Integrative Physiology

Ranolazine Improves Cardiac Diastolic Dysfunction Through Modulation of Myofilament Calcium Sensitivity

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Rationale: Previously, we demonstrated that a deoxycorticosterone acetate (DOCA)-salt hypertensive mouse model produces cardiac oxidative stress and diastolic dysfunction with preserved systolic function. Oxidative stress has been shown to increase late inward sodium current (I_{Na}), reducing the net cytosolic Ca^{2+} efflux.

Objective: Oxidative stress in the DOCA-salt model may increase late I_{Na}, resulting in diastolic dysfunction amenable to treatment with ranolazine.

Methods and Results: Echocardiography detected evidence of diastolic dysfunction in hypertensive mice that improved after treatment with ranolazine (E/E': sham, 31.9±2.8, sham+ranolazine, 30.2±1.9, DOCA-salt, 41.8±2.6, and DOCA-salt+ranolazine, 31.9±2.6; P=0.018). The end-diastolic pressure-volume relationship slope was elevated in DOCA-salt mice, improving to sham levels with treatment (sham, 0.16±0.01 versus sham+ranolazine, 0.18±0.01 versus DOCA-salt, 0.23±0.2 versus DOCA-salt+ranolazine, 0.17±0.1 mm Hg/L; P<0.005). DOCA-salt myocytes demonstrated impaired relaxation, τ, improving with ranolazine (DOCA-salt, 0.18±0.02, DOCA-salt+ranolazine, 0.13±0.01, sham, 0.11±0.01, sham+ranolazine, 0.09±0.02 seconds; P=0.0004). Neither late I_{Na} nor the Ca^{2+} transients were different from sham myocytes. Detergent extracted fiber bundles from DOCA-salt hearts demonstrated increased myofilament response to Ca^{2+} with glutathionylation of myosin binding protein C. Treatment with ranolazine ameliorated the Ca^{2+} response and cross-bridge kinetics.

Conclusions: Diastolic dysfunction could be reversed by ranolazine, probably resulting from a direct effect on myofilaments, indicating that cardiac oxidative stress may mediate diastolic dysfunction through altering the contractile apparatus. (Circ Res. 2012;110:841-850.)

Key Words: diastole ■ ranolazine ■ oxidative stress ■ myofilaments

Diastolic dysfunction is characterized by prolonged relaxation of the myocardium, and, untreated, can lead to the clinical syndrome of heart failure with preserved ejection fraction (HFpEF). HFpEF is an increasingly prevalent health burden accounting for significant morbidity, mortality, and health care expenditures.1–4 The underlying mechanisms in diastolic dysfunction are not clearly understood, limiting treatment options.5 Recent large clinical trials using the standard therapies for systolic heart failure have failed to demonstrate improvement, further emphasizing differences in the underlying pathophysiology of diastolic dysfunction.6–8

There are several potential mechanisms for diastolic dysfunction. One is increased diastolic Ca^{2+} resulting in a slowed ventricular relaxation and diastolic dysfunction. Ca^{2+} is removed from the cytosol during diastole by the sarcoplasmic reticular Ca^{2+}-ATPase (SERCA) and the Na^{+}/Ca^{2+} exchanger (NCX). The NCX couples Ca^{2+} extrusion to the transmembrane Na^{+} gradient.9 In the failing heart, a small number of the Na^{+} channels fail to inactivate creating a late Na^{+} current (I_{Na}).10–13 The late I_{Na} increases Na^{+} entry into the cell, reducing Ca^{2+} extrusion by NCX.14 Because late I_{Na} is increased with oxidative stress,15 this represents a possible mechanism underlying diastolic dysfunction. Alternatively, myofilament cross-bridge kinetics and response to Ca^{2+} are regulated6–19 and, if altered, could contribute to diastolic dysfunction.

Previously, we have reported a hypertensive mouse model (deoxycorticosterone acetate [DOCA]-salt) of diastolic dysfunction in which oxidative stress is central to the pathology.20 Ranolazine, a novel anti-ischemic medication, is used clinically to treat angina without lowering blood pressure or heart rate.21,22

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ROS-induced increases in cardiomyocyte late $I_{\text{Na}}$ have been shown to be attenuated in vitro with ranolazine treatment.\textsuperscript{15} Therefore, we tested if, in the DOCA-salt model of isolated diastolic dysfunction, treatment with the late $I_{\text{Na}}$ blocker ranolazine would improve cardiac relaxation, negating the deleterious effects of increased cardiac oxidative stress.

### Methods

An expanded Methods section is available in the online-only Data Supplement.

#### Generation of DOCA-Salt Mouse Model

Previously, we have shown that this model leads to mild hypertension, myocardial oxidative stress, and diastolic dysfunction.\textsuperscript{20} A gradual and mild elevation in blood pressure was induced by unilateral nephrectomy, subcutaneous implantation of a controlled-release DOCA pellet (0.7 mg/dl; Innovative Research of America, Sarasota, FL) and substituting drinking water with 1.05\% saline. Control animals underwent a sham operation, had placebo pellet implantation, and received water without salt.

#### Chronic Administration of Ranolazine

A special diet containing 5 mg ranolazine, 0.3 mg P450 inhibitor, and 0.25 mg red food color was pressed into a 1-g nutritionally complete, grain-based tablet (Harlan, Madison, WI). The control diet contained 0.3 mg P450 inhibitor and 0.25 mg yellow food color pressed into the same type of 1-g tablets. DOCA-salt and sham mice consumed daily ~1 g of the special diet from postoperative day 14–18 for DOCA-salt and control mice. All experiments were approved by the University of Illinois at Chicago Animal Care and Use Committee.

#### Noninvasive Assessment of Diastolic Dysfunction

Mice were anesthetized, maintained at 37\(^\circ\)C, and studied by echocardiography (Vevo 770, VisualSonics Inc, Toronto, Canada). M-mode images in the parasternal long-axis and the left ventricular (LV) short-axis views at the midpapillary level were taken. Measurements were averaged from 3 consecutive beats during expiration. LV inflow velocities (E and A waves) were interrogated by conventional pulsed-wave Doppler from the apical 4-chamber view. The mitral annulus longitudinal velocities ($S_m$, $E'$, and $A'$) were determined by pulsed-wave tissue Doppler from the apical 4-chamber view. Interpretation was done by 2 investigators blinded to the treatment groups. First, baseline images were acquired. Subsequently, to determine the acute effect, the mice were injected with 30 mg/kg ranolazine by intraperitoneal route, followed by a second echocardiogram 30 minutes later.

### Invasive Assessment of Diastolic Dysfunction

Mice were anesthetized with 1–1.5\% isoflurane and maintained at 37\(^\circ\)C. The pressure-volume (PV) catheter was inserted into the right common carotid artery and advanced into the LV. Inferior vena cava occlusion was performed via a diaphragm incision. Volume and parallel conductance calibration were performed as previously described.\textsuperscript{20} Baseline hemodynamic measurements were obtained, and, subsequently, to determine the acute effect, the mice received an intravenous injection of ranolazine (5 mg/kg) followed by an infusion at 4.8 mg/kg per hour while additional hemodynamic measurements were recorded. Blood samples were obtained during the last 5 minutes of the procedure to determine the plasma ranolazine concentration.

### Cell Shortening and Calcium Transient Measurements

The mechanical properties of the cardiomyocytes were assessed using an IonOptix Myocam System (Ionoptix Inc, Milton, MA). Unloaded cardiomyocytes were placed on a glass slide and allowed to adhere for 10 minutes at 37\(^\circ\)C. Cardiomyocytes were then imaged with an inverted microscope and perfused with a Tyrode buffer containing 1.2 mM/L calcium at room temperature. Cardiomyocytes were paced at 0.5, 1, or 2 Hz for 10-ms duration, and sarcomere shortening and relengthening were assessed using the following indices: peak fractional shortening (FS), time to 90\% peak shortening, and $\tau$, the relaxation time constant ($a_0 + a_1e^{\tau t}$, $t$=time). Cardiomyocytes were treated with 10 \text{µmol/L} ranolazine for 10 minutes before evaluation. Initial experiments were done at room temperature and subsequent studies at 37\(^\circ\)C showed no change in the effects of DOCA-salt or ranolazine. For calcium transient measurements, cardiomyocytes were loaded with 1 \text{µmol/L} Fura 2-AM for 10 minutes at 37\(^\circ\)C, and fluorescence measurements were recorded with a dual-excitation fluorescence photomultiplier system (IonOptix). After loading, the cells were washed and resuspended in normal Tyrode solution. The cardiomyocytes were placed then in the cell chamber, stimulated at 0.5, 1, or 2 Hz for 10-ms duration, and imaged through a Fluor \times40 objective lens. Cells were exposed to light emitted by a 75-W Xenon lamp and passed though either a 340- or 380-nm wavelength filter. The emitted fluorescence was detected at 510 nm. To take into account any interference, the background fluorescence for each cardiomyocyte was determined by moving the cardiomyocyte out of the view and recording the fluorescence from the bath solution alone.

### Studies With Detergent-Extracted (Skinned) Fiber Bundles

We measured myofilament response to $Ca^{2+}$ under 2 experimental conditions as described in detail in the online-only Data Supplement. In a first series of experiments, the skinned fiber bundles were mounted in a force measuring apparatus and sarcomere length (SL) was adjusted to 2.2 \text{µm}, using a laser diffraction pattern, and width and thickness were determined for calculation of cross-sectional area. Force was measured over a range of pCa values. Skinned fiber bundles were treated with 10 \text{µmol/L} ranolazine for 15 minutes before evaluation. In a second series of experiments under different experimental conditions, we determined steady-state Mg-ATPase activity while simultaneously measuring isometric tension in skinned fiber bundles as a function of pCa. In all experiments, skinned fibers were treated with ranolazine or DMSO vehicle for 15 minutes before beginning either tension or tension/ATPase rate measurements. Tension generated was computed from the force/cross-sectional area and data were analyzed in the GraphPad Prism software and fit with a sigmoidal modified Hill equation to generate tension-pCa curves, Hill coefficients, and pCa\textsubscript{50} (pCa value at half-maximum tension). We also determined cross-birefringence kinetics by using a quick release/restretch protocol. The rate constant for force redevelopment, Ktr, provides a measure of the rate of cross-birefringence entry into the force generating state. In all experiments, only fiber bundles retaining more than 80\% of their initial maximum tension were included in the
Table 1. Effect of Ranolazine on Echocardiographic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham + Ranolazine</th>
<th>DOCA-Salt</th>
<th>DOCA-Salt + Ranolazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF, %</td>
<td>52.6±1.5†</td>
<td>52.8±0.8†</td>
<td>49.6±1.3</td>
<td>46.9±1.6</td>
</tr>
<tr>
<td>Sm, cm/s</td>
<td>2.45±0.08</td>
<td>2.31±0.09</td>
<td>2.18±0.15</td>
<td>2.15±0.14</td>
</tr>
<tr>
<td>E/A</td>
<td>1.47±0.05</td>
<td>1.63±0.07*</td>
<td>1.21±0.10</td>
<td>1.62±0.11*</td>
</tr>
<tr>
<td>E′, cm/s</td>
<td>2.77±0.15†</td>
<td>2.67±0.11*</td>
<td>2.15±0.11</td>
<td>2.47±0.18</td>
</tr>
<tr>
<td>E′/A′</td>
<td>1.22±0.06*</td>
<td>1.16±0.02*</td>
<td>0.82±0.06</td>
<td>1.31±0.11*</td>
</tr>
<tr>
<td>E/E′</td>
<td>31.9±2.8*</td>
<td>30.2±1.9*</td>
<td>41.8±2.6</td>
<td>31.9±2.6*</td>
</tr>
</tbody>
</table>

EF indicates ejection fraction; Sm, systolic septal mitral annulus velocity measured by tissue Doppler imaging (TDI); E, early diastolic filling velocity, and A, late diastolic filling velocity measured by conventional Doppler; E′, early septal mitral annulus velocity (TDI); A′, early septal diastolic mitral annulus velocity (TDI).

Data are mean ± SEM; n=8–10.

*P<0.05 versus DOCA-salt.
†P<0.05 versus DOCA-salt + ranolazine.

Results

Ranolazine Attenuated Diastolic Dysfunction

In Vivo

As previously described, DOCA-salt mice had evidence of diastolic dysfunction with preserved systolic function by transthoracic echocardiography at postoperative days 14–18 (Table 1).20 Intrapерitoneal injection of ranolazine improved diastolic dysfunction without affecting systolic function. DOCA-salt mice had significant reductions in tissue mitral annulus early longitudinal (E′) velocities and the ratio of early annulus to late annulus (E′/A′) velocities, which improved to sham levels with ranolazine treatment (Online Figure I). The ratio of early diastolic filling velocity to the early diastolic mitral annulus velocity (E/E′) has been reported to have the highest correlation with invasive hemodynamic measures of diastolic dysfunction.20,23 Hypertensive mice had a higher E/E′ compared with controls, and ranolazine returned this ratio toward normal in hypertensive mice. The mitral inflow velocities, E and A, were similar among the groups, a pseudonormal pattern, as reported before.20 The changes in relaxation parameters occurred in the absence of valvular regurgitation, LV wall motion abnormalities, or hypertrophy. Despite a slight reduction in FS (%) when comparing sham with DOCA-salt+ranolazine groups, LVEF (%) and septal annulus systolic velocity (Sm) were statistically indistinguishable among the groups, suggesting the treatments had little effect on systolic function (Table 1).

Invasive hemodynamic evaluation confirmed the echocardiographic findings (Table 2). As expected, systolic blood pressure (SBP), diastolic blood pressure (DBP), and LV end-diastolic pressure were mildly elevated in DOCA-salt mice compared with sham and sham-treated mice, although DOCA-salt mice treated with ranolazine did not differ significantly from DOCA-salt mice in any of these parameters. As described before, the best fit for the end-diastolic pressure-volume relation (EDPVR) was by the following linear function: pressure_end diastole = EDPVR×volume_end diastole+intercept.20 Hypertensive DOCA-salt mice had a steeper EDPVR compared with DOCA-salt-treated and control groups (P<0.005; Figure 1B and 1C). The slopes were 0.23±0.026, 0.17±0.01, and 0.16±0.01 mm Hg/μL; for DOCA-salt, DOCA-salt+ranolazine, and sham, respectively. Additionally, the EDPVR in DOCA-salt mice demonstrated a linear response to serum ranolazine levels (correlation coefficient=0.70, P<0.05; Figure 1E).

In an additional set of experiments, we treated DOCA-salt mice with ranolazine for 7 days and measured diastolic function using echocardiography to evaluate the effect of chronic ranolazine therapy on myocardial function (Online Table I). Prolonged treatment of DOCA-salt mice with ranolazine significantly improved the E′/A′ ratio in DOCA-salt mice (DOCA-salt, 0.74±0.05 versus DOCA-salt+ranolazine, 1.10±0.08; P<0.05). Moreover, ranolazine decreased E/E′ in DOCA-salt mice when treated chronically (DOCA-salt, 43.69±2.73 versus DOCA-

Table 2. Effect of Ranolazine on Hemodynamics

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham + Ranolazine</th>
<th>DOCA-Salt</th>
<th>DOCA-Salt + Ranolazine</th>
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<tbody>
<tr>
<td>SBP, mm Hg</td>
<td>88±3*†</td>
<td>93±3*</td>
<td>109±7</td>
<td>102±3</td>
</tr>
<tr>
<td>DBP, mm Hg</td>
<td>52±5*†</td>
<td>58±2*†</td>
<td>78±4</td>
<td>73±2</td>
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<td>HR, bpm</td>
<td>618±24</td>
<td>620±5</td>
<td>588±16</td>
<td>588±16</td>
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<td>LVEF, mm Hg</td>
<td>83±2*</td>
<td>85±3*</td>
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<td>98±3</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>3.2±0.4*</td>
<td>4.3±0.5</td>
<td>5.1±0.9</td>
<td>3.5±0.4*</td>
</tr>
<tr>
<td>EF, %</td>
<td>71±6</td>
<td>73±5</td>
<td>67±6</td>
<td>67±4</td>
</tr>
<tr>
<td>EDPR, mm Hg/μL</td>
<td>0.16±0.01*</td>
<td>0.18±0.01*</td>
<td>0.23±0.02</td>
<td>0.17±0.01*</td>
</tr>
<tr>
<td>ESPVR, mm Hg/μL</td>
<td>3.0±0.6</td>
<td>3.9±0.5</td>
<td>4.7±0.8</td>
<td>4.1±0.2</td>
</tr>
<tr>
<td>dp/dt min, mm Hg/s</td>
<td>−10 420±594</td>
<td>−10 083±685</td>
<td>−12 181±641</td>
<td>−12 211±792</td>
</tr>
<tr>
<td>dp/dt max, mm Hg/s</td>
<td>12 818±490</td>
<td>12 032±787</td>
<td>13 585±832</td>
<td>12 495±555</td>
</tr>
</tbody>
</table>

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; LVEF, left ventricular end-systolic pressure; LVEDP, left ventricular end-diastolic pressure; EF, ejection fraction; EDPR, end-diastolic pressure-volume relationship; ESPVR, end-diastolic pressure-volume relationship.

Data are mean ± SEM; n=8.

*P<0.05 versus DOCA-salt.
†P<0.05 versus DOCA-salt + ranolazine.
Ranolazine Improved Relaxation in DOCA-Salt Cardiomyocytes

Silberman et al.20 showed that impaired relaxation of cardiomyocytes was the main contributor to diastolic dysfunction in the DOCA-salt hypertensive model, finding no increase in cardiac fibrosis or inflammation. To confirm that ranolazine was working directly on DOCA-salt cardiomyocytes to improve relaxation, freshly isolated ventricular cardiomyocytes were stimulated at 0.5, 1, and 2 Hz at 37°C and assessed. DOCA-salt cardiomyocytes had preserved contractile function, as previously reported20 (Figure 2A and 2B). Additionally, treatment with ranolazine did not affect contraction in sham and DOCA-salt mice (Figure 2D). Treatment with ranolazine improved resting sarcomere length (DOCA-salt, 1.75±0.01, DOCA-salt+ranolazine, 1.81±0.01, sham, 1.85±0.02, sham+ranolazine, 1.86±0.02 μm; P<0.0001). In the absence of changes in resting Ca²⁺ (see below), this indicated that myofilament response to Ca²⁺ may be altered in diastolic dysfunction. Comparable effects were seen at 1 and 2 Hz stimulation rates and physiological temperature (Online Figure II). The effect of ranolazine in these experiments was reversible on washout (data not shown).

Diastolic Dysfunction Was Independent of Intracellular Calcium Cycling

To elucidate the mechanism underlying the impaired diastolic function in hypertensive mice, Ca²⁺ transients were measured in freshly isolated ventricular myocytes. Ca²⁺ transients did not differ significantly between sham and DOCA-salt mice, and the addition of 10 μmol/L ranolazine did not affect either group (Figure 3). Baseline intracellular Ca²⁺ was similar in all groups (Figure 3A). Additionally, peak intracellular Ca²⁺ and the rate of Ca²⁺ release was similar among all 4 groups (Figure 3B and 3C). Surprisingly, there was no difference among the rates of intracellular Ca²⁺ egress among the 4 groups measured as the time constant τ (Figure 3D). This held true at pacing rates of 1 and 2 Hz as well (Online Figure III). We cannot rule out regional variations in myocyte properties may obscure changes in Ca²⁺.
handling, however. Online Figure IX, we show phase-plane plots of the averaged Fura-2 fluorescence dual excitation ratio (340/380) versus sarcomere length during the entire contraction-relaxation cycle in sham, DOCA-salt, and ranolazine-treated groups.

**Late INa Was Not Elevated in DOCA-Salt Mice**

Oxidative stress is known to induce late INa, that can be blocked by the antianginal drug ranolazine, and the DOCA-salt model is associated with increased cardiac oxidative stress.20,24 Nevertheless, voltage-clamp studies show no increase in late INa in DOCA-salt cardiomyocytes compared with sham (sham, 0.105±0.01; sham+ranolazine, 0.113±0.004; DOCA-salt, 0.101±0.008; DOCA-salt+ranolazine, 0.114±0.013; P=NS) (Figure 4A). Integrated late INa was measured starting at 5% of peak current and ending 40 ms after depolarization. DOCA-salt myocytes accumulated a similar amount of charge as sham. Extracellular addition of 10 µmol/L ranolazine did not

![Figure 2. Functional analysis of isolated cardiomyocytes. A, Fractional shortening of isolated cardiomyocytes paced at 0.5 Hz at 37°C represented as the peak shortening divided by the baseline sarcomere length (n=12, P=NS). B, Time to 90% peak contraction in isolated cardiomyocytes (n=12, P=NS). C, Isolated cardiomyocytes from DOCA-salt mice have a prolonged relaxation constant (τ) compared with control animals. The addition of ranolazine to isolated DOCA-salt cardiomyocytes normalizes relaxation kinetics (n=12, P<0.0001 versus all groups). D, The mean diastolic sarcomere length was significantly shorter in the DOCA-salt cardiomyocytes compared with the sham. The addition of ranolazine to the DOCA-salt cardiomyocytes significantly lengthened resting sarcomeres but had no effect on sham cardiomyocytes (n=12, *P<0.0001 versus all groups).](#)

![Figure 3. DOCA-salt mice have no difference in intracellular calcium cycling when compared with sham mice. A, Resting fluorescence. B, The peak Ca²⁺ fluorescence in isolated cardiomyocytes loaded with the ratiometric fluorescent dye, Fura 2-AM, and paced at 0.5 Hz (n=12, P=NS). C, The time to 90% peak Ca²⁺ fluorescence representing the rate of calcium entry into the cytosol (n=12, P=NS). D, The rate of relaxation measured as the time constant τ did not differ among groups (n=12, P=NS).](#)
affect the late accumulated charge in DOCA-salt cardiomyocytes, which was similar to that seen for sham and sham treated myocytes (Figure 4B).

Skinned Fibers From DOCA-Salt–Treated Hearts Demonstrated Increased Response to Ca\(^{2+}\) and Depressed Cross-Bridge Kinetics

To examine directly myofilament function, the Ca\(^{2+}\) sensitivity of steady-state isometric tension development was measured in a first series of experiments with skinned fiber bundles from sham and DOCA-salt heart preparations. With DOCA-salt treatment, the mean steady-state isometric tension demonstrated an increase in maximum tension and a small but significant leftward shift of the pCa-tension relation (Figure 5, left panel). Myofilament Ca\(^{2+}\) sensitivity, indexed by pCa\(_{50}\), was significantly (\(P<0.02\)) greater in DOCA-salt than in sham myofilaments (Figure 5). As indicated in Figure 5, ranolazine treatment reduced maximum tension and returned the DOCA-salt myofilament sensitivity to levels similar to sham fibers with little effect on fiber bundles from sham hearts. The changes in Ca\(^{2+}\) sensitivity are statistically significant and in a direction that explain the decreased diastolic function. Nevertheless, we cannot completely rule out other possible mechanisms for decreased diastolic relaxation. These results imply that resting tension in Ca\(^{2+}\)-free conditions should be increased, although this was not tested here.

To assess whether the fiber bundles from DOCA-salt–treated hearts demonstrated altered cross-bridge kinetics, we measured tension development and simultaneously the rate of ATP hydrolysis (Online Figure IV). As summarized in Table 3, there was a significant increase in maximum tension of fiber bundles from DOCA-salt–treated hearts compared with shams. Treatment of the fiber bundles from DOCA-salt–treated hearts with ranolazine restored tension to the control levels. Nevertheless, measurement of ATPase rates during the development of steady-state tension showed responses different from the tension measurements. There was no significant difference of maximum ATPase rate between fibers from sham controls compared with fibers from DOCA-salt–treated hearts. Treatment of the fibers from DOCA-salt hearts with ranolazine induced a significant increase in ATPase rate. Online Figure IV shows the ATPase rate plotted as a function of tension. The slope of this relation, which provides a measure of tension cost, was significantly depressed in fibers from DOCA-salt–treated hearts compared with shams, implying a slowing of myofilament exit from the cross-bridge cycle (Table 3).25 Moreover, although ranolazine had no significant effect on the skinned fibers from shams, treatment of the DOCA-salt fibers with ranolazine induced a significant increase in ATPase rate over that of the fibers from DOCA-salt–treated hearts. We also determined Ktr, which is a measure of the rate of reentry of cross-bridges into force generating states. No change in Ktr was observed under any of the conditions studied. In a separate set of experiments, we measured myofilament calcium sensitivity of fibers from hearts of DOCA-salt mice and DOCA-salt mice chronically treated with ranolazine and confirmed that ranolazine treatment significantly reduces maximal tension in response to Ca\(^{2+}\) in vivo. Although undetected changes in Na\(^{+}\) and Ca\(^{2+}\) currents under stretch cannot be completely eliminated as a cause of diastolic dysfunction, because these experiments were carried out in skinned fibers, our data suggest that a major cause
is alterations in the myofilaments that are ameliorated by ranolazine.

Myofilament Protein Phosphorylation and Oxidation Analysis

To understand the mechanism whereby hypertension generated changes in myofilament contractile properties, we performed an analysis of myofilament protein abundance and posttranslational modifications. Representative ProQ and Coomassie gels can be seen in Online Figures V and VII. There were no changes in the abundances of myofilaments or the levels of phosphorylation of major myofilament proteins such as myosin binding protein C, troponin I, and myosin light chain 2 (Online Figure VI). Similar findings were noted with chronic ranolazine administration. Additionally, there was an increase in S-glutathionylation of myosin binding protein C in DOCA-salt mice (Figure 6A and 6B). We determined S-glutathionylation levels in DOCA-salt and sham hearts from mice after or without chronic administration of ranolazine, which confirmed our previous results (Online Figure VIII). These data indicate that there are DOCA-salt–induced changes in the state of critical myofilament proteins, which may explain the changes in myofilament activity.

Discussion

Based on the idea that cardiac oxidative stress is involved in the pathophysiologies of diastolic dysfunction and the late INa,15,20 we investigated the effects of ranolazine, a late INa blocker.

Figure 5. Myofilament Ca$^{2+}$ responsiveness in skinned fiber bundles isolated from DOCA-salt and sham hearts with and without ranolazine. A through D, The mean steady-state isometric tension of skinned fiber bundles is plotted as a function of pCa. DOCA-salt fibers demonstrate a significant (P<0.05) increase in maximum tension (44.51±0.55 mN/mm$^2$; n=6) compared with shams (39.7±0.79 mN/mm$^2$; n=6) and DOCA-salt fibers treated with ranolazine (36.03±1.50 mN/mm$^2$). DOCA-salt fibers demonstrate a significant (P<0.05) increase in mean Ca$^{2+}$ sensitivity (pCa$_{50}$=6.09±0.01; n=6) compared with shams (pCa$_{50}$=6.02±0.01; n=6). Treatment with ranolazine normalized these changes. E, pCa-tension relations normalized to maximum tension. DOCA-salt fibers (pCa$_{50}$=6.1±0.02; Hill n=3.42±0.30) demonstrate a significant increase in mean Ca$^{2+}$ sensitivity (*P<0.05) as compared with shams without DOCA treatment (pCa$_{50}$=6.0±0.01; Hill n=3.80±0.61), shams with DOCA treatment (pCa$_{50}$=6.0±0.01; Hill n=3.91±0.50), and fibers from DOCA mice and treated with ranolazine (pCa$_{50}$=6.0±0.03; Hill n=3.71±1.0).

Table 3. Effect of Ranolazine on Tension and ATPase Rate of Skinned Fiber Bundles

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Sham + Ranolazine</th>
<th>DOCA-Salt</th>
<th>DOCA-Salt + Ranolazine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum tension, mN/mm$^2$</td>
<td>26.84±0.35†‡</td>
<td>36.80±0.16</td>
<td>33.46±0.38</td>
<td>27.19±0.54</td>
</tr>
<tr>
<td>Maximum ATPase, pmol·s$^{-1}$·mg$^{-1}$</td>
<td>233.2±2.9†‡</td>
<td>302.0±9.0*</td>
<td>234.6±0.7</td>
<td>326.9±8.7*</td>
</tr>
<tr>
<td>Tension cost, ΔATPase/Δtension</td>
<td>8.3±0.4*</td>
<td>7.1±0.4</td>
<td>6.4±0.5</td>
<td>10.8±0.8*</td>
</tr>
<tr>
<td>Passive tension, mN/mm$^2$</td>
<td>0.45±0.26</td>
<td>0.55±0.33</td>
<td>0.20±0.32</td>
<td>0.68±0.40</td>
</tr>
<tr>
<td>Ktr</td>
<td>10.9±0.7</td>
<td>11.2±0.5</td>
<td>11.2±0.9</td>
<td>11.0±1.0</td>
</tr>
</tbody>
</table>

Ktr indicates rate constant for force redevelopment. Data are mean±SEM; n=5–7.

*P<0.05 versus DOCA-salt.
†P<0.05 versus DOCA-salt + ranolazine.
‡P<0.05 versus sham + ranolazine.
inhibitor, on the mechanical derangements induced in the DOCA-salt hypertensive model of diastolic dysfunction. Mild hypertension in this model resulted in impaired relaxation that improved acutely after ranolazine treatment. Without changes in heart rate or blood pressure, ranolazine rapidly improved relaxation when measured both noninvasively and invasively. EDPPVR, the most widely accepted measure of relaxation, significantly improved in the DOCA-salt mice confirming our noninvasive echocardiographic results. In our study, the EDPPVR relationship but not dP/dt min was different between controls and DOCA-salt mice. This discrepancy has been noted in other studies and, given our results, may imply that actin-myosin cross-bridge cycling affects a latter phase of cardiac diastole. Additionally, ranolazine did not significantly affect hemodynamics in the sham mouse. At the cellular level, isolated DOCA-salt cardiomyocytes demonstrated impaired relaxation that improved with ranolazine.

Silberman et al. reported the DOCA-salt model of diastolic dysfunction was associated with cardiac oxidative stress and targeting of reactive oxygen species production improved diastolic function. We sought to investigate the downstream mediators of the increased oxidative load to better elucidate the mechanisms regulating diastolic function. Oxidative stress is known to modulate a number of proteins important in cardiac function, including the SR Ca\(^{2+}\) pump, the sarcolemmal L-type Ca\(^{2+}\) channel, the sodium-calcium exchanger, phospholamban, myofilaments, and the late I\(_{Na}\). Largely on the basis of the analogy to systolic dysfunction, we expected to find increased diastolic Ca\(^{2+}\) resulting in slowed myocyte relaxation and diastolic dysfunction, but surprisingly, we found no changes in Ca\(^{2+}\) cycling between DOCA-salt and sham mice. Instead, we noted changes in myofilament activity in the absence of changes in Ca\(^{2+}\) handling. This is consistent with other models of diastolic dysfunction including a model of diabetic cardiomyopathy in which similar changes in sarcomere length were reported with no correlating changes in Ca\(^{2+}\) cycling.

Previously, it has been reported that myocyte relaxation can be dissociated from the decline of intracellular Ca\(^{2+}\) and myofilament Ca\(^{2+}\) sensitivity is a consistent functional abnormality seen in dilated cardiomyopathy. These results suggest that although oxidative stress is associated with both systolic and diastolic dysfunction, mediators of these dysfunctions appear to differ. This may help explain the unimpressive results treating diastolic dysfunction when using drugs proven to be beneficial in systolic heart failure.

In vitro studies demonstrating the effectiveness of ranolazine to treat impaired relaxation have used isolated muscle strips, isolated cardiomyocytes, and working heart preparations. Previous studies in both rabbit and rat models have shown that ranolazine attenuates diastolic dysfunction in ischemia/reperfusion. Additionally, in a dog model of chronic heart failure, ranolazine reduces LVEDP. Finally, trials in humans with ischemic heart disease and type 3 long-QT syndrome have supported a role for ranolazine in the treatment of diastolic dysfunction. The mechanism for this effect was thought to be a reduction in late I\(_{Na}\) with a subsequent reduction in diastolic Ca\(^{2+}\). Despite the efficacy of ranolazine in the relief of diastolic dysfunction, no increase in late I\(_{Na}\) in the DOCA-salt cardiomyocytes was noted, nor were there changes in calcium cycling to indicate significant alterations in Ca\(^{2+}\) handling in this form of diastolic dysfunction. On the other hand, at rest, the DOCA-salt myocytes had a significant decrease in sarcomere length in the absence of changes in Ca\(^{2+}\) concentration, suggesting increased diastolic tension compared with sham mice. Myofilaments from diastolic dysfunction mice had increased maximum tension and sensitivity to Ca\(^{2+}\) as well as a slowing of cross-bridge exit kinetics compared with sham mice that normalized with ranolazine treatment. Taken together, these results suggest ranolazine improved diastolic function at the cardiomyocyte level through the modulation of myofilament cross-bridge kinetics and sensitivity to Ca\(^{2+}\). The decrease in sarcomere length in intact cells with no change in passive tension of the muscle strips is consistent with our hypothesis that alterations in cross-bridges are responsible for the changes in diastolic function. This result is consistent with the significance of molecular motors as determinants of relaxation in ejecting hearts.

Our investigation of the posttranslational modifications indicated that there was a significant increase in glutathionylation of myosin binding protein C in animals with diastolic dysfunction. Interestingly, accumulating evidence indicates a significant role of myosin binding protein C in control of cross-bridge kinetics. This posttranslational modification supports the hypothesis that hypertension-induced alterations in contractile parameters result from oxidative modification of myosin binding protein C. Inasmuch as acute and direct addition of ranolazine to the skinned fiber bundles enhanced cross-bridge kinetics and the effects of ranolazine were reversible with washout, it seems likely that this agent has direct effects on the myofilaments. Because the effect of ranolazine correlated with myosin binding protein C glutathionylation, the effect of ranolazine was present.
only in diastolic dysfunction, and glutathionylation was not altered by ranolazine, oxidative modification of myofilaments may create a binding site for ranolazine to relieve diastolic dysfunction. Future studies will determine the posttranslational modifications, not necessarily limited to phosphorylation and glutathionylation, which may occur to other myofilament proteins, how these modifications influence the modification of other proteins, and what targets are best for therapeutic interventions to obtain a desirable phenotypic response.

Measurement of diastolic dysfunction is complicated and controversial. There is no agreement on the best technique. The left ventricular pressure-volume relationship generated during venous occlusion was used in this study because it allows characterization of diastolic function independent of load conditions. Nevertheless, there can be errors in estimating volume because of nonlinearities in the conductance-volume relationship that are more prominent during large-volume deviations. Using only the first 6 pressure-volume loops during venous occlusion resulted in an identical EDPVR, so the likelihood of a sizable error is small. Moreover, in this study, we measured diastolic dysfunction in several different ways and with several different preparations. The effect of ranolazine on diastolic function was seen in vivo by conductance catheter measurements and echocardiography and in vitro using muscle strips and isolated myocytes.

In conclusion, the present study demonstrates that ranolazine treatment improves diastolic function through modulation of myofilament activity. Ranolazine dosing in human studies has shown the therapeutic concentration ranges between 2–6 μM/L. Therefore, we were able to produce an effect on diastolic function in the DOCA-salt mice with roughly the same concentrations used clinically.26 Our DOCA-salt model is one of the few animal models of diastolic dysfunction in the absence of hypertrophy or changes in ejection fraction, but these results may not be transferable to humans. On the other hand, a human trial suggests that ranolazine may have salutary effects in diastolic dysfunction associated with myocardial injury.43

Acknowledgments
We are grateful for excellent technical assistance by Chad M. Warren with gel analysis and by Amin Rmeileh with skinned fiber tension measurements.

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Disclosures
Patents are pending for Methods and Compositions for Treating Diastolic Dysfunction (60/840,368) and Sodium Current Blockade to Treat Diastolic Heart Failure (61/241,585 and 61/263,920) (S.C.D.).

References

What Is Known?
- Diastolic heart failure is increasing in incidence, and the cause is unknown.
- Diastolic dysfunction is associated with cardiac oxidative stress, and oxidation can cause prolonged sodium channel opening (i.e., a late current), leading to calcium loading of cardiomyocytes, possibly causing diastolic dysfunction.
- Ranolazine blocks the sodium channel late current and may treat diastolic dysfunction.

What New Information Does This Article Contribute?
- Ranolazine improved hypertension-mediated diastolic dysfunction when measured in vivo, in muscle strips, or in isolated myocytes.
- Ranolazine acted directly on myofilaments to improve relaxation, not through blocking the late sodium current.
- Diastolic dysfunction was correlated with oxidative modification of myosin binding protein C, suggesting cardiac oxidative stress may mediate diastolic dysfunction through altering the contractile apparatus.

Heart failure with preserved ejection fraction occurs in approximately half of all heart failure cases. This type of heart failure is caused by a failure of the myocardium to relax properly, but the mechanism of the diastolic dysfunction is unknown. Furthermore, no specific treatments are currently available. Previously, it has been shown that diastolic dysfunction is associated with cardiac oxidation and oxidative stress causes a prolonged cardiomyocyte sodium entry. This entry leads to diastolic calcium loading which may explain the impaired relaxation. Ranolazine is known to block this type of sodium entry, so we tested its ability to treat diastolic dysfunction. In a hypertension-mediated mouse model, diastolic dysfunction was correlated with oxidative modification of myosin binding protein C. Ranolazine was able to ameliorate diastolic dysfunction measured multiple different ways and in several preparations. This effect was a result of ranolazine acting directly on the myofilaments rather than blocking the late sodium current, however. In conclusion, ranolazine may be useful in the treatment of diastolic dysfunction.
Ranolazine Improves Cardiac Diastolic Dysfunction Through Modulation of Myofilament Calcium Sensitivity
Joshua D. Lovelock, Michelle M. Monasky, Euy-Myoung Jeong, Harvey A. Lardin, Hong Liu, Bindiya G. Patel, Domenico M. Taglieri, Lianzhi Gu, Praveen Kumar, Narayan Pokhrel, Dewan Zeng, Luiz Belardinelli, Dan Sorescu, R. John Solaro and Samuel C. Dudley, Jr

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Supplemental Material

Ranolazine improves cardiac diastolic dysfunction through modulation of myofilament calcium sensitivity


Supplemental Methods

Myocyte isolation: Cardiac ventricular myocytes were isolated from the hearts of DOCA-salt or age matched controls mice 14-18 d post-operatively using a modified enzymatic digestion protocol from the Alliance for Cellular Signaling as previously described.\(^1\)

Voltage clamping studies: Voltage-clamp experiments were performed on isolated murine ventricular myocytes with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) in whole cell configuration. Data acquisition was performed at a sampling rate of 20 kHz and filtered at 10 kHz. Data recording and analysis were done with the pClamp8 software suite (Molecular Devices) and OriginPro 8 (OriginLab, Northampton, MA). All experiments were carried out at room temperature. Myocytes were plated on glass cover slips and were perfused with a low-sodium Tyrode solution containing the following (in mM): N-methyl-D-glucamine 100 (titrated to pH 7.4 with HCl), NaCl 15, tetramethylammonium chloride 20, CsCl 5, MgCl\(_2\) 1, glucose 10, 4-aminopyridine 3, MnCl\(_2\) 2, HEPES 10, and CaCl\(_2\) 1 (final pH 7.4, CsOH). Patch electrodes were pulled from capillaries purchased from Harvard Apparatus (Holliston, MA) using a model P-97 puller from Sutter Instruments (Novato, CA). Electrodes were filled with an electrode solution containing (in mM): CsCl 20, tetraethylammonium chloride 20, glutamic acid 80, NaCl 10, MgCl\(_2\) 1, MgATP 5, Li\(_2\)GTP 0.3, HEPES 10, EGTA 10, MgCl\(_2\) 0.13 (corresponding to [Ca\(^{2+}\)]\(_{free}\) of < 10 nM)\(^2\). Electrode solution pH was adjusted to 7.2 with CsOH. Electrodes used for these experiments had access resistances between 1.0 and 1.5 MΩ.

Ranolazine was provided as a crystalline solid by Gilead Sciences. Prior to acute experiments, a DMSO stock solution was prepared and diluted (minimum 100:1) directly into the Tyrode solution used for perfusion. Cells that were treated with ranolazine were exposed to the drug for 15 min prior to beginning voltage-clamp experiments.

Studies with detergent extracted (skinned) fiber bundles: Mice were anesthetized with pentobarbital (50 mg/kg IP), and the hearts were rapidly excised and rinsed in a pH 7.0 ice-cold relaxing solution (HR) composed of (in mM) 10 EGTA, 41.89 K-Prop, 6.57 MgCl\(_2\), 100 BES, 6.22 ATP, 5 Na azide, and 10 creatine phosphate. The solution also contained 1 µg/ml leupeptin, 2.5 µg/ml pepstatin A, and 50 µM phenylmethylsulfonyl fluoride. Left ventricular papillary muscles were dissected and fiber bundles were prepared as previously described.\(^3\) The fiber bundles were extracted overnight in relaxing solution plus 1% Triton X-100 at 4°C.

In a first series of experiments the skinned fiber bundles were mounted with cellulose acetate glue in a force measuring apparatus and sarcomere length was adjusted to 2.2 µm using a laser diffraction pattern and width and thickness were determined for calculation of cross-sectional area.\(^4\) The skinned fibers were activated at 22°C over a series of pCa (-log of the M Ca\(^{2+}\) concentration) values between pCa 8.00 and pCa 4.5. Activating solutions were prepared by mixing HR with a pCa 4.50 solution of 10.00 mM EGTA, 9.99 mM CaCl\(_2\), 22.16 mM K-Prop (K-Prop stock had 1.00 M propionic acid and 1.00 M KOH), 6.20 mM MgCl\(_2\), 100.00 mM BES, 6.29 mM Na-ATP, 10.00 mM creatine phosphate (CrP), 5.00 mM Na-azide, 2.5 µg/mL pepstatin, 1 µg/mL leupeptin, and 50 µM PMSF, pH 7.0. HR and pCa8.00-pCa 4.50 solutions had one unit of creatine phosphokinase per 200 µL of solution.
In a second series of experiments force and ATPase rate were measured simultaneously using methods and an experimental apparatus previously described. The fiber bundles were mounted between a force transducer and displacement motor using aluminum T-clips, and the sarcomere length was set to 2.2 μm using He-Ne laser diffraction. The width and diameter were each measured at three points along the fiber bundle. Force per cross-sectional area was used to determine tension. The fiber was contracted initially at a saturating calcium concentration (pCa 4.5) and sarcomere length was again adjusted to 2.2 μm. Sarcomere length remained constant throughout the rest of the experiment.

ATPase activity was measured at 20°C as previously described and calibrated with rapid injections of ADP (0.5 nmol) with a motor-controlled syringe. The fiber was placed in relaxing solution for 2 min, then in the preactivation solution for 2-3 min each time before being placed in the activating solution for 1-2 min (until stabilization of force) and then quickly returned to the relaxing solution. Various contraction-relaxation cycles were carried out using different ratios of total calcium concentration to total EGTA concentration. The final contraction was again at a saturating calcium concentration. The relation between Ca²⁺ and tension or ATPase activity was fitted using a modified Hill equation as described previously.

Analysis of Post-Translational Modifications: Skinned fibers with 1% (v/v) Triton X-100 were solubilized in 15 μL sample buffer (8 M urea, 2 M thiourea, 0.05 M tris pH 6.8, 75 mM DTT, 3% SDS, and 0.05% bromophenol blue) by incubation on a shaker for 30 min followed by two cycles of 10 min incubations in a sonicating bath with 30 seconds vortexing between the incubations. Samples were heated at 100°C for three min and after 10 min spin clarification, all of the supernatant was loaded on to a 12% resolving 1D SDS-PAGE gel. The gels were stained and destained with Pro-Q Diamond (Invitrogen) according to the manufacturer's recommendations preceding imaging with a Typhoon 9410 scanner (GE Healthcare). Optical density of the proteins was determined using ImageQuant TL (GE Healthcare) software and Commassie R-250 stained gel was used to normalize protein load to both actin the whole lane. Results were exported to Excel and analyzed with JMP statistical software (Cary, NC).

Western blot analysis was used to detect for glutathionylated proteins. Myofibrils were prepared from DOCA and sham mouse hearts, and pellets were solubilized in a non-reducing 2X Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris HCl pH 6.8). 25 mM N-ethylmaleimide (NEM) was added to the standard rigor buffer with Triton X-100, the standard rigor wash buffer and the 2X Laemmli buffer. Using the protein concentration determined from an RC-DC (Bio-Rad) assay, 40 μg of total protein was applied to 1D 12% resolving SDS-PAGE gel and transferred onto a 0.2 μM Polyvinylidene fluoride (PVDF) membrane. The blot was blocked in 5% nonfat dry milk with 2.5 mM NEM for 1 hour. Anti-glutathione mouse monoclonal primary antibody (Virogen, Watertown, MA) was used at 1:1000 dilution along with anti-mouse HRP-conjugated secondary antibody (Sigma) at 1:100,000 dilution to detect for S-glutathionylation. Optical density of the bands was measured with ImageQuant TL (GE Healthcare) and exported to Excel for further analysis. An antibody to myosin binding protein C was used for identification of the band indentified as being modified.

Statistical analysis: Each value is expressed as mean ± SE. A one-way ANOVA was used to test for mean differences in invasive and noninvasive parameters. Where appropriate, post hoc ANOVA testing (Tukey's) was used to assess mean differences between groups at a given time point. A p value < 0.05 was considered significant.
References
Supplemental Figure Legends

**Online Figure I.** Representative echocardiographic assessments of LV diastolic function. Septal mitral annulus velocities interrogated with tissue Doppler imaging (TDI). The sham mouse has a higher E’ (early diastolic velocity), and lower A’ (late diastolic velocity) than the hypertensive DOCA-salt mouse (upper panel). Treatment with ranolazine increased the ratio of E’ to A’ in the DOCA-salt mouse. Treatment of the sham mouse with ranolazine had little effect on mitral annulus velocities in the sham mouse. Sm (systolic septal mitral annulus velocity) was similar among all four groups.

**Online Figure II.** Effects of rate on relaxation and resting sarcomere length in isolated cardiomyocytes. A: DOCA-salt cardiomyocytes paced at 1 Hz show a significantly prolonger relaxation time (τ) compared with the other groups. B: At 2 Hz, the effect on relaxation was similar to slower pacing with a significantly slowed relaxation time. Ranolazine improved relaxations times at both frequencies. C and D: DOCA-salt cardiomyocytes demonstrated a significantly shorter sarcomere length compared to the other groups and both 1 and 2 Hz. Ranolazine lengthened sarcomere length at both frequencies. *p < 0.05 DOCA-salt vs. all other groups.

**Online Figure III.** Effect of rate on resting Ca^{2+} levels and Ca^{2+} extrusion from isolated cardiomyocytes. A and B: There is no significant difference in resting Ca^{2+} levels at rest among the four groups at 1 and 2 Hz. C and D: Additionally, there were no significant differences in the rate of Ca^{2+} extrusion from the isolated cardiomyocytes at rates of 1 and 2 Hz.

**Online Figure IV.** Chemo-mechanical transduction in fiber bundles treated with ranolazine from sham and DOCA-salt treated hearts. The relationship between ATPase rate and tension development. Tension cost was determined as the slope of the relationship between tension and ATPase activity. *P < 0.05 for DOCA-salt + ranolazine compared to DOCA-salt + DMSO. †P<0.05 for DOCA-salt + DMSO compared to Sham + DMSO. See text for details.

**Online Figure V.** Representative ProQ (A) and Coomassie R-250 gels (B) showing phosphorylation states of various myofilament proteins of fibers treated acutely with either ranolazine or DMSO.

**Online Figure VI.** Representative comparisons of the post-translational modifications of the myofilaments from sham and DOCA-salt hearts. A: Comparison of myosin binding protein C (MyBP-C), B: Troponin I (TnI), and C: myosin light chain 2 (MLC2) phosphorylations in fiber bundles from sham and DOCA-salt hearts. There were no significant differences. Values are given as means ± SEM for 6-9 determinations.

**Online Figure VII.** Representative ProQ (A) and Coomassie R-250 gels (B) showing phosphorylation states of various myofilament proteins of hearts from DOCA-salt or sham mice after or without chronic administration of ranolazine.

**Online Figure VIII.** A: Representative gels showing changes in S-glutathionylation of myosin binding protein-C. B: Comparison of myosin binding protein C (MyBP-C) S-glutathionylation in hearts from DOCA-salt or sham mice after or without chronic adminstration of ranolazine. Values are given as means ± SEM for 5 determinations.
Online Figure IX. Phase-plane diagram between the fura-2 ratio (340/380) and sarcomere length during the entire contraction-relaxation cycle between sham and DOCA-salt cardiomyocytes in the presence or absence of ranolazine.

Online Figure X. The normalized contraction of individual cardiomyocytes illustrating the difference in relaxation between a DOCA-salt mouse and a ranolazine treated DOCA-salt mouse.
Online Table I. Chronic Effect of Ranolazine on echocardiographic parameters

Data are means ± SEM. EF, ejection fraction; Sm, systolic septal mitral annulus velocity measured by tissue doppler imaging (TDI); E, early diastolic filling velocity and A, late diastolic filling velocity measured by conventional doppler; E’, early septal mitral annulus velocity (TDI); A’, late diastolic septal mitral annulus velocity (TDI). n=7-10, *p < 0.05 vs. DOCA-salt.
Online Figure I

Sham

DOCA-salt

Sham + ranolazine

DOCA-salt + ranolazine
Online Figure II

A. 1 Hz

B. 2 Hz

C. Sarcomere length (µm)

D. Sarcomere length (µm)
Online Figure III

A

1 Hz

Resting fluorescence (340 nm / 380 nm)

Sham  Sham + ran  DOCA-salt  DOCA-salt + ran

0.0 0.5 1.0 1.5

B

2 Hz

Resting fluorescence (340 nm / 380 nm)

Sham  Sham + ran  DOCA-salt  DOCA-salt + ran

0.0 0.5 1.0 1.5

C

Extrusion of calcium τ (s)

Sham  Sham + ran  DOCA-salt  DOCA-salt + ran

0.0 0.1 0.2 0.3 0.4

D

Extrusion of calcium τ (s)

Sham  Sham + ran  DOCA-salt  DOCA-salt + ran

0.0 0.1 0.2 0.3 0.4
Online Figure IV

**Sham**
- Slope = 8.3 ± 0.4°

**DOCA-salt**
- Slope = 6.4 ± 0.5

**Sham + ranolazine**
- Slope = 7.1 ± 0.4

**DOCA-salt + ranolazine**
- Slope = 10.8 ± 0.8°
Online Figure V

A

B

Actin

MLC2

TnT-3

TnT-4

TnI

MyBP-C

Mwt (KDa) Sham + DMSO Sham + Ran DOCA-salt + DMSO DOCA-salt + Ran
Online Figure VI

(A) MyBP-C

(B) TnI

(C) MLC-2
Online Figure VII

A

B

Mwt (KDa) | Sham Control | Sham + Ran | DOCA-salt Control | DOCA-salt + Ran

25

75

MyBP-C

TnT-3

TnT-4

Tnl

MLC2

25

Actin
Online Figure VIII

A

Mwt (KDa)

Sham Control  Sham Ranolazine  DOCA Control  DOCA Ranolazine

Anti-Glutathione

Anti-MyBP-C

B

GSP/MyBP-C (%)

Sham  Sham  DOCA-Salt  DOCA-Salt + ran

* * *
Online Figure IX

![Graph showing sarcomere length vs. Fura-2 dual excitation ratio (340/380).]

- Sham
- Sham + ran
- DOCA-salt
- DOCA-salt + ran
Online Figure X

Sarcomere Length (normalized)

- DOCA-salt
- DOCA-salt + ran

400 msec