Materials and Methods

Animals and Infusion of Angiotensin Peptides. All animal protocols were approved by the Medical College of Wisconsin (MCW) Institutional Animal Care and Use Committee. Animals were housed and cared for at the MCW Animal Resource Center and were given food and water ad libitum. Sprague-Dawley (SD) rats fed a high salt diet (Dyets 4% NaCl) to suppress the native renin-angiotensin system, underwent 7 days of hind-limb electrical stimulation, and received different treatments during the entire stimulation protocol. Rats were randomly assigned to the following groups: vehicle infusion, AngII (3 ng · kg^{-1} · min^{-1} i.v.), AngII (3 ng · kg^{-1} · min^{-1} i.v.) with Losartan (50 mg · kg^{-1} · day^{-1}, in the drinking water), AngII (3 ng · kg^{-1} · min^{-1} i.v.) with [D-Ala(7)]-Ang-(1-7) (A779) (400 ng · kg^{-1} · day^{-1}, in the drinking water), Ang-(1-7) (2.6 ng · kg^{-1} · min^{-1} i.v.), Ang-(1-7) (2.6 ng · kg^{-1} · min^{-1} i.v.) with Losartan (50 mg · kg^{-1} · day^{-1}, in the drinking water), or Ang-(1-7) (2.6 ng · kg^{-1} · min^{-1} i.v.) with A779 (400 ng · kg^{-1} · day^{-1}, in the drinking water). All rats completed experimental protocols at 8-10 weeks of age. This AngII dose has been shown to be a low, subpressor dose in SD rats. We focused on using a subpressor infusion that restores plasma AngII to a level that is high enough to promote normal endothelial function and has been previously demonstrated to promote normal endothelial function in vivo[1, 2]. Ang-(1-7) dosage was chosen to be equimolar to AngII.

Electrical Stimulator Surgical Procedures. Electrical stimulator and jugular catheters were implanted as previously described[2]. Anesthetized rats received subcutaneous incisions over the thoracolumbar region and medial aspect of the right leg, and a miniature battery powered stimulator was implanted as previously described [3]. Incisions were also made in the ventral and dorsal thoracic regions. A Tygon catheter was implanted in the jugular vein, tunneled subcutaneously, and exteriorized at the back of the neck. The catheter was passed through metal spring to a swivel allowing the animal full range of motion. After 24 h of recovery, continuous infusion of AngII, AngII + A779, Ang-(1-7), Ang-(1-7) + A779, or saline vehicle was started at a rate of 0.12 ml/h as noted above. The stimulator was activated by magnetic reed switch and electrodes located near the common peroneal nerve in the lower leg produced square-wave impulses of 0.3 ms duration, 10-Hz frequency and 3-V potential, causing intermittent contractions of the tibialis anterior (TA) muscles for eight consecutive hours, daily for the remainder of the study. The contralateral leg was used as a control and all animals were euthanized after seven days of stimulation, followed by collection of the TA for morphological analysis.

Tissue Harvest and Morphological Analysis of Vessel Density. The animals were euthanized by an overdose of Beuthanasia solution according to approved protocols and the stimulated and contralateral unstimulated TA muscles were excised and weighed. Muscles were fixed overnight in a 0.25% formalin solution, microsectioned, and immersed in a solution of 30μg/mL rhodamine labeled *Griffonia simplicifolia* I (GS-I) lectin (Vector Labs) for 2 hours. The sections were

rinsed, mounted on microscope slides, and visualized with a fluorescent microscope system (Nikon E-80i microscope with Q-Imaging QIClick camera, 200x)[2, 3]. Images were taken from twenty representative fields from each muscle and analyzed using Metamorph software (Molecular Devices) for percent change in microvessel density[2, 3]. Vessel counts from all fields were averaged to a single vessel density defined as the mean number of vessel-grid intersections per microscope field (0.155 mm^2) for each muscle. Within experimental groups mean vessel densities of stimulated muscles were compared to contralateral unstimulated muscles, presented as mean ± SE, and evaluated using a paired *t*-test.

Vasodilation Experiments. Male SD rats, 8-10 weeks of age, had a femoral vein catheter implanted under aseptic conditions, tunneled subcutaneously to the midcapular region, externalized and attached to a swivel to allow free movement of the animal. After 3d recovery with saline infusion (0.5 ml/h) and low salt diet, animals were placed on 4% NaCl salt diet and received an intravenous infusion of AngII (5 $ng \cdot kg^{-1} \cdot min^{-1}$), Ang-(1-7) (4 $ng \cdot kg^{-1} \cdot min^{-1}$) or DMSO vehicle (20 μL/h). In addition, animals received pharmacological inhibitors Losartan (AT_1R, 20 $\mu g \cdot kg^{-1} \cdot min^{-1}$), A779 (Mas1 receptor, 400 $ng \cdot kg^{-1} \cdot min^{-1}$), PD-98050 (ERK1/2 pathway, 10μg/hr) or SB-203580 (p38MAPK pathway, 10μg/hr) for 3-5 days before acute *ex vivo* experiments as described in previous reports[1, 2, 4-6]. On the day of experiment, animals were anesthetized with pentobarbital sodium (60mg·kg ip) and brain was removed and immersed in physiological salt solution. The middle cerebral artery (MCA) was excised, prepared for vasoreactivity assays, and internal diameter was measured using television microscopy and a video micrometer[1]. Maximum vascular diameter changes were recorded in response to endothelium-dependent vasodilator agonist ACh (10^{-10}-10^{-5} M) or smooth muscle-dependent NO donor sodium nitroprusside (10^{-10}-10^{-5} M) as previously described[1, 5].

Angiogenesis Tube Formation Assay. Rat cardiac microvascular endothelial cells (RMVECs) (Cell Biologics, C# R1111), grown according to company protocol, were brought to 70-90% confluency, washed twice with Dulbecco's phosphate buffered saline (DPBS, Life Technologies), and lifted using Enzymatic Free Cell Dissociation Buffer (Millipore) with gentle agitation for 30 minutes at 37ºC. RMVECs were then centrifuged at 100 x g for 5 minutes, washed twice with DPBS, resuspended in 1 mL MCDB131 basal media plus 1% or 10% FBS depending on experiment, and counted using the cell countess system (Invitrogen). RMVECs were diluted for the addition of 10,000 or 20,000 cells in 1 mL of media per well of the four-well slides (Lab-Tek/Thermo Fisher Scientific) coated in 250 uL of Growth Factor Depleted Matrigel (BD Biosciences). Serum-starved and growth factor depleted conditions were utilized as a tool to stunt normal RMVEC tube formation stimulation to better decipher changes that would be observed through the addition of 100 nM Ang-(1-7) or AngII, plus or minus inhibitors. *In vitro* doses (10-100 fold in vivo concentrations) were selected to mimic the *in vivo* angiogenesis phenotype. Treatment conditions included RMVECs plus vehicle, RMVECs plus the 50 μM ERK1/2 inhibitor (Cell Signaling Technology, PD-98050), RMVECs plus the 10 μM p38MAPK

inhibitor (Cell Signaling Technology, SB-203580), and all three of those conditions plus 100 nM Ang-(1-7) or 100 nM AngII. Inhibitor concentrations were based on manufacturer recommendations and previously verified in the participating laboratories systems[4]. At 24 and 48 hours 4X and/or 10X magnification images were taken using a TS100 Inverted Microscope (Nikon Corporation) for analysis of the mean tube length per field (μm) using open-access PipeLine tube formation analysis software[7]. The results were averaged across biological and technical replicates followed by unpaired t-test analysis comparing groups as appropriate.

Isolation of Mas1 Receptor Signal Protein Complex. Conditions for immuno-precipitation were optimized in the presence and absence of dithiobis(succinimidyl propionate) (DSP) crosslinker (Thermo, #22586). Conditions without the DSP cross-linker were determined to be optimal due to DSP epitope inhibition. Therefore, cryolysis was used in place of crosslinking during the immuoprecipitations (IP) to stabilize the protein complex. RMVECs were divided into 100 nM Ang-(1-7) treated (5 minutes at 37^{o}C) or non-treated groups, washed 3 times with ice cold DPBS (non-treated) or DPBS plus 100 nM Ang-(1-7) (treated), cells were scraped, and supernatants were transferred to 50 mL conical tubes. Following centrifugation at 300 x g for 10 minutes at 4^{o}C, supernatant was aspirated, washed with 10 mL ice cold DPBS plus or minus Ang-(1-7) as before, and the process was repeated twice. RMVECs were resuspended in 20 mM Hepes/1.2% PVP buffer with protease inhibitors and kept on ice until the next step. Cryolysis of the RMVECs was then performed in liquid nitrogen according to the Life Technologies cryolysis protocol in the Dynabeads Co-IP Kit (#143.21D). Frozen cell pellets were place in a liquid nitrogen cooled 2 mL microcentrifuge tube with a sterile metal bead, frozen tubes were secured in an oscillating homogenizer, and samples were oscillated at 30 hertz for 1 minute three times or until a powder is formed; re-submerge in liquid nitrogen between each oscillation. Frozen cell pellet powders were then resuspended in solution with anti-Mas1antibody (Santa Cruz; #sc-135063) coupled Dynabeads, incubated 30 minutes at 4^{o}C in a thermomixer at 500 rpm, and immuno-precipitated according to the Invitrogen Dynabeads Co-Immunoprecipitation Kit (#143.21D) and M-270 Epoxy Dynabeads Antibody Coupling Kit (#143.11D) manufacturer protocols.

Liquid Chromatography and Mass Spectrometry (MS) Analysis. Isolated protein samples were dried using a vacuum centrifuge, resuspended in 100 μL 25 mM ammonium bicarbonate, and prepared for LC-MS/MS as described previously[8, 9]. Tryptic peptide mixtures (1.9 μl) were separated using a NanoAccuity UPLC system (Waters, Milford, MA) coupled with an in-house packed 5Å C18 resin (Phenomenex, Torrance, CA) column (15 cm long 50μm inner diameter). A 120 minute gradient from 98% HPLC water/2% ACN/0.1% formic acid to 98% ACN/2% HPLC water/0.1% formic acid was used and peptides were analyzed using an LTQ-Orbitrap Velos MS (Thermo Scientific, Waltham, MA). All Orbitrap Velos MS/MS settings utilized were as indicated in our previous studies [8, 10]. Raw mass spectra were searched against a Uniprot Rodent Database in both SEQUEST and MASCOT search algorithms, from which the best

match for each scan was kept after combining searches for individual runs. Variable modification of +57-Da for alkylation of cysteine and +16-Da for oxidation of methionine were included in search parameters. Utilizing in-house Visualize proteomic analysis software[11], protein matches were filtered to remove redundancies, to remove common contaminants, selected for a $P \geq 0.95$ (FDR<5%), and a comparison of groups was then run on the Ang-(1-7) treated versus non-treated immuno-precipitation protein data. Further stringent filters were applied to the comparison, including a scan count ≥ 25 in either condition, significant increase in Ang-(1-7) treated sample ($p \leq 0.05$), presence in ≥ 6 (of 8) biological replicates, and presence in ≥ 10 (of 16) total technical replicates of the Ang-(1-7) treated sample. This dataset was used for subsequent pathway mapping using a combination of UniprotKB, StringDB, Ingenuity Pathway Analysis, and Protein Center platforms.

Statistical Analysis of MS/MS Comparisons. All MS/MS statistical analyses were performed utilizing open source Visualize software with built-in statistical analysis for large proteomic dataset comparison[11]. These analyses utilized the G test, which is a log-likelihood ratio test, whose distribution can be approximated by a chi-squared distribution with a single degree of freedom[12]. For null hypotheses we assume that the expected proportion of scans for a given protein is directly related to the ratio of the total scans in each group. Each observed scan count for each protein is multiplied by this ratio, or its inverse depending on which group, to give us our expected frequency. Observed scans from Group 0 (S0) are multiplied by the ratio of total scans in Group 1 (T1) over Total Scans in Group 0 (T0) (E0=S0*T1/T0), likewise observed scans from Group 1 (S1) are multiplied by the ratio of T0 over T1 (E1=S1*(T0/T1)). The specific G value calculation is 2 * (S0 * ln(S0/E0) + S1 * ln(S1/E1)), and thus if our observed frequencies perfectly fit our expected frequencies, we would get a G-value of 0, and the larger the G-value the more our observed frequency departs from the expected. The distribution of G can be approximated by a chi-squared distribution with a degree of freedom of one to determine significance ($P < 0.05$). These calculations are performed as described in Sokal, Robert R., Rohlf, F.J. 1995. Biometry. New York: W.H. Freeman and Company.

Real-Time PCR Analysis of Ang-(1-7) and AngII Treated RMVECs. The RT^2 Profiler^{TM} PCR Array PARN-091E-4 (QIAGEN) designed for profiling the expression of 84 common angiogenesis related genes was used to examine expression changes induced in RMVECs by Ang-(1-7). Comparisons were made between RMVECS non-stimulated, Ang-(1-7) stimulated, low dose AngII stimulated, high dose AngII stimulated, and combinations of those treatments with the ERK1/2 and p38MAPK inhibitors. One 100 mm plate of RMVECs was incubated plus/minus 100 nM Ang-(1-7), 100 nM AngII, or 1 µM AngII in MCDB131 basal media plus 1% FBS at 37ºC for 30 minutes, scraped, and RNA was isolated using the RNeasy Mini-Kit (QIAGEN, #74104) according to manufacturer protocol. Additional groups were added for each treatment using the ERK1/2 and p38MAPK inhibitors as described for the tube formation assay. The RNase-Free DNase Set (QIAGEN, #79254) was used for elimination of DNA contamination. Isolated RNA concentration was measured using absorbance on the NanoDrop

System. ~400-700 ng of RNA was then converted to cDNA using the RT^2 First Strand Kit (QIAGEN, cat. # 330401) and diluted according the manufacturer protocol for the RT^2 Profiler^{TM} PCR Array PARN-091E-4 (QIAGEN). RT^2 SYBR Green ROX^{TM} (QIAGEN, #330522) was used for the array. Samples were run in the ABI 7900HT instrument (SA Biosciences), thresholded and normalized according to manufacturer protocol, and statistically analyzed using the QIAGEN online RT^2 Profiler PCR Array Data Analysis Software version 3.5. RT-PCR analyses were then compared with the proteomic pathway analysis data to formulate the influence of Ang-(1-7) on signaling in RMVECs and between groups to look at similarities/differences. Gene and protein lists were then analyzed using a combination of Ingenuity Pathways Analysis software, UniprotKB, and Protein Center software to develop pathways incorporating the data. Pathway figures were produced using Servier Medical Art (www.servier.com).

ERK1/2 and p38MAPK Immunoblotting and Analysis. Cultured endothelial cells were serum starved in basal media containing 1% FBS for 2 hours, then treated with 100 nM Ang1-7 for 0 minutes, 2 minutes, 5 minutes, and 15 minutes. Control cells were grown in complete media. Following treatment, media was aspirated and cells were scraped in MPER buffer (Pierce cat# 78501) containing Protease Inhibitor (Roche cat# 11697498001) and HALT phosphatase inhibitor (Pierce cat# 78420). Cells were lysed with a 21-gauge needle and assayed for protein concentration with MicroBCA kit (Pierce cat# 23235). Four micrograms of protein from each sample was loaded on a 10% TGX PAGE gel (Biorad) and transferred to PVDF. Blots were blocked overnight in 5% NFDM (Biorad cat# 1706404) and 1% BSA (Sigma cat# A7906). Blots were incubated in ERK1/2, pERK1/2, p38MAPK, and pp38MAPK primary antibody for 2 hours at 1:1000 dilution (Cell Signaling Technology cat# cs9102, cs9101, cs9212, and cs9211). Blots were rinsed and incubated with goat anti-rabbit HRP-conjugated secondary antibody (BioRad cat# 1706515) for 1 hour diluted 1:5000, and then detected using the SuperSignal West Dura Chemiluminescence Substrate (Pierce cat# 34075). Membranes were imaged on ImageQuant LAS 500 imager (GE) and analyzed with ImageJ software (http://imagej.nih.gov/ij/). Quantified values were then compared via a One-way ANOVA, Holm-Sidak post-test to determine significant alterations.

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