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Dantrolene, a RyR2 Stabilizer, Restores Impaired Diastolic Function in the Pressure-overloaded Hypertrophied Heart

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Abstract To investigate whether dantrolene (DAN), cardiac ryanodine receptor (RyR2) stabilizer, improves impaired diastolic function in an early pressure-overloaded hypertrophied heart, pressure-overload hypertrophy was induced by transverse aortic constriction (TAC) in mice. Wild-type (WT) mice were divided into four groups: sham-operated mice (Sham), sham-operated mice treated with DAN (DAN+Sham), TAC mice (TAC), and TAC mice treated with DAN (DAN+TAC). The mice were then followed up for 2 weeks. Left ventricular (LV) hypertrophy was induced in TAC, but not DAN+TAC mice, 2 weeks after TAC. There were no differences in LV fractional shortening among the four groups. Catheter tip micromanometer showed that the time constant of LV pressure decay, an index of diastolic function, was significantly prolonged in TAC but not in DAN+TAC mice. Diastolic function was significantly impaired in TAC, but not in DAN+TAC mice as determined by cell shortening and Ca^{2+} transients. An increase in diastolic Ca^{2+} leakage and a decrease in calmodulin (CaM) binding affinity to RyR2 were observed in TAC mice, while diastolic Ca^{2+} leakage improved in DAN+TAC mice. Thus, DAN prevented the progression of hypertrophy and improved the impairment of LV relaxation by inhibiting diastolic Ca^{2+} leakage through RyR2 and the dissociation of CaM from RyR2.

Key words: ryanodine receptor, cardiac hypertrophy, calmodulin, DAN, pressure overload

Introduction

Cardiac hypertrophy is usually considered a compensatory response of the heart to pressure overload.[1] In this compensatory phase, left ventricular (LV) diastolic function is largely impaired by abnormalities in LV relaxation and stiffness.[2] Active LV relaxation is affected by the rate of Ca^{2+} reuptake into the sarcoplasmic reticulum (SR), and abnormalities in LV relaxation are observed early in the course of all cardiac disease.[3] In the clinical setting, diastolic LV relaxation is characterized by a prolonged diastolic LV

relaxation time constant of LV pressure decay, which is calculated by the time constant of the exponential regression: $P(t)= P_0e^{-t/T}$ (P_0 is the LV pressure at max dP/dt, t is the time after onset of relaxation, and T is the time constant).[4] This diastolic dysfunction may be attributed to diastolic heart failure, where persistent pressure overload lead to systolic heart failure and lethal arrhythmia.[5-7] Furthermore, diastolic Ca^{2+} leakage through cardiac ryanodine receptors (RyR2) has been shown to be involved in the development of cardiac hypertrophy and subsequent heart failure.[5-7] Therefore, the suppression of Ca^{2+} leakage through RyR2 could potentially serve as a new treatment for LV diastolic heart failure.

One of the leading hypothesized mechanisms of diastolic Ca^{2+} leakage through RyR2 is a faulty domain switch (zipping/unzipping) as characterized in catecholaminergic polymorphic ventricular tachycardia (CPVT) and heart failure models.[8-13] According to this hypothesis, defective inter-domain interaction in mutation-prone domains between the N-terminal domain (amino acids 1-619) and central domain (amino acids 2000-2500), cause calmodulin (CaM) to dissociate from RyR2, and subsequently leads to diastolic Ca^{2+} leakage.[6,7,14,15] Recently, we developed RyR2(V3599K/+) knock-in (KI) mice and showed that a single mutation enhanced the binding affinity of CaM to RyR2 and inhibited diastolic Ca^{2+} leakage through RyR2, thus mitigating ventricular tachycardia (VT) when crossbred with heterozygous CPVT (R2474S/+) - associated KI mice.[15] Furthermore, in homogenous RyR2 (V3599K/V3599K) KI mice, TAC-induced hypertrophy and progressive heart failure were markedly inhibited by enhancing the affinity of CaM to RyR2, resulting in diastolic Ca^{2+} leakage inhibition through RyR2.[7]

Dantrolene (DAN), a therapeutic agent for malignant hyperthermia, markedly improves the function of failing cardiomyocytes by stabilizing the inter-domain interaction between the N-terminal and central domains within RyR2.[12,16-18] We reported that diastolic Ca^{2+} release due to CaM displacement from RyR2 is a primary cause of pressure overload-induced hypertrophy and that genetic modulation via a V3599K mutation in RyR2 completely inhibited hypertrophy by enhancing the binding affinity of CaM to RyR2 and inhibiting Ca^{2+} leakage.[7] Based on these findings, we investigated whether chronic DAN treatment can prevent LV hypertrophy and improve impaired diastolic function in a mouse TAC model.

Materials and methods

Animal models and study protocol

This study conformed 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). The care of the animals and the protocols used were in accordance with the guidelines of the Animal Ethics Committee of Yamaguchi University School of Medicine. All experimental protocols were approved by the Animal Ethics Committee of Yamaguchi University School of Medicine.[7]

C57BL/6 male mice (10-12 weeks of age) were used in this study. C57BL/6N mice were obtained from Japan SLC, Inc. (Hamamatsu, Japan). All *in vivo* experiments were performed in random order. Mice were anesthetized in an induction chamber with 2% isoflurane mixed with 0.5-1.0 L/min of 100% O_2. We used 7.0 silk to make two loose knots tied around the transverse aorta, and a small piece of a 27½ gauge blunt needle was placed parallel to the transverse aorta. The first knot was quickly tied against the needle, and the second needle was promptly removed to yield a constriction of 0.4 mm diameter constriction.[7] For sham control mice, the entire procedure was identical, except for ligation of the aorta.

DAN was purchased from Sigma Aldrich (St Louis, MO, USA). As shown in Fig. 1A, 20 mg/kg/day DAN was intraperitoneally injected 1 week before surgery and continued for 2 weeks. Wild-type (WT) mice were randomly divided into four groups: (1) mice with a sham operation and an intraperitoneal injection (i.p.) of solvent only (Sham); (2) mice with a sham operation and an i.p. of solvent and DAN (DAN+Sham, 20 mg/kg/day); (3) mice with a TAC operation and an i.p. of solvent only (TAC); and (4) mice with a TAC

operation and an i.p. of solvent and DAN (DAN+TAC).

Transthoracic echocardiography

Echocardiograms were obtained as previously described.[7] Cardiac function was analyzed using an F37 ultrasound machine (Hitachi Medical, Netherlands) equipped with a 7.5 MHz probe (Hitachi, UST-5413). The mice were initially anesthetized using 2-3% isoflurane (mixed with oxygen) and maintained with 1-2% isoflurane during echocardiography. Cardiac ventricular dimensions were measured using M-mode echocardiography.[6,7,15]

LV pressure analysis

We anesthetized mice with 1.5% isoflurane and fixed them on a heating pad (37℃). The abdominal cavity was opened and a small opening was made under the diaphragm. From the apex of the heart, a 1.4-F high-fidelity micromanometer catheter (Millar Instruments, Houston, TX, USA) was introduced into the left ventricular cavity to measure and record LV pressure, as described previously.[7]

Histology

Hearts from mice were fixed in 10% formalin. Hematoxylin-eosin and Masson's trichrome staining were performed for each section of the heart, as described previously.[7,15]

Isolation of cardiomyocytes

Cardiomyocytes were isolated from mouse hearts, as described previously.[5-7,15] The mice were anesthetized with pentobarbital sodium (70 mg/kg, i.p.) and heparin (i.p.). The chest cavity was opened, and the heart was quickly removed and perfused in retrograde with collagenase-free buffer via the aorta under constant flow in the perfusion system. Finally, isolated cardiomyocytes were transferred to laminin-coated glass culture dishes and incubated at 37℃ in a 5% CO_2 and 95% O_2 atmosphere.[5-7,15]

Analysis of Ca^{2+} sparks and sarcoplasmic reticulum (SR) Ca^{2+} content

We measured Ca^{2+} sparks using a laser-scanning confocal microscope (LSM-510, Carl Zeiss).[5-7,15] Briefly, isolated cardiomyocytes were loaded with Fluo-4 acetoxymethyl ester (Fluo-4 AM, 20 μM; Molecular Probes, OR, USA) for 20 min at 37℃ in a 5% CO_2 and 95% O_2 atmosphere. The microscope was set to line-scan mode, wherein a single cardiomyocyte was scanned repeatedly (520.8 Hz) along a line parallel to the longitudinal axis, avoiding the nuclei. To monitor Ca^{2+} sparks, cardiomyocytes were stimulated until the Ca^{2+} transient frequency reached a steady state, and then stimulation was stopped and Ca^{2+} sparks were recorded during the subsequent 10 second rest period.[5-7,15]

Data was analyzed using SparkMaster, an automated analysis program that allows for rapid and reliable Spark analysis.[5-7,15] To assess the SR Ca^{2+} content, caffeine (10 mM) was rapidly perfused into the cardiomyocytes to discharge SR-loaded Ca^{2+}, as described previously.[5-7,15]

Ca^{2+} transient monitoring in cardiomyocytes

Isolated ventricular myocytes were incubated with 2.0 μM Fluo-4 AM for 20 min at room temperature and washed twice with Tyrode's solution. All experiments were conducted at 28℃. Intracellular Ca^{2+} in cells stimulated by a field electric stimulator (IonOptix, MA, USA) was measured using a fluorescent digital microscope (BZ9000, Keyence, Japan).[5-7,15]

Binding affinity of endogenous CaM to the RyR2 in intact cardiomyocytes

Isolated cardiomyocytes were fixed with 4% paraformaldehyde for 5 min, as previously described.[5-7,15] The isolated cardiomyocytes were then incubated overnight at 4℃ with a monoclonal mouse anti-CaM antibody (EP799Y, Abcam) and anti-RyR2 antibody (Sigma-Aldrich, C3-33) in 1% BSA and 0.5% Triton X-100, followed by fluorescent labeling with an Alexa488-conjugated goat anti-rabbit secondary antibody (Molecular Probes) and Alexa633-conjugated goat anti-mouse secondary antibody (Molecular Probes), respectively. For colocalization of the anti-CaM antibody (Alexa488; green) and anti-RyR2 antibody (Alexa633; red), images were obtained at 633 nm and 488 nm excitation in conjunction with an LP 650 filter and a BP 505-530 nm filter, respectively.[5-7,15]

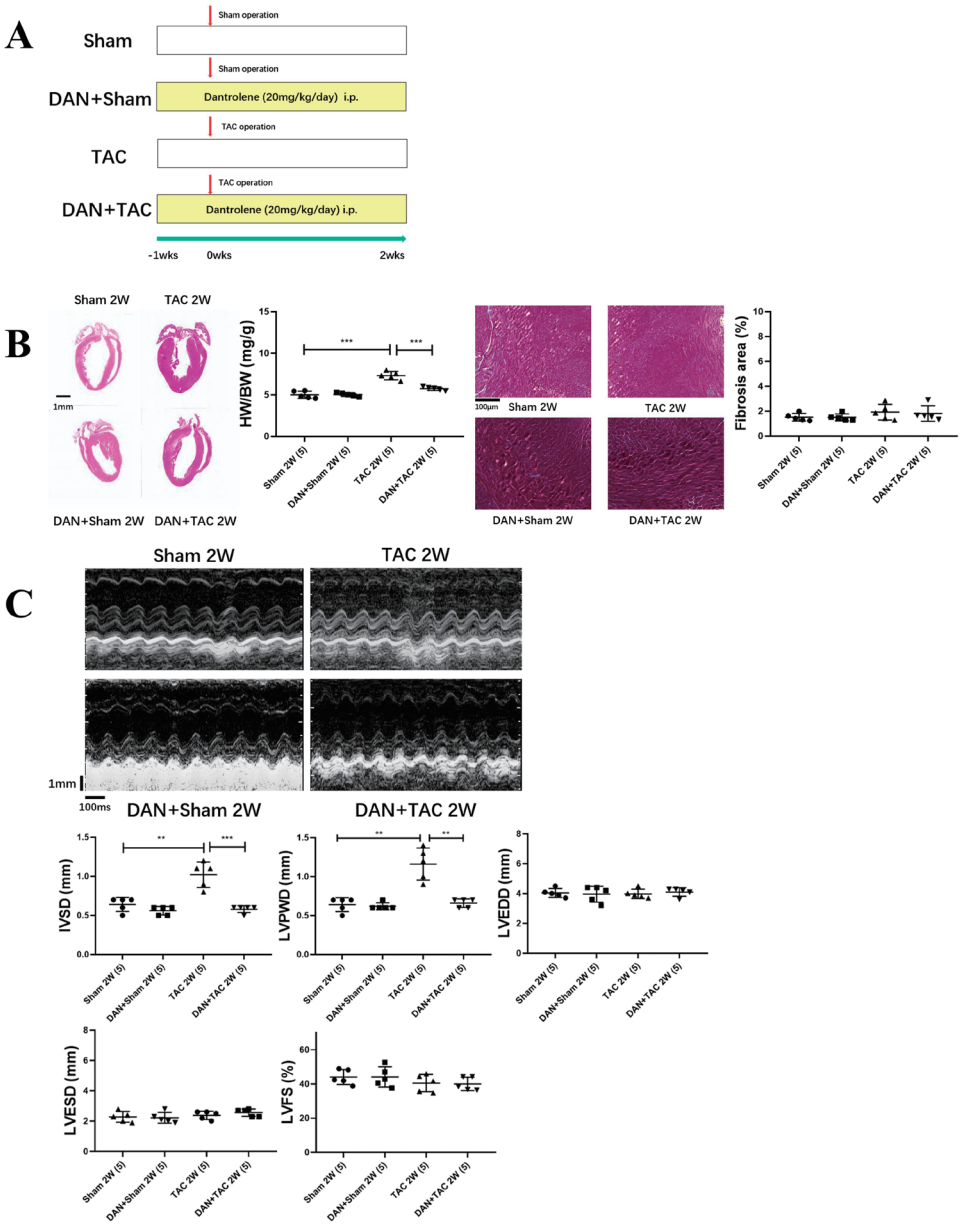

Fig. 1 Study design, structural and functional characteristics after TAC in mice.

A. Study design of the TAC model. B. Representative images of long axis sections of the hearts stained with hematoxylin eosin and summarized data of heart weight (HW)/body weight (BW). Representative images of left ventricular (LV) stained with Masson's trichrome and the summarized data of LV fibrosis area. C. Representative images of echocardiography and summarized data. LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV fractional shortening (LVFS) ([LVEDD − LVESD]/LVEDD × 100), intra-ventricular septum diastolic thickness (IVSD), left ventricular posterior wall diastolic thickness (LVPWD). Individual mouse values are plotted as the mean ± standard deviation (SD). The numbers in parentheses are the numbers of mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (one-way analysis of variance [ANOVA] with a post-hoc Tukey's multiple comparison test).

Western blot

We performed immunoblot analysis of SR Ca^{2+}-ATPase (SERCA2a: Affinity Bioreagents, Golden, CO, USA), phospholamban (PLN; Millipore, Billerica, MA, USA), phosphorylated PLN at serine 16 (P-Ser16-PLN: Fitzgerald, Acton, MA, USA), phosphorylated PLN at threonine 17 (P-Thr17-PLN: Santa Cruz Biotechnology, Dallas, TX, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; CHEMICON International, Inc.), as described previously.[19]

Results

LV hypertrophy was observed in the TAC mice, but not in the TAC mice treated with DAN

Histology and echocardiography at 2 weeks after the sham operation, showed no difference in the structural and functional features of the heart between Sham and DAN+Sham mice (Fig. 1B, C). After 2 weeks of TAC, the LV wall thickness (intra-ventricular septum diastolic thickness, left ventricular posterior wall diastolic thickness) and LV weight to body weight ratio increased in TAC mice, but not in DAN+TAC mice (Fig. 1B, C). Moreover, there was no difference in LV interstitial fibrosis among the four groups (Fig. 1B).

Catheter-tip micromanometer showed that LV relaxation was significantly impaired in TAC mice, but not in TAC mice treated with DAN

Both peak systolic pressures increased to the same level in the TAC and DAN+TAC groups (Fig. 2A, B). This indicates that a comparable degree of pressure overload was imposed on the LV. There was no difference in LV end-diastolic pressure (LVEDP) between the groups 2 weeks post-operation. Interestingly, 2 weeks after TAC, the +dP/dt max and -dP/dt min of LV pressure were preserved in TAC mice. The time constant (tau) of LV pressure decay, which represents an index of passive LV relaxation, was significantly prolonged in TAC mice but not in DAN+TAC mice (Fig. 2B).

Analyses of morphology, cell shortening and Ca^{2+} transient, Ca^{2+} sparks, SR Ca^{2+} content in intact cardiomyocytes

There was no difference in cell length among the four groups, but cell area and width were larger in TAC mice than in TAC mice treated with DAN (Fig. 3A, B), indicating that TAC-induced hypertrophy was significantly inhibited. Cell shortening and Ca^{2+} transient data showed that there were no differences in systolic function (% sarcomere from baseline, peak of Ca^{2+} transient) among the four groups (Fig. 3C, D). However, the time from peak to a 80% decline in cell shortening, and time from peak to an 80% decline in Ca^{2+} transient were significantly longer in TAC mice than in TAC mice treated with DAN (Fig. 3C, D), indicating that chronic DAN treatment restored the impaired LV relaxation in TAC cardiomyocytes (Fig.3C, D). Moreover, Ca^{2+} spark frequency was significantly increased in TAC mice, but not in Sham, DAN+Sham, and DAN+TAC mice (Fig. 3E). Lastly, sarcoplasmic reticulum (SR) Ca^{2+} content was not significantly different among the four groups (Fig. 3F).

Chronic DAN treatment inhibited the dissociation of CaM from RyR2 in the pressure overloaded heart

We evaluated whether chronic DAN administration improved CaM binding to RyR2 in TAC cardiomyocytes. In basal cardiomyocytes, only approximately 1% of CaM is freely diffused, bound CaM is concentrated on the Z line, and more than 90% is bound to RyR2.[13] Therefore, most of the CaM on the Z line is thought to be bound to RyR2. In the present study, endogenous CaM on the Z line was significantly reduced in the TAC group, but not in the DAN+TAC group (Fig. 4A, B).

There were no differences in the protein expression levels of SERCA, and PLN and the phosphorylation levels of RyR2 and PLN

Western blotting was performed to clarify whether the protein expression of SERCA, and PLN, as well as the phosphorylation of PLN (Ser 16 is a protein kinase A [PKA] phosphorylation site and Thr 17 is a Ca^{2+}/calmodulin-dependent protein kinase II [CaMKII] phosphorylation site) affect intracellular Ca^{2+} handling. As shown in Fig. 5, there were no differences in the total protein levels of SERCA and PLN, as well as the phosphorylated PLN at ser16 and Thr17, among all

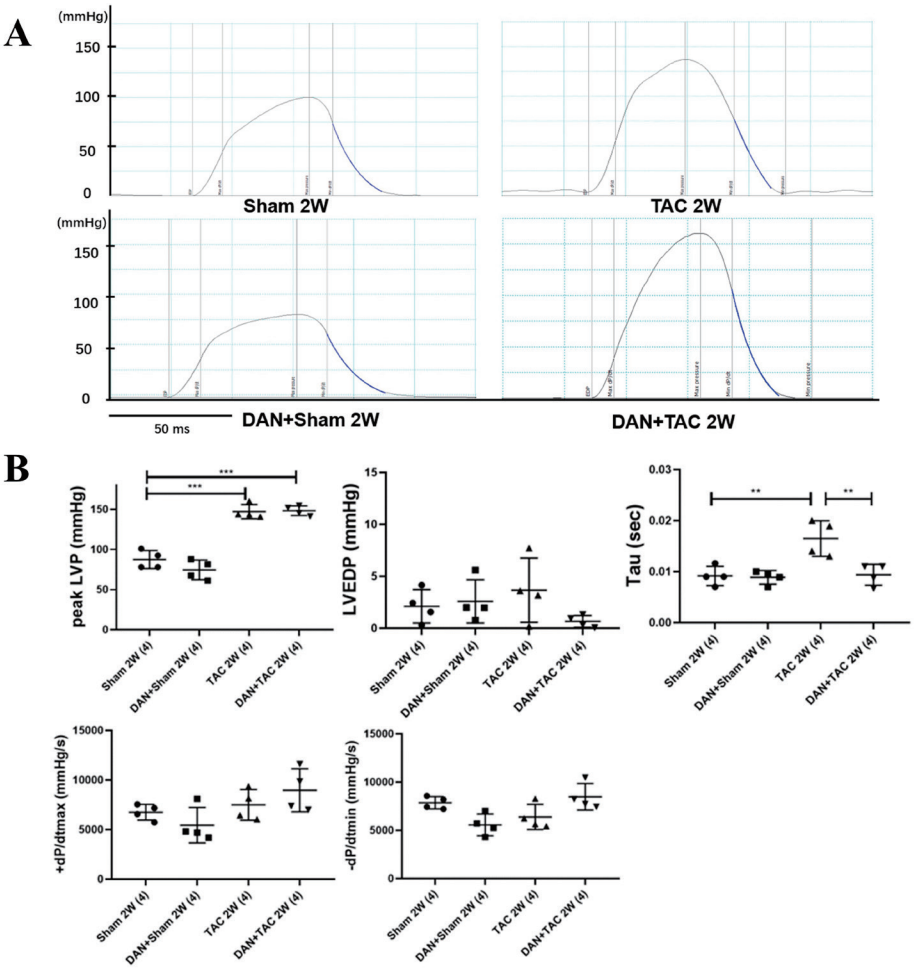

Fig. 2 Analyses of systolic and diastolic property from the left ventricular (LV) pressure waveform.

A. Representative images of LV pressure. B. Summarized of hemodynamic parameters: Peak left ventricular pressure (LVP), +dP/dt max of LVP, -dP/dt min of LVP, Tau, LV relaxation time constant of LV pressure decay; LV end-diastolic pressure (LVEDP). Individual mouse values are plotted as the mean ± standard deviation (SD). The numbers in parentheses are the numbers of mice. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance with post-hoc Tukey's multiple comparison test).

groups.

Discussion

General overview

Our study showed that RyR2-stabilization with DAN prevented LV hypertrophy and impaired LV relaxation in TAC-induced pressure-overloaded hearts. This is supported by the following observations: 1) LV hypertrophy developed 2 weeks after TAC in TAC mice, but not in TAC mice treated with DAN; 2) histological examination showed that 2 weeks of TAC-induced pressure overload did not cause an increase in myocardial fibrosis in either the TAC or TAC with DAN groups; and 3) measurement of LV pressure in vivo using a catheter tip micromanometer revealed that LV relaxation impairment (prolongation of tau) occurred in the TAC group, although the other systolic (dP/dt max) and diastolic (-dP/dt min) indices were not impaired. However, no LV relaxation impairments were observed in the TAC-treated DAN group. To our knowledge, this is the first report to demonstrate that chronic DAN treatment can

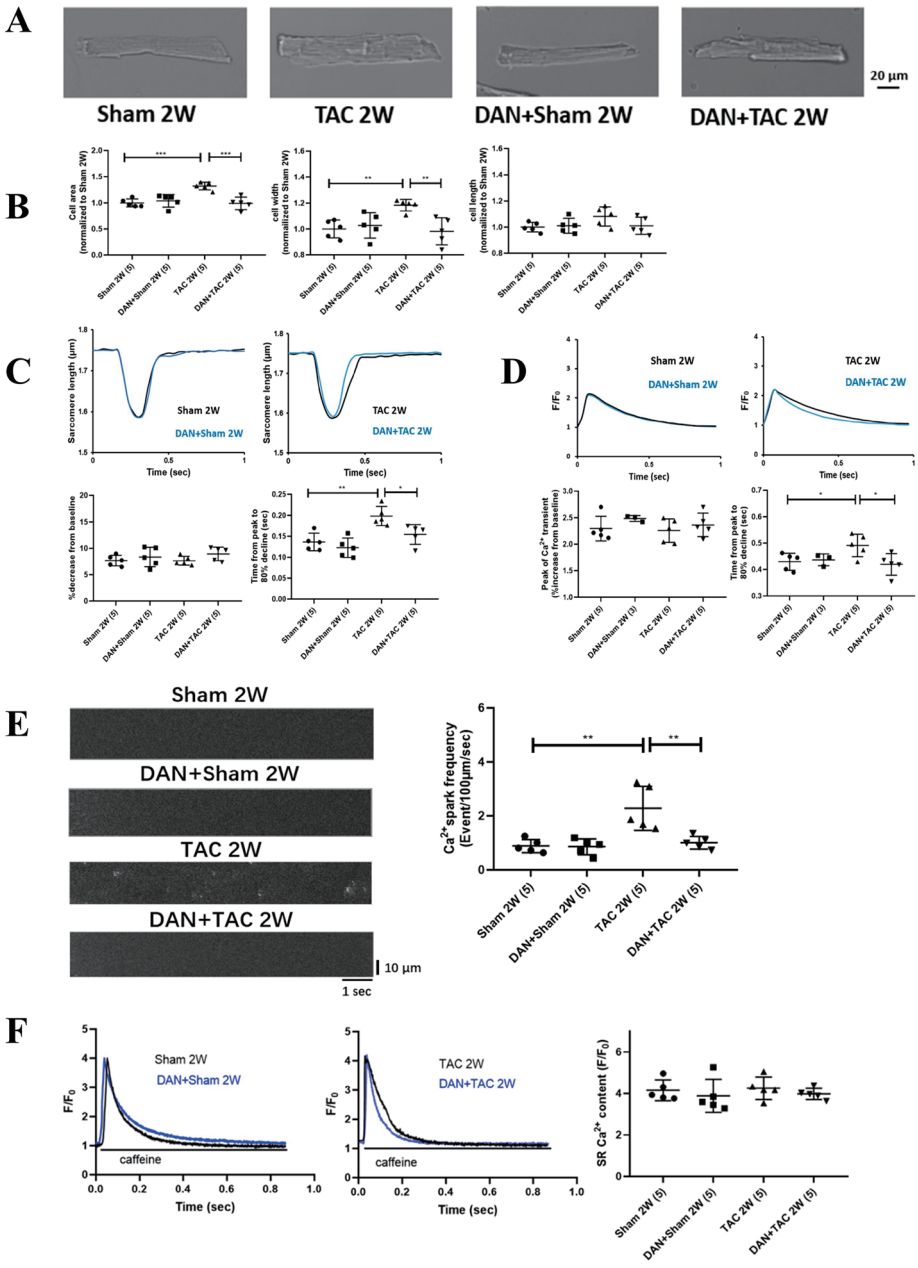

Fig. 3 Morphology, Ca^{2+} transients, sarcomere shortening, Ca^{2+} sparks, and content in intact cardiomyocytes.

A. Representative images of isolated cardiomyocytes. B. Summarized data of the cell area, cell width, and cell length in isolated cardiomyocytes (n = 250-400 cells from five hearts.) C. Representative recordings of sarcomere shortening at a pacing rate of 1 Hz, and summarized data oftime from peak to 80% decline (n = 20-30 cells from five hearts.) D. Representative recording of Ca^{2+} transients and the summarized data. Data was measured using Fluo-4 AM fluorescent signals at a pacing rate of 1 Hz (n = 20-30 cells from 3-5 hearts.) E. Representative recordings of spontaneous Ca^{2+} sparks and summarized data of spontaneous Ca^{2+} spark frequency (n=10-20 cells from five hearts.) F. representative recordings of SR Ca^{2+} content measured from caffeine-induced Ca^{2+} transient and summarized data of SR Ca^{2+} content (n=10-20 cells from five hearts). Values for individual mice are plotted together as mean ± standard deviation (SD). Parentheses indicate the number of mice. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance with post-hoc Tukey's multiple comparison test).

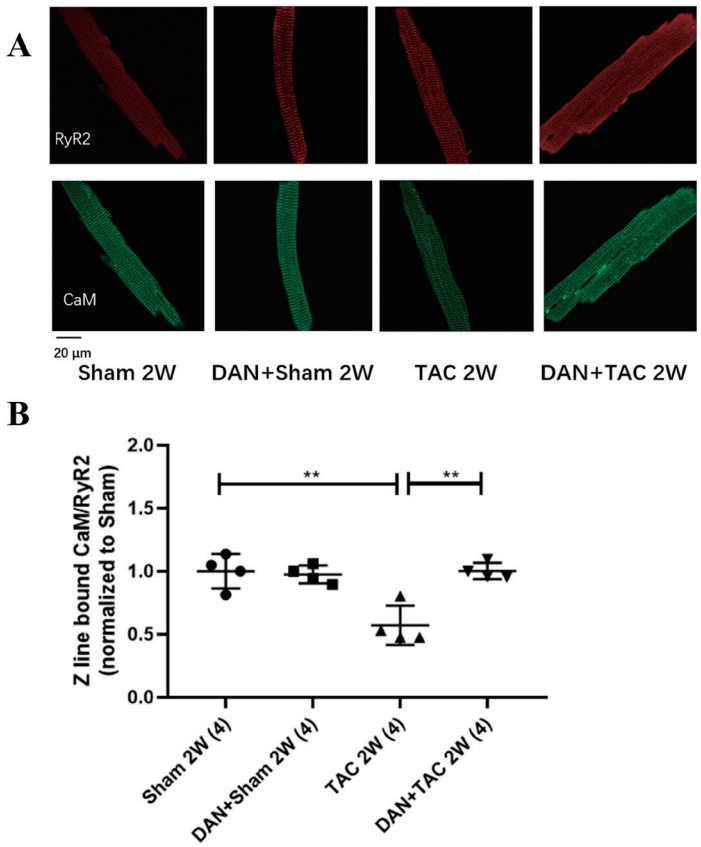

Fig. 4 CaM-RyR2 interaction in cardiomyocytes.

A. Representative images of endogenous CaM co-localized with RyR2. B. Summarized data of the Z-line bound CaM. The immuno-fluorescence signal of the Z-line bound CaM was divided by that of RyR2, normalized to control (baseline of wild-type [WT] Sham), and expressed as a ratio. N=20-38 cells from 4 hearts. Values for individual mice are plotted together as mean ± standard deviation (SD). Parentheses indicate the number of mice. *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance with post-hoc Tukey's multiple comparison test). CaM, calmodulin.

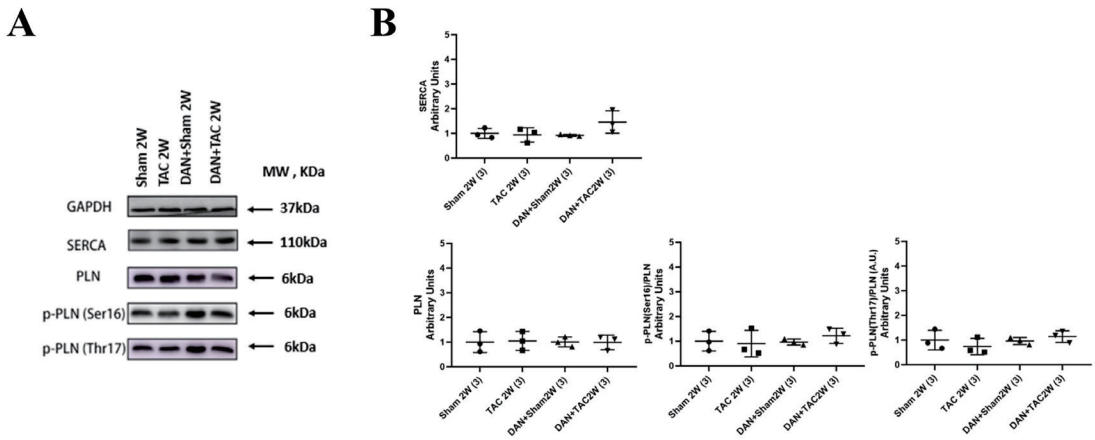

Fig. 5 Western blots of total RyR2, SERCA, total PLN, and phosphorylated RyR2, PLN.

A. Representative images of western blots of total SERCA, PLN, and phosphorylated PLN. B. Summarized data of western blots. Data represent mean ± standard deviation from three hearts. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PLN, phospholamban; SERCA, sarcoplasmic reticulum Ca^{2+}-ATPase.

inhibit hypertrophy and improve the impairment of LV relaxation in the early stage of a pressure-overloaded heart.

DAN as a stabilizer of RyR2 prevents the dissociation of CaM and the diastolic Ca^{2+} leakage from RyR2 in the pressure-overloaded hypertrophic heart

DAN binds to amino acids Leu^{590}-Cys^{609} of the N-terminal domain and restores the defective inter-domain interaction between the N-terminal and central domains in MH-type RyR1.[16,20] Interestingly, DAN specifically binds to the corresponding Leu^{601}-Cys^{620} domain in RyR2 which is completely preserved.[12,19] This causes a conformational change from unzipping to zipping between N-terminal and central domains to enhance the binding affinity of CaM to RyR2, and subsequently inhibits Ca^{2+} leakage in CPVT, and heart failure.[5,6,12-15,17,18] Tetrameric structure analysis of RyR2 using cryo-three-dimensional (3D) electron microscopy revealed that the zipping/unzipping interface between the N-terminal (1-220 amino acids) and central domain (2250-2500 amino acids) is proximal to both the CaM-binding domain (3583-3603 amino acids) and DAN-binding site (601-620 amino acids), suggesting that DAN structurally suppresses CaM dissociation and domain unzipping, thereby stabilizing channels.[7,21-23]

Postulated mechanism by which chronic DAN treatment prevents LV hypertrophy and impaired LV relaxation

Recently, we reported that a genetic mutation at V3599K of RyR2 that inhibited diastolic Ca^{2+} leakage by enhancing the binding affinity of calmodulin (CaM) to RyR2, markedly inhibiting LV hypertrophy.[7] While acute pressure overload to WT cardiomyocytes normally upregulated the hypertrophic signal transduction in the CaMKII-histone deacetylase, and the calcineurin-nuclear factor of activated T cells pathways, that to V3599K RyR2 cardiomyocytes did not.[7] DAN directly binds to Leu^{601}-Cys^{620} of the N-terminal domain,[12,24] and its binding corrects the defective inter- and intra-subunit interactions between the N-terminal domain and central domains within the tetrameric RyR2, which inhibits CaM dissociation and Ca^{2+} leakage from RyR2.[5-7,23] Therefore, DAN is thought to prevent LV hypertrophy due to the inhibition of CaM dissociation and Ca^{2+} leakage from RyR2.

Abnormalities in LV relaxation and stiffness play a crucial role in heart failure with preserved ejection fraction.[2] At the cellular level, abnormalities in Ca^{2+} uptake, possibly caused by dysfunction in SERCA, and PLN,[25] greatly contribute to the impairment of LV relaxation.[2-4] However, western blot analysis showed that there were no differences in the expression levels of total SERCA, PLN, and phosphorylated PLN. The present study showed that chronic DAN treatment prevented diastolic Ca^{2+} leakage through RyR2 and improved the prolonged decline of Ca^{2+} transient waves and the impaired relaxation of cell shortening, resulted in the improvement of the impaired LV relaxation in TAC mice.

Limitations

Although DAN did not affect the protein expression of SAECA2a, and PLN, or the phosphorylation levels of PLN, further examination is needed to clarify whether DAN affects Ca^{2+} handling through proteins other than RyR2.

Conclusion

Stabilization of RyR2 with chronic DAN treatment suppressed cardiac hypertrophy and restored the impaired diastolic function by enhancing CaM binding to RyR2 thus preventing diastolic Ca^{2+} leakage.

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Conflict of Interest

The authors declare no conflict of interest.

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