Calpain Inhibition Preserves Talin and Attenuates Right Heart Failure in Acute Pulmonary Hypertension

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Online Data Supplement

Details of experimental preparation and instrumentation

This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). It was approved by the Animal Studies Subcommittees of the Denver Department of Veterans Affairs Medical Center (#08008S) and the University of Colorado at Denver (#57802007).

Forty-three juvenile domestic farm pigs of either sex (25-35 kg Yorkshire strain) were sedated with ketamine HCl (25mg/kg IM), followed by deep general anesthesia induced with α-chloralose 100mg/kg/hr via an ear vein; anesthesia was then maintained throughout the remainder of the experiment with α-chloralose (30-50 mg/kg-hr IV). Following anesthesia, pigs were intubated via tracheotomy and mechanically ventilated to maintain physiologic pCO_2 between 35 and 45 mmHg, pH between 7.35 and 7.45, and pO_2 greater than 100 mmHg. Body temperature was maintained with a recirculating warm water heating blanket. Normal saline solution was infused continuously at 150-250 ml/hr IV.

After exposure via median sternotomy, the pericardium was opened and the heart was instrumented as illustrated in **Figure 1**. A solid-state micromanometer catheter (Millar Instruments, Houston, TX) was introduced into the RV via an internal jugular vein. Orthogonal pairs of piezoelectric crystals (indicated by *x* in **Figure 1**) were implanted in the central anterior RV wall for determination of segment shortening using a sonomicrometer (Sonometrics, London, Ontario, Canada). Ultrasonic transit-time flow probes (Transonic Systems, Ithaca, NY) were placed around the main pulmonary artery for measurement of cardiac output, and around the proximal right coronary artery for measurement of coronary artery flow. An umbilical tape

snare was placed around the pulmonary artery to produce RVPO, and a hydraulic occluder was placed around the inferior vena cava to alter preload. In addition to the instrumentation illustrated here, a Millar catheter was inserted into the left ventricle via apical puncture, pacing wires were affixed to the left atrial appendage, and a 26 g catheter was inserted into the proximal right coronary artery. A lead II electrocardiogram was recorded from needle electrodes placed subcutaneously in the four limbs.

Following surgical instrumentation, autonomic blockade was produced with atropine (0.2 mg/kg IV) and propranolol (1.0 mg/kg IV), and the heart was paced approximately 10 bpm faster than the spontaneous heart rate, to prevent potential alterations in indices of contractile function due to fluctuations in sympathetic tone or heart rate.

At the conclusion of the experimental protocol, surviving pigs were administered a supplemental dose of α-chloralose 100 mg/kg and then euthanized by induction of ventricular fibrillation using an IV bolus of 50 ml 10% KCl.

Details of hemodynamic data analysis

Hemodynamic data were digitized at 200 Hz and recorded using a computerized data acquisition system. *Global RV stroke work* was calculated as the instantaneous product of pulmonary artery flow and RV systolic pressure over a single cardiac cycle. *RV free wall area* was defined as the instantaneous area $\boldsymbol{k}$, subtended by four sonomicrometry crystals implanted in the central RV free wall, continuously determined by the formula

$$\boldsymbol{k} = \sqrt{4p^2q^2 - (b^2 + d^2 - a^2 - c^2)^2} \Big/ 4$$

where ***p***, ***q***, ***a***, ***b***, ***c***, and ***d*** are the chord lengths between crystals as indicated in Figure 1, determined continuously by the digital sonomicrometer. *End diastole* was defined as the point corresponding to the peak of the R wave of the electrocardiogram. *RV regional external work* was defined as the area of an RV pressure-wall area loop inscribed during a single cardiac cycle. RV regional Frank-Starling relations were determined by recording RV pressure-wall area loops during 10 s occlusions of the IVC with mechanical ventilation suspended. Three to six IVC occlusions were performed under baseline conditions and following release of RVPO, spaced at intervals of 1-2 minutes, and the slope and intercept of the regional Frank-Starling relations were calculated. IVC occlusion was not performed during RVPO because IVC occlusion during RVPO results in hemodynamic instability.

Regional external work normalized to baseline at matched end-diastolic area incorporates both regional Frank-Starling slope and intercept into a single index of contractile function. It was calculated from the regional Frank-Starling relation slope (M) and intercept (I) with preload (end-diastolic RV free wall area) set to its baseline, steady state value (P) by the formula

$$M * (P - I)$$

as previously described in Greyson CR, *J Mol Cell Cardiol* 2008;44(1):59-68.

For indices of function derived from regional Frank-Starling relations where the values depend upon the distance between implanted sonomicrometry crystals (i.e., a non-physiologic source of variability), values were normalized to baseline.

Details of gel electrophoresis

In approximately half of the surviving pigs, the central RV free wall excised and frozen on a liquid nitrogen cooled steel mortar for later analysis. Tissue from pigs that died before the end of the protocol was not analyzed since *ante mortem* RV ischemia was possible due to low perfusion pressure in those pigs and could have resulted in nonspecific protein degradation.

For polyacrylamide gel electrophoresis and Western immunoblotting, tissue was homogenized at 4°C using a glass-glass homogenizer in 10 vol of buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1 mM leupeptin, 1 mM AEBSF, and 10 μg/ml aprotinin). Homogenates were centrifuged at 4000 g for 5 min to pellet debris and the supernatant protein extracts were collected. Bradford protein assay was performed and protein concentrations equalized. Protein extracts were mixed with LDS sample buffer and sample reducing agent (Invitrogen), heated (70° C, 10 min), loaded at 20 μg total protein per sample well into a 4-12% gradient bis-tris or 3-8% tris-acetate mini gels (Invitrogen), and run using MES or TA buffer according to manufacturer recommendations. Proteins were transferred to PVDF membranes using NuPAGE transfer buffer at 30 V for 60 min (Invitrogen). Membranes were blocked in 5% nonfat milk/Tris buffered saline (50 mM Tris-HCl, 0.15 M NaCl, TBS; 2 hrs, RT) incubated with primary antibodies diluted in Tris-Tween buffer (TBS pH 7.4 plus 0.1% Tween-20 and 2% BSA, TTB; overnight, 4°C) and rinsed 3 times for 10 min in TTB. Membranes were simultaneously probed for tubulin (cat# ab7291, Abcam, Cambridge, MA, 1:10000) as a control for loading. Membranes were then incubated in HRP-conjugated anti-mouse Fab antibody diluted in TTB (1:5000, 1 hr, RT), rinsed 4 times in TTB for 10 min each, then visualized using a TMB chromogenic assay (Pierce, Woburn, MA).

Quantitation was performed by scanning and densitometric analysis (Phoretix, Nonlinear Dynamics, Durham, NC), with background subtraction and Gaussian fitting of band intensity. To permit comparisons across separate gels, a fiduciary set of samples was repeated on each gel; protein abundance of each individual band on a gel was then normalized to the mean of the fiduciary set on that gel; the resulting value for each sample then represents the deviation of a particular sample from the mean of the fiduciary set. Duplicate values of the fiduciary samples were averaged.

Antibodies used were spectrin, clone AA6, Research Diagnostics, Concord, MA, 1:1000; desmin, clone DEU-10, Sigma, St. Louis, MO, 1:7500; troponin-I, clone MAB I7, Research Diagnostics, 1:50000; SERCA2, clone IID8, CalBiochem, 1:7500; talin clone 8d4, Sigma, 1:1000; vinculin clone VIN-11-5, Sigma, 1:1333; α-actinin clone EA53, 1:4000, Sigma, 1:4000; α-tubulin: clone DM1A, Abcam, 1:10,000.

Details of histology procedures and analysis

Biopsies were flash frozen in optimal cutting temperature compound by immersion in dry-ice cooled isopentane and stored at -80 deg C in airtight containers until sectioning. Frozen blocks were oriented so that the predominant fiber direction of the specimen was orthogonal to the cryotome blade, and 16 μm cryosections were cut and mounted on SuperFrost/Plus slides. Sections were post-fixed with 4% freshly prepared paraformaldehyde for 15 min, permeabilized with 0.05% triton X-100 in PBS for 10 min, blocked in 100 mM glycine in PBS for 10 min, and incubated overnight in 0.01% triton X-100 + 0.1% carboxylated bovine serum albumin (BSA-c, Electron Microscopy Sciences) in PBS, pH 7.6. Next, slides were incubated in a moist chamber

at RT for 3 hrs with anti-talin (1:80), anti-vinculin (1:80) or anti-alpha-actinin (1:1400) antibody in 0.01% triton X-100 + 0.1% BSA-c in PBS, washed 5 times for 3 min with 0.01% triton X-100 in PBS, then labeled for 2 hrs at RT in the dark with donkey anti-mouse-cy3 (1:500) plus DAPI 1:500 (Jackson Labs). Next, slides were treated with 40 µl freshly prepared PBS-glycerol (1:3) + 0.1% p-phenylene diamene before applying cover slips and sealing with nail polish.

For each pair of baseline and pressure overload tissue, sectioning and staining were performed during a single session with the same reagents, and during imaging, all imaging parameters (e.g., exposure, fluorescence illumination intensity) were kept constant. Slides were imaged with a Leica digital deconvolution microscope using a 63x oil immersion objective (NA 1.4) and optically sectioned at 0.5 µm z-axis steps. Fields with predominantly transverse or longitudinal fiber direction and uniform staining were identified, and image stacks were obtained and post-processed using constrained iterative deconvolution with Slidebook 5 software (Intelligent Imaging Innovations, Denver, CO). All post-processing steps were identical for sets of samples sectioned and stained during a single session, after which overall image intensity was equalized among all images.

Figure E1. Global Hemodynamic Data (online). RV and LV systolic pressure, cardiac output, and global RV stroke work in Vehicle (VEH) and MDL-28170 treated pigs (MDL) under each hemodynamic condition (mean ± SEM). *19 of 23 vehicle pigs *vs* 9 of 20 MDL-28170 pigs developed hemodynamic instability (defined as death or SBP<85 mmHg, p=0.013 by Fisher's exact test). For RV systolic pressure, † p=0.011 for vehicle *vs* MDL-28170. For cardiac output normalized to baseline value, ‡p=0.078, §p=0.015, $^{\|}$p=0.042. For global RV stroke work normalized to baseline value, **p=0.021 for VEH *vs* MDL-28170. For un-normalized cardiac output, **p=0.040 for VEH *vs* MDL.

Figure E2. Regional Hemodynamic Data (online). All values are normalized to baseline, and are expressed as mean ± SEM. For regional external work normalized to baseline value, *p=0.020 for vehicle pigs *vs* MDL-28170 pigs. For regional Frank-Starling slope normalized to baseline value, †p=0.020 for vehicle pigs *vs* MDL-28170 pigs. "Regional Work, Matched EDA" is regional external work normalized to baseline at matched end-diastolic area--see Methods in the Online Supplement for details. Note that indices derived from the Frank-Starling relation were not determined during RVPO because IVC occlusion during severe RVPO results in hemodynamic instability.

Figure E3. α-Actinin (online). Representative photomicrographs obtained from 16 μm longitudinal sections of the RV free wall in Sham operated pigs, in pigs after 2 hrs acute RVPO, and in pigs subjected to 2 hrs RVPO but pretreated with MDL-28170. Cryosections were immunostained for α-actinin (red) and with DAPI (in blue, for nuclei). Under Sham conditions, α-actinin signal exhibited a regular striated appearance; following 2 hrs acute RVPO, there was patchy blurring of the striations (green arrow) and loss of the linear appearance of the striations (white arrow). Biopsies from pigs subjected to 2 hrs RVPO but pretreated with MDL-28170

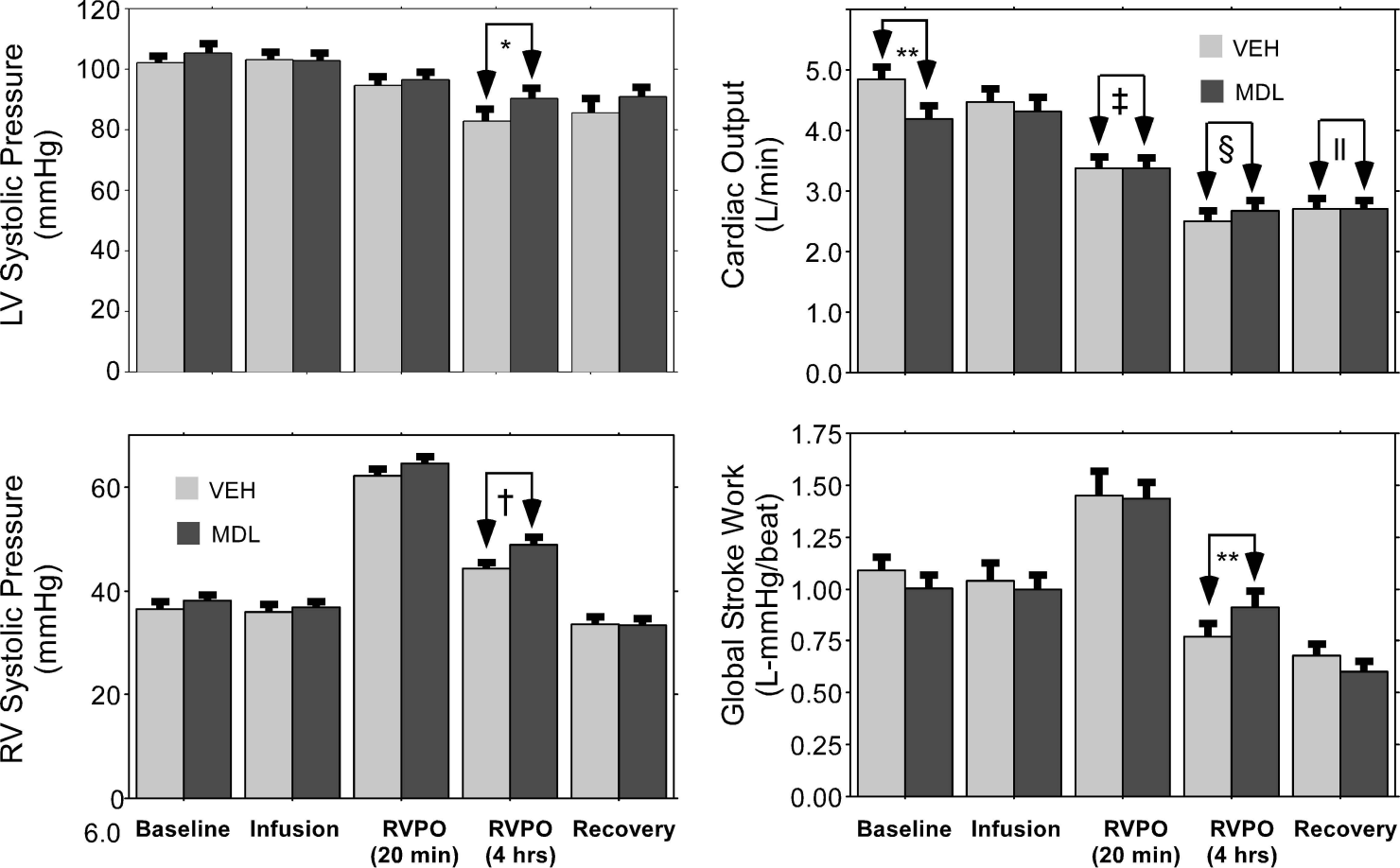
exhibited structure more like Sham than RVPO. Image sets were obtained as described for Figure 6. Scale bar is 20 μm.

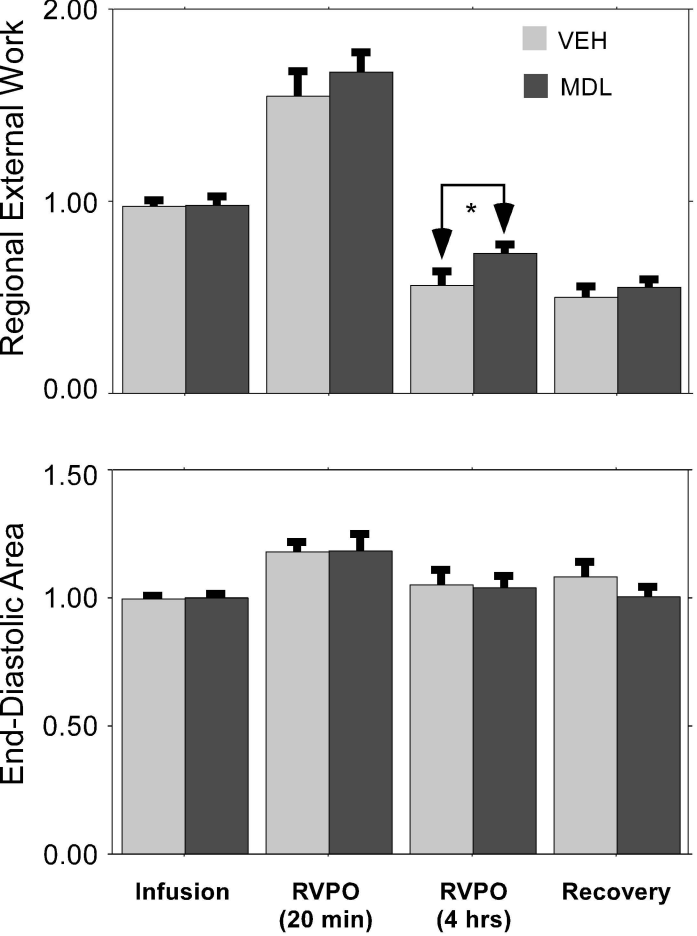
Figure E4. Vinculin (online). Representative photomicrographs obtained from 16 μm longitudinal or transverse sections of the RV free wall in Sham operated pigs, in pigs after 2 hrs acute RVPO, and in pigs subjected to 2 hrs RVPO but pretreated with MDL-28170. Cryosections were immunostained for vinculin (red) and with DAPI (in blue, for nuclei). Under Sham conditions, vinculin signal exhibited a regular punctate appearance localized to the cell surface; following 2 hrs acute RVPO, there was a suggestion of blurring of the signal (green arrow) and patchy loss (white arrow), similar to although less prominent than the findings for talin (Figure 6). Biopsies from pigs subjected to 2 hrs RVPO but pretreated with MDL-28170 were not significantly different from Sham. Image sets were obtained as described for Figure 6. Scale bar is 20 μm.

Legends for Video Files

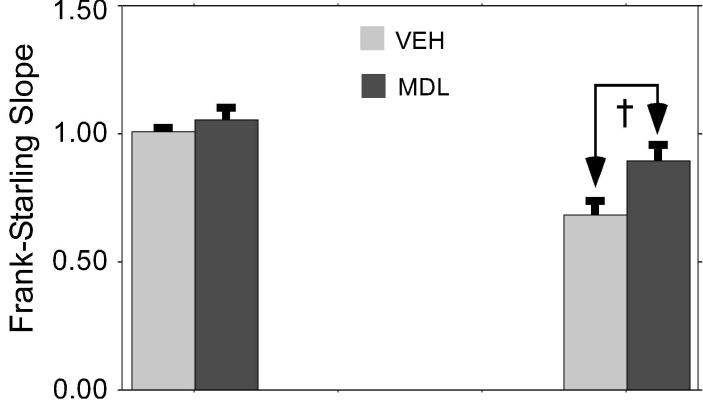
The image stacks obtained from the biopsies shown in Figure 7 were used to create video animations of three-dimensional reconstructions of right ventricular myocardium using Slidebook 4 software. **"Talin Structure at Baseline.mov"** shows a detail of a 10 μm section obtained from the right ventricular free wall of a pig under baseline conditions. **"Talin Structure after RVPO.mov"** shows a 10 μm section obtained from the same pig following 2 hrs acute RVPO. Tissue was immunostained for talin (red) and stained with DAPI for nuclei (blue). Under baseline conditions, talin exhibited a regular rib-like structure coaxial with the long

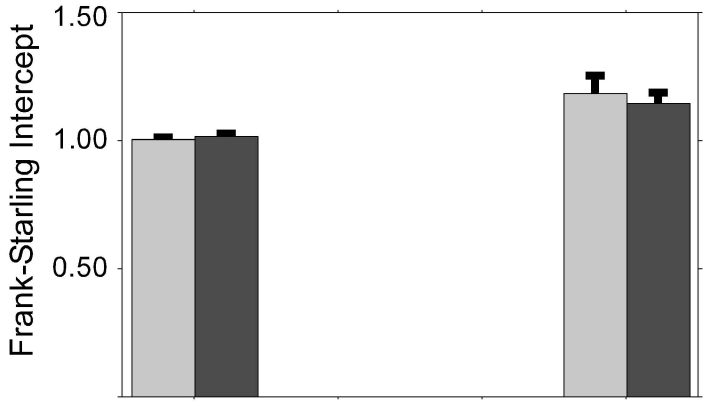
axis of the myocyte. Following 2 hrs acute RVPO, there was disruption of talin's regular rib-like appearance.

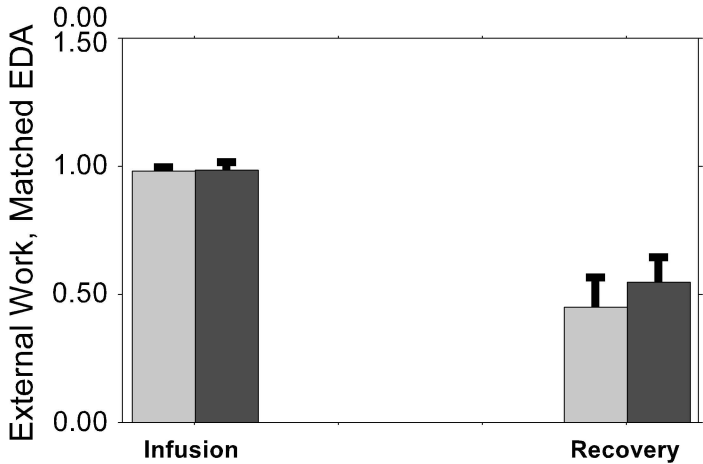

LV Systolic Pressure (mmHg)
120
100
80
60
40
20
0
*
Cardiac Output (L/min)
5.0
4.0
3.0
2.0
1.0
0.0
**
‡
§
ǁ
VEH
MDL
RV Systolic Pressure (mmHg)
60
40
20
0
†
Global Stroke Work (L-mmHg/beat)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
**
6.0
Baseline
Infusion
RVPO (20 min)
RVPO (4 hrs)
Recovery

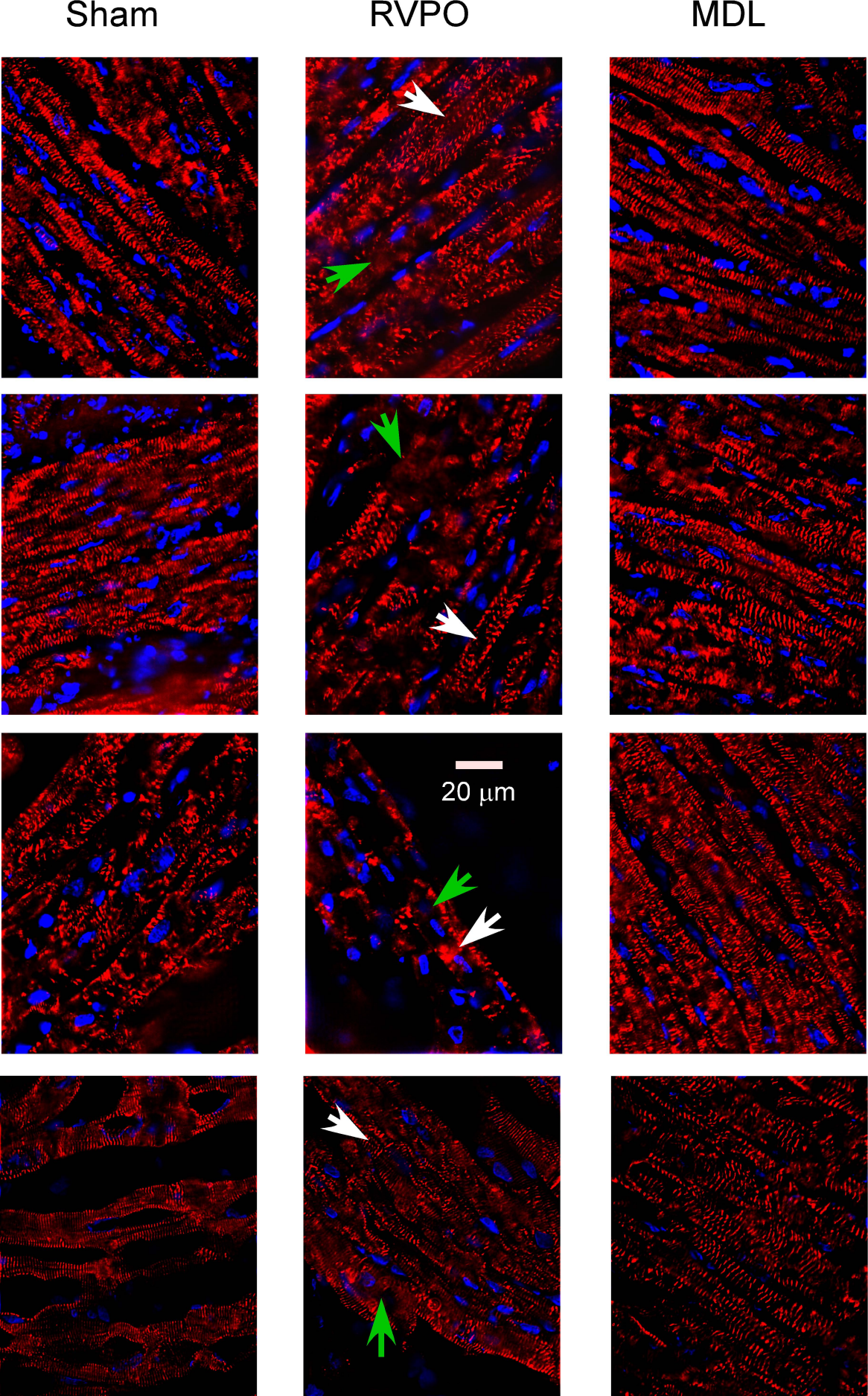

Regional External Work
2.00
1.00
0.00
VEH
MDL
*

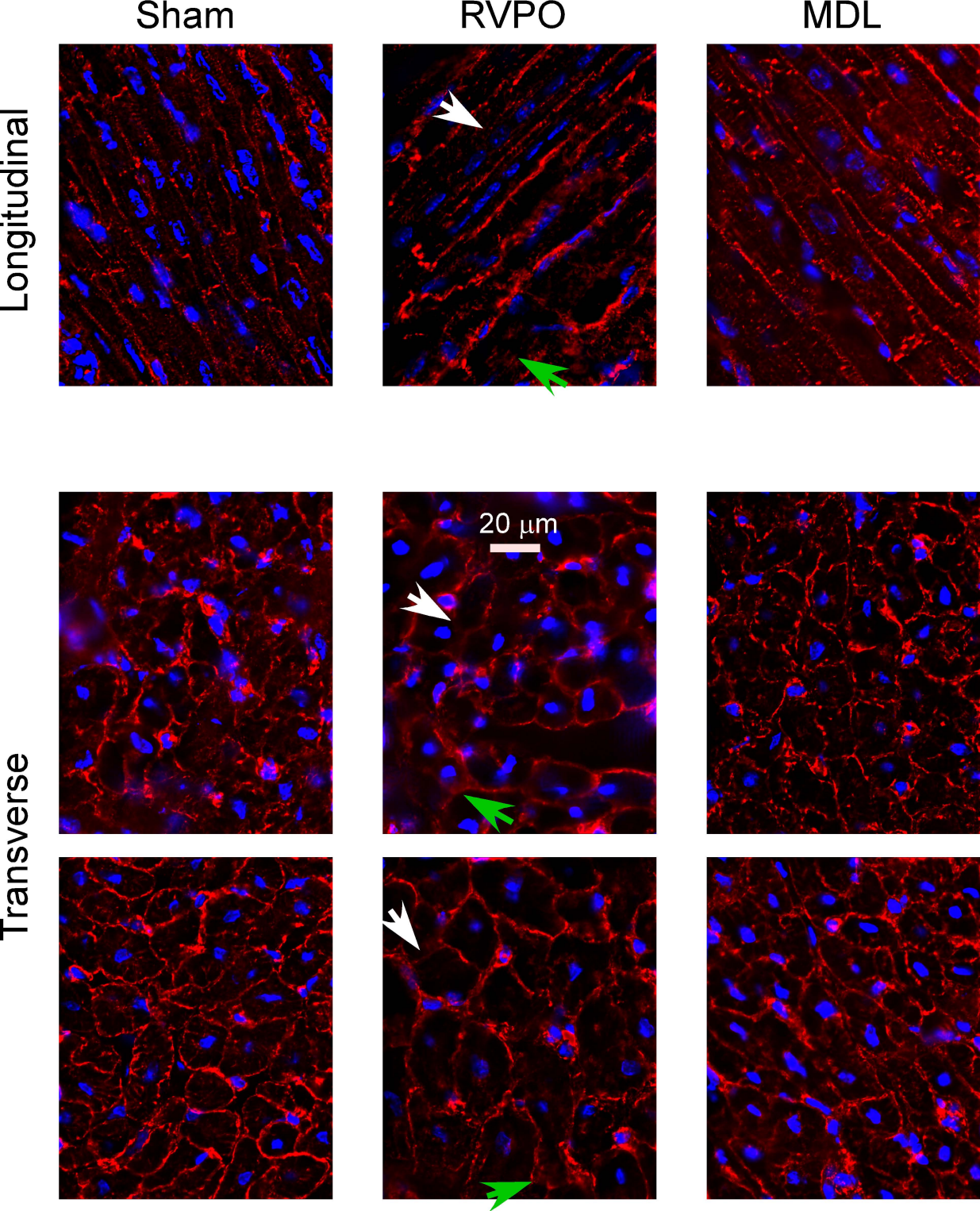
End-Diastolic Area
1.50
1.00
0.50
0.00
Infusion
RVPO
(20 min)
RVPO
(4 hrs)
Recovery


Frank-Starling Slope
1.50
1.00
0.50
0.00
VEH
MDL
†


Frank-Starling Intercept
1.50
1.00
0.50


External Work, Matched EDA
0.00
1.50
1.00
0.50
0.00
Infusion
Recovery


Sham
RVPO
MDL
20 μm


Sham
RVPO
MDL
Longitudinal
Transverse
20 μm