Cross talk between AT1 receptors and Toll-like receptor 4 in microglia contributes to angiotensin II-derived ROS production in the hypothalamic paraventricular nucleus

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Biancardi VC, Stranahan AM, Krause EG, de Kloet AD, Stern JE. Cross talk between AT1 receptors and Toll-like receptor 4 in microglia contributes to angiotensin II-derived ROS production in the hypothalamic paraventricular nucleus. Am J Physiol Heart Circ Physiol 310: H404–H415, 2016. First published December 4, 2015; doi:10.1152/ajpheart.00247.2015.—ANG II is thought to increase sympathetic outflow by increasing oxidative stress and promoting local inflammation in the paraventricular nucleus (PVN) of the hypothalamus. However, the relative contributions of inflammation and oxidative stress to sympathetic drive remain poorly understood, and the underlying cellular and molecular targets have yet to be examined. ANG II has been shown to enhance Toll-like receptor (TLR)4-mediated signaling on microglia. Thus, in the present study, we aimed to determine whether ANG II-mediated activation of microglial TLR4 signaling is a key molecular target initiating local oxidative stress in the PVN. We found TLR4 and ANG II type 1 (AT1) receptor mRNA expression in hypothalamic microglia, providing molecular evidence for the potential interaction between these two receptors. In hypothalamic slices, ANG II induced microglial activation within the PVN (~65% increase, P < 0.001), an effect that was blunted in the absence of functional TLR4. ANG II increased ROS production, as indicated by dihydroethidium fluorescence, within the PVN of rats and mice (P < 0.0001 in both cases), effects that were also dependent on the presence of functional TLR4. The microglial inhibitor minocycline attenuated ANG II-mediated ROS production, yet ANG II effects persisted in PVN single-minded 1- AT1a knockout mice, supporting the contribution of a non-neuronal source (likely microglia) to ANG II-driven ROS production in the PVN. Taken together, these results support functional interactions between AT1 receptors and TLR4 in mediating ANG II-dependent microglial activation and oxidative stress within the PVN. More broadly, our results support a functional interaction between the central renin-angiotensin system and innate immunity in the regulation of neurohumoral outflows from the PVN.

renin-angiotensin system in the regulation of sympathetic output from the paraventricular nucleus.

ANGIOTENSIN II (ANG II) plays a critical role in fluid balance and hemodynamic regulation (25). Within the central nervous system (CNS), ANG II acting through ANG II type 1a (AT1a) receptors (AT1aR) has been shown to be implicated in the regulation of sympathetic and neuroendocrine outputs from the brain (45, 49, 50). The paraventricular nucleus (PVN) of the hypothalamus has been recognized as a critical neuronal substrate mediating central ANG II actions, thus playing a critical role in ANG II-mediated neurohumoral responses (23, 78). Indeed, a large body of evidence supports enhanced angiotensinergic actions within the CNS, including the PVN, as a key pathophysiological mechanism involved in cardiovascular diseases, including hypertension and heart failure (37, 60, 78, 80, 88). However, despite its importance, the precise mechanisms and cellular/molecular targets underlying ANG II signaling within the CNS are incompletely understood.

Inflammation and oxidative stress have emerged as novel mechanisms by which central ANG II mediates its deleterious effects. For example, studies have shown that pressor and sympathetic responses to central ANG II are mediated by superoxide within the PVN (8, 22). Likewise, several studies have supported ANG II-mediated generation of ROS within the subfornical organ, PVN, and rostral ventrolateral medulla as an important contributor to sympathoexcitation in cardiovascular diseases (10, 33, 91, 92). Additionally, ANG II is known as a strong proinflammatory signal (46, 66). Systemic administration of ANG II stimulates expression of NF-kB and various proinflammatory cytokines, including IL-1β, IL-6, and TNF-α (11, 33), within the PVN. In addition, ANG II-mediated inflammation is now also recognized as an important pathophysiological mechanism in hypertension (65, 90). Thus, while both oxidative stress and inflammation are critical ANG II-mediated events within the PVN with major pathophysiological consequences, whether they are causally related and what the precise cellular and molecular targets mediating these actions are remain unknown.

Microglia are the resident immune cells in the brain and the first responders to injury and infection, turning into an activated, proinflammatory state (68), resulting in the release of a variety of proinflammatory and neuroactive substances, including cytokines, chemokines, and ROS (68). Importantly, ANG II has been shown to promote microglial cell activation in the PVN (32, 71).

NEW & NOTEWORTHY

This study supports microglia as a key cellular source of ANG II-mediated ROS production within the paraventricular nucleus and provides evidence for Toll-like receptor 4 as a key underlying molecular mechanism. These findings support a functional interaction between the innate immunity and central renin-angiotensin system in the regulation of sympathetic output from the paraventricular nucleus.
Along with microglia, Toll-like receptor (TLR4) is a signaling receptor that plays a major role in innate immunity (3, 76) and has been shown to also contribute to neuroinflammatory diseases (7, 13, 79). TLR4 is primarily expressed in microglial cells, and its activation leads to microglial cell activation and the release of proinflammatory signals (38, 47, 68). Moreover, TLR4 activation can lead to ROS production in neutrophils (1), and in vitro and in vivo studies have supported a functional/molecular link between ANG II-TLR4 activation and oxidative stress within the PVN. Our results show that ANG II induces microglial activation within the PVN, an effect that is blunted in the absence of functional TLR4. We also demonstrate that TLR4 protein and mRNA and AT_1a mRNA are expressed in hypothalamic microglia. In addition, we found that ANG II increased PVN ROS production, an effect that was also blunted in the absence of functional TLR4, was attenuated by the microglial cell inhibitor minocycline and, whereas it was dependent on AT_Rs, it did not involve receptors expressed in neurons. Taken together, the results from this work support a functional interaction between microglial AT_R and TLR4 in mediating ANG II-dependent microglial activation and oxidative stress within the PVN, ultimately suggesting a functional interaction between the central renin-angiotensin system and innate immunity in the regulation of neurohumoral outflows from the PVN.

METHODS

Animals and Experimental Groups

Male Wistar rats were purchased from Harlan Laboratories. Male TLR4-deficient C3H/HeJ and TLR4-sufficient C3H/OuJ mice were kindly donated by Dr. Dhandapani (Georgia Regents University) or purchased from The Jackson Laboratory. PVN single-minded (Sim)-1-AT_1a knockout (KO) mice and littermate control mice (AT_1a flox/flox) were donated by Dr. Krause (University of Florida); in these mice, AT_1a receptors were specifically deleted from PVN neurons through Cre recombinase-dependent excision driven by the Sim1 promoter (Sim1Cre mice were bread with AT_1a flox mice as previously described) (19). All male animals used in this study were 7–12 wk old at the time experiments began. All procedures were approved by the Institutional Animal Care and Use Committee of Georgia Regents University.

Acute Hypothalamic Slices and Ex Vivo ANG II Stimulation

Coronal hypothalamic slices (200 μm thick) containing the PVN were obtained using a vibroslicer. Ice-cold standard artificial cerebrospinal fluid (aCSF) (6, 57) was used in the preparation process. The hemispheres were separated and placed in two holding chambers at 32°C for 60 min. After this time, the chambers were transferred to room temperature, and aCSF or aCSF in the presence of ANG II (1 μM, Sigma-Aldrich) was added to each of the chambers for another 60 min. Sections were then postfixed in 4% paraformaldehyde (PFA) for 24 h, and immunohistochemistry was performed for the microglial marker ionized calcium-binding adaptor molecule (IBA1).

ROS Assessment

A modified dihydroethidium (DHE) staining method was used to evaluate in situ production of ROS (34, 81). Transverse sections (16 μm) of hypothalamic frozen tissue were collected on glass slides and equilibrated in either fresh aCSF, losartan (2 μM), or minocycline (100 μM, all Sigma-Aldrich) for 10 min in a humidified chamber at 37°C. Slides were then placed in an inverted Zeiss LSM780 confocal microscope stage (atmosphere and temperature controlled, Carl Zeiss), and sections were incubated with DHE (1 μM, Invitrogen) in the presence of either aCSF, ANG II (0.01–1 μM), or ANG II associated with losartan or minocycline. Immediately after the beginning of incubation, images were acquired every 2 min for a total of 10 min. Zeiss LSM780 software was used for analyses. Forty randomly selected cells per slice were manually traced, and the intensity of fluorescence of each cell was plotted over time. aCSF was used as the vehicle for all drug dilution.

Isolation of Microglia

Microglia from the hypothalamus were isolated by Percoll density gradient as previously described (21, 24). Animals were transcardially perfused with sterile saline, and the hypothalamus was macrodissected and homogenized using a Tenbroeck homogenizer with Dulbecco’s PBS (dPBS) supplemented with 2% glucose. The homogenate was filtered using a 40-μm cell strainer, and cell pellets were obtained by brief centrifugation. The Percoll gradient was created by suspending the cell pellet in 70% isotonic Percoll followed by additional layers of 30% isotonic Percoll and dPBS. Gradients were centrifuged at 1,200 g for 55 min to separate mononuclear cells from the other CNS elements, and the resulting 70%–30% interface was collected. The resultant suspension was washed in dPBS, and the cell pellet was acquired by centrifugation at 1,000 g for 15 min. The resulting pellet was resuspended in 100 μl dPBS. Cell counting and morphological analysis was performed using a handheld automatic cell counter (Scepter instrument, EMD Millipore). Fifty thousand cells were then used for PCR experiments. In a subset of animals, to account for viability after isolation of microglia, 50,000 cells were stained with the live cell DNA dye Vybrant DyeCycle green for 2 h under a CO2 incubator (1:1,000 in DMEM with 10% FBS, Invitrogen). After this incubation, fluorescence and phase-contrast images were taken in a DeltaVision high-resolution microscope (Applied Precision). In addition, to determine the purity of microglia and whether there was contamination of astrocytes and neurons, immunohistochemistry in isolated cells was performed and imaged as described below.

Fluorescence Immunohistochemistry

Standard immunofluorescence was performed as previously described (4, 6).

Immunofluorescence in hypothalamic tissue. Animals were transcardially perfused with 0.01 mol/l PBS (150 ml) and 4% PFA (350 ml). Brains were dissected and postfixed overnight in 4% PFA supplemented with 2% glucose. The homogenate was filtered using a 40-μm cell strainer, and cell pellets were obtained by brief centrifugation. The Percoll gradient was created by suspending the cell pellet in 70% isotonic Percoll followed by additional layers of 30% isotonic Percoll and dPBS. Gradients were centrifuged at 1,200 g for 55 min to separate mononuclear cells from the other CNS elements, and the resulting 70%–30% interface was collected. The resultant suspension was washed in dPBS, and the cell pellet was acquired by centrifugation at 1,000 g for 15 min. The resulting pellet was resuspended in 100 μl dPBS. Cell counting and morphological analysis was performed using a handheld automatic cell counter (Scepter instrument, EMD Millipore). Fifty thousand cells were then used for PCR experiments. In a subset of animals, to account for viability after isolation of microglia, 50,000 cells were stained with the live cell DNA dye Vybrant DyeCycle green for 2 h under a CO2 incubator (1:1,000 in DMEM with 10% FBS, Invitrogen). After this incubation, fluorescence and phase-contrast images were taken in a DeltaVision high-resolution microscope (Applied Precision). In addition, to determine the purity of microglia and whether there was contamination of astrocytes and neurons, immunohistochemistry in isolated cells was performed and imaged as described below.

Isolated microglia. Approximately 50,000 cells were fixed in 4% PFA for 30 min followed by a brief rinse with dPBS and placed to adhere in a coated glass slide. Cells were immunostained with microglia-specific (IBA1 anti-rabbit, 1:200), neuron-specific (NeuN, anti-mouse, 1:200), and astrocyte-specific (gial fibrillary acidic protein, anti-chicken, 1:200) primary antibodies in dPBS, 0.05% Tween, and 5% horse serum overnight. Hoechst counterstaining (1 μg/ml) was performed after the complete immunohistochemistry reaction.

Immunofluorescence for tissue and cells was revealed by a secondary reaction with anti-mouse Cy3 (1:250); anti-guinea pig Cy3 (1:250), anti-rabbit Cy3 or FITC (1:250), or anti-chicken Cy5 (1:50), accordingly (all secondary antibodies from Jackson Immunoresearch).
The specificity of each antibody was tested by the omission of the primary antibody.

Confocal Imaging Acquisition

Immunofluorescence was examined with an upright Zeiss LSM510 confocal microscope as previously described (4). Images from consecutive optical focal planes were taken (for hypothalamic 200-µm sections: 40 images, 2-µm intervals; for 30-µm sections: 20 images, 1-µm intervals; for isolated microglial cells: 6 images, 1-µm intervals), and a projection image of the sections was then generated. Each channel was acquired sequentially to minimize crossover artifacts among the channels. In hypothalamic slices, we quantified IBA1 density within the PVN based on a threshold paradigm as previously described (4–6), and the results were expressed as IBA percent areas. Images were evaluated with ImageJ software (National Institutes of Health), and the ImageJ plugin (http://rsbweb.nih.gov/ij/plugins/colocalization.html), which detects and displays pixels containing signals from both channels, was used to determine the colocalization between signals (5).

Real-Time RT-PCR

Microglial cells. cDNA from 50,000 cells/animal was acquired using the Power SYBR green cells-to-Ct kit (Ambion, Life Technologies). Lysis solution was added to the cells, and reverse transcription was performed using a RT master mix according to the manufacturer’s instructions. PCR amplification of cDNA was also performed using the Power SYBR green cells-to-Ct kit: cDNA (4 µl) was added to a PCR cocktail with gene-specific primers (200 nM each of forward and reverse primers), and PCR cycling conditions were set up as per the manufacturer’s instructions.

PVN punches. Brains from TLR4-deficient and -sufficient mice (n = 3 mice/group) were collected and immediately frozen in isopentane. Subsequent hypothalamic sections (200 µm) containing the PVN were obtained using a cryostat. The PVN was bilaterally microdissected using a Harris aluminum micropunch needle (1.0 mm, Ted Pella) following The Mouse Brain in Stereotaxic Coordinates (PVN: –0.82 to –1.22 from the bregma; Paxinos and Franklin, 2nd edition, 2001). Samples were collected with RNAlater reagent (Ambion), and total RNA was isolated using TRI reagent (Sigma-Aldrich). The RNA concentration was determined by a NanoDrop spectrophotometer (Thermo Scientific). cDNA synthesis was performed using 250 ng RNA for each sample using an iScript cDNA Synthesis Kit (Bio-Rad) per the manufacturer’s instructions. cDNA (1 µl) was added to a PCR cocktail with gene-specific primers (200 nM each of forward and reverse primers), and PCR cycling conditions were set up as per manufacturer’s instructions.

The following primer sequences were used: 1) CD11b (GenBank Accession No. NM_012711.1, forward 5'-ATCCGTAAGTATGTGAGAGAAC-3' and reverse 5'-TCTGCTACCTCACAATGACATC-3'), 2) AT1a (GenBank Accession No. NM_030985.4, forward 5'-CCTGTCACCTCCAAAAG-3' and reverse 5'-AACCTCTGTTTACCCGG-3'), and 3) TLR4 (GenBank Accession No. NM_019178.1, forward 5'-GATGGCTACAGACTGCGAGTCCC-3' and reverse 5'-CACTCGAGGTAAGTTTCTGCTA-3'). B-Actin (GenBank Accession No. NM_031144.3, forward 5'-CACAGGCTGATGAGGCTAGCTA-3' and reverse 5'-GACCAGATGTGTGTGACACCT-3') served as a housekeeping gene. All primers were obtained from Integrated DNA Technologies. For each experimental sample, duplicate reactions were conducted. The formation of the PCR product was monitored in real time using the 7500 Fast Real Time PCR System (Applied Biosystems). AT1a mRNA expression levels were expressed as percentages of AT1a mRNA levels relative to TLR4-sufficient mice.

Statistical Analyses

Data are presented as means ± SE. Unpaired Student t-tests or one-way or two-way ANOVAs followed by post hoc multiple-comparison tests were used as indicated (GraphPad Prism). P values of <0.05 were considered to indicate statistical significance.

RESULTS

ANG II-Mediated PVN Microglial Activation Requires Functional TLR4

Previous studies have shown that ANG II stimulates microglial cell activation within the PVN (32, 71). Here, we investigated whether this effect involves TLR4 and whether this leads to the production of superoxide. To this end, we performed ex vivo assays using acute hypothalamic slices containing the PVN, which were obtained from control rats as well as from TLR4-sufficient C3H/OuJ (TLR4-sufficient) and TLR4-deficient C3H/HeJ (TLR4-deficient) mice (56). We found that ANG II (1 µM) significantly increased the degree of activated microglia (shown as significantly enhanced staining density for IBA1 within the PVN) (15, 71) in rat slices compared with control aCSF (~65% increase from 9.8 ± 1.1% to 16.2 ± 1.4%, n = 10, P < 0.001; Fig. 1, A–E). Similarly, we found that ANG II increased IBA1 staining in TLR4-sufficient mice (~65% increase from 3.9 ± 0.1% to 6.5 ± 0.3%, n = 5, P < 0.05; Fig. 1F), effects that were blunted in TLR4-deficient mice (~20% increase from 4.3 ± 0.1% to 5.2 ± 0.5%, n = 3, Fig. 1F). To rule out the possibility that basal staining of microglia was different between TLR4 mouse strains, we measured basal IBA1 in a separate set of animals. In this case, we failed to observe significant differences in IBA1 staining between TLR4-sufficient mice (6.5 ± 0.5%) and deficient mice (7.2 ± 0.6%, n = 4 mice/group). Taken together, these results are consistent with the notion that ANG II-mediated microglial activation requires the presence of functional TLR4.

PVN Microglial Cells Express AT1a,Rs and TLR4

While these results support a contribution of TLR4 to ANG II-mediated microglial activation, whether ANG II receptors and TLR4 is at present unknown. To address this, we performed real-time PCR in isolated microglial cells from the hypothalamus along with immunohistochemistry in hypothalamic slices. Hypothalamic microglial cells were isolated following conventional Percoll density gradient procedures (see METHODS). The viability, identity, and purity of the extracted population were then assessed. To confirm the phenotype of the isolated cells, we used an automatic cell counting approach, which uses morphometry (i.e., cell body diameter) to differentiate isolated cells from debris (Fig. 2A). As previously reported, isolated microglia had a cell body diameter of ~6–9 µm (21). Analysis of viable living cells following this procedure was measured using a DNA-selective stain that is cell permeable and emits a fluorescent signal proportional to the DNA mass after binding double-stranded DNA of living cells (Vybrant DyeCycle green; Fig. 2, B–D). We found that in our samples, ~90% of cells were alive. In addition, isolated cells were stained with antibodies against microglia (IBA1), astrocytes (glial fibrillary acidic protein), and neurons (NeuN). As shown in Fig. 2, E–I, cells identified by Hoechst counterstaining (Fig. 2E) were positively immuno-
stained for IBA1 (Fig. 2F), whereas no staining was observed for astrocytes (Fig. 2G) or neurons (Fig. 2H), indicating that the cells collected were mainly microglia.

As shown in Fig. 2N, real-time PCR performed on cDNA obtained from the isolated hypothalamic microglial cell population showed robust amplification for the microglial marker CD11B (as well as for IBA1; not shown). Moreover, both AT1α and TLR4 mRNAs were also detected in the same samples of microglia. Similar results were observed in six independently performed experiments. Likewise, using immunohistochemistry, we found immunoreactivity and costaining for the microglial marker IBA1 and TLR4 (Fig. 2, J–M). Taken together, these experiments support the presence of mRNA for AT1α and mRNA and protein for TLR4 in hypothalamic microglia.

**ANG II-Mediated ROS Within the PVN Involves Activated Microglia**

We used DHE staining to evaluate in situ production of ROS (34, 81) and to determine whether microglia and TLR4 contributed to ANG II-mediated ROS production within the PVN. Experiments were run in pairs of slices: one slice incubated with regular aCSF and another slice in the presence of ANG II (0.01–1 μM). As previously observed, we found that even in the presence of aCSF, there was a progressive increase in background DHE fluorescence levels during imaging acquisition (89). Thus, to quantify the effect of ANG II, we calculated proportional changes (percentages of aCSF) in DHE staining fluorescence intensity at each particular time point and plotted this proportional change over time. We found that ANG II significantly increased DHE staining within the PVN of rats in a time-dependent manner (F = 15.3, P < 0.0001 by one-way ANOVA, Fig. 3, A–C) and concentration-dependent manner (F = 129.3, P < 0.0001 by one-way ANOVA, data obtained 10 min after the beginning of the different concentrations of ANG II incubation; Fig. 3D). The proportional changes of ANG II-dependent ROS production started within 2 min of incubation in ANG II, peaked at 4–8 min, and decreased thereafter (Fig. 3C).

Based on our data described above, we then evaluated whether activated microglia contribute to ANG II-evoked ROS production in the PVN. To this end, we performed similar DHE experiments in slices incubated in either 1) regular aCSF, 2) aCSF + ANG II (1 μM), or 3) aCSF + ANG II + the microglial inhibitor minocycline (100 μM) (15, 86). As previously observed, ANG II significantly increased DHE staining compared with control aCSF (n = 4/group, Fig. 4, A–D), an effect that was partially blunted in the presence of minocycline (n = 4, P < 0.0001 vs. ANG II).

**ANG II-Mediated ROS Production Within the PVN Is Dependent on TLR4**

To determine whether TLR4 contributes to ANG II-mediated ROS production in the PVN, experiments were repeated in TLR4-sufficient and -deficient mice. We observed that similar to our previous results in rats, ANG II (1 μM) significantly increased DHE staining in TLR4-sufficient mice (n = 4, P < 0.0001; Fig. 5, A, B, and E), an effect that was, however, absent in TLR4-deficient mice (n = 5; Fig. 5, C–E). To exclude the possibility that the results observed were due to basal differ-
ences in the expression pattern of AT1aRs between TLR4 mouse strains, we measured AT1a mRNA expression within the PVN of TLR4-sufficient and -deficient mice. We did not observe significant differences in AT1a mRNA expression between strains (TLR4-sufficient mice: 100 ± 17.3% and TLR4-deficient mice: 118 ± 9.8%, n = 3; Fig. 5F). Taken together, our results support a major contribution of microglia and TLR4 to ANG II-mediated ROS production within the PVN. However, in addition to their presence in microglia [as shown in the present study and elsewhere (38, 68)], TLR4 as well as AT1aRs have also been shown to be expressed in neurons (40, 48, 64). Thus, to further test the relative contribution of microglia versus neurons as cell targets mediating ANG II effects on PVN ROS production, we performed similar experiments in mice in which AT1aRs were specifically deleted from PVN neurons [(Sim1)-AT1a KO mice; see METHODS]. In

Fig. 2. Hypothalamic microglia express ANG II type 1 (AT1) receptors (AT1aRs) and TLR4. A: automatic measurement of cell number and diameter from the extracted cell population by Percol gradient permitting the distinction between microglia (M) and debris. B and C: live isolated cells (phase contrast; B) were stained with Vybrant DyeCycle (C). D: merged image. The arrow points to a representative dead cell. Manual counting showed ~90% live cells in our samples. E–H: isolated cells (Hoechst counterstaining; E) showed positive immunostaining for microglia (IBA1; F) but not for astrocytes [glial fibrillary acidic protein (GFAP); G] or neurons (NeuN; H). I: merged images from E–H. J and K: confocal photomicrographs showing the microglial marker IBA1 (red; J) and TLR4 (green; K). L: merged image showing the signal colocalization (white pixels). M: only colocalized pixels are shown. N: real-time PCR detection of microglia (CD11B), AT1a, and TLR4 mRNA in isolated hypothalamic microglia. Scale bars = 10 μm (isolated cells) and 20 μm (tissue).
in the absence of neuronal AT1Rs, ANG II continues to stimulate ROS production, likely via the activation of microglia signaling cascades.

**DISCUSSION**

The findings from the present study provide evidence that microglial TLR4 is a molecular candidate causally linking ANG II-AT1aR-mediated microglial cell activation and oxidative stress within the PVN. Our results show that 1) ANG II-induced microglial activation within the PVN is dependent on the presence of TLR4; 2) TLR4 and AT1a mRNA are expressed in hypothalamic microglia; 3) ANG II via AT1aRs induces ROS production within the PVN, in part from activated microglia; and 4) functional TLR4 is necessary for ANG II-induced ROS production within the PVN.

Functional TLR4 Is Required for ANG II-Induced Microglial Activation Within the PVN

Previous studies have demonstrated that overactivation of ANG II-mediated signaling within the PVN plays a major role in microglial activation, with the consequent production of proinflammatory cytokines (32, 71). Accordingly, we found in our preparation an increase in the degree of activated microglia in the presence of ANG II. However, the molecular targets mediating these actions remain incompletely understood. Because microglia are the major cell types expressing TLR4 within the CNS (38, 51, 54) and ANG II has been implicated in the induction of the inflammatory response via the TLR4 pathway (17, 29, 82), we further assessed the contribution of TLR4 to ANG II-induced microglial activation within the PVN.

ANG II, via AT1Rs, has been shown to induce inflammatory responses by increasing TLR4 mRNA and protein expression in mesangial cells, smooth muscle cells, and mesothelial cells in vitro and in vivo (29, 82, 84). Likewise, AT1R blockers reduced TLR4 protein and mRNA expression, with a subsequent decrease in proinflammatory effects in human monocytes (18). Within the CNS, gene and protein expression for TLR4 were found to be upregulated within the PVN of ANG II-infused rats, in which chronic blockade of brain TLR4 attenuates inflammatory molecules (17). In the present study, we found that ANG II-mediated microglial activation was blunted in mice lacking functional TLR4, supporting TLR4 as a molecular candidate by which ANG II mediates microglial activation. These data are in accordance with recent work showing that direct TLR4 activation via lipopolysaccharide, a specific ligand for TLR4 (44), promotes microglial activation within the PVN (47).

We need to acknowledge the possibility of microglial cell priming due to possible tissue damage during the tissue slicing procedure. This was minimized, however, by slicing the tissue under very cold conditions. This is a standard procedure that under normal and astrocyte activity in brain slices (6, 28, 62, 72). Even if microglia were partially activated, and considering that all experimental groups were subjected to the same dissection/slicing procedures, this potential drawback did not prevent us from detecting significant differences among groups. To provide anatomic evidence for ANG II-TLR4 cross talk within PVN microglia, we performed real-time PCR in isolated microglia of the hypothalamus. The expression of functional...
AT1Rs has been previously demonstrated in amoeboid cultured microglia in postnatal rats based on RT-PCR and lectin stain (41, 52). Expression of the AT1R, however, has been shown to progressively decrease with the transformation of amoeboid into ramified forms of microglia (41), and other studies have reported lack of AT1R protein and mRNA expression in adult microglia under normal conditions (52, 83). In our hands, however, besides finding TLR4 protein expression within PVN microglia, we found the presence of TLR4 and AT1a mRNA expression in isolated hypothalamic microglia. The discrepancies observed among these studies could be due in part to the material used (cultured cells vs. brain tissue or cells extracted in vivo). While we also observed AT1aR immunoreactivity in PVN microglia (not shown), the commercially available antibodies for AT1Rs are unreliable due to lack of specificity (26).

Thus, given these limitations, we based our conclusions regarding the presence of functional AT1aRs on PVN microglial cells on the combined results we obtained from the PCR experiments along with our functional and pharmacological experiments. In addition, although most of our isolated cells were positively stained with IBA1 (>90%) or showed robust expression of IBA1 and CD11B mRNA, we acknowledge that these markers are not able to distinguish microglia from macrophages. Thus, our experiments did not differentiate between innate microglia or possible infiltrating macrophages.

**TLR4 in Microglia Is Involved in ANG II-Mediated ROS Within the PVN**

ROS has been highlighted as a critical mechanism by which ANG II exerts its deleterious effects within the CNS (10, 35, 91, 92). For example, ANG II-mediated ROS production within the PVN contributes to cardiovascular diseases with high sympathetic drive, such as hypertension and heart failure (9, 43, 59, 74). However, the cellular sources and molecular targets involved in ANG II-ROS signaling dysregulation have not been fully elucidated. Because both ANG II-AT1R-mediated signaling and TLR4-mediated signaling share in common several downstream products, including the generation of pro-inflammatory and neuroactive substances such as ROS (1, 68), we sought to determine whether these two signaling mechanisms were interrelated.

As previously reported (22, 74), we found that ANG II stimulated ROS production within the PVN. Moreover, our results support that microglia significantly contributed to this effect. First, we found that in the presence of the microglial inhibitor minocycline (77, 86), ANG II-mediated ROS production was significantly blunted. The unblocked ROS production in the presence of minocycline may be indicative of an incomplete block by minocycline or, more likely, that an alternative cellular source (i.e., neuronal) also contributes to ANG II-ROS production in the PVN (see more below).

Interestingly, minocycline has been considered neuroprotective not only for inhibiting microglial activation itself but also to inhibit NADPH and proinflammatory cytokine expression, including nitric oxide synthase expression and IL-1α, from activated microglia (16, 84). Moreover, minocycline has also been shown to have cytoprotective effects by inhibiting enzymatic activity of MAPKs (31, 73), which may have also contributed to some of the effects observed here.

Second, we found that ANG II-mediated ROS production was almost completely absent in TLR4-deficient mice compared with TLR4-sufficient mice. Since TLR4 is predominantly expressed in microglia (38, 51, 54), these studies further support a contribution of microglia to ANG II-mediated ROS production in the PVN. These results are in accordance with previous studies showing that ANG II promotes the involvement of microglia in NADPH-derived ROS in primary mesencephalic cultures (63) and that lipopolysaccharide failed to induce ROS production in bone marrow-derived macrophages cells from the same TLR4-deficient mice (67). Nonetheless, TLR4 in the brain is not exclusively expressed in microglia, and, along with AT1aRs, TLR4 has been shown to be also present in neurons (40, 48, 64). Similarly, the NADPH oxidase family, a primary source contributing to ANG II-mediated superoxide production, is present in both microglia as well as neurons (12, 55, 70, 75, 85). Thus, to determine to what extent PVN neurons contribute to ANG II-mediated ROS production, at least under our conditions, we repeated experiments in mice in which AT1aRs were specifically deleted from PVN neurons as previously characterized [Sim1Cre PVN (Sim1)-AT1a KO mice] (19). Similar to what we observed in rats, ANG II-mediated ROS production in wild-type mice was partially blunted by minocycline and, in this case, completely blocked by the AT1R blocker losartan. In PVN (Sim1)-AT1a KO mice, ANG II-mediated ROS production was slightly but not significantly lower than in control AT1a flox/flox mice (~11% less), supporting a minor contribution of AT1aR-expressing PVN neurons to ANG II-mediated ROS production in the PVN.
Moreover, minocycline almost completely prevented ANG II-mediated ROS production in PVN (Sim1)-AT1a KO mice. In addition to AT1aRs, ANG II can also activate brain AT1bRs. However, whether AT1bRs are present in the PVN is controversial, with studies supporting (14, 58) or arguing against (30, 39) its presence within the PVN. Since both AT1R subtypes share similar intracellular signaling pathways, we cannot rule out at present a possible role for AT1bRs in ANG II-mediated ROS production in the PVN. Taken together, these results support the notion that microglia, in a TLR4- and AT1aR-dependent manner, are the major cellular substrates contributing to ANG II-mediated ROS production in the PVN.

The nature of the ANG II-AT1aR-TLR4 interaction on stimulating ROS production remains unknown. Our results show that ANG II-mediated ROS production in microglia was completely blocked by losartan. This argues against direct activation of TLR4, supporting, conversely, the notion that ANG II stimulates TLR4 signaling through a cascade of intracellular signals linked to AT1Rs. In this sense, AT1R activation has been reported to promote the transactivation of EGF receptors (EGFRs) (69). EGFR signaling is required for TLR4-mediated activation of NF-κB in response to lipopolysaccharide (20), and its activation by ANG II can occur within a similar time course (in minutes) as the effect we describe here for ANG II-TLR4-ROS production. Another possibility is that ANG II induces the upregulation and activation of the cytokine transforming growth factor-β by trombospondin-1 (36), which, in turn, activates TLR4 pathways in macrophages to induce TNF-α production (42). Clearly, future studies are warranted to more precisely characterize the signaling mechanisms underlying the ANG II-AT1aR-TLR4 cross talk in mediating microglial ROS production in the PVN. Moreover, given that the present study focused on characterizing the mechanistic links between ANG II-AT1aR-TLR4 cross talk in PVN microglial cells, future in vivo studies are also warranted to elucidate the cardiovascular consequences of ANG II activation of this microglial receptor cross talk.

**Functional Implications**

It is well established that the renin-angiotensin system plays a critical role in cardiovascular homeostasis as well as in the development and maintenance of cardiovascular-related diseases such as hypertension and heart failure (27, 78). Within the PVN, ANG II has been associated with increased microglial activation and increased expression of proinflammatory cytokines and the transcription factor NF-κB during these pathological conditions (11, 61, 71, 87). In addition, we recently showed that ANG II contributes to blood-brain barrier disruption within the PVN during hypertension, promoting leakage of circulating ANG II, which targets microglia within this nucleus (5). Moreover, a growing body of evidence supports the notion that microglia play a critical role in mediating the cardiovascular effects of ANG II.

Fig. 5. Absence of functional TLR4 blunts ANG II-mediated ROS production within the PVN of mice. A–D: confocal images showing DHE staining in the presence of aCSF (A and C) and ANG II (1 μM; B and D) in TLR4-sufficient mice (A and B) and TLR4-deficient mice (C and D). E: summary data showing increased ROS production in the presence of ANG II in TLR4-sufficient mice but not in TLR4 deficient mice. F: summary data showing lack of statistical differences in basal PVN AT1a mRNA expression between TLR4-sufficient and -deficient mice (expressed as the percentage of AT1a mRNA levels in TLR4-sufficient mice). AU, arbitrary units. Scale bars = 50 μm. n = 4 and 5 mice/group for ROS measurements and 3 mice/group for mRNA measurements. ***P < 0.0001 vs. aCSF at each given time point. (Main effect by two-way ANOVA: treatment: F = 11.9, P < 0.0001; time: F = 0.4, P < 0.05; interactions: F = 3.12, P < 0.0001.)
of evidence implicates TLR4 signaling within the CNS in exacerbated sympathoexcitation and in the development of cardiovascular diseases. For instance, it has been shown that TLR4 activation, via AT1Rs within the brain stem, contributes to sympathoexcitation drive in heart failure (53), and, recently, Dange et al. (17) have shown that brain TLR4 blockade improves cardiac function in ANG II-induced hypertensive rats. Thus, our study, along with previous studies (17, 53), supports a mechanistic cross talk between the central renin-angiotensin system and the microglia-TLR4 signaling cascade in critical brain areas involved in cardiovascular control. Given the importance of both ANG II and TLR4 in hypertension and heart failure, it will be important to investigate in the future whether the ANG
II-AT1a-R-TLR4-ROS pathway described here is exacerbated in these prevalent cardiovascular disorders.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.C.B., A.M.S., E.G.K., A.d.d.K., and J.E.S. edited and revised manuscript; V.C.B., A.M.S., E.G.K., A.D.d.K., and V.C.B. prepared figures; V.C.B. drafted manuscript; V.C.B., A.M.S., and V.C.B. approved final version of manuscript.

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