Supplementary material for Staiculescu *et al*

Prolonged Vasoconstriction of Resistance Arteries Involves Vascular Smooth Muscle Actin Polymerization Leading to Inward Remodeling

1. Supplementary Materials and Methods

1.1.Animals

All animal procedures complied with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011), and were approved by the Animal Care and Use committee at the University of Missouri-Columbia. Male Sprague-Dawley rats (150-250g) were used in these experiments. Before experimentation, rats were provided with standard rat chow and water for *ad libitum* consumption while housed in pairs and maintained with a 12hr/day-illumination cycle.

1.2.Isolation of arterioles

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (100mg/Kg). Surgical plane anesthesia was confirmed by loss of spinal reflexes. The right or left cremaster muscle was excised and pinned flat in a refrigerated (4ºC) dissecting chamber. The chamber contained physiological saline solution (PSS) of the following composition (in mM): 145.0 NaCl, 4.7 KCL, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 1.0 NaH_2P0_4, 5.0 dextrose, 3.0 3-(*N*-morpholino) propane sulfonic acid (MOPS) buffer, 2.0

pyruvate, 0.02EDTA, and 0.15 bovine serum albumin, pH=7.4. A first-order segment of the cremaster feed arteriole was isolated.

1.3.Vessel electroporation

Electroporation was used to introduce Actin-Alexa-488 (Invitrogen, California) into vascular smooth muscle cells in the intact isolated arterioles. First we determined the optimal electroporation parameters to be used by conducting a series of experiments based on previously published studies.[1, 2] Three different groups of arterioles were exposed to 8 pulses of 20, 40, or 60V for 8ms at 100ms intervals. There were no effects of electroporation on vascular function as determined by responses of the cannulated and pressurized arterioles to increasing concentrations of phenylephrine (Figure S1). Based on these experiments, the electroporation protocol we used to introduce actin molecules into the vascular smooth muscle of arterioles consisted of placing an isolated arteriole within a silicone chamber containing 70μl of a 10% Actin-Alexa-488 solution in Ca^{2+}-free PSS. A set of 3mm platinum tweezertrodes (Harvard Apparatus, Halliston, MA) separated by 3mm was placed within the chamber with the plates on both sides of the arteriole. Using a BTX ECM 830 Generator (Harvard Apparatus, Halliston, MA), 8 pulses of 60V (133.3V/cm^2) were applied for 8ms each at 100ms intervals. The electroporated vessel was then placed in a dish filled with PSS without albumin, covered with aluminum foil, and maintained overnight at 4ºC. This electroporation protocol consistently and effectively introduced Actin-Alexa-488 into 20-100% of smooth muscle cells within isolated arterioles without significant effects on vascular reactivity.

1.4.Visualization and quantification of fluorescent actin within smooth muscle cells of electroporated arterioles

Arterioles electroporated with Actin-Alexa-488 were placed in a cannulation and observation chamber (Living Systems Instrumentation, Burlington, VT) filled with PSS without albumin. The upstream end of the arteriole was tied onto an open pipette filled with PSS containing albumin using 12-0 nylon suture material. Any remaining red blood cells were flushed from the lumen by application of gentle positive pressure (≤20 mmHg). The downstream end of the arteriole was cannulated and tied onto a closed micropipette. All vessels were warmed to 34.5°C. Only arterioles without leaks that developed spontaneous tone at an intraluminal pressure of 60mmHg were used in the experiments. Each arteriole was examined for Actin-Alexa-488-dependent fluorescence with a confocal microscope (Leica TCS SP5) using a 63X water-immersion 1.2 numerical aperture objective. Excitation wavelength was 488nm while emission was detected between 495-580nm. Images were 1024x1024 pixels with a pixel size of 241nm^2. Z-sections were obtained at 1μm intervals through half of the vessel from the adventitial surface of the arteriolar segment to a depth where the maximal mid-diameter was observed. All images were digitally stored and subsequently analyzed using Imaris (Bitplane, CT, USA) imaging software. Single smooth muscle cells were individually segmented from the whole-arteriole images using a touch screen display monitor. Individual cells were segmented based on their intracellular fluorescence. Each smooth muscle cell chosen was segmented at each time point when images were taken during a vasoconstriction protocol designed to induce inward remodeling (see below). At least

two segmented cells per vessel were analyzed for florescence intensity at all imaging time points in the protocol.

1.5.Effects of prolonged exposure to norepinephrine (NE) + angiotensin-II (Ang-II) on actin fluorescence

We have previously shown that a prolonged (4hr) exposure of isolated arterioles to NE+Ang-II causes inward eutrophic remodeling.[3, 4] Here, to confirm that 4 hour treatment of arterioles with NE ($10^{-5.5}$M) + Ang-II (10^{-7}M) results in inward eutrophic remodeling, we performed measurements of internal diameter and calculated the wall's cross-sectional area, and wall to lumen ratio of arterioles treated with either control vehicle or the vasoconstrictor agents. We determined that a 4hr incubation with NE+Ang-II resulted in a significant increase in media to lumen ration (from 0.3±0.04 to 0.34±0.05) and a significant reduction in the internal diameter from (159.7±6.14μm to 153.05±5.7μm). As vessels had no significant change in cross-sectional area of the wall, these findings indicate arterioles developed inward eutrophic remodeling (Figure S2). In control vessels the changes in wall to lumen ratio and internal vascular diameter were not significant after 4 hours of incubation in PSS (Figure S2).

To determine the effects of prolonged exposure to NE+Ang-II on actin fluorescence, three-dimensional (3D) images of cannulated arterioles were reconstructed from the through-focus image sets obtained initially after the vessel developed spontaneous myogenic tone and then again every hour during 4hr of exposure to NE ($10^{-5.5}$M) + Ang-II (10^{-7}M). After 4hr of exposure to the agonists the vessels were washed three times with PSS to remove the vasoconstrictors and an additional image set was obtained.

Control arterioles in this experimental series were isolated, electroporated with Actin-Alexa-488 and cannulated, but not exposed to the vasoconstrictor agonists. Once control arterioles developed spontaneous myogenic tone, they were incubated in PSS without albumin for 4hr. As with the treated arterioles, through focus image sets were obtained using the same imaging parameters every hour during the 4hr incubation period and once after washing.

1.6.Effects of cytochalasin-D on inward remodeling and actin fluorescence in arterioles exposed to NE+ANG II for 4 hours

To determine the effects of preventing actin polymerization in vasoconstriction-induced inward remodeling and Actin-Alexa-488-dependent fluorescence, cannulated arterioles were allowed to develop spontaneous tone followed by exposure to cytochalasin-D (500nM) for 20 min and then imaged. After this initial image set was taken, arterioles were exposed 4hr to NE+Ang-II while in the presence of cytochalasin-D. An image set was taken every hour using the same confocal-microscope settings as outlined previously. The vessels were then washed three times with PSS without albumin to remove the vasoconstrictor agonists and cytochalasin-D, and a final image set was collected.

In an additional series of experiments, arterioles not electroporated were first dilated with 10^{-4}M adenosine and then exposed to calcium-free PSS with 2mM EGTA and 10^{-4}M adenosine to obtain maximal passive diameter. Subsequently, vessels were allowed to regain tone in PSS with calcium and then exposed to 500nM cytochalasin-D for 20min. This was followed by a 4hr exposure to either NE+Ang-II in the presence of

cytochalasin-D or in the case of control vessels to cytochalasin-D alone. After the prolonged exposure to vasoconstrictors, vessels were washed with PSS without NE, Ang-II, or cytochalasin-D for 30min and then exposed to adenosine followed by calcium-free PSS. In these experiments vascular diameter was continuously recorded using a video dimension analyzer (Living Systems Instrumentation, Burlington, VE).

1.7.*In vitro* polymerization of Actin-Alexa-488

In vitro experiments were conducted to determine the effects of Actin-Alexa-488 polymerization on fluorescence intensity. Experiments were conducted in mini-well slides (Ibidi GmbH, Munich, Germany) placed on a confocal microscope using the same objective and excitation/emission parameters as when imaging arterioles. First, 20µl of PSS without calcium was placed in the well and imaged. This was followed by the addition of Actin-Alexa-488 (1µg/µl). After imaging the solution containing Actin-Alexa-488, 10µl of actin polymerization buffer (Cytoskeleton, Denver, CO) was added to obtain a final concentration of 50mM KCl, 2 mM$MgCl_2$ and 1mM ATP. Images were then taken every 30s for 10min. Subsequently, 2µM mycalolide-B was added to depolymerize actin and images were taken every 30s for 10min. In control experiments, actin polymerization buffer was substituted for general actin buffer (Cytoskeleton, Denver, CO) to obtain final a concentration of 5mM Tris-HCl, 0.2mM $CaCl_2$, 0.2mM ATP, and 0.5mM DTT.

1.8.Effects of Rho/Rac/Cdc42 activation on inward remodeling

To determine the effects of Rho/Rac/CDC42 activation on actin polymerization and inward remodeling, isolated arterioles with tone were exposed 4hr to 1μg/ml CN04 (Cytoskeleton, Denver, CO, USA). As in the previously described protocols, maximal passive diameter was obtained before and after the prolonged exposure to CN04.

1.9.Analysis of actin polymerization by differential centrifugation and electrophoresis

Actin polymerization was also measured in rat mesenteric arterioles following 4 hrs of exposure to NE+Ang-II, jasplakinolide (100nM, EMD Chemicals, San Diego, CA), CN04 (1μg/ml), or vehicle control. In these vessels actin polymerization was determined as the relative amount of F- vs. total-actin using a commercially available kit (BK037, Cytoskeleton Denver, CO, USA). The procedure was slightly modified compared to previously published protocols.[5] Briefly, after 4hr of exposure to the agonists, the arterioles were homogenized with a micro tissue grinder (Kimble Kontes, Vineland, NJ) containing 80μl of lysis and F-actin stabilization buffer at 37°C. Immediately after the addition of the buffer the tubes were vortexed and incubated for 10min at 37°C in a water bath. The lysate was then centrifuged at 100,000g for 1hr at 37°C. The supernatant containing G-actin was removed into a different tube and the pellet containing F-actin was re-suspended in a solution containing 10μM cytochalasin-D. The pellet was incubated for 1hr on ice to allow for the dissociation of the F-actin into monomeric G-actin. During this time, the pellet solution was vortexed every 15min in order to aid in the dissociation of F-actin into monomeric actin. Equal volumes of the

supernatant and pellet were separated by SDS-PAGE and the blots were probed with rabbit polyclonal anti-actin antibody (1:1000, dilution; Cytoskeleton AAN01). Protein bands were visualized by chemiluminescence with a BioRad ChemiDoc Imager and quantified by densitometry.

1.10.Effects of Rho-Associated Protein Kinase and Rac-1 inhibition on inward remodeling

To determine the effects of Rho associated protein kinase (ROCK) inhibition on vasoconstriction-induced inward remodeling, isolated cremaster arterioles were exposed to 1 μM Y27632 (Sigma, Saint Louis, MO, USA) for 20min prior to and during a 4hr exposure to NE+Ang-II. As described above, maximal passive diameter was obtained before and after the prolonged exposure to the agonists. To determine the effects of Rac-1 inhibition on vasoconstriction-induced inward remodeling, isolated cremaster arterioles were exposed to 100μM NSC23766 (Tocris, Bristol, UK) for 20min prior to and during a 4hr exposure to NE+Ang-II. As described above, maximal passive diameter was obtained before and after the prolonged exposure to the agonists.

1.11.Immunohistochemistry

To determine the effects of cytochalasin-D and mycalolide-B on the structure of vascular smooth muscle actin stress fibers, cannulated and pressurized arterioles were exposed to cytochalasin-D (10μM), mycalolide-B (2μM), or vehicle control for 1hr and then fixed with 4% paraformaldehyde. After washing the paraformaldehyde from the arterioles, glycine buffer (0.1mM glycine) was added to quench any remaining

paraformaldehyde. Subsequently, vessels were permeabilized with 0.5% Triton X-100 in PBS and incubated at 4°C overnight with Alexa-546 phalloidin alone or in combination with mouse anti-α and anti-β tubulin antibodies (Invitrogen). Vessels were then washed and incubated with a combination of 4', 6-diamidino-2-phenylindole (DAPI) and goat anti-mouse Alexa-488 secondary antibody (Invitrogen). Labeled vessels were washed six times with cold buffer and imaged on a Leica SP5 confocal microscope.

1.12.Data analysis

Data are presented as means±SE. Diameters are expressed as raw data and either as percentages of the maximal diameter obtained during the first exposure to calcium-free PSS or as percentages of the diameter when vessels first developed myogenic tone. Fluorescence data was analyzed using the imaging software, Imaris (Bitplane, CT, USA). Mean fluorescence intensity from a minimum of two cells per vessel was expressed as percentages of the intensity obtained from the first image taken when vessels developed spontaneous myogenic tone. Linear regression analysis was performed using GraphPad Prism 5.0 as previously described[6, 7] to obtain the slope of change in fluorescence for each of the electroporated vessels over time. Slopes for the change in fluorescence were then categorized according to treatment group and differences between the mean slopes determined with ANOVA followed by Tukey's multiple range test.

For analysis of F/total-actin, ratios were normalized to those of vessels maintained under control conditions and expressed as fold change from control induced by the different treatments.

Statistically, unpaired Student's t-tests were used to compare the mean fluorescence intensity of Actin-Alexa-488 in vitro (control vs. polymerization buffer, Figure 1B), the fold change in F to total actin ratio in arterioles exposed to control conditions or CN04 (Figure 6A), and the change in passive diameter induced by prolonged exposure to CN04 compared to that of vessels kept under control conditions (Figure 6B); paired Student's t-tests were used to compare the mean passive diameters of vessels before and after prolonged exposure to different agonists or inhibitors (Figure 3A, 3B, 3C, 6C, and 6D) and the differences in cross-sectional area and wall to lumen ratio before and after prolonged exposure to NE+Ang-II or vehicle control (Figure S2); ANOVA followed by Tukey's test was used to compared more than two means as in Figures 1B, 4A, 4B, and 5. Values of $P \leq 0.05$ were considered significant.

Supplemental References

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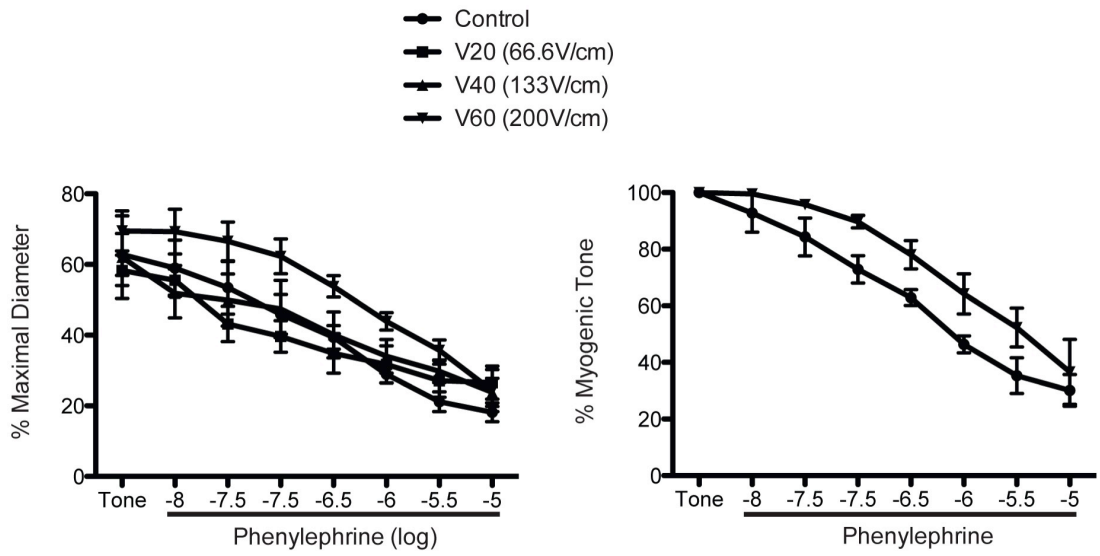
Supplemental Figure Legends

Figure S1.Electroporation of rat cremaster arterioles does not alter the maximal vasoconstrictor response to phenylephrine. *Left panel:* dose response curves for arterioles exposed to three different voltages (20, 40 and 60V) and controls (n=4 per condition). Data is expressed as percentage of the maximum passive diameter obtained under Ca^{+2} free conditions. *Right panel:* dose response curves for vessels electroporated with 60V and controls. Data are expressed as percentage (mean±SE) of the spontaneous myogenic tone diameter for each group. The EC50 is shifted in the electroporated vessels from -6.57±0.30 to -5.77±0.67 but is not statistically significant (P>0.05) while the maximum response to phenylephrine is not affected.

Figure S2. Exposure of isolated arterioles to NE+Ang-II for 4 hours induces changes in the wall's cross-sectional area (CSA) and passive wall to lumen ratio consistent with inward eutrophic remodeling. Panel ***A,*** shows the wall's CSA and mean wall to lumen ratio taken under passive condition (i.e., in calcium-free buffer) before and after a 4-hour exposure of vessels (n=6) to control conditions (i.e., vessels were cannulated, pressurized to 60mmHg and kept at 34.5C in PSS). Panel ***B,*** shows the wall's CSA and mean wall to lumen ratio taken under passive condition (i.e., in calcium-free buffer) before and after a 4-hour exposure of the vessels (n=7) to norepinephrine (NE, $10^{-5.5}$M) + Angiotensin-II (Ang-II, 10^{-7}M) while cannulated, pressurized to 60mmHg and kept at 34.5C. Data are means±SE. *P≤0.05 vs. before exposure to NE+Ang-II.


Control
V20 (66.6V/cm)
V40 (133V/cm)
V60 (200V/cm)
% Maximal Diameter
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Tone
-8
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-7.5
-6.5
-6
-5.5
-5
Phenylephrine (log)
% Myogenic Tone
100
80
60
40
20
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Tone
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-5.5
-5
Phenylephrine

Figure S1.

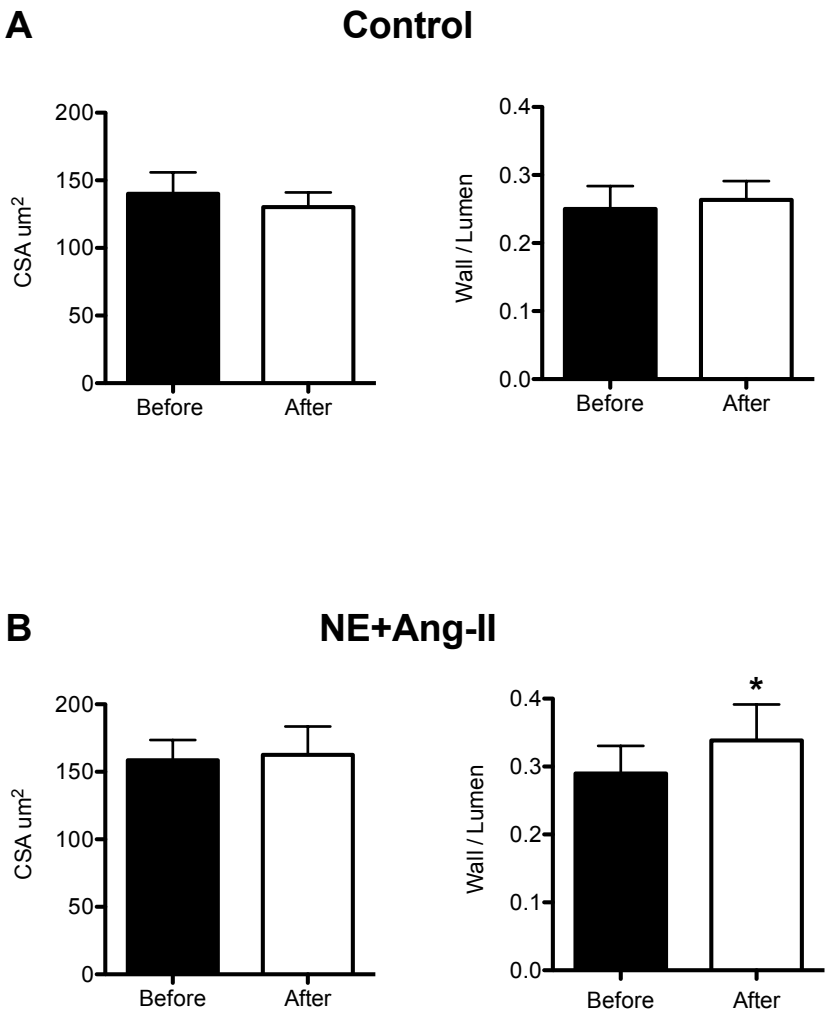
A
Control
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Figure S2.