Mitochondrial calcium overload is a key determinant in heart failure

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Calcium (Ca^{2+}) released from the sarcoplasmic reticulum (SR) is crucial for excitation–contraction (E–C) coupling. Mitochondria, the major source of energy, in the form of ATP, required for cardiac contractility, are closely interconnected with the SR, and Ca^{2+} is essential for optimal function of these organelles. However, Ca^{2+} accumulation can impair mitochondrial function, leading to reduced ATP production and increased release of reactive oxygen species (ROS). Oxidative stress contributes to heart failure (HF), but whether mitochondrial Ca^{2+} plays a mechanistic role in HF remains unresolved. Here, we show for the first time, to our knowledge, that diastolic SR Ca^{2+} leak causes mitochondrial Ca^{2+} overload and dysfunction in a murine model of postmyocardial infarction HF. There are two forms of Ca^{2+} release channels on cardiac SR: type 2 ryanodine receptors (RyR2s) and type 2 inositol 1,4,5-trisphosphate receptors (IP3R2s). Using murine models harboring RyR2 mutations that either cause or inhibit SR Ca^{2+} leak, we found that leaky RyR2 channels result in mitochondrial Ca^{2+} overload, dysmorphology, and malfunction. In contrast, cardiac-specific deletion of IP3R2 had no major effect on mitochondrial fitness in HF. Moreover, genetic enhancement of mitochondrial antioxidant activity improved mitochondrial function and reduced posttranslational modifications of RyR2 macromolecular complex. Our data demonstrate that leaky RyR2, but not IP3R2, channels cause mitochondrial Ca^{2+} overload and dysfunction in HF.

ryanodine receptor | heart failure | mitochondria | calcium | IP3 receptor

Type 2 ryanodine receptor/Ca^{2+} release channel (RyR2) and type 2 inositol 1,4,5-trisphosphate receptor (IP3R2) are the major intracellular Ca^{2+} release channels in the heart (1–3). RyR2 is essential for cardiac excitation–contraction (E–C) coupling (2), whereas the role of IP3R2 in cardiomyocytes is less well understood (3). E–C coupling requires energy in the form of ATP produced primarily by oxidative phosphorylation in mitochondria (4–8).

Both increased and reduced mitochondrial Ca^{2+} levels have been implicated in mitochondrial dysfunction and increased reactive oxygen species (ROS) production in heart failure (HF) (6, 7, 9–17). Albeit Ca^{2+} is required for activation of key enzymes (i.e., pyruvate dehydrogenase phosphatase, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase) in the tricarboxylic acid (also known as Krebs) cycle (18, 19), excessive mitochondrial Ca^{2+} uptake has been associated with cellular dysfunction (14, 20). Furthermore, the exact source of mitochondrial Ca^{2+} has not been clearly established. Given the intimate anatomical and functional association between the sarcoplasmic reticulum (SR) and mitochondria (6, 21, 22), we hypothesized that SR Ca^{2+} release via RyR2 and/or IP3R2 channels in cardiomyocytes could lead to mitochondrial Ca^{2+} accumulation and dysfunction contributing to oxidative overload and energy depletion.

Results and Discussion

Increased Mitochondrial Ca^{2+} in Failing Hearts. Cardiac mitochondrial Ca^{2+} (Fig. 1 *A*–*D* and Fig. S1) and ROS (Fig. 1*E*) were significantly elevated in mice following myocardial infarction (MI).

To determine whether the observed mitochondrial Ca^{2+} overload in failing hearts can be caused by SR Ca^{2+} leak via RyR2, we used a murine model harboring a mutation that renders the channels leaky (RyR2-S2808D) and a second model (RyR2-S2808A) with RyR2 channels protected against leak. Ca^{2+} sparks frequency (diastolic openings of RyR2 channels that reflect SR Ca^{2+} leak) was significantly increased (Fig. S2*A*), and SR Ca^{2+} load reduced (Fig. S2*B*) in cardiomyocytes from RyR2-S2808D mice compared with WT and RyR2-S2808A cardiomyocytes.

Notably, RyR2-mediated SR Ca^{2+} leak (Fig. S2) was associated with increased mitochondrial Ca^{2+} (Fig. 1 *A* and *D*) and ROS production (Fig. 1*E*). Constitutive cardiac SR Ca^{2+} leak via RyR2 (RyR2-S2808D mice) resulted in dysmorphic and malfunctioning mitochondria (Fig. S3). We observed a marked reduction in mitochondrial size (Fig. S3*D*), aspect ratio (Fig. S3*G*), and form factor (Fig. S3*H*) in left ventricular cardiomyocytes harboring leaky RyR2 channels, reflecting a low fusion-to-fission ratio. These data indicate that intracellular Ca^{2+} leak via RyR2 correlates with augmented mitochondrial fragmentation, strongly supporting a functional role for Ca^{2+} in regulating mitochondrial morphological dynamism.

Importantly, our data showing increased cardiac mitochondrial Ca^{2+} in HF, determined in absolute values in isolated organelles (Fig. 1*A*) and confirmed in dynamic evaluations at the cellular level (Fig. 1 *B*–*D* and Fig. S1), reconcile conflicting reports concerning mitochondrial Ca^{2+} in failing hearts (7, 10, 12, 13, 15, 17).

Significance

We demonstrate that intracellular Ca^{2+} leak causes mitochondrial Ca^{2+} overload and dysfunction in postischemic heart failure (HF). In particular, sarcoplasmic reticulum (SR) Ca^{2+} leak via type 2 ryanodine receptor (RyR2)—but not type 2 inositol 1,4,5-trisphosphate receptor (IP3R2)—channels plays a fundamental role in the pathophysiology of mitochondrial Ca^{2+} overload and dysfunction in HF. We present here a previously undisclosed molecular mechanism in HF with crucial implications in cardiac physiology. Indeed, our data establish a feedback loop between SR and mitochondria in which SR Ca^{2+} leak triggers mitochondrial dysfunction and increases the production of free radicals, which in turn lead to posttranslational modifications of RyR2 and enhance intracellular Ca^{2+} leak, thereby contributing to impaired cardiac function after myocardial infarction.

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Reviewers included: G.S.P., Duke University.

Conflict of interest statement: A.R.M. is a consultant and member of the board of ARMGO, which is targeting RyR channels for therapeutic purposes.

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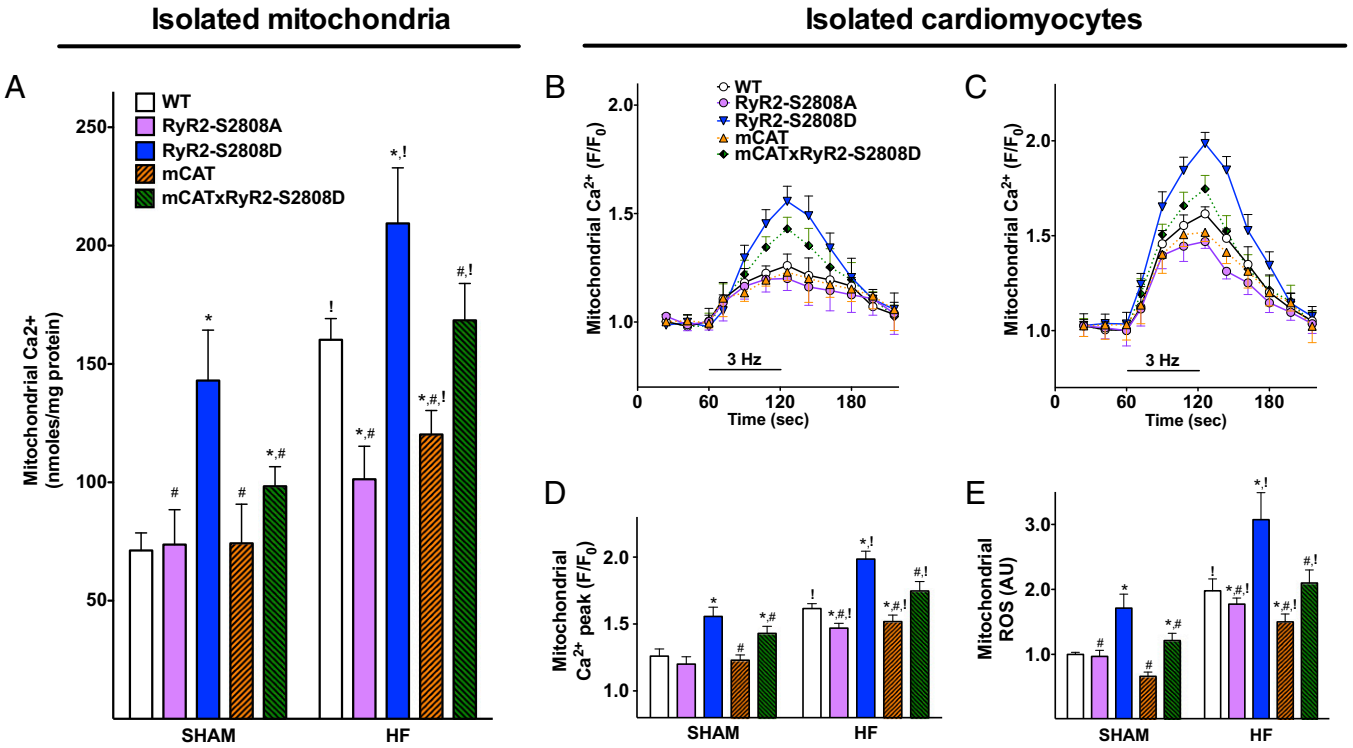


Fig. 1. Increased mitochondrial Ca^{2+} in post-MI heart failure. (*A*) Direct measurement of total Ca^{2+} content in mitochondria isolated from sham or failing ventricular samples of 6-mo-old WT, RyR2-S2808A, RyR2-S2808D, mCAT, and mCAT × RyR2-S2808D mice. Mitochondria were purified from ≥6 mice in each experimental group. (*B–D*) Mitochondrial Ca^{2+} dynamics in response to 3 Hz in cardiomyocytes (n = 22–35) enzymatically isolated from at least 7 mice per group isolated from the indicated groups. (*E*) Mitochondrial ROS generation in ventricular cardiomyocytes isolated from the indicated mice using the mitochondria-targeted fluorescent indicator of superoxide production MitoSOX Red; n > 120 ventricular myocytes from ≥4 mice in each group. Data shown represent mean ± SEM from triplicate experiments. *P < 0.05 vs. WT; $^{\#}P$ < 0.05 vs. RyR2-S2808D, ANOVA, Tukey–Kramer post hoc test; $^{!}P$ < 0.05 vs. SHAM, two-tailed t test. AU, arbitrary units; ROS, reactive oxygen species.

Effects of Redox Imbalance on RyR2 Channel in Postischemic HF. We have previously shown that protein kinase A (PKA) phosphorylation and oxidation of RyR2 channels cause SR Ca^{2+} leak and contribute to HF progression (1, 2). HF-related PKA phosphorylation—in part also attributable to decreased cAMP type 4 phosphodiesterase, PDE4D3, in the RyR2 channel complex (23)—nitrosylation, and oxidation of RyR2 were attenuated in a mouse model (mCAT) with decreased ROS levels obtained via targeted overexpression of human catalase in mitochondria (Fig. 2 *A–D*).

Reduced binding of the RyR2 stabilizing subunit calstabin2 (24) to the channel due to RyR2 oxidation and PKA phosphorylation causes spontaneous diastolic SR Ca^{2+} release contributing to cardiac dysfunction in HF (1, 2). Genetically reducing RyR2 oxidation (mCAT mice) or preventing RyR2 PKA phosphorylation (RyR2-S2808A mice harboring RyR2 channels that cannot be PKA-phosphorylated) improved calstabin2 and PDE4D3 binding to RyR2 (Fig. 2 *A–F*) and cardiac performance (Fig. 2*G* and Table S1) after MI. Mitochondrial morphology (Fig. 3 *A–I* and Fig. S4) and function (Fig. 3 *J–M* and Table S2) were also improved in mCAT mice.

Oxidative overload in cardiomyocytes originates from multiple sources, including mitochondria, NAD(P)H oxidase, xanthine oxidase, and uncoupled nitric oxide synthase (16, 25, 26). Mitochondrial-derived ROS are elevated during cardiac overload or ischemic stress (4, 26, 27). Mitochondrial membrane potential, $\Delta\psi_m$, is closely linked to Ca^{2+} levels and to mitochondrial ROS production; indeed, depolarized mitochondria produce more ROS, leading to further organelle depolarization, resulting in a vicious cycle. The decrease in $\Delta\psi_m$ observed in mitochondria from RyR2-S2808D ventricular cardiomyocytes (Fig. S3*J*) is consistent with a progressive decline in $\Delta\psi_m$ due to increasing [Ca^{2+}] in cardiac mitochondria and is most likely due to elevated cytosolic [Ca^{2+}] caused by RyR2-mediated SR Ca^{2+} leak (10). Supporting this view, mitochondria exposed to elevated [Ca^{2+}] exhibit reduced $\Delta\psi_m$, due to the large mitochondrial Ca^{2+} current generated during local [Ca^{2+}] transients (28).

Ventricular cardiomyocytes harboring constitutively leaky RyR2 channels exhibited a reduction in mitochondrial ATP content and generation (Fig. S3 *L* and *M*), consistent with previous observations in failing human hearts (6). Further studies are needed to investigate in detail other systems, including neurohormonal and (epi)genetic mechanisms, endoplasmic reticulum (ER) stress, necrosis/apoptosis, and autophagy, that might participate in the regulation of bioenergetic homeostasis in HF (5, 6, 19, 25, 29).

Distinctive Roles of RyR2 and IP3R2 in the Pathophysiology of Mitochondrial Dysfunction in HF. To determine the source of SR Ca^{2+} leak that causes mitochondrial overload in failing hearts, we investigated the roles of the two major Ca^{2+} release channels on myocardial SR: RyR2 and IP3R2 (1).

We generated a murine model ($IP3R2^{CVKO}$) in which IP3R2 expression was specifically ablated in ventricular cardiomyocytes via *Cre/Lox* recombination (Fig. S5 *A–E*). $IP3R2^{CVKO}$ mice survived to adulthood without alterations in baseline myocardial function, and there was no up-regulation of the other two isoforms of IP3R (IP3R1 and IP3R3) (Fig. S5 *F* and *G*). Ca^{2+} sparks, SR Ca^{2+} load (Fig. S6), mitochondrial Ca^{2+} level (Fig. S6*C* and Fig. S7 *A* and *B*), and ROS production (Fig. S6*D*) were not significantly changed in $IP3R2^{CVKO}$ ventricular cardiomyocytes evaluated both in sham or post-MI mice. Myocardial mitochondria from $IP3R2^{CVKO}$ mice were normal (Fig. 4), and there was no

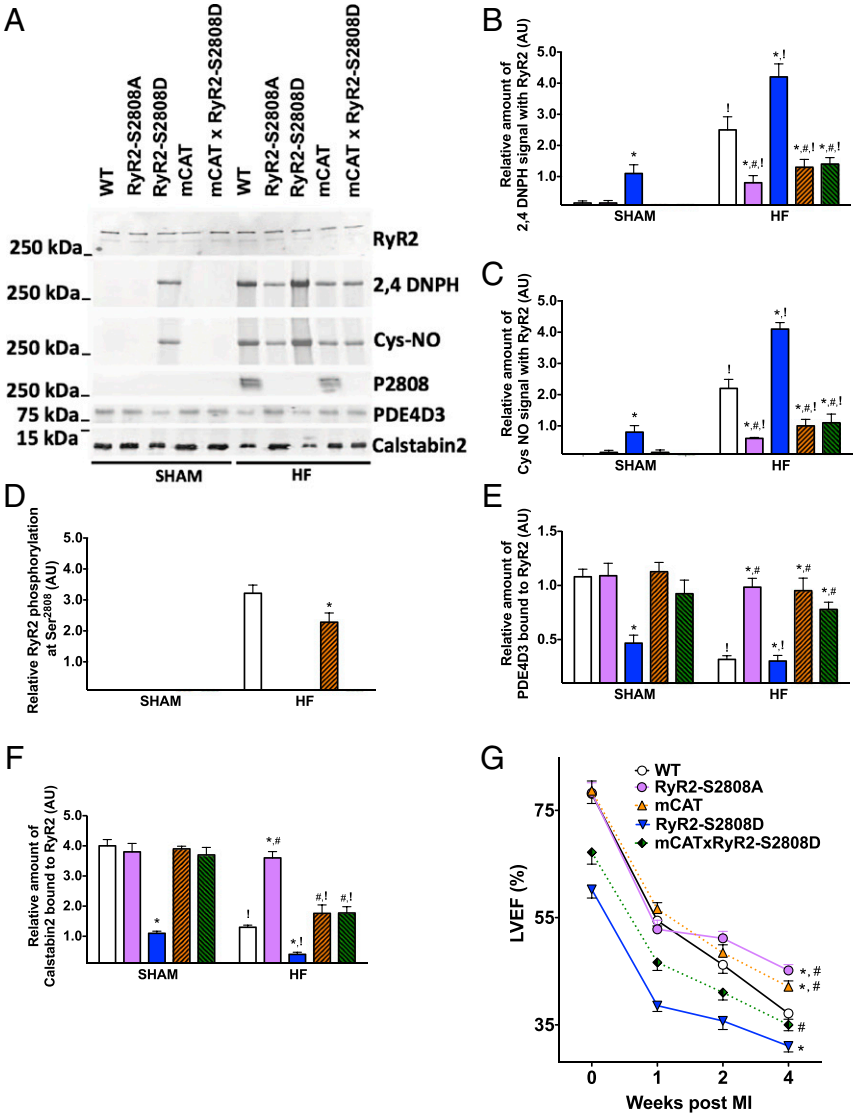


Fig. 2. Posttranslational modifications of RyR2 complex and HF progression post-MI. (*A*) Biochemical evaluation of RyR2 macromolecular complex in left ventricular samples from sham and heart failure (HF) mice. To determine channel oxidation, the carbonyl groups in the protein side chains of immunoprecipitated RyR2 were derivatized to 2,4 dinitrophenylhydrazone (2,4 DNPH) by reaction with 2,4 dinitrophenylhydrazine. The 2,4 DNPH signal associated with RyR2 was determined by anti-DNP antibody (specificity for RyR2 was achieved due to immunoprecipitation of the protein). Anti-Cys NO antibody analysis of immunoprecipitated RyR2 was used to measure RyR2 nitrosylation. Quantification of RyR2 oxidation (*B*), Cys-nitrosylation (*C*), phosphorylation at Ser^{2808} (*D*), PDE4D3 (*E*), and calstabin2 (*F*) bound to RyR2; note that constitutive phosphorylation of Ser^{2808}, mimicked by the aspartate residue substitution in RyR2-S2808D mice, cannot be detected. Data shown represent mean ± SEM from triplicate experiments. $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.05$ vs. RyR2-S2808D, ANOVA, Tukey–Kramer post hoc test; $^{!}P < 0.05$ vs. SHAM, two-tailed *t* test. (*G*) Progressive cardiac dysfunction after myocardial infarction (MI) assessed by serial echocardiographic analyses. LVEF, left ventricular ejection fraction. Data are shown as mean ± SEM; $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.05$ vs. RyR2-S2808D; ANOVA repeated measures; $n = 16–20$ per group. AU, arbitrary units. See also Table S1.

major effect on acute HF progression (Fig. S7*C* and Table S1). Further investigations are warranted to explore the potential role of IP3R2 in ischemia/reperfusion and in long-term ischemic HF, especially given the reported involvement of IP3R2 in advanced stages of HF (30).

Prevention of RyR2 Posttranslational Modifications Attenuates Mitochondrial Dysfunction in HF. Mitochondrial ROS levels were markedly reduced in cardiomyocytes isolated from mCAT × RyR2-S2808D mice [mice expressing leaky RyR2 channels (RyR2-S2808D) crossed with mCAT mice] compared with RyR2-S2808D littermates, both in HF and sham conditions (Fig. 1*E*). Moreover, RyR2 oxidation and nitrosylation were significantly decreased in left ventricular samples from mCAT × RyR2-S2808D mice compared with RyR2-S2808D littermates (Fig. 2 *A*–*C*). SR Ca^{2+} leak (Fig. S2) and mitochondrial Ca^{2+} accumulation (Fig. 1 *A*–*D* and Fig. S1) observed in RyR2-S2808D were significantly reduced after crossing with mCAT mice. Additionally, post-MI, HF progression was markedly attenuated in mCAT × RyR2-S2808D mice (Fig. 2*G* and Table S1). These data show that mitochondria are a critical source of ROS that oxidizes RyR2 and promotes SR Ca^{2+} leak in failing hearts although there are likely additional sources of ROS, such as xanthine oxidase, that are significantly increased in failing hearts (Fig. S8).

Genetic ablation of the RyR2 PKA phosphorylation site at Ser^{2808} attenuated cardiac mitochondrial dysmorphology after MI (Fig. 3 *B* and *F*–*I*) and reduced mitochondrial ROS levels (Fig. 1*E*), indicating that, in addition to oxidation, PKA phosphorylation

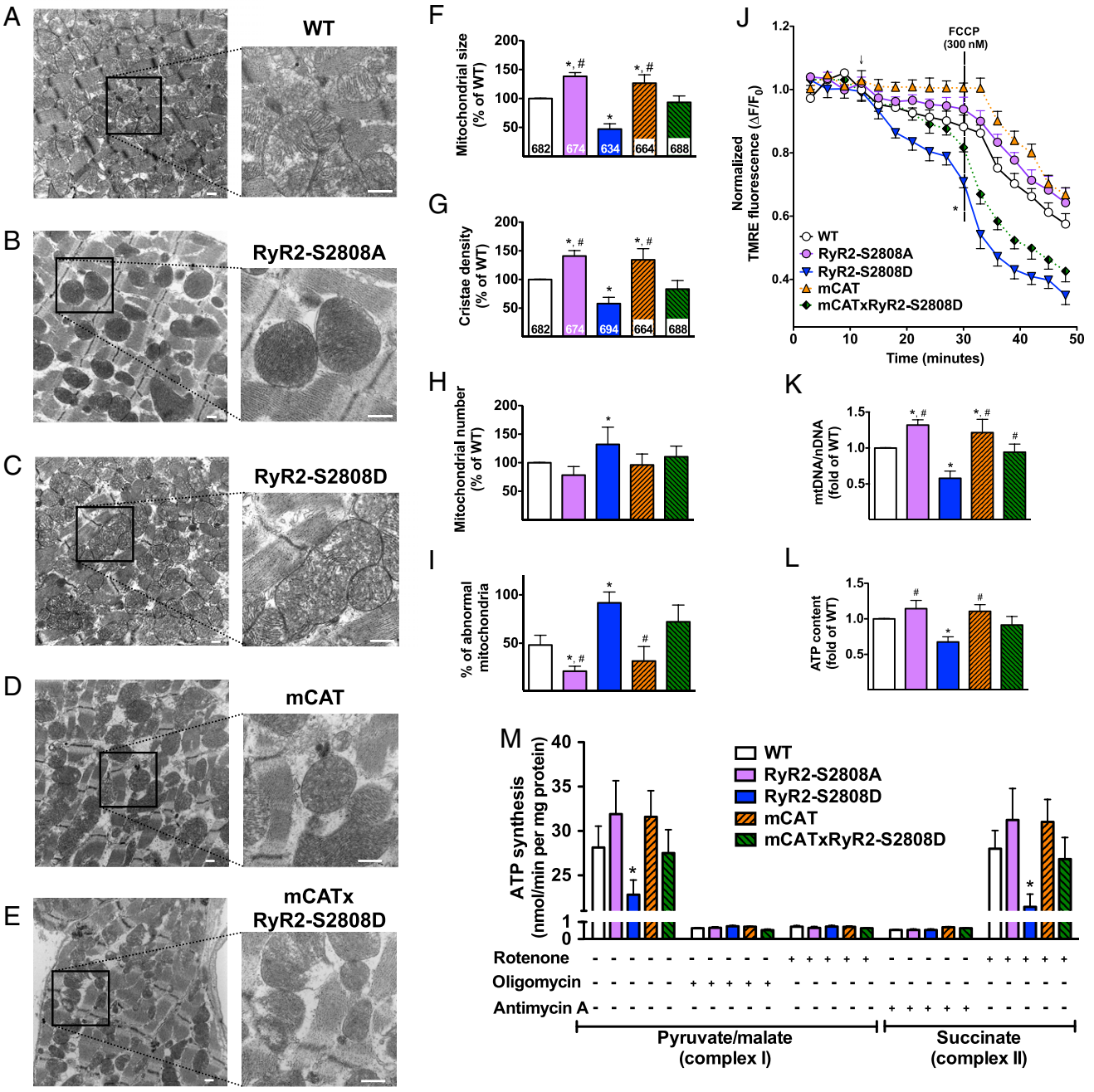


Fig. 3. Leaky RyR2 channels and mitochondrial dysfunction in heart failure. (*A–E*) Representative transmission electron micrographs of cardiac mitochondria post myocardial infarction from WT (*A*), RyR2-S2808A (no leak) (*B*), RyR2-S2808D (leaky) (*C*), mCAT (*D*), mCAT × RyR2-S2808D (*E*), $n = 5$ per group. (Magnification: *A–E*, 15,000×; *Insets*, 50,000×.) (Scale bars: 500 nm.) Note the diffuse myofibrillar disarray. (*F–I*) Quantification of ultrastructural mitochondrial alterations depicted in *A–E*. Mitochondrial size (*F*) and cristae density (*G*). Numbers in the bars indicate the number of mitochondria analyzed. Quantification of mitochondrial number per image (*H*) and percentage of abnormal mitochondria per image at 15,000× (*I*). Relative number of damaged mitochondria was quantified by blinded observers from 8 to 10 images from different fields. Data are shown as mean ± SEM, $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.05$ vs. RyR2-S2808D, ANOVA, Tukey–Kramer post hoc test. (*J*) Assessment of the inner mitochondrial membrane potential ($\Delta\psi_m$); the arrow denotes addition of H_2O_2 (100 μM). FCCP, carbonylcyanide-p-trifluoro-methoxy-phenyl-hydrazone. The * indicates significant difference ($P < 0.05$, ANOVA repeated measures, Tukey–Kramer post hoc test) of the WT group ($n = 8$) compared with RyR2-S2808D ($n = 7$), mCAT × RyR2-S2808D ($n = 7$), and mCAT ($n = 6$), or between RyR2-S2808D and mCAT × RyR2-S2808D groups. (*K*) Mitochondrial DNA (mtDNA)/nuclear DNA (nDNA) copy number and (*L*) ATP content assessed in left ventricle ($n = 8$ per group). (*M*) Measurement of ATP synthesis rates in cardiac mitochondria isolated from failing hearts of the indicated groups. ATP synthesis was driven by complex I (pyruvate/malate, 5 mM) and complex II (succinate 5 mM). The specificity of the measurements was verified using inhibitors (0.5 μM) of respiratory complex, as indicated ($n = 3$ per group, triplicate measurements per sample). All data are shown as mean ± SEM, $^{*}P < 0.05$ vs. WT; $^{\#}P < 0.05$ vs. RyR2-S2808D, ANOVA, Tukey–Kramer post hoc test.

of RyR2 channel promotes SR Ca^{2+} leak and mitochondrial dysfunction. Indeed, RyR2-S2808A ventricular cardiomyocytes exhibited reduced mitochondrial Ca^{2+} uptake (Fig. 1 *B–D* and Fig. S1) and increased mtDNA levels (Fig. 3*K*). We also observed a trend toward ameliorated $\Delta\psi_m$ dissipation (Fig. 3*J*) and increased ATP content and synthesis (Fig. 3 *L* and *M*).

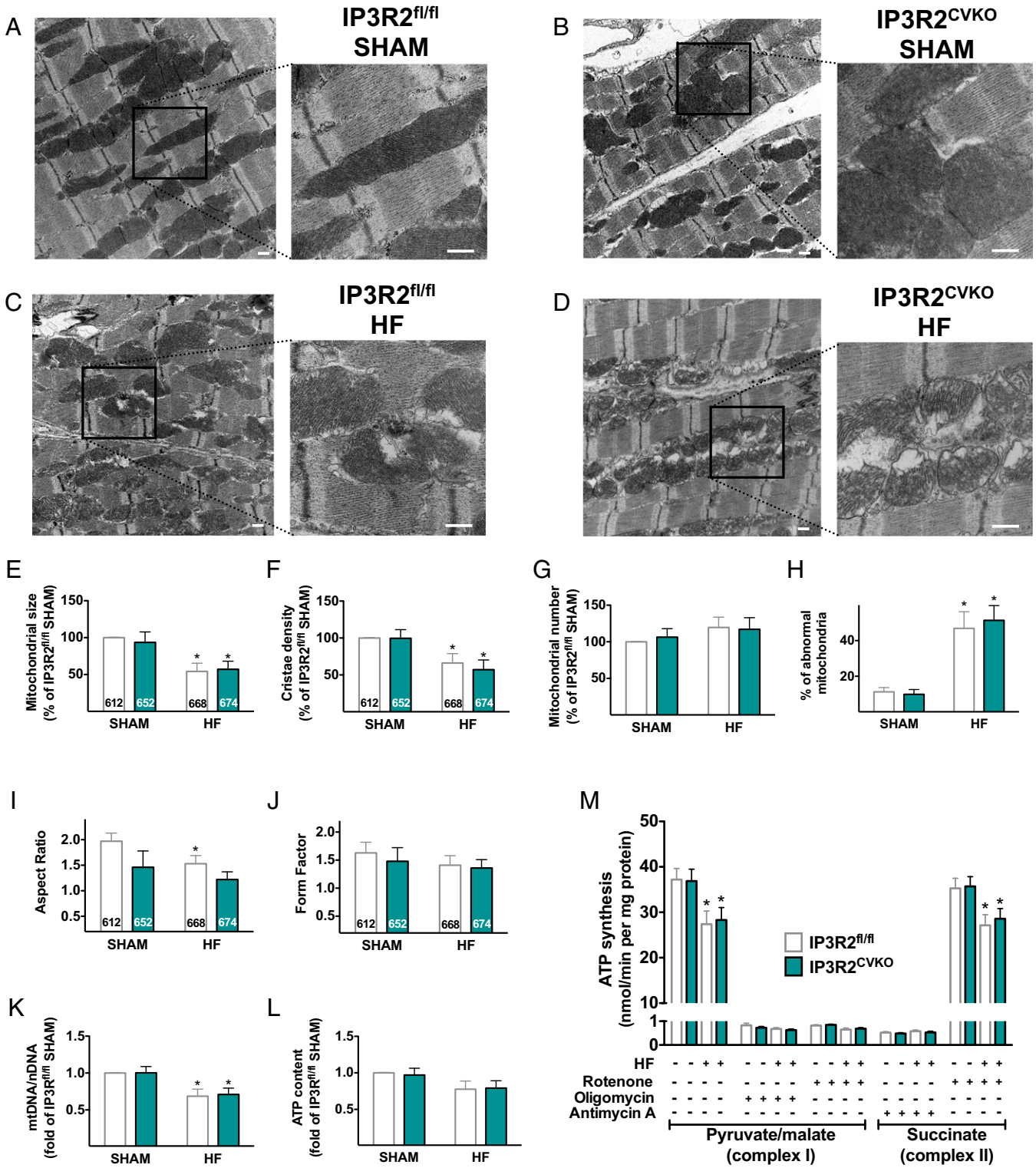


Fig. 4. Cardiac ablation of IP3R2 does not rescue mitochondrial abnormalities observed in failing hearts. (*A–D*) Representative transmission electron micrographs of cardiac mitochondria in SHAM conditions (*A* and *B*) and post myocardial infarction (*C* and *D*) from IP3R2$^{fl/fl}$ (*A* and *C*) and IP3R2^{CVKO} mice (*B* and *D*), $n = 5$ per group. (Magnification: *A–D*, 15,000×; *Insets*, 50,000×.) (Scale bars: 500 nm.) (*E–J*) Morphometric analysis of mitochondrial ultrastructure. Mitochondrial size (*E*) and cristae density (*F*). Quantification of mitochondrial number per image (*G*) and percentage of abnormal mitochondria per image at 15,000× (*H*). Evaluation of aspect ratio (*I*) and format form (*J*) (see *SI Materials and Methods* for details). (*K*) Mitochondrial DNA (mtDNA)/nuclear DNA (nDNA) copy number assessed in left ventricular tissue. (*L*) Assessment of ATP content in left ventricle ($n = 6$ per group) and (*M*) measurement of ATP synthesis rates in isolated mitochondria in sham conditions and 4 wk after coronary artery ligation, as described in Fig. 3*M*. All data are shown as mean ± SEM, $^*P < 0.05$ vs. SHAM; two-tailed *t* test. Numbers in the bars indicate the number of mitochondria analyzed.

RyR2-S2808A mice harboring nonleaky RyR2 channels exhibited reduced depletion of calstabin2 from the RyR2 complex in HF (Fig. 2 *A* and *F*), and significantly less RyR2 oxidation and nitrosylation (Fig. 2 *A*–*C*) and reduced post-MI HF progression (Fig. 2*G* and Table S1).

Taken together, our experimental findings demonstrate that SR Ca^{2+} leak via RyR2, but not IP3R2, channels plays a crucial role in the pathophysiology of mitochondrial Ca^{2+} overload and dysfunction in HF. Our data suggest a feedback loop between SR and mitochondria in HF in which SR Ca^{2+} leak triggers mitochondrial dysfunction and increases ROS production, which in turn can further oxidize RyR2 and enhance intracellular Ca^{2+} leak, contributing to impaired cardiac function post-MI.

Materials and Methods

The targeted deletion of IP3R2 in ventricular cardiomyocytes was obtained by flanking exon 3 of *IP3R2* with loxP sites (Fig. S2). Mice harboring the $IP3R2^{flox/flox}$ allele were bred with MHC-Cre transgenic mice to obtain a cardiac ventricular-specific ablation of IP3R2. A detailed description of materials and methods for in vivo experiments (31–35), isolation of adult cardiomyocytes (34, 36), isolation of mitochondria (37), assessment of mitochondrial dynamics, Ca^{2+} content, and membrane potential (34, 37), real-time RT-qPCR (38, 39), immunoprecipitation/immunoblot, and electron microscopy (40) can be found in *SI Materials and Methods*.

Ethical Approval. All studies were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Columbia University.

Statistics. All results are presented as mean ± SEM. Statistical analysis was performed using an unpaired two-tailed *t* test (for two groups) and one-way ANOVA with Tukey–Kramer post hoc test (for groups of three or more) unless otherwise indicated. *P* values of less than 0.05 were considered significant.

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