Angiotensin II–Induced Hypertension Is Modulated by Nuclear Factor-κB in the Paraventricular Nucleus

Jeffrey P. Cardinale, Srinivas Sriramula, Nithya Mariappan, Deepmala Agarwal, Joseph Francis

Abstract—Hypertension is considered a low-grade inflammatory condition, and understanding the role of transcription factors in guiding this response is pertinent. A prominent transcription factor that governs inflammatory responses and has become a focal point in hypertensive research is nuclear factor-κB (NFκB). Within the hypothalamic paraventricular nucleus (PVN), a known brain cardioregulatory center, NFκB becomes potentially even more important in ultimately coordinating the systemic hypertensive response. To definitively demonstrate the role of NFκB in the neurogenic hypertensive response, we hypothesized that PVN NFκB blockade would attenuate angiotensin II–induced hypertension. Twelve-week–old male Sprague-Dawley rats were implanted with radiotelemetry probes for blood pressure measurement and allowed a 7-day recovery. After baseline blood pressure recordings, rats were administered either continuous NFκB decoy oligodeoxynucleotide infusion or microinjection of a serine mutated adenoviral inhibitory-κB vector, or their respective controls, bilaterally into the PVN to inhibit NFκB at two levels of its activation pathway. Simultaneously, rats were implanted subcutaneously with an angiotensin II or saline-filled 14-day osmotic minipump. After the 2-week treatments, rats were euthanized and brain tissues collected for PVN analysis. Bilaterally inhibited NFκB rats had a decrease in blood pressure, NFκB p65 subunit activity, proinflammatory cytokines, and reactive oxygen species, including the angiotensin II type 1 receptor, angiotensin-converting enzyme, tumor necrosis factor, and superoxide in angiotensin II–treated rats. Moreover, after NFκB blockade, key protective antihypertensive renin-angiotensin system components were upregulated. This demonstrates the important role that transcription factor NFκB plays within the PVN in modulating and perpetuating the hypertensive response via renin-angiotensin system modulation.

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Key Words: angiotensin II ▪ hypertension ▪ cytokines ▪ transcription factors ▪ superoxide

Hypertension is a condition closely associated with the renin-angiotensin system (RAS) and increased expression of proinflammatory cytokines (PICs) and reactive oxygen species (ROS), in both systemic and local hypertensive responses.1-6 Studies from our laboratory and others have shown that, in hypertension, angiotensin II (Ang II), PICs, and ROS can increase the activity of the transcription factor nuclear factor-κB (NFκB), which, in turn, can further increase PIC and ROS expression in a positive feed-forward manner.5,7-10

Within the brain, multiple cardioregulatory regions exhibit a local RAS, including the hypothalamic paraventricular nucleus (PVN), which can synthesize and release both prohypertensive and antihypertensive RAS component peptides.2,11,12 The PVN is widely recognized as a central integration site for the coordination of autonomic and neuroendocrine responses that regulates thirst, salt appetite, and sympathetic outflow.13-15 Ang II is a large peptide that cannot cross the blood-brain barrier (BBB). Therefore, it exerts its roles by acting on the circumventricular organs, where the BBB is either weak or absent.16,17 Signals from these circumventricular organs subsequently activate neurons within the various cardioregulatory centers of the hypothalamus and brain stem, including the PVN,17 which can respond by locally producing components of the RAS and via sympathetic signals to the periphery.5

Findings from our laboratory and others have shown that, in hypertension, PICs are increased within discrete brain sites, such as the PVN, and that signals from both the systemic and local RASs increase PICs and oxidative stress.5,18 Within the PVN, RAS components, PICs, and ROS have been linked to increased sympathoexcitation and perpetuation of the hypertensive state.19,20 Based on the preceding evidence, we hypothesized that bilateral PVN blockade of NFκB would attenuate these observed regional changes, which propagate the Ang II–induced hypertensive response, including increases in PICs and ROS. To test this hypothesis, we blocked NFκB within the PVN using two approaches: bilateral PVN NFκB decoy oligodeoxynucleotide infusion or bilateral PVN microinjection of an adenoviral vector containing a serine mutated inhibitory-κB (IκB; AdIκB) insert. These techniques block separate locations in the NFκB...
transcription activation pathway. Our results demonstrate that blocking NFκB attenuates hypertension through a reduction of PIC and ROS actions within the PVN. NFκB also appears to mediate the balance between the prohypertensive and the antihypertensive arms of the RAS. These data indicate that PVN-specific NFκB plays a role in controlling hypertension through increased PICs and ROS via RAS modulation.

Materials and Methods

Animals

Male Sprague-Dawley rats (12 weeks old, 250–350 g) were used in this study. Animals were housed in a temperature- (25±1°C) and light-controlled (12:12-hour light:dark cycle) room with free access to water and normal rat chow (0.4% salt content). All of the animal procedures were reviewed and approved by the Louisiana State University Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines.

Experimental Protocol

All of the experimental rats were anesthetized and implanted with radiotelemetry transmitters. After a 7-day recovery, bilateral cannulae were placed into the PVN. Fourteen-day osmotic minipumps (ALZET, model 2002; infusion rate of 0.5 μL/h) were filled with Ang II (Bachem, 200 ng/kg per minute) dissolved in 0.9% saline, or saline alone, and implanted subcutaneously in the retroscapular area. Simultaneously, osmotic minipumps (ALZET, model 1004; infusion rate of 0.11 μL/h) were filled with NFκB decoy or control scrambled decoy oligodeoxynucleotide (2 ng/kg per minute; Sigma), dissolved in artificial cerebrospinal fluid (aCSF), implanted subcutaneously in the retroscapular area and connected to the cannula. The NFκB decoy concentration was determined from a previous pilot study in rats using 3 different doses, 200 pg/kg per minute, 2 ng/kg per minute, and 200 ng/kg per minute. The 2 ng/kg per minute dose was found to be optimal, whereas the highest dose caused increased mortality and the lowest dose did not produce complete NFκB inhibition as measured using an NFκB (p65) activity assay. Rats were divided into 4 groups: (1) no treatment (controls; n=11); (2) saline minipump + bilateral PVN NFκB decoy (saline+NFκB decoy; n=18); (3) Ang II minipump + bilateral PVN scrambled decoy (Ang II + scrambled decoy; n=18); and (4) Ang II minipump + bilateral PVN NFκB decoy (Ang II + NFκB decoy; n=20). To determine the potential effect of NFκB decoy leakage into the brain’s ventricular system, rats (n=7) were administered intra-cerebroventriculally (ICV) the same dose (2 ng/kg per minute) at the same flow rate.

Another group of rats were injected (2×10^10 pfu/mL, 100 nL) bilaterally intra-PVN with an adenoviral vector (Ad) containing IκBα serine mutated at the S23A/S36A positions (AdIκBα) or a control Ad with an empty cassette region (AdEmpty; both adenoviruses obtained from Gene Transfer Vector Core, University of Iowa, Carver College of Medicine) using a 1-μL Hamilton syringe, as described previously.12,21 Fourteen-day osmotic minipumps (ALZET, model 2002; 0.5 μL/h) were filled with Ang II dissolved in 0.9% saline or saline alone and simultaneously implanted subcutaneously into the retroscapular area. These rats were divided into 4 groups: (1) no treatment (controls; n=10); (2) saline minipump + bilateral PVN AdIκBα (saline+AdIκBα; n=19); (3) Ang II minipump + bilateral PVN AdEmpty (Ang II + AdEmpty; n=18); and (4) Ang II minipump + bilateral PVN AdIκBα (Ang II + AdIκBα; n=21). A final group of rats was treated with Ang II alone (n=15) and used for Western blot, immunohistochemical, and electron paramagnetic resonance analysis. After 14 days of blood pressure recordings, rats were euthanized using a high ketamine+xylazine dose, and brain tissue was collected for mRNA and protein analysis. Blood plasma was used to determine circulating norepinephrine (NE) via high-performance liquid chromatography. Rats that received treatment unilaterally into the PVN or had malfunctioning pumps (ie, tube detachment from pump or cannula, based on postmortem analysis) were excluded from the final analysis (success rate: bilateral cannulation, ∼78%; bilateral microinjection, ∼65%). Chow salt content did not appear to have any effect on pressure response. A P<0.05 was considered statistically significant. An expanded Methods section can be found in the online Data Supplement, available at http://hyper.ahajournals.org.

Results

NFκB Blockade in the PVN Reduces p65 Subunit Binding Activity

The PVN was bilaterally infused with NFκB decoy oligodeoxynucleotide via a fixed cannula (Figure 1A) or bilaterally microinjected with an adenovirus encoding serine mutated IκBα to overexpress IκB and inhibit NFκB within the PVN. Localization of injection sites of all rats are schematically represented in Figure S1 (please see the online Data Supplement at http://hyper.ahajournals.org), with those receiving unilateral or no treatment excluded from the subsequent final analyses. The localization of IκB gene overexpression specifically within the PVN after bilateral PVN AdIκBα micro-injection was indicated by enhanced IκB fluorescence (Figures 1B and S2A). To determine the efficacy of the two methods of inhibiting NFκB activity, an NFκB p65 subunit activity assay was conducted after the PVN 14-day treatments. The p65 subunit activity was dramatically increased in the PVN in the two Ang II–treated groups versus their respective controls (Figure 1C). This increase in activity was attenuated in both Ang II+NFκB decoy and Ang II+AdIκBα-treated rats. However, p65 subunit activity was unaltered in the lateral hypothalamus of any group (Figure S2B), indicating that the effect of NFκB and its blockade was localized to the PVN and not the surrounding regions. These data show that NFκB is increased within the PVN during Ang II–induced hypertension and that the use of NFκB decoy or AdIκBα can potently, and site specifically, inhibit NFκB activity.

NFκB Blockade in the PVN Attenuates the Ang II–Induced Blood Pressure Response

Chronic 14-day Ang II infusion significantly increased the mean arterial pressure (MAP) in rats that received scramble decoy or AdEmpty treatment versus their respective saline-infused controls (saline+NFκB decoy and saline+AdIκBα; Figure 2). Conversely, the MAP of Ang II+NFκB decoy and Ang II+AdIκBα rats had a significantly reduced MAP from their Ang II–treated counterparts, although the MAP was not normalized. Rats receiving either treatment unilaterally, while showing a reduced MAP versus the Ang II control groups, were not reduced as effectively as bilateral treatments (Figure S3), indicating compensation from the untreated side of the PVN. Furthermore, Ang II+ICV NFκB decoy-treated rats had a slightly, although not significantly, reduced MAP versus the Ang II+scramble decoy group (Figure S3A), demonstrating that the potential effect from ventricle decoy leakage on additional central cardioregulatory sites is minimal. These data indicate that PVN NFκB plays an important role in regulating blood pressure response in Ang II–induced hypertension.
NFκB Blockade Decreases PIC Expression in the PVN

Ang II infusion significantly increased the mRNA expression of tumor necrosis factor-α (TNF), interleukin-1B (IL-1B), IL-6, and the chemokine monocyte chemotactic protein (MCP)-1 in the PVN versus control rats. However, bilateral PVN NFκB decoy infusion or bilateral microinjection of AdIκB into the PVN attenuated these changes in PIC gene expression (Figure 3A). Furthermore, immunohistochemistry against TNF showed an increased staining in Ang II–treated rats (Ang II alone) versus controls (no treatment; Figures 3B and S4). This protein expression was reduced via bilateral PVN NFκB inhibition, PIC levels are reduced within this hypothalamic region.

NFκB Blockade Effects RAS Component Expression in the PVN

Ang II infusion significantly increased the mRNA expression of the prohypertensive Ang II type 1 receptor (AT1R) and angiotensin-converting enzyme (ACE) in the PVN when compared with control rat groups and decreased the antihypertensive ACE2 and MasR expression versus their respective control groups (Figure 4A). Bilateral NFκB decoy infusion or microinjection of AdIκB into the PVN reversed these gene expression changes. Ang II also decreased the Mas/AT1 R ratio within the PVN, which was reversed in Ang II+NFκB decoy and Ang II+AdIκB groups (Figure S5A). These results were further confirmed for AT1R and MasR protein levels in the PVN by Western blot (Figure 4B) and densitometric (Figure S5B and S5C) analysis. These results show that NFκB plays a modulatory role in differential RAS
component expression in the PVN during Ang II–induced hypertension.

NFκB Blockade Reduces ROS Production in the PVN

Total ROS, superoxide (O$_2^•^-$), and peroxynitrite (OONO$^•$) levels were significantly increased in the PVN of Ang II–Scramble decoy and Ang II–AdEmpty-infused rats are increased vs control groups. Expression is decreased after bilateral PVN NFκB decoy infusion or adenoviral inhibitory-κB (AdκB) microinjection (n=6–8 per group). B, Immunohistochemical staining for TNF is increased in the PVN in Ang II–infused rats (Ang II alone) vs controls (no treatment). Staining was decreased after NFκB decoy infusion or AdκB microinjection in rats treated bilaterally intra-PVN. Representative images from preparations of 5 to 6 rats. *P<0.05 vs respective Ang II–treated rats, #P<0.05 vs respective control-treated rats.

NFκB Blockade Increases Neuronal NO Synthase Expression in the PVN and Decreased Circulating Plasma NE

Neuronal NO synthase (nNOS) expression is an indirect indicator of neuronal activity. In Ang II–treated rat groups, nNOS mRNA was significantly decreased when compared with the control groups (Figure 6A). Bilateral NFκB decoy infusion or AdκB microinjection into the PVN reversed and elevated these Ang II–induced changes above that of the normotensive controls. These results were further confirmed with immunohistochemistry against nNOS (Figures 6B and S6A), where Ang II–infused rats (Ang II alone) had decreased nNOS presence versus controls (no treatment), which was reversed by NFκB blockade. Furthermore, plasma NE was increased in Ang II–treated rats when compared with controls, but normalized after bilateral NFκB decoy infusion or AdκB microinjection into the PVN (Figure S6B). These results indicate that NFκB within the PVN potentially augments neuronal activity and sympathoexcitation and that NFκB inhibition within this region can attenuate these Ang II–induced hypertensive changes.

Discussion

In the present study, we investigated the effects of bilateral hypothalamic PVN-specific blockade of NFκB on the Ang II–induced hypertensive response. The salient findings of this study are as follows: (1) peripheral Ang II infusion increases MAP, which is attenuated by bilateral PVN NFκB blockade;
(2) peripheral Ang II infusion increases PICs, ROS, and prohypertensive (ACE and AT1R) RAS components and decreases the antihypertensive (ACE2 and Mas) RAS components within the PVN; (3) bilateral PVN-specific NFκB inhibition of Ang II–infused rats not only decreases PVN PICs but also modulates RAS component expression, such as decreasing ACE and AT1R expression and increasing ACE2 and MasR expression; (4) PVN blockade of NFκB within the PVN was differentially regulated along with PIC and oxidative stress genes and proteins.5 This work presented the involvement of brain NFκB in regulating the hypertensive response, a previously novel proposal. However, ICV infusion of pyrroline dithiocarbamate, a known antioxidant,25 was used to study NFκB in the PVN, signifying that NFκB and its subsequent actions could have been reduced through an antioxidant-driven mechanism.

Figure 4. Effects of nuclear factor-κB (NFκB) inhibition on angiotensin II (Ang II)–induced changes in renin-angiotensin system (RAS) component expression. A, mRNA expression of Ang II type 1 receptor (AT1R) and angiotensin-converting enzyme (ACE) was increased, whereas ACE2 and the MasR were decreased in the Ang II–treated rat groups vs controls. Bilateral paraventricular nucleus (PVN) NFκB decoy infusion or adenoviral inhibitory-κB (AdIκB) microinjection reversed these changes. B, Western blots show increased AT1R and decreased MasR in Ang II–treated rats (Ang II alone) vs controls (no treatment). Bilateral PVN NFκB decoy infusion or AdIκB microinjection reversed these changes. n = 6 to 8 per group, *P < 0.05 vs respective Ang II–treated rats, #P < 0.05 vs respective control-treated rats.

On stimulation, NFκB is released after IκB phosphorylation, ubiquination, and degradation, freeing NFκB and allowing its nuclear translocation to act on κB binding sites and commence transcription.22 In this study, two approaches were used to block NFκB within the PVN. AdIκB binds to NFκB similar to endogenous IκB, but serine mutations at the S32A/S36A position prevent IκB phosphorylation and the ensuing NFκB release, thereby inhibiting its capability to translocate into the nucleus and transcribe target genes.22 Decoy oligodeoxynucleotides act by targeting and adhering to the cis-element binding sites of free NFκB, preventing its attachment to κB binding sites and blocking subsequent gene transcription.23,24 By blocking NFκB at two separate activation pathway locations, it effectively demonstrates the role of PVN NFκB in Ang II–induced hypertension and potentially signifies that there are no secondary pathways activated between NFκB/IκB release and its nuclear translocation/binding. Previous findings from our laboratory investigating the role of the PVN in Ang II–induced hypertension demonstrated that NFκB within the PVN was differentially regulated along with PIC and oxidative stress genes and proteins.5 This work presented the involvement of brain NFκB in regulating the hypertensive response, a previously novel proposal. However, ICV infusion of pyrroline dithiocarbamate, a known antioxidant,25 was used to study NFκB in the PVN, signifying that NFκB and its subsequent actions could have been reduced through an antioxidant-driven mechanism.
mechanism rather than through direct NFκB intervention. Also, because of ICV administration, we could not rule out the possible involvement of other affected cardioregulatory regions for the observed reduction in the hypertensive response. Therefore, the exact role and proposed involvement/mechanism of NFκB within the PVN remains uncertain. The current study looks at NFκB blockade specifically within the PVN in the Ang II–induced hypertensive response, delineates the involvement of PVN NFκB in regulating ROS and PIC expression and, perhaps more importantly, shows that NFκB serves as a potential tipping point between the injurious prohypertensive and protective antihypertensive RAS axes within the PVN.

Extensive evidence implicates the RAS within the brain in hypertension.2,12,26,27 In the PVN, Ang II is increased in multiple hypertensive models, and PVN blockade of the

Figure 5. Effects of nuclear factor-κB (NFκB) inhibition on angiotensin II (Ang II)–induced changes in reactive oxygen species (ROS) levels. Ang II–infused rats (Ang II alone) had an increase in total ROS, superoxide, and peroxynitrite within the paraventricular nucleus (PVN) vs control rats (no treatment). Bilateral PVN NFκB decoy infusion or adenoviral inhibitory-κB (AdIκB) microinjection decreased these ROS changes within the PVN. Electron paramagnetic resonance (EPR) spectra (right) for total ROS and superoxide. Graphic data when vs total protein per sample. n=5 to 6 per group, *P<0.05 vs Ang II–treated rats, #P<0.05 vs controls.

Figure 6. Effects of nuclear factor-κB (NFκB) inhibition on angiotensin II (Ang II)–induced increases in neuronal NO synthase (nNOS) expression. A, mRNA expression of nNOS in the paraventricular nucleus (PVN) of Ang II–infused rat groups is decreased vs control groups but increased after bilateral PVN NFκB decoy infusion or adenoviral inhibitory-κB (AdIκB) microinjection (n=6–8 per group). B, Immunohistochemical staining for nNOS is increased in the PVN in Ang II–infused rats (Ang II alone) vs controls (no treatment). Staining was increased in rats treated bilaterally intra-PVN via NFκB decoy infusion or AdIκB microinjection. Representative images from preparations of 5 to 6 rats. *P<0.05 vs respective Ang II–treated rats, #P<0.05 vs respective control–treated rats.
AT\(_1\)R partially inhibits the effects of Ang II–induced hypertension.\(^{5,14,26–28}\) In the current study, NF\(_{\kappa}\)B blockade within the PVN decreases ACE and the AT\(_1\)R, thereby limiting the effect that Ang II could have on perpetuating Ang II–induced hypertension. Moreover, NF\(_{\kappa}\)B inhibition also increased ACE2 and MasR expression, as well as improved the MasR/AT\(_1\)R ratio, indicating enhancement of the protective anti-hypertensive RAS axis. ACE converts Ang I to Ang II. ACE2, however, converts Ang I and Ang II to angiotensin (1-7),\(^{29}\) which acts on the MasR to elicit actions opposing those of ACE/Ang II, including vasorelaxation and decreased sympathetic activity.\(^{30–32}\) Our results agree with other findings that detail the antihypertensive actions of ACE2 and the MasR,\(^{30,32}\) including their decreased activity after, and interplay with, AT\(_1\)R activation within the brain.\(^{12,21,31,33}\) Combined, these results suggest NF\(_{\kappa}\)B as an important balance point between the protective (ACE2/Ang[1-7]/Mas) and non-protective (ACE/Ang II /AT\(_1\)R) arms of the RAS and that NF\(_{\kappa}\)B blockade promotes the more beneficial actions of ACE2 and the MasR.

The association between the RAS and elevated PICS in hypertension has often been explored.\(^{1,15,19,20}\) NF\(_{\kappa}\)B is a key regulator of the PIC expression and inflammatory response observed in hypertension.\(^{5,8,34}\) Here, we show that bilateral NF\(_{\kappa}\)B inhibition in the PVN reduces PIC expression, establishing not only the definitive involvement of NF\(_{\kappa}\)B within the PVN in regulating the Ang II–induced hypertensive pressure response, but also that PICS may play a role in RAS modulation. Recently, ROS, especially O\(_2^\bullet\)•, have been shown as important signaling factors within the brain for enhancement of the neurogenic hypertensive response through both Ang II and PIC mechanisms.\(^{9,10,35}\) Here, bilateral NF\(_{\kappa}\)B blockade reduced the ROS response, including O\(_2^\bullet\)•, thus potentially inhibiting one of the mechanistic pathways by which the hypertensive response and sympathoexcitation (as indicated by decreased plasma NE), is modulated. These results are further confirmed by previous experiments in our laboratory showing that ICV administration of the ROS scavenger, Tempol, reduced renal sympathetic nerve activity in Ang II–treated rats.\(^{5}\) This shows that PVN-specific NF\(_{\kappa}\)B blockade reduces the PIC and ROS reactions typically associated with Ang II–induced hypertension and highlights the central position that PVN NF\(_{\kappa}\)B plays in regulating the neurogenic component of hypertension.

Sympathoexcitation is a component of hypertension, and increased levels of Ang II in the central nervous system and PVN can enhance sympathetic outflow through increased ROS, AT\(_1\)R, and NE activation and a subsequent downregulation of nNOS.\(^{3,36–39}\) The expression and presence of nNOS, an indirect indicator of neuronal activity and downstream sympathetic activity, is inversely proportional to the level of sympathetic outflow,\(^{40–42}\) and NO, a well-known sympatho-inhibitory neurotransmitter, when blocked, results in elevated MAP and sympathoexcitation.\(^{37,43}\) Furthermore, increased superoxide can interact with the decreasing nNOS-produced NO, forming OONO,\(^*\) further reducing NO bioavailability and resulting in enhanced sympathoexcitation.\(^{44}\) Recently, Ang(1-7) and MasR activation have been shown to increase nNOS activity and NO release.\(^{45}\) The current study shows that, after Ang II treatment, nNOS is decreased within the PVN, potentially indicating increased sympathetic outflow. Also, bilateral NF\(_{\kappa}\)B inhibition decreased elevated Ang II–induced OONO.\(^*\). This OONO.\(^*\) decrease paralleled that of O\(_2^\bullet\)• and was concurrent with the nNOS increase, indicating that NF\(_{\kappa}\)B potentially plays a deciding role in regulating NO availability and sympathoexcitation. These results were reinforced by circulating NE, which was normalized in Ang II–infused rats after bilateral NF\(_{\kappa}\)B blockade. Therefore, bilateral NF\(_{\kappa}\)B inhibition increased nNOS and reduced O\(_2^\bullet\)•, OONO, and plasma NE, possibly through balancing RAS components, and thus potentially reducing sympathoexcitation.

In conclusion, this study shows that after the Ang II activation of the prohypertensive RAS arm, PICS are increased, which separately and together, can act to increase the activity of NF\(_{\kappa}\)B and lead to the transcription of additional prohypertensive modulators in a positive feed-forward manner. NF\(_{\kappa}\)B acts to increase, along with Ang II, the presence of ROS, such as O\(_2^\bullet\)•, which subsequently affects the present NO levels, thereby effecting neuronal activity/function and NE release. Blockade of NF\(_{\kappa}\)B at two separate locations in its activation pathway prevents these changes, restores the RAS balance, and promotes the antihypertensive RAS arm, including ACE2 and the MasR. It also reduces PIC and ROS expression and elevates nNOS, all of which contribute to a reduction in MAP and an improvement in the Ang II–induced hypertensive state. However, this signaling mechanism must be further studied to delineate the manner by which NF\(_{\kappa}\)B and O\(_2^\bullet\)• interact within the PVN in Ang II–induced hypertension. We propose that Ang II activation of NF\(_{\kappa}\)B increases PICs and O\(_2^\bullet\)•, tipping the balance of the RAS in favor of the prohypertensive arm and decreasing the antihypertensive arm, resulting in a further increase in PIC and ROS expression, in a vicious positive feed-forward mechanism (Figure S7). Limitations for this study include the use of the Ang II hypertensive model, because this does not represent all modes of hypertension. Also, we only explored the PVN region, although there are multiple cardiorelevant sites in the brain that can play a role in modulating the hypertensive response; however, we feel that the PVN is of importance because of its recognized integrative functions. Moreover, as the literature suggests, adenoviruses are well known for their lack of cell specificity and can be expressed by neurons and glia and participate in retrograde transport.\(^{46}\) For this reason, we used NF\(_{\kappa}\)B decoy oligodeoxynucleotides to further verify our results. Thus, in the current clinical environment where novel hypertensive therapeutic measures are continuously being sought, this study provides a conceptual basis for including NF\(_{\kappa}\)B inhibitors that can specifically act within the brain as a possible future pharmacological approach for the treatment of hypertension.

Perspectives
Increasing evidence indicates that central nervous system mechanisms play an important role in the pathogenesis of cardiovascular disease. In this study, we demonstrate that inflammatory molecules, specifically, transcription factor
NFκB, within the PVN, can modulate the hypertensive response. In addition, we demonstrate that inflammation is a double-edged weapon that not only upregulates the deleterious prohypertensive RAS axis but also downregulates the protective antihypertensive RAS axis. Thus, inflammatory-mediated modulation of the brain RAS might be an important critical contributor to neurogenic hypertension. Because inflammation and the RAS are potent inducers of oxidative stress and NFκB has been shown to respond to and induce oxidative stress, it may be advantageous to target NFκB to better treat hypertension. Although the current methods used within this study are impractical for current clinical administration, one can explore the use of NFκB small molecule inhibitors that cross the blood-brain barrier, thereby targeting the brain’s source of inflammation and oxidative stress, for controlling and treating this debilitating condition.

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Disclosures

None.

References


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Running Title: NFκB modulates RAS components in the PVN

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Materials and Methods

Animals:
Male Sprague-Dawley rats (12 weeks old, 250-350 grams) were used in this study. Animals were housed in a temperature-controlled room (25 ± 1°C) and maintained on a 12:12 hour light:dark cycle with access to water and food ad libitum. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Louisiana State University in accordance with NIH guidelines.

Experimental protocol:
Rats were anaesthetized and implanted with radio-telemetry transmitters. Following a 7-day recovery, bilateral cannulae were placed into the PVN. Fourteen-day osmotic minipumps (Alzet, model 2002) with an infusion rate of 0.5µl/h were filled with AngII (Bachem, 200ng/kg/min) dissolved in 0.9% saline, or saline alone, and implanted subcutaneously in the retroscapular area. Simultaneously, osmotic minipumps (infusion rate of 0.11µl/h; Alzet, model 1004) were filled with NFκB decoy or control scrambled decoy oligodeoxynucleotide (2ng/kg/min; Sigma), dissolved in aCSF. The NFκB decoy dose was determined from a previous pilot study in rats using three different doses, 200pg/kg/min, 2ng/kg/min and 200ng/kg/min. The 2ng/kg/min dose was found to be optimal, while the highest dose caused increased mortality and the lowest dose did not produce complete NFκB inhibition as measured using an NFκB (p65) activity assay. Pumps were also implanted subcutaneously in the retroscapular area and connected to the cannula. Rats were divided into 4 groups: 1) No treatment (Controls; n=11); 2) Saline minipump + bilateral PVN NFkB decoy (Saline+NFkB decoy; n=18); 3) AngII minipump + bilateral PVN scrambled decoy (AngII+Scramble decoy; n=18); and 4) AngII minipump + bilateral PVN NFκB decoy (AngII+NFκB decoy; n=20).

Another group of rats were also implanted with radio-telemetry transmitters and allowed a 7-day recovery. These rats were injected (2x10^{10} pfu/ml, 100nL) bilaterally intra-PVN with an Adenoviral vector (Ad) containing IκB serine mutated at the S23A/S36A positions (AdIκB), or a control Ad with an empty cassette region (AdEmpty; both vectors obtained from Gene Transfer Vector Core, University of Iowa, Carver College of Medicine) using a 1µl Hamilton syringe, as previously described with slight variations.1, 2 Fourteen-day osmotic minipumps (Alzet, model 2002; 0.5µl/h) were filled with AngII dissolved in 0.9% saline or saline alone and were implanted subcutaneously in the retroscapular area. These rats were also divided into 4 groups: 1) No treatment (Controls; n=10); 2) Saline minipump + bilateral PVN AdIκB (Saline+AdIκB; n=19); 3) AngII minipump + bilateral PVN AdEmpty (AngII+AdEmpty; n=18); and 4) AngII minipump + bilateral PVN AdIκB (AngII+AdIκB; n=21). To determine the potential effect of NFκB decoy leakage into the brain’s ventricular system, rats (n=7) were administered intracerebroventricularly (ICV) the same dose at the same flow rate. A final group of rats were treated with AngII alone (n=15) and used for western blot, immunohistochemical and EPR analysis. All rats were euthanized using a high ketamine+xylazine dose after 14 days of blood pressure recordings and brain tissue was collected for mRNA and protein analysis. All rats that received treatment unilaterally into the PVN or had malfunctioning pumps (i.e. tube detachment from pump of cannula, based upon post-mortem analysis) were excluded from the final analysis (success rate: bilateral cannulation ~ 78%; bilateral microinjection ~ 65%). A p<0.05 was considered statistically significant.
NFκB oligodeoxynucleotide sequences:
Sequences were constructed based upon the previous publications.3-6

Wild-type NFκB decoy oligodeoxynucleotide sequence:
5'-G*A*T*-CGA-GGG-GAC-TTT-CCC-T*A*G*-C-3'
3'-C*T*A*-GCT-CCC-CTG-AAA-GGG-A*T*C*-G-5'

NFκB scramble decoy oligodeoxynucleotide (bold sequences altered):
5'-G*A*T*-CGA-GGA-AGC-TTT-CCC-T*A*G*-C-3'
3'-C*T*A*-GCT-CCT-TCG-AAA-GGG-A*T*C*-G-5'

*Denotes bases with phosphorothioate modification in their sequences.

Blood pressure measurement:
Blood pressure was measured continuously in conscious rats implanted with radio-telemetry transmitters (Model TA11PA-C40, Data Sciences International, St. Paul, MN). Rats were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.) and placed dorsally on a heated surgical table. An incision was made on the ventral surface of the left leg, and the femoral artery and vein were exposed and bluntly dissected apart. The femoral artery was ligated distally, and a small clamp was used to temporarily interrupt the blood flow. The catheter tip was introduced through a small incision in the femoral artery, advanced ~6 cm into the abdominal aorta, such that the tip was distal to the origin of the renal arteries, and secured into place. The body of the transmitter was placed in to the abdominal cavity and sutured to the abdominal wall. The abdominal musculature was sutured and the skin layer was closed. Rats received benzathine penicillin (30000 U, i.m.) and buprenorphine (0.1 mg/kg, s.c.) immediately following surgery and 12 h postoperatively. The rats were allowed 7-day surgical recovery.

Bilateral cannula implantation or intra-PVN injections:
Rats to receive NFκB decoy or scrambled oligodeoxynucleotide decoy were anesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) mixture (i.p.). The rats were placed in a stereotaxic instrument (Kopf instruments; Tujunga, CA) and the skull was exposed through an incision on the midline of the scalp. After bregma was identified, the coordinates for the PVN were determined from the Paxinos and Watson (2007) rat atlas, at 1.8 mm posterior and 7.9 mm ventral to the zero level. Rats were implanted with custom designed bilateral cannulae (Plastics One; Roanoke, VA) and subsequently attached to osmotic mimipumps via sterile vinyl tubing. For Adenovirus microinjections, we followed the same surgical procedure except that rats were injected bilaterally intra-PVN with AdIkB or AdEmpty virus using a 1μl Hamilton syringe with a custom made needle/tip and injector plunger that advances flush with the needle tip (Hamilton Company USA) at ±0.4 mm lateral to the bregma.2 Cannulated rats were examined for cannula placement during cryostat slicing with crystal violet staining prior to PVN punching and immunohistochemical slicing (bilateral cannulation ~ 78%; n=22). Microinjection locations were also determined via crystal violet staining prior to PVN punching and immunohistochemical slicing (bilateral microinjection ~ 65%; n=24). At this point, animals identified as receiving
unilateral or no treatments into the PVN were removed from the final analysis.

**Detection of total ROS, superoxide and peroxynitrite in PVN tissue:**
One of the most sensitive and definitive methods of superoxide production is electron spin resonance (EPR). In this study, we utilized an established technique for ROS detection in tissue using EPR and spin traps as previously described.7 8

**RNA isolation and real-time RT-PCR:**
Total RNA was extracted from the PVN using TRI reagent (Invitrogen), and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad), as previously described.9 10 The mRNA expression levels of TNF, IL-1β, IL-6, MCP-1, AT1R, ACE, ACE2, the Mas receptor and nNOS were determined using previously published specific custom made primers.9-13 GAPDH was used as the housekeeping gene. Real-time RT-PCR (qRT-PCR) was performed in 384 well PCR plates using Bio-Rad PCR Master Mix (The iTaq SYBR™ Green Supermix with ROX) and the ABI Prism 7900 sequence detection system (Applied Biosystems). The PCR cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles (15 s at 95°C, 1 min, at 60°C). A dissociation step (15 s at 95°C, 15 s, at 60°C and 15 s at 95°C) was added to check the melting temperature of the specific PCR product.

**Western blot:**
Western blot analysis was performed according to standard protocols. The PVN tissue was homogenized with RIPA lysis buffer. Equal amounts of protein (5 µg) were separated by SDS-PAGE on 10% (wt/vol) gels and transferred on to PVDF membrane (Immobilon-P, Millipore), and blocked with 1% BSA in TBS-T at room temperature for 60 min. The membranes were subjected to immunoblot analyses with anti-AT1 (Abcam), anti-MasR (Santa Cruz) and anti-GAPDH (Santa Cruz) antibodies (1:200 dilution). Immunodetection was accomplished with a horse radish anti-rabbit or anti-goat secondary antibody (1:2000 dilution) using an enhanced chemiluminescence kit (Amersham) as previously described.2 9 The data were quantified by densitometry using ImageJ software and were normalized to GAPDH expression.

**Immunohistochemical analysis:**
Rats (n=5-6 in each group) were transcardially perfused with 200ml of ice-cold PBS (7.4 pH; 0.1M) followed immediately by 200 ml of 4% paraformaldehyde in PBS. The brains were removed, postfixed in 4% paraformaldehyde solution for 2 hours, and transferred to a phosphate buffer containing 20% sucrose (pH 7.4) and stored overnight. For immunostaining, 10µm coronal sections from paraffin embedded brains were collected on slides. First the sections were incubated with 0.3% H2O2 in methanol for 10 minutes. For antigen retrieval, citrate buffer with microwave heating technique is used. Then the sections were incubated with 1.5% goat or rabbit serum in PBS containing 0.3% Triton X100 for 30 minutes. The sections were incubated with primary antibodies (TNF, 1:100 dilution, anti-goat; Santa Cruz; nNOS, 1:100 dilution, anti-goat; Santa Cruz) overnight at 4°C followed by incubation with biotinylated goat-anti rabbit or rabbit-anti goat secondary antibodies for 60 minutes, and stained with Vectastain ABC kit (Vector Laboratories) according to the manufacturer’s instructions. Each step was followed by washing the sections with PBS containing 0.3% Triton X100. Sections incubated without primary antibody were used as negative controls. Graphical analysis was conducted based on positively
stained cell counts of sections from examined rats.

**Determination of IκB overexpression by immunofluorescence:**
For detection of IκB (red fluorescent protein) in PVN tissue, slides were incubated overnight at 4°C with a 1:100 dilution of goat polyclonal anti-IκB (Santa Cruz) as previously described.⁷

**p65 activity assay for assessment of NFκB binding activity:**
The NFκB/p65 Active ELISA kit (Active Motif) was used to measure the binding activity of free NFκB p65 in nuclear extracts. The extraction and analysis were done using a sandwich ELISA method and in accordance to the manufacturer’s instructions.

**Detection of plasma norepinephrine:**
Plasma norepinephrine (NE) was measured in plasma samples using an Eicom HTEC-500 system fitted with an HPLC-ECD using HPLC-EC as described previously.¹⁰, ¹⁴

**Statistical analysis:**
All results are expressed as mean±SEM. For statistical analysis of the data, student’s t test, one-way ANOVA or repeated measures ANOVA followed by Bonferroni’s correction post hoc was performed (GraphPad Prism v5.0 for Windows, GraphPad Software, San Diego California, USA) to determine differences among groups. A value of p<0.05 was considered statistically significant.

**References:**


**S1.** Localization of treatment sites within the PVN. Bilateral cannulae placement (A; n=22) and microinjection (B; n=24) sites as determined by post-mortem analysis of the brain using crystal violet staining prior to punching the PVN and immunohistochemical slicing. Rats demonstrating unilateral treatments also demonstrated an elevated MAP from the bilaterally treated AngII+Scramble decoy, AngII+AdEmpty, AngII+ NFκB decoy and AngII+ AdIκB groups.
S2. IκB expression and NFκB p65 activity assay in the lateral hypothalamus. (A) An additional representative sample of IκB red fluorescence expression localized to the PVN. (B) Activity of p65 was unchanged in the lateral hypothalamus of the AngII-treated groups when compared to control groups. Nor was the p65 activity altered in AngII-treated rats following bilateral PVN NFκB decoy infusion or AdIkB microinjection. n=5-6/group.
S3. Effects of unilateral and ICV NFκB inhibition on AngII-induced increases in MAP. AngII increased MAP in centrally treated control rats. Bilateral NFκB decoy infusion or AdIκB microinjection into the PVN decreased this AngII-induced increase in MAP. Unilaterally treated NFκB decoy infusion (A; n=5) or AdIκB microinjection (B; n=7) groups displayed a decrease versus AngII controls, but not to the level of bilaterally infused animals. ICV NFκB decoy treated rats (A; n=7) also showed a slight reduction in MAP, but not as effectively as bilateral PVN inhibition.
S4. Positive immunohistochemical staining for TNF as determined from equal PVN levels of rats per treatment group. n=5-6 in each group, *p<0.05 vs respective AngII-treated rats, #p<0.05 vs respective control-treated rats.
S5. Effects of NFκB inhibition on AngII-induced changes in RAS component expression. (A) Mas/AT₁R ratio and western densitometry for AT₁R (B) and the Mas receptor (C). Bilateral PVN NFκB decoy infusion or AdIkB microinjection decreased the AngII-induced increase in AT₁R and increased the AngII-induced decrease in Mas receptor protein expression. n=6-8 in each group, *p<0.05 vs respective AngII-treated rats, #p<0.05 vs respective control-treated rats.
S6. Effects of NFκB inhibition on AngII-induced changes on nNOS and in plasma NE. Positive immunohistochemical staining for nNOS (A) as determined from equal PVN levels of rats per treatment group. n=5-6 in each group, *p<0.05 vs respective AngII-treated rats, #p<0.05 vs respective control-treated rats. (B) Plasma NE as measured by HPLC is increased in AngII-treated rats versus their respective controls. This increase was attenuated in rats treated with NFκB decoy and AdIkB. n=6-7 in each group, *p<0.05 vs respective AngII-treated rats, #p<0.05 vs respective control-treated rats.
S7. Schematic showing the proposed signaling mechanism in the PVN whereby AngII leads to an increased hypertensive response via NFκB and modulation of the balance between the pro- and anti-hypertensive arms of the renin-angiotensin system (solid lines/arrows). NFκB inhibition attenuates this response (green lines/arrows).