Ero1α-Dependent ERp44 Dissociation From RyR2 Contributes to Cardiac Arrhythmia

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BACKGROUND: Oxidative stress in cardiac disease promotes proarrhythmic disturbances in Ca2+ homeostasis, impairing luminal Ca2+ regulation of the sarcoplasmic reticulum (SR) Ca2+ release channel, the RyR2 (ryanodine receptor), and increasing channel activity. However, exact mechanisms underlying redox-mediated increase of RyR2 function in cardiac disease remain elusive. We tested whether the oxidoreductase family of proteins that dynamically regulate the oxidative environment within the SR are involved in this process.

METHODS: A rat model of hypertrophy induced by thoracic aortic banding (TAB) was used for ex vivo whole heart optical mapping and for Ca2+ and reactive oxygen species imaging in isolated ventricular myocytes (VMs).

RESULTS: The SR-targeted reactive oxygen species biosensor ERroGFP showed increased intra-SR oxidation in TAB VMs that was associated with increased expression of Ero1α (endoplasmic reticulum oxidoreductase 1 alpha). Pharmacological (EN460) or genetic Ero1α inhibition normalized SR redox state, increased Ca2+ transient amplitude and SR Ca2+ content, and reduced proarrhythmic spontaneous Ca2+ waves in TAB VMs under β-adrenergic stimulation (isoproterenol). Ero1α overexpression in Sham VMs had opposite effects. Ero1α inhibition attenuated Ca2+-dependent ventricular tachyarrhythmias in TAB hearts challenged with isoproterenol. Experiments in TAB VMs and human embryonic kidney 293 cells expressing human RyR2 revealed that an Ero1α-mediated increase in SR Ca2+-channel activity involves dissociation of intraluminal protein ERp44 (endoplasmic reticulum protein 44) from the RyR2 complex. Site-directed mutagenesis and molecular dynamics simulations demonstrated a novel redox-sensitive association of ERp44 with RyR2 mediated by intraluminal cysteine 4806. ERp44-RyR2 association in TAB VMs was restored by Ero1α inhibition, but not by reducing agent dithiothreitol, as hypo-oxidation precludes formation of covalent bond between RyR2 and ERp44.

CONCLUSIONS: A novel axis of intraluminal interaction between RyR2, ERp44, and Ero1α has been identified. Ero1α inhibition exhibits promising therapeutic potential by stabilizing RyR2-ERp44 complex, thereby reducing spontaneous Ca2+ release and Ca2+-dependent tachyarrhythmias in hypertrophic hearts, without causing hypo-oxidative stress in the SR.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: cardiovascular diseases | constriction | heart failure | homeostasis | oxidoreductases

In This Issue, see p 691 | Editorial, see p 725

A mismatch between increased oxidative stress and defective antioxidant defenses in cardiovascular disease (CVD) contributes to aberrant Ca2+ homeostasis. As the main Ca2+ release channel of the sarcoplasmic reticulum (SR), the cardiac RyR2 (ryanodine receptor) provides a major pathway for untimely Ca2+ release that can precipitate Ca2+-dependent ventricular tachyarrhythmias causative of sudden cardiac death. It is well established that increased oxidative stress in CVD such as heart failure (HF), hypertrophy, diabetic cardiomyopathy, or aging enhances RyR2 activity via reversible posttranslational modifications including CaMKII (Ca2+/-
What Is Known?

- Oxidative stress is a major contributor to cardiac dysfunction in the diseased heart.
- Cardiac disease-associated redox modifications of the RyR2 (ryanodine receptor type 2) sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channel are implicated in arrhythmogenesis.
- Clinical trials with antioxidants to improve cardiac function have showed limited success.

What New Information Does This Article Contribute?

- We have identified a novel RyR2 interacting partner—ERp44 (endoplasmic reticulum protein 44)—that covalently associates with the last intraluminal loop of RyR2 Cysteine 4806 in a redox-sensitive manner, stabilizing RyR2 complex activity.
- Cardiac hypertrophy-mediated upregulation of ER stress-inducible Ero1\(\alpha\) (endoplasmic recticulum oxidoreductase 1 alpha) removes ERp44 from the complex, contributing to RyR2 dysfunction and thereby increasing propensity to Ca\(^{2+}\)-dependent ventricular tachyarrhythmias.
- Ero1\(\alpha\) inhibition restores ERp44-RyR2 association in myocytes from hypertrophic hearts, reducing proarrhythmic spontaneous SR Ca\(^{2+}\) release.

Novelty and Significance

Reduction of sudden cardiac death incidence due to ventricular tachyarrhythmias remains a major challenge in the postindustrial world. Hyperactivity of the RyR2 SR Ca\(^{2+}\) release channel complex due to posttranslational oxidative modifications plays a key role in Ca\(^{2+}\)-dependent arrhythmogenesis in cardiac hypertrophy and failure. However, treatment with antioxidants to reduce RyR2 reactive cysteines, primarily located at the cytosolic face of the channel, demonstrates only partial functional recovery, which might explain limited success of clinical trials. We have identified a novel RyR2 interacting partner, intra-SR protein ERp44, which covalently associates with the RyR2 at its luminal face and stabilizes RyR2 complex activity. Importantly, RyR2 association via cysteine 4806 with ERp44 can be achieved only at oxidizing conditions and antioxidant treatment precludes disulfide bond formation. We found that in cardiac hypertrophy, RyR2-ERp44 association is disrupted by ER stress-inducible oxidoreductase Ero1\(\alpha\), contributing to proarrhythmic spontaneous SR Ca\(^{2+}\) release. Our data suggest that Ero1\(\alpha\) may be a promising therapeutic target to reduce arrhythmogenesis and improve cardiac function during hypertrophy and heart failure, without disturbing the finely balanced intra-SR redox environment.

Nonstandard Abbreviations and Acronyms

| CVD         | cardiovascular disease |
|-------------|-----------------------|
| Ero1\(\alpha\) | endoplasmic reticulum oxidoreductase 1 alpha |
| ERp44       | endoplasmic reticulum protein 44 |
| HEK293      | human embryonic kidney 293 |
| HF          | heart failure |
| hRyR2       | human ryanodine receptor type 2 |
| IP3R1       | inositol triphosphate receptor type 1 |
| IP3R2       | inositol triphosphate receptor type 2 |
| PDI         | protein disulfide isomerase |
| PVC         | premature ventricular contraction |
| ROS         | reactive oxygen species |
| RyR2        | ryanodine receptor type 2 |
| SR          | sarcoplasmic reticulum |
| TAB         | thoracic aortic banding |
| VF          | ventricular fibrillation |
| VM          | ventricular myocytes |
| VT          | ventricular tachycardia |

Recent evidence suggests that SR oxidoreductase enzymes can modulate Ca\(^{2+}\) homeostasis in the heart,\(^{23-26}\) and the development of new oxidoreductase inhibitors may provide a novel therapeutic strategy to combat arrhythmias in hypertrophic hearts.
yet the molecular mechanisms remain largely unexplored. Of particular interest is the luminal oxidoreductase Ero1α (endoplasmic reticulum oxidoreductase 1 alpha), known to be induced during ER stress as a component of the PERK (protein kinase RNA-like ER kinase) branch of the unfolded protein response. This protein can dynamically regulate the redox environment of the SR, transferring electrons from PDIs to reduce molecular oxygen to H2O2. Of note, Ero1α directly interacts with PDI protein ERp44 (endoplasmic reticulum protein 44), and this interaction increases with oxidative stress. Furthermore, ERp44 was reported to inhibit Ca2+ release channel IP3R1 (inositol triphosphate receptor type 1) by a direct protein-protein interaction that was redox-dependent. Importantly, ERp44 interacts with an IP3R1 region that has sequence homology to the last intraluminal loop of RyR2. However, whether ERp44 interaction with RyR2 exerts a stabilizing influence on the SR Ca2+ release channel complex remains unknown.

In the present study, we investigated the role of dysregulated SR oxidoreductase system in aberrant RyR2 function and Ca2+-dependent arrhythmia using a rat model of pressure-overload cardiac hypertrophy. Our results implicate CVD-mediated upregulation of Ero1α in SR oxidative stress and dissociation of ERp44 from the RyR2 complex promoting spontaneous SR Ca2+ release in VMs from hypertrophic hearts. Genetic of pharmacological inhibition of Ero1α restored the intra-SR redox environment and, unlike the reducing agent diithiothreitol, restored the ERp44-RyR2 interaction, markedly improving Ca2+ homeostasis. Accordingly, Ero1α inhibition significantly reduced malignant ventricular tachyarrhythmias induced by β-adrenergic stimulation in ex vivo hypertrophic rat hearts.

METHODS

Data supporting findings of this study are available from corresponding authors upon reasonable request.
suggesting EN460 antiarrhythmic effect stems from suppressing proarrhythmic spontaneous Ca\(^{2+}\) release (Figure S7). Accordingly, hearts pretreated with EN460 did not show DAD-like spontaneous Ca\(^{2+}\) release during burst-pause protocol (Figure 2C, Figure S7) nor during PVC or initiation of transient VT, which is typical of TAB hearts even before isoproterenol treatment.

We next tested effects of modulating Ero1α activity at the cellular level. Adenoviral-mediated overexpression or knockdown of Ero1α in Sham and TAB VMs was confirmed by Western blot analysis (Figure 3A and 3B). Measured with SR-targeted redox probe ERroGFP, oxidation levels within the SR were increased in Sham VMs with overexpression of Ero1α (Figure 3 and 3D). Importantly, Ero1α knockdown in TAB VMs reduced the redox status to Sham levels. To test whether modulation of Ero1α activity can modulate RyR2-mediated Ca\(^{2+}\) release, we measured intracellular Ca\(^{2+}\) handling in Fluo-3 loaded Sham and TAB VMs. Assessment of Ca\(^{2+}\) transients and spontaneous Ca\(^{2+}\) waves induced by pace-pause protocol revealed Ero1α overexpression in Sham VMs recapitulates the TAB phenotype, reducing Ca\(^{2+}\) transient amplitude, spontaneous Ca\(^{2+}\) wave latency (Figure 3E and 3F) and caffeine-sensitive Ca\(^{2+}\) store load (Figure 3G and 3H). All of these changes are indicative of increased RyR2 activity. Conversely, knockdown of Ero1α in TAB VMs had opposite effects, reducing proarrhythmic RyR2-mediated spontaneous SR Ca\(^{2+}\) release. Treatment
of TAB VMs with 4PBA (500 nmol/L) for 24 hours to inhibit ER stress recapitulated findings obtained from loss-of-Ero1α experiments (Figure S8). While Brefeldin A treatment of Sham VMs (1 µmol/L, 24 hours) followed a similar trend to findings obtained with gain-of-Ero1α experiments (Figure S8), differences were not significant, perhaps indicating 24 hours was not sufficient to elicit an effect. These data strongly suggest that ER stress-linked SR oxidoreductase activity modulates SR Ca2+ release in addition to SR redox homeostasis.

**ERp44 Interacts With RyR2 and the Complex Is Reduced in Hypertrophic Myocytes**

We performed immunolocalization experiments with Sham and TAB VMs, probing for RyR2 and ERp44 expression (Figure 4A, negative controls Figure S9), and the extent of direct signal overlap was quantified using Manders split coefficients. RyR2/ERp44 signal overlap in TAB VMs was reduced compared with TAB VMs (Figure 4B). Western blot analysis demonstrated this is not attributable to altered expression of ERp44, since the RyR2/ERp44 ratio remained similar (Figure 4C and 4D). Of note, co-IP experiments using HF samples from canine hearts show a trend decreasing ERp44 complexed with RyR2, consistent with results in rats (Figure S10).

Oxidoreductase Ero1α is a binding partner of ERp44, and loss of Ero1α function in transgenic mice has been shown to be protective in pressure-overloaded hearts. Application of EN460 (20 µmol/L, 30 minutes) increased RyR2-ERp44 signal coincidence. Notably, treatment of TAB VMs with general ER stress inhibitor 4PBA also increased RyR2-ERp44 coincidence (Figure S11). In contrast, thiol-reducing and disulfide bridge-breaking agent dithiothreitol (5 mmol/L, 10 minutes) did not restore protein colocalization. These findings were mirrored in assessment of native RyR2-ERp44 protein complexes of Sham, TAB and EN460/dithiothreitol

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**Figure 2. Inhibition of Ero1α (endoplasmic reticulum oxidoreductase 1 alpha) oxidoreductase reduces arrhythmogenic potential in hypertrophic rat hearts.**

**A.** ECG recordings of ventricular fibrillation (VF) induction in thoracic aortic banding (TAB) hearts under isoproterenol (ISO; 50 nmol/L). After EN460 (10 µmol/L, 30 min), transient ventricular tachycardia (VTs) were observed rather than long-lasting VFs. **B.** Propagation maps of Sham heart (left), VF in TAB heart (center), and transient VT in the presence of EN460 (right). Arrows represent reentry. **C.** Rapid pacing followed by pause-induced spontaneous Ca2+ release that triggered delayed after depolarizations. EN460 suppressed spontaneous Ca2+ release. **D.** Number of ex vivo Sham, TAB, and TAB+EN460 hearts exhibiting VF. N=7 Sham+ISO, N=7 TAB+ISO, N=8 TAB+ISO+EN460 hearts. *P=2.1×10−4, obtained using Freeman-Halton extension of the Fisher exact test.
treated TAB VMs in blue native gel electrophoresis (BN-PAGE, Figure 4E and 4F). A reduced amount of ERp44 was observed along with RyR2 in TAB versus Sham group, indicative of decreased protein-protein interaction. The amount of ERp44 that migrated with RyR2 in TAB VMs was restored by treatment with EN460 but not with dithiothreitol. These data suggest that association of ERp44 with RyR2 is redox- and Ero1α-sensitive.

Disruption of the RyR2-ERp44 Interaction Enhances RyR2 Channel Activity in a Heterologous Cell System

Although IP3R2 (inositol triphosphate receptor type 2) is established as the major isoform in VMs,41 the expression of IP3R1 has been reported as increased in end-stage human HF.42 However, IP3R inhibition with xestospongin C43 in Ero1α-overexpressing Sham VMs did not fully restore Ca²⁺ handling (Figure S12). Furthermore, IP3R inhibition was not as effective as Ero1α knockdown in TAB VMs, suggesting aberrant RyR2 complex assembly plays a significant role in Ero1α-mediated disruption in Ca²⁺ homeostasis. To test this further, and avoid confounding effects of IP3R1-ERp44 interaction,31 we investigated Ca²⁺ release dynamics in human embryonic kidney 293 (HEK293) cells lacking all 3 isoforms of IP3R (HEK293 IP3R-3KO).44 Spontaneous luminal Ca²⁺ release events monitored with R-CEPIAer45 were used as an assessment of RyR2 channel function in cells expressing recombinant hRyR2 (human RyR2) constructs46,47 (Figure 5A). Co-expression of short hairpin RNA to knockdown endogenous ERp44 (Figure 5B) increased

Figure 3. Altered expression levels of Ero1α (endoplasmic reticulum oxidoreductase 1 alpha) modulate intrasarcoplasmic reticulum (SR) redox state and RyR2 (ryanodine receptor type 2)-mediated Ca²⁺ release.
A, Representative Western blot demonstrating adenoviral overexpression of Ero1α, and shRNA-mediated Ero1α knockdown in cultured Sham and thoracic aortic banding (TAB) ventricular myocytes (VMs), respectively. B, Mean±SEM Ero1α signal, normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). N=4 Sham, N=4 TAB animals. P values calculated using Kruskal-Wallis with Dunn posthoc. C, Representative SR redox probe ERroGFP fluorescence traces. VMs were treated with isoproterenol (ISO; 50 nmol/L) and paced at 2 Hz (5 min). Dithiothreitol (DTT; 5 mmol/L) and 2,2′-dithiodipyridine (DTDP; 200 µmol/L) were used to obtain minimum and maximum fluorescence. D, Mean±SEM ERroGFP fluorescence (%). N=6 Sham, N=5 TAB animals, n=11 Sham, n=12 Sham+Ero1α, n=9 TAB, n=13 TAB+Ero1α-shRNA VMs. P values obtained using 2-level random intercept model with Tukey posthoc. E, Flu-3 fluorescence (F/F₀) profiles of ISO-treated VMs undergoing 2 Hz pace-pause protocol. F, Mean±SEM Ca²⁺ transient amplitude at 2 Hz (ΔF/F₀) and spontaneous Ca²⁺ wave (SCW) latency (s). N=9 Sham, N=9 TAB animals, n=25 Sham, n=18 Sham+Ero1α, n=29 TAB VMs, n=14 TAB+Ero1α-shRNA VMs. P values obtained using 2-level random intercept model with Tukey posthoc. G, Representative traces of caffeine-induced Ca²⁺ transients (10 mmol/L). H, Mean±SEM caffeine-sensitive Ca²⁺ store load. n=15 Sham, n=14 Sham+Ero1α, n=15 TAB, n=15 TAB+Ero1α-shRNA VMs; from the same animals used in E and F. P values obtained using 2-level random intercept model with Tukey posthoc.
hRyR2-WT (wild type)-mediated Ca^{2+} release events (Figure 5D and 5E). To corroborate the hypothesis that increased expression/activity of oxidoreductase Ero1α promoted dissociation of ERp44 from RyR2, Ero1α was overexpressed (Figure 5C). hRyR2-WT-mediated Ca^{2+} release events increased with Ero1α overexpression (Figure 5D and 5E), indicative that modulation of PDI proteins within the SR effectively modulate RyR2 activity.

We reasoned that interaction of specific Cysteine residues of RyR2 with ERp44 underlies luminal Ca^{2+} regulation of the channel, and this interaction is disrupted with Ero1α overexpression. Figure 6A shows Cysteine 4806 (Cys4806, human nomenclature) within the last luminal loop of RyR2 that we posit is the primary Cysteine residue mediating RyR2-ERp44 interaction. Cys4806Ser substitution (hRyR2-MUT, Figure 6B) eliminated any putative thiol group-mediated covalent bond...
formation with ERp44. Confocal microscopy revealed that the frequency of Ca<sup>2+</sup>-release events in cells expressing hRyR2-MUT was increased when compared with hRyR2-WT (Figure 6C–6E). These data highlight a putative role for luminal Cys4806 in regulating RyR2-mediated Ca<sup>2+</sup> release. Critically, increased Ero1α or reduced ERp44 expression levels significantly modulated Ca<sup>2+</sup> release via hRyR2-WT (Figure 5D and 5E) but were unable to change Ca<sup>2+</sup> release via hRyR2-MUT (Figure 6F and 6G).

**RyR2-ERp44 Interaction Is Redox-Dependent**

We next performed in silico calculations to model redox-dependent RyR2-ERp44 interaction (Figure 7). ERp44 forms mixed disulfides with other proteins via cysteine 29. Yang et al. resolved the crystal structure of ERp44 in complex with SR-resident protein PRXD4 (peroxiredoxin 4), and showed that ERp44 binds the oxidized, but not reduced, form. The reduced segment of PRXD4 involved in thiol-disulfide interchange reactions with ERp44 structurally aligned with reduced structures of RyR2 (PDB ID: 6jh6, amino acids 4751–4882) and IP3R1 (PDB ID: 3jav; Figure 7A). High degree of structural homology with the ERp44-PRXD4 crystal structure allowed us to estimate how the ERp44-RyR2 complex might form using molecular docking software (Figure 7B). We repeated multiple iterations of docking and short molecular dynamics simulations (up to 100 ns) of both the reduced and oxidized RyR2 forms in complex with the opened state of ERp44. Docking studies revealed that RyR2-Cys4806 and ERp44-cysteine 29 could proximally associate to form a disulfide bridge (Figure 7C), and predicted oxidized RyR2 retained RyR2-ERp44 interaction, whereas the reduction of RyR2 caused the dissociation of proteins within picoseconds (Figure 7D, Videos S4 and S5). Cys4806Ser substitution resulted in more pronounced RyR2-ERp44 distance fluctuations but not complete dissociation. These data suggest RyR2-Cys4806 is an important mediator of interaction with ERp44, possibly forming a disulfide bridge with cysteine 29 of ERp44 during normal luminal redox states.

**DISCUSSION**

In the present study, we aimed to unravel molecular determinants of the RyR2 luminal redox sensor. The basal SR redox potential is higher than that in the cytosol, and we have shown that this promotes association of the SR-resident protein ERp44 with the last intraluminal loop of RyR2 to modulate SR Ca<sup>2+</sup> release. Activation of oxidoreductase Ero1α in cardiac hypertrophy, in addition to increasing SR oxidation, dissociates ERp44 from the
complex and augments RyR2-mediated spontaneous SR Ca\(^{2+}\) release. We also showed that inhibition of Ero1\(\alpha\) in VMs from hypertrophic hearts restored SR ROS levels and RyR2 association with ERp44, improving intracellular Ca\(^{2+}\) handling and most importantly, reducing the propensity for spontaneous Ca\(^{2+}\) release following \(\beta\)-adrenergic stimulation. In ex vivo hypertrophic hearts challenged with \(\beta\)-adrenergic agonist, inhibition of Ero1\(\alpha\) produced striking attenuation of Ca\(^{2+}\)-dependent ventricular arrhythmia.

**Ero1\(\alpha\) Upregulation Promotes Proarrhythmic Spontaneous SR Ca\(^{2+}\) Release in Cardiac Hypertrophy**

Abnormally high RyR2 channel activity has a central role in contractile deficiency and arrhythmogenesis underlying sudden cardiac death\(^{34,49}\) and posttranslational modifications of the channel including PKA/CaMKII-mediated phosphorylation or oxidation are involved, as well as their interplay (ie, ROS-CaMKII-RyR2) at the cytosolic face of the channel.
Here, we show a novel regulatory axis for RyR2 ROS modulation involving the luminal ROS sensor Ero1α and the luminal RyR2-binding protein ERp44. Our previous studies showed that β-adrenergic stimulation induces Ca²⁺-dependent VT/VF in 100% of ex vivo hearts from rats with pressure-overload induced hypertrophy. Our results show increased Ero1α expression as part of the ER stress response in VMs from TAB rat hearts (Figure 1) and human and canine HF samples (Figures S1 and S2). These data are consistent with previous findings from a mouse model of HF induced by thoracic aortic constriction. Ero1α upregulation is expected to increase H₂O₂ production within the ER, and we confirmed this in TAB rat VMs using the SR-targeted redox state biosensor ERroGFP (Figures 1 and 3). Both acute Ero1α inhibition by small molecule inhibitor EN460 and more chronic short hairpin RNA-mediated knockdown normalized SR oxidation levels in TAB VMs. Conversely, Ero1α overexpression in Sham VMs increased SR oxidation levels. These data implicate Ero1α as an important mediator of dysregulated SR redox potential in cardiac pathology. Furthermore, as H₂O₂ is capable of crossing intracellular membranes and traveling substantial distances within the cell, our results support the notion that the SR is a significant source of ROS, in addition to well-established sources such as NOX2 (NADPH oxidase 2) or mitochondria. The possibility also exists that H₂O₂ may directly modulate reactive cysteines located in the cytosolic domains of RyR2.
of RyR2. Indeed, immunoprecipitation experiments demonstrated a significant reduction of RyR2 oxidation level in TAB VMs treated with EN460 (Figure S13). Moreover, Ero1α knockdown in TABs reduced mitochondrial matrix H₂O₂ levels measured using mitochondria-targeted biosensor MLS-HyPer7 (Figure S14). While the latter might be a direct result of reduced H₂O₂ emission from the SR, our recent demonstration of RyR2 Ca²⁺-leak-dependent disruption of mitochondrial Ca²⁺ and ROS homeostasis would favor a mechanism that involved RyR2 channel stabilization.

Ero1α overexpression in Sham VMs treated with isoproterenol decreased Ca²⁺ transient amplitude and SR Ca²⁺ content and shortened spontaneous Ca²⁺ wave latency, consistent with increased RyR2 activity (Figure 3). In TAB VMs, Ero1α knockdown normalized Ca²⁺ homeostasis, increasing Ca²⁺ transient amplitude, loading of the SR, and reducing the propensity for proarrhythmic spontaneous Ca²⁺ waves. Further investigations of WT- and Cys4806Ser hRyR2 in HEK293 cells devoid of IP3R isoforms revealed that Ero1α overexpression potently increases RyR2 activity (Figure 5). Accordingly, treatment of ex vivo TAB hearts with an Ero1α inhibitor attenuated Ca²⁺-dependent tachyarrhythmia evoked by β-adrenergic challenge (Figure 2, Figure S5). Previous work using VMs from transgenic mouse model of Ero1α loss-of-function showed a marked decrease in Ca²⁺ transient amplitudes and SR Ca²⁺ content, both in baseline conditions and under isoproterenol. However, pressure-overloaded Ero1α mutant hearts exhibited significant preservation of cardiac function in comparison to WTs, pointing to Ero1α’s key role in aberrant excitation-contraction coupling during HF development. Our data extend these findings and indicate that strategies to reduce Ero1α activity, which is increased in CVD, can be of benefit in therapeutically improving contractility and limiting proarrhythmic spontaneous RyR2-mediated SR Ca²⁺ release.

A recent study established antiarrhythmic effects induced by PERK knockout in myocardial infarct mice. Of note, Ero1α is upregulated as part of the ER stress-induced unfolded protein response, downstream of PERK. Indeed, modulation of ER stress produced similar effects to altering Ero1α levels on Ca²⁺ handling (Figure 3, Figures S8 and S10). These data support that Ero1α upregulation is a key factor contributing to Ca²⁺-dependent arrhythmogenesis in conditions associated with ER stress and the unfolded protein response including hypertrophy, myocardial infarct and HF.

**RyR2 Interacts With ERp44 in a Redox-Sensitive Manner**

Besides regulating the redox environment, Ero1α was shown to modulate function of SR Ca²⁺ channel multimolecular complexes, such as IP3R1, by disrupting tethering of luminal protein ERp44 to the complex. While our results do not exclude the importance of Ero1α-mediated IP3R1-ERp44 disassociation in hypertrophic VMs (Figure S12), we demonstrate that Ero1α inhibition in TAB VMs restores the association of ERp44 with RyR2 (Figure 4), stabilizing channel activity.

RyR2 is established as a major redox sensor in cardiac myocytes. Reversible modifications of reactive cysteines modulate channel activity. Increased disulfide bond formation has been linked to pathological increase in RyR2 activity in cardiac disease, and reducing agents that attenuated this increase were associated with improvements in Ca²⁺ handling. However, clinical studies did not substantiate a therapeutic effect of antioxidants in improving outcomes for patients with HF. In line with this, reducing agents such as dithiothreitol or mercaptopyrropropionyl glycine produced only partial recovery in Ca²⁺ handling in VMs from HF or infarcted hearts. Furthermore, sequential application of oxidizing agent 2,2-dithiodipyridine, which promotes disulfide bond formation, followed by dithiothreitol in VMs from healthy hearts did not result in full restoration of SR Ca²⁺ content, implying only partial stabilization of RyR2 activity when reactive cysteines at both cytosolic and luminal RyR2 sides are reduced. Our new data demonstrates that oxidative stress promotes the dissociation of important regulatory molecular component(s) from the RyR2 complex, which cannot be reversed by application of a reducing agent. Given the major difference in redox potential between the cytosol and the SR, we surmised this regulatory component was most likely a resident SR protein that associates with a small luminal portion of RyR2 in a redox-sensitive manner. ERp44 expression is restricted to endoplasmic reticulum and its major role is thought to be in forming disulfide bonds with the target proteins in the Golgi and for transport to its final location in the ER.

Higo et al showed that ERp44 associates with an IP3R1 intraluminal loop, a region homologous to the last intraluminal loop of RyR2, to affect IP3R1 regulation. This mechanism was subsequently shown to operate in VMs from Ero44 knockout mice. Our immunolocalization studies showed spatial colocalization between RyR2 and ERp44 in VMs from Sham hearts was lost in VMs from TAB hearts (Figure 4). Furthermore, treatment of TAB VMs with dithiothreitol did not reverse this pattern. Further evidence of RyR2-ERp44 interaction was obtained using BN-PAGE, which confirmed the presence of ERp44 in the RyR2 macromolecular complex of healthy VMs, as well as its dissociation in hypertrophic VMs that was not reversible by dithiothreitol (Figure 4). Using HEK293 cells, we showed that ERp44 knockdown with short hairpin RNAs increases RyR2 activity (Figure 5). Moreover, substitution of RyR2 Cys4806Ser disrupted putative disulfide bond formation with ERp44 and led to an increase in RyR2 activity. ERp44 knockdown failed to produce additional effects (Figure 6). Molecular docking computer simulations confirmed that RyR2-ERp44 association is highly unstable in reducing conditions requiring high redox potential (Figure 7).
Importantly, given the very high redox potential in the SR of TAB VMs, which must favor RyR2-ERp44 association, the fact that ERp44 is lost from the complex implies involvement of active enzymatic reaction to break the bond between RyR2 and ERp44. It has been shown that activated oxidoreductase Ero1α associates with ERp44, removing it from its binding partners.28,29 Indeed Ero1α inhibition in TAB VMs, unlike dithiothreitol treatment, restored RyR2-ERp44 colocalization and the abundance of ERp44 in the RyR2 complex (Figure 4). Moreover, the importance of the Ero1α effect on ERp44-RyR2 association versus its effect on ROS was underscored by the lack of additional effects of Ero1α overexpression in HEK293 cells expressing hRyR2-MUT (Figure 6).

Conclusions
To summarize, we have identified a novel RyR2 interacting partner—ERp44—that covalently associates with the last intraluminal loop of RyR2 in a redox-sensitive manner, stabilizing RyR2 complex activity. In addition to increasing SR redox potential, Ero1α upregulation removes ERp44 from the complex, contributing to RyR2 dysfunction and thereby increased propensity to Ca2+ dependent ventricular tachyarrhythmias in hypertrophic hearts. Our data suggest that ER stress-induced Ero1α may be a promising therapeutic target to reduce arrhythmogenesis and improve cardiac function during the development of HF, without compromising the finely balanced intra-SR redox environment (Figure 8).

ARTICLE INFORMATION
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