**Supplemental Text**

# Transient Receptor Potential Melastatin 7 Cation Channel (TRPM7) Kinase: a new player in hypertension

Tayze T Antunes[1], Glaucia E Callera[1], Ying He[1], Alvaro Yogi[1], Alexey G Ryazanov[2], Lillia V Ryazanova[2], Alexander Zhai[3], Duncan J Stewart[3], Alvin Shrier[4],

Rhian M Touyz[1,5].

[1]Kidney Research Centre, Department of Cellular and Molecular Medicine, Ottawa Hospital Research Institute, University of Ottawa, Canada;[2]Department of Pharmacology, Rutgers Robert Wood Johnson Medical School, USA;[3]Sprott Centre for Stem Cell Research and Regenerative Medicine Program, Dept. of Medicine, Ottawa Hospital Research Institute, University of Ottawa, Canada;[4]Department of Physiology and Groupe de Recherche Axé sur la Structure des Protéines, McGill University, Canada;[5]Institute of Cardiovascular & Medical Sciences, BHF Glasgow Cardiovascular Research Centre, University of Glasgow, UK.

**To whom correspondence should be addressed:**

Rhian M Touyz, MD, PhD

Institute of Cardiovascular and Medical Sciences,
BHF Glasgow Cardiovascular Research Centre, University of Glasgow,
126 University Place, Glasgow, G12 8TA
Phone: + 44 (0)141 330 7775/7774, Fax: + 44 (0)141330-3360,
Email: Rhian.Touyz@glasgow.ac.uk

## Methods

### Animals

The study was approved by the Animal Ethics Committee of the Ottawa Hospital Research Institute, University of Ottawa and carried out according to the recommendations of the Canadian Council for Animal Care. Male, 3 month old wild type ($TRPM7^{+/+}$) mice (C57BL/6J and SV129 background) and mice heterozygous for the deletion of the TRPM7kinase domain (TRPM7Δkinase), generated by the gene-targeting vector technique (1) were maintained in controlled room temperature (25°C) with a 12-h light/dark cycle and had free access to food and water. At 8 weeks of age, mice were infused with Ang II (400 ng/Kg/min) by osmotic minipumps for 4 weeks. Vehicle (saline)-infused mice were used as a control group. Systolic blood pressure (BP) was measured by tail-cuff plethysmography. Basal BP was measured on 3 different occasions before minipumps were surgically implanted in mice. After receiving the minipumps, BP was measured 1-2 times weekly. Spot urine samples were obtained a day prior to

sacrifice. At the end of the study, mice were killed by decapitation under anesthesia and mesenteric arteries, aorta, heart and blood samples collected for analysis.

**Genotyping of TRPM7$^{+/+}$ and TRPM7Δkinase mice**

A small piece of mouse ear was excised and DNA was extracted with a 100 µl of tissue extractor plus 25 µl of tissue preparation solution (REDExtract-N-Amp$^{TM}$ Tissue PCR Kit, Sigma XNATR-1KT). Samples were incubated at room temperature for 10 minutes and then heated at 98 °C for 3 minutes, following by the addition of 100 µl of neutralizer. Samples were spun down and the supernatant obtained was kept at -20$^{o}$C for further PCR reaction. 4 µl of supernatant was used as a template in a 20 µl PCR reaction mix. Primers used to detect TRPM7kinase deletion (5' tgc gag gcc aga ggc cac ttg tgt agc 3'; and : 5' tgc gag gcc aga ggc cac ttg tgt agc 3') were designed to amplify the neighboring sequence regions of TRPM7kinase domain. Therefore, only in the absence of TRPM7kinase domain these primers were able to amplify the designated template during the PCR cycle specified. For this reason, when using these specificTRPM7 kinase primer set for genotyping TRPM7$^{+/+}$ mice, these samples do not show any product amplification since the extension time in our PCR conditions do not allow amplification of such a large sequence (TRPM7 plus neighbor sequence).

To guarantee that the absence of PCR bands with these primers were truly TRPM7$^{+/+}$ mice and not a result obtained due to a poor DNA extraction and/or PCR performance, a control primer set (5' aaa tct tag gct ggt aga cag tg 3'; and 5' ctt atc tct caa gcc aat tta gga g 3') independent of TRPM7kinase deletion was generated to accurately interpret the data. Therefore with the control primer set both TRPM7$^{+/+}$ and TRPM7Δkinase samples must show DNA amplification. PCR protocol was as following: 1 cycle of 94$^{o}$C (heat start), and 35 cycles of: 10 sec at 94$^{o}$ C (denaturation), 30 sec at 62$^{o}$ C (annealing), 2 min at 68$^{o}$ C (extension). One final cycle of 5 min at 68$^{o}$ C was performed, following by a hold on temperature of 4$^{o}$ C. Agarose gel electrophoresis was used for visualizing the PCR products.

**Validation of TRPM7kinase antibody in human embryonic kidney (HEK) cells expressing full length TRPM7 and in HEK cells deficient in TRPM7 mutant (Δ kinase)**

Human TRPM7 WT and TRPM7 lacking a kinase domain (Δ kinase) cDNA cloning and the establishing of stable inducible expression of the corresponding proteins in HEK-293 T-Rex cells (Invitrogen) have been previously described (2). The stably transfected 293-HEK cells (expressing WT hTRPM7 and hTRPM7 Δ kinase under tetracycline control) were grown in in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 5 µg/ml Blasticidin S and 0.4 mg/ml Zeocin. Cells were grown in culture dishes treated with poly-Lysine (Sigma, P4832) in order to guarantee cell adherence once TRPM7 expression is induced upon addition of tetracycline. Briefly, to induce TRPM7 expression, cells were grown in the culture medium described above containing 1 µg/ml tetracycline for 24h. In the following day, cells were placed on starvation medium (DMEM containing 5 µg/ml Blasticidin S, 0.4 mg/ml Zeocin) and 1 µg/ml tetracycline overnight. Analysis of TRPM7 expression was performed by Western blotting using TRPM7 antibody (Epitomics Abcam, Rabbit mAb # 3828-1) (described below) in control cells (without tetracycline treatment) and in cells exposed to tetracycline to induce gene expression (48h).

**Biochemical analysis**

Plasma was collected immediately following sacrifice and kept at -80°C until further analysis. Electrolyte, cholesterol, urea, glucose, creatinine, and protein concentrations were determined using an automated analyzer (Beckman Coulter AU5800) by IDEXX Laboratories (Ontario, Canada).

**Urinary nitric oxide measurement**

Spot urine was kept at -80°C until further analysis. Total nitrate/nitrite levels were measured in the urine as nitric oxide metabolites using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, USA).

**Urinary Albumin and Creatinine measurement**

Assessment of albumin (µg/mL) and creatinine (mg/mL) excretion was performed in urine samples with commercial kits (Albuwell M and Creatinine Companion, Exocell) according to the manufacturer's instructions. Results were expressed as albumin:creatinine ratio (ACR).

**Echocardiography**

Cardiac geometry and function were performed using a Vevo 2100 imaging system with a 40-MHz transducer. Vehicle and Ang II-infused TRPM7$^{+/+}$ and TRPM7Δkinase mice were analyzed at the end of the study (25-27 days following Ang II infusion). Mice were lightly sedated by continuous gas inhalation (isoflurane, 1.5-2%) throughout the examination. Mice were secured in the supine position on a warming pad, the chest hair was shaved, and a warmed echo gel was placed on the shaved chest. Echo images were downloaded afterwards and analyzed offline using the Vevo 2100 software. 7 beats were measured for each mouse and averaged for the interpretation of any given measurements. For all the measurements shown, the hearts were examined in M-mode in the parasternal long axis view. All calculated parameters were automatically computed by the Vevo 2100 standard measurement package as previously reported (3).

**Histological analyses**

For histological and immunohistochemical analyses, the heart and aorta were fixed in 4 % paraformaldehyde and processed for paraffin embedding. Sections (5 µm thick) were stained with Sirius Red and Masson's Trichrome. Hematoxylin and eosin staining was performed for LV wall analysis as well as heart's gross morphology. For the morphometric analysis in cross-sections of heart, the left ventricle wall was measured in three regions and the values were averaged. Sirius red staining was performed to examine collagen deposition and Masson's trichrome to determine fibrosis.

For morphometric analysis in cross-sections of aorta, wall thickness was measured in four regions and the values were averaged. For immunohistochemical staining in aorta sections, heat-induced epitope retrieval was performed at 110$^{o}$ C for 12 min with tris-EDTA buffer (pH 9.0). To localize TRPM7, anti-TRPM7-C-terminal (#135817, Abcam, Cambridge, UK) primary antibody (dilution 1/50, rabbit) was used. The specificity of the staining was determined upon absence of immunoreactivity when the primary antibody was omitted.

**Wire myography to assess vascular function**

Vascular function in second order branches of mesenteric resistance arteries were studied with a wire myograph (DMT, Danish Myo Technology, Denmark) as previously described (4). Mesenteric resistance arteries were placed in Krebs Henseleit solution (mM: 118 NaCl, 4.65 KCl, 1.18 $MgSO_4$, 1.18 $KH_2PO_4$, 25 $NaHCO_3$, 2.5 $CaCl_2$, 0.026 EDTA, and 5.5 glucose) under a

dissecting microscopy, carefully cleaned of connective tissue, and second order branches (corresponding with resistance arteries) were isolated and mounted. Arterial segments (≤ 2 mm long) were cannulated by threading them onto two stainless steel wires (25 μm) and tying the wires to two supports. Arteries were continuously bubbled with 95% $O_2$ and 5 % $CO_2$ to achieve a pH of 7.4 at 37°C, allowed to equilibrate for 20 minutes, and normalized prior to experiments. Vessel contractility was accessed by cumulative concentration-response curves ($10^{-9}$ to 3 x $10^{-5}$ M) to norepinephrine (NE). Endothelium-dependent and -independent relaxation was assessed by measuring dilatory responses to cumulative concentration-response curves ($10^{-9}$ to 3 x $10^{-5}$ M) of acetylcholine (ACh) and diethylamine NONOate (DEA-NO), respectively, in vessels pre-contracted with NA at a concentration to achieve approximately 80% of maximal response.

**Pressure myography to assess vascular structure and mechanics**

Structural and mechanical properties of mesenteric resistance arteries were studied with a pressure myograph (Living Systems, Burlington, Vermont) (4,5). Mesenteric resistance arteries were placed in Krebs Henseleit solution, cleaned and dissected as described above. Arterial segments (2 to 3 mm long) were slipped onto 2 glass microcannulae, one of which was secured until the vessel walls were parallel, set to a pressure of 45 mmHg and allowed to equilibrate for 30-60 min at 37 °C in a calcium-free Krebs Henseleit solution (with the addition of 10 mM EGTA) to eliminate myogenic tone. Arteries were gassed with a mixture of 95 % $O_2$ and 5 % $CO_2$ at 37°C. Lumen diameter and vessel thickness were measured during a pressure-diameter curve by increasing intraluminal pressure in 20 mmHg steps between 0 and 120 mmHg. Lumen diameter, cross-sectional area, media to lumen ratio (structure), stress, strain and mechanics were calculated as previously reported (3, 4).

**Vascular tissue preparation for Western Blotting analysis**

Total protein was extracted from vascular homogenates. Frozen aortas and mesenteric arteries were homogenized in 50 mM Tris-HCl (pH 7.4) lysis buffer (containing 1% Nonited P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM $Na_3VO_4$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1μg/mL pepstatin A, 1 μg/mL leupeptin and 1 μg/mL aprotinin). Protein supernatant was separated by centrifugation at 13,000 x g for 10 min and pellet was discarded. The particular supernatant was kept at -80°C.

Mesenteric resistance arteries were fractionated to obtain cytosol and membrane-enriched fractions. Frozen mesenteric resistance arteries were pulverized (TP-103 Amalgamator) in 80 ul of cold buffer A, which consisted of 50 mM Tris-HCl pH 7.4, 2.5 mM EDTA, 5 mM EGTA, 2 mM$Na_3VO_4$, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin. The homogenate was centrifuged at 100,000 x *g* for 60 min at 4°C (ultracentrífuge Optima MAX (Beckman Coulter, CA, EUA), thereby isolating the cytosolic fractions in the supernatant. The pellet was resuspended in lysis buffer B; which consisted of lysis buffer A with the addition of 1% triton X-100; homogenized again, and centrifuged at 13,000 *g* for 10 min at 4°C. The supernantant obtained contained the membrane fractions. The cytosolic and membrane fractions obtained were kept at -80°C.

**Western Blotting**

Total protein from aorta homogenates (30 μg), mesenteric arteries (25 μg) and membrane-enriched fractions (4 μg) from mesenteric arteries were used for Western Blot analysis. Proteins were separated by electrophoresis on a polyacrylamide gel (10%), and transferred onto a

nitrocellulose membrane. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline solution with 0.1% Tween for 1 hour at room temperature. Membranes were then incubated with specific antibodies overnight at 4°C. Antibodies were as follows: TRPM7 (Epitomics, Abcam, Burlingame, CA); annexin-1, calpain, caveolin-1, VCAM-1, p47 phox (Santa Cruz Biotechnology, Dallas, Texas); phospho-JNK, phospho-ERK1/2, phospho Akt, phospho eNOS and eNOS (Cell Signaling, Danvers, MA). With the exception of VCAM-1 (1:500) and TRPM7 (1:250), all the antibodies were used in a 1:1000 dilution. β-actin (1:5000 dilution; Sigma, Oakville, ON) was used as an internal housekeeping control. After incubation with secondary antibodies, signals were revealed with chemiluminescence, visualized by autoradiography and quantified densitometrically.

**Reactive Oxygen Species (ROS) Assessment**

Vascular ROS generation was measured by lucigenin-enhanced chemiluminescence assay, with lucigenin as the electron acceptor and NADPH as the substrate. Aortic segments from $TRPM7^{+/+}$ and TRPMΔkinase mice treated with Ang II or vehicle were homogenized in assay buffer (50 mmol/L of $KH_2PO_4$, 1 mmol/L of EGTA, and 150 mmol/L of sucrose, pH 7.4) with a glass-to-glass homogenizer. The assay was performed with 100 µL of sample, 1.25 µL of lucigenin (5 µmol/L), 25 µL of NADPH (0.1 mmol/L) and assay buffer to a total volume of 250 µL. Luminescence was measured for 20 cycles of 18 seconds each by a luminometer (Lumistar Galaxy, BMG Lab Technologies, Germany). Basal readings were obtained prior to the addition of NADPH to the assay. The reaction was initiated by the addition of the substrate. Basal and buffer blank values were subtracted from the NADPH-derived luminescence. Superoxide anion production was expressed as relative luminescence unit (RLU)/µg protein.

Plasma hydrogen peroxide ($H_2O_2$) was assessed with Amplex Red assay kit (Molecular Probes by Invitrogen). $H_2O_2$ was detected spectrophotometrically in 10 µL of plasma sample. Absorbance was measured at multiple time points (0 to 60 min).

## References


1. Ryazanova LV, Rondon LJ, Zierler S, Hu Z, Galli J, Yamaguchi TP, Mazur A, Fleig A, Ryazanov AG. TRPM7 is essential for Mg(2+) homeostasis in mammals. *Nat Commun.* 2010;1:109-113.
2. Yogi A, Callera GE, O'Connor S, Antunes TT, Valinsky W, Miquel P, Montezano AC, Perraud AL, Schmitz C, Shrier A, Touyz RM.Aldosterone signaling through transient receptor potential melastatin 7 cation channel (TRPM7) and its α-kinase domain. *Cell Signal.* 2013;25:2163-2175.
3. Vinhas M, Araújo AC, Ribeiro S, Rosário LB, Belo JA. Transthoracic echocardiography reference values in juvenile and adult 129/Sv mice. *Cardiovasc Ultrasound*. 2013;11:12-14
4. Briones AM, Nguyen Dinh Cat A, Callera GE, Yogi A, Burger D, He Y, Corrêa JW, Gagnon AM, Gomez-Sanchez CE, Gomez-Sanchez EP, Sorisky A, Ooi TC, Ruzicka M, Burns KD, Touyz RM. Adipocytes produce aldosterone through calcineurin-dependent signaling pathways: implications in diabetes mellitus-associated obesity and vascular dysfunction. *Hypertension*. 2012;59:1069-1078.

5. Endemann D, Touyz RM, Li JS, Deng LY, Schiffrin EL. Altered angiotensin II-induced small artery contraction during the development of hypertension in spontaneously hypertensive rats. *Am J Hypertens*. 1999;12:716-723.

## Figure legends

**Supplemental figure S1. Hematoxylin and eosin staining of the heart of Ang II-infused TRPM7$^{+/+}$ and TRPM7Δkinase mice.** Hearts were isolated from TRPM7$^{+/+}$ and TRPM7Δkinase mice infused with vehicle or Ang II (400 ng/Kg/min). (A) Panels are representative photomicrographs of paraffin-embedded heart sections of 8-10 mice per group processed for hematoxylin and eosin staining with original magnification of x5, scale bar 200 μm. (B) For the morphometric analysis of left ventricle (LV) wall size, the LV wall thickness was measured in three regions and the values were averaged. Results are presented as mean ± SEM of 8-10 mice per group. $^{*}p<0.05$ vs vehicle-infused TRPM7Δkinase mice.

**Supplemental figure S2. Collagen accumulation and interstitial fibrosis in the heart of Ang II-infused TRPM7$^{+/+}$ and TRPM7Δkinase mice.** Hearts were isolated from mice infused with vehicle or Ang II. Panels are representative photomicrographs of paraffin-embedded of heart sections of 8-9 mice per group processed for Sirius Red (A) and Masson's trichrome (B) and, with original magnification of x20, scale bar 20 μm. Insets depict collagen accumulation (A) and fibrosis (B) of the arteries.

**Supplemental figure S3. Aortic hypertrophy in Ang II-infused mice.** Aortas were isolated from TRPM7$^{+/+}$ and TRPM7Δkinase infused with vehicle or Ang II. Paraffin-embedded thoracic aorta sections were processed for Masson's trichrome staining. For the morphometric analysis in cross-sections of aorta, the wall thickness was measured in four regions and the values were averaged. Upper panels, representative photomicrographs of Masson's trichrome-stained thoracic aorta sections, with original magnifications x200. Bar graph, wall thickness of aortic segments. Results are presented as mean ± SEM of 6-7 mice per group.*P <0.05 vs. respective vehicle-infused mice.

**Supplemental figure S4. Structural changes in mesenteric arteries from Ang II-infused TRPM7$^{+/+}$ and TRPM7Δkinase mice.** Mesenteric arteries were isolated from TRPM7$^{+/+}$ and TRPM7Δkinase mice infused with vehicle or Ang II and mounted as pressurized systems. Lumen diameter (A), media cross-sectional area (B), and media lumen ratio (C) were analyzed by pressure myography. Mesenteric arteries were incubated in 0 $Ca^{2+}$ physiological salt solution containing 10 mmol/L EGTA and were subjected to increasing levels of intraluminal pressure as indicated. Line graphs represent mean ± SEM of 5 mice per group. *p<0.05 vs vehicle-infused counterparts.

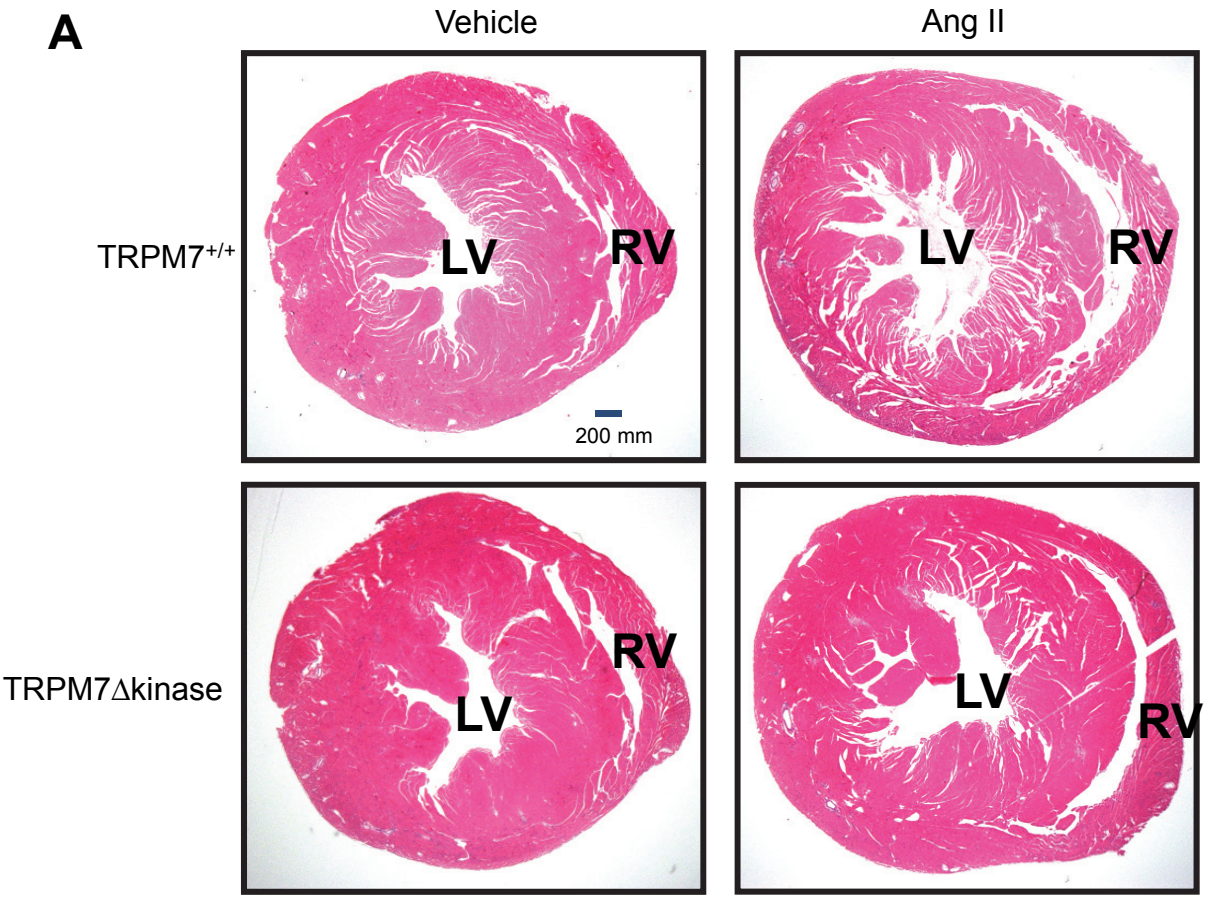
**Supplemental figure S5. Expression of eNOS and caveolin-1 in membrane-enriched vascular fractions from Ang II-infused TRPM7$^{+/+}$ and TRPM7Δkinase mice.** Mesenteric arteries were isolated from TRPM7$^{+/+}$ and TRPM7Δkinase mice infused with vehicle or Ang II. Protein expression of eNOS (A) and caveolin-1 (B) was analyzed in membrane-enriched fractions. Data are presented as the expression of eNOS (A) and caveolin-1 (B) relative to the expression of β-actin. Results are presented as mean ± SEM of 5-9 mice per group. *p<0.05 vs vehicle-infused TRPM7$^{+/+}$: $^{+}p<0.05$ vs Ang II-infused TRPM7$^{+/+}$.

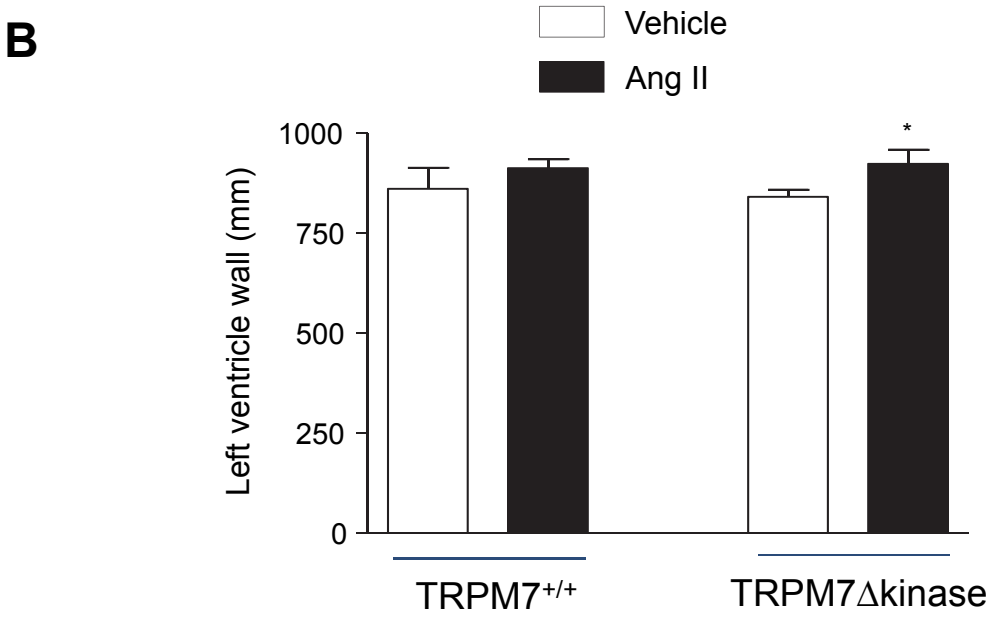
**Supplemental figure S6. Ang II effects on JNK phosphoryltion in aortas.** Aortas were isolated from TRPM7$^{+/+}$ and TRPM7Δkinase mice infused with vehicle or Ang II.

Phosphorylation levels of JNK are presented relative to expression of β actin. Upper panels, representative immunoblots of phospho-JNK and β-actin. Results are presented as mean ± SEM of 5-9 mice per group. *$p<0.05$ vs vehicle-infused counterparts.

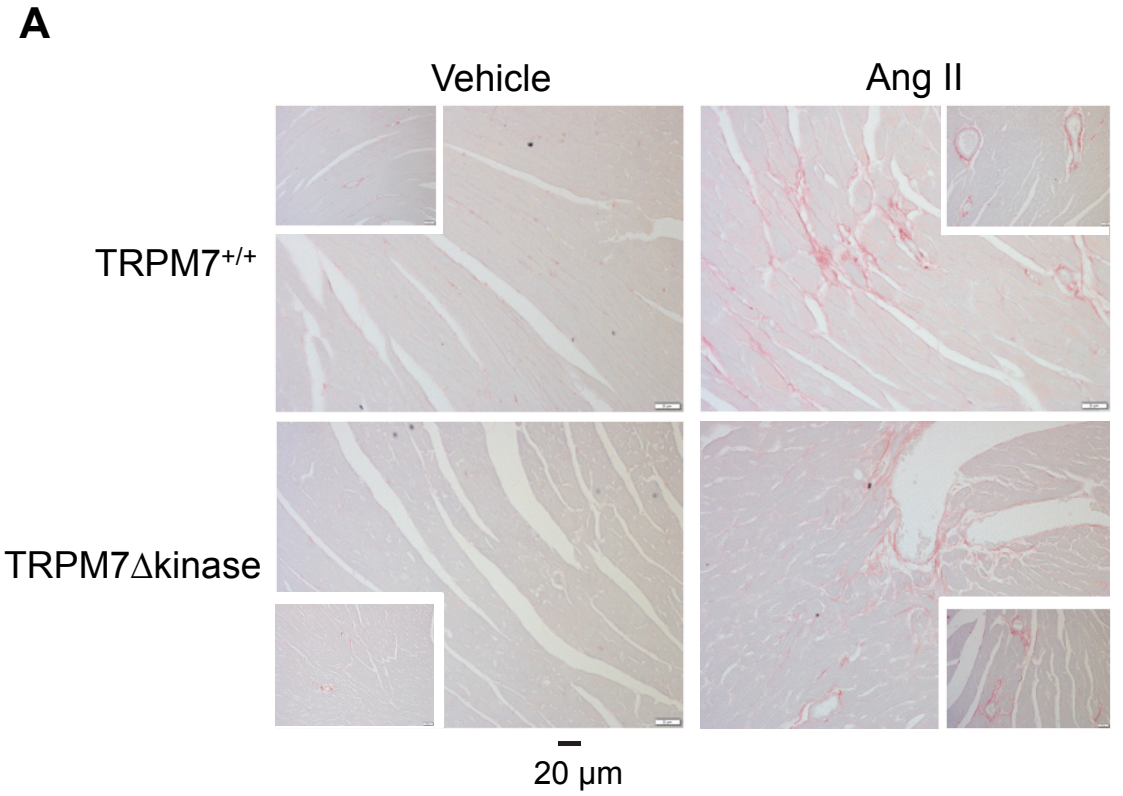
**Supplemental figure S7. Vascular and systemic oxidative stress in Ang II-infused TRPM7$^{+/+}$ and TRPM7Δkinase mice.** Plasma, aorta, and mesenteric arteries were obtained from TRPM7$^{+/+}$ and TRPM7$^{+/-}$ mice infused with vehicle or Ang II. (A) plasma $H_2O_2$ status accessed by Amplex red following a 30 min reaction time; (B) time-course of Amplex red reaction (0 to 60 min). (C) Superoxide anion generation accessed in aorta homogenates by lucigenin-enhanced chemiluminescence. (D) Protein expression of p47phox analyzed in membrane-enriched fractions and presented as a ratio to β actin. Upper panels, representative immunoblots of p47phox and β actin. Bar and line graphs represent mean ± SEM of 5-14 mice per group.

Supplemental Figure S1

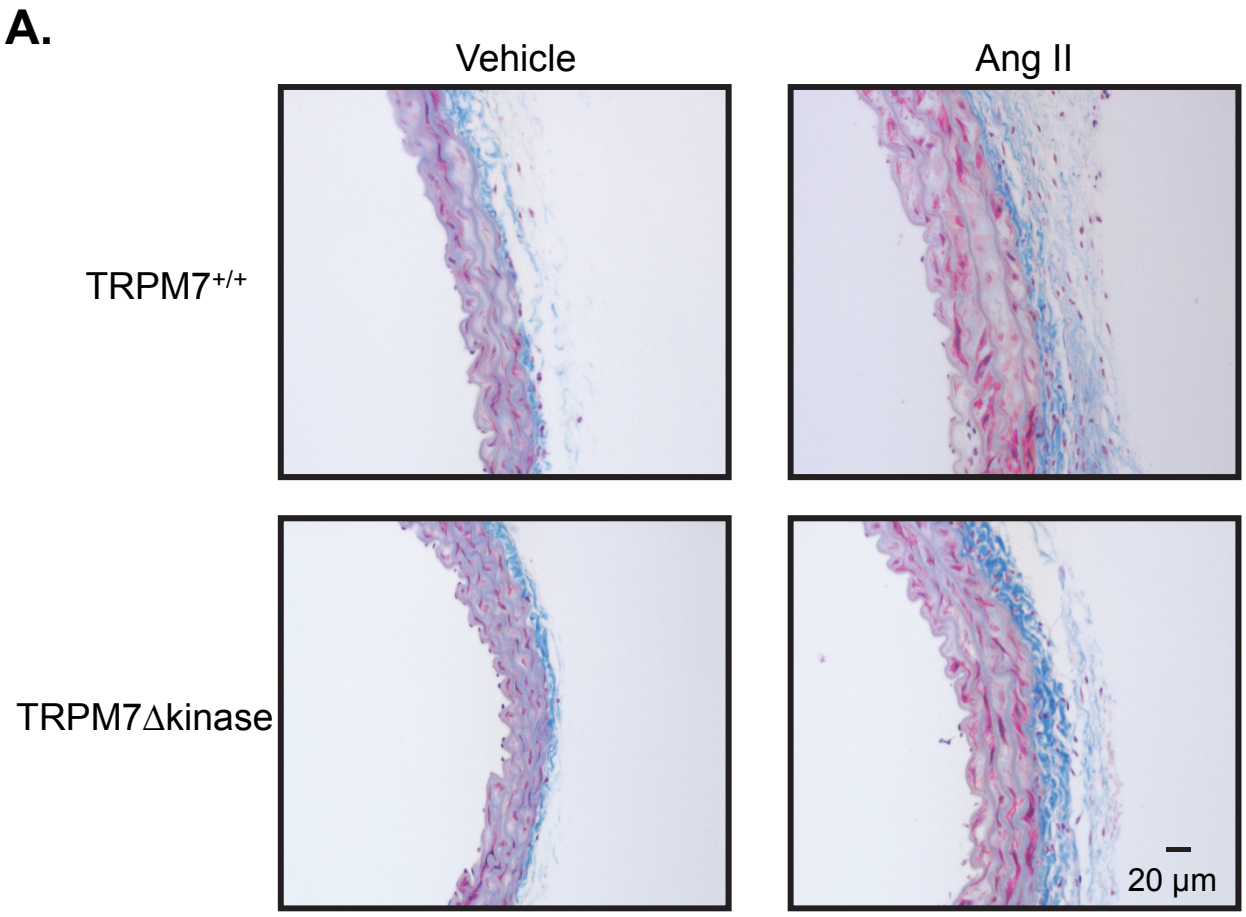


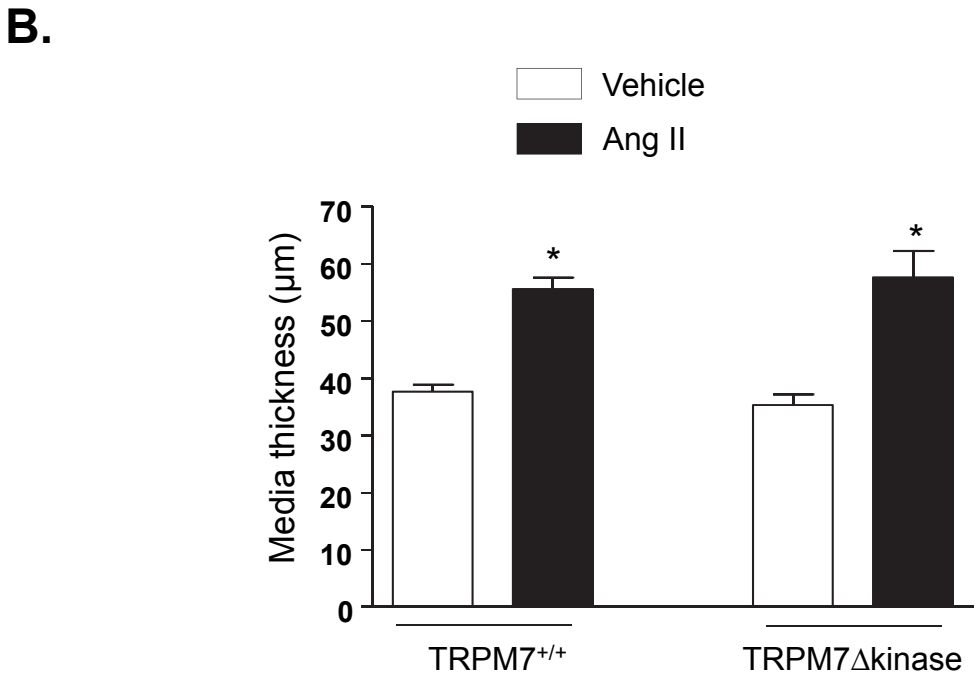



Supplemental Figure S2



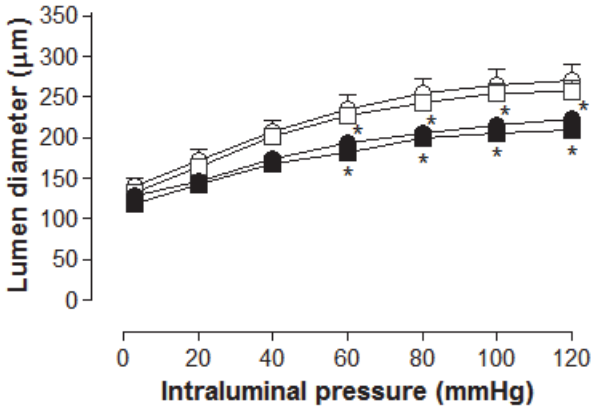
Supplemental Figure S3

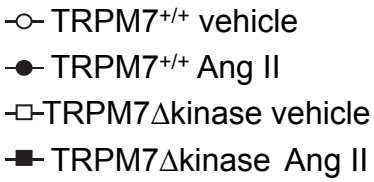


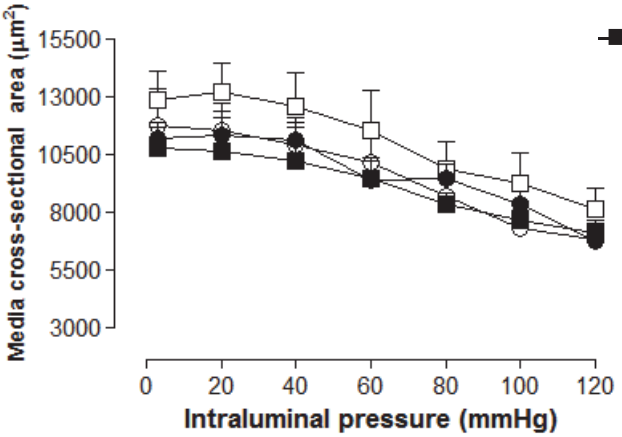
Supplemental Figure S4

**A.**

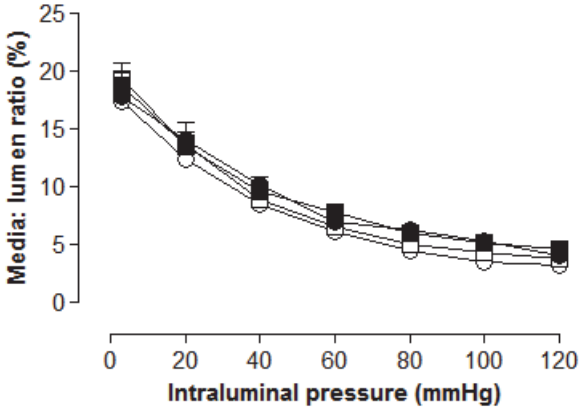



**B.**

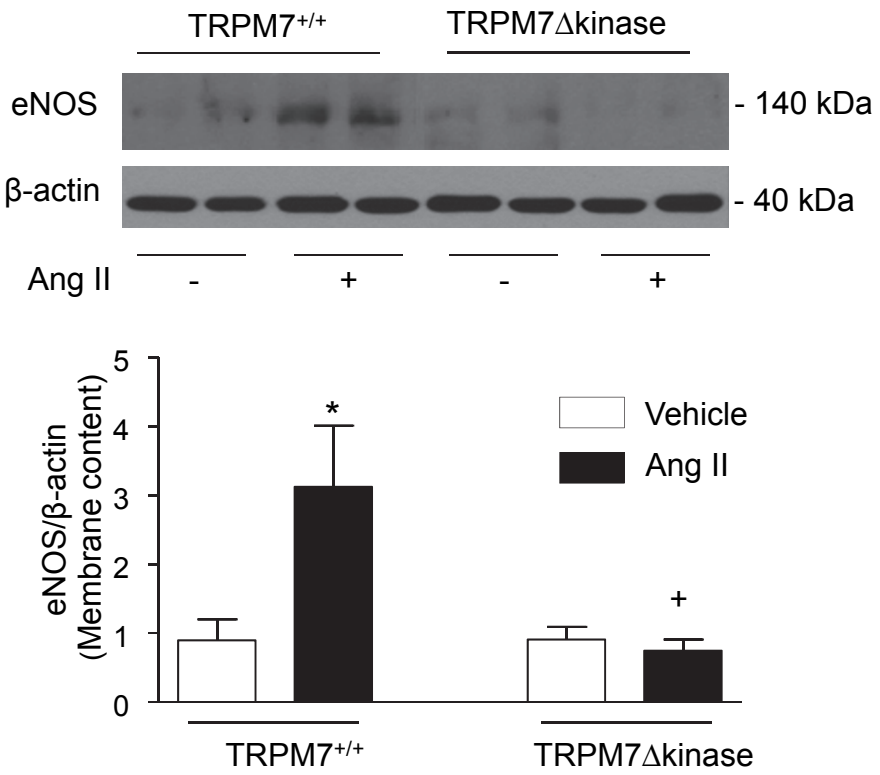



**C.**

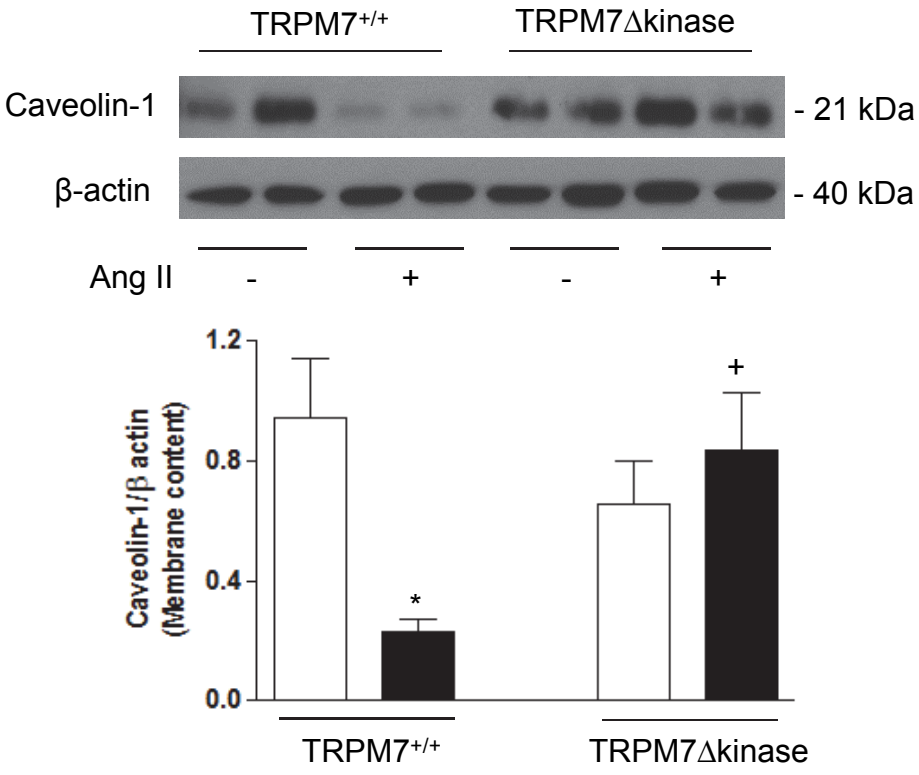


Supplemental Figure S5

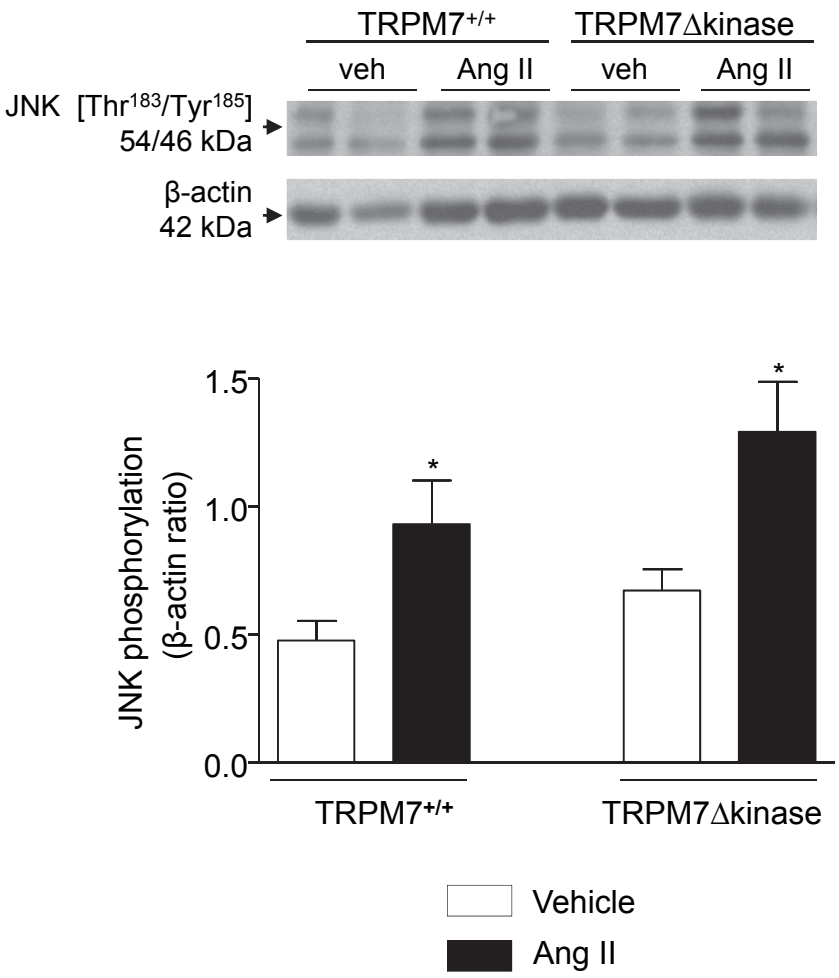
**A**




**B**



Supplemental Figure S6



Supplemental Figure S7