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Redox Modification of Ryanodine Receptors Contributes to Sarcoplasmic Reticulum Ca^{2+} Leak in Chronic Heart Failure

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Abstract

Abnormal cardiac ryanodine receptor (RyR2) function is recognized as an important factor in the pathogenesis of heart failure (HF). However, the specific molecular causes underlying RyR2 defects in HF remain poorly understood. In the present study, we used a canine model of chronic HF to test the hypothesis that the HF-related alterations in RyR2 function are caused by posttranslational modification by reactive oxygen species generated in the failing heart. Experimental approaches included imaging of cytosolic ($[Ca^{2+}]_c$) and sarcoplasmic reticulum (SR) luminal Ca^{2+} ($[Ca^{2+}]$SR) in isolated intact and permeabilized ventricular myocytes and single RyR2 channel recording using the planar lipid bilayer technique. The ratio of reduced to oxidized glutathione, as well as the level of free thiols on RyR2 decreased markedly in failing versus control hearts consistent with increased oxidative stress in HF. RyR2-mediated SR Ca^{2+} leak was significantly enhanced in permeabilized myocytes, resulting in reduced $[Ca^{2+}]_{SR}$ in HF compared to control cells. Both SR Ca^{2+} leak and $[Ca^{2+}]_{SR}$ were partially normalized by treating HF myocytes with reducing agents. Conversely, oxidizing agents accelerated SR Ca^{2+} leak and decreased $[Ca^{2+}]_{SR}$ in cells from normal hearts. Moreover, exposure to antioxidants significantly improved intracellular Ca^{2+}-handling parameters in intact HF myocytes. Single RyR2 channel activity was significantly higher in HF versus control because of increased sensitivity to activation by luminal Ca^{2+} and was partially normalized by reducing agents through restoring luminal Ca^{2+} sensitivity oxidation of control RyR2s enhanced their luminal Ca^{2+} sensitivity, thus reproducing the HF phenotype. These findings suggest that redox modification contributes to abnormal function of RyR2s in HF, presenting a potential therapeutic target for treating HF.

Keywords

ryanodine receptor; heart failure; disulfide oxidation; Ca^{2+}-induced Ca^{2+} release

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Heart failure (HF) is a disease state in which the muscle of the heart becomes too weak to adequately pump blood through the body. Although HF is very complex, altered intracellular Ca^{2+} handling, and, in particular, depletion of intracellular Ca^{2+} stores, is recognized as an important feature of myocytes from failing hearts.[1–5] In cardiac muscle,

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Disclosures None.

Ca^{2+} release from the intracellular storage compartment, the sarcoplasmic reticulum (SR), is central to the process of excitation– contraction (EC) coupling.[6] During each heart beat, in response to a small Ca^{2+} entry via sarcolemmal voltage-dependent Ca^{2+} channels, SR Ca^{2+} is released through specialized Ca^{2+}-gated SR Ca^{2+} channels called ryanodine receptors (RyR2s) causing shortening of the contractile filaments. Evidence from human HF and various models of HF suggests that RyR2s become excessively active ie, "leaky" in the failing heart.[7–9] This increase in RyR2 activity has been linked to defective modulation of the channel by SR luminal Ca^{2+}, a mechanism that normally operates to terminate SR Ca^{2+} release and keep the RyR2 channels closed (ie, refractory) during diastole.[10] Uncontrolled RyR2 gating is expected to result in increased diastolic SR Ca^{2+} leak causing a reduction of the SR Ca^{2+} content, thus limiting the ability of cardiac muscle to contract in systole. Consistent with this scenario, we recently found that increased SR Ca^{2+} leak is a major determinant of the reduced SR Ca^{2+} content and suppressed Ca^{2+} signaling in myocytes from a chronic canine model of HF.[11,12]

The underlying biochemical causes of HF-related abnormalities in RyR2 function are a subject of intense debate. Marks and colleagues have reported that increased phosphorylation of RyR2 by protein kinase A (on Ser2809) results in dissociation of FKBP12.6 from the RyR2 complex, causingRyR2s to become hyperactive.[7,13] However, various aspects of this proposed mechanism have been questioned by others.[14–16] More recently, Bers and colleagues[17,18] presented data suggesting that RyR2 phosphorylation by Ca^{2+}/calmodulin-dependent protein kinase Ca^{2+}/calmodulin-dependent protein kinase (CaMK II on Ser2815) contributes to elevated SR Ca^{2+} leak in HF. However, other investigators failed to detect a stimulatory influence of CaMKII phosphorylation on SR Ca^{2+} release.[19]

Alterations in phosphorylation status is not the only type of biochemical modification that could affect RyR2s in HF. HF is accompanied by increased oxidative stress resulting from formation of reactive oxygen species (ROS) (eg, superoxide anion, hydrogen peroxide, and hydroxyl radical).[20–23] These compounds are capable of reacting with reactive cysteines (ie, thiols susceptible to redox-based modifications), causing changes in RyR2 function. Sulfhydryl oxidation by various oxidizing agents has been shown to increase RyR2 open probability.[24–26] Accordingly, the goal of the present study was to test the hypothesis that increased RyR2-mediated leak in the failing heart is caused by abnormal modification(s) of the RyR2s by ROS generated in the course of HF.

Materials and Methods

Chronic left ventricular dysfunction was induced by right ventricular tachypacing in a canine model for ≈4 months.[27] Single myocytes were isolated as previously described.[9] Cytosolic and intra-SR [Ca^{2+}] changes were monitored by using confocal microscopy. Single-channel recordings were performed using the lipid bilayer technique. Redox balance in control and HF tissue samples were assessed by measuring levels of reduced (GSH) and oxidized (GSSG) forms of glutathione with high-performance liquid chromatography, whereas in myocytes, the fluorescent indicator 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA) and confocal microscopy were used to measure ROS. The amounts of oxidized thiols in RyR2s were determined with monobromobimane (mBB).

An expanded Material and Methods section can be found in the online data supplement at http://circres.ahajournals.org.

Results

Increased Oxidative Stress and RyR2 Oxidation in HF

We assessed redox balance and oxidative stress in HF versus control myocardium. Although the reduced glutathione levels were not different, the ratio of reduced to oxidized glutathione decreased significantly in HF compared to controls confirming that oxidative stress is elevated in HF (Figure 1A and 1B). Consistent with increased oxidative stress, imaging of myocytes loaded with the ROS indicator DCFDA showed a significant increase in ROS in HF compared to control cells (Figure 1C and 1D). Importantly, incubation of HF myocytes with the cell-permeable ROS scavenger 2-mercaptopropionylglycine (MPG) restored normal ROS levels in these cells. Furthermore, measurements with mBB revealed an increased amount of oxidized thiols in RyR2s from failing hearts (Figure 1E and 1G and Figure I in the online data supplement).

Normalization of Ca^{2+} Transients in HF Myocytes by Antioxidants

Next, we performed measurements of cytosolic Ca^{2+} transients in intact cardiac myocytes isolated from control and failing hearts (Figure 2). Figure 2A depicts representative line-scan images and corresponding time-dependent profiles of Ca^{2+} transients recorded in field-stimulated control and HF myocytes under baseline conditions and after treatment of the cells with oxidizing or reducing reagents. Characteristic of HF, the amplitude of the Ca^{2+} transients was significantly lower in HF than in control myocytes. In control myocytes, application of the oxidizing agent 2,2′-dithiodipyridine (DTDP) depressed the amplitude of Ca^{2+} transients making them similar to the HF phenotype. Conversely, incubation of HF myocytes with the antioxidant MPG increased the amplitude of the Ca^{2+} transients rendering them similar to controls. Summary data are shown in Figure 2B. Exposure to MPG had no significant effect in control myocytes (supplemental Figure II, A and B), consistent with the notion that reversible oxidation of components of the EC coupling machinery is minimal in myocytes from healthy hearts. Thus, treatment with antioxidants improves the parameters of Ca^{2+} transients in HF, whereas the impact of HF on Ca^{2+} transients can be mimicked by oxidative agents.

Normalization of $[Ca^{2+}]_{SR}$ and SR Ca^{2+} Leak in HF Myocytes by Antioxidants

To further investigate the role of redox modifications in mediating the HF-associated alterations in intracellular Ca^{2+} handling, we performed measurements of intra-SR $[Ca^{2+}]$in saponin-permeabilized myocytes using the low-affinity Ca^{2+} indicator Fluo-5N loaded to the SR. Recently, we demonstrated that $[Ca^{2+}]_{SR}$ is reduced in this particular model of HF because of increased Ca^{2+} leak via RyR2s in the absence of significant changes in SERCA-mediated SR Ca^{2+} uptake.[9,11] Consistent with our previous results, baseline $[Ca^{2+}]_{SR}$ was substantially lower in HF than in control myocytes (Figure 3A and 3B). Furthermore, SR Ca^{2+} leak, assessed as the difference in $[Ca^{2+}]_{SR}$ measured in the presence or absence of the RyR2 inhibitor ruthenium red (RR) (30 μmol/L), was significantly higher in HF than in control myocytes (Figure 3C). Application of the oxidizing agent DTDP (1 to 200 μmol/L) to control cells caused a rapid decline in $[Ca^{2+}]_{SR}$, and increased SR Ca^2 leak. Subsequent applica tion of dithiothreitol (DTT) (1 mmol/L) caused a partial recovery of $[Ca^{2+}]_{SR}$ and normalization of leak in DTDP-treated cells. These results are documented in Figure 3A, 3B, and 3D. At the same time, treatment of $[Ca^{2+}]_{SR}$-depleted HF cells with DTT (0.01 to 10 mmol/L) produced an increase in $[Ca^{2+}]_{SR}$ and lowered Ca^{2+} leak toward control values (Figure 3A, 3B, and 3E). Similar results in control and HF myocytes were obtained using the oxidizing and reducing reagents thimerosal and MPG instead of DTDP and DTT, respectively (online-only Data Supplement Figure III).

Consistent with the lack of effects of MPG in intact control cells, DTT did not affect intra-SR $[Ca^{2+}]$ in permeabilized cells from the control group (supplemental Figure II, C and D), again suggesting that RyR2s are not substantially modified by reversible oxidation in myocytes from normal hearts. At the same time, DTDP decreased $[Ca^{2+}]_{SR}$ in HF myocytes in a manner similar to that in control cells (supplemental Figure II, E and F), indicating that augmenting oxidative stress can further elevate SR Ca^{2+} leak in HF myocytes. Interestingly, the extent of recovery of SR Ca^{2+} leak by DTT in HF myocytes treated with DTDP was significantly less than in untreated HF myocytes, suggesting that excessive oxidation, resulting from both HF and DTDP, can result in irreversible modification of RyR2s or that DTDP additionally modifies RyR2 at sites inaccessible to DTT.

In a separate series of experiments, we directly measured the RyR2-mediated SR Ca^{2+} leak following inhibition of SR Ca^{2+} uptake by thapsigargin (Tg) (Figure 4A and 4B). On application of the SERCA inhibitor Tg, luminal Fluo-5N fluorescence decreases with time, providing a measure of the rate of SR Ca^{2+} leak. Consistent with the results of steadystate $[Ca^{2+}]_{SR}$ measurements, the SR Ca^{2+} leak rate was markedly enhanced in HF myocytes compared to controls. We were able to mimic the HF phenotype by treating the control cells with the oxidizing agent DTDP. As shown in Figure 4A, incubating control myocytes with DTDP lowered the basal level of intra-SR $[Ca^{2+}]$ and accelerated the SR Ca^{2+} leak, which was unmasked by Tg. Conversely, application of DTT normalized SR Ca^{2+} leak in HF myocytes. Pooled data for time constants of decay in these experiments are presented in Figure 4B. Taken together, these results suggest that the HF-related changes in myocyte Ca^{2+} handling could be attributed, in part, to redox modifications of components of the SR Ca^{2+} release machinery including the RyR2 channel.

Changes in Single RyR2s

Consistent with our previous results,[9] RyR2s from failing hearts exhibited higher functional activity than RyR2s from normal hearts (Figure 5). This difference was especially large at low luminal $[Ca^{2+}]$ because of the apparent HF-induced loss of ability of RyR2s to deactivate at reduced $[Ca^{2+}]_{SR}$. Oxidation by DTDP (10 μmol/L) increased the activity of RyR2 from the control group (Figure 5A, top, and 5B). Importantly, oxidation of control RyR2s resulted in a significant reduction in the ability to increase activity in response to elevation of luminal Ca^{2+}, reminiscent of the behavior of HF channels. On the other hand, treatment of HF channels with the reducing agent DTT (5 mmol/L) decreased channel activity and at the same time restored their ability to respond to luminal Ca^{2+} (Figure 5A, bottom, and 5C). These results are summarized in Figure 5D, which shows relative values of open probabilities (P_o) at low and high luminal Ca^{2+} for control and HF RyR2s at baseline and after exposure to DTDP or DTT, respectively. As the graph demonstrates, we were able to "convert" the control and HF RyR2 phenotypes from one to another using oxidizing and reducing reagents, respectively.

It has been previously shown that RyRs contain different groups of thiols, the oxidation of which exerts different effects on channel function, ie, activation or inactivation.[28–30] In agreement with the previous studies, high doses of the oxidant DTDP (200 μmol/L) caused a rapid and nearly complete activation of RyR2s, followed by reduction of P_o back toward baseline levels in samples from both control and failing hearts (supplemental Figure IV, A and B). At the same time, low doses of DTDP (10 μmol/L) resulted in a less initially pronounced but sustained stimulation of RyR2s (supplemental Figure IV, B). Additionally, DTT caused a concentration-dependent reduction of P_o in hyperactive HF channels toward normal (control) P_o levels (supplemental Figure IV, C). These results support the notion that multiple functionally relevant thiols exist in individual RyR2s that become progressively modified with increasing levels of oxidative stress. Considering the concentration dependence of the effects of DTDP and the ability of high doses of DTDP to further increase

the activity of HF RyR2s in our experiments, it appears that oxidative modifications of RyR2 in HF do not necessarily involve all the different classes of thiols and are mostly limited to those promoting channel openings at moderate oxidation. Collectively, our findings suggest that redox modifications are involved in acquisition of the HF phenotype by RyR2s.

Role of Different Types of Redox Modifications

Three types of reversible redox modifications have been described for the RyR2 channel, including *S*-nitrosylation, *S*-glutathionylation, and disulfide oxidation. To probe the involvement of these specific reactions in HF-related changes in SR Ca^{2+} leak, we used modification-specific reducing agents. Whereas DTT reverses all 3 types of modification, glutaredoxin reverses *S*-nitrosylation and *S*-glutathionylation without affecting oxidized disulfides, and ascorbate reverses only *S*-nitrosylation.[31,32] Both glutaredoxin (1 units/mL +0.5 mmol/L GSH) and ascorbate (1 mmol/L) failed to produce measurable effects on $[Ca^{2+}]_{SR}$ in failing myocytes as Fluo-5N $\Delta F_{CAF}/\Delta F_{max}$ on application of glutaredoxin was 0.624 ± 0.027 (n=7) and in the presence of ascorbate was 0.627 ± 0.031 (n=8) compared to this parameter under basal conditions 0.630 ± 0.011 (n=12). At the same time, DTT significantly increased $[Ca^{2+}]_{SR}$ (Figure 3A and 3B). Therefore, it appears that disulfide oxidation is the predominant type of modification involved in elevated RyR2-mediated SR Ca^{2+} leak in HF.

Discussion

Recently, we demonstrated using a chronic canine model of HF that increased leakage of Ca^{2+} from the SR via hyperactive RyR2s is a primary factor in determining the reduced SR Ca^{2+} content and decreased amplitude of cytosolic Ca^{2+} transients in HF myocytes.[11] Our present study in the same model provides evidence that abnormal RyR2 activity in HF is at least partly attributable to modification of RyR2 by reactive oxygen species generated in the failing heart.

Multiple studies in human HF and various animal models of HF have demonstrated that the level of oxidative stress is elevated in HF because of increased production of ROS.[20–23,33] In accordance with those previous studies, ROS were increased in failing hearts (Figure 1). Moreover, the number of oxidized thiols on RyR2s increased in HF as compared to control. These changes in redox balance were paralleled by alterations in myocyte Ca^{2+} cycling, including reduced cytosolic Ca^{2+} transients (Figure 2), the elevated SR Ca^{2+} leak and reduced $[Ca^{2+}]_{SR}$ (Figures 3 and 4), abnormalities characteristic of HF. Treatment of HF myocytes with reducing reagents (DTT and MPG) normalized these parameters of intracellular Ca^{2+} cycling, by restoring normal redox balance. In contrast, treatment of normal myocytes with oxidizing agents (DTDP and thimerosal; Figure 3 and supplemental Figure III) produced alterations in SR Ca^{2+} leak and $[Ca^{2+}]_{SR}$ similar to those observed in HF cells. Likewise, at the single channel level, RyR2s from failing hearts exhibited abnormally high open probability that could be partially normalized by DTT, whereas oxidation of normal RyR2 channels enhanced their activity, reproducing the HF phenotype (Figure 5).

Previously, we demonstrated that alterations in RyR2 activity in HF are associated with altered modulation of the channel by luminal Ca^{2+}.[9] In normal RyR2 channels, lowering luminal Ca^{2+} causes a decrease in open probability.[34,35] This deactivation process is believed to be responsible for termination of systolic SR Ca^{2+} release and for the transient diastolic refractoriness of Ca^{2+} signaling that follows Ca^{2+} release.[10] In HF, the ability of RyR2 to deactivate by reduced luminal $[Ca^{2+}]$ becomes compromised, accounting for the elevated diastolic SR Ca^{2+} leak characteristic of HF myocytes.[9] If the HF-related changes in RyR2s were attributable to redox modification of the RyR2 channels, restoring normal redox

balance would be expected to normalize RyR2 responsiveness to luminal Ca^{2+}. Consistent with this expectation, application of reducing agents to restore redox balance normalized luminal $[Ca^{2+}]$ dependence of the RyR2s. Moreover, oxidizing normal RyR2s compromised their luminal Ca^{2+} modulation in a manner similar to that observed in HF RyR2s. Thus, by oxidizing and reducing normal and HF RyR2s, we were able to convert the channels between HF and normal phenotypes, respectively, providing strong evidence for the role of oxidative modifications in HF-related changes in RyR2s.

Because DTT is membrane-impermeant (at pH 7.2), the effects of this compound on RyR2 channels in HF, includingimprovement of luminal Ca^{2+} dependence, most likely occurred at the cytosolic side rather than the luminal side of RyR2s. The ability of cytosolic ligands to influence luminal Ca^{2+} sensitivity of the channel is consistent with the notion that RyR2 is an allosteric protein.[30] Other examples of such allosteric effects include modulation of RyR2 responsiveness to cytosolic ligands, such as Ca^{2+}, Mg^{2+}, and tetracaine, by luminal Ca^{2+} and calsequestrin.[34] Additionally, there is evidence that protein kinase A phosphorylation at Ser2030 alters the sensitivity of RyR2 to luminal Ca^{2+}.[36] Thus, it appears that cytosolic signals other than redox modulation are capable of influencing RyR2 luminal Ca^{2+} regulation, pre-sumably through allosteric mechanisms that traverse the channel protein. At the same time, given the partial restorative effects of DTT on SR Ca^{2+} leak and RyR2, it is also possible that some of the redox-mediated alterations of RyR2s in HF involve luminal sites. Consistent with this possibility, the activity of ryanodine receptors has been shown to be influenced by oxidation of the channel from the luminal side of the SR.[37]

RyR2 contains 89 cysteines, of which only approximately 21 are normally free,[38] with even fewer remaining unmodified in HF.[33] The redox modification of reactive cysteines is thought to involve disulfide crosslinking, *S*-nitrosylation, and/or *S*-glutathionylation.[31] The failure of the *S*-nitrosylation– and *S*-glutathionylation–specific reducing reagents glutaredoxin and ascorbate to influence the RyR2-mediated leak in a manner consistent with that of DTT suggests that disulfide oxidation is the most likely form of redox-sensitive modulation in our experimental settings. Oxidation of reactive cysteines results in disulfide bonds between neighboring subunits, which are thought to drive a conformational change, promoting RyR2 opening by sensitizing the channel to Ca^{2+}.[38,39] Being the most stable of the 3 types of modification, disulfide oxidation may competitively replace[40] *S*-nitrosylation and *S*-glutathionylation of susceptible reactive thiols during chronic HF, where there is likely to be long-term elevated oxidative stress.

Although treatment with antioxidants resulted in significant improvement of RyR2-mediated leak and single RyR2 function, the normalization was only partial in our experiments. The residual leak could be attributable to inaccessibility of modified sites to DTT, as discussed above; irreversible oxidative modification(s) of the RyR2 channel complex2;[29,41] or to other types of posttranslational modifications such as phosphorylation at either protein kinase A[7] or CaMKII sites.[17] That is, although oxidative modification(s) appear(s) to be a major causal factor in abnormal RyR2 function in our particular HF model, our results do not exclude the possibility that changes in RyR2 phosphorylation status play a role in the alterations in cellular Ca^{2+} cycling and RyR2 activity in other models, stages, or durations of HF. Interestingly, elevated circulating catecholamines (which occur in the canine tachypacing model of HF[42]) while increasing phosphorylation, can also result in oxidative stress[43,44] through autooxidation or other mechanisms. Previously, it has been proposed[33] that altered interdomain interactions coupled with dissociation of FKBP12.6 caused by redox modification of the RyR2 contributes to the enhanced RyR2-mediated leak in HF. Although our data support the role of oxidative modification of RyR2s in HF, the role of FKBP12.6 is not supported by our experiments. Indeed, in our experiments normalization of SR Ca^{2+} leak

in permeabilized cells and normalization of open probability of reconstituted RyR2s by reducing agents occurred in the absence of available FKBP12.6 to reassociate with RyR2s.

Limitations

Our studies performed in isolated myocytes and lipid bilayer experiments are limited by the fact that they may not fully represent redox modulation occurring in an intact organism. Our experiments were conducted at ambient (20%) oxygen concentrations. Increased ROS production at elevated oxygen levels could lead to more extensive redox-mediated alterations of Ca^{2+} handling in HF myocytes because of their reduced resistance to oxidative stress. Similarly, laser illumination and/or loading of myocytes with acetoxymethyl ester (AM) dyes could influence results by causing production of ROS. In separate experiments with physiological (2%) or ambient oxygen concentrations, there were no appreciable alterations in Ca^{2+} transients, SR Ca^{2+} leak, or ROS production when comparing the two oxygen concentrations in either HF or control myocytes (supplemental Figure V). Additionally, ROS levels were not affected by up to 2 hours of incubation with 10 μmol/L DCFDA or by incubation with 10 μmol/L DCFDA and 10 μmol/L Rhod-2AM (supplemental Figure VI). Nevertheless, because of limitations inherent to all experiments in ex vivo systems, our findings need to be interpreted cautiously. Although our results show that redox effects can account in part for the HF phenotype of abnormal Ca^{2+} handling in isolated myocytes and RyR2s, the applicability of these findings to in vivo settings will require further verification. Additionally, although our study focuses on redox-mediated changes in RyR2s, redox signaling has multiple targets, and additional mechanisms (eg, modification of other ion channels and transporters and of contractile filaments) are likely to contribute to alterations of cardiac contractility in HF.

Summary and Conclusions

Our study shows that abnormal oxidative modification of RyR2s by ROS contributes to the enhanced RyR2 activity and elevated SR Ca^{2+} leak that underlie the characteristic alterations of cytosolic Ca^{2+} transients and $[Ca^{2+}]_{SR}$ in chronic HF. Importantly, enhanced oxidation of reactive cysteines results in altered modulation of RyR2s by luminal Ca^{2+}. Luminal control is thought to contribute to Ca^{2+}-release termination and Ca^{2+}-signaling refractoriness following systolic release and thus plays an important role in preventing abnormal diastolic Ca^{2+} release. By linking redox effects to abnormal luminal regulation, our study provides new insights into understanding abnormal RyR2 behavior in HF. Targeted improvement of RyR2 redox-balance may present a rational therapeutic strategy for treating HF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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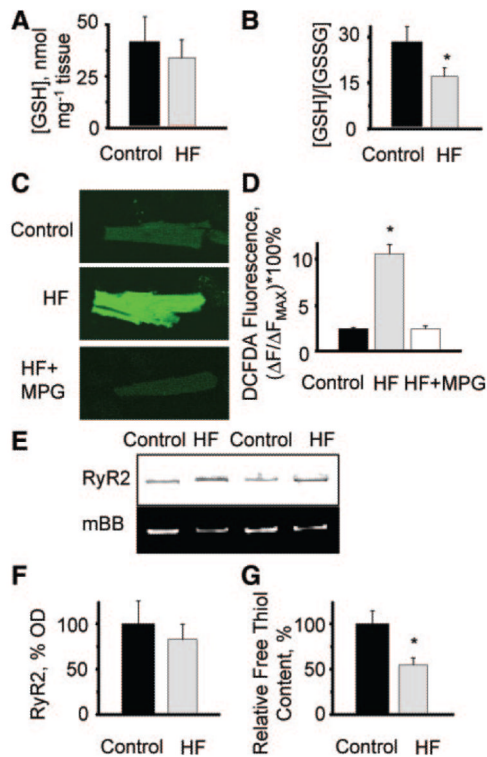
Figure 1.
Increases in oxidative stress, ROS formation, and RyR2 oxidation in heart failure. A, Averages of the levels of reduced form of glutathione (GSH) measured in tissue homogenates from normal (38.9±14.6 nmol per mg tissue) and failing hearts (29.4±11.2 nmol per mg tissue). B, Averages of ratios of reduced to oxidized glutathione (GSH/GSSG) measured in tissue homogenates from normal (28.6±10.4) and failing hearts (17.7±5.1). *P<0.05 (n=9 and 8 for control and HF, respectively). C, Fluorescence of the ROS-sensitive indicator DCFDA loaded into myocytes from normal and failing hearts. Representative images of DCFDA-loaded cells from control (top) and failing hearts under basal conditions (middle) and after 30 minutes of incubation with 800 μmol/L MPG (bottom). D, Summary graph for averages of DCFDA fluorescence normalized to the signal in the presence of 10 mmol/L H_2O_2 (F_{max}) measured in myocytes from control hearts (2.47±0.09) and myocytes from failing hearts under basal conditions (10.77±1.01) and in the presence of MPG (2.48±0.28). *P<0.05 vs control (n=21 to 32). E, Representative images of Coomassie-stained gels and mBB fluorescence intensity of RyR2 from normal and failing hearts. F, Pooled data for RyR2 content in samples from normal vs failing hearts. G, Relative free thiol content (%) of RyR2s from control vs HF samples obtained by normalizing mBB fluorescence to RyR2 level. *P<0.05 vs control (n=10).
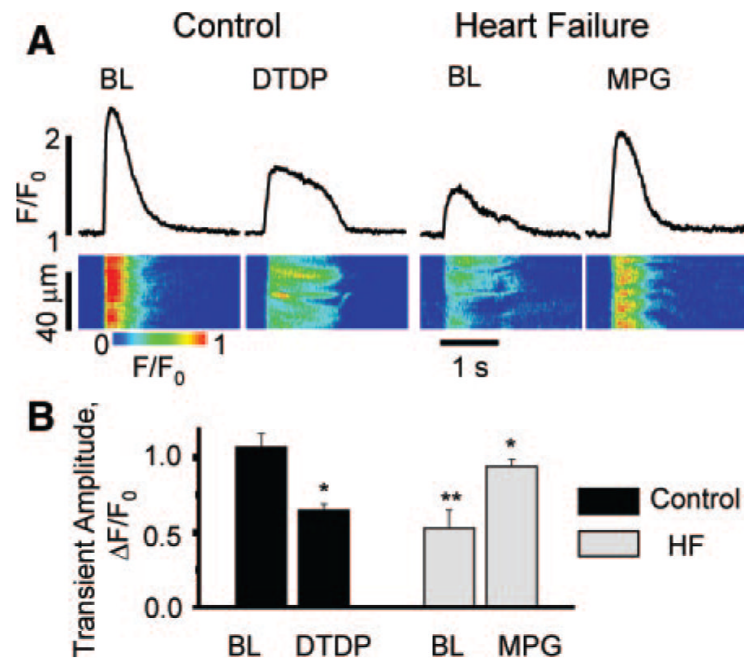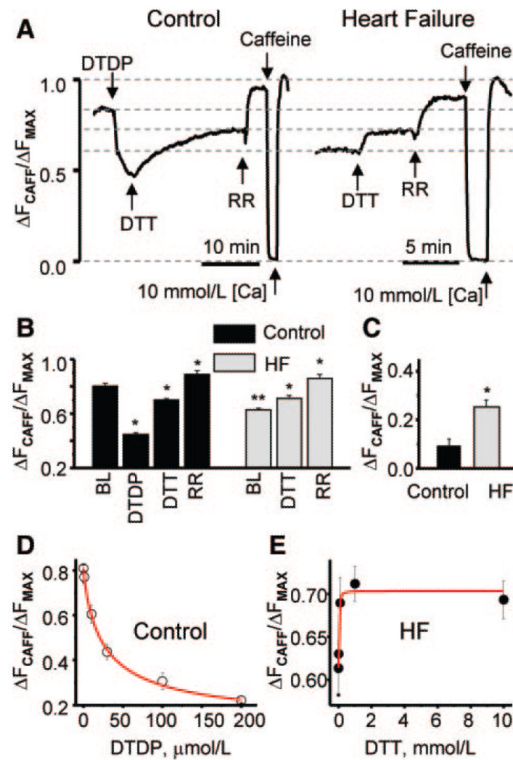
Figure 2.
Effects of oxidizing and reducing reagents on Ca^{2+} transients in control and HF myocytes. A, Representative linescan images and corresponding time-dependent profiles of cytosolic Ca^{2+} transients recorded during field stimulation (0.3 Hz) of control (left) and HF (right) myocytes under baseline (BL) conditions and after treatment of the cells with DTDP (100 μmol/L) or reducing MPG (800 μmol/L for 30 minutes), respectively. B, Summary graph for averages of amplitudes of Ca^{2+} transient. Rhod-2 $\Delta F/F_0$ values were 1.07±0.09 for control, 0.65±0.04 for control+DTDP, 0.53±0.12 for HF, and 0.94±0.05 for HF+MPG (n from 5 to 9). *$P<0.05$ vs baseline, **$P<0.05$ vs control.

Figure 3.
Effects of oxidizing and reducing agents on intra-SR Ca^{2+} in saponin-permeabilized myocytes from normal and failing hearts. A, Representative traces of time-dependent changes of cell-averaged luminal Fluo-5N signals in control (left) and HF permeabilized (right) myocytes. The experimental protocol involved consecutive applications of DTDP (50 μmol/L), DTT (1 mmol/L) and RR (30 μmol/L) in control cells and consecutive applications of DTT (1 mmol/L) and RR (30 μmol/L) in HF cells, as indicated. The values of F_{min} and F_{max} in both control and HF myocytes after removal of RR were obtained on application of 10 mmol/L caffeine with low Ca^{2+} and high Ca^{2+} (10 mmol/L), respectively. B, Summary graph of averaged Fluo-5N signals. The values of normalized Fluo-5N signal were 0.812±0.016, 0.456±0.012, 0.688±0.019, and 0.895±0.028 for baseline and in the presence of DTDP, DTT, and RR, respectively, in myocytes from normal hearts. In myocytes from failing hearts, the values for baseline and in the presence of DTT and RR were 0.610±0.012, 0.712±0.021, and 0.860±0.029, respectively. *$P<0.05$ vs baseline (BL), **$P<0.05$ vs control (n=10 to 12 for control and HF, respectively). C, SR Ca^{2+} leak determined as the difference in $[Ca^{2+}]_{SR}$ measured at the baseline conditions and in the presence of the 30 μmol/L RR in myocytes from healthy (0.083±0.026) and diseased hearts (0.25±0.017). D and E, Dose dependencies of DTDP and DTT effects on intra-SR $[Ca^{2+}]$ in myocytes from healthy (control) and failing hearts, respectively (n was from 7 to 14 for each data point). Data sets were fitted by logistic functions (red lines) with IC_{50} values of 26 μmol/L for DTDP and 28 μmol/L for DTT.
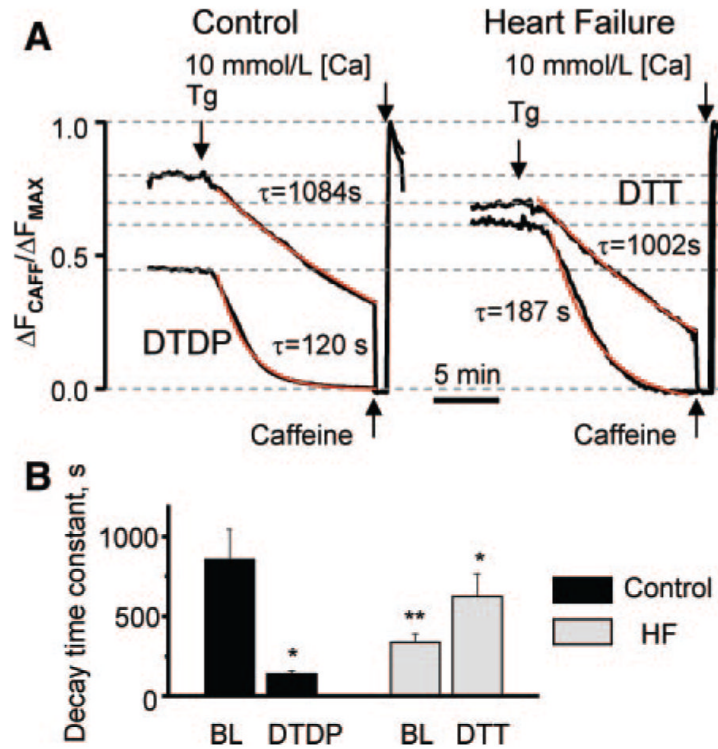
Figure 4.
Effects of oxidizing and reducing reagents on SR Ca^{2+} leak in control and HF permeabilized myocytes. A, Representative recordings of intra-SR Fluo-5N fluorescence as measures of SR Ca^{2+} leak unmasked by inhibition of the SERCA pump with Tg (10 μmol/L) in control (left) and HF permeabilized myocytes (right) under baseline conditions and after treatment of the control and HF cells with DTDP (50 μmol/L) or DTT (1 mmol/L), respectively. The decline of Fluo-5N signal in the presence of Tg was fitted by exponential functions (red lines). B, Average time constants (from exponential fits) of SR Ca^{2+} leak for control cells at baseline (BL) conditions (856±191 s, n=12) or in the presence of DTDP (139±20 seconds; n=8) (black bars) and for myocytes from failing hearts at baseline conditions (337±52 seconds; n=10) or in the presence of DTT (625±140 seconds; n=6) (light bars). *$P<0.05$ vs baseline (BL), **$P<0.05$ vs control.
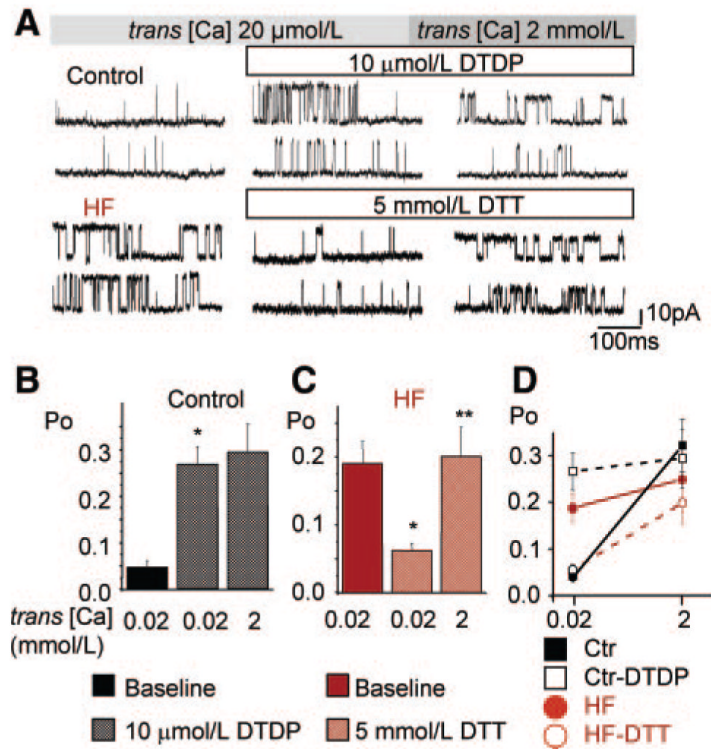
Figure 5.
Effects of oxidizing and reducing reagents on activity of RyRs from control and failing hearts. A, Representative recordings of single RyR2 channels from control and HF samples (top and bottom recordings) at low and high *trans* [Ca^{2+}] and in the presence of either DTDP (10 μmol/L) or DTT (5 mmol/L) as indicated. Single-channel activities were recorded in 350 mmol/L symmetrical $CsCH_3SO_3$ solutions at 40 mV. Channel openings are shown as upward deflections from the closed level. B, Summary data of relative values of P_o in control RyR2s before and after the addition (to the *cis* side) of 10 μmol/L DTDP at 20 μmol/L Ca^{2+} *trans* [Ca^{2+}] and following elevation of *trans* [Ca^{2+}] to 2 mmol/L. *$P<0.05$ vs baseline (n=6 channels). C, Summary data of relative values of P_o in HF RyR2s before and after the addition (to the *cis* side) of 5 mmol/L DTT with 20 μmol/L [Ca^{2+}] *trans* and following elevation of *trans* [Ca^{2+}] to 2 mmol/L. *$P<0.05$ vs baseline, **$P<0.05$ vs 20 μmol/L Ca^{2+} *trans* [Ca^{2+}] (n=7 channels). D, Summary data of relative values of P_o at low and high luminal Ca^{2+} as in B and C.