Supplemental Material

Ranolazine improves cardiac diastolic dysfunction through modulation of myofilament calcium sensitivity

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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_2GTP 0.3, HEPES 10, EGTA 10, $CaCl_2$ 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.[5, 6] 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.[7] 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.[4, 6] 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 Ca^{2+} and tension or ATPase activity was fitted using a modified Hill equation as described previously.[8]

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.[9] 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.[6, 10] 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.[9] 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.[10] 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

1. Silberman GA, Fan TH, Liu H, Jiao Z, Xiao HD, Lovelock JD, Boulden BM, Widder J, Fredd S, Bernstein KE, Wolska BM, Dikalov S, Harrison DG, Dudley SC, Jr. Uncoupled cardiac nitric oxide synthase mediates diastolic dysfunction. *Circulation.* 2010;121:519-528.

2. Patton C, Thompson S, Epel D. Some precautions in using chelators to buffer metals in biological solutions. *Cell Calcium.* 2004;35:427-431.

3. Wolska BM, Vijayan K, Arteaga GM, Konhilas JP, Phillips RM, Kim R, Naya T, Leiden JM, Martin AF, de Tombe PP, Solaro RJ. Expression of slow skeletal troponin I in adult transgenic mouse heart muscle reduces the force decline observed during acidic conditions. *J Physiol.* 2001;536:863-870.

4. de Tombe PP, ter Keurs HE. Force and velocity of sarcomere shortening in trabeculae from rat heart. Effects of temperature. *Circ Res.* 1990;66:1239-1254.

5. de Tombe PP, Stienen GJ. Protein kinase A does not alter economy of force maintenance in skinned rat cardiac trabeculae. *Circ Res.* 1995;76:734-741.

6. Wolska BM, Keller RS, Evans CC, Palmiter KA, Phillips RM, Muthuchamy M, Oehlenschlager J, Wieczorek DF, de Tombe PP, Solaro RJ. Correlation between myofilament response to Ca^{2+} and altered dynamics of contraction and relaxation in transgenic cardiac cells that express beta-tropomyosin. *Circ Res.* 1999;84:745-751.

7. Vahebi S, Kobayashi T, Warren CM, de Tombe PP, Solaro RJ. Functional effects of rho-kinase-dependent phosphorylation of specific sites on cardiac troponin. *Circ Res.* 2005;96:740-747.

8. Canton M, Neverova I, Menabo R, Van Eyk J, Di Lisa F. Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. *Am J Physiol Heart Circ Physiol.* 2004;286:H870-877.

9. Layland J, Cave AC, Warren C, Grieve DJ, Sparks E, Kentish JC, Solaro RJ, Shah AM. Protection against endotoxemia-induced contractile dysfunction in mice with cardiac-specific expression of slow skeletal troponin I. *FASEB J.* 2005;19:1137-1139.

10. Hill BG, Ramana KV, Cai J, Bhatnagar A, Srivastava SK. Measurement and identification of S-glutathiolated proteins. *Methods Enzymol.*473:179-197.

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. $^{\dagger}P<0.05$ 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 (MLC_2) 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.

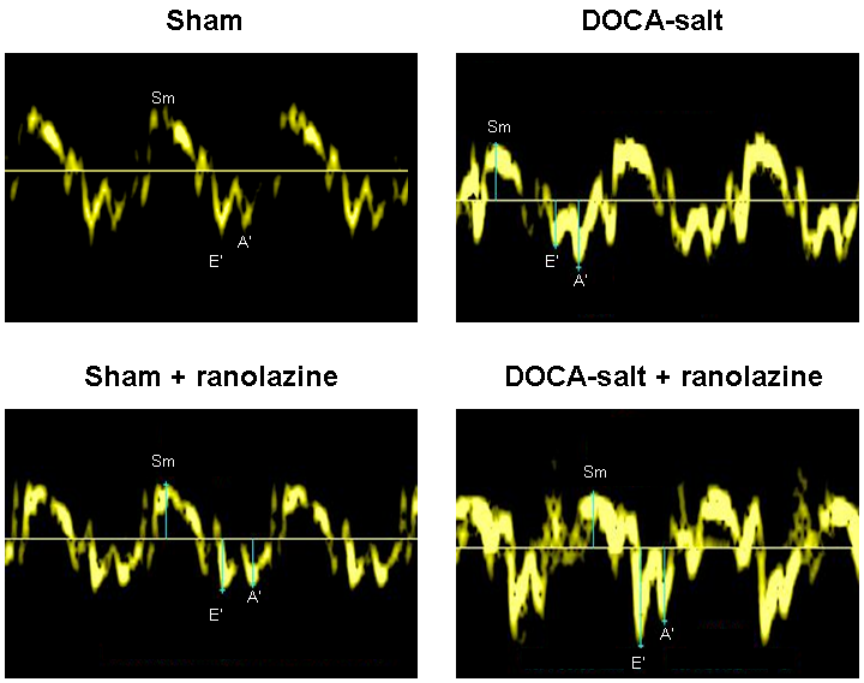
Online Figure X. The normalized contraction of individual cardiomyocytes illustrating the difference in relaxation between a DOCA-salt mouse and a ranolazine treated DOCA-salt mouse.

Online Table I. Chronic Effect of Ranolazine on echocardiographic parameters

	Sham	Sham + ranolazine	DOCA-salt	DOCA-salt + ranolazine
ES (%)	55.1 ± 4.7	49.7 ± 4.9	50.8 ± 4.3	53.3 ± 1.3
Sm (cm/s)	2.85 ± 1.87	2.04 ± 0.12	1.85 ± 0.17	1.82 ± 0.05
E/A	2.31 ± 0.31	2.42 ± 0.42	3.11 ± 0.85	1.84 ± 0.35
E’ (cm/s)	2.23 ± 0.17*	2.11 ± 0.21	1.43 ± 0.08	1.86 ± 0.13*
E’/A’	1.20 ± 0.09*	1.03 ± 0.07	0.74 ± 0.05	1.10 ± 0.08*
E/E’	30.4 ± 1.17*	28.0 ± 3.45*	43.7 ± 2.73	29.3 ± 5.07*

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



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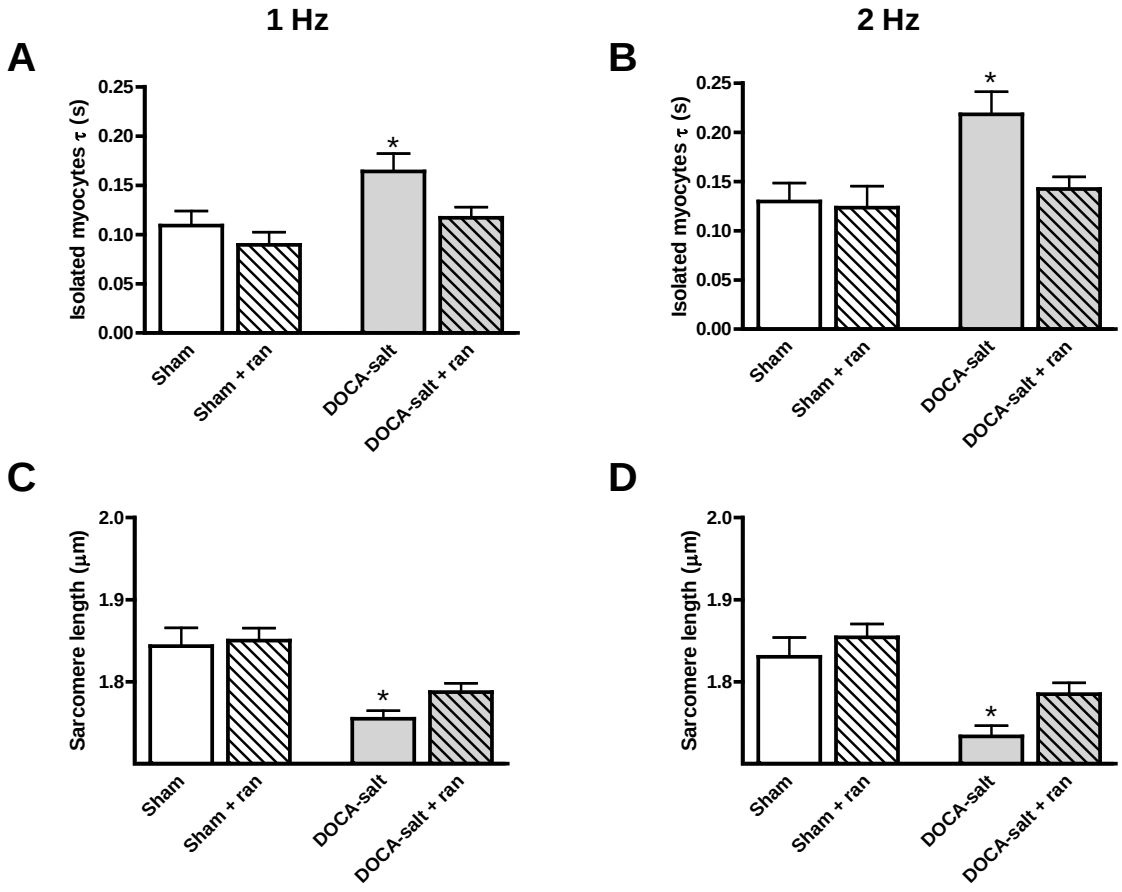

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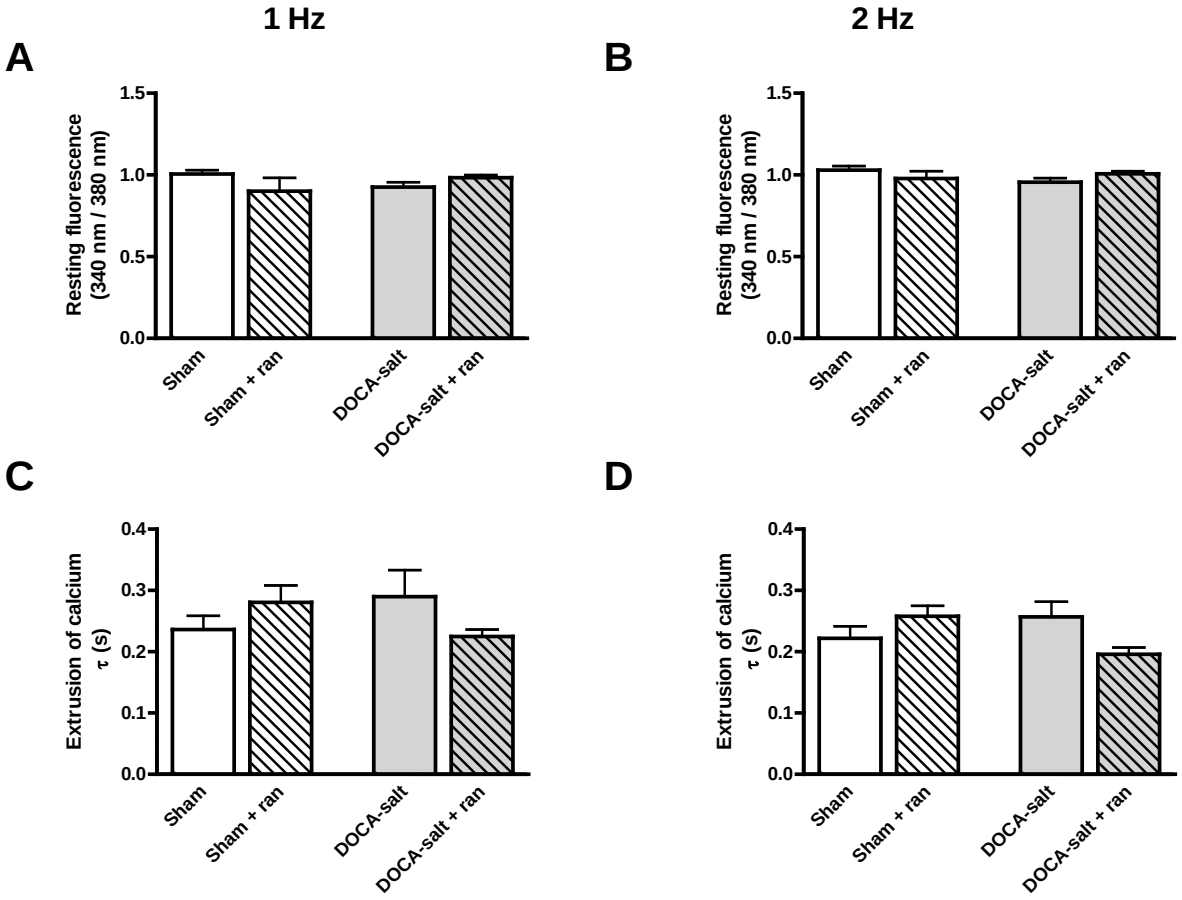

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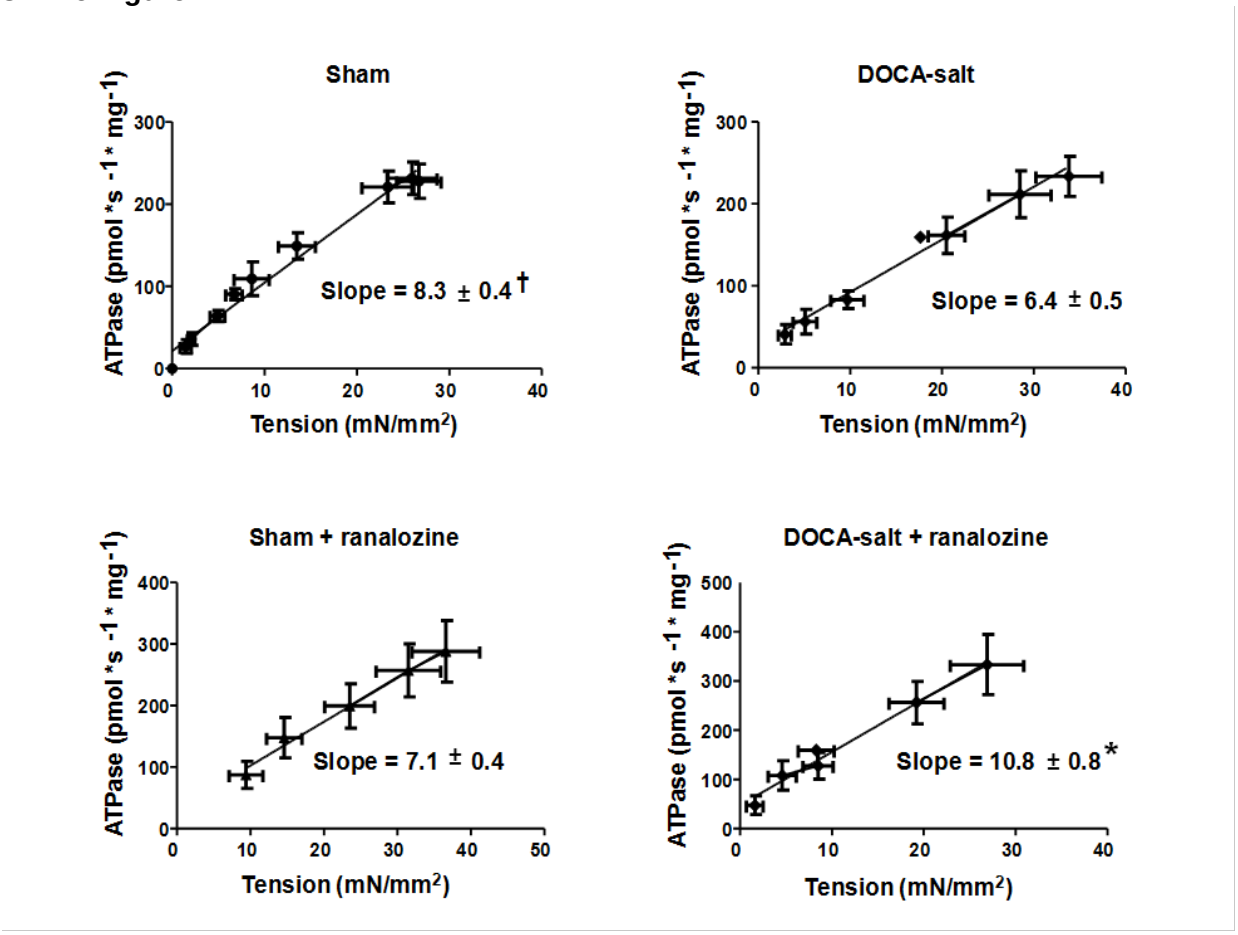

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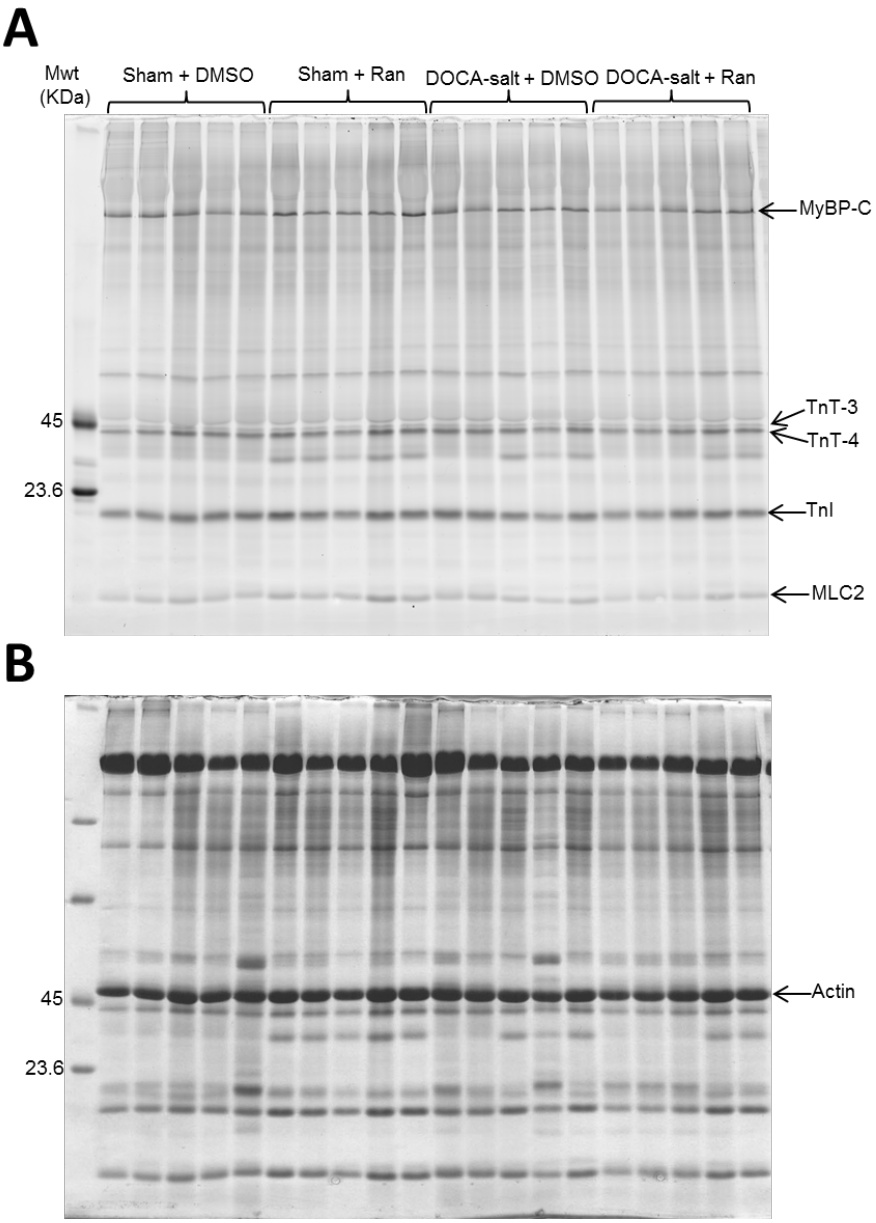

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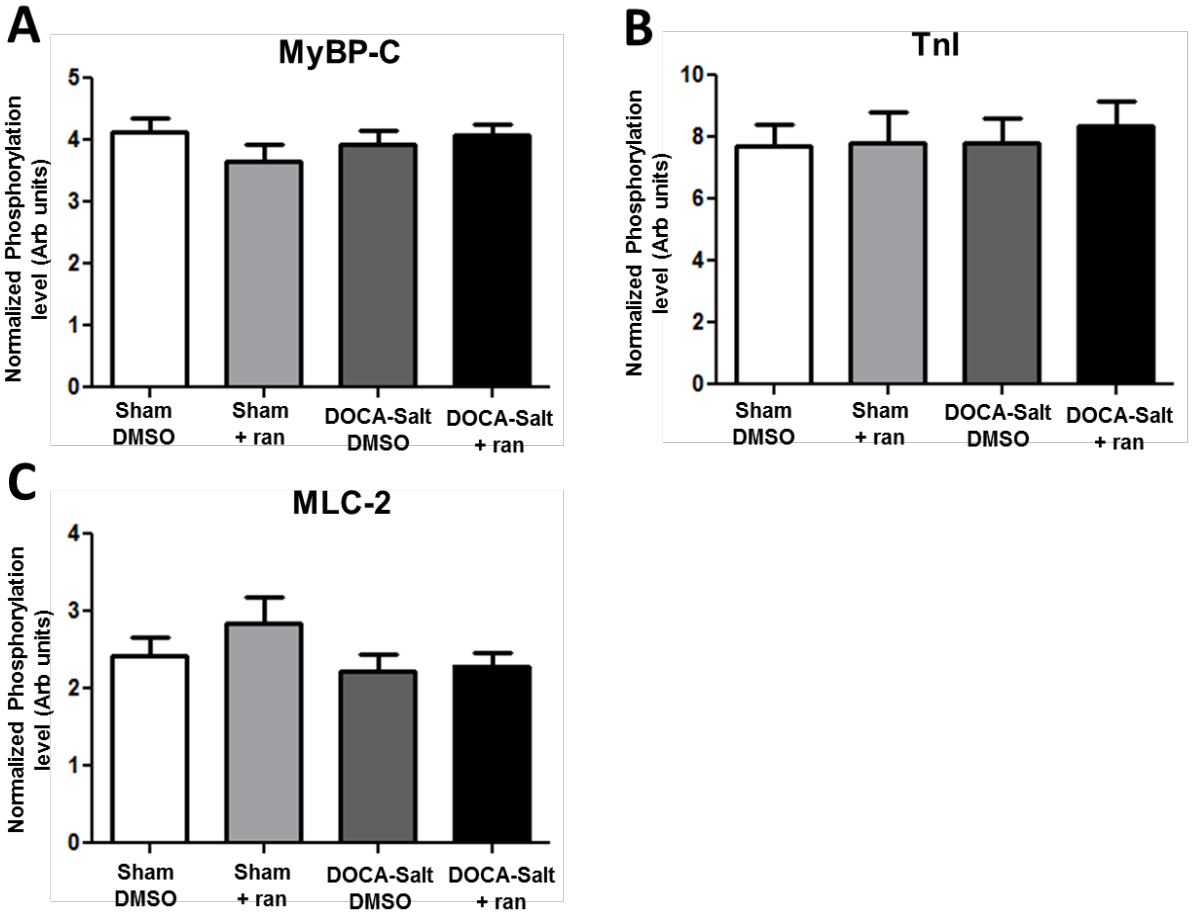

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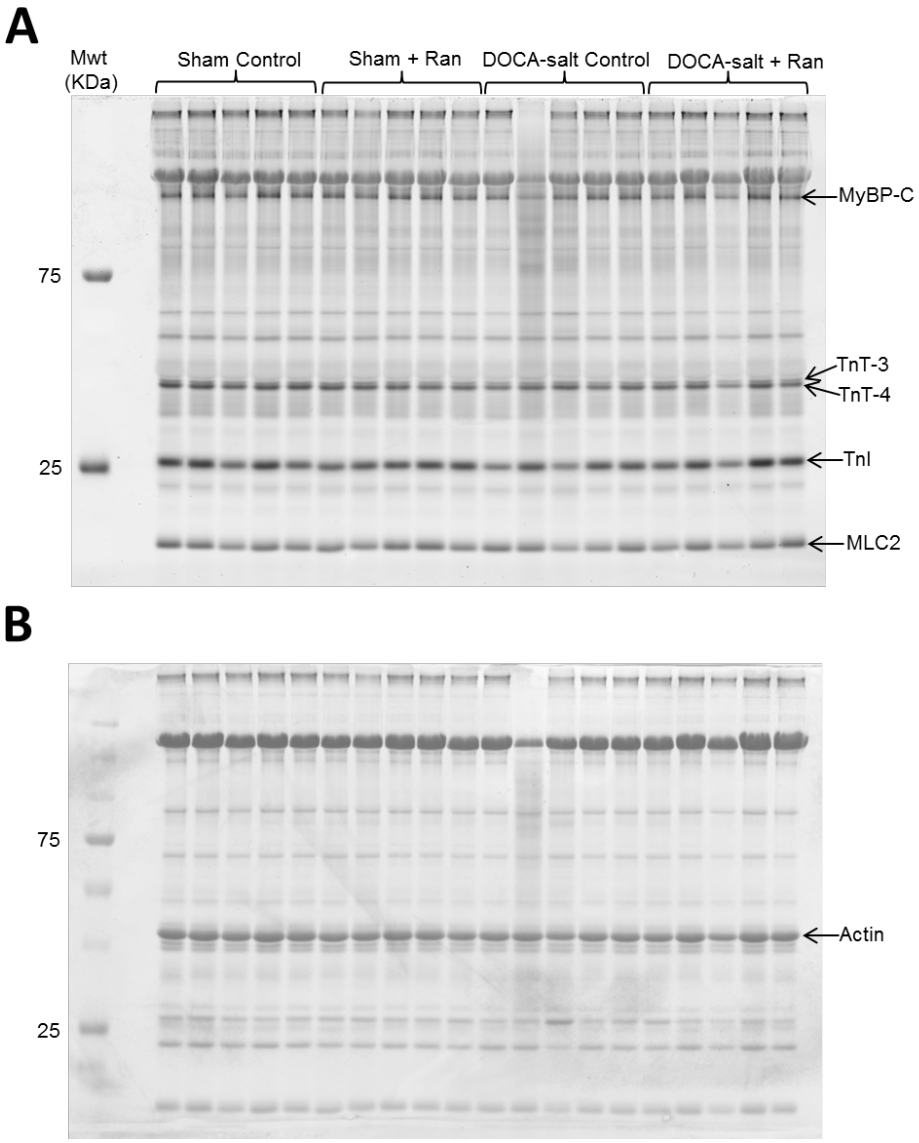

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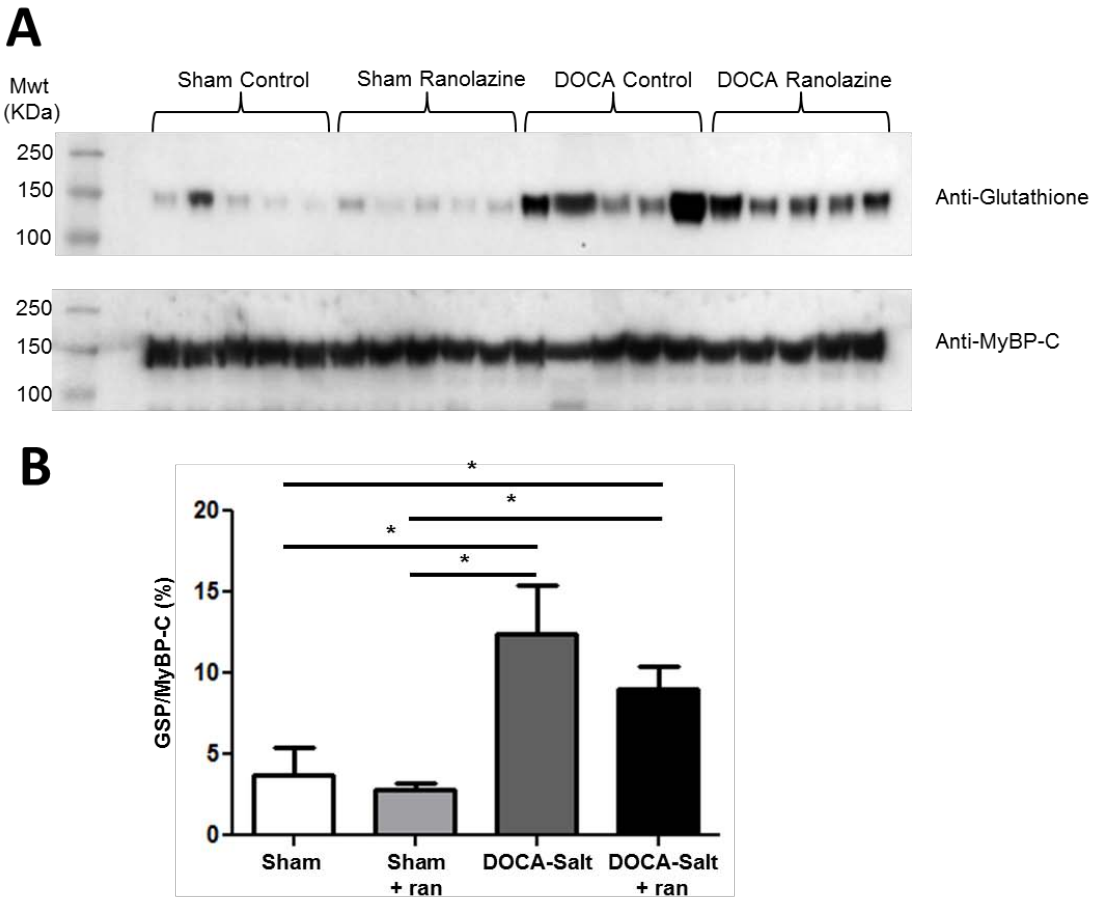
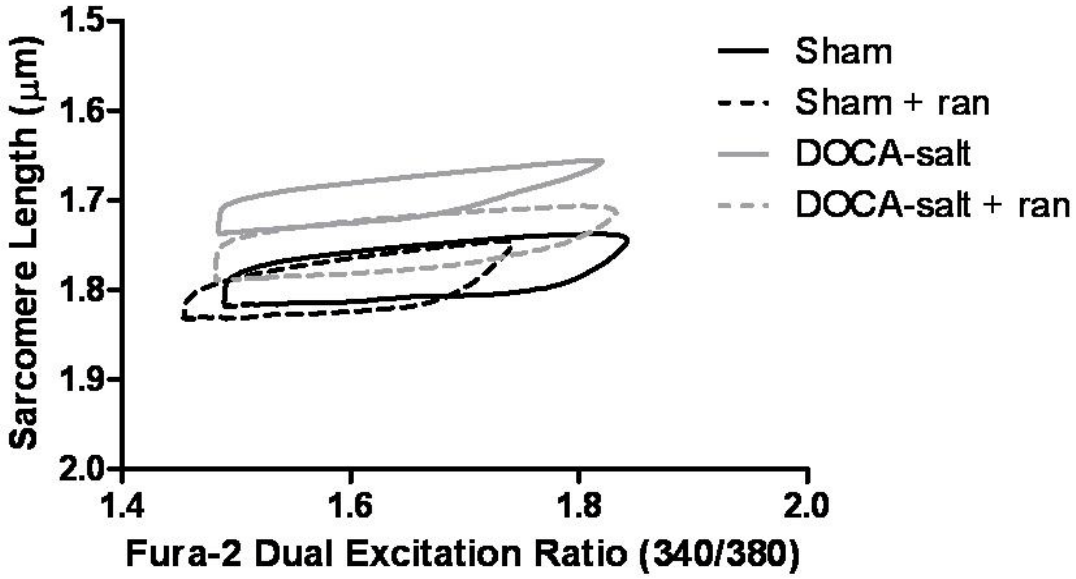


Online Figure IX



Online Figure X