ONLINE SUPPLEMENT

Brain-mediated Dysregulation of the Bone Marrow Activity in Angiotensin II-induced Hypertension

Joo Yun Jun1*, Jasenka Zubcevic1*, Yanfei Qi1, Aqeela Afzal2, Jessica Marulanda Carvajal1, Jeffrey Thinschmidt3, Maria B. Grant3, J Mocco2, and Mohan K Raizada1

1Department of Physiology and Functional Genomics, University of Florida College of Medicine, Gainesville, FL

2Department of Neurosurgery, University of Florida College of Medicine, Gainesville, FL

3Department of Pharmacology and Therapeutics, University of Florida College of Medicine, Gainesville, FL

*These authors contributed equally to this study.

Short title: Brain-bone marrow communication in hypertension

Corresponding Author:
Mohan K. Raizada, Ph.D.
Department of Physiology & Functional Genomics
University of Florida
Gainesville, FL 32610
Email: mraizada@ufl.edu
Phone: 352-392-9299
Fax: 352-294-0191
Preparation of primary neuronal culture

Neuronal cells in primary culture from the brainstem and hypothalamus of one day-old SD were established as described previously. Briefly, tissues from brain were dissected, combined and dissociated, and plated in poly-L-lysine pre-coated culture dishes. As prepared, the neuronal cultures contained more than 90% neurons. The cultures were maintained for 12-14 days prior to use in the experiments.

Measurement of ROS production

Cultured neurons were treated with AngII (500nM), or co-treated with mitoTEMPO (2 and 5μmol/L, Enzo lifescience, ALX-430-150) for 4 hours. Cellular \( \text{O}_2^- \) was measured by DHE (dihydroethidium, Invitrogen) fluorescent staining, and mitochondrial \( \text{O}_2^- \) was measured by MitoSOX Red (Invitrogen). DHE fluorescence (Ex 510 nm/Em 580 nm) was measured by microplate reader (Synergy Mx, BioTek). The mitochondrial specific marker mitoTracker green (Invitrogen) was used to establish the mitochondrial subcellular location of MitoSOX. Images were obtained with a Zeiss Axioplan 2 Fluorescent Microscope.

Animal

Adult male Sprague-Dawley (SD) rats aged between 6 weeks and 7 weeks (Charles River Laboratories) were individually housed in a temperature-controlled room (22°C to 23°C) with a 12:12-hour light-dark cycle. All experimental procedures were approved by the University of Florida Institute Animal Care and Use Committee.

Telemetric Recordings of Arterial Pressure

Male SD rats (7 to 8 weeks, n=40) were anesthetized with a mixture of O\(_2\) (1 L/min) and isoflurane (3% to 4%). A radiotransmitter (TA11PAC40, Data Sciences International) was implanted to record arterial pressure and heart rate from the abdominal aorta, as described previously. A bolus injection of buprenorphine (0.03 mg/kg SC) was administered after each surgery. Rats were allowed to recover for 7 to 10 days before baseline telemetric measurements were taken. A full spectral analysis was performed on the BP signal to reveal potential mechanisms as described previously.

Implantation of Subcutaneous Osmotic minipump

Rats were further assigned to subgroups (n=5-8) to receive either Ang II (200 ng/kg/minute) or 0.9% saline with/without mitoTEMPO (100 or 170 ng/kg/min) or with/without phenylephrine (1ug/kg/min) delivered via an osmotic minipump (No. 2004, 2006, ALZET) implanted subcutaneously between the scapulae. Pump
lasted for 4, 6, or 12 weeks from the day of the drug preparation, depending on the experiment.

**Intracerebroventricular mitoTEMPO Infusion**

Ten to fourteen days after implantation of telemetry transmitters, rats were implanted with intracerebroventricular (ICV) cannulae for infusion of mitoTEMPO on day 0. Rats were anesthetized with a 4% isoflurane/O2 mixture, and the head was positioned in a Kopf stereotaxic apparatus. An infusion cannula (Brain infusion kit 1 3-5mm, ALZET) was implanted into the left cerebroventricle (1.3 mm caudal to bregma, 1.5 mm lateral to the midline, and 4.5 mm ventral to the dura). A 4-week osmotic minipump was connected to the infusion cannula via the catheter tube to deliver mitoTEMPO (Enzo Life Science, 100 or 170 ng/kg/min).

**Immunohistochemistry**

Brains were cut into 20-μm coronal sections including PVN and incubated with anti-Iba-1 antibody (1:500, Waco, cat # 019-19741) as specific marker for microglia and the anti-rabbit IgG (1:200, VECTOR, cat# BA-1000) as a secondary antibody. The secondary antibody is conjugated with 3,3'-diaminobenzidine (DAB). Staining procedure and microglia quantification is described elsewhere. An Olympus BX41 microscope was used to obtain images from DAB stained sections for microglia.

**Cardiac Pathology**

Hearts were collected at the end of the experiment, and processed for cardiac morphology and histology as described previously. Briefly, left ventricles were weighed and fixed in 10% paraformaldehyde before they were embedded in paraffin. Four μm cross-sectioned left ventricles were stained with hematoxylin-eosin for the myocyte diameter measurement, or stained with Picro-sirius red dye for interstitial fibrosis. Twenty-five images were taken from each section and analyzed using image J software.

**RNA isolation and Real-Time PCR**

To analyze the mRNA levels, both hypothalamic and brain stem tissues including the PVN, SFO, RVLM, and NTS were dissected, as described previously. Briefly, coronal segments were sliced according to the coordinates by Paxinos and Watson, and small blocks of each area were excised (2.0 mm wide and high). Total RNA was prepared using RNeasy kit (Qiagen) according to the manufacturer’s instruction. About 200 to 300 ng of purified RNA were reverse transcribed using high-capacity cDNA reverse transcription kit (Bio-Rad Laboratories). Quantitative Real-Time PCR was performed with specific primers and probes of IL1b, TNF-a, and CD11b by using PRISM 7000 sequence
detection system (Applied Biosystem). Data were normalized to 18s ribosomal RNA or GAPDH.

**MNC isolation from BM**

Intact femur and tibia were collected into PBS+2% FBS+1 mM EDTA buffer, followed by cleaning and removal of muscle and fat as described elsewhere. The samples were collected at 11 am, as it has been shown that the EPCs are at their highest at this time of the day. The tips of the bones were cut to flush bone marrow cells with 20 ml of the PBS+2% FBS+1mM EDTA buffer, using a 10 ml syringe into a 50ml conical tube. Cells were spun down at 1200 rpm for 15mins at room temperature. To remove the residual red blood cells (RBCs), ammonium chloride (STEM CELL technology, Cat # 07850) was added and cells were incubated for 10 min on ice followed by 2 times washing with PBS+2% FBS+1mM EDTA, to remove the residual RBCs. The resulting MNCs were re-suspended in 1 ml of PBS+2% FBS+1mM EDTA and kept on ice until use.

**Tube formation assay**

Isolated MNCs were plated in fibronectin pre-coated 6-well plates, and maintained with endothelial basal medium for up to 3 weeks, or until they differentiated into endothelial cells. Cells were then transferred to 96-well Matrigel matrix plate (BD BioCoat TM Angiogenesis System Endothelial Cell Tube Formation, Cat #: 354149) at 2.5~3x10^3 cells/ml and incubated for 12 hours at 37°C, 5% CO2. Cells were monitored under microscope (bright field) every 2-3 hours to identify the ability of tube formation. The length of tubes and the number of branches from the images were measured using image J software.

**Direct flow cytometry (FACS) analysis**

To profile the levels of BM and blood ICs and EPCs, the MNCs from BM and blood were prepared in a concentration of 0.5-1x10^6 cells/100ul in PBS+2% FBS+1mM EDTA mixture media. CD3+/45+, CD4/8+, CD4/8+/CD25+ were used as representative of T cells prominent in Ang II-induced hypertension, and CD68+ cells were used as representative of macrophages, which are also activated by Ang II. CD90+/CD4/5/8- cells were used as representative for EPCs, as these have previously shown angiogenic and endothelial reparative properties in the rat. Antibodies were purchased from AbD Serotec (Alex647 conjugated CD4/5/8/3/68, RPE conjugated CD25, FITC conjugated CD45, Perpendicular conjugated CD90), and used as recommended by the company. Cells were incubated with antibodies for 45 minutes at 4°C. Individual antibodies were prepared in each cell suspension and used as control. After spinning down and washing twice, cells were fixed with 2% paraformaldehyde for later analysis. All samples were read on an LSR-II (BD Biosystems) in University of Florida Interdisciplinary Center for Biotechnology Research (ICBR) and the data were analyzed with FACS Diva software, version 6.1.2.
Pseudo Rabies Virus Tracing

Pseudo Rabies Virus (PRV-152) tagged with GFP was a gift from Dr. L. W. Enquist, Princeton University, and was used as a retrograde tracer. This viral strain is extensively used in neuronal circuit tracing experiments due to its specific neuroinvasive ability and the ability of retrograde transportation across the neuronal synapses. The replication-competent virus was injected into the femur bone marrow, and green fluorescence was examined in various brain regions 6 days later. The surgical site was shaved and prepared with sterile scrub and the left femur was exposed using a scalpel blade. The bone surface was cleaned using 3% H2O2 and a hole was burred into the distal epiphysis. To avoid any viral contamination during injections, the femoral bone was isolated from the surrounding tissue using sterile gauze soaked with 3% H2O2. PRV was injected into the bone marrow using a 10 ul Hamilton syringe (PRV-152: 6-8ul of 1.5 x 10^10 PFU virus recombinants). The needle was kept in place for 10 min to avoid the reflux of the inoculums along the needle track. The place of injections was stamped with Ethicon bone wax and the surface of the bone was wiped with 70% ethanol. The muscle surrounding the femur was sutured, and the skin was closed with surgical nylon. Analgesics were administered prior to surgery and for 48 hours after as needed. Two separate control experiments were performed in which the virus was delivered either to the adjacent muscle or in the BM after the ablation of sciatic and femoral nerves. No positive GFP cells were observed in any of the CV relevant regions in the brain.

Data and Statistical Analysis

Data were expressed as mean±SEM 2-way ANOVAs or 1-way ANOVAs, and the Bonferroni post-test was used to allow multiple comparisons of cardiovascular variables across time and between different groups. Paired/unpaired Student t tests were used for further comparisons between 2 groups where applicable, with P<0.05 considered significant.
References


12. Smith BN, Banfield BW, Smeraski CA, Wilcox CL, Dudek FE, Enquist LW, Pickard GE. Pseudorabies virus expressing enhanced green fluorescent
Figure S1: Effects of mitoTEMPO on autonomic nerve activity in Ang II-induced hypertension. A, $\Delta$LF(SBP): Sympathetic vasomotor drive. B, $\Delta$HF(PI): Cardiac parasympathetic drive. C, $\Delta$sBRG(PI): Cardiac spontaneous baroreflex gain. D, $\Delta$LF/HF: Vasovagal balance. Ang II (200ng/kg/min) and mitoTEMPO ICV and SC (170ng/kg/min), mitoTEMPO ICV only (100ng/kg/min) *$P<0.05$ vs control, #$P<0.05$ vs Ang II.
Figure S2: Effect of chronic Ang II infusion on blood EPCs and ICs. A, Decrease in blood EPC at week 6 and 12 of Ang II infusion. B, Increase in blood inflammatory cells at 12 weeks of Ang II infusion (IC; CD4+/8+: T lymphocytes, CD4+/8+/25+: T regulatory cells, CD45+/3+: T lymphocytes, CD68+: macrophages) by chronic Ang II infusion. C, The ratio of EPCs to ICs. *P<0.01 vs control. N=6
Figure S3: Effects of chronic Ang II infusion on tube formation ability of mononuclear cells (MNC) derived from BM. A, The representative image of cultured MNC tube formation from control group at 5 hours of matrigel matrix plate. B, The representative image of cultured MNC tube formation from Ang II infusion group at 5 hours of matrigel matrix plate. C, Tube lengths were measured using image J. D, Number of branches were counted using image J. Scale bars=100μm. *P<0.05 vs control, N=8
Figure S4: The effects of chronic phenylephrine infusion on EPCs and ICs. A, MAP was increased by 6 weeks infusion of SC phenylephrine. B, HR was not changed by phenylephrine infusion. C, ICs and EPCs were not significantly altered by phenylephrine infusion. D, The ratio of EPC to CD4/8+ was decreased but not in other ICs (CD4/8/25+, CD45/3+, CD68+). *P<0.05
Figure S5: Effects of mitoTEMPO on Ang II-induced cardiac hypertrophy, myocyte diameter and interstitial fibrosis. A, The ratio of heart to body weight. B, Cardiac myocyte diameter measured from H&E-stained left ventricle section. C, Representative left ventricle sections of picro-sirius dye staining-positive
fibrotic areas. D, Quantification graph generated from Image J software. *$P<0.05$, **$P<0.01$ vs control, #$P<0.05$ vs Ang II. Scale bars=100μm. N=6-7