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The myosin super-relaxed state is disrupted by estradiol deficiency

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ABSTRACT

We have used quantitative epifluorescence microscopy of fluorescent ATP to measure single-nucleotide turnover in skinned skeletal muscle fibers from mouse models of female aging and hormone treatment. Aging causes declines in muscle strength, often leading to frailty, disability, and loss of independence for the elderly. Female muscle is additionally affected by age due to reduction of ovarian hormone production with menopause. Estradiol (E_2) is the key hormonal signal to skeletal muscle in females, and strength loss is attenuated by E_2 treatment. To investigate E_2 mechanisms on skeletal muscle, single fibers were isolated from sham-operated or ovariectomized (OVX) mice, with or without E_2 treatment, and were incubated with 2′-(or-3′)-*O*-(*N*-methylanthraniloyl) adenosine 5′-triphosphate (mantATP). We measured decay of mantATP fluorescence in an ATP-chase experiment, as pioneered by Cooke and coworkers, who unveiled a novel regulated state of muscle myosin characterized by slow nucleotide turnover on the order of minutes, termed the super-relaxed state (SRX). We detected a slow phase of nucleotide turnover in a portion of the myosin heads from sham fibers, consistent with SRX. Turnover was substantially faster in OVX fibers, with a turnover time constant for the slow phase of 65 ± 8 s as compared to 102 ± 7 s for sham fibers. 60-days E_2 treatment in OVX mice substantially reversed this effect on SRX, while acute exposure of isolated muscles from OVX mice to E_2 had no effect. We conclude that E_2-mediated signaling reversibly regulates slow ATP turnover by myosin. Age- and hormone-related muscle functional losses may be targetable at the level of myosin structure/function for strategies to offset weakness and metabolic changes that occur with age.

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1. Introduction

1.1. Muscle weakness, aging, and myosin

The loss of skeletal muscle strength is an undesirable consequence of aging, since it consistently predicts falls, mortality, and functional status such as mobility in elderly individuals [1]. Skeletal muscle of females is dually affected by age due to the simultaneous loss of ovarian hormone production. Declines in strength are accelerated around the time of menopause in women and following ovariectomy in rodents, and our meta-analyses showed that post-menopausal women on estrogen-based hormone therapy and ovariectomized rodents treated with estradiol are stronger than those that remain hormone deficient [2].

We have shown that in females, estradiol deficiency is detrimental to myosin and muscle functions [2–7]. Furthermore, we established that the strong-binding state of myosin during contraction is perturbed by aging, disease, and estradiol deficiency and is a major factor causing strength loss [4–6,8–12]. However, the molecular mechanism underlying estradiol's impact on myosin's molecular force-generating capacity is not known. Myosin consists of two heavy chains and two pairs of light chains and the structural arrangement on the thick filament is stabilized by myosin binding protein C (MyBP-C), the regulatory light chain (RLC), and titin. The light-chain-binding domain of myosin serves as a lever arm, transmitting the free energy of ATP hydrolysis in the actin-binding catalytic domain to produce force and movement during contraction [13]. Phosphorylation of RLC influences this molecular force generation in striated muscle [14] and is such a critical event that specific mutations disrupting RLC phosphorylation in cardiac myosin reduce force and cause cardiomyopathy [15,16]. In skeletal muscle, RLC phosphorylation modulates myosin structure, force, and power output [17,18]. Estradiol has recently been implicated in

Abbreviations: E_2, estradiol; EDL, extensor digitorum longus; KAc, potassium acetate; mantATP, 2′-(or-3′)-*O*-(*N*-methylanthraniloyl) adenosine 5′-triphosphate; MOPS, 3-(N-morpholino) propanesulfonic acid; MyBP-C, myosin binding protein C; RLC, regulatory light chain; P1, population one; P2, population two; pRLC, phosphorylated RLC; Sham, sham-operated; s, seconds; SRX, super-relaxed state; T1, turnover time constant one; T2, turnover time constant two.

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the regulation of RLC phosphorylation and contractility of cardiomyocytes [19,20]. In human skeletal muscle, a proteomic study showed age-dependent reduction of RLC phosphorylation [21], and it was recently shown that RLC phosphorylation is affected in muscle fibers of old women but not old men, relative to young counterparts [22]. Phosphorylation of RLC is also enhanced by estradiol in rodent skeletal muscle [23].

1.2. The super-relaxed state of myosin

A fascinating implication of myosin phosphorylation in aged female muscle relates to the newly discovered myosin super-relaxed state (SRX), which utilizes ATP much more slowly than in the conventional relaxed state, and is disrupted by phosphorylation of myosin RLC [24–26]. These insights have led us to test the hypothesis that estradiol deficiency disrupts the normal structure and function of myosin SRX in female muscle.

In the present study we have detected the SRX state in permeabilized mouse muscle fibers, using fluorescent ATP (mantATP: 2′-(or-3′)-*O*-(*N*-methylanthraniloyl) adenosine 5′-triphosphate) and fluorescence microscopy to detect the kinetics of ATP turnover, as developed by Cooke and coworkers [26]. Skeletal muscle fibers were prepared from adult ovary-intact and ovariectomized mice treated with placebo or estradiol. We hypothesize that estradiol deficiency, as occurs in aged females, stabilizes the SRX state, decreasing isometric force, thus leading to muscle weakness. Here we provide the first report of SRX ATP turnover in a mouse model for human pathophysiological mechanisms using isolated single skeletal muscle fibers and confocal epifluorescence. Furthermore, we have identified a regulatory mechanism that is highly relevant to biological aging and the problem of muscle weakness that occurs due to age in females.

2. Materials and methods

2.1. Animals

Female C57BL/6 mice aged 3–4 months (National Institute on Aging) underwent bilateral ovariectomy surgeries (OVX) or sham operations (Sham), which consisted of the same procedures as the OVX except that the ovaries were not removed. For some experiments, subgroups of OVX mice immediately received a treatment of 17β-estradiol (OVX + E_2) via 60-days time release pellets as described previously [5]. Mice resumed normal cage activities for 6–8 weeks. On the day of sacrifice, mice were anesthetized by an i.p. injection of pentobarbital sodium (100 mg/kg body mass), and psoas and extensor digitorum longus (EDL) muscles were harvested. Psoas muscles were immediately prepared for SRX experiments, and EDL muscles were flash frozen in liquid nitrogen. Uteri were dissected and weighed as an indicator of successful ovariectomy surgery and E_2 treatment (average uterine mass for Sham, OVX, OVX + E_2: 105.9 mg, 14.0 mg, and 136.3 mg, respectively). While still under anesthesia, mice were euthanized by exsanguination. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Minnesota and complied with the American Physiological Society guidelines.

2.2. SRX experiments

Freshly dissected mouse psoas fibers were dissected into ~1 mm bundles and chemically skinned as described previously [26,27]. Single muscle fibers were mounted in 35 mm glass bottom culture dishes (Bioptechs, Butler, PA) to be used with the confocal microscope. In the SRX experiment, fibers were first incubated in a solution containing 250 μM mantATP. After 5 min, this solution was exchanged for one containing 4 mM ATP (“chase”). The solution also contained 120 mM KAc, 5 mM K-phosphate, 5 mM magnesium acetate, 4 mM EGTA, and 50 mM MOPS pH 6.8 [26]. Experiments were performed at 23 ± 1 °C with an Olympus FV1000 IX2 confocal microscope and 60× UPlanApo N oil immersion objective lens (1.42 NA) for differential interference contrast and epifluorescence imaging. Fluorescence was acquired with 405 nm excitation and a 460/50-nm emission filter. Images were scanned in a 512 × 512 pixel grid with a total exposure time of 1.1 s and an effective pixel size of 414 nm. Chase experiments were performed during time-lapse imaging for 400–600 s. Fiber fluorescence was acquired and analyzed as described previously [26]. Images were imported into ImageJ and fluorescence intensities within a circular region (20 μm diameter) were recorded. Fluorescence signal was analyzed as the average of three separate regions in each fiber minus the average of three regions outside the fiber in the same image. The data were fit to a two-exponential decay using a nonlinear least-squares algorithm in Origin 9.0 (OriginLab Corp.). Two exponentials were sufficient to achieve $r^2 > 0.99$; a third exponential did not substantially improve the fit.

Phosphorylation of RLC, measured for intact muscle fibers from the same mice, showed a similar pattern to the SRX data. OVX-treated fibers showed a 35 ± 4% decrease (compared to Sham) in the fraction of phosphorylated RLC, and E_2 treatment restored the phosphorylation level to within experimental error of the Sham value, as we reported previously using Western immunoblots [23]. In the present study, the SRX experiments were performed on skinned fibers with uniformly low RLC phosphorylation due to endogenous phosphatases, thus these results reflect the effects of estradiol on SRX without confounding effects of RLC phosphorylation.

2.3. Statistical analysis

Data are expressed as mean ± SEM of independent experiments (one fiber per experiment.) Data were analyzed by one-way ANOVA using Origin 9.0. Holm-Sidak post hoc tests were performed with significance accepted at $p < 0.05$.

3. Results

3.1. Measuring single-nucleotide turnovers in permeable mouse muscle fibers

Quantitative epifluorescence microscopy was used to measure ATP turnover kinetics in permeable skeletal muscle from mice (Figs. 1 and 2). During the chase experiment, the fluorescence intensity decreases as bound mant nucleotides (mantATP hydrolyzed into mantADP and P_i) are released from myosin and replaced by ATP (Fig. 2). Thus, the fluorescence intensity decays as the fluorescence from mantATP is eliminated by competition from ATP. We first validated our assay with rabbit fibers obtaining results similar to those established in the literature (data not shown) [26]. We proceeded to measure turnover in mouse fibers and found that ATP turnover in mouse is similarly biphasic (Fig. 2), with a slow time constant (T2, see Table 1) associated with super-relaxed myosin heads (SRX). Although the T2 in mouse was about half as long as observed in rabbit, the mouse T2 was decreased by ADP chase or low temperature, effects demonstrated previously for the SRX in rabbit [26].

3.2. SRX experiments in Sham and OVX mice

The ATP turnover kinetics in single fibers from Sham and OVX mice was determined by ATP chase (representative data in Fig. 2A, all data analyzed in Table 1). T1, the exponential lifetime

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