

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 S107 (“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.

calcium | excitation-contraction coupling | heart | sudden cardiac death | myopathy

Duchenne muscular dystrophy (DMD), the most common X-linked disorder in males, results from dystrophin deficiency due to nonsense mutations (1–3). Dystrophin contributes to maintenance of cellular architecture and permits signal transduction between the cytoskeleton and the extracellular matrix. DMD affects 1 in 3,500 newborn males and usually leads to death from respiratory or cardiac failure by age 30 years (1). The severity of cardiomyopathy is not proportional to the severity of the skeletal muscle disorder. Among DMD patients, the cardiac phenotype varies with age, from no discernible cardiac left ventricular enlargement or dysfunction to dilated cardiomyopathy (DCM) with heart failure (4). The incidence of DCM in DMD patients has been estimated to be 25% by age 6 years, 59% at age 10 years, and ~100% in adults (4). In addition, DMD patients often exhibit electrocardiographic abnormalities and frequent premature ventricular contractions (PVCs) (5). These abnormalities have been found to occur in 85% of the patients before age 10 years and in 91% before age 18 years (6). As cardiomyopathy progresses, ventricular arrhythmias increase, often leading to sudden cardiac death (SCD) (7). Most of the electrical and functional abnormalities have been attributed to cardiac fibrosis; however, electrical abnormalities may occur in the absence of overt cardiac histopathology (5), and ECG changes are similar in patients with DMD regardless of the presence or absence of DCM (8).

Although several cellular mechanisms have been proposed to account for these cardiac abnormalities, the definitive mechanism underlying SCD in DMD has not yet been established. Dystrophin deficiency has been linked to altered Ca²⁺ homeostasis due to defects in plasma membrane integrity in skeletal muscle (9, 10) and the heart (10–12). It has been proposed that dystrophin deficiency could increase the activity of stretch-activated channels (SACs), including the TRPC1 channel in the heart (13) and the

TRPC3 (14) and TRPV2 (15) channels in skeletal muscle. Jung et al. (11) recently proposed another explanation for the altered Ca²⁺ homeostasis, based on stretch-induced production of Ca²⁺-dependent reactive oxygen species (ROS) by mitochondria, which in turn activates sarcoplasmic reticulum (SR) Ca²⁺ release, resulting in elevated resting Ca²⁺. Indeed, ROS production and oxidative damage may be involved in the pathogenesis of heart failure in DMD (16). Interestingly, the NO/cGMP signaling pathway also has been reported to exhibit early signs of alteration before any overt evidence of cardiomyopathy (17). Thus, a consensus is emerging that dystrophin deficiency leads to abnormal intracellular Ca²⁺ homeostasis in cardiac and skeletal muscle, but the mechanisms underlying the altered Ca²⁺ homeostasis remain to be fully elucidated.

In a recent study, we showed that SR Ca²⁺ leak via release channel/ryanodine receptor (RyR2) caused by progressive S-nitrosylation of the channel and calstabin1 depletion likely contributes to muscle weakness in DMD (18). Moreover, S-nitrosylation of the channel is associated with a significant up-regulation of inducible nitric oxide synthase (iNOS) in *mdx* skeletal muscle and its association with the RyR1 macromolecular complex (18). In the heart, decreased neuronal NOS isoform (nNOS) activity and increased iNOS activity have been reported and correlated with abnormal ECG activity (19). We reasoned that early changes in cardiomyocyte Ca²⁺-dependent signaling could provide particular insight into the critical initiating events in the development of cardiac disease in DMD. Therefore, the goal of this study was to investigate the role of RyR2 in the genesis of cardiac arrhythmias in *mdx* mice occurring before any sign of overt cardiac contractile dysfunction is apparent.

Results

Cardiac RyR2 Channels Are S-Nitrosylated and Depleted of Calstabin2 in *mdx* Mice. We hypothesized that defects in the RyR2 macromolecular complex might be observed in the heart, and thus assessed the biochemical composition of the RyR2 macromolecular complex in cardiac muscle of *mdx* mice at age 35 days, a stage at which early signs of muscular dystrophy in skeletal muscle are detectable but no evidence of overt cardiomyopathy is apparent (Table 1). We compared these data with that for cardiac muscle from *mdx* mice and age-matched WT littermates

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Conflict of interest statement: A.R.M. is on the scientific advisory board and owns shares in ARMGO Pharma, Inc., a startup company developing RyR-targeted drugs for clinical use in the treatment of heart failure and sudden death.

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

	WT mice (n = 5)	<i>mdx</i> mice (n = 5)
HW/BW, mg · g ⁻¹	3.78 ± 0.37	4.26 ± 0.32
Fractional shortening, %	52.0 ± 5.2	60.4 ± 2.7
Relative wall thickness	0.75 ± 0.10	0.83 ± 0.06
E wave, m/s	0.74 ± 0.04	0.71 ± 0.05
VTI, cm	1.43 ± 0.19	1.47 ± 0.25

HW/BW, heart weight:body weight ratio; VTI, velocity time integral of aortic flow.

at age 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-2808 (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 (O_2^-).

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 nNOS coimmunoprecipitated with RyR2 in the 35-day-old *mdx* mice, whereas iNOS coimmunoprecipitated with RyR2 in the older *mdx* mice (Fig. 1F and G).

Diastolic SR Ca²⁺ Leak and SR Function in *mdx* Mice. Spontaneous SR Ca²⁺ release events (Ca²⁺ sparks) correspond to stochastic release of Ca²⁺ arising from small clusters of SR Ca²⁺ RyR release channels (22). The occurrence of Ca²⁺ sparks reflects the open probability of RyR in situ and can be used as an index of diastolic Ca²⁺ leak (23). Cardiomyocytes from *mdx* mice exhibited a significantly higher frequency of spontaneous Ca²⁺ sparks than those from WT mice (Fig. 2A and B). The amplitude (Fig. 2C) and spatial spread (Fig. 2E) of Ca²⁺ sparks were similar in *mdx* and WT mice, whereas their duration was slightly shorter in *mdx* mice (Fig. 2D). Treating *mdx* mice with S107 prevented this increase in the frequency of sparks. Of note, treatment with S107 did increase the

amplitude of the sparks, possibly reflecting an increase in SR Ca²⁺ content. Together, these data indicate that RyR2 were abnormally “leaky” in *mdx* mice during diastole. This leaky behavior was prevented when isolated cardiomyocytes were preincubated with NAC (Fig. S2), strongly supporting a direct role of ROS/reactive nitrogen species (RNS) on RyR2 function in *mdx* hearts.

Global Ca²⁺ signals (i.e., Ca²⁺ transients) were triggered in isolated ventricular cardiomyocytes loaded with indo-1 AM. Diastolic Ca²⁺ was significantly higher in the *mdx* mice; this was prevented by treatment with either S107 or NAC (Fig. 3A and B). Ca²⁺ transient amplitude were slightly, but not significantly, reduced in *mdx* mice (Fig. 3A and C). Cardiomyocyte contraction was unaffected in the 35-day-old mice, because cell shortening was similar in the *mdx* and WT mice (reduced cell length of $9.72\% \pm 0.82\%$ and $9.13\% \pm 0.70\%$, respectively).

Initiation of ventricular tachycardia may result from DADs and ectopic Ca²⁺ transients. To evaluate the propensity of *mdx* cardiomyocytes to generate DADs, cardiomyocytes were field-stimulated for 1 min at 1 Hz. Diastolic Ca²⁺ was then recorded during a subsequent rest period of 30 s. Whereas diastolic Ca²⁺ remained constant in WT cardiomyocytes, Ca²⁺ oscillations and waves were seen in ~50% of the *mdx* cardiomyocytes these were fully prevented by treatment with S107 and NAC (Fig. 3A and C). DADs are known to be enhanced by β-stimulation; thus, when isoproterenol was applied to cardiomyocytes, spontaneous (i.e., not driven by electrical stimulation) Ca²⁺ transients due to DADs were observed in 40% of cells from *mdx* mice, but in <10% of cells from WT mice (Fig. S3). Treatment with S107 reduced the incidence of spontaneous Ca²⁺ transients in *mdx* mice (Fig. S4).

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, Left). 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 given by the RR interval was similar in WT, *mdx*, and S107-treated *mdx* 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). PVCs are ectopic contractions that originate from the ventricles. 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.

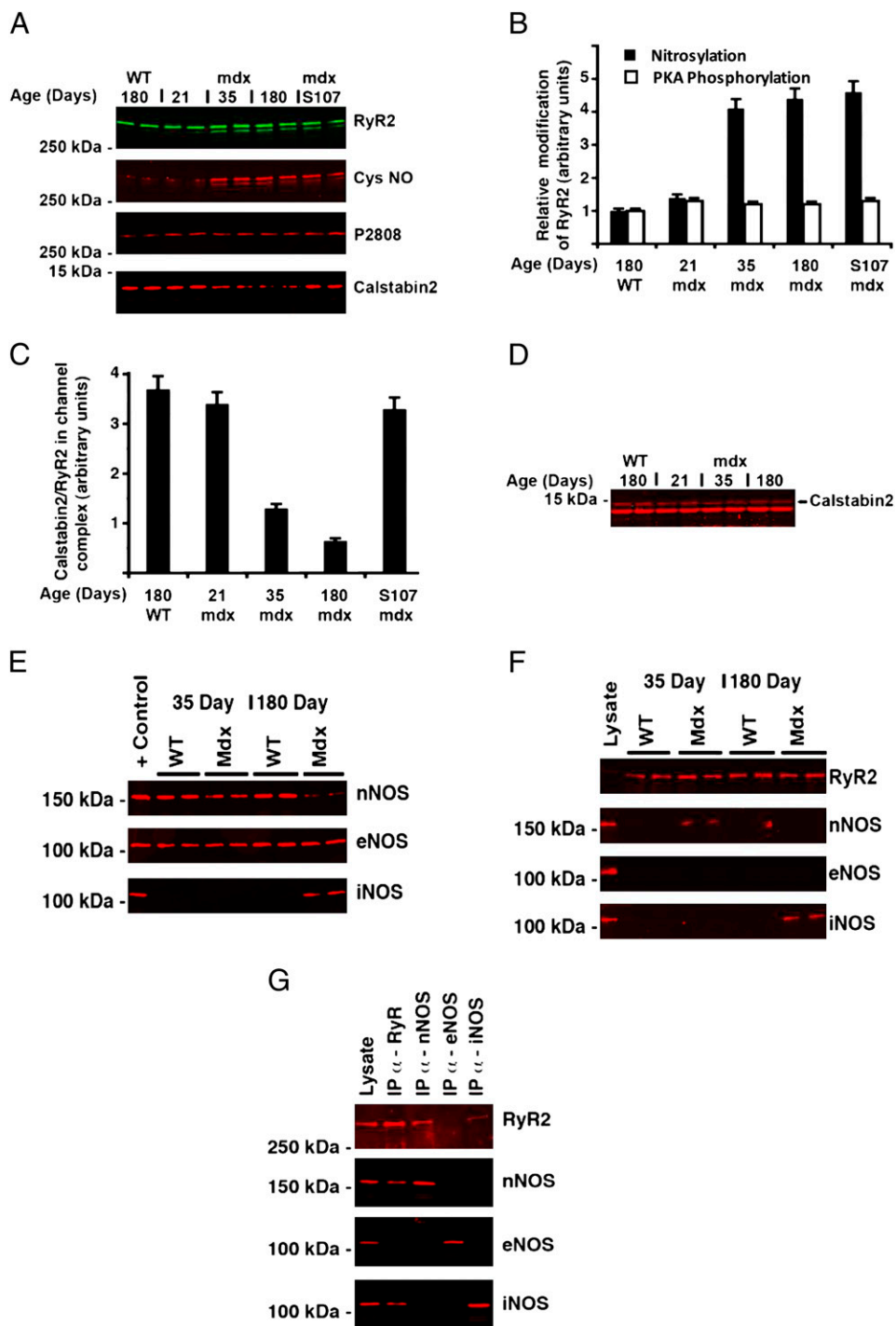


Fig. 1. RyR2 is S-nitrosylated and depleted of calstabin2 in *mdx* mice hearts. RyR2 was immunoprecipitated from heart homogenate of *mdx* and WT littermates at 21, 35, and 180 days after birth, as described in *SI Materials and Methods*. In addition, a group of 5-week-old *mdx* mice were treated for 2 weeks with S107. (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 μ g) separated by 15% PAGE, prepared using an anti-calstabin2 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 μ g 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 μ g 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 μ g of 180-day-old WT heart lysate for RyR2, nNOS, and eNOS and 100 μ g of 180-day-old *mdx* heart lysate for iNOS.

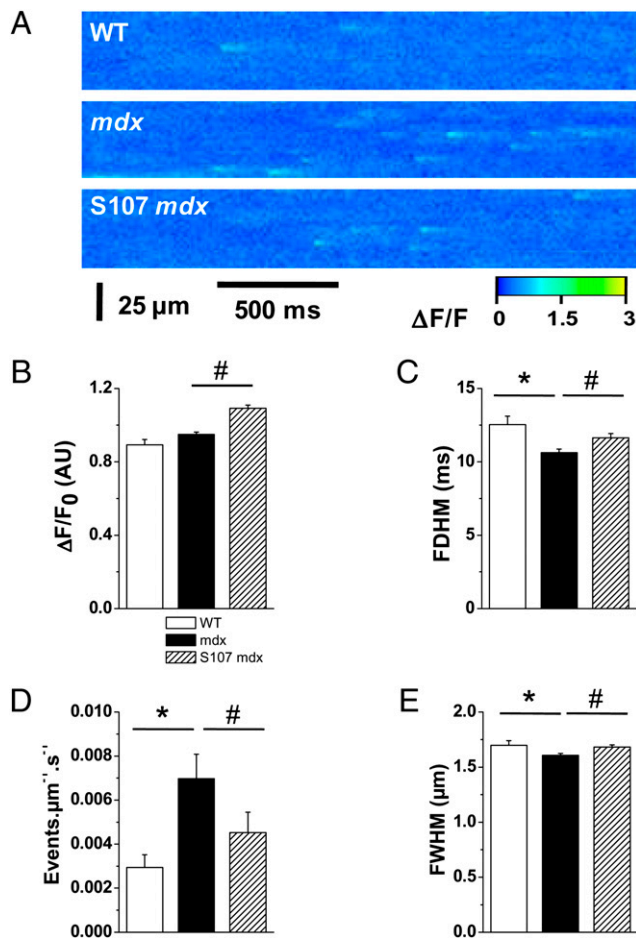


Fig. 2. SR Ca^{2+} leak assessed by Ca^{2+} sparks analysis in *mdx* mice. Spontaneous SR Ca^{2+} release events were recorded in fluo-4 AM-loaded intact cardiomyocytes by laser scanning confocal microscopy, as described in *S1 Materials and Methods*. (A) Representative $\Delta\text{F}/\text{F}$ line scan images (1.54 ms/line) were recorded in WT (Top) and *mdx* mice without (Middle) and with (Bottom) S107 treatment. Diastolic SR Ca^{2+} leak is estimated by the average sparks frequency (D). Average spatiotemporal properties of Ca^{2+} sparks, such as amplitude (B), full duration at half maximum (C), and spatial spread (full width at half maximum) (E). Data are expressed as mean \pm SEM. * $P < 0.05$ WT vs. *mdx*; # $P < 0.05$ *mdx* vs. S107-*mdx*. $n = 195$ sparks in 20 cells in WT, 1,272 sparks in 58 cells in *mdx*, and 889 sparks in 60 cells in S107-treated *mdx* mice.

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 SR Ca^{2+} 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,

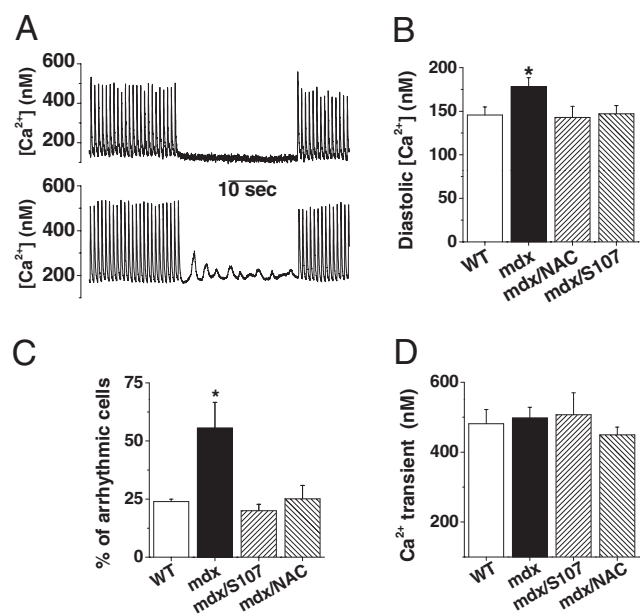


Fig. 3. Elevated diastolic Ca^{2+} concentration in *mdx* mice. Isolated cardiomyocytes, loaded with indo-1 AM as described in *S1 Materials and Methods*, were paced at 1 Hz. After 1 min, cells were maintained quiescent for 30 s before a second train of stimulation. (A) Global Ca^{2+} was recorded simultaneously, as illustrated by two typical recordings in WT and in *mdx* cardiomyocytes. (B) During the stimulation, diastolic Ca^{2+} was elevated in *mdx* cardiomyocyte. This was prevented by NAC or S107 treatment. (C) During the 30-s rest period, ~50% of *mdx* cardiomyocytes exhibited Ca^{2+} waves that were not observed in WT or in *mdx* after NAC or S107 treatment. (D) The peak amplitude of the Ca^{2+} transients did not differ significantly in all conditions. Data are expressed as mean \pm SEM. WT, $n = 24$; *mdx*, $n = 29$; *mdx*-S107, $n = 15$; *mdx*-NAC, $n = 28$. * $P < 0.05$.

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 Ca^{2+} load and thus, in the presence of a leaky RyR2 channel, will increase the driving force for a diastolic SR Ca^{2+} 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

might, however, be mediated by enhanced cGMP signaling (45) or by a compensatory down-regulation of iNOS, as suggested by diminished inflammatory macrophage infiltration (44).

In addition to gene therapy (46) and the recently reported cardioprotective effect of a membrane sealant (47), which offer promising new therapeutic approaches, we provide here an additional target against sudden fatal arrhythmia in DMD. Normalizing RyR2 function and inhibiting diastolic SR Ca²⁺ leak by preventing depletion of calstabin2 from the RyR2 complex and stabilizing a closed state of the channel without interfering with normal channel function have antiarrhythmic effects in *mdx* mice. QT dispersion has been shown to be a good marker of arrhythmias in patients with DMD and represents a major risk factor for the development of ventricular arrhythmias (48). Our results suggest that early antiarrhythmic treatment, through specific modulation of RyR2 properties, could be beneficial in preventing SCD in DMD. Indeed, because multiple therapies have the potential to improve exercise capacity in DMD patients, the risk of cardiovascular pathology and particularly arrhythmias likely will increase. Accordingly, this finding also should be

explored in milder muscular dystrophies, such as Becker muscular dystrophy, that are characterized by cardiac manifestations, including arrhythmias and/or SCD (49).

Materials and Methods

For a detailed description of the methods, see *SI Materials and Methods*.

Animals. Male mice of strain C57BL/10ScSc-Dmdmdx1J, designated *mdx*, were obtained from Dr. D. Mornet (Inserm Eri25, Montpellier, France). Male mice of strain C57BL/10J, designated WT, were obtained from Harlan (Gannat, France). Thirty-five-day-old and 180-day-old weight-matched mice were randomized to treatment with either S107 (20) or vehicle (H₂O). S107 was diluted in drinking water (25 mg/100 mL). The structure and purity of S107 were confirmed by NMR, MS, and elemental analysis, and the specificity of S107 for RyR channels was assessed by extensive testing against a panel of >250 receptors and kinases (20).

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