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Alterations in the subcellular distribution of NADPH oxidase p47^{phox} in hypothalamic paraventricular neurons following slow pressor Angiotensin II hypertension in female mice with accelerated ovarian failure

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Abstract

At younger ages, women have a lower risk for hypertension than men, but this sexual dimorphism declines with the onset of menopause. These differences are paralleled in rodents following “slow-pressor” angiotensin II (AngII) administration: young male and aged female mice, but not young females, develop hypertension. There is also an established sexual dimorphism both in the cardiovascular response to the neurohypophyseal hormone arginine vasopressin (AVP) and in the expression of oxidative stress. We examined the relationship between AngII-mediated hypertension and the cellular distribution of the superoxide generating NADPH oxidase (NOX) in AVP-expressing hypothalamic paraventricular nucleus (PVN) neurons in “menopausal” female mice. Dual labeling immunoelectron microscopy was used to determine if the subcellular distribution of the organizer/adaptor NOX p47^{phox} subunit is altered in PVN dendrites following AngII administered (14 days) during the “postmenopausal” stage of accelerated ovarian failure (AOF) in young female mice treated with 4-vinylcyclohexene diepoxide. Slow-pressor AngII elevated blood pressure in AOF females and induced a significant increase in near plasmalemmal p47^{phox} and a decrease in cytoplasmic p47^{phox} in PVN AVP dendrites. These changes are opposite to those observed in AngII-induced hypertensive male mice (Coleman et al., *J. Neuroscience* 33: 4308–16, 2013), and may be ascribed in part to baseline differences between young females and males in the near plasmalemmal p47^{phox} on AVP dendrites seen in the present

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study. These findings highlight fundamental differences in the neural substrates of oxidative stress in the PVN associated with AngII hypertension in postmenopausal females compared with males.

Keywords

menopause; 4-vinylcyclohexene diepoxide; arginine-vasopressin antibody (catalogue number T-5048, RRID: AB_2313978); electron microscopy; p47^{phox} NADPH oxidase subunit antibody (catalogue number sc-7660, RRID: AB_2298320); Graphpad Prism (<http://www.graphpad.com/scientific-software/prism/> RRID: rid_000081)

INTRODUCTION

After menopause, hypertension and cardiovascular disease risk increase in women (Abramson and Melvin, 2014; Cheng et al., 2012; Maric-Bilkan et al., 2014; Martins et al., 2001). Similarly, in rodent models of hypertension, such as slow-pressor angiotensin II (AngII) infusion, a comparable sexual dimorphism in blood pressure in mice is observed; young males and aged females, but not young females become hypertensive (Marques-Lopes et al., 2014; Xue et al., 2005). However, the mechanisms that underlie the sexually dimorphic susceptibility to hypertension are not well understood.

Systemic administration of low doses of AngII does not produce a rapid pressor response, but results in a slow-developing increase in blood pressure (“slow pressor” AngII hypertension) (Kawada et al., 2002; Reckelhoff and Romero, 2003; Zimmerman et al., 2004). The paraventricular nucleus of the hypothalamus (PVN) is crucial to slow-onset AngII-induced hypertension (for review (Braga et al., 2011)). A significant sexual dimorphism is found in neuroendocrine systems implicated in blood pressure regulation. In particular, arginine vasopressin (AVP), a neurohypophyseal hormone important for cardiovascular function, is known to be associated with differing effects on blood pressure in males and females (Share and Crofton, 1993).

Hypertension linked to AngII is strongly associated with superoxide production by the canonical Nox2 expressing NADPH oxidase (NOX2) in the PVN (Coleman et al., 2013; Wang et al., 2013; Zimmerman et al., 2004). The Nox2 expressing NOX is composed of membrane-associated subunits (p22^{phox} and gp91^{phox}) and cytoplasmic (p40^{phox}, p67^{phox}, and p47^{phox}) subunits (Bedard and Krause, 2007). The NOX2 isoform requires mobilization of cytoplasmic p47^{phox} to dock with the membrane bound proteins for superoxide production (Brandes et al., 2014). Originally characterized as a specialized enzymatic source of phagocytic superoxide production within neutrophils (for review see (Babior, 1999)), NOX2 is now known to be expressed within multiple cell types (Jackson et al., 2004; Lassegue et al., 2012; Li and Shah, 2002), and is found in brain vascular cells, glia, and neurons (Nayernia et al., 2014). At the subcellular level, NOX2 is known to be active in diverse subcellular organelles (Li and Shah, 2002; Souabni et al., 2014; Zhang et al., 2008).

AngII-induced reactive oxygen species (ROS) production has been demonstrated in neurons implicated in cardiovascular regulation (Wang et al 2008; Xue et al., 2008; Zimmerman et al., 2004). In particular, PVN neurons containing the AngII type 1 receptor (Marques-Lopes

et al., 2015; Wang et al., 2008) or projecting to the spinal cord (Marques-Lopes et al., 2014) show an increase in ROS production in response to AngII in males. Moreover, in males, slow-pressor AngII hypertension results in a repartitioning of p47^{phox} to the plasmalemma of PVN neurons that do not express AVP, along with a concomitant increase in ROS production in these neurons (Coleman et al., 2013). In males, slow-pressor AngII hypertension also results in increased AVP production, stimulation of the posterior pituitary to increase plasma levels of AVP, and excitation of projections to the rostral ventrolateral medulla (Capone et al., 2012; Ferguson and Latchford, 2000; Kawano and Masuko, 2010; Veltmar et al., 1992). In male mice, despite AngII-induced activation of AVP-containing PVN neurons, slow-pressor AngII does not result in increased NOX2-dependent ROS production in these neurons, and, consistent with this, is associated with decreased plasmalemmal p47^{phox} (Coleman et al., 2013).

Compared to males, young adult female rodents show lower ROS production in neural cardiovascular regulatory circuits (Marques-Lopes et al., 2014; Marques-Lopes et al., 2015; Pierce et al., 2009). Moreover, estradiol administration to ovariectomized females improves redox balance by reducing NOX2 activity and inhibiting ROS production (Campos et al., 2014; Xue et al., 2008). However, it is not known whether the subcellular distribution of p47^{phox} in AVP and non-AVP neurons in the PVN is altered in “postmenopausal” mice following slow-pressor AngII-induced hypertension. This study used dual label quantitative electron microscopic dual immunolabeling immunoelectron microscopy to assess this possibility using a recently established mouse model of accelerated-ovarian failure (AOF) that uniquely recapitulates hormonal changes seen in human menopause (for reviews (Van Kempen et al., 2011; Van Kempen et al., 2014)). Light and electron microscopic immunolabeling also was used to determine if there is a baseline sexual dimorphism in the subcellular distribution of p47^{phox} in AVP and non-AVP PVN neurons.

METHODS

Animals

Experiments were approved by the Weill Cornell Medicine Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Adult female and male C57BL/6 mice (N = 12, 2–3 month old) were obtained from Jackson Laboratory (Bar Harbor, Maine). Female mice used for AOF studies (N = 15) were bred in the Weill Cornell Medical College facilities from C57BL/6 mice originally obtained from Jackson laboratories. All mice were kept on a 12:12 light/dark cycle (lights on at 6:00 AM) and housed in groups of 3–4 with *ad libitum* access to food and water. Estrous cycle stage was determined using vaginal smear cytology (Byers et al., 2012). In studies comparing young adult female and male mice, females were cycled for 10 days prior to perfusion-fixation. Males were comparably handled for 10 days prior to perfusion-fixation.

AOF induction

The validity, predictability, and reproducibility of a menopause-like state with AOF has been demonstrated in previous studies (for reviews see (Van Kempen et al., 2011; Van Kempen et

al., 2014). Low doses of 4-vinylcyclohexene diepoxide (VCD) selectively eliminate primary follicles in the ovary and, following ovarian failure, result in undetectable levels of estradiol, decreased levels of progesterone, increased levels of luteinizing and follicle stimulating hormones and androstenedione (Lohff et al., 2005; Mayer et al., 2004). Several studies have demonstrated that VCD administration does not negatively affect peripheral tissues, including organ weights and liver and kidney function (Haas et al., 2007; Mayer et al., 2005; Sahambi et al., 2008; Wright et al., 2008). Recently, we have shown that VCD does not have any direct effects on brain areas inside (i.e., hippocampus, PVN, nucleus of the solitary tract) or outside (i.e., area postrema, subfornical organ) the blood brain barrier (Van Kempen et al., 2014).

For AOF induction, postnatal day 55 intact female mice were injected intraperitoneally (i.p.) with vehicle (N = 3; VEH: 0.5% DMSO in sesame oil) or VCD (N = 12; 130 mg/kg; Sigma-Aldrich, St. Louis, MO) sequentially over 5 days for 3 weeks, as previously described (Van Kempen et al., 2014). Regular estrous cyclicity of the mice was verified by taking vaginal smears during the first 5 injection days and observing at least one day of proestrus and a normal sequence of stages [i.e., proestrus, 1–2 days estrus, 2–3 days diestrus I/II; (Byers et al., 2012; Goldman et al., 2007)]. The same investigator (TAVK) made all of the injections and blood pressure measurements (see below), as well as all of the cage changes during those times. Mice were weighed weekly. Based on the literature and our experience with the AOF model, mice receiving VCD were considered “post-menopausal” 127 days after the first VCD injection (Lohff et al., 2005; Van Kempen et al., 2014). At this time point, mice were cycled for 10 days to verify cyclicity of VEH mice and acyclicity of AOF mice (Van Kempen et al., 2014). Only mice that were appropriately cyclic (young females) or acyclic (Post-menopausal mice) were implanted with mini-osmotic pumps.

Slow pressor AngII and tail cuff plethmography

As previously described (Coleman et al., 2013; Marques-Lopes et al., 2014), osmotic minipumps (Alzet, Durect Corporation, Cupertino, CA) were filled (~ 100 µl) with either AngII dissolved in vehicle (0.9% NaCl in 0.01% bovine serum albumin; BSA) or the vehicle alone. Prior to osmotic minipump preparation, mice were weighed to determine the appropriate amount of AngII (600 ng/kg/min) for each pump. Filled pumps were stored in sterile saline, and incubated in a 37°C water bath overnight prior to pump implantation. Pumps were implanted subcutaneously in anesthetized mice (isofluorane: 5% induction, 1.5–2% maintenance), and remained in place for the duration of the experiment (14 days).

Systolic blood pressure (SBP) was measured in awake mice using tail-cuff plethysmography (Model MC4000, Hatteras Instruments, Cary, NC) as previously described (Coleman et al., 2010). Tail-cuff plethysmography is a reliable non-invasive measurement for comparing SBP between groups (Capone et al., 2010; Coleman et al., 2010; Coleman et al., 2013; Marques-Lopes et al., 2014; Wang et al., 2013). Unlike radiotelemetric recordings (Butz and Davisson, 2001), tail-cuff phethysmography does not compromise the carotid artery, and is particularly suited to the brain perfusion required for electron microscopy-level tissue fixation (Milner et al., 2011). The final SBP measurement was taken 12 days following the

osmotic minipumps implantations. Mice were euthanized 14 days after minipump implantations.

Antibody Characterization

Please see Table 1 for a list of all antibodies used.

A goat polyclonal antiserum raised against a peptide mapped to the C terminus of human p47^{phox} (Cat. # sc-7660; RRID: AB_2298320) was obtained from Santa Cruz Biotechnology (Table 1). On western blots, this affinity purified antibody specifically recognizes a band ~47 kDa in lysate from HEK293T cells transfected with the p47^{phox} gene (Santa Cruz Biotechnology). In acrolein/paraformaldehyde fixed PVN sections from p47^{phox} (−/−) knock-out mice, no immunolabeling using this antibody is detected (Coleman et al., 2013).

The guinea pig polyclonal antiserum raised against AVP (Cat # T-5048; RRID: AB_2313978) was obtained from Peninsula Laboratories (Table 1). By radioimmunoassay, this antiserum recognizes vasopressin, has less than 1% cross-reaction with other vasopressin derivatives, and does not recognize oxytocin (Peninsula Laboratories Technical Services). No immunolabeling is seen with this antibody in Brattleboro rats, which do not express vasopressin (Drouyer et al., 2010).

Light microscopic immunocytochemistry

We used light microscopic immunocytochemistry to determine if there were baseline sex differences in the distribution or size of AVP neurons in the PVN. For this, adult diestrus female and male mice (N=3/group) were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused intracardially with ~5 ml saline (0.9%) containing 2% heparin followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB, pH 7.4). Following removal from the skull, the brain was placed overnight in the fixative, sectioned coronally (40 µm thick) on a Vibratome (VT 1000S, Leica, Buffalo Grove, IL) and collected in PB. Sections were stored in cryoprotectant (30% sucrose, 30% ethylene glycol in PB) at −20°C until immunocytochemical processing (Milner et al., 2011).

For each animal, every other section extending through the rostrocaudal extent of the PVN [+0.5 mm to −1.1 mm from bregma (Hof et al., 2000)] was selected. Sections were then punch coded and pairs of female and male mice were pooled into single containers, ensuring identical exposure to the antisera (Milner et al., 2011; Pierce et al., 1999). Sections were then processed for light microscopic immunoperoxidase localization of AVP using previously described methods (Milner et al., 2011). Sections were rinsed in 0.1 M Tris-saline (TS; pH7.6) and then incubated for 30 min in 0.5% bovine serum albumin (BSA) in TS. Sections then were placed in guinea pig anti-AVP antiserum (1:4000) in BSA/TS for one day at room temperature and 1 day at 4°C. Sections then were incubated in goat anti-guinea pig biotinylated IgG (1:400; Jackson Immunoresearch Laboratories, West Grove, PA) for 30 min followed by a 30 min incubation in avidin-biotin complex (ABC; 1:100 dilution; Vectastain Elite Kit, Vector Laboratories, Burlingame, CA) in TS. Sections were developed in 3,3'-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis MO) and H₂O₂ in TS. All antibody incubations were performed in 0.1% BSA/TS and separated by washes in TS.

Sections were mounted on gelatin-coated slides and dehydrated through an ascending series of alcohol to xylene. The slides were coverslipped with DPX (Sigma-Aldrich). Sections were analyzed under a Nikon Microphot light microscope. The area and minimum diameter of AVP-labeled neurons in the PVN was compared between females and males. Investigators blinded to experimental condition performed all analyses. One section through the middle of the PVN ($\sim$ Bregma -0.90) from each animal was photographed. The area and minimum diameter of 20 randomly selected neurons per animal were then measured using imageJ software.

Dual label immunocytochemistry for electron microscopy

Dual labeling immunocytochemical studies are a powerful tool for determining relative changes in the subcellular distribution of proteins in dendrites following experimental manipulations (Milner et al., 2011). Two studies dually labeling p47^{phox} and AVP in PVN sections were performed. The first study determined the effect of slow pressor AngII hypertension on the subcellular distribution of p47^{phox} in the PVN of AOF post-menopausal mice. For comparison, young vehicle-treated female mice, which are known not to become hypertensive following slow pressor AngII (Marques-Lopes et al., 2014)(Marques-Lopes et al., 2015 submitted), also were included in this study. The second study compared intact adult diestrus female and male mice ($N = 3/\text{group}$) to determine if there were baseline differences in subcellular distribution of p47^{phox} in the PVN. Vehicle injected mice (study 1) and young female mice (study 2) were all in diestrus at the conclusion of the study.

For both studies, mice were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and perfused intracardially with ~ 5 ml saline (0.9%) containing 2% heparin followed by 3.75% acrolein in 2% paraformaldehyde in PB (Milner et al., 2011). To maintain consistency between both studies, the same investigator (TAM) perfused all mice. The whole brain was post-fixed for 30 min in 1.85% acrolein and 2% paraformaldehyde in PB before being sectioned and then stored in cryoprotectant at 0°C as described above. For each animal, 2–3 PVN sections (-0.70 to -0.90 mm from Bregma; (Hof et al., 2000)) were rinsed in PB and coded with hole-punches. Tissue from experimental groups for each study were then pooled into single crucibles and processed in tandem for all of the immunocytochemical procedures.

Sections were processed for dual immunolabeling by a pre-embedding protocol as previously described (Milner et al., 2011). Sections were first incubated in 1% sodium borohydride for 30 min to remove free aldehyde sites, and then rinsed in PB (Milner et al., 2011). Tissue sections were then “freeze-thawed” to enhance tissue penetration of the antibodies as previously described (Coleman et al., 2013). Sections were next rinsed in PB followed by a rinse in TS prior to a 30 min incubation in 0.5% BSA in TS. Sections were rinsed in TS and then incubated in a cocktail of guinea pig anti-vasopressin (1:4000) and goat anti-p47^{phox} (1:50) antisera for 24 hours at room temperature and 24 hours at 4°C . Sections then were processed for immunoperoxidase localization of AVP as described for light microscopic immunocytochemistry.

Following the DAB reaction, sections were then processed for immunogold labeling of p47^{phox} as previously described (Coleman et al., 2013). For this, sections were rinsed in 0.01M PB with 0.001% gelatin and 0.08% BSA and then incubated overnight at 4°C in

donkey anti-goat IgG conjugated to one nm colloidal gold particles (1:50; Electron Microscopy Sciences, Fort Washington, PA; EMS) diluted in the same solution. The nanogold-conjugated IgG was fixed by incubating sections in 2% glutaraldehyde, and the gold particles were enhanced using a Silver IntenSE M kit (RPN491; GE Healthcare, Waukegan, WI) for 7 minutes (Milner et al., 2011).

Sections were post-fixed in 2% osmium tetroxide for one hr, dehydrated, and flat embedded in Embed-812 (EMS) between two sheets of Aclar plastic (Milner et al., 2011). Ultrathin sections (70 nm thick) through the PVN were cut with a diamond knife (EMS) on a Leica EM UC6 ultratome, and collected on 400-mesh, thin-bar copper grids (EMS). Grids were then counterstained with uranyl acetate and Reynold's lead citrate (Milner et al., 2011).

Ultrastructural data analysis

An investigator blinded to animal condition collected and analyzed all the electron microscopic (EM) data on a CM10 electron microscope. Sections were examined first at low magnification (3400x) to select fields having good morphological preservation and the presence of immunolabeling. From these fields, images were randomly photographed at 13,500x. These images were obtained exclusively at the plastic-tissue interface to ensure even antibody tissue penetration (Milner et al., 2011). Profiles were identified by defined morphological criteria (Peters et al., 1991). Dendritic profiles contained regular arrays of microtubules and were generally found post-synaptic to axon terminals. Immunoperoxidase labeling for AVP was evident as a characteristic, electron-dense peroxidase reaction product precipitate. Silver-intensified immunogold (SIG) labeling for p47^{phox} is visible as black, electron-dense particles. Profiles were considered dual labeled if they contained immunoperoxidase and at least one SIG particle, and single labeled if they only contained at least one SIG particle, but there was no evidence of immunoperoxidase labeling.

Single and dually labeled dendrites in the PVN (n = 50 for each type of dendrite per mouse) were collected from 3 animals per experimental condition using established methods (Milner et al., 2011; Sesack et al., 2006). Dendrite size can be related to relative distance from the cell body, and distal (small) and proximal (large) dendrites may receive different synaptic inputs (Froemke et al., 2005; Lovett-Barron et al., 2014). Thus, dendrites were classified as small (average diameter $\leq 1 \mu\text{m}$) or large (average diameter $> 1 \mu\text{m}$). Subcellular localization of p47^{phox} SIG particles was defined as either plasmalemmal (onPM), near plasmalemmal (nrPM; within 70 nm, but not touching the plasma membrane; referred to as "endomembranes near the plasma membrane" by Coleman et al. 2013), or cytoplasmic (Cy; (Coleman et al., 2013; Pierce et al., 2009). Morphometry of single- and double-labeled dendrites was performed using Microcomputer Imaging Device software (MCID, Imaging Research Inc., Ontario, Canada) to determine the form factor, perimeter (i.e., plasma membrane), area, average diameter, and minor axis length (Coleman et al., 2013). Dendrites were included in the analysis only if the plasma membrane was visible around the entire dendrite. Dendrites with an oblong or irregular shape were indicated with a form factor lower than 0.5, and were excluded from the analysis.

The number, density, and partitioning ratio of SIG particles in each subcellular compartment and in Total (sum of SIG particles onPM, nrPM, and Cy) were compared statistically by

condition. SIG particle density accounts for differences in dendritic size. The SIG particle density for onPM and nrPM was obtained by dividing the number of SIG particles per μm of plasmalemma. The SIG particle density for Cy and total was obtained by dividing the number of SIG particles by the area (μm^2) of the dendritic profile. The partitioning ratio of SIG particles is defined as the number of SIG particles in a subcellular compartment as a proportion of Total, and indicates the relative distribution of SIG particles in each subcellular compartment. Dendritic profile cross sectional perimeter, area, and average diameter were also compared across all groups. As other papers have recently described (Coleman et al., 2013; Marques-Lopes et al., 2014), morphometric measures, such as average diameter, demonstrate substantial variation based on AngII administration and/or sex. Thus, the number, density, and partitioning ratio of SIG particles in each subcellular compartment were also statistically compared (1) in dendrites of all sizes (2) in small (average diameter $< 1.0 \mu\text{m}$) and (3) in large (average diameter $> 1.0 \mu\text{m}$) dendrites (Marques-Lopes et al., 2014).

Statistics

One-way ANOVA was used to compare the means between treatment groups (oil/saline, VCD/saline and VCD/AngII) using JMP8 software (SAS Institute, Cary, NC). Significant ANOVAs then were further analyzed with a Tukey post-hoc test. Data are expressed as mean $\pm$ SEM. Analyses were considered statistically significant if $P < 0.05$. The analysis of SBP measurements was conducted by separate ANOVAs on day 6 and day 12 instead of a repeated measures design to avoid the potential confounding effect of variability in day-to-day conditions that could affect blood pressure measurements. Although this analysis does not allow us to make statistical comparisons across days, it does allow for post-hoc analyses to be run between treatment groups.

For analyzing sex differences in size in which there were only 2 groups (females and males) student's t-tests followed by a Holm correction (Holm 1979) was used. For analyzing sex differences in $p47^{\text{phox}}$ distribution, which was our pre-planned analysis variable, we performed a Mann-Whitney test on the near plasmalemmal densities, as this data set did not follow a normal distribution (determined by D'Agostino-Pearson normality test).

Cytoplasmic densities and total densities were analyzed by student's t-tests followed by a Holm correction. The difference in location was expected as previously observed (Coleman et al., 2013). Analyses were considered statistically significant if $P \leq 0.05$.

Figure preparation

Microsoft PowerPoint 2010 was used to crop images and make final adjustments to brightness, contrast, and sharpness. Graphs were generated using Prism 6 (<http://www.graphpad.com/scientific-software/prism/> RRID: rid_000081; GraphPad Software, La Jolla, CA).

RESULTS

Slow-pressor AngII administration increases blood pressure in post-menopausal AOF mice

Prior to implanting osmotic mini-pumps, there was no significant difference in baseline blood pressure of VEH and VCD-injected mice ($P > 0.05$; Fig. 1A). On day 6 and 12 after osmotic mini-pump implantation, analysis with one-way ANOVA showed a significant effect of AngII administration [Day 6: $F(2,330) = 67.23$, $P < 0.0001$; Day 12: $F(2,330) = 45.60$, $P < 0.0001$; Fig. 1B]. Post-hoc analysis demonstrated significantly higher blood pressure in the VCD group receiving AngII relative to either saline group on day 6 ($P < 0.001$) and day 12 ($P < 0.001$), but no significant differences ($P > 0.05$) between VCD and VEH injected groups receiving saline.

Slow pressor AngII in AOF females alters subcellular distribution of p47^{phox} in AVP dendrites of the PVN

In VEH and VCD-injected females, the light microscopic distribution of AVP-immunoreactive neurons throughout the rostrocaudal extent of the PVN was similar to previous studies (Biag et al., 2012). A dense cluster of AVP-labeled neurons was located in the lateral zone of the posterior magnocellular region of the PVN (Fig. 2). In addition, a smaller grouping of AVP-labeled neurons was present in the medial zone of the posterior magnocellular region of the PVN. Scattered AVP-labeled neurons also were observed in the remainder of the PVN. EM samples were taken through all subregions of the PVN.

In both AOF hypertensive and VEH-injected female controls, immunoelectron microscopic analysis revealed that p47^{phox} SIGs were observed in cellular profiles with and without AVP immunoreactivity, including dendrites, as well as axon terminals and glia. In all groups, numerous dendritic profiles of PVN neurons showed dual immunoperoxidase and immunogold-silver labeling for AVP and p47^{phox}, respectively (Fig. 3). In dendrites, AVP immunoperoxidase labeling was diffusely distributed throughout the cytoplasm, whereas p47^{phox} SIG particles were associated with discrete subcellular compartments.

p47^{phox} in non-AVP dendrites: morphology and subcellular distribution—

Analysis with one-way ANOVA demonstrated no significant ($P > 0.05$) main effect of group (VEH/saline, VCD/saline, VCD/AngII) on morphometric measures (area, perimeter, average diameter) in p47^{phox} single labeled dendrites. Likewise, there was no significant main effect of group ($P > 0.05$) on the density of p47^{phox} SIG particles in total, or in any subcellular compartment (on plasma membrane, near plasma membrane, cytoplasm) in non-AVP dendrites (Fig. 4A–C). While, there was no significant main effect of group on the partitioning ratio of p47^{phox} in the non-AVP dendrites (Fig. 4A–C), there was a trend for a main effect of group on the partitioning ratio of p47^{phox} SIG particles near the plasma membrane of non-AVP dendrites [$F(2,330) = 2.91$, $P = 0.056$; Fig. 5A]. This trend may have been driven by the lower partitioning ratio in the VEH/Sal group.

p47^{phox} in AVP dendrites: morphology and subcellular distribution—In dual labeled dendritic profiles, analysis with one-way ANOVA demonstrated a significant main

effect of treatment group (VEH/saline, VCD/saline, VCD/AngII) on average diameter ($F(2,330) = 5.54$, $P = 0.0043$). Post-hoc analysis showed that the average diameter of p47^{phox}/AVP dendrites in VEH/Sal group ($0.081 \pm 0.037 \mu\text{m}$) was significantly lower ($P = 0.0038$) than the VCD/Sal group ($0.96 \pm 0.025 \mu\text{m}$), but not significantly different than the VCD/AngII group ($0.88 \pm 0.030 \mu\text{m}$). Analysis with oneway ANOVA did not demonstrate significant differences ($P > 0.05$) in the area or perimeter of dual-labeled dendritic profiles. Analysis with one-way ANOVA demonstrated a significant main effect of group (VEH/saline, VCD/saline, VCD/AngII) on the density of p47^{phox} in the near plasma membrane compartment of AVP dendrites [$F(2,330) = 6.16$, $P = 0.0024$; Fig. 4A). Post-hoc analysis showed a significantly greater density of near plasmalemmal p47^{phox} in the VCD/AngII group relative to both the VCD/Sal ($P = 0.0041$) and VEH/Sal ($P = 0.017$) groups. However, there was no main effect of group on either the cytoplasmic or total density of p47^{phox} in AVP dendrites.

The partitioning ratio of near plasma membrane [$F(2,330) = 8.05$, $P = 0.0004$] and cytoplasmic [$F(2,330) = 3.61$, $P = 0.028$] p47^{phox} SIG particles in AVP-labeled dendrites significantly differed between treatment groups (VEH/saline, VCD/saline, VCD/AngII) as analyzed by one-way ANOVA (Fig. 5). Post-hoc analysis showed a significantly greater near plasma membrane partitioning ratio on AVP dendrites in the VCD/AngII group relative to both the VCD/Sal ($P = 0.0015$) and the VEH/Sal ($p = 0.028$) groups (Fig. 5A). In contrast, in the cytoplasmic compartment, post-hoc analysis showed that VCD/AngII mice had a significantly lower p47^{phox} SIG cytoplasmic partitioning ratio in AVP-labeled dendrites than the VCD/Sal group ($P = 0.028$; Fig. 5B). The cytoplasmic partitioning ratio of p47^{phox} SIGs in AVP-labeled dendrites was not significantly different between VEH/Sal and VCD/Sal groups ($P > 0.05$),

Baseline sex differences in p47^{phox} localization in AVP containing PVN dendrites

The pattern of p47^{phox} SIG particle distribution in non-AVP and AVP dendrites in the hypertensive VCD mice was the opposite of what we observed previously in hypertensive males (Coleman et al., 2013). Thus, to assess whether the difference in dendritic p47^{phox} localization in hypertensive male and female mice was related to basal sex-dependent processes, the distribution of p47^{phox} was compared in young adult female and male mice in the absence of AngII and VCD treatment.

By light microscopy, we found that the qualitative pattern of AVP-immunoreactive neurons throughout the rostrocaudal extent of the PVN was similar in females and males. We also found that the size of AVP-labeled neurons was similar. Neither the area (females: $114.7 \pm 4.2 \mu\text{m}^2$; males: $112.9 \pm 4.1 \mu\text{m}^2$) nor the minimum diameter (females: $8.8 \pm 0.2 \mu\text{m}$; males: $8.3 \pm 0.2 \mu\text{m}$) of AVP-labeled neurons was significantly different ($P > 0.05$) in females and males.

In contrast with the light microscopic analysis, an electron microscopic visual assessment showed qualitative differences in the distribution of p47^{phox} SIG particles in AVP containing dendrites within the PVN of female and male mice (Fig. 6A,B). Quantitative analysis revealed significant differences between females and males both in the morphometry of dual labeled dendrites and in the subcellular distribution of p47^{phox} in dual labeled dendrites. The

area [$t(263) = -3.2, P = 0.0051$], perimeter [$t(263) = -2.7, P = 0.0076$], and average diameter [$t(263) = -3.1, P = 0.0051$] of dual labeled dendrites were significantly larger in females than males (Fig. 6C). Compared to males, females also had a significantly greater number of total p47^{phox} SIG particles [$t(263) = -2.7, P = 0.021$ (not shown)]. Dual labeled dendritic profiles were segregated by size into small ($\leq 1\mu\text{m}$) and large ($> 1\mu\text{m}$) categories. The density of p47^{phox} SIG particles near the plasmalemma of large [Mann-Whitney U: 2115, $P = 0.05$; Fig. 6D)] but not small ($p > 0.05$) AVP-labeled dendrites was significantly higher in females compared to males (not shown). There were no significant sex differences in the density of cytoplasmic or total p47^{phox} SIG particles in AVP dendrites (Fig. 6E).

In contrast to AVP-labeled dendrites, the morphometric measures (e.g., area, perimeter, average diameter) of non-AVP dendrites containing p47^{phox} SIG particles were not significantly different between females and males (not shown). Moreover, the number of p47^{phox} SIG particles in any of the subcellular compartments of non-AVP dendrites did not differ significantly in females and males (not shown).

DISCUSSION

We have shown that AOF-treated “postmenopausal” females respond to slow pressor AngII with an increase in blood pressure and a redistribution of p47^{phox} in AVP dendrites in the PVN (Fig. 7). We also provide new evidence for baseline sex differences in the subcellular distribution of p47^{phox} within AVP-containing dendrites (Fig. 7). These differences may contribute to the divergent effects of hypertension-associated changes in p47^{phox} partitioning in the PVN of postmenopausal females compared with hypertensive males (Coleman et al., 2013). These findings support evidence for fundamental sex differences in the hypothalamic mechanisms underlying neurohumoral and/or sympathetic regulation of pressor responses.

Slow-pressor AngII elevates blood pressure in post-menopausal AOF mice

Our results demonstrate that slow-pressor AngII induces an increase in blood pressure in post-menopausal AOF mice. The magnitude of the increase in blood pressure following slow pressor AngII administration is similar to that observed by several studies in males (Capone et al., 2012; Coleman et al., 2013; Marques-Lopes et al., 2014; Marques-Lopes et al., 2015) as well as ovariectomized females (Xue et al., 2005; 2009). However, in contrast to ovariectomy, in which all ovarian hormones are depleted, the AOF model recapitulates more closely the hormonal changes seen in human post-menopause (Van Kempen et al., 2011). In the AOF model, at the post-menopausal time-point (i.e., 127 days after the first VCD injection) serum levels of estradiol are undetectable, as observed in human menopause (Lohff et al., 2005; Mayer et al., 2004). Importantly, our studies support previous studies demonstrating that the lack of estrogens is crucial for these AngII-induced elevations in blood pressure (Xue et al., 2005; 2009).

The redistribution of p47^{phox} in AVP dendrites in hypertensive AOF females is opposite to that observed previously in hypertensive males

In the present study, we found significant differences in the subcellular distribution of p47^{phox} SIG in dendrites of AVP-containing PVN neurons in estrogen-depleted hypertensive

female mice. AngII-administered VCD mice had approximately twice as much p47^{phox} SIG near the plasma membrane of AVP-containing dendrites as their saline counterparts as well as oil-injected saline-infused female mice. AngII administration also decreased the proportion of cytoplasmic p47^{phox} SIG particles in AVP-containing dendrites in VCD mice. Together, these findings suggest that AngII-induced hypertension in AOF mice results in the redistribution of p47^{phox} towards the plasma membrane of AVP-containing neurons. These findings are in contrast to those observed previously in male mice (Coleman et al., 2013), in which AngII-induced hypertension decreased p47^{phox} SIGs on the plasma membrane in PVN AVP-containing dendrites (Coleman et al., 2013).

The sex differences in p47^{phox} redistribution in AVP-containing neurons following AngII administration are paralleled by baseline differences present in male and female mice. Under basal conditions, young females, compared to young males, had more p47^{phox} SIGs near the plasmalemma in large AVP dendrites (Fig. 7). The greater proximity of p47^{phox} to plasma membranes in these dendrites in females could facilitate their mobilization toward the plasma membrane in the AOF hypertensive mice. However, the basal sex difference in the subcellular distribution of p47^{phox} SIG particles in large AVP dendrites appears not to be influenced by hormonal state as both young and AOF non-AngII-treated females have similar distributions of p47^{phox} SIG particles in AVP-containing dendrites. This is consistent with our previous work (Pierce et al., 2009) in rat rostral ventrolateral medulla showing that the levels and subcellular distribution of p47^{phox} in tyrosine hydroxylase containing dendrites do not vary over the estrous cycle.

AVP neurons in the PVN project to the rostral ventrolateral medulla (Gomez et al., 1993; Sawchenko and Swanson, 1982) and to the posterior lobe of the pituitary where they can secrete AVP into the general circulation (Biag et al., 2012). Thus, the baseline increases in p47^{phox} near the plasma membrane of AVP dendrites in young females could prime sympathoexcitatory and/or neurohypophyseal neurons for increased oxidative stress when estrogen is depleted at postmenopause. Future studies will address this issue.

Lack of p47^{phox} redistribution in non-AVP dendrites in hypertensive AOF females

Our present findings indicate that AngII-induced hypertension in AOF females does not affect the subcellular distribution of p47^{phox} in non-AVP neurons in the PVN. This is in contrast to a prior report that p47^{phox} is increased in vesicular endomembranes near the plasma membrane in hypertensive males (Coleman et al., 2013). The difference in hypertensive male and hypertensive postmenopausal female mice also contrasts with our present finding showing a lack of baseline sex differences between males and females in the expression level or the distribution of p47^{phox} in non-AVP dendrites.

Non-AVP expressing PVN neurons are heterogeneous both hodologically and neurochemically (Benarroch, 2005; Biag et al., 2012). Previous studies have shown that some PVN neurons lacking AVP project to the spinal cord (Biag et al., 2012; Coleman et al., 2013; Gonzalez et al., 2012; Marques-Lopes et al., 2015). Moreover, numerous non-AVP neurons contain estrogen receptor β (Marques-Lopes et al., 2014) or angiotensin type 1A receptor (Marques-Lopes et al., 2015). The similar basal p47^{phox} distribution in non-AVP dendrites of in male and female mice, and the stable p47^{phox} distribution in non-AVP

dendrites of hypertensive AOF mice are consistent with our earlier findings that young adult female and male mice do not demonstrate baseline differences in ROS production in PVN neurons projecting to the spinal cord, or in neurons containing angiotensin type 1A receptors (Marques-Lopes et al., 2014; Marques-Lopes et al., 2015; Wang et al., 2008). However, it is important to note that because the non-AVP neurons comprise a mixed population of cells, potential differences in the subcellular distribution of p47^{phox} in young and menopausal females as well as between the sexes could be masked in the absence of phenotypic markers for cell-type identification.

Functional considerations

Hypertension is a known risk factor for cardiovascular disease, cerebrovascular dysfunction, and neurocognitive degenerative diseases (Faraco and Iadecola, 2013). Both the increased incidence of hypertension in women at ages associated with menopause (Cheng et al., 2012), and the relationship between vasomotor symptoms and hypertension point to the critical role of ovarian hormones in blood pressure regulation (Sadeghi et al., 2012; Thurston et al., 2012; Van Kempen et al., 2015).

In rodent models, young males show increased signs of hypertension-associated p47^{phox} expression and NOX activity compared to young females (Dantas et al., 2004), however, females show increasing blood pressure and oxidative stress during estrogen depletion (Lopez-Sepulveda et al., 2008; Yanes et al., 2011). In a rat model of polycystic ovary syndrome there is an increase in renal p47^{phox} expression and NOX activity (Yanes et al., 2011). In addition, spontaneous hypertension in ovariectomized rats is associated with increased p47^{phox} gene and protein expression (Lopez-Sepulveda et al., 2008), as well as phosphorylation and NOX activity (Ceravolo et al., 2013). Estrogen replacement has been shown to blunt NOX activity and to regulate p47^{phox} gene expression (Ceravolo et al., 2013; Sumi et al., 2003).

The altered distribution of p47^{phox} in AVP-containing PVN neurons in hypertensive menopausal mice, but not in hypertensive male mice, suggests that vasopressinergic neurons experience adaptations in oxidative signaling accompanying hypertension with ovarian failure. These results are consistent with a growing literature demonstrating numerous sex differences in the mechanisms underlying blood pressure regulation (Zimmerman and Sullivan, 2013). The ability of estrogen to counteract AngII mediated hypertension and oxidative stress (Ceravolo et al., 2013; Muehlfelder et al., 2012; Sankar et al., 2015; Xue et al., 2013), as well as to improve function in cardiovascular disease models (Arias-Loza et al., 2013) point to the potential utility of manipulating estrogen signaling in blood pressure control, at least early in the development of menopause (i.e. perimenopause). An improved understanding of sex differences, neural cardiovascular regulation, and gonadal hormones may aid in bridging the gender gap in hypertensive treatments, particularly during menopause, and improve hypertensive outcomes in women.

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The results demonstrate that in a mouse model of postmenopause, Angiotensin II-mediated slow-pressor hypertension alters the subcellular distribution of the NADPH oxidase p47^{phox} subunit in vasopressinergic hypothalamic neurons. These findings provide novel evidence for fundamental differences in the hypothalamic mechanisms driving Angiotensin II-mediated hypertension post-menopausal females and males.

Fig. 1. Slow-pressor AngII increases systemic blood pressure in post-menopausal AOF females
 Prior to pump implantation (A) systolic blood pressure (SBP) did not differ between vehicle (VEH) and 4-vinylcyclohexene diepoxide (VCD) treated mice. Compared to saline (Sal), slow-pressor AngII administration significantly increased SBP on days 6 and 12 (B) after pump implantation in VCD treated mice. N = 3/group * P < 0.05

Fig. 2. Distribution of AVP neurons in the PVN of young female mice

Light micrograph shows representative AVP labeling in the PVN of a young female mouse. A dense cluster of AVP-labeled neurons is found in the lateral zone of the posterior magnocellular region (pml). A smaller cluster of AVP neurons is found in the posterior medial region (pmm). Scattered AVP-labeled neurons are found in the remainder of the PVN. Bar: 100 μ m.

Fig. 3. AOF mice infused with AngII show qualitative differences with saline infused mice with respect to p47^{phox} localization in non-AVP and AVP dendrites

Representative electron micrographs show p47^{phox} localization in vehicle (VEH) injected saline (SAL) treated mice (A, B), in VCD/ saline treated mice (C, D) and in VCD/AngII treated mice (E, F). In non-AVP dendrites, p47^{phox} SIG particles are detected on the plasma membrane (chevron) in VEH/ saline (A) and VCD/ saline (C) treated mice, and near the plasma membrane (arrowhead) in VCD/AngII treated mice (E). Arrows: p47^{phox} SIG particles in the cytoplasm. In AVP-labeled dendrites, p47^{phox} SIG particles are in the

cytoplasm (arrows) in VEH/ saline (**B**) and VCD/ saline injected mice (**D**). p47^{phox} SIG particles are found near the plasma membrane (arrowhead) and in the cytoplasm of an AVP-labeled dendrite from a VCD/AngII treated mouse (**F**). Bar: 500 nm

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Fig. 4. Slow pressor AngII administration in AOF females alters subcellular densities of p47^{phox} in PVN dendrites
(A) The density of p47^{phox} near the plasma membrane in AVP dendritic profiles of VCD/AngII treated mice is increased compared to VCD/saline and VEH/saline administered mice.
(B, C) There are no differences in either the densities of cytoplasmic **(B)** or total **(C)** p47^{phox} SIG particles in either the non-AVP or AVP dendrites across groups (VEH/saline, VCD/saline, VCD/AngII). Grey areas of the graph indicate densities in dendrites containing AVP labeling. N = 3 mice/group; N = 50 dendrites each phenotype/mouse; * P < 0.05

Fig. 5. Slow pressor AngII administration in AOF females alters the partitioning ratio of p47^{phox} in PVN dendrites

The partitioning ratio is shown as the number of SIG particles near the plasma membrane (A), or in the cytoplasm (B) relative to the total number of particles. The ratio of p47^{phox} SIG particles near the plasma membrane and in the cytoplasm is differentially affected by AngII administration in non-AVP and AVP-containing dendrites. There are increases in both the ratio of near plasma membrane p47^{phox} in AVP-labeled dendritic profiles of VCD/AngII mice compared to VEH/saline mice. A concomitant decrease was seen in cytoplasmic

p47^{phox} SIGs in AVP dendrites from VCD/AngII treated animals when compared to VEH/saline and VCD/saline (B). Grey areas of the graph indicate densities in dendrites containing AVP labeling. N = 3 mice/group; N = 50 dendrites each phenotype/mouse; * P < 0.05; †: P = 0.056

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Fig. 6. Baseline sex differences in the size of dendrites and levels of p47^{phox} in dual labeled PVN neurons

Representative electron micrographs illustrate the distribution of p47^{phox} SIG particles in AVP dendrites (p47^{phox} + AVP-D) of PVN neurons in female (A) and male (B) mice. Chevron: SIG on plasma membrane; arrow: SIG in cytoplasm. Bar: 500 nm. (C) Dual p47^{phox}/AVP-labeled dendrites had a significantly larger area, perimeter and average diameter in females compared to males. (D) The density of p47^{phox} SIG particles near the plasma membrane of large AVP dendrites was significantly lower (* P < 0.05) in females compared to males. (E) The density of p47^{phox} SIG particles in the cytoplasm and in total in

AVP dendrites was not significantly different between females and males. N = 3 mice/group;
N = 50 dendrites each phenotype/mouse

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Fig. 7. Summary of subcellular distribution of p47^{phox} SIGs in PVN dendrites at baseline and following AngII induced hypertension in females and males
At baseline, young females have a greater density of p47^{phox} SIG near the plasma membrane of AVP-containing dendrites than males. Compared to young females, AOF females have increased plasmalemmal p47^{phox} SIG particles on non-AVP dendrites. Following slow pressor AngII, both AOF females and males have elevated blood pressure. In postmenopausal females, AngII does not alter the distribution of p47^{phox} SIG in non-AVP dendrites; however, AngII increases the near plasmalemmal p47^{phox} SIG in AVP-containing dendrites. Previous studies (Coleman et al., 2013) have shown the opposite effects in males. In hypertensive males, more p47^{phox} SIG particles are found near plasmalemmal in non-AVP dendrites, and fewer plasmalemmal p47^{phox} SIG particles are seen in AVP-containing dendrites.

Table 1

Table of Primary Antibodies used

Antigen	Description of Immunogen	Source, host species, Cat. #, Clone or Lot# RRID	Concentration used
p47 ^{phox}	Raised against C terminus peptide of human p47 ^{phox}	Santa Cruz Biotechnology, Goat Polyclonal, Cat# sc-7660, RRID: AB_2298320	1:50
Arginine-vasopressin	Synthetic Arg ⁸ -vasopressin	Peninsula Laboratories, Guinea pig Polyclonal, Cat. # T-5048, RRID: AB_2313978	1:4000