Brain-Mediated Dysregulation of the Bone Marrow Activity in Angiotensin II–Induced Hypertension

Joo Yun Jun*, Jasenka Zubcevic*, Yanfei Qi, Aqeela Afzal, Jessica Marulanda Carvajal, Jeffrey S. Thinschmidt, Maria B. Grant, J. Mocco, Mohan K. Raizada

Abstract—Oxidative stress in the brain is implicated in increased sympathetic drive, inflammatory status, and vascular dysfunctions, associated with development and establishment of hypertension. However, little is known about the mechanism of this impaired brain-vascular communication. Here, we tested the hypothesis that increased oxidative stress in the brain cardioregulatory areas, such as the paraventricular nucleus of the hypothalamus, is driven by mitochondrial reactive oxygen species and leads to increased inflammatory cells (ICs) and decreased/dysfunctional endothelial progenitor cells (EPCs), thereby compromising vasculature repair and accelerating hypertension. Chronic angiotensin II infusion resulted in elevated blood pressure and sympathetic vasomotor drive, decreased spontaneous baroreflex gain, and increased microglia activation in the paraventricular nucleus. This was associated with 46% decrease in bone marrow (BM)-derived EPCs and 250% increase in BM ICs, resulting in 5-fold decrease of EPC/IC ratio in the BM. Treatment with mitochondrial-targeted antioxidant, a scavenger of mitochondrial O$_2^·$ intracerebroventricularly but not subcutaneously attenuated angiotensin II–induced hypertension, decreased activation of microglia in the paraventricular nucleus, and normalized EPCs/ICs. This functional communication between the brain and BM was confirmed by retrograde neuronal labeling from the BM with green fluorescent protein–tagged pseudorabies virus. Administration of green fluorescent protein–tagged pseudorabies virus into the BM resulted in predominant labeling of paraventricular nucleus neurons within 3 days, with some fluorescence in the nucleus tractus solitarius, the rostral ventrolateral medulla, and subfornical organ. Taken together, these data demonstrate that inhibition of mitochondrial reactive oxygen species attenuates angiotensin II–induced hypertension and corrects the imbalance in EPCs/ICs in the BM. They suggest that an imbalance in vascular reparative and ICs may perpetuate vascular pathophysiology in this model of hypertension.

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Key Words: mitochondrial reactive oxygen species ■ endothelial progenitor cells ■ mean arterial pressure ■ neurogenic hypertension

The autonomic nervous system (ANS) and the immune system (IS) play major roles in the pathophysiology of hypertension and other cardiovascular diseases. Increased sympathetic drive has been shown to precede hypertension and, together with the decreased parasympathetic drive and the baroreceptor reflex, is a consequence of the elevated renin-angiotensin system activity in the central cardioregulatory regions, such as the paraventricular nucleus (PVN) and subfornical organ, among others. Angiotensin II (Ang II)–dependent hypertension is also associated with an overactive IS, because the elevated levels of inflammatory cells (ICs) and cytokines contribute to both the pressor effects of Ang II, as well as the cardiovascular pathophysiology. A functional link between the ANS and bone marrow (BM) cells has long been appreciated, and a cellular association between BM stromal cells and hematopoietic stem cells and nerve terminals has been described as the neuroreticular complex.

Recent studies support that the ANS can modulate the effects of the IS and influence the pathophysiology of cardiovascular diseases. A direct sympathetic innervation of the immune organs, such as the spleen, and increased splenic sympathetic nerve activity by central Ang II infusion are directly related to the enhanced splenic immune response. Moreover, stimulation of the vagus nerve exerts anti-inflammatory effects by decreasing levels of the inflammatory cytokines and suppressing the activation of ICs. Therefore, the possibility exists that Ang II–dependent dysfunction of ANS precedes the activation of the IS and that the ANS-IS synergetic action initiates the development of hypertension, as inferred by the recent observations of Abboud et al and Harrison et al.

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Endothelial dysfunction is a well-accepted hallmark of early hypertension. Because mature endothelial cells have limited reparative capacity, the BM-derived endothelial progenitor cells (EPCs) contribute to the repair and maintenance of the damaged endothelium. However, EPC numbers and function are inversely correlated in patients with hypertension and other cardiovascular diseases.14–17 Similar to the ICs, BM EPCs also appear to be neuroregulated.18 Evidence exists that the BM is densely innervated by the sympathetic and parasympathetic fibers,19–22 and dysregulation of the neuronal input to the BM impairs EPC function in diabetes mellitus.18 Moreover, both the EPC function and the numbers are improved by some renin-angiotensin system blockers,23,24 suggesting that one of the consequences of the overactive renin-angiotensin system in hypertension may also be the impairment of the endothelial reparative processes, which could lead to accelerated vascular dysfunction and hypertension-associated pathophysiology.

In line with this, we recently proposed a hypothesis of a dysfunctional autonomic-immune-vascular mechanism in neurogenic hypertension.25 This involves Ang II–mediated neuroinflammation as exhibited by activation of the brain microglia, primarily in the PVN, resulting in excessive production of reactive oxygen species (ROS), elevated sympathetic activity, and modulation of BM inflammatory and vascular reparative cells in the periphery, all playing important roles in the development and establishment of neurogenic hypertension.25–27 In the brain, mitochondria are one of the major sources of intracellular ROS production, resulting in excessive oxidative stress, and altering neuronal redox state, which disrupts the sympathetic drive.26 Collectively, we propose that increase in mitochondrial ROS is central in the dysfunctional neural-vascular communication associated with neurogenic hypertension. Furthermore, we postulate that impaired central-BM regulation of ICs and EPCs will impact the levels of activated ICs, as well as the vasoreparative potential of EPCs, and that this shift in the cardiovascular damage/repair ratio will contribute to cardiovascular pathophysiology in hypertension. Hence, we investigated the effect of central administration of mitochondrial-targeted antioxidant (mitoTEMPO) on the brain inflammatory status and the behavior of BM-derived EPCs and ICs after a chronic Ang II infusion in the rat model of neurogenic hypertension.

Methods

All experimental protocols are presented in the Methods section and are available in the online-only Data Supplement. All animal procedures were approved by the University of Florida Institute Animal Care and Use Committee.

Results

Chronic Ang II Causes Hypertension, Perturbs the Autonomic Nervous System, and Impairs BM Cell Activity

Ang II infusion resulted in an increase in mean arterial pressure (MAP) to 177 ± 6 mm Hg (n=8) at 4 weeks and to 202 ± 4 mm Hg (n=4) at 12 weeks of infusion, compared with the MAP of 98 ± 2 mm Hg in the age-matched controls (n=8) (Figure 1A). Spectral analysis of telemetry blood pressure signal was performed, and the variables low frequency (systolic blood pressure) and spontaneous baroreflex gain (pulse interval) indicated 6-fold increase in the sympathetic vasmotor drive and 3-fold decrease in the cardiac spontaneous baroreflex gain, respectively, after 4 weeks of Ang II infusion (Figure S1A and S1B in the online-only Data Supplement). The cardiac parasympathetic drive measured by high frequency (pulse interval) did not show changes among the groups (Figure S1C), yet the ratio of low frequency: high frequency, which is an indicator of vasovagal balance, was 2-fold elevated by Ang II (Figure S1D). This was associated with a 46% to 70% decrease in EPCs in the BM (Figure 1B). In addition, there were significant increases in CD4+8+ (200%), CD4+8+25+ (230%), CD3+45+ (50%), and CD68+ (220%) macrophages. 

Figure 1. Effect of chronic angiotensin II (Ang II) infusion on bone marrow-derived endothelial progenitor cells (BM EPCs) and inflammatory cells (ICs). A, Mean arterial pressure (MAP) measured by tail cuff after 4 to 12 weeks of Ang II infusion. B, Decreased BM-derived EPCs by chronic Ang II infusion. C, Increase in BM inflammatory cells (IC; CD45+/3+; T lymphocytes, CD45+/8+25+; T regulatory cells, CD45+8+/25+; T lymphocytes, CD68+; macrophages) by chronic Ang II infusion. D, The ratio of EPCs to ICs. *P<0.01, #P<0.001 vs control, n=6.
cells in the BM of Ang II–infused rats (Figure 1C). As a result, a 2- to 5-fold decrease in the EPC/IC ratio was observed in the BM of the Ang II-infused rats (Figure 1D). Similar trends were observed in blood EPCs and IC (Figure S2 in the online-only Data Supplement). To determine whether Ang II hypertension was associated with dysfunctional BM mononuclear cells, tube formation assays were performed. We found that both the tube length and the number of branches in the Ang II–infused rats were 20% to 30% lower than those grown from the control rats’ mononuclear cells (Figure S3 in the online-only Data Supplement).

Six-week subcutaneous infusion of phenylephrine, a vasoconstrictor that increases MAP without crossing the blood-brain barrier,27 increased MAP by ≈22 mm Hg (Figure S4A and S4B in the online-only Data Supplement). This chronic increase in MAP was associated with a trend of decreases in EPC/IC ratios in the BM, which were not significant for the most part (Figure S4C and S4D in the online-only Data Supplement). Only EPC/CD4/8+ ratio showed a significant (20%) decrease in the BM of the phenylephrine-infused animals. These data suggest that Ang II–dependent elevation in BP alone is not sufficient for inducing this imbalance.

Intracerebroventricular But Not Subcutaneous MitoTEMPO Infusion Prevents Hypertension, Balances the Autonomic Nervous System, and Restores BM Cell Activity

Ang II treatment of the rat neuronal cultures resulted in a 30% increase in cellular O$_2^•−$; which was completely inhibited by cotreatment with 2 to 5 μmol/L of mitoTEMPO, a selective scavenger of mitochondrial O$_2^•−$ (Figure 2A). This inhibition occurred primarily within the mitochondria, as evidenced by the costaining with mitoTracker Green (a mitochondrial specific fluorescence dye) and mitoSOX Red (a superoxide marker) (Figure 2B). Furthermore, chronic intracerebroventricular (ICV) infusion of mitoTEMPO (4 weeks) attenuated hypertension induced by Ang II (Figure 3A). This attenuation was significant by 18 days of mitoTEMPO ICV infusion and was dose dependent (100ng/kg per minute; 146±12 mm Hg; n=6, 170ng/kg per minute; 112±13 mm Hg; n=8). In contrast, subcutaneous infusion of 100ng/kg per minute of mitoTEMPO did not influence Ang II–induced increases in MAP. However, 170ng/kg per minute dose of mitoTEMPO administrated subcutaneously showed a trend toward a decrease in MAP, but this effect was not significant (Figure 3B).

Inhibition of MAP by ICV mitoTEMPO (170ng/kg per minute) was associated with the attenuation of heart weight/body weight ratio, cardiac myocyte diameter as an indicator of hypertrophy, and cardiac fibrosis (Figure S5 in the online-only Data Supplement).

Spectral analysis of telemetry recording was performed to investigate whether mitochondrial ROS influenced autonomic nerve activity. ICV mitoTEMPO (170ng/kg per minute) was able to normalize the Ang II–perturbed low frequency (systolic blood pressure) and spontaneous baroreflex gain (pulse interval) (Figure S1A and S1B in the online-only Data Supplement), as well as the elevated low frequency/high frequency ratio (Figure S1D in the online-only Data Supplement). Together, these observations demonstrate that scavenging of brain mitochondrial O$_2^•−$ inhibits sympathetic vasomotor drive, normalizes vasovagal balance, and attenuates high BP and cardiac hypertrophy induced by chronic Ang II infusion.

Our next objective was to determine effects of ICV mitoTEMPO on PVN microglial activation by determining the increase in microglial numbers and changes in their size and shape, which are the main indicators of microglial activation.28 Ang II caused a 180% increase in the number of microglia and a 1.5-fold increase in CD11b mRNA (Figure 4B and 4C). Furthermore, the microglia appeared enlarged and their shape changed after Ang II infusion (Figure 4A). Additionally, 1.6-fold and 2.3-fold increases in interleukin-1β and tumor necrosis factorα mRNA levels were observed, respectively.

Figure 2. Cellular and mitochondrial superoxide scavenging by mitochondrial-targeted antioxidant (mitoTEMPO) in angiotensin II (Ang II)-treated cultured neuron. A, Cellular superoxide stained by dehydroethidium. Relative fluorescence unit was detected by microplate reader. *P<0.05 vs control, #P<0.05 vs Ang II. B, Representative images of neurons. mitoSOX (red) staining is used to detect mitochondrial superoxide and mitoTracker (green) is used for mitochondrial localization. Scale bars=50 μmol.
Finally, the effects of ICV mitoTEMPO on BM EPCs and ICs were studied to confirm a brain-BM communication in this neurogenic model of hypertension. Ang II–induced decrease in the number of BM EPCs was completely restored by ICV mitoTEMPO treatment (Figure 5A). In addition, BM ICs were normalized by ICV mitoTEMPO (Figure 5B). As a result, there was a 5-fold decrease in the EPC/IC ratio in the BM of Ang II–treated animals, and ICV mitoTEMPO normalized this imbalance (Figure 5C). In contrast, subcutaneous infusion of mitoTEMPO failed to normalize the decrease in BM EPCs/ICs ratio.

**BM-PVN Connection Traced by PRV-GFP**

Retrograde tracing with a replication-competent strain of pseudorabies virus containing green fluorescent protein (GFP) was carried out to confirm brain-BM connections. Microinjection of pseudorabies virus-GFP in the femur BM resulted in GFP-positive neurons in the PVN, predominantly. However, modest GFP fluorescence was also observed in the nucleus tractus solitarius, the rostral ventrolateral medulla, and subfornical organ (Figure 5D). This retrograde labeling was specific to BM because a similar microinjection of pseudorabies virus–GFP in the adjacent skeletal muscle did not show any GFP in the PVN. Furthermore, severing sciatic and femoral nerves completely attenuated BM-PVN retrograde transport of this virus.

**Discussion**

The present study for the first time indicates the functional interactions between the cardiovascular relevant brain regions, particularly the PVN and the BM. We have shown that Ang II–induced hypertension, which exhibits a neurogenic component, is associated with a decrease in the EPC/IC ratio. This may compromise the ability of EPCs to repair vasculature and perpetuate hypertension-linked vascular pathophysiology. Retrograde labeling of the neurons in the cardio regulatory brain regions in general and the PVN in particular after pseudorabies virus-GFP administration into the BM further supports a neural-BM connection, albeit an anatomical one. The functional interaction between the brain and BM is, however, implied by specific inhibition of the brain mitochondrial ROS, which apparently restores the imbalance of the EPC/IC ratio within the BM to control levels, as well as attenuates the Ang II–induced hypertension. To confirm that this effect is specific, we used another vasoconstrictor, phenylephrine, to increase blood pressure without the central nervous system involvement, because phenylephrine is not known to cross the blood-brain barrier. For the most part, we found no significant effect of chronic phenylephrine infusion on the BM EPCs and ICs or their ratio. Therefore, we conclude that the effect we see on the EPC/IC ratio in the BM is not the consequence of the hypertension, per se, but it may directly be linked to the elevated sympathetic drive caused by brain Ang II–dependent mitochondrial ROS. We would predict that this brain-mediated dysfunction of the BM activity would be inhibited by blocking the sympathetic drive to the BM. However, this would be difficult to demonstrate at present because it would require either a specific nerve ablation or a complete sympathectomy, both of which may be too severe for the timeline in the present study. We have, however, recently been able to isolate and record activity from a specific nerve entering the rat femur via the nutrient foramen, which has characteristics of a sympathetic nerve (unpublished preliminary data). Our future studies will attempt to isolate and characterize the sympathetic activity of
this nerve in hypertensive rats to relate it to the BM EPC/IC activity.

We have demonstrated that ICV but not subcutaneous infusion of mitoTEMPO attenuates hypertension by modulating the sympathetic vasomotor drive and cardiac baroreflex gain. Furthermore, mitoTEMPO treatment prevented the development of Ang II–induced cardiac hypertrophy, presumably through an indirect effect on attenuation of the blood pressure increase. It is also possible that the mitoTEMPO-induced prevention of cardiac hypertrophy may have been a result of a direct effect of lowering of the cardiac sympathetic drive; however, because we did not measure the sympathetic drive to the heart, we are unable to make this conclusion at the present time. However, Dikalova et al.29 observed an attenuation of hypertension even by subcutaneous administration of mitoTEMPO in mice, which is in contrast to our present results. This discrepancy may likely be attributed to an increased accessibility of mitoTEMPO in the brains of mice, as a result of the previously reported altered permeability of the blood-brain barrier in mice subjected to twice the concentration of Ang II compared with the one used in the rats in the present study.30 This is particularly relevant in view of evidence that autonomic regions of the brain are highly vascularized.31 However, we cannot at this point rule out the possibility of functional differences between the ICV and subcutaneous mitoTEMPO delivery, because the higher levels of subcutaneous mitoTEMPO showed some albeit insignificant response, which could have resulted from the mitoTEMPO effect on the peripheral tissue mitochondria (eg, smooth muscle of the blood vessels). Additional differences in other humoral responses and metabolic processing between the 2 species cannot be ruled out at the present time.

Dysfunctional/decreased EPCs and increased ICs in hypertension and cardiovascular disease are well established based on both clinical and animal studies.32–34 Harrison’s group was among the first to demonstrate the role of T cells and adaptive IS in the pathogenesis of hypertension.34 Our present study appears to support the role of the CD4+ T-cell inflammatory lymphocytes in Ang II–induced hypertension. As it has been reported, the activation of a subset of the proinflammatory CD4+ cells by Ang II is directly dependent on the activation of the third ventricle regions of the brain, because targeted lesions of this brain area prevented the Ang II–induced CD4+ activation.34 Therefore, the ANS is implicated in the activation of the immune response in hypertension. This is in line with our present observations, because ICV infusion of mitoTEMPO inhibits Ang II–induced sympathetic drive, as well as normalizes the CD4+ levels. On the other hand, the CD4+CD25+ regulatory T cells (T Regs) are able to suppress innate and adaptive immune responses by suppressing proinflammatory actions of other T lymphocyte subtypes, as well as those of macrophages.35 Thus, the increase in the CD4+CD25+ T Regs that we observed in the present study may be compensatory to the elevation in other ICs, as the system seeks to restore its balance. Furthermore, we observed elevation in the CD8+ cells, which have been implicated in the process of killing of adjacent cells, as well as in the release of
proinflammatory cytokines and the activation of macrophages, thereby contributing to the Ang II–dependent hypertensive pathophysiology. Similarly, the Ang II–dependent increase in the CD3+CD45- inflammatory leukocytes has previously been shown in hypertension, as these cells infiltrate the vasculature and damage the endothelium. Accumulation of inflammatory leukocytes in the nucleus tractus solitarius of the spontaneously hypertensive rat apparently contributes to the dysfunctional baroreflex processing. Similarly, activation of macrophages (CD68+ cells) contributes to Ang II–dependent vascular dysfunction, hypertension, and oxidative stress. Peripheral circulating macrophages may also infiltrate specific brain regions and contribute to the central oxidative stress by releasing ROS and proinflammatory cytokines, which could lead to modulation of the sympathetic drive. It is possible that the activation of ICs and the subsequent ROS and the proinflammatory cytokine release may directly influence the BM EPCs in Ang II–dependent hypertension. However, future studies are needed to adequately address this issue.

The novelty of the current study is that it provides evidence of the existence of a brain-BM axis for which the dysfunction seems to be apparent in the pathogenesis of neurogenic hypertension in our model. The question of whether the deleterious effects result from ICs restricted to the peripheral system or the ability of these cells to infiltrate into the central nervous system and participate in neuroinflammation remains to be answered. However, based on recent evidence, it is tempting to suggest that both extravasation of ICs and their progenitors into the PVN and their differentiation into microglia together with resident microglial activation are critical in neural dysregulation in hypertension. Thus, the feed-forward loop between the brain and BM, in addition to local activation of microglia and generation of mitochondrial ROS, may contribute to this pathophysiology. We postulate that Ang II infusion activates resident microglia in the PVN and induces production of mitochondrial ROS initiating a cascade of signaling events involving cytokines/chemokines leading to impaired BM activity. Extravasation of BM-derived IC progenitors into the PVN perpetuates these deleterious effects. However, alternate sources of ROS and targets of Ang II cannot be ruled out at the present time in view of observations that Ang II also stimulates neuronal ROS. Further studies on Ang II–mediated interactions between microglia and neurons are needed to establish the precise mechanism.

**Perspectives**

In this study, we present evidence for the existence of a brain-BM axis, the dysfunction of which may perpetuate the vascular pathophysiology in hypertension. We present the hypothesis that the central activation of angiotensinergic pathways...
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Disclosures

None.

References


**What Is New?**

- The hypothesis of a dysfunctional brain-bone marrow communication in hypertension is proposed.
- Mitochondrial oxidative stress in the brain autonomic areas is responsible for angiotensin II hypertension.
- An imbalance between the vascular reparative cells (endothelial progenitor cells) and the damaging inflammatory cells is associated with increased sympathetic drive and hypertension.

**What Is Relevant?**

- First evidence that a dysfunctional brain-bone marrow communication may be responsible for the vascular pathophysiology of hypertension.
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Brain-mediated Dysregulation of the Bone Marrow Activity in Angiotensin II-induced Hypertension

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Short title: Brain-bone marrow communication in hypertension

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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 O$_2^{-}$ was measured by DHE (dihydroethidium, Invitrogen) fluorescent staining, and mitochondrial 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/O_2_ 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™ 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, Perpcy5.5 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 10¹⁰ 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