Early Interference With p44/42 Mitogen-Activated Protein Kinase Signaling in Hypothalamic Paraventricular Nucleus Attenuates Angiotensin II–Induced Hypertension

Yang Yu,* Bao-Jian Xue,* Zhi-Hua Zhang,* Shun-Guang Wei, Terry G. Beltz, Fang Guo, Alan Kim Johnson, Robert B. Felder

Abstract—Blood-borne angiotensin II (ANG II) can upregulate p44/42 mitogen-activated protein kinase (MAPK) signaling and ANG II type-1 receptors in the hypothalamic paraventricular nucleus (PVN), a critical cardiovascular and autonomic center. We tested the hypothesis that brain p44/42 MAPK signaling contributes to the development of ANG II–induced hypertension. The ANG II infusion (120 ng/kg per min, subcutaneously) induced increases in phosphorylated p44/42 MAPK and ANG II type-1 receptors in the PVN after 1 week, before the onset of hypertension, that were sustained as hypertension developed during a 2- or 3-week infusion protocol. Bilateral PVN microinjections of small interfering RNAs for p44/42 MAPK, at the onset of the ANG II infusion or 1 week later, prevented the early increase in p44/42 MAPK activity. The early treatment normalized ANG II type-1 receptor expression in the PVN and attenuated the hypertensive response to the 2-week infusion of ANG II. The later small interfering RNA microinjections had a transient effect on ANG II type-1 receptor expression in PVN and no effect on the hypertensive response to the 3-week infusion of ANG II. The early treatment also normalized the pressure response to ganglionic blockade. The ANG II infusion induced increases in mRNA for proinflammatory cytokines that were not affected by either small interfering RNA treatment. These results suggest that the full expression of ANG II–induced hypertension depends on p44/42 MAPK-mediated effects. A potential role for p44/42 MAPK in modulating the ANG II–induced central inflammatory response might also be considered. MAPK signaling in PVN may be a novel target for early intervention in the progression of ANG II-dependent hypertension. (Hypertension. 2013;61:842-849.) * Online Data Supplement

Key Words: autonomic regulation • brain • proinflammatory cytokines • renin-angiotensin system

Hypertension is associated with augmented renin–angiotensin system (RAS) activity and an increase in proinflammatory cytokines (PICs) in the periphery and in the brain.1–5 Overactivity of the brain RAS and PICs has been implicated in the development and the maintenance of hypertension in multiple experimental and genetic animal models, via alterations in body fluid homeostasis, neurohormonal release, and sympathetic outflow.2,4,6 Interventions that reduce the expression of RAS or PICs in the brain can significantly ameliorate these effects and attenuate hypertension.2,4,8

Recent studies from our laboratory and others have demonstrated that p44/42 mitogen-activated protein kinase (MAPK) signaling regulates the expression of RAS and PICs in the brain.9,11 p44/42 MAPK is expressed in several brain regions associated with cardiovascular and autonomic regulation, including the paraventricular nucleus of hypothalamus (PVN) and the subfornical organ (SFO).9,10 Blood-borne angiotensin II (ANG II), which is increased in heart failure and many forms of hypertension, increases p44/42 MAPK activity in the PVN and SFO.11 Activation of p44/42 MAPK can upregulate the PVN expression of ANG II type-1 receptor (AT1R)9–11 and of PICs.12,13 Pharmacological inhibition of p44/42 MAPK signaling in the brain can reduce AT1R expression in the PVN of normal rats subjected to a continuous low dose of ANG II, sympathetic activity in rats with heart failure induced by myocardial infarction, and the pressor response to acute central administration of ANG II in normal rats.5,11 The prominent involvement of brain p44/42 MAPK signaling in ANG II–mediated cardiovascular and sympathetic responses led us to hypothesize an important role for p44/42 MAPK signaling in the PVN in the development of ANG II–induced hypertension.

Methods

Experimental Protocols

The slow ANG II–infusion protocol was used to induce hypertension in adult Sprague-Dawley rats, as previously described.2 Some
animals (n=33) underwent continuous monitoring of mean blood pressure (MBP) and heart rate (HR) by telemetry. These rats were anesthetized with ketamine–xylazine (100 and 10 mg/kg), and under sterile conditions a telemetry probe (TA11PA-C40, Data Science International) was implanted in a femoral artery. After a 1-week recovery period, baseline MBP and HR were recorded for 5 days. They were then reanesthetized with ketamine–xylazine, and under sterile conditions, an osmotic minipump (model 2002 for 2-week infusion; model 2004 for 3-week infusion, Alzet) was implanted subcutaneously to deliver ANG II (120 ng/kg per min). Others (n=84) received the ANG II infusion without telemetry monitoring.

To test the role of p44/42 MAPK signaling in the PVN in the development of ANG II–induced hypertension, rats received bilateral PVN microinjections of p44/42 small interfering RNA (siRNA), scrambled siRNA, or vehicle, at 1 of 2 time points during the ANG II infusion. The early treatment group (n=60, including 18 rats with telemetry probes), received the PVN microinjections and implantation of the osmotic minipump at the same time. The duration of the ANG II infusion in this group was 2 weeks. The late treatment groups (n=57, including 15 rats with telemetry probes) received the PVN microinjections in a separate sterile surgical procedure under ketamine–xylazine anesthesia 1 week after starting the ANG II infusion. The duration of the ANG II infusion in this group was 3 weeks. In animals with telemetry probes, sympathetic tone was assessed at baseline and 2 weeks after the PVN microinjections by examining the MBP response to ganglionic blockade with hexamethonium bromide (30 mg/kg, intraperitoneal).

One week or 2 weeks after the PVN microinjections, animals in both groups were euthanized with an overdose of urethane to collect brain tissue for molecular studies. The thoracic aorta and heart tissues were also collected for molecular or anatomic studies in some animals after 2 or 3 weeks of ANG II infusion. Untreated rats (n=22) were used as controls.

Additional animals were used in control studies to determine the specificity and optimal dose of the p44/42 siRNA (n=15), the effects of the siRNA microinjections on central indicators of inflammation (n=12), and the accuracy of the PVN microinjections (n=6).

### Additional Methods

Please see the online-only Data Supplement.

### Results

#### Hemodynamic and PVN Molecular Effects of the Slow Pressor ANG II Infusion

In rats receiving the 2-week infusion of ANG II+vehicle (n=6), in the early treatment protocol, MBP increased slightly but not significantly during the first week of the ANG II infusion, but increased dramatically during week 2, peaking at 136±6 mm Hg (Figure 1A). Rats receiving the 3-week infusion of ANG II+vehicle (n=5), in the later treatment protocol, had a similar rise in MBP in week 2 and a further increase in week 3, peaking at 159±5 mm Hg (Figure 1C).

There were no significant changes in HR in either protocol. Hexamethonium bromide administered at week 2 (Figure

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**Figure 1.** The effect of bilateral paraventricular nucleus (PVN) microinjections of p44/42 mitogen-activated protein kinase (MAPK) small interfering RNA (siRNA) on angiotensin II (ANG II)-induced hypertension in rats. Daily mean blood pressure (MBP) and heart rate (HR) before and during systemic infusion of ANG II in rats treated early (A and B) or late (C and D) with p44/42 siRNA, a scrambled siRNA, or vehicle. Values are mean±SEM (n=5–6 for each group). *P<0.05, vs baseline; and †P<0.05, ANG II+p44/42 siRNA vs ANG II+vehicle or ANG II+scrambled siRNA.
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2A) or week 3 (Figure 2B) to assess sympathetic tone elicited a \( \approx 60 \) mmHg drop in MBP, substantially larger than that observed at baseline.

In the PVN of rats infused with ANG II+vehicle, compared with control rats, phosphorylated (ph-) p44/42 MAPK (Figure 3) and AT\( \text{R} \) (Figures 4 and 5) expression had increased at the end of week 1, before the rise in MBP, and there were small, but significant, increases in mRNA for interleukin (IL)-1\( \beta \) (Figure 6) and cyclooxygenase (COX)-2 (Figure 7). At that time point, there were no changes in mRNA expression of AT\( \text{R} \) or mRNA for tumor necrosis factor (TNF)-\( \alpha \), IL-6, IL-4, or COX-1. After 2 or 3 weeks of ANG II+vehicle infusion, as pressure was rising, the increases in ph-p44/42 and AT\( \text{R} \) were sustained, the increases in mRNA for IL-1\( \beta \) and COX-2 were larger, mRNA for TNF-\( \alpha \) and IL-6 had also increased, and mRNA for IL-4 had decreased. There were no changes in mRNA for AT\( \text{R} \) or COX-1.

**Effects of PVN Microinjections of p44/42 SiRNA**

Early treatment with bilateral PVN microinjections of p44/42 siRNA, delivered at the onset of the ANG II infusion, had no effect on MBP in week 1, but significantly attenuated the rise in MBP in week 2 (Figure 1A). Sympathetic tone, as measured by the response to hexamethonium bromide (Figure 2A), was normalized by the p44/42 siRNA. Later treatment with p44/42 siRNA, at the end of week 1 of ANG II infusion but still in the prehypertensive phase, had no significant effect on the subsequent ANG II–induced rise in MBP (Figure 1C) or the response to hexamethonium bromide (Figure 2B). Neither treatment affected HR. Treatment with a scrambled siRNA control had no effect on ANG II–induced increases in MBP or HR.

At the end of week 1, the ANG II–infused rats treated early with p44/42 siRNA had reduced total p44/42 MAPK and normal ph-p44/42 MAPK (Figure 3A and 3B) and AT\( \text{R} \) levels (Figures 4A and 5A) in the PVN, compared with normal control rats. At the end of week 2, the reductions in p44/42 MAPK and ph-p44/42 MAPK were no longer present, but AT\( \text{R} \) mRNA and protein remained at normal levels. The early p44/42 siRNA treatment had no effect on mRNA for AT\( \text{R} \), IL-1\( \beta \), TNF-\( \alpha \), IL-6, IL-4, COX-1, or COX-2 (Figures 4B, 6, and 7).

The ANG II–infused rats that were treated later with p44/42 siRNA had similar reductions in total p44/42 and ph-p44/42 (Figure 3C and 3D) and in AT\( \text{R} \) levels (Figures 4C and 5B) expression in the PVN 1 week after receiving the PVN microinjections. However, 2 weeks after the PVN microinjections, both p44/42 and AT\( \text{R} \) expression in these rats was similar to that in the ANG II–infused rats treated with vehicle or a scrambled siRNA. The later p44/42 siRNA treatment also had no
Effect on the mRNA for AT₁R or the inflammatory mediators (Figures 4D, 6, and 7).

Effects of ANG II Infusion and PVN Microinjections of p44/42 siRNA on Heart and Vascular Tissues

Two weeks of ANG II infusion significantly increased mRNA expression of IL-1β, TNF-α, and IL-6, and decreased mRNA expression of IL-4 in the thoracic aorta of rats receiving PVN microinjections of vehicle or scrambled siRNA, compared with control rats. These changes were similar to those observed in the brain of ANG II–infused rats (Figure S2 in the online-only Data Supplement). The early treatment with PVN microinjections of p44/42 siRNA had no effect on these ANG II–induced inflammatory changes.

As seen in Table S2, the heart weight (HW) and HW/body weight (BW) ratio were significantly higher in the ANG II–infused rats treated with vehicle or scrambled siRNA. The ANG II–infused rats that received early PVN microinjections of p44/42 siRNA had significantly lower HW/BW ratio. The later PVN microinjections of p44/42 siRNA had no effect on the HW/BW ratio. There was no significant difference in BW across the experimental groups.

Control Studies

In the absence of ANG II infusion, PVN microinjections of p44/42 siRNA, scrambled siRNA, or vehicle alone had no effect on the expression of inflammatory mediators in the PVN (Figure S3 in the online-only Data Supplement). The average cycle threshold values for each gene in control rats were similar at the 2- and 3-week time points (data not shown).

In a separate group of rats (n=6), microinjections of sky blue dye validated the coordinates used for the PVN microinjections of siRNA (Figure S4 in the online-only Data Supplement).
The novel finding of this study is that the full expression of ANG II–induced hypertension requires the early engagement of p44/42 MAPK signaling in the PVN. A previous study from our laboratory demonstrated that a chronic (4-week) subcutaneous administration of a low dose of ANG II increased both ph-p44/42 MAPK and AT1R expression in PVN and SFO. Intracerebroventricular administration of the AT1R blocker losartan prevented the phosphorylation of p44/42 MAPK, and intracerebroventricular administration of either losartan or the p44/42 MAPK inhibitor PD98059 prevented the increase in AT1R. Those findings demonstrated that systemically administered ANG II upregulates AT1R expression in PVN and SFO in a p44/42 MAPK-dependent manner. In the present study, a slow pressor dose of ANG II that induced hypertension increased ph-p44/42 MAPK and AT1R mRNA and protein in the PVN, and early intervention to reduce p44/42 MAPK activity in PVN significantly attenuated the ANG II–induced increases in AT1R expression, sympathetic nerve activity, blood pressure, and indices of cardiac remodeling. The ANG II infusion also upregulated mRNAs for the inflammatory mediators IL-1β, IL-6, TNF-α, and COX-2, but these were not affected by interrupting p44/42 MAPK signaling. There was no apparent effect of the ANG II infusion or of p44/42 MAPK activity on AT1R expression in the PVN. These results demonstrate that p44/42 MAPK signaling in the PVN is a pivotal mechanism in the prehypertensive phase of ANG II–induced hypertension.

Considering the ability of ANG II to upregulate its own receptors in cardiovascular regions of the brain and the well-recognized role of the brain RAS in hypertension, the most likely explanation for the salutary effect of early intervention in PVN p44/42 MAPK signaling is the observed reduction in AT1R expression. The early siRNA treatment was more effective in that regard, reducing AT1R at weeks 1 and 2, during which MBP remained significantly lower than expected. This is particularly interesting because the effect of the early treatment on AT1R expression outlasted the transient effect of the siRNA to suppress the ph-p44/42 levels. SiRNA treatment a week after starting the ANG II infusion also reduced AT1R expression in the PVN, when measured at ANG II infusion week 2, but did not attenuate the ANG II–induced rise in blood pressure. These findings suggest that there is a narrow therapeutic window for effective intervention in the upregulation of brain RAS activity in the prehypertensive phase of ANG II hypertension. Notably, at the 2-week time point, the ANG II infusion had induced a broader and more vigorous inflammatory response in the PVN that was unaffected by the p44/42 siRNA treatment and was sustained for the remainder of the 3-week infusion protocol. This ANG II–induced rise in PICs may explain the failure of later intervention to affect the rise in MBP or the expression of AT1R at week 3.

**Figure 6.** Quantitative comparison of the mRNA expression for interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-4, and IL-6 in the paraventricular nucleus (PVN) of angiotensin II (ANG II)-infused rats treated early (A–D) or late (E–H) with PVN microinjections of p44/42 mitogen-activated protein kinase (MAPK) small interfering RNA (siRNA), a scrambled siRNA, or vehicle. Untreated rats served as Control. Values are mean±SEM (n=6–8 for each group). *P<0.05, vs Control.

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**Discussion**

The novel finding of this study is that the full expression of ANG II–induced hypertension requires the early engagement of p44/42 MAPK signaling in the PVN. A previous study from our laboratory demonstrated that a chronic (4-week) subcutaneous administration of a low dose of ANG II increased both ph-p44/42 MAPK and AT1R expression in PVN and SFO. Intracerebroventricular administration of the AT1R blocker losartan prevented the phosphorylation of p44/42 MAPK, and intracerebroventricular administration of either losartan or the p44/42 MAPK inhibitor PD98059 prevented the increase in AT1R. Those findings demonstrated that systemically administered ANG II upregulates AT1R expression in PVN and SFO in a p44/42 MAPK-dependent manner. In the present study, a slow pressor dose of ANG II that induced hypertension increased ph-p44/42 MAPK and AT1R mRNA and protein in the PVN, and early intervention to reduce p44/42 MAPK activity in PVN significantly attenuated the ANG II–induced increases in AT1R expression, sympathetic nerve activity, blood pressure, and indices of cardiac remodeling. The ANG II infusion also upregulated mRNAs for the inflammatory mediators IL-1β, IL-6, TNF-α, and COX-2, but these were not affected by interrupting p44/42 MAPK signaling. There was no apparent effect of the ANG II infusion or of p44/42 MAPK activity on AT1R expression in the PVN. These results demonstrate that p44/42 MAPK signaling in the PVN is a pivotal mechanism in the prehypertensive phase of ANG II–induced hypertension.

Considering the ability of ANG II to upregulate its own receptors in cardiovascular regions of the brain and the well-recognized role of the brain RAS in hypertension, the most likely explanation for the salutary effect of early intervention in PVN p44/42 MAPK signaling is the observed reduction in AT1R expression. The early siRNA treatment was more effective in that regard, reducing AT1R at weeks 1 and 2, during which MBP remained significantly lower than expected. This is particularly interesting because the effect of the early treatment on AT1R expression outlasted the transient effect of the siRNA to suppress the ph-p44/42 levels. SiRNA treatment a week after starting the ANG II infusion also reduced AT1R expression in the PVN, when measured at ANG II infusion week 2, but did not attenuate the ANG II–induced rise in blood pressure. These findings suggest that there is a narrow therapeutic window for effective intervention in the upregulation of brain RAS activity in the prehypertensive phase of ANG II hypertension. Notably, at the 2-week time point, the ANG II infusion had induced a broader and more vigorous inflammatory response in the PVN that was unaffected by the p44/42 siRNA treatment and was sustained for the remainder of the 3-week infusion protocol. This ANG II–induced rise in PICs may explain the failure of later intervention to affect the rise in MBP or the expression of AT1R at week 3.
The contribution of central interactions between ANG II and the PICs to the pathophysiology of hypertension is well described in the extant literature. Microglial cells express AT1R receptors, and chronic ANG II infusion stimulates the production of inflammatory mediators in PVN—including TNF-α, IL-1β, and IL-6, the PICs measured in the present study. Reducing the expression of these mediators by inhibiting microglial activation or by overexpressing the anti-inflammatory cytokine IL-10 significantly attenuates the blood pressure response. In the present study, the more substantial expression of TNF-α, IL-1β, and IL-6 at 2 and 3 weeks may well have contributed to the continued rise in MBP. The associated increase in the expression of COX-2, which is induced by the PICs, may also be a contributing factor. COX-2 is the limiting enzyme in the synthesis of prostaglandin E2, which is known to disinhibit parvocellular PVN neurons. We have demonstrated a role for cytokine-induced COX-2 activity in sympathetic activation in heart failure. And, although the present study provided no evidence for upregulation of COX-1 activity in PVN, a role for constitutively expressed COX-1, an alternative route for prostaglandin E2 production that has been reported to contribute to hypertension in this model, might also be considered.

The increase in inflammatory mediators may have contributed to upregulation of AT1R at the later time points, independent of ph-p44/42 MAPK activity. The tight link between inflammatory mediators and the brain RAS is emphasized by recent work in the heart failure model, demonstrating that expression of PICs and AT1R is affected by agents that block either pathway. More pertinent to the present study is the observation that blocking TNF-α in the PVN of rats with heart failure reduced the PVN expression of AT1R. The crucial link between these 2 systems appears to be nuclear factor κB—the PICs, acting through nuclear factor κB, are known to upregulate the expression of AT1R and angiotensinogen, and ANG II apparently upregulates both brain RAS components and PICs via this transcription factor. Finally, a potential role for inflammation as an alternative stimulus to MAPK activity deserves mention. Although the PICs are more commonly associated with activation of p38 MAPK, in unpublished work, we have found that an acute intracarotid artery injection of TNF-α can activate p44/42 MAPK signaling in PVN, and that intracerebroventricular administration of a p44/42 MAPK inhibitor reduces the associated sympathetic response. It is therefore conceivable that at least some of the observed effects of the p44/42 siRNA to reduce ANG II–induced increases in blood pressure and sympathetic activity may be attributed to inhibiting the downstream effects of PICs.

The ANG II infusion induced peripheral cardiovascular effects, including vascular inflammation and cardiac remodeling. The vascular inflammatory effects were unaffected by a reduction in PVN ph-p44/42 MAPK, suggesting their dependence on the local effects of circulating ANG II. In contrast, the increase in HW/BW ratio was significantly reduced by the PVN microinjections of p44/42 MAPK small interfering RNA (siRNA), a scrambled siRNA, or vehicle. Untreated rats served as Control. Values are means±SEM (n=6–8 for each group). *P<0.05, vs Control.

Limitations of the Study

The present study did not determine the extent to which ANG II–induced hypertension is dependent on brain p44/42 MAPK signaling. A partial reduction in p44/42 activity in PVN attenuated, but did not prevent, the ANG II–induced hypertensive response. The residual blood pressure response might be explained by the incomplete knockdown of p44/42 MAPK. The effectiveness of microinjected siRNA is limited, in our hands, reducing p44/42 MAPK by only about 38%, and the reduction occurs in close proximity to the injection site.
site. Moreover, in the present study, the effect of the p44/42 siRNA to silence p44/42 expression was no longer present at week 2 after the microinjections, consistent with a previous report indicating that the maximal silencing effect of siRNA transfection typically occurs 5 to 7 days after injection. Viral transfection would likely produce a more widespread and sustained reduction in p44/42 MAPK, with greater impact on the measured variables.

Other possible explanations for the residual ANG II–induced rise in blood pressure might also be entertained, including the potential involvement of MAPK signaling and AT1R upregulation in other central cardiovascular nuclei (eg, SFO, rostral ventrolateral medulla) that contribute to sympathetic activation in hypertension. The PVN was singled out for these microinjections, but the effects of MAPK on angiotensinergic signaling in other key regions in which it has been identified (eg, in SFO) have yet to be studied. In addition, whereas p44/42 MAPK signaling was the focus of this investigation, a role for other MAPK signaling pathways (eg, p38 MAPK, c-Jun N-terminal kinase) cannot be excluded.

Finally, the influence of other excitatory mediators (eg, aldosterone, PICs) that may be present in the PVN and in other cardiovascular-related regions of the brain must also be considered. We recently demonstrated that aldosterone activates p44/42 MAPK in PVN via an interaction with AT1R that apparently mediates its central effects on sympathetic drive. Other recent studies have shown that ANG II–induced hypertension can be largely blocked by a mineralocorticoid receptor antagonist, reinforcing the concept of a central interdependence of ANG II and aldosterone effects.

Perspectives
This study identifies an intracellular signaling mechanism in the PVN that is activated in the prehypertensive phase of ANG II–induced hypertension, and that can be manipulated early to prevent the full expression of the hypertensive state. Blocking brain p44/42 MAPK signaling has also been shown to ameliorate sympathetic activation in an animal model of systolic heart failure. Although the present study focused on the effects of ANG II–induced p44/42 MAPK signaling in the upregulation of the AT1R and brain RAS activity, as illustrated in Figure 8, this mechanism can also be activated by aldosterone and the PICs, either directly or indirectly via their effects on RAS. As an inducible downstream signaling pathway for several key sympathoexcitatory mediators present in the brain in heart failure and hypertension, p44/42 MAPK signaling seems an ideal target for intervention in cardiovascular disease states.

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Disclosures
None.

References

Novelty and Significance

What Is New?
- Activation of the p44/42 mitogen-activated protein kinase (MAPK) pathway in the hypothalamic paraventricular nucleus (PVN) is necessary for the full expression of the slow pressor response to angiotensin II (ANG II).

What Is Relevant?
- In ANG II–induced hypertension, p44/42 MAPK signaling upregulates ANG II type-1 receptor (AT1) expression in PVN.
- ANG II–induced upregulation of inflammatory mediators in PVN is independent of p44/42 MAPK signaling.

What Is Important?
- Early interference with p44/42 MAPK signaling in PVN ameliorates ANG II–induced hypertension.

Summary
Pretreatment of PVN with p44/42 small interfering RNA (siRNA) reduces the ANG II–induced increase in AT1R expression in the PVN, and the full expression of ANG II–induced hypertension. Pretreatment with p44/42 siRNA does not affect the PVN expression of inflammatory mediators, but may reduce their downstream influences on blood pressure and sympathetic activity.
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to

EARLY INTERFERENCE WITH P44/42 MAPK SIGNALING IN HYPOTHALAMIC
PARAVENTRICULAR NUCLEUS ATTENUATES ANGIOTENSIN II - INDUCED
HYPERTENSION

*Yang Yu¹, *Bao-Jian Xue², *Zhi-Hua Zhang¹, Shun-Guang Wei¹, Terry G Beltz², Fang Guo²,
Alan Kim Johnson² and Robert B Felder¹,³

¹Department of Internal Medicine, Roy J and Lucille A Carver College of Medicine
²Department of Psychology
University of Iowa, Iowa City, IA, 52242
and
³Veterans Affairs Medical Center, Iowa City, IA, 52246

*These three authors contributed equally to this work.

Running Title: Brain p44/42 MAPK signaling in ANG II hypertension
SPECIFIC METHODS

Animals
Adult male Sprague-Dawley rats (250 to 300 g; Harlan Sprague-Dawley, Indianapolis, IN) were housed in temperature- (23±2°C) and light-controlled animal quarters and were provided with rat chow ad libitum. All experimental procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

Dose-response relationship for paraventricular nucleus (PVN) microinjection of p44/42 MAPK siRNA
Extrapolating from doses of siRNA used in vivo to silence target genes in the brain in rodents, reported by us1 and others,2,3 we tested the ability of three different concentrations of the pooled p44/42 siRNA to reduce p44/42 MAPK expression in the PVN of normal rats (Figure S1). A dose-response curve was generated in normal rats (n=15). A 5.6 ng, 28 ng or 56 ng pooled siRNA (Thermo Fisher Scientific Inc.) targeting p44/42 MAPK in 0.5 µl 10 mM JetSI™ (Polyplus-transfection, Inc) was microinjected bilaterally into PVN. Scrambled siRNA or vehicle (artificial cerebrospinal fluid) were used as controls. These siRNAs are specifically designed for in vivo use in animal models. JetSI™ 10 mM, a cationic amphiphile molecule that has been used successfully for delivery of siRNA into the brain by intracerebroventricular or hypothalamic injection4, 5 was used as the transfection reagent in this study. Five days later, rats were euthanized with an overdose of urethane to collect the brain for molecular study.

Western blot revealed that the smallest dose of p44/42 siRNA (5.6 ng) had no significant effect on p44/42 MAPK expression in the PVN, compared with scrambled siRNA or vehicle. A modest dose of p44/42 siRNA (28 ng) resulted in a significant reduction in both total and phosphorylated p44/42 MAPK (Figure S1). A higher dose of p44/42 siRNA (56 ng) had no additional effect. The p44/42 siRNA had no effects on either total p38 MAPK or c-Jun N-terminal kinase (JNK) (Figure S1), the two other major MAPK signaling pathways, indicating the specificity of p44/42 siRNA. We used the lower of the two effective doses of p44/42 siRNA (28 ng) to evaluate the role of p44/42 MAPK in the PVN in ANG II-induced hypertension.

PVN microinjections
PVN microinjection was performed as previously described1,6 Briefly, a 29-gauge guide cannula was inserted 0.5 mm above the PVN region. A 35-gauge (128 μm OD; 51.2 μm ID) stainless steel injection cannula was attached to PE-10 tubing, which was then connected to a 0.5-μl Hamilton microsyringe. The tip of the injection cannula was inserted into the guide cannula and then adjusted to a length extending 0.5 mm beyond the tip of the guide cannula. Bilateral microinjections were made in a volume of 0.5 μl over 30 sec. The PVN injection cannulae were positioned 1.8 mm posterior to bregma, 0.4 mm from midline, 7.6 mm ventral to dura.

To verify the accuracy of the PVN microinjection, 0.5 μl 2% pontamine sky blue was microinjected bilaterally into the same stereotaxic location (n=6). One hour after the dye microinjection, the animal was perfused. The brain was removed and postfixed in 4% paraformaldehyde for 24 hours. Coronal brain sections (40 μm) were made with a microtome and the needle track was microscopically examined. In all cases, the microinjection sites were located within the PVN.
To control for the possibility that the siRNA microinjections might independently elicit an inflammatory response, untreated rats underwent bilateral PVN microinjections of p44/42 siRNA, scrambled siRNA or vehicle (n=4 for each group). One week later, rats were euthanized with an overdose of urethane to collect the brains for real-time PCR to assess pro-inflammatory cytokine expression.

**Dissection of brain tissue for molecular study**

The PVN tissue was obtained as previously described. Briefly, brain tissues were stored at -80°C. The brain was cut into 500-μm coronal sections. The PVN region was punched with a 15-gauge needle stub (ID: 1.5 mm). This method necessarily includes a small amount of surrounding hypothalamic tissue.

**Western blot analysis**

Protein was extracted using cell lysis buffer (Cell Signaling Technology Inc, Beverly, MA). Protein level for p-p44/42 and total p44/42 were measured by Western blot analysis, as previously described, using primary antibodies to p-p44/42 (Thr202/Tyr204; no. 4377; 1:250), total p44/42, p38 and JNK (no. 4695, 9212 and 9252, respectively; 1:1,000, Cell Signaling Technology, Danvers, MA) and angiotensin II (ANG II) type-1 receptors (AT1R; SC-1173, 1:500, Santa Cruz, CA). The density of the bands was quantified using Image Lab analysis software (Bio-Rad, Hercules, CA).

**Quantification of mRNA expression**

The total RNA was extracted using TRI Reagent (Molecular Research Center, Inc). mRNA levels for renin-angiotensin system components (AT1R and ANG II type-2 receptor) and inflammatory mediators [interleukin-1β, tumor necrosis factor-α, cyclooxygenase (COX)-1 and COX-2] were analyzed with TaqMan or SYRB Green real-time PCR following reverse transcription of total RNA. The sequences for the primers and probes used are summarized in Table S1. Primers and probes for TaqMan GAPDH were purchased from Applied Biosystems (Foster City, CA). Real-time PCR was performed using the ABI prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA). The values were normalized to GAPDH and the final concentration of mRNA was calculated using the formula $x=2^{-\Delta\Delta Ct}$, where $x$=fold difference relative to control.

**Statistical Analysis**

All data are expressed as mean±SEM. The significance of differences in mean values was analyzed by one-way or two-way repeated-measure ANOVA followed by Fisher's post hoc test. $P<0.05$ was considered statistically significant.
REFERENCES


<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers and Probes</th>
</tr>
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<tbody>
<tr>
<td>AT1R (SYBR)</td>
<td>Forward primer: 5'- GGATGTTCTCAGAGAGAGTACAT-3'</td>
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<td>Reverse primer: 5'- CCTGCCCTTGTACCTGTGTG-3'</td>
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<td>IL-6 (SYBR)</td>
<td>Forward primer: 5'- TCCTACCCCAACTTCCATGCTC-3'</td>
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<td>Reverse primer: 5'- TTGGATGCTCTGGCTCTTAGCC-3'</td>
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<td>IL-4 (SYBR)</td>
<td>Forward primer: 5'- ATGGGTCAGCCCCCACCTTG-3'</td>
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<td>Reverse primer: 5'- ATCCGTGATACTCTCCTCCGTT-3'</td>
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<td>GAPDH (SYBR)</td>
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<td>Reverse primer: 5'- ATGTAGGCAATGAGGTCCAC-3'</td>
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<td>AT2R (TaqMan)</td>
<td>Forward primer: 5'- CAATCTGGCTTGGCTGACCT-3'</td>
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<td>Reverse primer: 5'- TGCACATCAGCTCAAAAGA-3'</td>
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<td>Probe: 5'- CAACCCCTTCTCTCTCTGGGCAACCTATTACTCTTATA-3'</td>
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<td>IL-1β (TaqMan)</td>
<td>Forward primer: 5'- CACCTCTCAAGCAGACACAG-3'</td>
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<td>Probe: 5'- TGTCGCCAATTTCTGGTTCCTTCTGGG-3'</td>
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<td>TNF-α (TaqMan)</td>
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<td>Probe: 5'- AGAGGCCCCTTCCGTAAGACCCCTTTC-3'</td>
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<td></td>
<td>Probe: 5'- TCTTTGCCAGCAGCTCCTCAGTCTT-3'</td>
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</table>

AT1R: angiotensin II type-1 receptors; IL-6: interleukin-6; IL-4: interleukin-4; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; AT2R: angiotensin II type-1 receptors; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; COX-1: cyclooxygenase-1; COX-2: cyclooxygenase-2
Table S2. Effects of p44/42 MAPK activity in PVN on ANG II-induced cardiac remodeling

<table>
<thead>
<tr>
<th>Anatomical Variables</th>
<th>Control (n=8)</th>
<th>ANG II + vehicle (n=10)</th>
<th>ANG II + p44/42 siRNA (n=10)</th>
<th>ANG II + scrambled siRNA (n=10)</th>
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</thead>
<tbody>
<tr>
<td>Two weeks after early siRNA treatment</td>
<td></td>
<td></td>
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<tr>
<td>BW (g)</td>
<td>383 ± 3</td>
<td>379 ± 4</td>
<td>378 ± 4</td>
<td>382 ± 5</td>
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<td>HW (mg)</td>
<td>997 ± 23</td>
<td>1093 ± 23*</td>
<td>1029 ± 24</td>
<td>1085 ± 20*</td>
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<td>HW/BW (mg/g)</td>
<td>2.60 ± 0.06</td>
<td>2.88 ± 0.12*</td>
<td>2.72 ± 0.06†</td>
<td>2.84 ± 0.06*</td>
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<tr>
<td>Two weeks after late siRNA treatment</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>396 ± 4</td>
<td>385 ± 6</td>
<td>390 ± 7</td>
<td>387 ± 8</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>996 ± 21</td>
<td>1124 ± 30*</td>
<td>1119 ± 35*</td>
<td>1127 ± 33*</td>
</tr>
<tr>
<td>HW/BW (mg/g)</td>
<td>2.52 ± 0.04</td>
<td>2.92 ± 0.07*</td>
<td>2.87 ± 0.06*</td>
<td>2.91 ± 0.07*</td>
</tr>
</tbody>
</table>

BW: body weight; HW: heart weight. Values are expressed as mean ± SEM. *P < 0.05, vs. Control; †P < 0.05, ANG II + p44/42 siRNAs vs. ANG II + vehicle or ANG II + scrambled siRNA.
Figure S1. Dose-response relationship and specificity for p44/42 MAPK siRNAs. Pooled siRNAs targeting p44/42 at doses of 5.6 ng, 28 ng or 56 ng in 0.5 µl delivery reagent were microinjected bilaterally into PVN of normal rats. Non-targeting scrambled siRNA or vehicle (aCSF) were used as controls. 5 days later, Western blot from PVN, including some immediately surrounding tissue, revealed significant reduction in both total and phosphorylated p44/42 MAPK with the 28 ng dose. Notably, p44/42 siRNA had no effects on total p38 or JNK, two other major components of MAPK family. Values are mean ± SEM (n = 3 for each group). *P< 0.05 vs. vehicle or scrambled siRNA.
**Figure S2.** The effect of bilateral PVN microinjection of p44/42 MAPK siRNA on mRNA expression for IL-1β, TNF-α, IL-4 and IL-6 in thoracic aorta of ANG II-infused rats treated early with p44/42 MAPK siRNA, a scrambled siRNA, or vehicle. Data was obtained at 2 weeks after PVN microinjection. Untreated rats served as Control. Values are mean ± SEM (n = 4 for each group). *P< 0.05, vs. Control.
Figure S3. The effect of bilateral PVN microinjection of p44/42 MAPK siRNA, a scrambled siRNA, or vehicle alone on mRNA expression for IL-1β, TNF-α and COX-2 in the PVN in rats without ANG II infusion. Untreated rats served as Control. Values are mean ± SEM (n = 4-8 for each group).
Figure S4. A representative photomicrograph of bilateral PVN microinjections of 2% pontamine sky blue dye in a rat. The arrows indicate needle tracts penetrating the PVN. Technical artifact is present.