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Oxidative Capacity and Fatigability in Run Trained Malignant Hyperthermia Susceptible Mice

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Abstract

Introduction—The purpose of this study was to test the hypothesis that Malignant Hyperthermia model mice (RyR1Y522S/wt) are more vulnerable to exercise-induced muscle injury and fatigability and adapt less to run training.

Methods—Following 6 weeks of voluntary wheel running, we measured anterior crural muscle fatigability, muscle injury, and cytochrome oxidase (COX) and citrate synthase (CS).

Results—Although RyR1Y522S/wt mice ran without experiencing MH episodes, they ran 42% less distance than wild type (WT) mice. Muscles from WT mice exhibited increased fatigue resistance and COX content after training. Muscles from RyR1Y522S/wt mice demonstrated no significant change in fatigability or COX and CS after training. However, muscles from RyR1Y522S/wt mice displayed less intrinsic fatigability and greater COX/CS content and muscle damage than WT mice.

Discussion—RyR1Y522S/wt mice can run without experiencing rhabdomyolysis, and their inability to adapt to training appears to stem from intrinsic enhancement of mitochondrial enzymes and fatigue resistance.

Keywords

ryanodine receptor; injury; adaptation; mitochondria; calcium

INTRODUCTION

Malignant Hyperthermia (MH) is an inherited skeletal muscle disorder in which susceptible persons experience muscle rigidity, fever, tachycardia, hypoxia, lactic acidosis, and death when exposed to halogenated inhalation anesthetics and certain depolarizing muscle relaxants.^{1–3} When not associated with another underlying myopathy, no apparent symptoms of MH susceptibility are evident outside of anesthesia. However, MH susceptibility is also known to be associated with Central Core Disease (CCD), a common congenital myopathy that is characterized by discrete regions of muscle fibers containing disrupted mitochondria, sarcoplasmic reticulum (SR), and myofibrils, and mild-to-moderate muscle weakness.⁴ The known genetic loci associated with MH and CCD tend to localize to genes that code for proteins involved in excitation-contraction (E-C) coupling. In skeletal muscle, E-C coupling is characterized by the interaction between the sarcolemmal voltage-

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gated L-type Ca^{2+} channel and the SR Ca^{2+} release channel, also known as the ryanodine receptor (RyR1).⁵

Although the exact mechanisms responsible for the pathophysiology associated with MH episodes and CCD are unknown, studies using an MH mouse model indicate that an RyR1 mutation (RyR1^{Y522S/wt}) promotes nitrosative and oxidative modifications of RyR1 that predispose the channel to exhibit sustained opening and to leak Ca^{2+} when it is exposed to halogenated anesthetics or heat.⁶ Skeletal muscle from this mouse model of MH susceptibility has also been shown to start developing core regions of the muscle fiber that have abnormal mitochondria and triads at 2–4 months of age and progress to unstructured cores (i.e., severe myofibril and organelle disruption) at one year of age.^{7,6} In fact, the corresponding RyR1^{Y522S/wt} mutation in humans known to promote MH susceptibility is also associated with a high incidence of central cores in skeletal muscle.⁸

RyR1^{Y522S/wt} mice have also been shown to be a model for environmental heat stroke (EHS), as these mice experience RyR1 nitrosative and oxidative stress, muscle contracture, rhabdomyolysis, and death when exposed to elevated ambient temperatures (i.e., 37–41°C).⁶ Hyperthermia associated with EHS can be triggered by long periods of exercise in warm weather, as often occurs with athletes, military personnel, and laborers. The reported associations among MH, EHS, and exercise-induced rhabdomyolysis^{9–11} with RyR1 mutations prompted our previous study of the susceptibility of skeletal muscle from RyR1^{Y522S/wt} mice to exercise-induced rhabdomyolysis.¹² Although we showed that the RyR1^{Y522S/wt} mutation did not result in excessive muscle damage when muscles from mice performed high-force eccentric contractions in thermal neutral conditions, some mice became hypermetabolic and died when body core temperatures rose above normal.¹²

Although it has been suggested that MH susceptible individuals should not change their lifestyle in general,¹³ very little is known about the risks and benefits of exercise in this population.^{14,15,11} It is well known that aerobic endurance training improves cardiovascular health and promotes numerous skeletal muscle adaptations such as improved skeletal muscle oxidative capacity.¹⁶ However, the metabolic stress and increases in muscle and body temperatures that result from endurance exercise may place individuals with certain forms of MH susceptibility (i.e., RyR1^{Y522S/wt} mutation) at an increased risk for an MH episode or skeletal muscle damage. Additionally, it is important to determine if MH susceptible skeletal muscle is capable of adapting to aerobic training, like healthy skeletal muscle. Therefore, in this study we used RyR1^{Y522S/wt} mice to study the vulnerability of skeletal muscle to injury and the adaptive potential of aerobic metabolism after voluntary wheel running. We hypothesized that muscle from RyR1^{Y522S/wt} mice would exhibit more muscle fiber damage than wild-type (WT) mice after 6 weeks of voluntary wheel running in a thermal neutral environment. We also hypothesized that markers of aerobic metabolism (i.e., citrate synthase and cytochrome oxidase) of skeletal muscle from RyR1^{Y522S/wt} mice would adapt less than those of skeletal muscles from healthy WT mice after endurance training.

MATERIALS AND METHODS

Animals

Founder RyR1^{Y522S/wt} and C57/Bl6 WT mice were originally obtained from Dr. Susan Hamilton at Baylor College of Medicine. RyR1^{Y522S/wt} (n=16) and WT (n=16) mice, 17–21 weeks of age at sacrifice, in this study were bred at Georgia State University, and genotypes were confirmed using PCR analysis. Equal numbers of males and females of each genotype were randomly assigned to a voluntary wheel running group (RUN) and a sedentary control group (SED). The room temperature was maintained at 20–22 °C with a 12-h photoperiod, and all animals were supplied with food and water *ad libitum*. Thermal neutral conditions

are operationally defined as the normal housing temperature (20–22 °C) of the mice. All animal care and use procedures were approved by the Georgia State University institutional animal care and use committee and met the guidelines set by the American Physiological Society.

Experimental design and voluntary wheel running

Voluntary wheel running was performed for 6 weeks by RyR1^{Y522S/wt} (n=8) and WT (n=8) mice. Voluntary wheel running was chosen as the endurance exercise training model because the background strain (C57BL/6J) used to generate RyR1^{Y522S/wt} mice has been reported to consistently exhibit the lowest level of run performance on the treadmill compared to other strains and the highest level of performance during voluntary wheel running.¹⁷ Furthermore, the external stimuli (e.g., shocking grid, compressed air, bottle brushes) used to motivate mice to run on a treadmill would likely cause excessive stress and possibly induce MH episodes in RyR1^{Y522S/wt} mice. Mice in the wheel running group were housed individually with free access to the running wheel and were weighed every week during the experimental period. Each running wheel (12.5 cm diameter) was equipped with a digital cycling computer (Sigma Sports BC 800) and placed into standard plastic cages with a wire mesh top. Each morning, maximum running speed, average running speed, total distance run, and total run time were recorded for each wheel runner, and the digital cycling computer was reset. Age-matched RyR1^{Y522S/wt} (n=8) and WT (n=8) served as sedentary controls and were housed in groups of 5–10 animals per cage without running wheel access. After 6 weeks, anterior crural muscle strength, fatigability, oxidative capacity (i.e., citrate synthase activity and cytochrome oxidase content), and injury were assessed. Skeletal muscle injury was assessed by loss of strength, histological evidence of degeneration and regeneration, and immunoblotting of embryonic isoform of myosin heavy chain (MHC_{emb}).

In vivo strength and fatigue tests

Contractile function of the left anterior crural muscles was assessed by measuring peak isometric tetanic torque *in vivo* after 6 weeks of voluntary wheel running or sedentary cage activity.^{18,12,19–22} The anterior crural muscles (e.g., tibialis anterior, extensor digitorum longus) were studied, because our muscle testing apparatus is designed for this muscle group, these muscles are recruited heavily during running,²³ and 4-weeks of voluntary wheel running has been shown to increase the oxidative capacity in the tibialis anterior (TA) muscle.²⁴ After mice were anesthetized (0.3 mg kg⁻¹ fentanyl citrate, 16.7 mg kg⁻¹ droperidol, 5.0 mg kg⁻¹ diazepam; IP), the left hindlimb was aseptically prepared, and the mouse was placed on a heated platform with the left knee clamped and left foot secured to an aluminum “shoe” that is attached to the shaft of an Aurora Scientific 300B servomotor. Sterilized platinum needle electrodes were inserted through the skin for stimulation of the left common fibular nerve. Stimulation voltage and needle electrode placement were optimized with 5 to 15 isometric contractions (200 ms train of 0.1 ms pulses at 300 Hz). The strength of the anterior crural muscles was assessed by the performance of an isometric torque–stimulation frequency test. Isometric contractions (200 ms train of 0.1 ms pulses) were performed once every 45 s at the following stimulation frequencies: 20, 40, 60, 80, 100, 125, 150, 200, 250, 300, 350, and 400 Hz. Fatigability of the anterior crural muscles was assessed by the performance of 360 concentric contractions (38° angular movement at 400 °/s starting from a 19° plantarflexed position occurring once per second at a stimulation frequency of 300Hz). The fatigue index was calculated as the percent decline of absolute concentric torque from the 1st to the 360th contraction. Additionally, concentric torque measurements during the fatigue protocol were normalized to peak concentric torque for comparative analyses among groups.

***In vitro* analysis of extensor digitorum longus (EDL) muscle**

EDL muscles from mice were dissected free and studied at 30°C using an *in vitro* preparation as previously described.^{18,12,19-22} EDL muscles were studied at 30°C, because resting force from RyR1^{Y522S/wt} muscle exhibited a steady increase and active force was reduced at incubation temperatures of >35°C.²⁵ EDL muscles were mounted in a chamber containing a Krebs-Ringer bicarbonate buffer (pH 7.4) with (in mM) 144 Na⁺, 126.5 Cl⁻, 6 K⁺, 1 Mg²⁺, 1 SO₄²⁻, 1 PO₄³⁻, 25 HCO₃⁻, 1.25 Ca²⁺, 0.17 leucine, 0.10 isoleucine, 0.20 valine, 10 glucose, and 10 µg ml⁻¹ gentamicin sulfate and 0.10 U ml⁻¹ insulin (the buffer was equilibrated with 95% O₂-5% CO₂ gas). The distal tendon was attached by silk suture and cyanoacrylate adhesive to a fixed support, and the proximal tendon was attached to the lever arm of a servomotor system (Aurora Scientific 300B). Optimal physiological muscle length (L_o) in the chamber was set with a series of twitch contractions (0.2 ms pulse at 150 V). Next, peak isometric force as a function of stimulation frequency (10–300 Hz) was measured during isometric contractions (200 ms trains of 0.2 ms pulses), with 3 min between contractions. MH susceptibility was assessed with a caffeine test by measuring changes in baseline force during exposure to increasing caffeine concentrations (1, 2, 4, 8, 16, and 50 mM) and twitch contractions at a rate of 0.2 Hz. The force produced by the EDL muscle was normalized to physiological cross-sectional area (N cm⁻²) as described previously.²⁰

Muscle dissection and wet weight

After the muscle torque measurements, mice were given an anesthetic supplement of sodium pentobarbital (~50 mg/kg IP), and TA and EDL muscles were dissected. Muscle wet weight was measured using a standard analytical scale. Left TA muscles were embedded in Tissue Tek OCT (Miles Scientific), frozen in liquid nitrogen chilled 2-methylbutane, and rapidly stored at -80°C for later analyses. The right TA muscles were frozen in liquid nitrogen and kept at -80°C for future biochemical assays (i.e. citrate synthase enzyme activity assay).

Histology

TA muscle cryosections (13 µm) were obtained from the midbelly of the muscle and were subsequently stained with hematoxylin and eosin (H&E) to estimate the extent of cellular disruption caused by voluntary wheel running as previously described.^{12,19,26} Using Bioquant Nova Prime image analysis software in conjunction with an Olympus CK40 microscope (×20) connected to a microcomputer, muscle fiber cross sectional area (CSA) was determined by tracing the outline of muscle fibers with a computer mouse. Additionally, discolored muscle fibers containing 3 internal mononuclear cells were classified as injured, whereas muscle fibers containing 1 or 2 centrally located mononuclear cells with normal staining were classified as central nucleated muscle fibers. Finally, total fiber number, injured fiber number, and central nucleated fiber number were estimated for each muscle.

Citrate synthase enzyme activity

Citrate synthase (CS) enzyme activity was determined spectrophotometrically at 30°C according to a method similar to that described by Srere²⁷. Briefly, right TA muscles were transferred into 500 µl of ice-cold buffer containing 100 mM KPO₄ (pH 7.4) and 10 mM glutathione and homogenized on ice. After initial homogenization, the homogenate was sonicated and freeze-thawed two times to further disrupt the mitochondrial membrane and expose the CS. Subsequently, 50 µl of muscle homogenate were diluted further in 450 µl of 100 mM Tris buffer (pH 8.0). Aliquots (50 µl) of the diluted homogenate were added to a reaction mix containing 700 µl of 100 mM Tris buffer (pH 8.0), 100 µl of 1 mM DTNB solution, 50 µl of 3 mM acetyl CoA solution, and 100 µl 5 mM oxaloacetate (omitted for control) in a 12×75 mm glass culture tube. The optical density (OD) of the reaction solution

was measured at a wavelength of 412 nm at 15 seconds intervals for a period of 3 minutes. Samples were assayed in triplicate, and CS activity (in $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}$ wet weight⁻¹) was calculated assuming an extinction coefficient of 13.6.

Immunoblotting of cytochrome oxidase and MHC_{emb}

Cytochrome C oxidase (COX; subunit IV) content was measured in mitochondrial fractions from EDL muscle. Mitochondrial fractions were obtained using similar methods described in Bhattacharya and coworkers,²⁸ with some variations. Muscles were washed twice with muscle washing buffer (in mM: 200 mannitol, 70 sucrose, 0.1 NaEDTA, & 10 TrisCl, pH 7.4), minced, diluted with 300 μL of homogenization buffer (in mM: 100 sucrose, 46 KCl, 10 NaEDTA, 10 Tris-HCl, pH 7.4), and then homogenized with a PowerGen 125 tissue homogenizer (Fisher Scientific). Whole muscle homogenate was centrifuged (2000 g for 10 min). The pellet was re-suspended in 50 μL of homogenization buffer and centrifuged again. The supernatants from both centrifugations were combined and centrifuged at 10,000 g. The “mitochondrial” pellet was re-suspended in 20 μL of homogenization buffer following 1.5 minutes of sonication. 40 μg of mitochondrial protein diluted in denaturing sample buffer (66 mM Tris-HCl pH 6.8, 13.2% (v/v) glycerol, 10% (w/v) SDS, 1.0% (w/v) bromophenol blue, and 5.3% (v/v) β -mercaptoethanol) were loaded onto a 10% polyacrylamide gel, separated using SDS-PAGE, and then transferred to a nitrocellulose membrane. Membranes were blocked overnight at 4°C (5% non-fat dried milk in TBS-Tween) and then probed subsequently with anti-COXIV antibody (Molecular Probes, A21348, 1:800) at ~22°C for 2 hours and then a horseradish peroxidase conjugated secondary antibody (Jackson ImmunoResearch, 1:15,000) at ~22°C for 1 hour. The membrane was washed with TBS-Tween (3 $\times$ 15 min) before and after incubation with the secondary antibody. COXIV protein was detected using an enhanced chemiluminescence kit (Thermo Scientific). The total protein content in whole muscle homogenates and mitochondrial fractions was determined using the Bradford assay. Total COXIV protein content was determined by multiplying the banding intensity in 40 μg of mitochondrial protein by the total mitochondrial protein content. Total COXIV protein content was then normalized to whole muscle homogenate total protein content for comparative analysis. MHC_{emb} was analyzed by separating 20 μg of myofibrillar protein in an 8% polyacrylamide gel and transferring proteins to a nitrocellulose membrane. Membranes were blocked overnight and probed with a myosin (embryonic) antibody raised in mouse (F1.652; 1:100 in TBS-T) and a donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc.; 1:20,000 in TBS-T). The monoclonal MHC_{emb} antibody developed by H. M. Blau was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biology at the University of Iowa.

Statistical analysis

Pre-fatigue isometric strength, normalized concentric torque, CS activity, COX content, and histological variables were analyzed with separate activity (run vs. sedentary) by genotype (RyR1^{Y522S/wt} vs. WT) by sex (male vs. female) ANOVAs. Running variables (i.e., average and peak velocity, distance and time run) were assessed with a genotype (RyR1^{Y522S/wt} vs. WT) by time (Day 1, 7, 14, 21, 28, 35, 42) ANOVA with repeated measures on time. *In vitro* contractile variables and *in vivo* fatigue index were analyzed with separate factorial ANOVAs. In the event of a significant interaction, simple main-effects analysis and Bonferroni *post-hoc* tests were conducted. The alpha level was set at $p = 0.05$ for all analyses. All statistical analyses were made using either SPSS 17.0 or SigmaStat 3.0.

RESULTS

Morphological characteristics of RyR1^{Y522S/wt} and WT animals

Basic descriptive data of the mice belonging to each experimental group are shown in Table 1. There were no differences in age and final body weight (BW) between RyR1^{Y522S/wt} and WT mice at sacrifice (Table 1). Compared to female mice, male mice had 16.2% larger final BW ($p=0.014$), and 18.9% larger TA muscle wet weights ($p=0.005$; data not shown). The sex difference in muscle wet weight was explained by the greater BW of male mice, as there was no statistical difference between sexes when TA muscle wet weight was normalized to body weight (data not shown). Despite these sex differences, when experimental group means were compared, there were no statistical differences in TA muscle wet weight expressed in absolute (mg) or normalized (mg/g BW) terms (Table 1). Based on H&E staining, there was no difference in estimates of total fiber number or muscle fiber CSA between TA muscles from RyR1^{Y522S/wt} and WT mice (Table 1). Therefore, 4–5 month old RyR1^{Y522S/wt} mice do not exhibit any overt significant differences from WT mice based on gross morphological analysis.

Voluntary wheel running

At the start of the voluntary training (day 1), all RUN animals were 85–119 days old and had similar body weights (RyR1^{Y522S/wt} vs. WT: 24.3 ± 2.6 vs. 24.3 ± 3.3 g). All mice assigned to the RUN groups successfully completed the voluntary training, and the RyR1^{Y522S/wt} animals ran without experiencing a fatal MH episode. However, RyR1^{Y522S/wt} mice spent less time running and ran less distance than WT mice. RyR1^{Y522S/wt} mice ran on average 4.0 ± 1.1 km·d⁻¹ and an average total of 167.14 ± 47.0 km, whereas WT mice ran an average 6.9 ± 1.0 km·d⁻¹ and an average total of 288.2 ± 43.2 km over the 6-week training period. RyR1^{Y522S/wt} mice spent less time running in contrast to WT mice at day 1 (RyR1^{Y522S/wt} vs. WT: 2.8 ± 0.7 vs. 6.9 ± 1.1 h·day⁻¹), week 1 (RyR1^{Y522S/wt} vs. WT: 2.9 ± 0.7 vs. 5.2 ± 0.6 h·day⁻¹), and week 4 (RyR1^{Y522S/wt} vs. WT: 2.6 ± 0.5 vs. 4.5 ± 0.6 h·day⁻¹). Also, running time tended to be less in RyR1^{Y522S/wt} compared to WT mice during weeks 2, 3, 5, and 6 ($p=0.057$ – 0.11). There was no difference in average (RyR1^{Y522S/wt} vs. WT: 22.3 ± 1.8 vs. 24.9 ± 1.0 m·min⁻¹) or peak (RyR1^{Y522S/wt} vs. WT: 63.5 ± 2.6 vs. 64.4 ± 0.6 m·min⁻¹) wheel running velocity between RUN groups.

Effects of voluntary wheel running on injury markers

***In vivo* anterior crural muscle isometric torque**—Exercise-induced muscle injury results in immediate and prolonged strength deficits.^{18,12,19–22} Although we did not expect to see strength deficits in WT mice associated with voluntary wheel running (Figure 1A), elevated oxidative stress and modest core formation in relatively young RyR1^{Y522S/wt} mice⁷ would be expected to result in weakened skeletal muscle if exercise compounded these preexisting conditions. In lieu of the sex differences observed in body and TA muscle wet weights, *in vivo* muscle function was normalized to body weight for comparative analyses. Isometric muscle strength was lower in run trained RyR1^{Y522S/wt} than WT mice at almost all stimulation frequencies (Figure 1A; Table 2). However, torque values from RyR1^{Y522S/wt} mice were not different between the sedentary and run trained groups, indicating that these functional deficits are not associated with voluntary run training. Additional evidence against run training causing strength deficits in RyR1^{Y522S/wt} mice comes from the observation that both sedentary and run trained RyR1^{Y522S/wt} mice displayed identical leftward shifts in the isometric torque-stimulation frequency curves compared to WT mice (Figure 1B). Exercise-induced muscle injury is known to result in transient (i.e., 5 days) rightward shifts in the isometric torque-stimulation frequency relationship.¹⁸

Exercise-induced muscle injury is also known to decrease peak rates of force development (+dP/dt) and relaxation (−dP/dt), as well as half-relaxation times (HRT) during contractions in mice.²⁹ However, +dP/dt and −dP/dt were unaffected by run training in WT and RyR1^{Y522S/wt} mice (Table 2). Although HRT was slowed by wheel running in WT and RyR1^{Y522S/wt} mice (Table 2), there were no differences in HRT between WT and RyR1^{Y522S/wt} mice, suggesting this change reflects an adaptation to wheel running. Compared to WT mice, sedentary and run trained RyR1^{Y522S/wt} mice exhibited significant decreases in +dP/dt and −dP/dt, which is similar to the findings we reported earlier for sedentary RyR1^{Y522S/wt} mice.¹²

In vitro EDL muscle isometric force—Although peak isometric specific force is less in EDL muscles from RyR1^{Y522S/wt} mice than from WT mice (Figure 1C; Table 2), the sensitivity to caffeine is significantly higher in RyR1^{Y522S/wt} mice (Figure 1D), a hallmark of MH susceptibility. We have shown that exercise-induced muscle injury is associated with immediate and prolonged reductions in caffeine-induced force.²¹ However, run training did not alter isometric specific force or caffeine-induced force in either the RyR1^{Y522S/wt} or WT mice. Finally, EDL muscles from both sedentary and run trained RyR1^{Y522S/wt} mice exhibited a significant leftward shift in the normalized force-frequency responses (data not shown) similar to that observed *in vivo*, an observation inconsistent with exercise-induced muscle injury.

Muscle fiber damage—Before any voluntary wheel running, it appears that the RyR1^{Y522S/wt} genotype has an effect on the degree of muscle fiber degeneration. Sedentary RyR1^{Y522S/wt} mice showed higher basal levels of both injured ($0.52 \pm 0.21\%$, $p=0.007$) and centrally nucleated muscle fibers ($2.13 \pm 0.63\%$, $p<0.001$) in comparison with their WT counterparts ($0.19 \pm 0.11\%$ and $0.55 \pm 0.20\%$ for injured and regenerating muscle fibers, respectively; Figure 2). However, this level of damage did not result in marked levels of regeneration as assessed by the presence of MHC_{emb} (Fig. 2). As expected, six weeks of voluntary wheel running did not result in skeletal muscle damage in WT mice. In contrast to our hypothesis, wheel running did not induce or exacerbate muscle fiber damage in RyR1^{Y522S/wt} mice (Figure 2). In addition, 6 weeks of wheel running did not result in any differences between WT and RyR1^{Y522S/wt} in TA muscle fiber CSA and estimates of total fiber number (Table 1).

Effects of voluntary wheel running on fatigue resistance

Six weeks of wheel running enhanced fatigue resistance in WT mice (Figure 3; Table 2). Trained WT mice exhibited significantly less force loss than sedentary WT mice from contraction number 300 to 360 (Figure 3). In contrast, six weeks of voluntary wheel running failed to improve fatigue resistance in RyR1^{Y522S/wt} mice compared to their sedentary counterparts (Figure 3; Table 2). However, anterior crural muscles from RyR1^{Y522S/wt} mice were intrinsically more fatigue resistant than muscle from WT animals. Relative force output was greater in RyR1^{Y522S/wt} than WT mice from contraction number 30 through 360 (Figure 3).

Effects of voluntary wheel running on mitochondrial enzymes

Six weeks of voluntary wheel running increased COX but not CS in anterior crural muscle from WT mice (Figure 4). Although six weeks of running did not alter CS and COX content in RyR1^{Y522S/wt} mice (Figure 4, Run), anterior crural muscle from these mice had greater intrinsic levels of CS and COX than WT mice (Figure 4, Sed).

DISCUSSION

The primary novel finding of this study is that young adult RyR1^{Y522S/wt} mice can perform endurance exercise for 6 weeks without experiencing severe skeletal muscle damage or fatal MH episodes. Furthermore, anterior crural muscles from RyR1^{Y522S/wt} mice exhibit enhanced intrinsic oxidative capacity and fatigue resistance, however, their muscles apparently adapted very little to 6 weeks of voluntary wheel running.

The mouse RyR1^{Y522S/wt} mutation has been shown to be a good model of MH, environmental heat illness, and possibly CCD.^{30,6,7} We have demonstrated that skeletal muscle from RyR1^{Y522S/wt} mice can safely perform high-force resistance-like exercise *in vivo* as long as body temperature does not increase significantly.¹² In fact, the performance of eccentric contractions partially protected EDL muscles from temperature-induced elevations in baseline tension and deterioration of active force,²⁵ which are thought to result from uncontrolled SR Ca²⁺ release in this MH/CCD mouse model.⁶ Nevertheless, recent studies have reported that mitochondria and SR in focal regions within muscle fibers from these mutant mice progressively deteriorate over the course of the first year of life,^{7,6} suggesting that activities such as running may compound cellular damage due to increased redox stress. Contrary to our hypotheses, voluntary wheel running did not appear to induce skeletal muscle injury based on strength loss and histological evidence of damage (i.e., discolored fibers and monocellular infiltration) in RyR1^{Y522S/wt} mice three to five months of age. Whether more stressful endurance exercise training (i.e., increased mileage or forced work similar to military training) would precipitate rhabdomyolysis remains unclear. Although the data support the hypothesis that the oxidative capacity (i.e., CS and COX) of skeletal muscle from mutant mice is less adaptable to run training than wild type mice, we were surprised to discover that oxidative capacity and fatigue resistance are intrinsically higher in the mutant than in wild type muscle.

In the current and previous study,¹² we report that anterior crural muscles from RyR1^{Y522S/wt} mice exhibit intrinsically higher levels of cellular damage and strength deficits than wild type mice (Fig. 1 and 2). Furthermore, the cellular abnormalities observed in these studies were associated with a greater prevalence of central nuclei, an observation often used as a marker of regeneration.^{12,19,31} However, the lack of developmental myosin isoforms appears to suggest that the presence of central nuclei in the muscle from RyR1^{Y522S/wt} mice represents a pathological condition and not necessarily regeneration. This observation agrees with other studies that report higher values of internal nuclei in skeletal muscle fibers of MH susceptible individuals compared with normal patients.^{32–34} It is possible that the cellular damage observed in this study originates from the focal ultrastructural abnormalities (i.e., swollen and misshaped mitochondria, and disrupted triads and sarcomeres) that have been reported in RyR1^{Y522S/wt} mouse skeletal muscle,^{7,6} as well as from MHS individuals.³³ Although chronic elevations in intracellular Ca²⁺ are associated with apoptosis and the presence of central nuclei in some neuromuscular diseases,^{35,36} it is unknown whether apoptosis contributes to the reported muscle fiber abnormalities in RyR1^{Y522S/wt} mice.

Given the preexisting level of cellular damage in RyR1^{Y522S/wt} mice, it is surprising that daily running activity, which stresses intracellular Ca²⁺ handling and oxidative systems, did not compound the existing muscle fiber damage (Fig. 2) or existing strength deficits (Fig. 1) in RyR1^{Y522S/wt} mice. It is possible that the amount of exercise performed by RyR1^{Y522S/wt} mice was insufficient to stress the muscle fibers enough to exacerbate the damage. RyR1^{Y522S/wt} mice spent less time running (~39%) and ran less distance (~42%) than WT mice, although both groups exercised at equivalent running velocities. Alternatively, chronic SR Ca²⁺ leak from the RyR1^{Y522S/wt} and increased redox stress may have triggered

increases in anti-oxidant scavenging and/or Ca^{2+} handling systems that are capable of managing the stress caused by this level of exercise. Although anti-oxidant capacity is known to be higher in females than in males, we did not observe any sex specific differences in anterior crural muscle strength between RyR1^{Y522S/wt} and WT mice (data not shown). However, this observation should be interpreted cautiously due to the relatively low sample size and statistical power of this specific analysis.

Six weeks of voluntary wheel running increased oxidative capacity (Fig 4) and fatigue resistance (Fig.3) in wild type mice, whereas wheel running did not alter these characteristics in RyR1^{Y522S/wt} mice. Although the training stimulus to enhance oxidative capacity is significantly less in RyR1^{Y522S/wt} mice, it has been shown to be sufficient to produce significant increases in CS activity, succinate dehydrogenase staining, and PGC-1 α content in mice training between 2.1 and 4.6 km/d.³⁷

Unexpectedly, fatigue resistance and markers of oxidative capacity (i.e., COXIV content and CS activity) are intrinsically higher in RyR1^{Y522S/wt} than wild-type mice (Figures 3 & 4; Table 2). The mechanisms for the enhanced fatigue resistance in mature RyR1^{Y522S/wt} mice are unknown. Significant ATP and CP depletion in fast-twitch muscle is associated with skeletal muscle fatigue, and therefore, an increase in oxidative capacity would likely contribute to the preservation of force output during repeated tetanic contractions.³⁸ We and others have documented muscle cells increased indices of oxidative stress, oxygen consumption, and cytosolic [Ca^{2+}] in resting MHS,^{30,12,6} which are all known to activate cell signaling cascades involved in mitochondrial biogenesis,³⁹ and thus to associate with improved fatigability.^{40,16,41,42} Moreover, specific alterations in Ca^{2+} handling via manipulation of parvalbumin expression are known to promote changes in contractile rates, fatigue resistance, and oxidative capacity.^{43,44} In lieu of these findings, we offer that the altered resting cellular environment in RyR1^{Y522S/wt} mice aged 3 – 5 months promotes an enhanced oxidative and slow-twitch contractile phenotype in anterior crural muscles, which manifests in improved fatigability. This supposition is further supported by the presence of enlarged mitochondria in young adult RyR1^{Y522S/wt} mice reported by Boncompagni and colleagues⁷, reflecting an upregulation of mitochondrial enzymes and volume driven by the enhanced SR Ca^{2+} leak in this model. Although enhanced fatigue resistance in muscles with known mitochondrial abnormalities is somewhat counterintuitive, Wrendenberg and colleagues⁴⁵ reported that certain mitochondrial myopathies fail to exhibit enhanced fatigability due to an upregulation in mitochondrial mass to compensate for a respiratory chain deficiency.

Although skeletal muscle from RyR1^{Y522S/wt} mice experiences less force loss during repeated concentric contractions, *in vivo* torque and specific force of non-fatigued anterior crural muscles from RyR1^{Y522S/wt} mice are lower than wild type mice. Weaker skeletal muscles from RyR1^{Y522S/wt} mice do not result from stunted growth or atrophied muscle as evidenced by normal body and muscle weights, and muscle fiber number and diameter (Table 1). However, it is possible that the early core formation with its concomitant sarcomere disruption⁷ contributes to the strength deficits. Alternatively, it is also possible that reduced voltage-gated SR Ca^{2+} release associated with this RyR1 mutation^{46,30,6} contributes to the strength deficits, especially considering the extremely small and infrequent (1/500 μm length of fiber segment) nature of the cores involving sarcomeric disruption at ages 2 – 4 months⁷ and the ability of muscle fibers to effectively transmit force laterally around focal areas of damage.^{47,48}

The observation that RyR1^{Y522S/wt} mice ran approximately 4.0 km/d for six weeks without suffering apparent MH episodes or rhabdomyolysis is encouraging for individuals with this mutation. However, the reason for their reduced running volume compared to wild type

mice of the same strain is unknown. Observations that skeletal muscle from RyR1^{Y522S/wt} mice display higher resistance to fatigue and ran at equivalent velocities as wild type mice suggest that factors other than peripheral muscle tissue may influence endurance capacity in these mice. One possible explanation of the reduced running volume in RyR1^{Y522S/wt} mice involves disruptions in brain mediated neuroendocrine responses to exercise. In support of this idea, the hypothalamus and hippocampus are known to express RYR and influence physical activity⁴⁹ and exercise tolerance.⁵⁰ Moreover, malignant hyperthermia susceptible pigs exhibit lower concentrations of serotonin in the hippocampus⁵¹ and lower basal levels of corticotrophin and cortisol in the plasma compared to wild type pigs.⁵²

Considering that MH is associated with a high number of RyR1 mutations and its potential interaction with environmental heat stress, it is not surprising that reports of episodic manifestations (e.g., cramping, loss of consciousness, elevated serum creatine kinase, prolonged elevated core temperature) of MH in humans indicate a varied response to single or multiple bouts of exercise.^{53-55,14,15,11} The findings of the current and our previous study¹² that MH susceptible mice can perform a six week voluntary wheel running or four week resistance-like training regimen under thermal neutral conditions without inducing rhabdomyolysis, suggest, with caution, that young adults with the RyR1^{Y522S/wt} mutation can perform submaximal endurance and resistance exercise under mild environmental conditions without increased risk of adverse complications.

In summary, RyR1^{Y522S/wt} mice can exercise in thermal neutral conditions without experiencing a MH episode or developing central cores but fail to exercise voluntarily to the same degree as wild type mice. Following six weeks of voluntary wheel running, anterior crural muscle fatigue resistance improved in wild type mice but not in RyR1^{Y522S/wt} mice. Interestingly, anterior crural muscles from RyR1^{Y522S/wt} mice exhibit an intrinsically enhanced oxidative and slow-twitch contractile phenotype and reduced fatigability compared to wild type mice. This is a likely result of the altered resting cellular environment (e.g., elevated resting cytosolic Ca²⁺) induced by the RyR1^{Y522S/wt} mutation. It remains to be determined whether RyR1^{Y522S/wt} muscle can safely further adapt to a higher intensity or more prolonged aerobic exercise regimen, however, under thermal neutral conditions mice with the RyR1^{Y522S/wt} mutation can perform daily voluntary aerobic exercise and weekly electrically stimulated resistance-like exercise¹² without adverse complications.

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ABBREVIATIONS

CCD	central core disease
COX	cytochrome oxidase IV
CS	citrate synthase
E-C	excitation-contraction
EDL	extensor digitorum longus
EHS	exertional heat stroke
H&E	hematoxylin and eosin
L_o	optimal muscle length

MH	malignant hyperthermia
MHC_{emb}	myosin heavy chain embryonic isoform
OD	optical density
PCR	polymerase chain reaction
RYR1	ryanodine receptor skeletal muscle isoform
RyR1^{Y522S/wt}	heterozygous mutation of the ryanodine receptor that results in MH susceptibility
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SED	sedentary
SR	sarcoplasmic reticulum
WT	wild type
TA	tibialis anterior

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Figure 1. Isometric torque and force in trained and sedentary wild type (WT) and RyR1^{Y522S/wt} mice. *In vivo* isometric torque (A); *in vivo* isometric torque normalized to peak isometric tetanic torque (B); *in vitro* isometric specific force (C); *in vitro* caffeine contracture specific force (D). *, RyR1^{Y522S/wt} mice (Sed and Run) significantly different than WT (Sed and Run). Sed, sedentary mice; Run, trained mice.

Figure 2.

Skeletal muscle from Malignant Hyperthermia susceptible mice (RyR1^{Y522S/wt}) exhibits small but significant increases in cellular degeneration (F) and centrally located nuclei (G) than muscle from wild type (WT) mice. Cellular damage and central nuclei were detected via hematoxylin and eosin stain in sedentary (Sed) WT mice (A), trained (Run) WT mice (B), sedentary RyR1^{Y522S/wt} mice (C), and trained RyR1^{Y522S/wt} mice (D). Increased central nuclei in muscle fibers from RyR1^{Y522S/wt} mice does not stem from increased regeneration as judged by the lack of embryonic myosin heavy isoforms (MHC_{emb}), a common marker for muscle regeneration (E). Muscle injured 7d before by 150 eccentric

contractions is shown as a positive control for MHC_{emb} . * indicates cellular damage above the symbol; # indicates central nuclei above the symbol.

Figure 3.

In vivo concentric torque of trained (Run) and sedentary (Sed) wild type (WT) and RyR1^{Y522S/wt} mice during a fatigue contractile protocol. Concentric torque is expressed relative to peak torque produced during the first contraction of the protocol (*See methods*). Values are means $\pm$ SEM. *, Relative torque produced by both sedentary and trained RyR1^{Y522S/wt} mice is significantly greater than for WT mice ($p < 0.05$); #, WT trained produced greater torque than sedentary mice ($p < 0.05$).

Figure 4.

Anterior crural muscle from RyR1^{Y522S/wt} mice exhibits greater mitochondrial enzyme content than wild type (WT) mice. Cytochrome oxidase (COXIV) protein content (A and B) and citrate synthase activity (C) were measured in anterior crural muscles from sedentary (Sed) and trained (Run) wild type (WT) and RyR1^{Y522S/wt} mice. Bars with different letters indicate a statistically significant difference ($p < 0.05$). Values are expressed as means $\pm$ SEM.

Table 1

Final age and body weight (BW), unexercised right tibialis anterior (TA) muscle wet weights, right TA muscle wet weight normalized to body weight, muscle fiber cross sectional area (CSA) and estimate of total fiber number of sedentary control animals.

	Sedentary mice		Wheel running mice	
	WT	RyR1 ^{Y522S/wt}	WT	RyR1 ^{Y522S/wt}
	(n=8)	(n=8)	(n=8)	(n=8)
Age (d)	137.4 (4.3)	135.3 (5.2)	145.6 (2.5)	144.5 (4.6)
Body weight (g)	24.9 (1.4)	24.4 (0.8)	24.8 (1.0)	25.2 (1.1)
Right TA muscle wet weight (mg)	45.5 (2.2)	43.6 (1.9)	47.8 (1.9)	46.0 (0.8)
Right TA/body weight (mg g ⁻¹)	1.84 (0.05)	1.79 (0.06)	1.93 (0.05)	1.82 (0.04)
Left TA muscle fiber CSA (μm ²)	1889 (87)	2022 (99)	1890 (75)	2007 (141)
Left TA muscle fiber number	2584 (79)	2511 (157)	2675 (116)	2251 (121)

Values are means (± standard deviation). WT, wild type; RyR1^{Y522S/wt}, Malignant Hyperthermia susceptible mice; TA, tibialis anterior muscle; CSA, cross-sectional area.

Table 2

Basic functional characteristics of anterior crural muscles.

	Sedentary		Wheel Running	
	WT	RyR1 ^{Y522S/wt}	WT	RyR1 ^{Y522S/wt}
<i>Anterior crural muscles: In vivo</i>				
Sample Size (n)	8	8	8	8
Isometric Torque				
Twitch (Nmm•kg bdy wt ⁻¹)	29.1 (1.9)	21.7 [†] (1.2)	30.6 (1.4)	22.9 [†] (2.1)
Twitch +dP/dt (Nmm•s ⁻¹)	114.4 (12.5)	82.4 [†] (7.6)	124.7 (8.5)	83.6 [†] (18.4)
Twitch -dP/dt (Nmm•s ⁻¹)	58.1 (4.5)	40.5 [†] (3.7)	63.5 (4.7)	40.4 [†] (8.3)
Twitch HRT (ms)	7.8 (0.6)	7.4 (0.7)	9.8 [*] (0.9)	9.2 [*] (1.8)
Tetanic (Nmm•kg bdy wt ⁻¹)	101.9 (3.8)	89.7 [†] (3.1)	110.6 (3.8)	92.6 [†] (4.6)
Tetanic +dP/dt (Nmm•s ⁻¹)	173.8 (13.5)	145.5 [†] (8.6)	185.6 (10.9)	144.8 [†] (33.8)
Tetanic -dP/dt (Nmm•s ⁻¹)	113.0 (9.7)	79.3 [†] (6.5)	146.4 [*] (11.8)	73.5 [†] (17.7)
Tetanic HRT (ms)	34.5 (1.3)	43.1 [†] (2.0)	35.6 (0.9)	49.1 ^{†*} (8.1)
Concentric Torque Fatigue Index (% decline)	28.9 (3.1)	13.4 [†] (2.7)	19.2 [*] (1.6)	12.0 [†] (1.3)
<i>EDL muscle: In Vitro</i>				
Sample Size (n)	8	7	8	5
Wet Weight (mg)	11.0 (0.4)	10.9 (0.5)	11.2 (0.4)	11.4 (0.8)
Length (cm)	1.44 (0.2)	1.44 (0.1)	1.50 [*] (0.01)	1.50 [*] (0.01)
PCSA (cm ²) X 1000	16.5 (0.5)	16.3 (0.8)	16.1 (0.5)	16.4 (1.0)
Specific twitch force (N•cm ⁻²)	4.8 (0.2)	4.4 (0.4)	4.6 (0.2)	4.4 (0.2)
Specific tetanic force (N•cm ⁻²)	23.2 (0.8)	21.9 [†] (1.3)	22.9 (0.3)	20.5 [†] (0.8)

Values are expressed as group means (± SEM). Isometric torques were derived from pre-fatigue testing. WT, wild-type; RyR1^{Y522S/wt}, MH/CCD susceptible; PCSA, physiological cross-sectional area.

* Different from genotype-matched sedentary mice;

[†] different from activity-matched WT mice (p < 0.05).