Essential Role of Smooth Muscle STIM1 in Hypertension and Cardiovascular Dysfunction

Modar Kassan[1,2,4], Karima Ait-Aissa[2], Eman Radwan[2], Vishal Mali[2], Samuel Haddox[2], Mohanad Gabani[4], Wei Zhang[3], Souad Belmadani[2], Kaikobad Irani[4], Mohamed Trebak[3*], Khalid Matrougui[1,2*]

Short title: STIM1 and cardiovascular dysfunction

[1]Department of Physiology, Hypertension and Renal Center of Excellence, Tulane University, New Orleans, LA 70112 (M.K and K.M); [2]Department of Physiological Sciences, EVMS, Norfolk, VA 23501 (M.K, K.M and K.A); [3]Penn State University College of Medicine, Department of Cellular and Molecular Physiology, Hershey, PA 17033 (M.T); [4]University of Iowa, Department of Internal Medicine, Iowa City, IA 52242.

[*]Co-senior authors: correspondence should be addressed to either Khalid Matrougui, PhD., Department of Physiological Sciences, EVMS, Norfolk, VA 23501; phone: 757-446-5278; email: matrouk@evms.edu; or Mohamed Trebak, PhD., Penn State Hershey College of Medicine, Department of Cellular and Molecular Physiology, Hershey, PA 17033-0850, phone: 717-531-8152; email: mtrebak@hmc.psu.edu

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MATERIALS AND METHODS

Mice

All experiments were performed according to the American Guidelines for the Ethical Care of Animals and were approved by institutional Animal Care and Use Committees at Tulane University and EVMS. Wild type littermates (8 weeks-old males) and CHOP knockout mice (8 weeks-old males) were purchased from Jackson Laboratories (Bar Harbor, ME). Male STIM1 smooth muscle-specific knockout mice ($Stim1^{SMC-/-}$, 8 to 10 week-old) as well as heterozygote mice ($Stim1^{SMC-/+}$, 8 to 10 week-old) were generated in our laboratory using stim1 floxed mice provided by Stefan Feske (NYU)[1] and SM22α-Cre obtained from Jackson Laboratories.[1, 2] All mice were housed in groups of five, maintained at a temperature of 23 ºC with 12 h light/dark cycles and fed a solid standard diet (Na^+ content 0.4%) and water. Mice were divided into 8 groups: 1) wild type mice infused with saline, (WT, n=10); 2) wild type mice infused with Ang II (400 ng/kg/min) for 4 weeks (WT + Ang II, n=10); 3) $Stim1^{SMC-/-}$ mice infused with saline ($Stim1^{SMC-/-}$, n=10); 4) $Stim1^{SMC-/-}$ mice infused Ang II ($Stim1^{SMC-/-}$ + Ang II, n=10); 5) $Stim1^{SMC-/+}$ mice infused with saline ($Stim1^{SMC-/+}$, n=10); 6) $Stim1^{SMC-/+}$ mice infused Ang II ($Stim1^{SMC-/+}$ + Ang II, n=10); 7) $CHOP^{-/-}$ mice infused with saline ($CHOP^{-/-}$, n=10); 8) $CHOP^{-/-}$ mice infused with Ang II ($CHOP^{-/-}$ + Ang II, n=10). The infusion was performed using subcutaneous mini-osmotic pumps and the body weight and systolic blood pressure were recorded weekly. Systolic blood pressure (SBP) was measured using the CODA tail-cuff blood pressure system (Kent Scientific Torrington, USA). Arterial blood pressure measurements were performed at the same time of the day (between 9 am and 11 am) in order to avoid the influence of the circadian cycle, and the value of SBP was obtained by estimating the average of 10 measurements. At the end of the treatment period, mice were sacrificed and tissues (Thoracic aorta, mesenteric resistance artery and heart) were harvested immediately, placed in PSS solution (composition in mM: NaCl 118; KCl 4.7; CaCl2 2.5; KH2PO4 1.2; MgSO4x7H2O 1.2; NaHCO3 25 and glucose 11, pH=7.4) and processed appropriately for further studies. Blood samples were centrifuged at 2500 rpm for 10 min at 4 ºC to obtain plasma, which was immediately stored at -80 ºC. Heart was used to determine hypertrophy and fibrosis. Vascular reactivity was evaluated in aorta and mesenteric resistance arteries.

In another set of experiments, we used eight-weeks-old male C57/BL6 wild-type mice, STIM1 smooth muscle-specific knockout mice ($Stim1^{SMC-/-}$, 8 to 10 week-old) and their homologous heterozygous ($Stim1^{SMC-/+}$, 8 to 10 week-old). Mice were divided into 6 groups: 1) wild-type received vehicle, (WT, n=10); 2) wild type group received intra-peritoneal injection of Tunicamycin (Tunica, 1 mg/kg, 2 injections/week for two weeks, WT + Tunica, n=10); 3) $Stim1^{SMC-/+}$ group received vehicle ($Stim1^{SMC-/+}$, n=10); 4) $Stim1^{SMC-/+}$ group received Tunicamycin ($Stim1^{SMC-/+}$ + Tunica, n=10); 5) $Stim1^{SMC-/-}$ group received vehicle ($Stim1^{SMC-/-}$, n=10); 6) $Stim1^{SMC-/-}$ group received Tunicamycin ($Stim1^{SMC-/-}$ + Tunica, n=10). Body Weight and Systolic Blood pressure were measured weekly. At the end of treatment, mice were sacrificed, and aorta and mesenteric resistance arteries (MRA) were immediately harvested and placed in PSS solution for vascular reactivity studies and biochemical assays.

Cardiac Fibrosis and hypertrophy

The atrium was removed from the heart; all epicardial fat was removed, and the right and the left ventricles were separated. Transverse sections of the left ventricle were fixed in 4% of formalin, embedded in paraffin and cut into 4μm thick sections. Slices were stained with the collagen-specific Sirius-red (Sigma-Aldrich, USA). At least eight areas of the left ventricle from each heart were captured using a high-resolution digital camera (Olympus DP50, Japan). Cardiac hypertrophy was determined by evaluating heart weight/tibia length ratios as previously described[3].

Vascular Reactivity
MRA and thoracic aorta from WT, $CHOP^{-/-}$, $Stim1^{SMC-/-}$ and $Stim1^{SMC-/+}$ mice, infused with either saline or Ang II or injected with Tunicamycin, were carefully cleaned of fat and connective tissue and then cut into rings (2 mm in length). MRA and thoracic aorta were mounted in a small vessel dual chamber myograph for measurement of isometric tension. After a 30 min equilibration period in PSS solution bubbled with carbogen at 37°C and pH=7.4, arteries were stretched to their optimal lumen diameter for active tension development. After one-hour incubation, cumulative concentration responses to phenylephrine (PE, 3.10^{-8}-10^{-4} M) and thromboxane analog (U46619, 10^{-10}-10^{-5} M) were obtained. In another series of experiments, rings were pre-constricted with U46619 (3.10^{-7} mol/L) and when a steady maximal contraction was reached, cumulative concentration-response curves were obtained for acetylcholine (ACh, 10^{-8}-3.10^{-5}) and sodium nitroprusside (SNP, 10^{-8}-3.10^{-5}).

To determine the role of NADPH oxidase and TGFβ1 in impaired endothelium-dependent relaxation in hypertensive mice, aorta and MRA from all groups were incubated with NADPH oxidase and TGFβ1 inhibitors: gp91 ds-tat (100 μM) for 30 minutes and SB431542 (10 μM) for 1h respectively, then endothelium-dependent relaxation was determined after pre-contraction with U46619. The same protocol was used for mice treated with Tunicamycin (1 mg/Kg).

Western blot analysis
Western blot analysis was determined in lysates from mesenteric arteries and hearts using specific antibodies against phosphorylated (Serine 1177) and total-eNOS, eNOS P-T495 (#9574S), phosphorylated and total PKCα/β (# 9375S, # 2056S), CHOP (# 2895S), STIM1 (# Ab108994), ATF6 (# Ab 37149) and Bip (# 3177S) (1:1000 dilution, Cell Signaling Technology, Inc, USA), p47phox (sc-17845), P-Smad2,3 (# 8822Sß) and T-Smad1,2,3 (# Sc7960) (1:500 dilution, Santa Cruz Biotechnology, Inc) as previously described [3, 4].

Immunohistochemistry
Hearts, mesenteric resistance arteries and thoracic aorta were fixed in 4% of paraformaldehyde followed by zinc-saturated formalin and paraffin-embedding for either immunostaining or immunoperoxidase staining using the Vectastain ABC Kit. The hearts sections were incubated overnight with the anti-STIM1 antibody (1:200, Cell Signaling Technology, Inc) and anti-CHOP antibody (1:200, Cell Signaling Technology). Mesenteric resistance and thoracic aorta sections were incubated with anti 8-hydroxy-2-deoxyguanosine (8-OHD), marker of oxidative stress (1:200, Abnova; #MAB1998). At least eight sections from each sample were captured using a high-resolution digital camera (Olympus DP50, Japan).

Nitrites and Nitrates levels
A number of nitrites, the end product of NO metabolism was measured in aorta and MRA tissue samples by the Griess reaction. Optical density at 550 nm wavelengths was measured using a Spectramax 250 microplate reader (Molecular Devices, CA). Nitrite concentrations were calculated by establishing a standard curve with known sodium nitrite concentrations.

Colorimetric Determination of cGMP
The cGMP levels were measured in MRA lysates in all groups of mice. Measurements were performed using a sandwich enzyme-linked immunosorbent assay (ELISA; Cayman Chemical, MI) according to the manufacturer instructions.

NADPH oxidase activity assay
Superoxide anion levels generated by NADPH oxidase activity were measured in lysates of aorta and MRA using lucigenin chemiluminescence. Briefly, lysates were prepared in a sucrose buffer

containing KH2PO4 50 mM, EGTA 1 mM, sucrose 150 mM; pH=7.0 and the "Complete-C mini" protease inhibitor cocktail (Roche Diagnostics, IN) in a Tissue Dounce homogenizer on the ice, and aliquots of the homogenates were used immediately. To start the assay, a volume of 100 µL of each lysate was used in a total volume of 1 mL PBS buffer preheated at 37^{o}C, containing lucigenin (5 µM) and NADPH (100 µM). Blank samples were prepared using 100 µL of sucrose buffer. Lucigenin activity was measured every 30 seconds for 10 min in a luminometer (Turner biosystem 20/20, single tube luminometer) or till enzymatic activity reached a plateau. The NADPH oxidase activity was performed in the presence or absence of a direct inhibitor of NOS (L-NAME) or an inhibitor of NADPH oxidase (Apocynin). Data are expressed as % of the area under the curve of relative light units (RLU) normalized to protein content (µg protein) compared to WT.

eNOS ELISA

Total eNOS were determined in all groups by an ELISA Kit according to the manufacturer's guidelines (Mouse-eNOS kit, Qayee-Bio, China).

Reverse Transcription Polymerase Chain Reaction Real-Time Assay

ER stress markers (CHOP, BIP, ATF6), NOX isoforms (Nox 2 and 4) and STIM1 mRNA levels were determined in MRA as previously described [3, 4].

Drugs

Phenylephrine hydrochloride, acetylcholine, NADPH, Apocynin and angiotensin II were obtained from Sigma-Aldrich. U46619, Tunicamycin, and SB431542 were obtained from Tocris Bioscience. The gp91-ds-tat was obtained from AnaSpec, Inc. Stock solutions of drugs were prepared in ultrapure water, stored at -20^{o}C and appropriate dilutions were made on the day of the experiment.

Statistical analysis

Data are expressed as mean ± SEM. Concentration-response curves were analyzed using the GraphPad Prism 4.0 software (GraphPad, USA) and adjusted to a logistic equation. Statistical calculations for significant differences were performed using Student's t-test, one-way followed by Post-Hoc test or two-way ANOVA as appropriate. Comparisons are considered significant when $p< 0.05$.

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