Physical activity impacts resting skeletal muscle myosin conformation and lowers its ATP consumption

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Physical activity impacts resting skeletal muscle myosin conformation and lowers its ATP consumption

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It has recently been established that myosin, the molecular motor protein, is able to exist in two conformations in relaxed skeletal muscle. These conformations are known as the super-relaxed (SRX) and disordered-relaxed (DRX) states and are finely balanced to optimize ATP consumption and skeletal muscle metabolism. Indeed, SRX myosins are thought to have a 5- to 10-fold reduction in ATP turnover compared with DRX myosins. Here, we investigated whether chronic physical activity in humans would be associated with changes in the proportions of SRX and DRX skeletal myosins. For that, we isolated muscle fibers from young men of various physical activity levels (sedentary, moderately physically active, endurance-trained, and strength-trained athletes) and ran a loaded Mant-ATP chase protocol. We observed that in moderately physically active individuals, the amount of myosin molecules in the SRX state in type II muscle fibers was significantly greater than in age-matched sedentary individuals. In parallel, we did not find any difference in the proportions of SRX and DRX myosins in myofibers between highly endurance- and strength-trained athletes. We did however observe changes in their ATP turnover time. Altogether, these results indicate that physical activity level and training type can influence the resting skeletal muscle myosin dynamics. Our findings also emphasize that environmental stimuli such as exercise have the potential to rewire the molecular metabolism of human skeletal muscle through myosin.

Introduction

The role of myosin class II as a vital motor molecule in the contraction of striated muscle has been the subject of intense research over recent decades (Trivedi et al., 2018). What remains unclear is how myosin can regulate muscle metabolism at rest. In 2010, it was reported that myosin can exist in two different resting conformations (Stewart et al., 2010). These different conformations, or states, are termed the disordered-relaxed (DRX) and super-relaxed (SRX) states (Cooke, 2011). These states differ by the relative spatial positioning of myosin heads between thick and thin filaments (Toepfer et al., 2020). In the DRX state, one of the two myosin heads is free to sway within the interfilament space of the sarcomere and thus both the actin-binding and ATP-binding motifs on this myosin head are sterically open (Alamo et al., 2016; Alamo et al., 2017; Lee et al., 2018). In the SRX state, both myosin heads are folded back onto the thick-filament backbone and, therefore, the ATP-binding motif on both myosin heads in this molecule is sterically inhibited (Naber et al., 2011; Alamo et al., 2016; McNamara...
et al., 2015). This inhibition of the ATP-binding motif on the myosin head causes a substantial functional difference between the DRX and SRX conformations, with the SRX conformation demonstrating a 5- to 10-fold reduction in ATP turnover compared with the DRX conformation (Cooke, 2011; Toepfer et al., 2020; Schmid and Toepfer, 2021). This key difference between the two resting myosin conformations has led to the theory that the SRX may have evolved as an energy-saving mechanism to reduce ATP consumption in cardiac and skeletal muscles (Lee et al., 2018; Hooijman et al., 2011).

As the discovery of the SRX state is still relatively novel, little is known about the role of resting myosin conformations in the context of disease or the adaptation to environmental stimuli. Much of our knowledge of the regulation and functional significance of resting myosin head conformations comes from the discovery that dysregulation of myosin head conformation in cardiac muscle is pathogenic in hypertrophic cardiomyopathy (HCM; Alamo et al., 2017). HCM is a genetic cardiomyopathy caused by mutations in sarcomeric proteins, including myosin (Debold et al., 2007; van Dijk et al., 2009). As a disease, it results in myocardial hypercontractility, hypermetabolic rate, and reduced relaxation, which leads to progressive heart failure and may cause sudden cardiac arrest (Maron and Maron, 2013). It has been observed that in the various mutations that cause HCM, there are significantly higher numbers of DRX myosins in the cardiac muscle of these patients (Schmid and Toepfer, 2021; Toepfer et al., 2020; Toepfer et al., 2019).

To date, the involvement of myosin heads in disease and their dysregulation has all been attributed to genetic mutations in either myosin or neighboring partners. Therefore, an important question that remains to be addressed is how dynamic these different conformations are in their nature and if they can be influenced by lifestyle changes such as diet or physical activity/training. Given that exercise has vast influence on the function of skeletal muscle tissue and its molecular determinants, particularly in the context of the metabolic rate, we aimed to explore whether the training status in humans would be linked to altered proportions of SRX and DRX skeletal myosins in muscle fibers expressing either type I/slow or type II/fast myosin heavy chains (MyHCs). We hypothesized that increases in the habitual level of skeletal muscle activity would shift relaxed myosin molecules toward their SRX conformation to conserve energy and thereby optimize energy recharge rates in both the general population and in highly trained athletes. To test this hypothesis, we analyzed muscle fibers extracted from muscle biopsy specimens from four distinct groups: sedentary controls, moderately physically active (PA) controls, endurance-trained athletes (EA), and strength-trained athletes (SA). We observed that moderately PA individuals have a higher amount of myosin molecules in the SRX state in type II muscle fibers compared with sedentary individuals. Furthermore, we did not find any difference in the proportions of SRX and DRX myosins in myofibers between highly trained endurance and strength athletes. However, we did observe differences in the ATP turnover time of the myosin molecules between these groups. Together, these data indicate that physical activity level and training type are able to influence the conformation and activity of myosin in skeletal muscle.

**Materials and methods**

**Preparation of single muscle fibers from skeletal muscle**

Human vastus lateralis biopsy specimens were obtained upon informed consent from four different population groups. All biopsies were obtained from male subjects. Two sets of groups were used in this study. The first group comprised sedentary control individuals, who were untrained young males that completed less than three sessions of cardiopulmonary exercise per week, and moderately PA individuals, who were young male subjects engaged in leisure exercise, typically completing three or more cardiopulmonary exercise sessions (45–60 min/session) per week (Sahl et al., 2018). Using these non-elite groups allowed for the observation of potential differences in the myosin head conformation between the two groups with only modest differences in their level of physical activity versus very active athletes (120–180 min/d). Two contrasting athlete groups were also recruited, comprising EA and SA. EA subjects were recruited from local groups of highly trained endurance cyclists (V̇O2max 62.3 ± 3.6 ml/kg/min training load 9.1 ± 3.7 h/wk; Table 1; Frandsen et al., 2022). SA subjects were recruited from local weight training clubs. SA subjects were required to have resistance-trained for a minimum of 3 yr in the years preceding the study and have the ability to squat at least 1.5 times their own body weight (Hokken et al., 2021). These two athlete groups represented individuals that, whilst highly trained, both have vastly different training regimes and goals (maximizing cardiovascular fitness and muscle strength/mass, respectively). This strategy was chosen to identify if specific training regimes would be more efficient than others to alter myosin head conformation and affect the metabolism of resting skeletal muscle. The Greater Copenhagen Region Science Ethical Committee approved the use of sedentary, PA, and EA biopsies (project IDs: H-16049145, H-15002266). The ethics committee in the Region of Southern Denmark approved the use of strength athlete biopsies (project ID: S-20160116).

**Physical performance testing**

For EA, maximal oxygen uptake rate (V̇O2max) was assessed with breath-by-breath measurements of pulmonary V̇O2 and V̇CO2 by an online system (COSMED Quark CPET, COSMED) during a graded exercise test performed on a cycle ergometer. The

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**Table 1: Subject characteristics for non-athletes**

<table>
<thead>
<tr>
<th></th>
<th>Sedentary (n = 5)</th>
<th>Physically active (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>27.6 ± 3.5</td>
<td>24.3 ± 3.7</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>177.1 ± 5.5</td>
<td>189.3 ± 7.0*</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.1 ± 5.1</td>
<td>90.8 ± 9.9**</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.5 ± 1.4</td>
<td>25.3 ± 2.9</td>
</tr>
<tr>
<td>V̇O2max (ml/kg/min)</td>
<td>N/A</td>
<td>44.8 ± 4.0</td>
</tr>
</tbody>
</table>

Mean subject characteristics for the participants in the studies from which biopsies samples were obtained. Shown parameters are age (years), height (cm), weight (kg), and BMI (kg/m²) for all subjects. V̇O2max (ml/kg/min) is shown for physically active subjects. Data are displayed as mean ± SD. * = P < 0.05 vs. sedentary. ** = P < 0.01 vs. sedentary.
protocol was adapted from the Achten35/3 protocol and described in detail elsewhere (Achten et al., 2002; Frandsen et al., 2022). In brief, the VO_{2max} protocol was performed in continuation of a submaximal graded exercise test for determination of substrate oxidation and VO_{2max} was elicited through workload increments of 35 W every minute until volitional exhaustion. The self-reported squat, deadlift, and benchpress one-rep max values from the recruited strength athletes are reported in Table S1.

Mant-ATP chase experiments

All biopsies were immediately snap-frozen in liquid nitrogen and stored at −80°C. At the time of analysis, single-muscle fibers were chemically skinned as previously described (Ochala et al., 2021). They were stored at −20°C in a buffer solution containing glycerol and a relaxing solution mixed 50/50. This relaxing solution contained 0.1 M KCl, 10 mM imidazole, 1 mM MgCl₂, 2 mM EGTA, 0.25% (w/v) ATP, and KOH to pH 7.0. As described elsewhere, single myofibers were individually attached to a TEM-grid (G4103-IVL; Sigma-Aldrich, grids for transmission electron microscopy), which was positioned on a microscopy slide (Thermo Fisher Scientific, Superfrost Plus; Ochala et al., 2021).

Single nucleotide turnover was measured in flow chambers using fluorescent Mant-ATP (250 μM) as described previously (Stewart et al., 2010; Ochala et al., 2021). Single muscle fibers with a relaxed sarcomere length of 2.2 μm were incubated in a rigor buffer containing 100 μl Mant-ATP buffer for 5 min. Rigor buffer contained 120 mM potassium acetate, 5 mM Mg acetate, 2.5 mM K₂HPO₄ (dibasic), 2.5 mM KH₂PO₄ (monobasic), 50 mM MOPS, 5 mM EGTA, 100 mM TCEP, and KOH to pH 6.8. Mant-ATP or ATP was added to the rigor buffer to make the Mant-ATP buffer for 5 min. Rigor buffer contained 120 mM potassium acetate, 5 mM Mg acetate, 2.5 mM K₂HPO₄ (dibasic), 2.5 mM KH₂PO₄ (monobasic), 50 mM MOPS, 5 mM EGTA, 100 mM TCEP, and KOH to pH 6.8. Mant-ATP or ATP was added to the rigor buffer to make the Mant-ATP buffer for ATP chase buffer, respectively. Following incubation, fibers were positioned in the microscope and chased with 100 μl of rigor buffer containing ATP (4 mM). Fluorescence decays due to the mant-nucleotide dissociation from myosin was monitored over 300 s. The excitation wavelength for Mant-ATP was 385 nm. All experiments were performed at room temperature, 25°C. Fluorescence images were acquired on a Zeiss AXIO Lab A1 microscope (Carl Zeiss AG, GE). The average fluorescence intensities within a selected region of the fiber were determined using the ImageJ software. Background intensity measurements were also taken. Fiber fluorescence values were determined by subtracting the background intensity from the fiber intensity.

Mant-ATP chase experiment data analysis

Data were plotted using GraphPad Prism 8 after fitting with a two-phase exponential decay function using the following equation:

\[ \text{Normalised Fluorescence} = 1 - P_1 \left[ 1 - \exp \left( \frac{-T_1}{T_1} \right) \right] - P_2 \left[ 1 - \exp \left( \frac{-T_2}{T_2} \right) \right], \]

where P1 is the amplitude of the rapid decay approximating the DRX. T1 (seconds) is the time constant for the life of this P1 phase. P2 is the second phase approximating the SRX with the corresponding T2 is the time constant for the life of this P2 phase. As T1 and T2 denote the time constants for their corresponding phase, they are the calculated ATP turnover lifetimes for the corresponding myosin head conformation that they represent.

Fiber typing

After the completion of the Mant-ATP chase experiments, individual fibers were stained with an anti-MyHC slow/type I antibody (A4.951; IgM isoform: 1:50, DSHB). Fibers were then washed in PBS and incubated with a secondary antibody conjugated to Alexa 647 in a goat serum (Thermo Fisher Scientific, dilution 1:1,000). After washing, the muscle fibers were mounted in Fluoromount, and images were taken with a Zeiss AXIO Lab A1 microscope (Carl Zeiss AG, GE, objectives ×20 and ×10). Positive staining with the MyHC β-slow/type I antibody indicated a type I muscle fiber (Fig. 1a) and negative staining with the MyHC β-slow/type I antibody indicated a type II muscle fiber (Fig. 1b).

Statistical analysis

All measurements for Mant-ATP assays were performed on six isolated muscle fibers per individual, and these fibers were then fiber-typed. Single fiber data were averaged per individual. Statistical significance was calculated using unpaired Student’s t test. P < 0.05 was assumed to be significant (two-tailed). Parametric tests were used due to the normal distribution of the data. Data are presented as mean ± SEM in figures and as mean ± SD in Tables 1 and 2 containing subject characteristics. Sample number n refers to the number of individuals in each subject group who were analyzed and plotted. In T2 measurements, values of >300 s were omitted due to this value being longer than the experimental duration. T2 fiber omissions occurred on <5% of analyzed fibers. Statistical testing was performed using GraphPad Prism Version 9.3.1 (471; Insight Partners).

Online supplementary material

Table S1 reports the self-reported mean one-repetition maximum for squat, deadlift, and bench press for the strength athlete which were recruited and analyzed in this study.

Results

We investigated whether differences in the proportions of myosin-relaxed states, namely SRX and DRX, exist in skeletal muscles from sedentary controls, moderately PA controls, EAs, and SAs (Hokken et al., 2021; Frandsen et al., 2022; Sahl et al., 2018). Age, height, weight, and body mass index (BMI) for these individuals as well as VO_{2max} values for PA individuals are presented in Table 1. Importantly for this study, none of the subjects were overweight despite a considerable heterogeneity in the level of physical activity. Hence, BMI for the sedentary and PA individuals remained within a normal range (22.5 ± 1.4 and 25.3 ± 2.9 kg.m², respectively).

Reduced proportion of DRX myosins in the type II muscle fibers of moderately PA individuals compared with sedentary individuals

To test our initial hypothesis of a higher proportion of myosin heads in the SRX state in the PA group compared with the
sedentary individuals, we isolated myofibers (expressing the type I or II MyHC) from both subject groups. We then ran a loaded Mant-ATP chase protocol. Interestingly, the amount of DRX myosins was only significantly lower in the type II fibers of the PA group when compared with the sedentary group (Fig. 2, and b). These changes were matched by equally significant higher levels of SRX myosins for PA when compared with the sedentary group (Fig. 2 c). These results support our initial hypothesis eluding the fact that the regulation of skeletal myosin resting conformations may be influenced by the training level.

Using the same data obtained from our loaded Mant-ATP experiments, we next determined the ATP turnover times of both DRX and SRX myosins of both groups (T1 and T2 values). We did not observe any significant difference in the ATP turnover time of DRX or SRX myosins in either type I or type II muscle fibers (Fig. 2, d and e). These findings show that despite changes in the conformation of myosin expressing the type II MyHC, there are no changes in the rate of ATP turnover in these myosin molecules.

Following these observations, we wished to assess whether cardiopulmonary fitness is a major contributor to such molecular remodeling. To avoid any confounding effects of physical activity levels, we focused our attention on trained athlete groups who had two very distinct training profiles (EA vs. SA), both of which had been published previously (Hokken et al., 2021; Frandsen et al., 2022). Age, height, weight, and BMI for the PA group when compared with the sedentary group (Fig. 2, and b). These changes were matched by equally significant higher levels of SRX myosins for PA when compared with the sedentary group (Fig. 2 c). These results support our initial hypothesis eluding the fact that the regulation of skeletal myosin resting conformations may be influenced by the training level.

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### Table 2. Subject characteristics for athletes

<table>
<thead>
<tr>
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<th>Endurance athlete (EA; n = 6)</th>
<th>Strength athletes (SA; n = 5)</th>
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<tbody>
<tr>
<td>Age (yrs)</td>
<td>30.5 ± 4.0</td>
<td>24.4 ± 2.3*</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>181.9 ± 4.5</td>
<td>180.42 ± 3.2</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77.4 ± 4.5</td>
<td>95.4 ± 8.9**</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4 ± 0.6</td>
<td>29.0 ± 2.9***</td>
</tr>
<tr>
<td>VO₂max (ml/kg/min)</td>
<td>62.3 ± 3.6</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Mean subject characteristics for the participants in the studies from which biopsy samples were obtained. Shown parameters are age (years), height (cm), weight (kg), and BMI (kg/m²) for all subjects. VO₂max (ml/kg/min) is shown for endurance athlete subjects. Data are displayed as mean ± SD. * = P < 0.05 vs. EA. ** = P < 0.01 vs. EA. *** = P < 0.0001 vs. EA.
these groups as well as \( \text{VO}_{2\max} \) values for EA individuals are presented in Table 2. The SA group had a significantly higher BMI compared with the EA group (29.0 ± 2.9 and 23.4 ± 0.6 kg.m\(^{-2} \), respectively; \( P < 0.001 \)) due to a higher lean mass (Westcott, 2012). The reported \( \text{VO}_{2\max} \) of the EA (63.0 ± 3.4 ml/min/kg) underlines the high level of cardiopulmonary training (Joyner and Coyle, 2008).

**Discussion**

The present study is the first to examine to which extent myosin head conformations in relaxed type I and type II human skeletal myofibers differ between individuals of different physical activity levels and between highly trained athlete populations with vastly different training backgrounds (muscular endurance vs. strength). Our data indicate that in the type II myofibers of PA individuals, there is a significant reduction in the proportion of resting myosin heads in the DRX conformation compared with matched sedentary individuals. Furthermore, we observed that EA have a reduced ATP turnover lifetime in their DRX myosin heads in type I myofibers compared with type I and type II myofibers of SAs.

Physical activity is well known to have vast whole-body metabolic benefits including, but not limited to, improvements in mitochondrial function, removal of reactive oxygen species, rewiring of insulin signaling in skeletal muscle, and prevention of obesity (Holten et al., 2004; Needham et al., 2022; Richter and Hargreaves, 2013; Memme et al., 2021). However, how different types and levels of chronic physical exercise may influence DRX and SRX conformations in human myofibers thus affecting the basal metabolic rate of skeletal muscle has not been examined previously. Chronic physical activity and different training regimes have previously been shown/suggested to induce skeletal muscle remodeling notably via switching of MyHC expressions and thus fiber types (Aagaard and Andersen, 1998). In particular, preferential expression of MyHC type I and thus a higher proportion of slow, oxidative fibers is associated with endurance training (Andersen and Aagaard, 2010), whereas an increase in MyHC type II expression, denoting fast muscle fibers, is associated with strength training and an increase in the maximal force and power, which is able to be produced by a muscle (Harridge et al., 1996). The underlying mechanisms by which chronic exercise leads to such remodeling remain incompletely known. Based on the present findings, we suggest that relaxed myosin conformations play a role. Indeed, here, individuals with modestly higher cardiopulmonary physical activity levels than
the sedentary subjects (PA group) display a lower percentage of myosin molecules in the DRX state in their type II myofibers. This is likely to substantially reduce the molecular ATP consumption and energy demand of the glycolytic type II fibers. Such phenomenon-inducing "energy-preserving" type II muscle fibers may be part of a complex cascade of events favoring/shifting toward the type I, slow-oxidative and mitochondria-rich myofibers when the cardiopulmonary system is chronically activated. If "energy-dormant" myofibers are beneficial or necessary when the cardiopulmonary system is exercise-activated, it is possible that small molecules/compounds which target myosin or its binding partners could be designated as performance enhancing. Although the recently FDA-approved compound Mavacamten specifically targets cardiac myosin, the success of this compound demonstrates that resting myosin conformation is an effective therapeutic target and could be further targeted for the modulation of skeletal muscle function (Heitner et al., 2019). A longitudinal study that could observe changes to resting myosin conformation in a group of individuals undertaking a cardiopulmonary or strength training regime would be of benefit to this field. This would allow for the testing of the hypothesis that type II myofibers become increasingly energy-preserving during a progressive increase in cardiopulmonary performance level. Furthermore, it would be important to establish if changes to the myosin conformation ratio following changes in training status are observed on a whole-body level or if these changes are specific to specific muscles or muscle group, which has been trained. A study that could look at differences between trained and untrained legs in the same individual would be beneficial in furthering our understanding of the regulation of myosin conformation.

To define the extent of cardiopulmonary fitness on myosin conformations and to avoid any confounding factors, here, we also studied highly trained athlete groups who had two very distinct training profiles. Surprisingly, we found an increase in the ATP turnover lifetime of the DRX conformation in the elite strength-trained (SA group) type I and type II myofibers demonstrating that each ATP molecule is turned over at a slower rate than in the type I myofibers of elite endurance-trained individuals (EA group). The exact consequence of changes to resting myosin ATP turnover lifetime is currently unclear; however, an increase in turnover lifetime in a muscle fiber would contribute to a reduction in its resting metabolic rate. An increase in DRX ATP turnover lifetime may be of benefit to SAs as an energy-saving mechanism in between bouts of performance. Particularly as rapid force development, a key factor of enhanced performance in SA, has been observed to have a very high metabolic cost (Maffiuletti et al., 2016; van der Zee and Kuo, 2021). Further investigation into the consequences of training-induced changes to resting myosin ATP turnover lifetimes is of importance to the field of myosin research.

As mentioned above, mechanisms underlying the chronic exercise-induced changes in myosin SRX vs. DRX conformations remain unknown; however, previous studies have demonstrated the importance of posttranslational modifications in the regulation of myosin function and its neighboring proteins (Alamo et al., 2016; Brito et al., 2011; McNamara et al., 2019). It has been established that exercise can induce vast protein phosphorylation, including that of sarcomeric proteins, and that different training methods can induce distinct training-specific phosphorylation profiles (Needham et al., 2022; Bódi et al., 2021). The fiber-type specific differences in SRX/DRX conformations...
observed in the present study may be due to posttranslational modifications occurring on fiber-type specific sarcomeric proteins, such as myosin or myosin-binding protein C1/2 (MYBPC1/ MYBPC2). The significance of myosin-binding protein C has yet to be investigated in the context of exercise physiology, despite its known critical functions in regulating the function of both myosin and actin (McNamara and Sadayappan, 2018). Skeletal muscle myosin-binding protein C paralogs have been demonstrated to regulate the speed and force of muscle contraction ex vivo (Song et al., 2021). If this protein is influenced by training status, then it could have a significant functional impact on exercise performance and thus further investigation into this aspect is required. Furthermore, developing techniques that are able to measure protein modifications at single-fiber resolution would be beneficial in delineating the mechanisms of fiber-type specific metabolic adaptation during exercise or in disease.

This study faced some limitations in its design. The absence of VO2max measurements for both the sedentary and SA groups make it difficult for the exact level of cardiopulmonary levels of performance to be compared between the groups. Access to a greater amount of EA samples may have allowed for an increase in the number of isolated type II fibers from this population and the observation of the myosin conformation ratio in this subpopulation of fibers. Furthermore, the fiber-typing method performed in this study allowed us to distinguish between type I and type II fibers but not to distinguish between type Ila and type IIX myofibers. Type IIX fibers may show differences in their myosin dynamics when compared with type I and type Ila fibers, and this may have important physiological implications (very fast shortening speeds, high power, and rate of tension rise), particularly in the SA group, which would be expected to have a higher percentage of this fiber type (Phung et al., 2020).

In summary, the present study indicates that resting myosin dynamics are influenced by training level and by specific training regimens. We observed a lower percentage of myosin heads in the DRX state in the type II muscle fibers of PA compared with sedentary individuals. Additionally, we found that EA had reduced ATP turnover lifetime of DRX myosins when compared with SA. These findings are important as they suggest a dynamic plasticity of resting myosin conformations linked to training background (exercise discipline and performance level).

Data availability
All data from this manuscript are available from the corresponding authors upon request.

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Author contributions: C.T.A. Lewis: conceived study, performed experiments, analyzed data, interpreted results, wrote manuscript, and managed project. L. Tabrizian: performed experiments, analyzed data, interpreted results, and wrote manuscript. J. Laitila, T.N. Beck, M.S. Olsen, and M.M. Ognjanovic: performed experiments and critically reviewed manuscript. J. Nielsen and P. Aagaard: conceived study, provided subject samples, and critically reviewed manuscript. J.L. Andersen, C. Soendelnbroe, J.W. Helge, F. Dela, S. Larsen, R.E. Sahl, R. Hokken, S. Laugesen, A. Ingersen, T. Romer, M.T. Hansen, and C. Sutta: provided subject samples, critically reviewed manuscript.

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