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Oxidized CaMKII is Essential for Ventricular Arrhythmia in a Mouse Model of Duchenne Muscular Dystrophy

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Abstract

Background—Duchenne muscular dystrophy (DMD) patients are prone to ventricular arrhythmias, which may be caused by abnormal calcium (Ca^{2+}) homeostasis and elevated reactive oxygen species (ROS). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is vital for normal Ca^{2+} homeostasis, but excessive CaMKII activity contributes to abnormal Ca^{2+} homeostasis and arrhythmias in cardiomyocytes. ROS induces CaMKII to become autonomously active. We hypothesized that genetic inhibition of CaMKII oxidation (ox-CaMKII) in a mouse model of DMD can alleviate abnormal Ca^{2+} homeostasis, thus preventing ventricular arrhythmia. The objective of the study was to test if selective loss of ox-CaMKII affects ventricular arrhythmias in the *mdx* mouse model of DMD.

Methods and Results—5-(6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate staining revealed increased ROS production in ventricular myocytes isolated from *mdx* mice, which coincides with elevated ventricular ox-CaMKII demonstrated by Western blotting. Genetic inhibition of ox-CaMKII by knockin replacement of the regulatory domain methionines with valines (MM-VV) prevented ventricular tachycardia in *mdx* mice. Confocal calcium imaging of ventricular myocytes isolated from *mdx*:MM-VV mice revealed normalization of intracellular Ca^{2+} release events compared to cardiomyocytes from *mdx* mice. Abnormal action potentials

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assessed by optical mapping in *mdx* mice were also alleviated by genetic inhibition of ox-CaMKII. Knockout of the NADPH oxidase regulatory subunit $p47^{phox}$ normalized elevated ox-CaMKII, repaired intracellular Ca^{2+} homeostasis, and rescued inducible ventricular arrhythmias in *mdx* mice.

Conclusions—Inhibition of ROS or ox-CaMKII protects against pro-arrhythmic intracellular Ca^{2+} handling, and prevents ventricular arrhythmia in a mouse model of DMD.

Journal Subject Terms

Arrhythmias; Animal Models of Human Disease; Cell Signaling/Signal Transduction; Oxidant Stress; Cardiomyopathy

Keywords

Duchenne muscular dystrophy; calcium/calmodulin-dependent protein kinase II; oxidation; reactive oxygen species

Introduction

Duchenne muscular dystrophy (DMD) is the most prevalent forms of muscular dystrophy. It is an X-linked recessive disorder, caused by mutations within the dystrophin gene.[1] DMD is a fatal disease, affecting 1 in 3,500 male births, characterized by degeneration of muscle tissue with no definitive treatment. Patients with DMD often suffer from cardiomyopathy and may develop cardiac arrhythmias. Cardiomyopathy is found in 25% of DMD patients under the age of 6, 59% of 10 year-old patients, and virtually all adult DMD patients. Moreover, 26% of DMD patients also exhibit ventricular tachycardia (VT) and 51% exhibit an increased heart rate variability.[2]

Recent studies suggest that increased production of reactive oxygen species (ROS) may contribute to the development of cardiomyopathy in DMD. Studies in *mdx* mice, a mouse model of DMD, revealed enhanced ROS production in the hearts of these mice.[3, 4] Abnormal ROS production is one of the key factors leading to altered excitation-contraction coupling in *mdx* mice, which may contribute to contractile dysfunction and enhanced arrhythmogenesis.[4, 5] ROS can alter cardiac excitation-contraction coupling through oxidation of various phosphatases and kinases, including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII).[6, 7] CaMKII is a serine/threonine-kinase essential for intracellular Ca^{2+} homeostasis in cardiomyocytes. CaMKII activity is modulated by several post-translational modifications including autophosphorylation and oxidation, and aberrant CaMKII regulation contributes to development of heart failure and arrhythmias.[8, 9] Moreover, our studies have revealed that CaMKII-mediated phosphorylation of RyR2 promotes VT in *mdx* mice.[10]

Oxidation of two methionine residues (M281 and M282) within the CaMKII regulatory domain locks CaMKII into a constitutively active, Ca^{2+}- and calmodulin-independent conformational state.[7] Previous studies suggest that oxidized CaMKII (ox-CaMKII) can cause sinus node dysfunction.[11] Ox-CaMKII levels were found to be significantly increased

in pacemaker tissues from diabetic patients compared with non-diabetic patients after myocardial infarction. Moreover, activation of a mitochondrial/ox-CaMKII pathway contributes to increased sudden death in diabetic patients after myocardial infarction.[12]

In this study, we tested the hypothesis that increased ROS production promotes ventricular arrhythmias in a mouse model of DMD as a result of CaMKII oxidation. Our findings revealed increased oxidation of M281/M282 on CaMKII in *mdx* mice. Genetic inhibition of oxidation of these residues prevented VT induction in *mdx* mice. We found that ox-CaMKII promoted spontaneous sarcoplasmic reticulum (SR) Ca^{2+} release events, and Ca^{2+} waves that can lead to ectopic activity in the hearts of *mdx* mice. Finally, genetic ablation of $p47^{phox}$, a regulatory subunit of NADPH oxidase type 2 (NOX2), prevented CaMKII oxidation and VT, suggesting that NOX2 is involved in pathological ROS production in *mdx* mice.

Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure.

Experimental Animals

Mdx mice were obtained from Jackson laboratories (C57BL/10ScSn-Dmd*mdx*/J). CaMKII M281/282V(MM-VV) mice, in which methionine residues 281 and 282 are substituted by valines and thereby cannot undergo oxidation, were previously reported.[12] *Mdx:*MM-VV mice were generated by crossing *mdx* mice with MM-VV. All studies were performed on male mice according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Mouse Ventricular Myocyte Isolation

Mouse ventricular myocytes were isolated as described.[4] Briefly, mice were anesthetized and hearts were removed into 0 Ca^{2+} Tyrode solution (137 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM glucose, 3 mM NaOH, pH 7.4). The heart was cannulated through the aorta and connected to a Langendorff system, perfused with 0 Ca^{2+} Tyrode for 5 minutes, followed by 0 Ca^{2+} Tyrode containing 20 μg/ml Liberase (Roche, Indianapolis, IN) for 10 to 15 minutes at 37 °C. After digestion, the heart was removed in Krebs-bicarbonate buffer (90 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 5 mM pyruvic acid, 5 mM β-hydroxybutyric acid, 5 mM creatine, 20 mM taurine, 10 mM glucose, 0.5 mM EGTA, 5 mM HEPES, pH 7.2). The digested heart was minced and agitated in Krebs-bicarbonate buffer, then filtered through a 210 mm polyethylene mesh. The isolated ventricular myocytes were kept in Krebs-bicarbonate buffer before use.

Confocal Imaging

Confocal imaging was performed as described.[4, 13] Isolated ventricular myocytes were incubated in 2 mM Fluo-4-acetoxymethyl ester (Fluo-4 AM, Invitrogen, Carlsbad, CA) in

normal Tyrode's solution containing 1.8 mM Ca^{2+} for 1 hour at room temperature, followed by 15 min for de-esterification with dye-free normal Tyrode's and loaded on a laser scanning confocal microscope (LSM 510, Carl Zeiss, Thornwood, NY). Fluorescence images were recorded in line-scan mode with 1024 pixels per line at 500 Hz. After myocytes reached steady state with pacing at 1 Hz, pacing was stopping and Ca^{2+} sparks were counted. 10 mM caffeine was used to induce caffeine-induced Ca^{2+} transient to calculate SR Ca^{2+} content.

Western Blotting

Heart lysates were prepared from flash-frozen mouse ventricles as described.[14] Ox-CaMKII was detected by using an antibody specific for oxidized M281/M282 on CaMKII, developed by Dr. Anderson (1:1,000).[710] Refer to supplemental methods for further details.

CaMKII Activity Assay

Total CaMKII and Ca^{2+}/CaM-independent CaMKII activity from mouse ventricular lysate were assessed using the SignaTECT CaMKII activity kit (Promega) in conjunction with γ-32P radio-labeled ATP (Perkin Elmer) according to the manufacturer's instructions as described.[15] See supplemental methods for details.

Programmed Electrical Stimulation

Atrial and ventricular intracardiac electrograms were recorded using a 1.1-F octapolar electrode catheter (EPR-800, Millar Instruments, Houston, TX) inserted into the right ventricle via the right jugular vein, as described.[16] VT inducibility was determined using a combination of overdrive pacing and single, double and triple extrastimuli pacing protocols. Sustained VT was defined as ≥10 beats of VT.

Optical mapping

After euthanasia, the mouse heart was quickly removed and rinsed in oxygenated (95% O_2, 5% CO_2) cold normal Tyrode's solution containing 2.5 mmol/L Ca^{2+} and 250 nmol/L isoproterenol. A pacing electrode (Harvard Apparatus, MA, USA) was placed on the surface of the heart, connected to a stimulator (PowerLab 26T, AD Instruments, Sydney, Australia). The anterior epicardial surface was excited using an LED light source centered at 530nm and band pass-filtered from 511 to 551 nm (LEX-2, SciMedia, CA, USA). Blebbistatin (Sigma-Aldrich, B0560-5mg, 50 ul of 2.5 mg/ml in dimethyl sulfoxide) was added to eliminate motion artifacts. The voltage-sensitive dye RH237 was added to the perfusate (Invitrogen, S-1109, 20ul of 2.5mg/ml in dimethyl sulfoxide). The emitted fluorescence Vm signal was collected through a 50mm lens camera (Leica Plan APO 1.0x, SciMedia, CA, USA) and filtered at 700nm. The right atrium was paced at 10-Hz (S1) followed by a single extra stimulus (S2) after 70ms to 40ms, decreasing by 2ms every time.

Statistical Analysis

Results are expressed as mean ± SEM. Continuous variables were evaluated using SPSS or Prism using an unpaired Student *t* test or ANOVA, after performing the D'Agostino-Pearson normality test for normal distribution of the data. Categorical variables were evaluated with Fisher's exact test. $P<0.05$ was considered statistically significant.

Results

Elevated ox-CaMKII increases autonomously activated CaMKII in *mdx* mice

In recent studies, we demonstrated enhanced ROS production in the hearts of 3-month-old *mdx* mice.[4] Consistent with these findings, we found enhanced production of ROS by quantifying 5-(6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate fluorescence in ventricular myocytes of 5-month-old *mdx* mice compared to wild-type (WT) littermates (Supplemental Fig. 1). Western blotting revealed unaltered protein levels of CaMKII in the ventricles of *mdx* mice compared to WT littermates (Fig. 1A). We previously demonstrated unaltered CaMKII autophosphorylation levels of CaMKII in 5-month-old *mdx* mice.[10] In contrast, ox-CaMKII increased by 39±6 % in *mdx* mice compared to WT controls ($P<0.001$; Fig. 1B). This enhanced CaMKII oxidation was abrogated by genetic inhibition of CaMKII oxidation in *mdx*:MM-VV mice, in which M281 and M282 on CaMKII are mutated to valine (MM-VV) to selectively ablate activation by oxidation,[12] while maintaining other pathways for activation including autophosphorylation (Fig. 1B). Previously, it has been shown that CaMKII oxidation at M281/M282 can cause constitutive activation of CaMKII independent of Ca^{2+}/CaM activation.[15] In order to determine whether this increase in ox-CaMKII indeed enhanced Ca^{2+}/CaM-independent activation of CaMKII, we performed a CaMKII activity assay. Consistent with the finding that total CaMKII protein was unaltered, we found that total Ca^{2+}/CaM-activatable CaMKII activity was not changed between groups (Fig. 1C, upper panel). Conversely, the Ca^{2+}/CaM-independent activity was significantly increased 1.8-fold in *mdx* hearts compared to WT ($P=0.037$), and was ameliorated in *mdx*-MMVV hearts (Fig. 1C, lower panel; $P=0.037$). These findings suggest that CaMKII activity is increased in the hearts of *mdx* mice due to enhanced ROS-mediated oxidation of M281/M282.

Genetic inhibition of ox-CaMKII prevents ventricular arrhythmia in *mdx* mice

We previously demonstrated that *mdx* mice develop VT in response to programmed electrical stimulation. Moreover, pharmacological and genetic inhibition of CaMKII prevented VT induction in *mdx* mice.[10] To assess whether oxidation of CaMKII underlies the enhanced susceptibility to arrhythmias, we performed arrhythmia induction studies in *mdx*:MM-VV mice. While 50% of *mdx* mice developed reproducible sustained VT following intracardiac stimulation, none of the *mdx*:MM-VV mice developed VT ($P<0.05$; Fig. 2). These results suggest that oxidation of CaMKII underlies susceptibility to ventricular arrhythmias in *mdx* mice.

Inhibition of ox-CaMKII normalizes intracellular Ca^{2+} handling in *mdx* mice

Prior studies suggest that ventricular arrhythmias in *mdx* mice are in part caused by aberrant Ca^{2+} release events from the SR.[10] Here, we investigated whether ox-CaMKII contributes to abnormal SR Ca^{2+} handling in *mdx* mice. First, confocal imaging was performed to quantify spontaneous SR Ca^{2+} release events known as Ca^{2+} sparks in ventricular myocytes from WT, *mdx*, and *mdx*:MM-VV mice. Compared to WT littermates (1.4 ± 0.4 sparks/100μm/s), *mdx* mice exhibited a three-fold increase in Ca^{2+} spark frequency (CaSpF; 4.2 ± 0.5 sparks/100μm/s; $P<0.001$; Fig. 3A,B). In contrast, the CaSpF was significantly reduced in *mdx*:MM-VV mice (2.1 ± 0.3 sparks/100μm/s) compared to *mdx* mice. A similar reduction

in CaSpF (1.8 ± 0.4 sparks/100μm/s; P<0.01) was found in *mdx* cardiomyocytes treated with KN-93, a pharmacological inhibitor of CaMKII. Further analysis of spark properties revealed an increased Ca^{2+} spark amplitude in *mdx* mice (0.46 ± 0.008 a.u.) compared to WT mice (0.34 ± 0.005; P<0.001; Fig. 3C). Genetic inhibition of ox-CaMKII significantly reduced the spark amplitude in *mdx*:MM-VV mice (0.31 ± 0.004; P<0.001 vs. *mdx*). Again, a similar reduction in spark amplitude was found in *mdx* cardiomyocytes treated with KN-93 (0.23 ± 0.003; P<0.001).

To further examine the potential pro-arrhythmic potential of aberrant SR Ca^{2+} release event, ventricular myocytes were first subjected to a low frequency pacing train (1-Hz) followed by a pause. There was a trend towards an increased frequency of spontaneous Ca^{2+} waves (SCaWs) in *mdx* mice (1.5 ± 0.4 events/min) compared to WT mice (0.4 ± 0.3; Supplemental Fig. 2A). The SCaW frequency was significantly lower in *mdx*:MM-VV mice (0.2 ± 0.2 events/min) compared to *mdx* mice (P<0.01). In addition, there was a lower Ca^{2+} transient amplitude in *mdx* mice compared to WT (P<0.01), although this was not rescued in *mdx*:MM-VV mice (Supplemental Fig. 2B). There were no significant changes in sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a) (Supplemental Fig. 2C) or Na^{+}/Ca^{2+}-exchanger (NCX) activity among the three groups of mice (Supplemental Fig. 2D). Finally, pharmacological inhibition of CaMKII using KN-93 had effects on Ca^{2+} handling similar to genetic inhibition of CaMKII oxidation (which prevents activation).

Next, similar experiments were performed in myocytes paced at 3-Hz. Following a 3-Hz pacing train, the incidence of SCaWs was significantly higher in *mdx* mice (2.6 ± 0.8 events/min) compared to WT littermates (0.6 ± 0.4; P<0.05; Fig. 4A,B). This was reversed by genetic inhibition of ox-CaMKII in *mdx*:MM-VV mice (0.5 ± 0.3; P<0.05 vs *mdx*). Pharmacological inhibition of CaMKII using KN-93 also suppressed Ca waves. To determine whether enhanced SR Ca^{2+} leak altered SR Ca^{2+} storage, 10 mM caffeine was applied to measure SR Ca^{2+} content. Myocytes from *mdx* mice had significantly reduced SR Ca^{2+} content (3.2 ± 0.2 F/Fo) compared to those from WT mice (4.9 ± 0.4, P<0.001; Fig. 4C). In contrast, inhibition of ox-CaMKII in *mdx* mice normalized SR Ca^{2+} content (4.2 ± 0.4; P<0.05 vs *mdx*). Together, these data demonstrate that CaMKII oxidation is a key determinant of aberrant Ca^{2+} homeostasis in myocytes from *mdx* mice.

Inhibition of ox-CaMKII prevents triggered activity in isolated *mdx* mouse hearts

To gain more insight into the mechanisms underlying ventricular arrhythmias in *mdx* mice, optical mapping with simultaneous ECG monitoring was conducted (Fig. 5). Optical mapping of membrane potential was performed on perfused hearts *ex vivo* to visualize action potentials and epicardial activation sequences. In hearts from WT mice, sinus rhythm and normal epicardial activation patterns were observed. Following atrial depolarization, ventricular epicardial activation started from the apex of the right ventricle and spread towards the base. These studies revealed that none of the 5 WT mice developed ventricular arrhythmias (Fig. 5,A,B,G). In contrast, 5 of 7 *mdx* mice (P<0.05 vs WT) exhibited ventricular arrhythmias, initiated by ectopic focal activity (Fig. 5A,C,D,G). In one of these 5 mice, a clear rotor pattern could be seen following arrhythmia initiation. In the other 4 mice, arrhythmias appear to be caused by repeat focal activity. There was a trend towards a

reduced ventricular arrhythmia incidence in *mdx*:MM-VV mice (Fig. 5G; 1 of 6 mice; P=0.10 vs *mdx*).

Next, we assessed the incidence of delayed afterdepolarizations (DADs) following a pacing train. WT mouse hearts did not exhibit any DADs (0 of 5 WT mice, Fig. 5F,H). In contrast, 5 of 7 *mdx* mouse hearts developed DAD (P<0.05; arrow in Fig. 5F, and Fig. 5H). There was a trend towards a reduction in DAD incidence in isolated *mdx*:MM-VV hearts (1 of 6 *mdx*:MM-VV, P=0.10; Fig. 5F,H). KN-93, which is a small molecule inhibitor of CaMKII, also inhibited ventricular arrhythmia induction in *mdx* mice (Supplemental Fig. 3), confirming that CaMKII is a key factor responsible for ventricular arrhythmias in *mdx* mice.

NADPH oxidase mediates CaMKII activation in *mdx* mice

In a final set of experiments, we sought to determine whether NADPH oxidase is the source of ROS in *mdx* mice.[4] p47^{phox} is a key regulatory subunit of the NOX2 complex required for its activity.[17] The oxidation level of CaMKII was reduced in *mdx*:p47$^{-/-}$ mice compared to *mdx* mice, suggesting that NOX2 mediates increased ox-CaMKII in *mdx* mice (Fig. 6A). The incidence of inducible VT was also reduced in *mdx*:p47$^{-/-}$ mice (0 of 8 mice) compared to *mdx* mice (10 of 20 mice; P<0.05; Fig. 6B). Finally, NOX2 inhibition by p47^{phox} ablation also reduced the frequency of Ca^{2+} sparks frequency in *mdx*:p47$^{-/-}$ mice (2.2 ± 0.5 sparks/100μm/s) compared to *mdx* (4.2 ± 0.5, P<0.01; Fig. 6C). These results further support that NOX2-mediated oxidation of CaMKII is responsible for arrhythmogenic Ca^{2+} release in mdx mice.

Discussion

Enhanced CaMKII activity levels have been reported in several cardiovascular and pulmonary diseases, as well as cancer.[8, 18, 19] Since CaMKII is one of the key protein kinases that regulate intracellular Ca^{2+} homeostasis in cardiac myocytes, altered CaMKII activity can facilitate the development of arrhythmias. Indeed, CaMKII hyperactivity has been associated with atrial fibrillation and ventricular arrhythmias in heart failure and dystrophic cardiomyopathy.[10, 20–22]

CaMKII can be activated by several mechanisms including an increased heart rate which leads to autophosphorylation of threonine 287 on neighboring CaMKII subunits within the holoenzyme complex,[23, 24] by ROS which leads to oxidation of methionine residues 281 and 282,[7] or by *O*-GlcNAcylation of serine 279.[25] In a prior study, we demonstrated that cardiac CaMKII autophosphorylation was not increased in 5-month old *mdx* mice, whereas ox-CaMKII levels were increased.[10] Moreover, in the present study we found that autonomous Ca^{2+}/CaM-independent CaMKII activity was significantly increased in *mdx* mice (Fig. 1). Together, these findings suggest that ox-CaMKII is responsible for enhanced CaMKII enzymatic activity at least during early stages of dystrophic cardiomyopathy.

CaMKII can phosphorylate several Ca^{2+} channels and transporters involved in excitation-contraction coupling, including both sarcolemmal and SR channels. In a previous study, we demonstrated that inhibition of CaMKII phosphorylation of RyR2 prevents ventricular arrhythmias and abnormal SR Ca^{2+} leak in *mdx* mice.[10] KN-93 or the transgenic inhibitory

peptide AC3I both suppressed CaMKII activity, and reduced phosphorylation of S2814 on RyR2 in *mdx* mice.[10] RyR2 was proven to be a critical downstream target of CaMKII because mutation of the CaMKII phosphorylation site S2814 on RyR2 to alanine was sufficient to prevent the induction of ventricular arrhythmias in *mdx* mice. Similar to CaMKII post-translational modifications, enhanced oxidation preceded increased phosphorylation of RyR2 in *mdx* mice as described before.[4] Other post-translational modifications may contribute to enhanced RyR2-mediated SR Ca^{2+} leak, including enhanced ROS-dependent nitrosylation,[26] and increased oxidation of RyR2.[5] This may promote a feed-forward mechanism by which enhanced RyR2 channel activity promotes increased ROS production and further protein oxidation. While direct RyR2 oxidation represents one possible mechanism contributing to mdx pathogenesis, our data show that CaMKII inhibition by KN-93 and oxidation site ablation (*mdx*:MM-VV, Fig. 3) ameliorate aberrant SR Ca^{2+} release. This demonstrates that targeting CaMKII activity is sufficient to normalize SR Ca^{2+} in *mdx* mice.

Another SR channel that can be affected by CaMKII phosphorylation is SERCA2a. CaMKII phosphorylation of phospholamban a negative regulator of SERCA2a de-represses SERCA2a activity. However, there is no evidence of enhanced SERCA2a activity in *mdx* mice. On the contrary, two prior publications showed reduced SERCA2a activity in *mdx* mice,[27, 28] whereas in our study we did not find significant differences between SERCA2a activity comparing WT and *mdx* mice (Supplemental Fig. 1). One possible explanation for the discrepancy between our study and the previous studies is that we used *mdx* mice on a mixed Bl6/Bl10 background, whereas the previous studies were performed on *mdx* mice on a Bl10 background.

Sarcolemmal ion channels can also be phosphorylated by CaMKII. The L-type Ca^{2+} channel, Na^{+} channel, and NCX activity can be enhanced by CaMKII phosphorylation. However, in this study we did not find a significant increase in NCX activity (Supplemental Fig. 1), and did not assess L-type Ca^{2+} channel or Na^{+} channel activity. The lack of change in NCX activity may be due to more pronounced ox-CaMKII activity near the SR as compared to the sarcolemma, at least in hearts of 5-month old *mdx* mice.

In the present study, we found a 3-fold increase in the frequency of Ca^{2+} sparks and spontaneous Ca^{2+} waves, consistent with diastolic Ca^{2+} leak through RyR2.[10] Diastolic release of Ca^{2+} can cause DADs in myocytes from *mdx* mice, which in turn can lead to triggered ventricular arrhythmias (Fig. 5). In isolated hearts from *mdx* mice, we observed bidirectional VT and sustained VT, which are also seen in patients with CPVT, a genetic condition caused by gain-of-function RyR2 mutations.[29] In addition, in some *mdx* mice a re-entry pattern with ‘rotor’-like patterns was observed. These findings suggest that triggered arrhythmias may evolve into re-entrant arrhythmias, which may be facilitated by fibrotic structural remodeling,[30] or temporary changes in Ca^{2+} homeostasis as a result of the arrhythmia activity.[22]

In this study, increased ROS production was detected in cardiomyocytes isolated from *mdx* mice, consistent with prior studies.[4, 31] Jung *et al.*[31] suggested that enhanced fragility of the cell membrane resulting from the lack of dystrophin increases susceptibility to mechanical

stress, which in turn causes more ROS production by membrane-associated NADPH. NOX2 and NOX4 are the most abundantly expressed isoforms in adult cardiomyocytes, and increased expression of both has been reported in hearts of *mdx* mice.[28, 32] Prosser *et al.*[33] elegantly demonstrated that NOX2 is activated by disrupted membranes and mechanical stress. Enhanced stretch-dependent ROS production in *mdx* mice promotes intracellular Ca^{2+} waves.[33] Our data are in agreement and show that ROS produced by NOX2 promotes SR Ca^{2+} release abnormalities. Previously, we demonstrated increased ROS in mdx cardiomyocytes, which was abrogated by NOX2 inhibitor gp91ds.[4] Moreover, this is corroborated by the finding that p47^{phox} knockout reduced ox-CaMKII levels in *mdx* mice (Fig. 6). Taken together, these data suggest that in mdx mouse cardiomyocytes increased NOX2-mediated ROS production increases ox-CaMKII, thereby promoting aberrant SR Ca^{2+} release.

Conclusions

In this study, we demonstrated that *mdx* mice – a small animal model of DMD – are susceptible to pacing-induced ventricular arrhythmias. Ox-CaMKII promotes aberrant SR Ca^{2+} release through RyR2, which leads to DADs and triggered ventricular arrhythmias. On the other hand, genetic inhibition of ox-CaMKII normalized intracellular Ca^{2+} handling and prevented ventricular arrhythmias in *mdx*:MM-VV mice. Knockout of the NADPH oxidase regulatory subunit p47^{phox} normalized elevated ox-CaMKII levels, VT, and Ca^{2+} homeostasis in *mdx* mice, suggesting that ox-CaMKII is increased by NADPH oxidase activity in *mdx* mice. Therefore, targeting this oxidation signaling pathway may represent a novel treatment strategy to prevent ventricular arrhythmias in DMD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What Is Known?

- Patients with Duchenne's muscular dystrophy develop life-threatening ventricular arrhythmias.
- Enhanced CaMKII activity is associated with heart disease and arrhythmias, and can be activated by multiple mechanisms including ROS-mediated CaMKII oxidation.

What the Study Adds?

- NOX2 mediated ROS-induced CaMKII activation in the *mdx* mouse model of Duchenne muscular dystrophy promotes aberrant Ca^{2+} release and triggers ventricular arrhythmias
- Genetic inhibition of NOX2 ROS production or CaMKII oxidation, or pharmacological inhibition of CaMKII activity prevents ventricular arrhythmias in the *mdx* mice.
- Targeting CaMKII oxidation may be an effective strategy for treatment of ventricular arrhythmias in Duchenne muscular dystrophy.

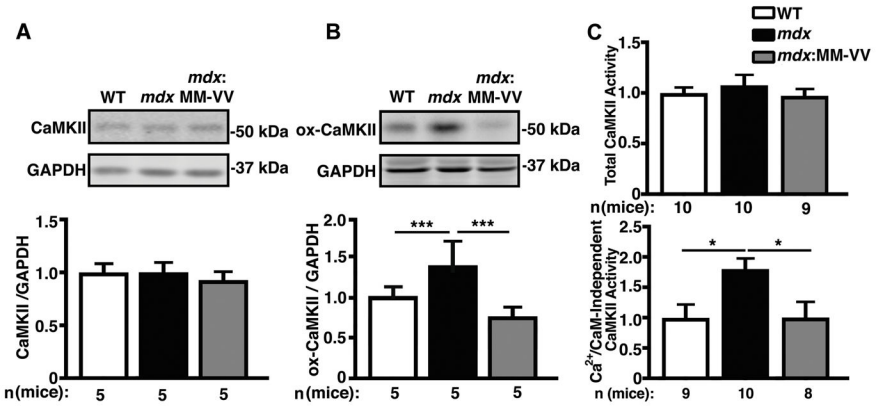

Figure 1. Elevated ox-CaMKII increases autonomously activated CaMKII in *mdx* mice

(A) Representative Western blots and quantification of total CaMKII expression level in *mdx* mouse ventricular lysates compared to WT and *mdx*:MM-VV. **(B)** Representative Western blots and quantification of oxidation at M281/M282 (ox-CaMKII) demonstrating significantly increased expression of oxidized CaMKII in *mdx* mouse ventricular lysates compared to WT and mdx:MM-VV. **(C)** Fold change in total CaMKII activity induced by Ca^{2+}/CaM (top), and Ca^{2+}/CaM-independent CaMKII activity (bottom). *P<0.05, ***P<0.001.

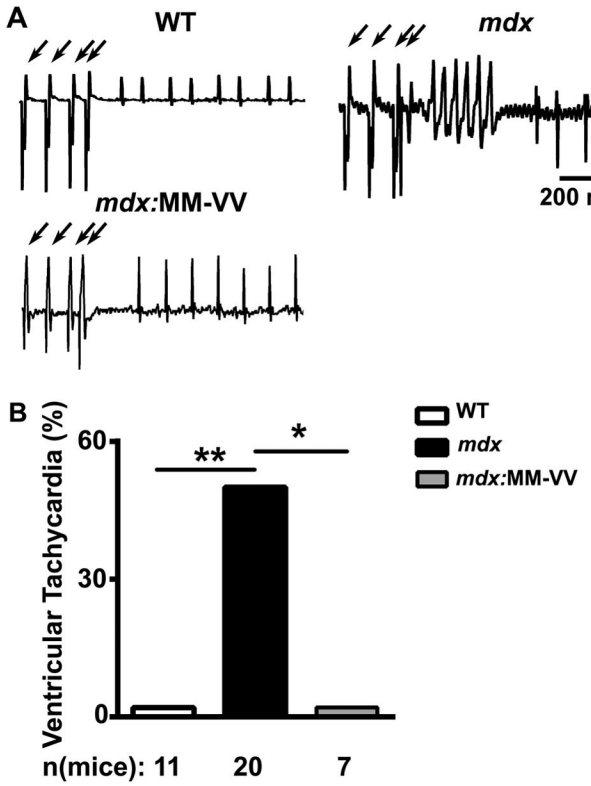

Figure 2. Genetic inhibition of ox-CaMKII inhibits ventricular tachycardia in *mdx* mice

(A) Representative electrograms were recorded from WT, *mdx*, and *mdx:*MM-VV mice following programmed electrical stimulation (PES; arrows). **(B)** Bar graph showing incidence of ventricular tachycardia following PES. *P<0.05, **P<0.01.

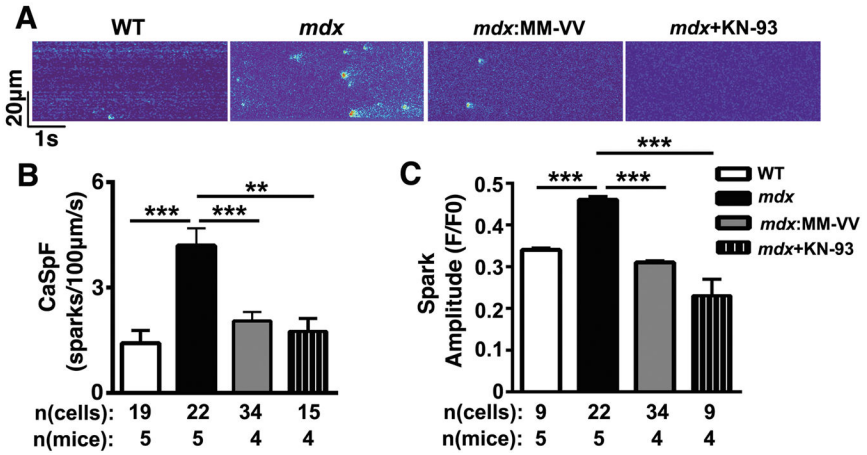

Figure 3. Genetic inhibition of ox-CaMKII normalizes Ca^{2+} sparks in *mdx* mice

(A) Representative line scan images after 1-Hz pacing recorded from isolated ventricular myocytes from WT, *mdx, mdx*: MM-VV mice, and *mdx* myocytes treated with CaMKII inhibitor KN-93. **(B)** Bar graph of averaged Ca^{2+} spark frequencies and **(C)** Ca^{2+} spark amplitude. **P<0.01,***P<0.001.

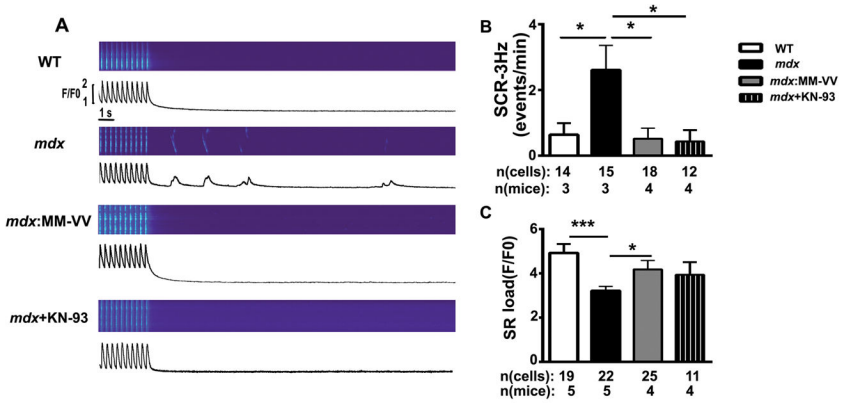

Figure 4. Genetic inhibition of ox-CaMKII normalizes Ca^{2+} waves in *mdx* mice

(A) Representative line scan images after 3-Hz pacing recorded in ventricular myocytes from WT, *mdx, mdx*: MM-VV mice, and *mdx* mice treated with KN-93. **(B)** Average frequency of spontaneous Ca^{2+} waves following 3-Hz pacing and **(C)** quantification of caffeine-induced SR Ca^{2+} load. *P<0.05, *** P<0.001.

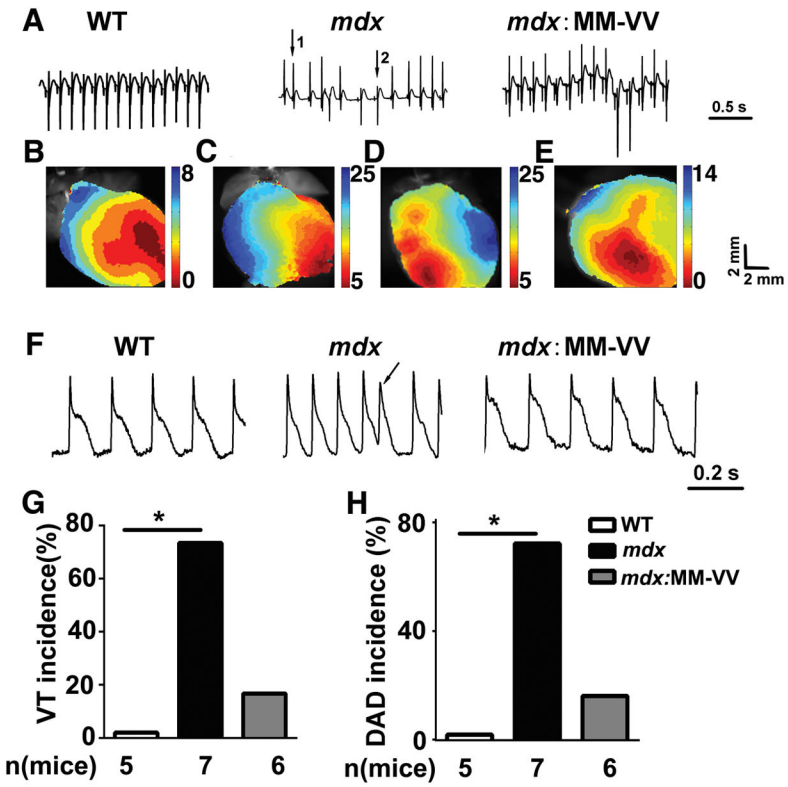

Figure 5. Genetic inhibition of ox-CaMKII attenuates ventricular arrhythmias in isolated *mdx* heart

(A) Representative electrogram traces in *ex vivo* hearts from WT, *mdx* and *mdx*:MM-VV mice. Following sinus rhythm (arrow 1), ventricular tachycardia (VT) beats emerged spontaneously (arrow 2) in the *mdx* mouse heart. **(B)** Normal activation map of WT mouse heart. **(C)** Activation maps of *mdx* mouse heart corresponding to normal beat (arrow 1) and (**D**) the ventricular tachycardia beat (arrow 2). **(E)** Representative activation map of heart from *mdx*: MM-VV mice showing normal activation map. **(F)** Representative action potential (AP) tracings from WT, *mdx*, and *mdx*:MM-VV hearts. Arrow denotes delayed after depolarization (DAD). **(G–H)** Quantification of VT **(G)** and DAD **(H)** incidence in ex vivo WT, *mdx*, and *mdx*:MM-VV hearts.

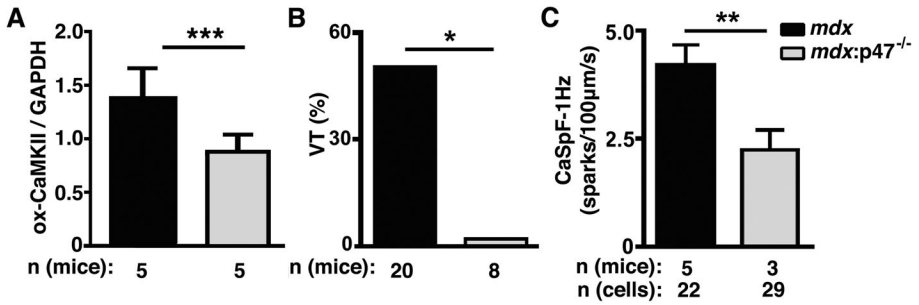

Figure 6. NADPH oxidase activity is required for aberrant ox-CaMKII, heart rhythms, and calcium handling in *mdx* mice

(A) Quantification of Western blots demonstrating ox-CaMKII level in *mdx* mice in which the NADPH oxidase 2 (Nox2) subunit p47^{phox} was absent (mdx:p47$^{-/-}$). **(B)** Incidence of ventricular tachycardia (VT) following programmed electrical stimulation in *mdx* and *mdx*:p47$^{-/-}$ mice. **(C)** Reduced Ca^{2+} spark frequency in myocytes from *mdx*:p47$^{-/-}$ mice compared to *mdx*. *P<0.05, **P<0.01, ***P<0.001.