached, various amplitudes of slack ($\Delta L$) were rapidly introduced (within 1-2 ms) at one end of the fiber. The time ($\Delta t$) required to take up the imposed slack was measured from the onset of the length step to the beginning of the tension redevelopment. For each amplitude of $\Delta L$ the fiber was re-extended while relaxed to minimize nonuniformity of the sarcomere length. A straight line was fitted to a plot of $\Delta L$ vs. $\Delta t$ using a least-squares regression, and the slope of the line was recorded as $V_o$ for that fiber. Relaxing and activating solutions were prepared as previously described (Larsson & Moss 1993). All contractile measurements were carried out at 15 °C. The contractile recordings were accepted in subsequent analyses only if $P_o$ did not change more than 10% from first to final activation, if SL during isometric tension development did not change by more than 0.10 μm compared with SL when the fiber was relaxed or if the $V_o$ value based on linear regression included four or more data points, and the data was discarded if the coefficient of reliability ($r^2$) for the fitted line was less than 0.96 (Moss 1979).

**Stiffness**

Once steady-state isometric force was reached, small-amplitude sinusoidal changes in length ($\Delta L$: ± 0.2% of fiber length), were applied at 500 Hz at one end of the fiber (Martyn et al. 2007). The resultant force response ($\Delta F$) was measured, and the mean of 20 consecutive readings of $\Delta L$ and $\Delta F$ was used to determine stiffness. The actual elastic modulus ($E$) was calculated as the difference between $E$ in activating solutions and resting $E$ measured in the same segment in the relaxing solution. $E$ was determined as follows (McDonald & Fitts 1995):

$$E = (\Delta F/\Delta L) \times (\text{fiber length/CSA})$$

**Fluorescent labeling, image acquisition and analyses of myonuclear organization**

Skinned single fiber segments were mounted at a fixed sarcomere length corresponding to optimal filament overlap for force generation. Actin and myonuclei were stained with Rhodamine Phalloidin and DAPI, respectively. Confocal images were analyzed by means of a novel algorithm. The volume $G$ of a General Elliptical Cylinder (GEC) was developed and used to calculate the volumes of the MNDs and the CSA of the fiber. The 3D parameters of every nucleus were determined manually and
the MND size determined by means of automatic image analysis. A detailed description of procedures
is given elsewhere (Cristea et al. 2010).

**Single fiber gel electrophoresis**

The procedure is described in detail elsewhere (Larsson & Moss 1993). In short, the MyHC
composition of single fibers was determined by SDS-PAGE. The total acryl amide and bis
concentrations were 4% (w/v) in the stacking gel and 6% in the running gel, and the gel matrix
included 30% glycerol. Polymerization was activated by adding TEMED to the stacking (0.1%) and
separation gels (0.07%). Sample loads were kept small to improve the resolution of the MyHC bands
and electrophoresis was performed at 120 V for 22–24 h with a Tris-glycine electrode buffer (pH 8.3)
at 10° C.

**Statistical analysis**

The paired-sample t-test was used to compare fiber CSA, MND and contractile recordings means
between HRT discordant twin pairs. The data normality was tested according to Kolmogorov-
Smirnov test. All values are expressed as mean ± standard error of mean (SEM). Statistical
significance was set at p < 0.05 for all analysis.
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FIGURE LEGENDS

Figure 1.
Confocal microscopy images of single muscle fibers and their representative myonuclei from non-users (A, C) and HRT users (B, D). Type I fibers (A, B) typically characterized by deep groove like structures harboring long chain of nuclei. Type IIa fibers (C, D) with more ordered organization of both spherical and elliptical myonuclei. DAPI (blue) stained myonuclei while rhodamine (red) labeled actin. Horizontal bar denoted 50μm (fiber) and 8μm (nuclei).

Figure 2.
Myonuclear domain size (A) and nuclei number per unit length (B) in type I and type IIa fibers in vastus lateralis muscles from hormone user and non-user twins. Asterisk denotes statistically significant difference from non-users (p<0.05). All values are mean ± SEM.

Figure 3.
Single fiber specific tension and stiffness recordings in type I (A) and type IIa (B) fibers in vastus lateralis muscles from hormone user and non-user twins. Asterisk (*) and Cyrillic (ж) denotes statistically significant difference for the specific force and stiffness respectively (p<0.05). All values are mean ± SEM.
Figure 2.
Table 1.

Cross-sectional area (CSA), specific tension (ST), stiffness and maximum velocity of unloaded shortening ($V_0$) in skinned single muscle fibers expressing different MyHC isoforms in hormone users and non-user twins.

<table>
<thead>
<tr>
<th></th>
<th>Type I</th>
<th>Type I/IIa</th>
<th>Type IIa</th>
<th>Type IIax</th>
<th>Type IIx</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonusers (N=6)</td>
<td>Users (N=6)</td>
<td>Nonusers (N=4)</td>
<td>Users (N=6)</td>
<td>Nonusers (N=4)</td>
</tr>
<tr>
<td>CSA (um$^2$)</td>
<td>2550 ± 110 (n=97)</td>
<td>2460 ± 140 (n=79)</td>
<td>2630 ± 450 (n=13)</td>
<td>1860 ± 190 (n=16)</td>
<td>2240 ± 170 (n=39)</td>
</tr>
<tr>
<td>ST (N/cm$^2$)</td>
<td>28.5 ± 2 (n=71)</td>
<td>36.4 ± 2.7 * (n=48)</td>
<td>30.1 ± 5.3 (n=9)</td>
<td>39.1 ± 4 (n=11)</td>
<td>31.2 ± 2.2 (n=25)</td>
</tr>
<tr>
<td>Stiffness (N/cm$^2$)</td>
<td>2550 ± 40 (n=71)</td>
<td>2870 ± 170 (n=48)</td>
<td>2450 ± 900 (n=7)</td>
<td>2880 ± 460 (n=8)</td>
<td>2140 ± 130 (n=20)</td>
</tr>
<tr>
<td>$V_0$(ML/s)</td>
<td>1 ± 0.1 (n=59)</td>
<td>0.90 ± 0.1 (n=46)</td>
<td>1.6 ± 0.6 (n=8)</td>
<td>1.8 ± 0.3 (n=10)</td>
<td>2.1 ± 0.3 (n=20)</td>
</tr>
</tbody>
</table>

Asterisk denotes significant difference from non-user group (p < 0.05) according to paired t-test. Values are mean ± SEM. Statistical analysis has been restricted to muscle fibers expressing type I or type IIa MyHC isoform, because of small sample size in fibers expressing other isoforms or combinations of isoforms. (N = number of subjects; n= number of fibers).
Table 2.

MyHC isoform expression measured in single muscle fibers and biopsy cross-sections from HRT users and non-users.

<table>
<thead>
<tr>
<th></th>
<th>Total number of fibers</th>
<th>Type I</th>
<th>Type I/IIa</th>
<th>Type IIa</th>
<th>Type IIax</th>
<th>Type IIx</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRT non-users</td>
<td>162</td>
<td>60 (47 ± 6)</td>
<td>8</td>
<td>24 (51 ± 6)</td>
<td>6</td>
<td>2 (12 ± 1)</td>
</tr>
<tr>
<td>Users</td>
<td>164</td>
<td>49 (43 ± 5)</td>
<td>10</td>
<td>30 (52 ± 4)</td>
<td>10</td>
<td>1 (15 ± 3)</td>
</tr>
</tbody>
</table>

Values for cross-sections (in parenthesis) are expressed as mean ± SEM. No statistically significant differences were observed in MyHC isoform expression between HRT users and non-users.