Leaky RyR2 trigger ventricular arrhythmias in Duchenne muscular dystrophy

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 Patients with Duchenne muscular dystrophy (DMD) have a progressive dilated cardiomyopathy associated with fatal cardiac arrhythmias. Electrical and functional abnormalities have been attributed to cardiac fibrosis; however, electrical abnormalities may occur in the absence of overt cardiac histopathology. Here we show that structural and functional remodeling of the cardiac sarcoplasmic reticulum (SR) Ca2+ release channel/ryanodine receptor (RyR2) occurs in the mdx mouse model of DMD. RyR2 from mdx hearts were S-nitrosylated and depleted of calstabin2 (FKBP12.6), resulting in “leaky” RyR2 channels and a diastolic SR Ca2+ leak. Inhibiting the depletion of calstabin2 from the RyR2 complex with the Ca2+ channel stabilizer ST707 (“rycal”) inhibited the SR Ca2+ leak, inhibited aberrant depolarization in isolated cardiomyocytes, and prevented arrhythmias in vivo. This suggests that diastolic SR Ca2+ leak via RyR2 due to S-nitrosylation of the channel and calstabin2 depletion from the channel complex likely triggers cardiac arrhythmias. Normalization of the RyR2-mediated diastolic SR Ca2+ leak prevents fatal sudden cardiac arrhythmias in DMD.

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at 180 days, at which time characteristic dilation of the myocardium was detected by echocardiography (3.55 ± 0.12 in mdx mice (n = 5) vs. 3.05 ± 0.08 mm in WT (n = 6); P = .015). We found a significant increase in S-nitrosylation of cysteine in RyR2 from mdx mice compared with that from WT littermates, as well as partial depletion of calstabin2 from the RyR2 complex, but no change in phosphorylation of Ser-2805 (Fig. 1A–C). Interestingly, RyR2/calstabin2 dissociation increased progressively with age. Nevertheless, the overall expression of calstabin2 was similar in hearts from young and old mice and also similar in WT and mdx mice (Fig. 1D). We have previously reported that in vivo treatment with S107, an orally available, RyR-specific “rycal” (20), inhibited calstabin1 depletion from the RyR1 macromolecular complex in skeletal muscle (18, 20). In mdx hearts, treatment with the rycal S107 inhibited depletion of calstabin2 from the RyR2 complex without affecting the S-nitrosylation state of the channel (Fig. 1A–C). Interestingly, treatment of the mice with N-acetyl cysteine (NAC) for 2 weeks also prevented RyR S-nitrosylation and calstabin2 depletion (Fig. S1), supporting the idea that S-nitrosylation of RyR2 occurs through the transformation of NO to peroxynitrite in the presence of superoxide anion (O2).

In early disease stages, while cardiac pathology is still not readily detectable, mdx mice exhibit abnormal susceptibility to mechanical stress and workload-induced damage (21). Alterations in NO and/or cGMP signaling have been reported despite minimal histological and echocardiographic changes in mdx mice, suggesting that these abnormalities precede overt pathology in the heart (17). In mdx skeletal muscle, RyR1 S-nitrosylation is correlated with an up-regulation of iNOS–RyR2 interaction. Here the total level of NOS isoforms was first determined by immunoblot analysis in hearts of 35-day-old and 180-day-old mice (Fig. 1E). Levels of nNOS were similar and unchanged in young and old WT mice and in young mdx mice; however, in older mdx mice, nNOS was down-regulated and iNOS was up-regulated. Expression of the endothelial isoform (eNOS) was constant in all groups (Fig. 1E). In contrast to the findings in skeletal muscle (18), coimmunoprecipitation analysis of NOS in the RyR2 macromolecular complex showed that NOS coimmunoprecipitated with RyR2 in the 35-day-old mdx mice, whereas iNOS coimmunoprecipitated with RyR2 in the older mdx mice (Fig. 1F and G).

Table 1. Hemodynamic parameters of WT and mdx hearts in 35-day-old mice, as measured by Doppler echocardiography (SI Materials and Methods)

<table>
<thead>
<tr>
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<th>WT mice (n = 5)</th>
<th>mdx mice (n = 5)</th>
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<tbody>
<tr>
<td>HW/BW, mg · g⁻¹</td>
<td>3.78 ± 0.37</td>
<td>4.26 ± 0.32</td>
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<tr>
<td>Fractional shortening, %</td>
<td>52.0 ± 5.2</td>
<td>60.4 ± 2.7</td>
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<tr>
<td>Relative wall thickness</td>
<td>0.75 ± 0.10</td>
<td>0.83 ± 0.06</td>
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<tr>
<td>E wave, m/s</td>
<td>0.74 ± 0.04</td>
<td>0.71 ± 0.05</td>
</tr>
<tr>
<td>VTI, cm</td>
<td>1.43 ± 0.19</td>
<td>1.47 ± 0.25</td>
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HW/BW, heart weight/body weight ratio; VTI, velocity time integral of aortic flow.

ECG Abnormalities and Ventricular Arrhythmias in mdx Mice. ECG monitoring was done in conscious nonanesthetized animals using telemetric recordings in WT, mdx, and S107-treated mdx mice for 24 h and analyzed specifically during the 12-h overnight period (Fig. 4A, Right). ECGs also were recorded after a 2-h isoproterenol challenge performed in each animal at the same time (Fig. 4A, Right). As shown by these representative traces, repolarization of the ventricles was slower in the mdx mice. This property was significantly exacerbated after isoproterenol challenge and prevented by treatment with S107. The heart rate variability, indicated by the standard deviation of heart rate (SDNN), was significantly lower in mdx mice than in WT mice (Fig. 4B). Reduced heart rate variability has been demonstrated to be a good predictor of increased mortality (24). Heart rate variability, indicated by the standard deviation of all normal RR intervals (SDNN), was significantly reduced in the mdx mice (Fig. 4C). Treating mdx mice with S107 restored the SDNN to control values (Fig. 4C). PVC frequency was significantly increased in mdx mice, and was fully reversed by S107 treatment (Fig. 4D). Long QT syndrome has been associated with a high occurrence of PVCs and SCD (25). The QT interval was prolonged in mdx mice and was restored to normal by S107 treatment (Fig. 4F). The ECG abnormalities observed in mdx animals are consistent with prolonged action potential duration (Fig S2), which has been reported to result from diastolic Ca²⁺ elevation (26, 27). These electrophysiological disturbances were prevented in cardiomyocytes obtained from mdx mice by treatment with S107 and acute application of NAC (Fig S2). The QT interval also was prolonged in mdx mice treated with isoproterenol (Fig. 4G). Furthermore, when challenged with isoproterenol 80% of the mdx mice developed sustained ventricular tachycardia (VT) (Fig. 4H). Importantly, VT was not triggered by isoproterenol stimulation in either WT or S107-treated mdx mice. Together, these findings indicate that even before the development of significant skeletal muscle pathology or cardiac contractility dysfunction, significant and potentially fatal ventricular arrhythmias exist that are exacerbated by sympathetic stimulation, likely triggered by abnormally leaky RyR2 channels.
Fig. 1. RyR2 is S-nitrosylated and depleted of calstabin2 in mdx mice hearts. RyR2 was immunoprecipitated from heart homogenate of mdx and WT littermates at 21, 35, and 180 days after birth, as described in SI Materials and Methods. In addition, a group of 5-week-old mdx mice were treated for 2 weeks with 5107. (A) Immunoblots prepared for RyR2, S-nitrosylation of cysteine residues on RyR2 (Cys-NO), and calstabin2 bound to RyR2. The blots are representative of three independent experiments. (B) Bar graph depicting the relative amount of RyR2 S-nitrosylation for each group, determined by dividing the Cys-NO signals by the total amount of RyR2 immunoprecipitated. (C) Bar graph depicting the relative amount of calstabin2 associated with the channel complex for each group, determined by dividing the calstabin2 signals by the total amount of RyR2 immunoprecipitated. (D) Immunoblots of heart lysates (100 μg) separated by 15% PAGE, prepared using an anti-calstabin antibody. (E) Immunoblots for nNOS, eNOS, and iNOS in 35- and 180-day-old mdx and WT littermate heart muscle lysate. (F) Coimmunoprecipitation of NOS enzymes with RyR2. RyR2 was immunoprecipitated from 500 μg of WT or mdx heart lysate and probed for RyR2 and the NOS enzymes. (G) Coimmunoprecipitation of RyR2 with NOS enzymes. NOS enzymes were immunoprecipitated separately from 500 μg of mdx heart lysate (35 days for nNOS; 180 days for eNOS and iNOS) and probed for RyR2 and the NOS enzymes. In F and G, positive controls for immunoblotting were 100 μg of 180-day-old WT heart lysate for RyR2, nNOS, and eNOS and 100 μg of 180-day-old mdx heart lysate for iNOS.
However, ECGs from mdx mice also revealed a significantly higher number of sinus arrests (21.1 ± 2.3 vs. 7.0 ± 2.0 in WT) and atrioventricular blocks (33.2 ± 3.7 vs. 4.0 ± 1.4 in WT) that were not corrected by S107 treatment.

To explore this finding further, we recorded ECGs in older mdx mice (~6 months) with early overt signs of DCM. At this age, mdx mice exhibited a slower repolarization phase (Fig. 5A), consistent with QT prolongation (Fig. 5B and C), as well as increased PVCs (Fig. 5D). When mdx mice were challenged with isoproterenol, they exhibited sustained VT leading to SCD (e.g., Fig. 5E). The mdx mice were extremely sensitive to isoproterenol-induced arrhythmias; 100% exhibited VT after isoproterenol. S107 treatment prevented the development of VT and sudden death in these mdx mice as well.

**Discussion**

The present study demonstrates the relationship among S-nitrosylation of RyR2, diastolic Ca2+ leak, and the development of fatal cardiac arrhythmias in the dystrophin-deficient heart. Sympathetic stimulation has been shown to exacerbate RyR2-dependent ventricular arrhythmias (28 31). Interestingly, compared with the WT mice, the mdx mice were more sensitive to sympathetic stimulation–induced VT, likely due to the remodeling of the RyR2 channel complex characterized by S-nitrosylation and partial depletion of calstabin2. Indeed, whereas the arrhythmias in the mdx mice were elicited by isoproterenol, the same isoproterenol treatment failed to elicit arrhythmias in age-matched WT mice. Isoproterenol increases the SR Ca2+ load and thus, in the presence of a leaky RyR2 channel, will increase the driving force for a diastolic SR Ca2+ leak that triggers the arrhythmias in mdx mice.

Isoproterenol treatment causes transient Protein Kinase A (PKA) phosphorylation of RyR2 (32). Because the RyR2 channels in WT mice are not leaky, treatment with isoproterenol typically does not elicit arrhythmias. This contrasts with pathological situations such as heart failure, in which the RyR2 channels are chronically PKA-phosphorylated and undergo remodeling such to deplete them of calstabin2, PDE4D3, PP1, and PP2A (32 34). The role of RyR2 leak in the etiology of VT in mdx mice is similar to that seen in catecholaminergic polymorphic VT (CPVT), in which a single point mutation of RyR2 reduces the affinity of calstabin2 for RyR2, and, under sympathetic stimulation leaky RyR2 channels, provides the trigger for fatal VT (28), which also can be prevented by rycals (29).

The role of the NO pathway in regulating cardiac excitation-contraction coupling remains controversial (35). A direct activation of RyR2 by S-nitrosylation has been reported (36). The effect of NO on the closely related RyR1 channel appears to be biphasic (37). NO also has a biphasic effect on the regulation of cardiac contractility (38). NO is produced in the heart by three different isoforms of NOS: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). iNOS is expressed in response to

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**Fig. 2.** SR Ca2+ leak assessed by Ca2+ sparks analysis in mdx mice. Spontaneous SR Ca2+ release events were recorded in fluo-4 AM-loaded intact cardiomyocytes by laser scanning confocal microscopy, as described in SI Materials and Methods. (A) Representative ΔF/F line scan images (1.54 ms/line) were recorded in WT (Top) and mdx mice without (Middle) and with (Bottom) S107 treatment. Diastolic SR Ca2+ leak is estimated by the average amplitude (D), full duration at half maximum (C), and spatial spread (full width at half maximum) (E). Data are expressed as mean ± SEM. WT, n = 24; mdx, n = 29; mdx-S107, n = 15; mdx-NAC, n = 28. *P < 0.05.

**Fig. 3.** Elevated diastolic Ca2+ concentration in mdx mice. Isolated cardiomyocytes, loaded with indo-1 AM as described in SI Materials and Methods, were paced at 1 Hz. After 1 min, cells were maintained quiescent for 30 s before a second train of stimulation. (A) Global Ca2+ was recorded simultaneously, as illustrated by two typical recordings in WT and in mdx cardiomyocytes. (B) During the stimulation, diastolic Ca2+ was elevated in mdx cardiomyocyte. This was prevented by NAC or S107 treatment. (C) The peak amplitude of the Ca2+ transients did not differ significantly in all conditions. Data are expressed as mean ± SEM. WT, n = 24; mdx, n = 29; mdx-S107, n = 15; mdx-NAC, n = 28. *P < 0.05.
cardiac stress, such as ischemia or inflammation, and is thought to remain in the cytoplasm (35). In normal hearts, eNOS is located in membrane caveolae, whereas some nNOS is thought to be located in the RyR2 macromolecular complex (35). Ablation regulation and recollection of nNOS may contribute to degeneration of muscle fibers in DMD (39). Thus, NO-mediated signaling depends on both the NOS isoform and compartmentalization (40). For instance, nNOS-deficient mice have decreased cardiac inotrophy, whereas eNOS-deficient mice have enhanced contractility, owing to corresponding changes in SR Ca\(^{2+}\) release, potentially indicating opposing effects of these two isoforms on cardiac function. By analogy with our results, increasing NO production by eNOS activation also has been shown to increase spontaneous Ca\(^{2+}\) sparks activity in isolated cardiomyocytes via a cGMP-independent pathway and presumably direct S-nitrosylation of RyR2 (41). This seems to disagree with a recent report that hyponitrosylation of RyR2 mediated a proarhythymogenic Ca\(^{2+}\) leak (42). This apparent contradiction might be explained by the fact that in the transgenic models used in that previous study, the interaction between RyR2 and calstabin2 was unchanged, whereas in the present study, S-nitrosylated RyR2 was partially depleted of calstabin2. Another possible explanation for this discrepancy is that decreased nNOS and increased iNOS have been observed in the mdx model (19). We also found (as we did previously in mdx skeletal muscle; ref. 18) increased iNOS in the RyR macromolecular complex; however, in contrast to the situation in skeletal muscle, here this occurred at an advanced disease stage (180 days). Thus, the severity of the pathology seems to be linked to the degree of iNOS overexpression in both cardiac and skeletal muscle (18). Because iNOS expression is related to inflammatory processes (35) and based on the differential progression of skeletal and cardiac muscle, it is possible that an inflammatory response contributes to the development of cardiac RyR2 dysfunction via an up-regulation of iNOS. Furthermore, iNOS is significantly increased in the skeletal muscle of both DMD patients and mdx mice, and rescue of the mdx phenotype by adenoviral-mediated dystrophin or utrophin expression has been found to normalize iNOS activity (43). Nevertheless, overexpression of nNOS in mdx mouse heart was found to partially rescue cardiac function in older mice (44). These beneficial effects

Fig. 4. ECG recordings in young mdx mice. ECGs were recorded as described in SI Materials and Methods. (A) Typical ECGs acquired by telemetry in 35-day-old vigil mice over 24 h and analyzed specifically during the 12-h overnight period in WT (Top; n = 5), mdx (Middle; n = 5), and S107-treated (Bottom; n = 5) mice in normal condition (Left) and after i.p. injection of isoproterenol (2.5 mg · kg\(^{-1}\)) (Right). (B–F) ECG analysis provides various functional parameters, including cardiac frequency given by the RR interval (B), heart rate variability expressed as SDNN (C), PVCs (D), QRS duration (E), and QT interval (F). (G) The QT interval also was measured after isoproterenol challenge. (H) Typical spontaneous sustained VT recorded in mdx mice after isoproterenol challenge. Note that VT was never triggered by isoproterenol treatment in either WT or S107-treated mice. *P < 0.05 control vs. mdx; #P < 0.05 mdx vs. S107-treated mdx.

Fig. 5. ECG recordings in older mdx mice. (A) Typical ECGs acquired by telemetry in 180-day-old vigil mice over 24 h and analyzed specifically during the 12-h overnight period in WT (Top; n = 5), mdx (Middle; n = 5), and S107-treated mdx (Bottom; n = 5) mice in normal condition. (B and C) As in young mice, QT intervals in baseline condition (B) and after injection of isoproterenol (C) were enlarged in mdx mice (black bars) compared with WT mice (open bars). (D) PVCs also were increased in mdx mice. These electrophysiological abnormalities were fully abolished after S107 treatment (dashed bars). (E) Typical spontaneous sustained VT recorded in mdx mice after isoproterenol challenge. This mouse died from SCD during the acquisition. Note that VT was never triggered under the same treatment in either WT or S107-treated mdx mice. *P < 0.05 control vs. mdx; #P < 0.05 mdx vs. S107-treated mdx.


Supporting Information

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SI Materials and Methods

ECG Recording and Analysis. Mice were monitored by ECG performed with a signal transmitter-receiver (RPC-1; DSI) connected to a data acquisition system (IOX2; EMKA Technologies). The data were collected continuously over 24 h at a sampling rate of 1,000 Hz. Continuous digital recordings were analyzed offline using the ECG-auto, version 1.5.12.22 (EMKA Technologies). Telemetric data were scanned using software by blinded observers to measure heart rate variability (HRV) parameters, as described previously (1). In brief, ECG signals were digitally filtered between 0.1 and 1,000 Hz and analyzed manually to detect arrhythmias identified by RR intervals two times greater or smaller than the mean, and then quantified. RR intervals were calculated from nocturnal ECG recordings.

HRV was analyzed similarly on the same recordings. For HRV analyses, the RR values not included between RR ± 2 SD (95.5% confidence intervals) were removed from the analyses. They were not replaced by any averaged or interpolated beat (2). The indexes used were the mean RR interval and SDNN, which reflects total autonomie variabilty.

RyR2 and NOS Biochemistry. Hearts from WT and mdx age-matched littersmates were lysed isotonically in 1.0 mL of a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20 mM NaF, 1.0 mM Na3VO4, and protease inhibitors. The levels of nNOS, eNOS, and iNOS were measured using the Odyssey infrared imaging system (LICOR Biosystems) and infrared-labeled secondary antibodies.

An anti-RyR antibody (4 mg; 5029 Ab) or the NOS isofrom antibodies (4 mg; BD Biosciences) were used to immunoprecipitate RyR2, nNOS, eNOS, or iNOS from 500 mg of heart homogenate. The samples were incubated with the appropriate antibody in 0.5 mL of a modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 0.9% NaCl, 5.0 mM NaF, 1.0 mM Na3VO4, 1% Triton X-100, and protease inhibitors] for 1 h at 4°C. The immune complexes were incubated with protein G Sepharose beads (Sigma-Aldrich) at 4°C for 1 h, washed three times with buffer. Proteins were separated on SDS/PAGE gels (6% for RyR2; 10% for NOS enzymes) and transferred onto nitrocellulose membranes for 1 h at 200 mA (SemiDry transfer blot; Bio-Rad). Immunoblots were prepared as described above. RyR2 S-nitrosylation was measured using antibodies against nNOS, eNOS, and iNOS from 500 mg of heart homogenate.

Measurement of Intracellular Ca2+ and Ca2+ Sparks. Cells were loaded for 30 min at RT with fluo-4 AM (5 µmol/L; Molecular Probes), then field-stimulated at 1 Hz with 1-ms current pulses delivered via two platinum electrodes, one on each side of the perfusion chamber. Changes in fluo-4 AM fluorescence were recorded using an LSM510 Meta confocal microscope equipped with a 63× water-immersion objective (numerical aperture: 1.2; Zeiss). All measurements were performed in line-scan mode (1.5 ms/line), and scanning was carried out along the long axis of the cell. An excitation wavelength of 488 nm was used, and emitted light was collected through a 505-nm long-pass filter. The laser intensity used (3%–6% of the maximum) had no noticeable deleterious effect on the fluorescence signal or cell function over the course of the experiment. To enable comparisons between cells, the change in fluorescence (ΔF) was divided by the fluorescence detected immediately before the 0.5-Hz stimulation pulse (F0). Spontaneous Ca2+ sparks were recorded in quiescent cells following 5-min stimulations to reach steady-state SR-Ca2+ content.

Calcium Imaging. To quantitatively monitor intracellular Ca2+ concentration, cardiomyocytes were loaded for 30 min at RT with 10 µM indo-1 AM (Invitrogen), then field-stimulated at 1 Hz (20 V, 1 ms), and simultaneously illuminated at 305 nm using a xenon arc bulb light. Indo-1 AM fluorescence emitted at 405 nm and 480 nm was recorded simultaneously using IonOptix acquisition software (Hilton). To record spontaneous Ca2+ waves in the resting condition, stimulation was stopped for 30 s once a Ca2+ transient steady-state was reached. Indo-1 AM calibration was performed as described previously (4), using the following equation:

$$\left[ \text{Ca}^{2+} \right] = K_d \times \beta \times (R - R_{\text{min}}) / (R_{\text{max}} - R)$$

where R is the fluorescence ratio (F405/F480), Rmin is the fluorescence ratio in the absence of [Ca2+], Rmax is the fluorescence ratio in the presence of [Ca2+], Kd is the dissociation constant, and β is the ratio of the F480 values at 0 and saturating level of Ca2+.

Measurement of Membrane Potential. Action potentials (APs) were recorded in rod-shaped Ca2+-tolerant myocytes under current-clamp conditions using a whole-cell patch-clamp technique at 22 ± 2°C with an Axopatch 200B (Axon Instruments), as described previously (5). Pipettes (2–3 MΩ) were filled with a recording solution [130 mM KCl, 25 mM Heps, 3 mM ATP(Mg), 0.4 mM GTP(Na), 0.5 mM EGTA, pH adjusted to 7.2 with KOH]. The myocytes were superfused with Tyrode’s solution (135 mM NaCl, 1 mM MgCl2, 4 mM KCl, 11 mM glucose, 2 mM Heps, 1.8 mM CaCl2, pH adjusted to 7.4 with KOH) (6). APs were elicited by 0.2-ms current injections of suprathreshold intensity. Signals were acquired at 10 kHz and filtered at 5 kHz using low-pass Bessel filter. Cells were stimulated routinely at 0.1 Hz until APs stabilized (3–5 min). Once steady state was established, myocytes were paced at 5 Hz for 1 s every 5 s, to record DADs. Data acquisition and analyses were performed using Pclamp version 10.1 (Axon Instruments). Resting membrane potential (RMP) and AP duration (APD) at 20% (APD20), 30% (APD30), 50% (APD50), 90% (APD90), and 95% (APD95) of repolarization were measured.

Statistical Analysis. Statistical comparison was performed with Sigmasstat version 3.5 (Systat Software). For spatiotemporal properties of Ca2+ sparks, normality tests failed, and thus data were statistically compared using Kruskal-Wallis one-way ANOVA on ranks. Other statistical comparisons were done with ANOVA. Data are expressed as mean ± SEM, and differences were considered significant at P < 0.05.

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Fig. S1. Treatment with NAC prevents RyR2 S-nitrosylation and depletion of calstabin2 in mdx hearts. RyR2 was immunoprecipitated from heart homogenate of 35-day-old mdx mice and WT littermates. In addition, a group of 3-week-old mdx mice were treated for 2 weeks with NAC. Immunoblots were prepared for RyR2, S-nitrosylation of cysteine residues on RyR2 (Cys-NO), and calstabin2 bound to RyR2. The blots are representative of three independent experiments.

Fig. S2. Effect of NAC on RyR function in mdx mice. Ventricular cardiomyocytes were isolated from 35 day-old mdx mice (n = 3) and separated into two batches. One batch was maintained in a normal external buffer, and the other was maintained for 1 h before use in experiments in the same medium containing the ROS/RNS scavenger NAC (20 mM). Cells were then loaded for 30 min with fluo-4 AM, with fluorescence monitored by laser scanning confocal microscopy. RyR2-induced diastolic SR Ca2+ leak was estimated based on the average frequency of sparks. The high sparks frequency observed in mdx cells was reversed to a value comparable to that in WT cells (Fig. 2D). *P < .05 mdx vs. NAC-treated mdx. n = 651 sparks in 30 cells in mdx; n = 175 sparks in 30 cells in NAC-treated mdx.
**Fig. S3.** Consequences of SR Ca\(^{2+}\) leak on AP duration and RMP. APs were recorded on isolated cardiomyocytes from 35-day-old mice using the patch-clamp technique with an internal medium containing a low EGTA concentration to avoid excessive Ca\(^{2+}\) buffering, which could interfere with Ca-dependent regulatory mechanisms on RMP. APs were recorded at 5 Hz. The stimulation was interrupted to challenge resting membrane potential and occurrence of DADs. (A–C) Typical train of APs followed by a rest period recorded in mdx cardiomyocytes (A), S107-treated mdx cardiomyocytes (B), and cardiomyocytes incubated with 20 mM NAC (C, Left). The arrow in A indicates a DAD in the mdx cardiomyocytes. This behavior was not observed after S107 treatment or NAC incubation. The first APs recorded in a train are shown in the right panel in an expanded time scale. AP durations were measured at different phases of AP repolarization. (D) Data summary showing a shortening effect of both S107 treatment and NAC incubation that appear to be significant at 90% and 95% repolarization, as denoted by the arrow in A (Right). RMP depolarization occurring in mdx cardiomyocytes was prevented by S107 treatment or NAC incubation. Data are expressed as mean ± SEM. n = 15 in mdx (black), n = 11 in S107-mdx (red), and n = 10 in NAC-mdx (blue). *P < 0.05 mdx vs. S107-treated mdx; #P < 0.05 mdx vs. NAC-treated mdx.

**Fig. S4.** Isoproterenol-induced ectopic Ca\(^{2+}\) transients in mdx mice. Isolated cardiomyocytes were paced at 1 Hz. Ca\(^{2+}\) fluorescence was recorded in line-scan mode (x vs. time; 1.5 ms/line) using confocal microscopy. (A) Typical sequences of Ca\(^{2+}\) transients obtained in cardiomyocytes after acute application of 100 nM isoproterenol. (B) This treatment significantly increased the number of ectopic Ca\(^{2+}\) transients, which were rare in WT and S107-treated mdx mice. Data are expressed as mean ± SEM. *P < .05 WT vs. mdx; #P < .05 mdx vs. S107-treated mdx.