Hormone replacement therapy improves contractile function and myonuclear organization of single fibres from postmenopausal monozygotic female twin pairs

Rizwan Qaisar [1], Guillaume Renaud[1], Yvette Hedstrom [1], Eija Pöllänen [2], Paula Ronkainen [2], Jaakko Kaprio [4], Markku Alen [3], Sarianna Sipilä [2], Konstantin Artemenko[5], Jonas Bergquist[5], Vuokko Kovanen [2], Lars Larsson [1]

Department of Neuroscience, Clinical Neurophysiology, Uppsala University, Sweden[1];

Gerontology Research Center, Department of Health Sciences, University of Jyväskylä, Jyväskylä, Finland[2],Department of Medical Rehabilitation, Oulu University Hospital and Institute of Health Sciences, University of Oulu, Finland[3],Department of Public Health; Institute for Molecular Medicine, University of Helsinki, & National Institute for Health and Welfare, Helsinki, Finland[4], Department of Chemistry – Biomedical Center, Uppsala University, Uppsala, Sweden[5]

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Address correspondence to:

Prof. Lars Larsson
Department of Neuroscience, Clinical Neurophysiology,
Uppsala University
SE-751 85 Uppsala, Sweden

Phone: +46 18 611 3445
Fax: +46 18 55 6106
E-mail: Lars.larsson@neurofys.uu.se

Key Point Summary:

- The ageing-related impairment of muscle function and consequent falls and fall-related injuries have severe negative effects on morbidity and mortality in old age, with women being more negatively affected than men.
- The effects of hormone replacement therapy (HRT) on regulation of muscle contraction and myonuclear organization were investigated in monozygous postmenopausal twin pairs where only one twin was a HRT-user..
- HRT treatment improved single fibre force-generating capacity (specific force), without affecting fibre size and speed of contraction, due to fibre type specific effects on force and number of force generating cross-bridges.
- HRT had a significant effect on the myonuclear organization in slow-twitch muscle fibres, improving the synthetic capacity of the myonuclei and optimizing transport of proteins.
- Significant positive effects on regulation of muscle contraction and myonuclear organization were observed at the cellular level in response to HRT with consequences for quality of life in postmenopausal women.

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SUMMARY

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 in parallel with the decrease in female sex steroid hormone production. We recruited six monozygous female twin pairs (55 – 59 years old) where only one twin pair was on hormone replacement therapy (HRT use = 7.8 ± 4.3 years) to investigate the association of HRT with the cytoplasmic volume supported by individual myonuclei (myonuclear domain size, MND) together with specific force at the single fibre level. HRT use was associated with a significantly smaller (~27%; $p < 0.05$) mean MND size in muscle fibres expressing the type I but not the IIa myosin heavy chain (MyHC) isoform. In comparison to non-users, higher specific force was recorded in HRT users both in muscle fibres expressing type I (~27%; $p < 0.05$) and type IIa (~23%; $p < 0.05$) MyHC isoforms. These differences were were fibre-type dependent, i.e., the higher specific force in fast-twitch muscle fibres was primarily caused by higher force per cross-bridge while slow-twitch fibres relied on both a higher number and force per cross-bridge. HRT use had no effect on fibre cross-sectional area (CSA), velocity of unloaded shortening (V_0) and relative proportion of MyHC isoforms. In conclusion, HRT appears to have significant positive effects on both regulation of muscle contraction and myonuclei organization in post-menopausal women.

(Summary length: 237 words)

ABBREVIATIONS

CSA	Cross-sectional area
HRT	Hormone Replacement Therapy
MZ twins	Monozygous twins
MND	Myonuclear Domain
MyHC	Myosin heavy chain
PTM	Post-translation modification
SEM	Standard error of mean
SD	Standard deviation

INTRODUCTION

Sarcopenia is the ageing-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 ageing-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; Yu *et al.*, 2007). These changes are attributed to a complex interface of many factors ultimately leading to a progressive loss of mobility in old age (Ryall *et al.*, 2008).

Current theories attribute an altered endocrine activity as an important contributor to the ageing-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 older 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, a case control study was recently performed in postmenopausal monozygotic (MZ) twin pairs where only one of the twins was a HRT user (Ronkainen *et al.*, 2009; Finni *et al.*, 2011). In that study, HRT users had better walking speed and jumping height (Ronkainen et al. 2010) as well as *in vivo* force production (Finni et al.2011) compared with their non-user genetically identical co-twins. 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; 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, a). Recently, positive anti-catabolic effect and an improved regulatory action on the cytoskeleton and extracellular matrix were observed in response to HRT treatment in MZ twin pairs., leading to better muscle quality (Ronkainen *et al.*, 2010; Ahtiainen *et al.*, 2012). 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 in general and/or in specific conditions like postmenopausal rehabilitation. Further, myonuclear organization is affected by ageing 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 fibres (Cristea, 2010; Qaisar *et al*., 2012)

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 MZ twin pairs described above by studying the force generation capacity, contractile speed and the 3D myonuclear organization in single muscle fibre segments.

MATERIALS & METHODS

Ethical Approval

The ethics committee of the Central Finland Health Care District approved the study, and it was conducted according to the guidelines laid down by the World Medical Association in the declaration of Helsinki (2000). Written informed consent was provided by the participants before taking the biopsy and the measurements.

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). Subjects with chronic musculoskeletal diseases, type 1 diabetes, type 2 diabetes with medication, diagnosed mental disorder, asthma with oral cortisol treatment, acute cancer, known drug or alcohol abuse/dependence, or Crohn's disease were excluded. All measurements were done on the same day in a given twin pair. 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 for the current study from participants in the Finnish Twin Cohort Study. The mean age of the 12 subjects was 56.6 ± 1.3years (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 fibres and weighed 50-120 mg. Specimens were placed in relaxing solution at 4 ^{0}C, and bundles of ~50 fibres were carefully dissected free and then tied with surgical silk to glass capillary tubes at slightly stretched lengths. The muscle fibre bundles were chemically skinned for 24 h in relaxing solution 50% (v/v) glycerol at 4 ^{0}C, cryoprotected (Frontera & Larsson, 1997) and subsequently stored at -180 ^{0}C before use. The relaxing solution contained (in

mM): 4 MgATP, 1 free Mg^{2+}, 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 Ca^{2+} concentration, expressed as pCa (-log[Ca^{2+}]), was 10^{-9} M. Apparent stability constant for Ca^{2+}-EGTA was corrected for temperature and ionic strength (Fabiato & Fabiato, 1979).

Muscle biopsy samples from 13 healthy male control subjects (25-89 yrs.) were included for comparison of myosin protein post-translational modifications (PTMs).

Single fibre contractile recordings

The experimental procedure has been described in detail elsewhere (Larsson & Moss, 1993). Briefly, membrane permeabilized muscle fibres 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-fibre segment was set to 2.77 ± 0.05 μm (range 2.71–2.85 μm) by adjusting the overall segment length. Fibre 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). Fibres were activated at pCa 4.5 and, once steady state tension was reached, various amplitudes of slack (ΔL) were rapidly introduced (within 1-2 ms) at one end of the fibre. The time (Δt) 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 ΔL the fibre was re-extended while relaxed to minimize nonuniformity of the sarcomere length. A straight line was fitted to a plot of ΔL vs. Δt using a least-squares regression, and the slope of the line was recorded as V_o for that fibre. 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 fibre 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 (ΔL: ± 0.2% of fibre length), were applied at 500 Hz at one end of the fibre (Martyn *et al.*, 2007). The resultant force response (ΔF) was measured, and the mean of 20 consecutive readings of ΔL and ΔF 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):

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

Fluorescent labeling, image acquisition and analyses of myonuclear organization

Skinned single fibre 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 fibre. 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 fibre gel electrophoresis

The procedure is described in detail elsewhere (Larsson & Moss, 1993). In short, the MyHC composition of single fibres 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.

Post-translational modifications

Vastus lateralis muscle biopsy cryo-sections from from HRT using and their non-using co-twis as well as from controls were run on 6% SDS-PAGE gel. Gel bands corresponding to type I, IIa and IIx MyHC isoforms were extracted. Samples were digested in-gel, separated with 40-min gradient RP-nanoHPLC and analyzed online using a 7-tesla LTQ-FT Ultra tandem mass spectrometer (ThermoFisher Scientific) modified with a nano electrospray ion source (ProxeonBiosystems). High-resolution survey scan followed by low-resolved MS/MS scans of the five most abundant peaks was used. Peptide identification was performed using the Mascot search engine allowing two missed cleavages and a set of variable PTMs (i.e. multiple oxidations, methylations, and phosphorylations (Artemenko *et al.*, 2011).

Statistical analysis

Dfferences between the means in body composition parameters between HRT using and non-using co-twins were tested using Wilcoxon's signed rank test. Intra-pair differences are expressed as percentages (IPD %) and calculated as follows: (HRT user – non-user) : (non-user) x100. In addition, 95 % confidence interval (95 % CI) was calculated for each IPD%.. The paired-sample *t*-test was used to compare fibre CSA, MND and contractile recordings between HRT using and their non-using co-twins. The data normality was assessed by the Kolmogorov-Smirnov test. Values are expressed as mean ± standard error of mean (SEM) with the exception of body composition values which are expressed as means ± standard deviations (SD). Statistical significance was set at $p < 0.05$ for all analysis.

RESULTS

Life style characteristics and body composition

Life style characteristics and body composition of the original study population with 15 54-62-yrs-old postmenopausal MZ twin pairs discordant for long-term HRT have been described in detail by Ronkainen et al. (Ronkainen *et al.*, 2009). In summary, there were no differences in the leisure or work physical activity, occupation, smoking behavior, alcohol use or daily energy intake between the HRT uusing and non-using co-twins. Furthermore, body composition in terms of body weight, BMI, waist or hip circumference as well as body fat percentage did not differ between HRT using and their non-using co-twins either in the original (n=15 pairs) nor in the current study population with six twin pairs (Table 1) .

Single fibre cross-sectional area and MyHC isoform expression

The CSA of individual muscle fibres was measured in a total of 326 fibre segments from HRT non-users (n= 162) and users (n = 164) at a fixed sarcomere length assuming an elliptical circumference (Table 2). Statistical analysis was restricted to fibres expressing the type I (n = 176) and type IIa MyHC isoforms (n = 89) because of the scarcity and unequal distribution across subjects of fibres 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 fibres expressing type I or type IIa MyHC isoform between twin pairs, resulting in a similar type IIa/I fibre area ratio between twin sisters. In young adults, type II fibres are typically larger than type I fibres independent of gender demonstrating that the preferential type II fibre atrophy reported in old age in skinned muscle fibres (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 of MyHC isoforms expressed in dissected single muscle fibres used in contractile measurements and in muscle biopsy cross-sections are presented in Table 3. In short, no statistically significant difference was found in the relative proportion of different MyHC isoforms between twin sisters, neither in the analyzed muscle fibres 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 fibre segments irrespective of MyHC isoform and HRT status (Fig. 1). The longitudinal axis of elliptical nuclei was parallel with the longitudinal axis of muscle fibre 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 *et al.*, 2010), fibres 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 organization of myonuclei was typically observed in fibres expressing the type I MyHC isoform while it was relatively scarce in fibres 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 fibres expressing the type I MyHC isoform in the HRT non-users (3 out of 31 fibre segments) and users (3 out of 29 fibre segments). Further, internal nuclei were infrequent in type I/IIa (1 of 7, in HRT user only) and in type IIa fibres (2 of 26, in non-users only), but they were not observed in fibres 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 fibre segment.

Nuclei number per unit length and MND size

The MNDs at the terminal part of the fibre segment may extend outside the fibre 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 fibres expressing type I (n = 61) and type IIa (n = 36) MyHC isoforms were considered for statistical analysis, because of the small number of fibres expressing other MyHC isoforms or a combination of MyHC isoforms. No significant difference was found in myonuclear number per unit length in muscle fibres expressing type I or type IIa MyHC isoforms between the HRT user and non-user groups (Fig. 2B).

In muscle fibres 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 fibres and extra myonuclei in the HRT users. In fibres expressing the type IIa MyHC isoform, MND size did not differ significantly between HRT users and non-users. .

Contractile Properties

A total of 216 fibres 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 2). Fibres 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 fibre types. Maximum force normalized to muscle fibre cross-sectional area, i.e., specific force was higher in the HRT using than in their non-using co-twins both in muscle fibres expressing the type I MyHC isoforms ~ 27% ($p<0.05$) and in fibres expressing the type IIa MyHC isoform ~23% ($p<0.05$) (Fig. 2C, Table 4). The higher specific force may accordingly reflect HRT associated differences in the regulation of muscle contraction, i.e., a larger number of strongly attached cross-bridges in series or force produced by each cross-bridge (Regnier *et al.*, 2004). Stiffness recordings (E_o) represent a good index of the number of strongly attached cross-bridges in series. In muscle fibres expressing the type I MyHC isoform, the slightly higher stiffness (~13%) in the HRT group was not statistically significant (Fig. 2C). Accordingly, it is unlikely that the higher 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 fibres from HRT users. In fibres expressing type IIa MyHC isoform, on the other hand, the ~17% higher stiffness ($p < 0.05$) suggests that larger number of cross-bridges contribute significantly to ~23% higher specific force among HRT users (Fig. 2C). Maximum velocity of unloaded shortening did not differ between users and non-users independent on MyHC isoform expression.

Post-translational myosin modifications

In an attempt to improve our understanding of the mechanisms underlying the HRT-induced effects on myosin function, a mass spectrometry approach was taken to determine myosin post-translational modifications (PTMs) in response to HRT treatment. Analyses were restricted to the type I and IIa MyHC isoform due to the paucity of the type IIx MyHC isoform in some of the HRT twin pairs. Type I and IIa MyHC isoforms were separated on 6% SDS-PAGE gels extracted and screened for acetylation, carbonylation, deamidation, glucosylation, methylation, nitration, ubiquitination, and phosphorylation by liquid chromatography–mass spectrometry (LC/MS).

Eight myosin modifications were observed in both HRT users and non-users. Although none of the myosin PTMs were specific to HRT treatment three out of four carbonylations dominated in the HRT group (Table 4). Deamidations, on the other hand, dominated among non-users and one of

these deaminations (position 1079) was only observed among the old controls and not in middle-aged or young (Table 5). One modification was situated in the myosin motor domain (carbonylation aa 413), and was more prominent in the non-users, the seven others were located in the tail region of the myosin. In addition, six modifications were specific for the type IIa MyHC isoform (carbonylation aa 853, deamidation aa 940, 1079, 1293 and methylation aa 1449, 1493) and the two other modifications were found in both type I and IIa myosin isoforms (carbonylation aa 413 and 1623/1627).

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 fibre CSA, (*ii*) stiffness values reflected the change in specific force in fibres expressing type IIa MyHC isoform, but not in type I fibres. (*iii*) smaller MND size was observed in fibres expressing the type I MyHC isoform in response to hormone use while MND size was unaffected in type IIa fibres. (*iv*) no significant change was observed in the velocity of unloaded shortening (V_0) and myonuclei number with HRT use.

Fibre 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 fibres expressing the fast myosin isoform, since human muscle fibres expressing fast myosin isoforms generate higher specific forces than fibres 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, fibre CSA or proportion of MyHC isoforms did not differ between users and non-user twins from HRT using and their non-using co-twins (Table 2 and 3). These observations are in accordance with a previous publication comparing hormone replacement with non-replacement in postmenopausal women (Widrick *et al.*, 2003).

HRT affects myosin function to preserve single fibre 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 fibres 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 fibres 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 fibre type specific effect of the HRT treatment in the post-menopausal women where the positive effects are mainly due to a quantitative effect in fibres expressing the fast myosin isoform while the effect is both quantitative and qualitative in fibres expressing the slow myosin isoform. The higher metabolic rate, mitochondrial density and formation of reactive oxidative species in slow versus fast muscle fibres suggest that posttranslational protein modifications are a significant source underlying the qualitative changes in the force generation capacity of the type I fibres in post-menopausal women (McArdle *et al.*, 2002).

The concentration of estrogen receptors is higher in slow- than fast-twitch fibres (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 ageing-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 fibres by a specific protection against post-translational modifications. In this context it is interesting to note that the only aging-specific modification observed among the twin-pairs (deamidation in the 1079 position in the tail region of the motor protein) dominated among non-users, but this PTM was specific for the IIa MyHC isoform. The modifications specific for type I and IIa MyHC isoforms, carbonylations in the 1623/1627 and 413 positions, dominated in the users (1623/1627) or non-users (413), respectively. Overall, the PTMs were mainly situated on the more accessible tail region of the myosin. Despite not interacting directly with actin, the myosin rod is essential for the molecular motor function of myosin and mutations in this region have been shown to disrupt the structure of the protein, resulting in myopathies (Meredith *et al.*, 2004; Tajsharghi *et al.*, 2005; Armel & Leinwand, 2010). The PTMs we observed in young individuals tended to be preserved in HRT users while non-users gained new PTMs found only in aged individuals.. However, multiple PTMs may go undetected and lost during enzymatic digestion when analyzed by mass spec. Post-translational modifications may also involve protein O-GlcNAcylation via which several cellular processes are regulated, which is comparable and partially competitive to protein phosporylation and acting as cellular sensor for nutritional status and glucose metabolism. Concerning the effects of HRT on protein O-GlcNAc modification Pöllänen et al. (2007) observed that the gene expression of OGT-enzyme catalyzing O-GlcNAcylation was down-regulated in skeletal muscle

of early postmenopausal women during one year HRT intervention in comparison to the placebo using controls. Consequently, other protein modifications undetected by mass spec may have affected regulation of muscle contraction at the contractile protein level.

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 among the HRT using twin pairs (Ronkainen *et al.*, 2009) was primarily due to the higher specific force.

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

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 fibre types in the non-user group (Fig. 2). This is probably related to the ageing-related preferential type IIa fibre atrophy (Tomonaga, 1977; Larsson *et al.*, 1978; Larsson, 1982) since we previously found no change in myonuclei count per unit fibre length with ageing (Cristea *et al.*, 2010). In the HRT user group, MND size was significantly smaller in muscle fibres expressing the type I MyHC isoform (Fig. 2A), but it did not differ between users and non-users in fibres expressing the type IIa MyHC isoform (Fig. 2B). A possible explanation for this discrepancy is the observation that the ageing-related oxidative stress has a more profound effects on slow-twitch fibres (McArdle *et al.*, 2002) in part due to a decreased production of HSP70 (Broome *et al.*, 2006). Slow-twitch fibres are also transcriptionally more active than fast-twitch fibres (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 fibres, estrogen not only arrests the ageing-related oxidative damage but also reduces myonuclear domain size to restore force generating capacity in ageing fibres. This also optimizes transport distances for cellular proteins and synthetic capacity of the ageing myonucleus. Further, recent experimental results from our group in "double-muscle mice" have shown that MND size does play an important role for maintenance of specific force (Qaisar *et al.*, 2012). The smaller MND size in HRT users may accordingly compensate for ageing-related changes in myonuclear organization (Cristea, 2010).

In the fibres expressing the type IIa MyHC isoform, on the other hand, HRT seems to have no effect on MND size. We have recently reported an ageing-related decline in CSA and MND size in type IIa fibres (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.

Gene set enrichment analyses of the muscle transcriptome of these postmenopausal monozygotic twins have revealed subtle, but significant differences in expressions in nine gene sets including "regulation of anatomical structure and morphogenesis" (Ronkainen et al. 2010). In order to investigate the contribution of HRT on muscle transcriptome changes, we are currently running analysis on muscle miRNA arrays to find out, whether posttranscriptional regulation via miRNAs is one mechanism of estrogen containing hormone replacement therapy to regulate muscle gene expression, possibly also the enzyme-catalyzed posttranslational modifications of proteins.

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 fibres 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 fibre-type dependent and the force per actin-myosin interaction plays a stronger role in fast- than slow-twitch fibres, with slow-twitch fibres 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.

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AUTHORS CONTRIBUTION

All experiments were performed in the University of Uppsala, Sweden while biopsies were taken at the University of Jyväskylä in Finland. R.Q, S.S, V.K and L.L designed the experiments. R.Q, Y.H and G.R. performed the analyses. K.A. and J.B. conducted the high resolution nLC-FTICR MS, interpreted MS and revised the paper. R.Q analyzed data and R.Q and L.L wrote the paper. E.P, P.R, J.K, M.A, S.S & V.K revised the paper. All authors approved the final version of the manuscript.

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Table 1.

Anthropometry and body compositionin HRT using twins and their non-using co-twins (n=6 pairs).

Variable	HRT Users	HRT non-users	IPD % (95 % CI)	P Value
Body height, cm	162.7±2.1	162.2±2.2	0.31 (-0.11 to 0.74)	0.250
Body weight, kg	70.3±4.5	78.4±6.8	-9.0 (-19.6 to 1.6)	0.156
BMI, kg/m^2	26.7±2.0	30.1±3.2	-9.5 (-20.6 to 1.6)	0.156
Waist circumference, cm	89.4±4.1	98.0±5.5	-8.3 (-16.3 to 0.3)	0.094
Hip circumference, cm	102.3±3.0	106.6±4.2	-3.8 (-8.7 to 1.1)	0.156
Body fat, %	33.1±3.5	37.7±3.8	-12.8 (-26.2 to 0.59)	0.094

Mean±SEM, mean intrapair difference % (IPD %) with 95 % confidence interval (95 % CI) are given. Wilcoxon Signed Ranks Test was used testing the significances for the mean intrapair differences.

Table 2.

Cross-sectional area (CSA), specific tension (ST), stiffness and maximum velocity of unloaded shortening (V_o) in skinned single muscle fibres expressing different MyHC isoforms in HRT using amd non-using co-twins.

	Type I		Type I/IIa		Type IIa		Type IIax		Type IIx	
	HRT Nonusers (N=6)	HRT Users (N=6)	HRT Nonusers (N=4)	HRT Users (N=6)	HRT Nonusers (N=6)	HRT Users (N=6)	HRT Nonusers (N=4)	HRT Users (N=6)	HRT Nonusers (N=2)	HRT Users (N=1)
CSA (um^2)	2550 ± 110 (n=97)	2460 ± 140 (n=79)	2630 ± 450 (n=13)	1860 ± 190 (n=16)	2240 ± 170 (n=39)	2260 ± 130 (n=50)	1850 ± 120 (n=10)	1560 ± 280 (n=17)	2050 ± 100 (n=3)	1320 (n=2)
ST (N/cm^2)	28.5 ± 2 (n=71)	36.4 ± 2.7* (n=48)	30.1 ± 5.3 (n=9)	39.1 ± 4 (n=11)	31.2 ± 2.2 (n=25)	38.2 ± 1.3* (n=39)	29.3 ±3 (n=9)	31.8 ± 4.8 (n=15)	23 ± 2.3 (n=3)	30.8 (n=2)
Stiffness (N/cm^2)	2550 ± 40 (n=71)	2870 ± 170 (n=48)	2450 ± 900 (n=7)	2880 ± 460 (n=8)	2140 ± 130 (n=20)	2500 ± 70* (n=32)	2200 ± 270 (n=8)	2050 ± 230 (n=6)		
V_o (ML/s)	1 ± 0.1 (n=59)	0.90 ± 0.1 (n=46)	1.6 ± 0.6 (n=8)	1.8 ± 0.3 (n=10)	2.1 ± 0.3 (n=20)	2.3 ± 0.2 (n=32)	2.5 ± 0.2 (n=7)	2.7 ± 0.1 (n=12)	2.3 ± 0.1 (n=3)	2.5 (n=2)

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 fibres expressing type I or type IIa MyHC isoform, because of small sample size in fibres expressing other isoforms or combinations of isoforms. (N = number of subjects; n = number of fibres).

Table 3.

MyHC isoform expression measured in single muscle fibres and biopsy cross-sections from HRT using and non-using co-twins.

	Total number of fibres	Type I (%)	Type I/IIa (%)	Type IIa (%)	Type IIax (%)	Type IIx (%)
HRT non-users	162	60 (47 ± 6)	8	24 (51 ± 6)	6	2 (12 ± 1)
HRT users	164	49 (43 ± 5)	10	30 (52 ± 4)	10	1 (15 ± 3)

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

Table 4.

Specific force and MND size in the six twin pairs.

Twins			Type I fibers		Type IIa Fibers	
Twin Pairs	**HRT Status**	**Total number of Fibers**	**ST (N/cm^2)**	**MND ($x10^3$ μm^3)**	**ST (N/cm^2)**	**MND ($x10^3$ μm^3)**
Pair 1	HRT Non-User	42	21.9 ± 2.3 (n = 23)	35.7 ± 4.7 (n = 8)	22.3 ± 2.1 (n = 9)	26.3 ± (n = 2)
	HRT User	34	28.4 ± 4.3 (n = 18)	34.3 ± 5.1 (n = 4)	33.4 ± 6.7 (n = 7)	12.7 ± (n = 3)
Pair 2	HRT Non-User	26	32.5 ± 5.7 (n = 9)	29.5 ± 4.3 (n = 7)	27.2 ± 3.7 (n = 7)	53.1 ± (n = 3)
	HRT User	25	39.9 ± 4.9 (n =9)	16.9 ± 3.1 (n = 5)	40.7 ± 8.3 (n = 5)	27.7 ± (n = 2)
Pair 3	HRT Non-User	21	34.9 ± 6.4 (n = 8)	33.1 ± 3.5 (n = 4)	34.7 ± 3.7 (n = 6)	20.9 ± (n = 2)
	HRT User	24	38.7 ± 4.1 (n = 9)	19.8 ± (n = 3)	40.5 ± 5.3 (n = 7)	60.6 ± 8.2 (n = 3)
Pair 4	HRT Non-User	22	23.4 ± 4.1 (n = 8)	31.7 ± 7.1 (n = 4)	32.1 ± 5.5 (n = 5)	27.4 ± 4.4 (n = 5)
	HRT User	27	29.1 ± 6.6 (n = 7)	18.7 ± 4.4 (n = 7)	41.6 ± 9.1 (n = 6)	22.7 ± 4 (n = 3)
Pair 5	HRT Non-User	19	29.9 ± 4.1 (n = 6)	29.4 ± 5.1 (n = 4)	36 ± 3.9 (n = 5)	26.6 ± 3.9 (n =4)
	HRT User	26	46.3 ± 6.3 (n = 7)	24.3 ± 5.4 (n = 4)	35.3 ± 3.3 (n = 8)	19.9 ± 3.1 (n = 4)
Pair 6	HRT Non-User	32	28.3 ± 5.3 (n = 17)	33.7 ± 7.3 (n = 4)	34.5 ± 4.1 (n =9)	28.9 ± (n=2)
	HRT User	28	35.9 ± 5 (n = 8)	25.4 ± 2.1 (n = 7)	37.8 ± 4.2 (n = 9)	27.7 ± (n = 3)

Values are expressed as mean ± SEM and the total number of fibres analyzed in each individual is given within parentheses.

Table 5.

Myosin protein post-translational modifications in HRT using (U) and non-using (NU) monozygotic twin pairs. The modified MyHC isoform, amino acid , amino acid position, and the domain of the modified myosin. I additionalso found in all healthy controls or only in old healthy controls.

Modification type	**Peptide sequence**	**Amino acid modified**	**HRT user or non-user**	**Myosin isoforms**	**Amino acid Position**	**Domain**
Carbonylation	***K****MEG****D****LNEMEIQLNHANR*	*D/K*	*5U 1NU*	I/IIa	*1623/1627*	Tail-domain
Carbonylation	*LQTESGE****F****SR*	*F*	*5U 2NU*	IIa	*1293*	Tail-domain
Carbonylation	*SAETE****K****EMATMKEEFQK*	*K*	*4U 2NU*	IIa	*853*	Tail-domain
Carbonylation	*VKVGNE****F****VTK*	*F*	*3U 6NU*	I/IIa	*413*	S1 myosin head (CM-loop)
Deamidation	AEDEEEI**N**AELTAK	N	1 U 5 NU	IIa	940	Tail-domain
Deamidation	LAQESIMDIE**N**EK	N	2U 4 NU	IIa	*1079*	Tail-domain*
Methylation	*QAEEAE****E****QANTNLSK*	*E*	*4 U 1 NU*	IIa	*1893*	Tail-domain
Methylation	TNAACAAL**D**KK	D	2 U 4 NU	IIa	1449	Tail-domain

*This deamidation was observed in observed in old but not in young controls.

FIGURE LEGENDS

Figure 1.

Confocal microscopy images of single muscle fibres and their representative myonuclei from HRT users and HRT non-users. Type I fibres typically characterized by deep groove like structures harboring long chain of nuclei. Type IIa fibres with more ordered organization of both spherical and elliptical myonuclei. DAPI (blue) stained myonuclei while rhodamine (red) labeled actin. Horizontal bars denote 50*u*m (fibre) and 8*u*m (nuclei).

Figure 2.

Myonuclear domain size (A), nuclei number per unit length (B), and specific tension and stiffness recordings (C) in type I and type IIa fibres in vastus lateralis muscles from HRT using and HRT non-using co-twins. In A and B, asterisk denotes statistically significant difference from non-users ($p<0.05$). In C, asterisk (*) and cyrillic (ж) denote statistically significant difference for the specific force and stiffness respectively ($p<0.05$). All values are mean ± SEM.