Ryanodine Receptor Oxidation Causes Intracellular Calcium Leak and Muscle Weakness in Aging

Daniel C. Andersson,1,2 Matthew J. Betzenhauser,1,2 Steven Reiken,1,2 Albano C. Meij,1,2 Alisa Umanskaya,1,2 Wenjun Xie,1,2 Takayuki Shiomi,3 Ran Zalk,1,2 Alain Lacampagne,4 and Andrew R. Marks1,2,3,*

1Department of Physiology and Cellular Biophysics
2The Clyde and Helen Wu Center for Molecular Cardiology
3Department of Medicine
College of Physicians and Surgeons of Columbia University, New York, NY 10032, USA
4INSERM, U-1046, Universités Montpellier, 34295 Montpellier, France
*Correspondence: arm42@columbia.edu
DOI 10.1016/j.cmet.2011.05.014

SUMMARY

Age-related loss of muscle mass and force (sarcopenia) contributes to disability and increased mortality. Ryanodine receptor 1 (RyR1) is the skeletal muscle sarcoplasmic reticulum calcium release channel required for muscle contraction. RyR1 from aged (24 months) rodents was oxidized, cysteine-nitrosylated, and depleted of the channel-stabilizing subunit calstabin1, compared to RyR1 from younger (3–6 months) adults. This RyR1 channel complex remodeling resulted in “leaky” channels with increased open probability, leading to intracellular calcium leak in skeletal muscle. Similarly, 6-month-old mice harboring leaky RyR1-S2844D mutant channels exhibited skeletal muscle defects comparable to 24-month-old wild-type mice. Treating aged mice with S107 stabilized binding of calstabin1 to RyR1, reduced intracellular calcium leak, decreased reactive oxygen species (ROS), and enhanced tetanic Ca2+ release, muscle-specific force, and exercise capacity. Taken together, these data indicate that leaky RyR1 contributes to age-related loss of muscle function.

INTRODUCTION

A hallmark of aging is the progressive decline in skeletal muscle function, characterized by reduced force generating capacity and loss of muscle mass (Rolland et al., 2008; Vijg and Campisi, 2008). Moreover, age-dependent deterioration of muscle function is not restricted to mammals as it is also observed in many animals including the nematode Caenorhabditis elegans (Hernando et al., 2002). Indeed, loss of muscular strength is highly predictive of frailty, disability, and mortality with increased age (Marzetti and Leeuwenburgh, 2006; Metter et al., 2002; Rantanen et al., 1999). Progressive development of muscle dysfunctions is common in aged humans and in animal models of aging (Marzetti and Leeuwenburgh, 2006), and affects as many as 30%–50% of 80-year-olds leading to profound loss of function in the elderly (Rolland et al., 2008).

Much attention has been focused on understanding how to reverse age-related muscle wasting, but there are no established treatments for sarcopenia at this time (Marzetti and Leeuwenburgh, 2006; Saini et al., 2009; Thomas, 2007). In contrast, improving specific force production, which is also significantly reduced in aged muscle (Brooks and Faulkner, 1988; González et al., 2000), has received less attention.

The observed loss of specific force in aged muscle suggests that the Ca2+ dependent process known as excitation-contraction (EC) coupling may be impaired in aging. During muscle contraction, membrane depolarization activates voltage-sensing Ca2+ channels in the transverse tubules (Cav1.1) that in turn activate the sarcoplasmic reticulum (SR) Ca2+ release channel, ryanodine receptor 1 (RyR1). The subsequent rise in cytoplasmic [Ca2+]cyt is critical for activation of actin-myosin cross-bridging, shortening of the sarcomere and muscle contraction (Allen et al., 2008; Andersson and Marks, 2010). The RyR1 is a homotetrameric macromolecular protein complex that includes four RyR1 monomers (~365,000 Da each), the RyR1 channel-stabilizing subunit calstabin1 (FKS06 binding protein 12, FKBP12), kinases, a phosphatase (PP1), phosphodiesterase (PDE4D3), and calmodulin (Zalk et al., 2007). Moreover, maladaptive cAMP-dependent protein kinase A (PKA)-mediated phosphorylation and redox-dependent modifications (cysteine-nitrosylation and oxidation) of the RyR1 have been linked to impaired Ca2+ handling and contractile dysfunction in chronic muscle fatigue, heart failure and muscular dystrophy (Allen et al., 2008; Bellinger et al., 2008, 2009; Zalk et al., 2007). Defective SR Ca2+ release has been reported in age-dependent muscle weakness (Gonzalez et al., 2003; Jiménez-Moreno et al., 2008) and maladaptive modifications of the RyR1 macromolecular complex have been implicated in this condition (Russ et al., 2011). However, the mechanism underlying impaired SR Ca2+ release in aging muscle remains to be elucidated.

Oxidative stress occurs in aged muscle (Jang et al., 2010; Moylan and Reid, 2007) and stress-induced protein oxidation increases with age (Jackson, 2009; Muller et al., 2007). Two important redox-dependent cellular mediators are cysteine-nitrosylation (SNO) and carbonyl modifications of proteins. Both nitrosylation and oxidation have been shown to affect skeletal muscle RyR1 function and Ca2+ signaling (Aracena-Parks et al., 2006; Barreiro and Hussain, 2010; Hidalgo, 2005). Jang et al. (2010) showed that ablation of the antioxidant enzyme superoxide dismutase 1 (SOD1) increased superoxide levels in...
Cell Metabolism
Calcium Leak and Muscle Weakness in Aging

murine skeletal muscle resulting in reduced specific force and accelerated age-dependent muscle pathology. Increased levels of reactive oxygen species (ROS) in the aged muscle are associated with altered cellular Ca\(^{2+}\) handling. Mice with a malignant hyperthermia mutation (Y522S) in RyR1 exhibit SR Ca\(^{2+}\) leak, which promotes mitochondrial dysfunction and oxidative stress-mediated changes of the RyR1 (Durham et al., 2008). Moreover, oxidation-dependent modifications of RyR1 can increase SR Ca\(^{2+}\) leak and a vicious cycle between RyR1-mediated SR Ca\(^{2+}\) leak and mitochondrial ROS production has been proposed (Durham et al., 2008). Furthermore, SNO-modification of the RyR1 has been shown to disrupt the interaction between RyR1 and calstabin1, resulting in channels that can leak SR Ca\(^{2+}\), leading to reduced SR Ca\(^{2+}\) release and muscle function (Bellinger et al., 2009; Zalk et al., 2007).

In the present study, we explore the role of RyR1 dysfunction as an underlying mechanism for defective Ca\(^{2+}\) handling in age-dependent and stress-induced muscle weakness. Fast twitch muscles from aged mice (\(\geq 2\) years old) exhibited reduced specific force and cytoplasmic Ca\(^{2+}\) transients. Moreover, RyR1 from aged rodents was oxidized, cysteine-nitrosylated, and depleted of calstabin1, resulting in “leaky” RyR1 manifested as increased single-channel open probability and Ca\(^{2+}\) spark frequency. Six-month-old mice genetically engineered to express constitutively leaky RyR1 channels (RyR1-S2844D) exhibited defective muscle function comparable that observed in 24-month-old wild-type (WT) mice. Mice with a muscle-specific deletion of calstabin1 had reduced muscle-specific force and impaired exercise capacity. Furthermore, WT flexor digitorum brevis (FDB) muscle fibers that were acutely treated with rapamycin to dissociate calstabin1 from RyR1 (Ahern et al., 1997; Brillantes et al., 1994) exhibited increased resting mitochondrial [Ca\(^{2+}\)], loss of mitochondrial membrane potential, and increased ROS and reactive nitrogen species (RNS) production. Muscle from aged mice with mitochondrially targeted catalase, and increased ROS and reactive nitrogen species production (Brookes et al., 2004; Duchen, 2000), whereas prolonged and excessively elevated mitochondrial [Ca\(^{2+}\)] impairs mitochondrial function due to dissipation of the mitochondrial membrane potential (\(\Delta$$\Psi$$m\)) and increased ROS production (Brookes et al., 2004; Duchen, 2000). To test whether RyR1-mediated SR Ca\(^{2+}\) leak can lead to mitochondrial dysfunction, we measured changes in mitochondrial/cytosol [Ca\(^{2+}\)] and mitochondrial membrane potential in FDB muscle fibers in the presence of rapamycin (15 \(\mu M\)), which causes SR Ca\(^{2+}\) leak by disrupting RyR1-calstabin1 interactions (Ahern et al., 1997; Brillantes et al., 1994). FDB fibers loaded with Rhod-2 were used to measure mitochondrial Ca\(^{2+}\) accumulation in the mitochondria (Figures S4A–S4C). Rapamycin treatment caused a time-dependent increase in the Rhod-2 signal, indicating Ca\(^{2+}\) accumulation in the mitochondria (Figure 2A). We next measured mitochondrial membrane potential in FDB fibers using the fluorescent indicator tetra-methyl rhodamine ethyl ester (TMRE) (Aydin et al., 2009; Duchen, 2004; Nagy et al., 2011). Rapamycin induced a progressive reduction in the TMRE signal (Figure 2B). At the end of each experiment, an uncoupler of the mitochondrial membrane potential (carbonyl cyanide p-trifluoromethoxyphenylhydrazone [FCCP]) was added to the muscle fiber resulting in further reduction in TMRE fluorescence (Figure 2B). Rapamycin has

RESULTS

Oxidation-Dependent Remodeling of RyR1 and Defective SR Ca\(^{2+}\) Release in Aged Muscle

We examined extensor digitorum longus (EDL) muscles from aged (24-month-old) C57BL/6 mice to explore the potential role of RyR1 dysfunction in aging skeletal muscle. EDL from aged mice exhibited reduced specific force compared to EDL from young (3- to 6-month-old) controls (mean specific force at 70 Hz tetanic contraction \(\pm\) SEM: aged, 251 \(\pm\) 21 kNm\(^{-2}\), versus young, 388 \(\pm\) 14 kNm\(^{-2}\), \(n = 5\) aged, 7 young, \(p < 0.001\)) (Figures 1A–1C). Isolated fast twitch muscle FDB fibers from aged mice also exhibited significantly reduced tetanic Ca\(^{2+}\) transients compared to FDB fibers from young mice (mean \(\Delta$$F$/F\(0\) at 70 Hz tetanic contraction \(\pm\) SEM: aged, 4.4 \(\pm\) 0.6, versus young, 6.8 \(\pm\) 0.9; \(n = 8\) aged, 10 young, \(p < 0.05\)) (Figures 1D–1F).

ROS were measured in FDB fibers using a cell-permeant form of the fluorescent indicator 2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCF) (Aydin et al., 2009; Durham et al., 2008). Muscle fibers from aged mice exhibited oxidative stress with significantly increased levels of DCF fluorescence compared to fibers from young adult mice (see Figures S1A and S1B available online). To determine whether the observed reductions in muscle-specific force and tetanic Ca\(^{2+}\) release were associated with oxidation-dependent remodeling of the RyR1 macromolecular complexes, RyR1 from aged and young adult murine muscles was immunoprecipitated and immunoblotted for components of the RyR1 complex (Bellinger et al., 2009). Skeletal muscle RyR1 complexes from aged mice exhibited significantly increased nitrosylation and oxidation, and depletion of calstabin1, compared to channels from young adult mice (Figures 1G and 1H). Similar remodeling of the RyR1 complex was observed in skeletal muscles from 24-month-old rats (Figures S2A and S2B). Aging is known to be associated with oxidative stress and mitochondrial abnormalities (Haigis and Yankner, 2010). EDL muscle from 24-month-old WT mice examined by electron microscopy exhibited a significant increase in mitochondria with disorganized or absent cristae compared to EDL muscle from young adult mice (Figures S3A, S3B, and S3E).

RyR1 Ca\(^{2+}\) Leak Causes Mitochondrial Dysfunction in Skeletal Muscle

Transient increases of mitochondrial [Ca\(^{2+}\)] enhance ATP production (Brookes et al., 2004; Duchen, 2000), whereas prolonged and excessively elevated mitochondrial [Ca\(^{2+}\)] impair mitochondrial function due to dissipation of the mitochondrial membrane potential (\(\Delta$$\Psi$$m\)) and increased ROS production (Brookes et al., 2004; Duchen, 2000). To test whether RyR1-mediated SR Ca\(^{2+}\) leak can lead to mitochondrial dysfunction, we measured changes in mitochondrial/cytosol [Ca\(^{2+}\)] and mitochondrial membrane potential in FDB muscle fibers in the presence of rapamycin (15 \(\mu M\)), which causes SR Ca\(^{2+}\) leak by disrupting RyR1-calstabin1 interactions (Ahern et al., 1997; Brillantes et al., 1994). FDB fibers loaded with Rhod-2 were used to measure mitochondrial Ca\(^{2+}\) accumulation in the mitochondria (Figures S4A–S4C). Rapamycin treatment caused a time-dependent increase in the Rhod-2 signal, indicating Ca\(^{2+}\) accumulation in the mitochondria (Figure 2A). We next measured mitochondrial membrane potential in FDB fibers using the fluorescent indicator tetra-methyl rhodamine ethyl ester (TMRE) (Aydin et al., 2009; Duchen, 2004; Nagy et al., 2011). Rapamycin induced a progressive reduction in the TMRE signal (Figure 2B). At the end of each experiment, an uncoupler of the mitochondrial membrane potential (carbonyl cyanide p-trifluoromethoxyphenylhydrazone [FCCP]) was added to the muscle fiber resulting in a further reduction in TMRE fluorescence (Figure 2B). Rapamycin has
other activities in addition to disrupting RyR1-calstabin1 interactions including inhibition of mTOR signaling. FK506 similarly depletes calstabin1 from RyR1 channels, but does not inhibit mTOR (Brillantes et al., 1994) and caused a reduction in the TMRE signal similar to rapamycin (Figure S4D). Moreover, rapamycin had no effect on mitochondrial membrane potential in FDB muscle from mice with a skeletal muscle targeted deficiency in FKBP12 (calstabin1) (Tang et al., 2004) (Figure S4E). Together, these results indicate that inhibiting RyR1-calstabin1 binding leads to Ca2+ leak and mitochondrial dysfunction. Mitochondrial dysfunction is typically associated with increased ROS production. We used the fluorescent indicator MitoSOX Red to measure mitochondrial superoxide (O2•-) production (Aydin et al., 2009). Rapamycin caused an increase in the MitoSOX Red signal (control: 114% ± 6%, N = 5, rapamycin: 171% ± 8%, N = 10, p < 0.01) (Figure 2C). At the end of each experiment the electron transport chain inhibitor Antimycin A (10 μM) was applied as a positive control (Mukhopadhyay et al., 2007). Ca2+ leak and ROS production in muscle are associated with increased reactive nitrogen species (RNS) (Durham et al., 2008) and increased nitrosylation of the RyR1 was observed in skeletal muscle from aged mice (Figure 1G). We loaded FDB fibers with the fluorescent RNS indicator DAF-FM (DAF) to examine the effects of leaky RyR1 on RNS production. In the presence of rapamycin, DAF fluorescence increased significantly compared to untreated controls (rapamycin: 121% ± 3%, baseline: 102% ± 5%, p < 0.001, N = 6) (Figure 2D), consistent with increased RNS production. Taken together, our data indicate that acute induction of RyR1-mediated SR Ca2+ leak leads to defective mitochondrial function associated with elevated ROS and RNS production.

The small molecule S107 inhibits SR Ca2+ leak by reducing the stress-induced depletion of calstabin from the RyR channel complex (Andersson and Marks, 2010; Bellinger et al., 2009; Lehnart et al., 2008). Therefore, we sought to determine whether the rapamycin-induced Ca2+ leak and the consequent detrimental effects on mitochondrial membrane potential could be prevented by treatment with S107. FDB fibers from young WT mice were incubated with S107 (5 μM) for 2–3 hr before the start of the experiment (Shan et al., 2010). The rapamycin-induced increase in Rhod-2 fluorescence as well as the loss of mitochondrial membrane potential and increase in MitoSOX Red and DAF signals were prevented by S107 (Figures 2A–2D) whereas intermittent twitch stimulation of the FDB fibers (that causes large but transient increases in cytoplasmic [Ca2+]) did not alter the mitochondrial membrane potential (Figure 2B). These data indicate that pathologic SR Ca2+ leak, but not action potential-mediated SR Ca2+ release, have detrimental effects on mitochondrial function and that the effects of pathologic Ca2+ leak on mitochondrial membrane potential could be prevented by S107 treatment.

Inhibiting RyR1 Ca2+ Leak Improves Muscle Force and Exercise Capacity

Aged mice were housed individually in cages equipped with running wheels, and voluntary running time and distance were continuously recorded. Fifty percent of the aged mice (n = 13) were supplied with S107 (~50 mg kg−1 d−1) in their drinking water for a 4 week period, whereas the other half (n = 14) served as the control group. Water consumption was not different between the S107 and vehicle groups (average daily consumption in ml, aged + S107, 8.4 ± 0.63; aged, 7.9 ± 0.4; ± SEM, p = NS). The S107-treated mice exhibited significantly increased running distance (aged + S107, 94 ± 14 km; aged, 57 ± 7 km; mean total distance in 4 weeks, ± SEM, p < 0.05) (Figure 3A) and average speed (S107, 155 ± 20 m/hr;
Thus, the improved tetanic SR Ca\(^{2+}\) release in muscles from S107-treated mice (mean force at 70 Hz tetanic contraction ± SEM: aged, 201 ± 21 kN/m\(^2\); aged + S107, 320 ± 19 kN/m\(^2\); p < 0.001) (Figures 3C–3E). The average tetanic forces for EDL muscles from untreated aged mice at 70 Hz in the experiments shown in Figures 1 and 3 were not significantly different (aged muscles from untreated aged mice at 70 Hz in the experiments shown in Figure S5). Furthermore, EDL muscle mass was not different between S107-treated versus untreated aged mice (aged + S107, 12.7 ± 0.6 mg; aged, 13.8 ± 0.6 mg; p = 0.22).

EDL muscles used in the force measurements were also analyzed for posttranslational modifications of RyR1. There were no differences in the levels of nitrosylation, oxidation or PKA phosphorylation of RyR1 from muscles from mice treated with S107 versus control. This suggests that the oxidative protein modifications are irreversible, as has been reported (Palmese et al., 2011), and protein nitrosylation may have a half-life longer than the 4 weeks of S107 treatment, which has also been reported (Hess et al., 2005). Muscles from the S107-treated mice did, however, show significantly more calstabin1 in the RyR1 complexes compared to skeletal muscle from control mice (Figures 3F and 3G).

To determine whether the improvement in exercise and muscle-specific force were associated with improved SR Ca\(^{2+}\) release, Ca\(^{2+}\) responses to tetanic stimulation were recorded in FDB myocytes from S107-treated and control mice. Tetanic Ca\(^{2+}\) transients were significantly increased in FDB myocytes from S107-treated mice compared to those from the control group (mean ΔF/F\(_0\) at 70 Hz tetanic contraction ± SEM: aged, 3.7 ± 0.3; S107, 5.9 ± 0.5; n = 13, p < 0.01) (Figures 4A–4C). Thus, the improved tetanic SR Ca\(^{2+}\) release in muscles from S107-treated aged mice accounts for the observed increase in muscle force production (Figures 3C–3E). In order to assess the effects of S107 treatment on the single-channel properties of isolated skeletal muscle RyR1 channels, SR membranes were prepared from EDL muscles and fused to planar lipid membrane bilayers, and Ca\(^{2+}\) fluxes through RyR1 channels were recorded as previously described (Brillantes et al., 1994) using conditions that simulate resting muscle (150 nM Ca\(^{2+}\) on the cis, “cytosolic” side of the channel). The open probability (P\(_o\)) of skeletal muscle RyR1 channels from young mice was low, as expected for normal skeletal muscle RyR1 channels (Figures 4D and 4E). In contrast, skeletal muscle RyR1 channels...
from the aged mice exhibited a significantly increased $P_o$, whereas channels from S107-treated aged mice displayed normal, low $P_o$ (Figures 4D and 4E). Taken together, these data suggest that S107 treatment improves exercise capacity in aged mice by reducing the loss of calstabin1 from the channel complexes, and restoring normal (nonleaky) channel function, which in turn results in improved tetanic Ca$^{2+}$ and muscle-specific force production.

To further test the hypothesis that the RyR1 channels in aged muscle are leaky we recorded spontaneous releases of SR Ca$^{2+}$, i.e., Ca$^{2+}$ sparks (Bellinger et al., 2009; Shirokova and Niggli, 2008; Ward et al., 2003), in permeabilized EDL muscle fiber bundles from S107-treated and muscles from untreated adult and aged mice. Ca$^{2+}$ spark frequency was significantly increased in muscle fibers from aged compared to young mice examined under identical conditions by blinded observers (Figures 5A and 5B). Furthermore, S107 treatment in vivo resulted in significantly reduced Ca$^{2+}$ spark frequencies in EDL muscle from aged mice (Figures 5A and 5B). Thus, both the increased Ca$^{2+}$ spark frequency and RyR1 $P_o$ in muscle from the aged mice support a model in which leaky RyR1 channels are associated with defective SR Ca$^{2+}$ release and reduced muscle force production. These defects can be reversed using S107 that inhibits the RyR1-mediated SR Ca$^{2+}$ leak by preventing depletion of the calstabin1 subunit from the channel complex, resulting in stabilization of the closed state of the channel in resting muscle (Brillantes et al., 1994).

Extreme exercise and heart failure are both associated with leaky RyR1 due to phosphorylation of RyR1 at serine 2844 resulting in skeletal muscle weakness (Shan et al., 2010; Bellinger et al., 2008; Reiken et al., 2003). To further test whether leaky RyR1 can cause muscle weakness we developed a knock-in mouse model with a leaky RyR1 due to substitution of aspartic acid for serine 2844 (RyR1-S2844D mouse). Compared to WT muscles, EDL muscles from 6-month-old RyR1-S2844D mice displayed increased Ca$^{2+}$ spark frequency (Figure 5B) and increased single RyR1 channel open probability under resting conditions (Figures S6A–S6C). These abnormalities observed in muscle from 6-month-old RyR1-S2844D mice were comparable to those observed in 24-month-old WT muscle. RyR1 from RyR1-S2844D mice was progressively oxidized, nitrosylated, and depleted of calstabin1 by 6 months of age (Figures 6A and 6B), again comparable to changes observed in RyR1 complexes from 24-month-old WT mice. EDL muscles from the RyR1-S2844D mouse exhibited a significant increase in mitochondria with abnormal morphology (Figures S3C and S3E). Muscle-specific force and action potential-triggered Ca$^{2+}$ transient amplitudes were reduced in the 6-month-old RyR1-S2844D mouse compared to WT mice (Figures 6C and 6D). Four weeks of S107 treatment in vivo reduced the elevated Ca$^{2+}$ spark frequency (Figure 5B) and improved muscle force in 6-month-old RyR1-S2844D mice, both to levels comparable to those observed in muscle from young WT mice (Figure 6C). In summary, the SR Ca$^{2+}$ leak and impaired muscle force production observed in skeletal muscle from 6-month-old RyR1-S2844D mice were comparable to those found in 24-month-old WT muscle.

**S107 Requires Calstabin1 to Reduce Ca$^{2+}$ Leak and Improve Muscle Function**

Extreme exercise and heart failure are both associated with leaky RyR1 due to phosphorylation of RyR1 at serine 2844 resulting in skeletal muscle weakness (Shan et al., 2010; Bellinger et al., 2008; Reiken et al., 2003). To further test whether leaky RyR1 can cause muscle weakness we developed a knock-in mouse model with a leaky RyR1 due to substitution of aspartic acid for serine 2844 (RyR1-S2844D mouse). Compared to WT muscles, EDL muscles from 6-month-old RyR1-S2844D mice displayed increased Ca$^{2+}$ spark frequency (Figure 5B) and increased single RyR1 channel open probability under resting conditions (Figures S6A–S6C). These abnormalities observed in muscle from 6-month-old RyR1-S2844D mice were comparable to those observed in 24-month-old WT muscle, consistent with leaky RyR1. RyR1 from RyR1-S2844D mice was progressively oxidized, nitrosylated, and depleted of calstabin1 by 6 months of age (Figures 6A and 6B), again comparable to changes observed in RyR1 complexes from 24-month-old WT mice. EDL muscles from the RyR1-S2844D mouse exhibited a significant increase in mitochondria with abnormal morphology (Figures S3C and S3E). Muscle-specific force and action potential-triggered Ca$^{2+}$ transient amplitudes were reduced in the 6-month-old RyR1-S2844D mouse compared to WT mice (Figures 6C and 6D). Four weeks of S107 treatment in vivo reduced the elevated Ca$^{2+}$ spark frequency (Figure 5B) and improved muscle force in 6-month-old RyR1-S2844D mice, both to levels comparable to those observed in muscle from young WT mice (Figure 6C). In summary, the SR Ca$^{2+}$ leak and impaired muscle force production observed in skeletal muscle from 6-month-old RyR1-S2844D mice were comparable to those found in 24-month-old WT muscle.
To further test whether the beneficial effects of S107 in aged mice can be attributed to the restored RyR1-calstabin1 binding with the consequent reduction in SR Ca\textsuperscript{2+} leak, we conducted a series of experiments in which we treated muscle-specific calstabin1-deficient (calstabin1 KO) mice with S107. In agreement with the results from Tang et al. (2004), we found that muscle-specific calstabin1 KO mice exhibited reduced EDL muscle-specific force (Figure 6D) compared to WT mice. Calstabin1 KO muscles had a “leaky RyR1” phenotype as indicated by enhanced frequency of Ca\textsuperscript{2+} sparks compared to young WT muscle (Figure 5B). Electron microscopy of EDL muscle from the muscle-specific calstabin1 KO mouse revealed a significant increase in mitochondria with abnormal morphology (Figures S3D and S3E). Muscle-specific calstabin1 KO FDB muscle fibers loaded with the ROS indicator DCF had increased fluorescence compared to the young WT, consistent with oxidative stress (Figure S1). However, S107 treatment, which reduced DCF fluorescence in aged WT mice, did not alter the DCF signal in muscle-specific calstabin1 KO muscle, suggesting that the ability of S107 to reduce SR Ca\textsuperscript{2+} leak and oxidative stress requires calstabin1 (Figure S1). Consistent with a reduction in muscle-specific force, action potential triggered Ca\textsuperscript{2+} release measured in Fluo-4 loaded FDB fibers from muscle-specific calstabin1 KO mice exhibited reduced Ca\textsuperscript{2+} transient amplitudes compared to young WT (Figure 6D). Moreover, exercise capacity was reduced in the calstabin1 KO mice compared to WT (Figure 6E). Treatment with S107 in the drinking water (~50 mg kg\textsuperscript{-1} d\textsuperscript{-1}) for 4 weeks did not restore specific force, Ca\textsuperscript{2+} transient amplitudes, Ca\textsuperscript{2+} spark frequency or the DCF signal in calstabin1 KO muscle (Figures 5B, 6D, 6F, S1A, and S1B). Furthermore, exercise capacity in young WT or calstabin1 KO mice was not improved by S107 treatment (Figure 6F). Taken together, these data indicate that the beneficial effects of S107 on Ca\textsuperscript{2+} handling and muscle function observed in both aged mice and in RyR1-S2844D mice require calstabin1 in skeletal muscle. Moreover, RyR1 from calstabin1 KO mice was oxidized and nitrosylated (Figures S2C and S2D). This suggests that ROS, produced downstream of the SR Ca\textsuperscript{2+} leak, feeds back to the RyR1 where it causes oxidative modifications.

To directly test the hypothesis that mitochondrial-derived ROS causes age-dependent RyR1 oxidation, we examined RyR1 oxidation in muscles from young (3 months) and aged (18 months) transgenic mice with mitochondrial targeted overexpression of catalase (MCAT) (Lee et al., 2010; Schriner et al., 2005). RyR1 oxidation was substantially reduced, nitrosylation slightly reduced and calstabin1 binding was preserved in samples from aged MCAT mice compared to age-matched WT controls (Figures 6G and 6H). We treated SR microsomes from WT muscle with oxidizing (H\textsubscript{2}O\textsubscript{2}) and nitrosylating (Noc-12) compounds alone and in combination to examine the effects of these modifications in more detail. Either nitrosylation or oxidation of the RyR1 led to partial depletion of calstabin1, whereas a combination of these two posttranslational modifications led to more extensive depletion (Figures S2E and S2F). Thus, oxidation and nitrosylation of RyR1 appear to work additively with respect to calstabin1 depletion.

**DISCUSSION**

In the present study, we show that oxidized RyR1 in muscle from aged mice is depleted of calstabin1, resulting in leaky channels, reduced tetanic Ca\textsuperscript{2+}, decreased muscle-specific force, and impaired exercise capacity. To confirm that leaky RyR1 can cause the defects in function observed in aged muscle, we generated a leaky RyR1 model (RyR1-S2844D mice) and used muscle-specific calstabin1-deficient mice. Both strains prematurely...
develop a skeletal muscle phenotype similar to that observed in 24-month-old WT mice. Moreover, the small molecule rycal drug S107, which preserves RyR1-calstabin1 binding and stabilizes RyR1 channels, reduced Ca\(^{2+}\) spark frequency, improved tetanic Ca\(^{2+}\) release, restored muscle-specific force, and improved exercise capacity in aged WT mice. S107 had no beneficial effects in muscle-specific calstabin1 KO mice, indicating that the mechanism of action of the drug involves calstabin1.

Our present study is consistent with previous reports showing impaired Ca\(^{2+}\) release in aged muscle (Jiménez-Moreno et al., 2008), reduced SR Ca\(^{2+}\) release in SR vesicles (Russ et al., 2007), and reduced caffeine-induced release of the SR Ca\(^{2+}\) store (Romero-Suarez et al., 2010). In addition to the pathologic SR Ca\(^{2+}\) leak via remodeled RyR1 that we report in the present study, other defects such as uncoupling between the voltage sensor and RyR1 (Jiménez-Moreno et al., 2008) may also contribute to muscle weakness in aging.

Mitochondria are the primary source of superoxide (O\(_2^-\)), and in the presence of NO, O\(_2^-\) facilitates the production of reactive nitrogen species (RNS) and protein nitrosylation (Szabó et al., 2007). Skeletal muscle RyR1 is sensitive to redox changes (Xia et al., 2000), and we have shown previously that leaky RyR1 in muscular dystrophy and in muscle fatigue after extreme exercise is cysteine-nitrosylated and depleted of calstabin1. In this study we show that the skeletal muscle RyR1 from aged mice are oxidized and nitrosylated. Moreover, the leaky RyR1 from RyR1-S2844D mice becomes progressively oxidized with age. Furthermore, acute induction of SR Ca\(^{2+}\) leak with rapamycin or FK506 or using muscle-specific calstabin1-deficient mice increased ROS and RNS production. Taken together, these findings suggest that SR Ca\(^{2+}\) leak may exacerbate mitochondrial dysfunction by causing mitochondrial Ca\(^{2+}\) overload, which in turn leads to increased RNS and ROS production. The increase in oxidative stress would further promote RyR1 leak by further oxidizing the channel and depleting it of the stabilizing subunit calstabin1.

The 4 week S107 treatment partially reduced oxidative stress in the aged muscle (Figure S1) but did not reduce oxidation or cysteine-nitrosylation of the RyR1. Oxidation-dependent carbonyl modifications of proteins have been reported to be irreversible (Palmese et al., 2011). Thus, inhibiting SR Ca\(^{2+}\) leak would decrease additional oxidation of the RyR1, but would not necessarily reverse the oxidation of RyR1. SNO protein modification is critically dependent on local production of NO, e.g., by NO synthases (NOS). The stability of SNO protein modifications are highly variable and can be influenced by multiple factors including the protein topology, redox state and pH in the vicinity of the affected protein (Hess et al., 2005). Moreover, although the effects of Ca\(^{2+}\) overload on mitochondrial dysfunction and increased ROS production are multifactorial, some of them are believed to be irreversible (Feissner et al., 2009; Jekabsone et al., 2003). The half-life of the mitochondria is 2–4 weeks (Kowald, 2001; Menzies and Gold, 1971) and the half-life of the RyR1 protein is ~10 days in muscle from aged rats (Ferrington et al., 1998). Thus, the continued ROS production from mitochondria and the slow turnover of the RyR1 protein contribute to the observation that RyR1 oxidation is not reduced during a 4 week course of S107 treatment, despite inhibition of the intracellular Ca\(^{2+}\) leak. Moreover, ROS generation from non-mitochondrial sources, e.g., nonphagocytic NAD(P)H oxidase (NOX), could contribute as they have been reported to activate RyR channels (Hidalgo et al., 2006; Xia et al., 2003). Our results, however, are consistent with a “vicious cycle” whereby SR Ca\(^{2+}\) leak and mitochondrial ROS are locally amplified and lead to progressive age-dependent muscle dysfunction (Figure 7). Moreover, our data show that despite persistent RyR1 oxidation and nitrosylation, S107 treatment inhibits the loss of calstabin1 from RyR1 resulting in stabilization of the channel closed state. Thus, S107 inhibits the pathologic SR Ca\(^{2+}\) leak, as long as calstabin1 is present and ameliorates age-dependent loss of muscle function, despite persistent oxidation and nitrosylation of RyR1.

Our data showing increased Ca\(^{2+}\) spark frequency in skeletal muscle from aged WT, RyR1-S2844D, and muscle-specific calstabin1-deficient mice are at odds with a previous study that reported reduced Ca\(^{2+}\) spark activity in aged skeletal muscle (Weisleder et al., 2006). The discrepancy between our results and those of Weisleder et al. (2006) could be due to differences in methodology because Weisleder et al. (2006) used...
Figure 6. Improved Muscle Function and Exercise Capacity in S107-Treated Mice Requires Calstabin1
(A) Immunoblot of immunoprecipitated RyR1 from WT, 1-month-old (1 m), 6-month-old (6 m) RyR1-S2844D mice, and 6-month-old (6 m) RyR1-S2844D mice that were treated with S107 (from the same animals as in C).
(B) Quantification of band intensities in (A) (mean ± SEM, n = 3, **p < 0.01 compared to WT, ##p < 0.01 compared to S2844D 1 m, ANOVA). RyR1 from RyR1-S2844D mice is progressively oxidized (DNP) and depleted of calstabin1 with age.
(C) EDL muscle force-frequency curves in 6-month-old RyR1-S2844D mice and young WT mice. S107 treatment (4 weeks) significantly increased muscle force in the RyR1-S2844D mice (mean ± SEM).
(D) Peak Ca²⁺ transient amplitudes at 70 Hz tetanic stimulation (peak Fluo-4 fluorescence [F] was normalized to resting fluorescence [F₀], ΔF/ΔF₀).
(E) EDL muscle from muscle-specific calstabin1 KO mice produce significantly less force compared to young WT. S107 treatment (4 weeks) did not restore EDL muscle force in muscle-specific calstabin1 KO mice.
(F) Daily voluntary running distance in young WT mice ± S107 treatment and in muscle-specific calstabin1 KO mice ± S107 treatment (mean ± SEM; *p < 0.05 [ANOVA]). The arrow indicates start of the S107 treatment.
(G) Immunoblot of immunoprecipitated RyR1 from young WT, aged (18 months) WT, young transgenic mice with mitochondrial targeted overexpression of catalase (MCAT), and aged (18 months) MCAT mice.
(H) Quantification of band intensities in (G) (mean ± SEM, n = 4 all groups; ***p < 0.001, ##p < 0.01 compared to aged WT [ANOVA]). The pooled data in the figure are mean ± SEM; *p < 0.05, ***p < 0.001 (ANOVA); the number of samples are indicated in parentheses in the figure legend. See also Figure S2.
intact muscle fibers treated with a hypotonic solution that causes the muscle cells to swell resulting in Ca\(^{2+}\) sparks. In contrast, we used saponin permeabilized muscle fibers in order to control the intracellular conditions (e.g., pH, [Ca\(^{2+}\)], [Mg\(^{2+}\)], and the Ca\(^{2+}\) dye concentration), which is not possible using intact muscle fibers (Isaeva et al., 2005; Ríos et al., 1999; Shirokova and Niggli, 2008). We have previously used this method to study pathological conditions where RyR1 exhibited a leaky phenotype (Bellinger et al., 2009; Reiken et al., 2003; Ward et al., 2003). In addition, the increased Ca\(^{2+}\) spark frequency is consistent with the increased RyR1 P_o observed in the aged muscle.

Much of the focus in the field of aging is on therapeutics that target anabolic pathways with hormones, including testosterone, growth hormone, and insulin-like growth factor-1 (Lynch, 2008; Rolland et al., 2008), to improve muscle mass. Some studies have demonstrated increased muscle mass without increased muscle strength or power (Lynch, 2008; Rolland et al., 2008). Inhibition of the endogenous negative regulator of myogenes, myostatin (growth differentiation factor-1) leads to a dramatic increase of muscle mass in mice and cattle (Lynch, 2006). However, muscular dystrophy patients that were treated with an antymostatin recombinant human antibody failed to improve muscle power (Wagner et al., 2008). Thus, increasing skeletal muscle mass is not necessarily accompanied by improved muscle function. Indeed, treating aged mice with S107 enhances muscle strength without increasing the size of the muscle, at least during the 4 week period of treatment examined in the present study.

Despite the important role of oxidative stress in aging-dependent pathologies, the use of dietary antioxidants as an experimental treatment for sarcopenia has not demonstrated improvement in muscle function (Kim et al., 2010). A complicating factor is that systemic antioxidants could impair beneficial effects of ROS (Jackson, 2009; Vijg and Campisi, 2008). By inhibiting the SR Ca\(^{2+}\) leak via RyR1 that is due to oxidation, S107 appears to be able to improve muscle function without blocking systemic ROS-dependent signaling and may represent a promising therapeutic option for reducing age-dependent loss of muscle function.

The regulatory mechanisms of aging are likely multifactorial and several signaling pathways seem to contribute, e.g., changes in insulin/insulin like growth factor (IGF), sirtuins, AMP kinase, and inflammatory signaling (Kenyon, 2010; Marzetti and Leeuwenburgh, 2006). Moreover, mitochondrial dysfunction

---

**Figure 7. Model of RyR1-Mediated SR Ca\(^{2+}\) Leak and Mitochondrial Dysfunction in Aging Skeletal Muscle**

(A) Sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak due to oxidation-dependent modifications of RyR1 exacerbates mitochondrial dysfunction and production of reactive oxygen species (ROS). This causes remodeling of RyR1 resulting in SR Ca\(^{2+}\) leak, which impairs muscle force production. (B and C) The RyR1 from young mice is not “leaky,” the SR Ca\(^{2+}\) stores are filled, and activation of the myocyte leads to SR Ca\(^{2+}\) release, which triggers muscle contraction. (D and E) In aging, ROS and reactive nitrogen species (RNS)-mediated remodeling of RyR1 results in dissociation of the RyR1-stabilizing subunit calstabin1 and SR Ca\(^{2+}\) leak. Under these conditions, muscle activation will lead to reduced SR Ca\(^{2+}\) release and impaired muscle force. [Ca\(^{2+}\)]_m, mitochondrial [Ca\(^{2+}\)]; ΔΨ_m, mitochondrial membrane potential; RyR1, ryanodine receptor type 1; ROS, reactive oxygen species; SR, sarcoplasmic reticulum.
is strongly implicated in the aging mechanism (Balaban et al., 2005; Larsson, 2010). In the present study, we show that leaky RyR1, mitochondrial dysfunction, and oxidative stress conspire to produce age-dependent muscle weakness and reduced exercise capacity.

**EXPERIMENTAL PROCEDURES**

A detailed description is found in the Supplemental Information.

**Animal Models**

Aged mice (23–26 months; C57BL/6) came from the National Institute of Aging, and young mice (3–6 months, C57BL/6) came from our own breeding. Generation of RyR1-S2844D mice (Figure S7) is described in the Supplemental Information. Muscle-specific cal1−/− (calstabin1) mice were bred in the lab after a breeding pair was generously provided to us by Susan Hamilton (Baylor College of Medicine) (Tang et al., 2004). Muscle lysates from young and aged mice with mitochondrial targeted overexpression of catalase (MCAT) were generously provided to us by Gerald Shulman (Yale University), who used mice provided by Peter S. Rabinovitch (University of Washington). The mice in the drug treatment experiments were randomized into two groups. The first group received S107 (50 mg/kg/day) in the drinking water, whereas the second group received water only. Voluntary exercise (running wheel) intensity was recorded continuously. All experiments were performed by blinded observers and were conducted in accordance with Columbia University Institutional Animal Care and Use Committee regulations.

**Force and Ca2+ Measurements**

EDL and FDB muscles were dissected. Force measurement was done on a carrier. RyR1 openings were quantified and open probability (P_o) was measured using Ca2+ spark measurements, saponin-permeabilized fiber bundles from whole EDL muscle using a system from Aurora Scientific (Ontario, Canada). EDL and FDB muscles were dissected. Force measurement was done on a carrier. RyR1 openings were quantified and open probability (P_o) was measured using Ca2+ spark measurements, saponin-permeabilized fiber bundles from whole EDL muscle using a system from Aurora Scientific (Ontario, Canada). RyR1 Immunoblotting

**Measurements of Mitochondrial Ca2+, Mitochondrial Membrane Potential, ROS, and RNS**

Enzymatically isolated FDB muscle fibers were loaded with the fluorescent indicators Rhod-2 and the mitochondrial markers Mitotracker green, TMRE, MitoSOX Red, DCF, and DAF-FM to measure mitochondrial Ca2+, mitochondrial membrane potential, ROS, and RNS, respectively.

**RyR1 Immunoblotting**

Muscle samples were prepared as described (Bellinger et al., 2009). Immunoblots were developed with anti-RyR (Affinity Bioreagents, Boulder, CO; 1:2000), anti-phospho-RyR1-pSer2844 (1:5000), an anti-Cys-NO antibody (Sigma, St. Louis; 1:2000), or an anti-calstabin antibody (1:2500). To determine channel oxygenation, the carbonyl groups in the protein side chains within the immunoprecipitate are derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP signal associated with RyR is determined using an anti-DNP antibody.

**Single RyR1 Channel**

Crude SR vesicles containing RyR1 were fused to planar lipid bilayers. Conductance through the RyR1 was measured using planar Ca2+ as the charge carrier. RyR1 openings were quantified and open probability (P_o) was calculated.

**Histology and Electron Microscopy**

Preparation of tissue samples and microscopy was performed according to established methods as described in the Supplemental Experimental Procedures.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, Supplemental References, and seven figures and can be found with this article online at doi:10.1016/j.cmet.2011.05.014.

**ACKNOWLEDGMENTS**

This project was supported by grants from the National Heart, Lung, and Blood Institute to A.R.M. and the Swedish Research Council and the Swedish Heart-Lung Foundation to D.C.A. The authors thank Susan Hamilton (Baylor College of Medicine) for providing the muscle-specific FKBP12 (calstabin1)-deficient mice. We thank Peter S. Rabinovitch (University of Washington) and Gerald Shulman (Yale University) for generously providing us with muscles from MCAT mice. A.R.M. is a consultant for a start-up company, ARMO Pharma, that is targeting RyR1 to improve exercise capacity in muscle diseases.

Received: December 15, 2010

Revised: March 18, 2011

Accepted: May 19, 2011

Published: August 2, 2011

**REFERENCES**


Xia, R., Stangler, T., and Abramson, J.J. (2000). Skeletal muscle ryanodine receptor is a redox sensor with a well defined redox potential that is sensitive to channel modulators. J. Biol. Chem. 275, 36556–36561.