Central Mineralocorticoid Receptors and the Role of Angiotensin II and Glutamate in the Paraventricular Nucleus of Rats With Angiotensin II–Induced Hypertension

Alexander Gabor, Frans H.H. Leenen

Abstract—A chronic increase in circulating angiotensin II (Ang II) activates an aldosterone–mineralocorticoid receptor-ouabain neuromodulatory pathway in the brain that increases neuronal activation in hypothalamic nuclei, such as the paraventricular nucleus (PVN) and causes progressive hypertension. Several models of chronic sympathetic hyperactivity are associated with an increase in AT1 and glutamate receptor activation in the PVN. The current study evaluated whether increased angiotensin type 1 (AT1) and glutamate receptor-dependent signaling in the PVN contributes to the maintenance of blood pressure (BP) in Ang II-hypertensive Wistar rats, and the role of aldosterone–mineralocorticoid receptor pathway in this enhanced signaling. After subcutaneous infusion of Ang II for 2 weeks, in conscious rats BP and heart rate were recorded after (1) 10-minute bilateral infusions of candesartan and kynurenate in the PVN; (2) 1 hour intracerebroventricular infusion of eplerenone, and (3) candesartan and kynurenate after eplerenone. Candesartan or kynurenate in the PVN fully reversed the increase in BP from circulating Ang II. Kynurenate after candesartan or candesartan after kynurenate did not further lower BP. Intracerebroventricular infusion of eplerenone at 16 hours after its infusion fully reversed the increase in BP from circulating Ang II. After eplerenone, candesartan and kynurenate in the PVN did not further decrease BP. These findings suggest that increased mineralocorticoid receptor activation in the brain activates a slow neuromodulatory pathway that maintains enhanced AT1 and glutamate receptor-dependent signaling in the PVN, and thereby the hypertension from a chronic increase in circulating Ang II. (Hypertension. 2013;61:1083-1090.) ● Online Data Supplement

Key Words: aldosterone ■ angiotensin II ■ blood pressure ■ glutamate ■ mineralocorticoid receptor ■ paraventricular nucleus

A chronic increase in circulating angiotensin II (Ang II) increases neuronal activation in hypothalamic nuclei, such as the paraventricular nucleus (PVN),1,2 and causes progressive hypertension.2,3 Presumably by increasing sympathetic activity,4,5 a central neuromodulatory pathway involving aldosterone–endogenous ouabain (EO) seems to play a critical role in these responses to Ang II.2,27 Chronic subcutaneous infusion of Ang II increases plasma and hypothalamic aldosterone content.2 Intracerebroventricular infusion of an aldosterone synthase (AS) inhibitor prevents increase in hypothalamic, but not in plasma aldosterone, and intracerebroventricular infusion of an AS inhibitor or a mineralocorticoid receptor (MR) blocker markedly attenuate the neuronal activation in the PVN.2 Intracerebroventricular infusion of an AS inhibitor,2 MR blocker,27 or an angiotensin type 1 (AT1)–receptor blocker4 largely prevents the Ang II–induced hypertension. These findings suggest that a chronic increase in circulating Ang II increases local hypothalamic aldosterone production, and activates an MR–EO pathway in the brain, which is essential for the Ang II–induced hypertension.

Several models of chronic sympathetic hyperactivity are associated with an increase in glutamate receptor and AT1–receptor activation in the PVN. A glutamate or AT1–receptor blocker in the PVN decreases sympathetic nerve activity, blood pressure (BP), and heart rate (HR) in rats with chronic heart failure8,10 and in spontaneously hypertensive rats.11 Glutamate and AT1–receptor blockers in the PVN decrease BP in water-deprived rats12 and in Dahl S rats on high-salt diet.13 In Dahl S rats on a high-salt diet, at the peak BP decrease by a glutamate receptor blocker, an AT1–receptor blocker in the PVN does not further decrease BP.13 These findings suggest that the effects of increased AT1–receptor activation in the PVN of hypertensive Dahl S rats are fully mediated by local glutamate release. Consistent with these findings, Ang II increases glutamatergic signaling in the PVN, either by increasing glutamate release from interneurons,14,15 or by decreasing gamma-aminobutyric acid (GABA)-mediated inhibition of the PVN.16,17 No studies have yet evaluated the role of the central aldosterone neuromodulatory pathway in Ang II and glutamate receptor activation in the PVN of hypertensive rats. We hypothesized...
that increased MR activation in the brain contributes to enhanced AT₁-receptor activation in the PVN via increased local glutamate release, and thereby to the maintenance of hypertension from a chronic increase in circulating Ang II.

The current study first evaluated the effects of acute blockade of glutamate receptors or AT₁ receptors in the PVN on BP and HR after subcutaneous infusion of Ang II for 2 weeks. To assess the interaction of these 2 mechanisms, 1 blocker was infused at the peak BP response to the other blocker. Second, we evaluated the effects of intracerebroventricular infusion of an MR blocker on BP and HR of rats with subcutaneous infusion of Ang II, and the BP and HR responses to glutamate and AT₁ receptor blockade in the PVN after MR blockade.

Methods

For Animals and Diet and Surgical Procedures, see the online-only Data Supplement.

Experimental Procedures

For all experiments, rats were placed in a small cage and intra-arterial catheters were connected to a pressure transducer for recordings of BP and HR via a personal computer equipped with software AcqKnowledge (ACQ 3.9). Mean arterial pressure (MAP) and HR were extracted from the raw BP signal by software analysis as previously described.13

For all intracerebral infusions, L-shaped injection cannulas (30 gauge) were lowered unilaterally into the left lateral ventricle, or bilaterally into the PVN through the guide cannulas placed on day 7 of subcutaneous infusion, and extending 0.5 mm past the guide. Injection cannulas were connected to either a 10- or 500-µL Hamilton microsyringe mounted on a Harvard infusion pump (model No. 2400-003). Infusions were performed intracerebroventricular at 3.8 µL/min and into the PVN at 300 nL/min as in our previous studies.11,12 Animals were allowed to settle for ≥30 minutes before recording of baseline BP and HR levels for 10 minutes. For multiple infusions into the PVN, at the peak effect of the first drug, cannulas were removed, reloaded with the second drug, and reinserted into the PVN within 5 minutes of the end of the first infusion. At the end of the experiment, rats were euthanized in a CO₂ chamber, and Evans Blue dye (1%) was injected into the infusion sites. Brains were removed, frozen, sectioned using a Leica cryostat, and stained with neutral red. Infusion sites were considered to be inside the lateral ventricle if the dye was confined to the ventricles and not in brain tissue, and inside the PVN if the midpoint of the dye circle was inside the borders of the PVN.13 Only data from rats with injection sites inside the PVN (Figure S1 in the online-only Data Supplement) or lateral ventricle were used for analysis. The distribution of the volume infused into the PVN was described previously (Figure 2B).15

Drugs

Intracerebroventricular infusion of eplerenone was performed at 20 µg/3.8 µL for 1 minute, followed by 0.83 µg/3.8 µL for 60 minutes (online-only Data Supplement). Infusion of candesartan (0.5 µg/300 nL/min) or kynurenic acid (0.14 µg/300 nL/min) was performed bilaterally in the PVN (5 or 1.4 µg total on each side) for 10 minutes.13

Experimental Protocols

Kynurenic Acid and Candesartan in the PVN

Three experimental protocols were used in 3 different groups of rats. Arterial cannulations were performed in the afternoon of day 12 to 13 of subcutaneous infusion, and all BP recordings were conducted the following morning.

Kynurenic Acid in the PVN

Bilateral infusion of vehicle (300 nL/min for 10 minutes per side) was performed in the PVN of rats with subcutaneous Ang II at 150 or 500 ng/kg per minute. Twenty minutes after vehicle infusion, kynurenic acid was infused into the PVN. To assess whether the effects of the kynurenic acid may result from leakage into the ventricles, in an additional group of rats with subcutaneous Ang II at 500 ng/kg per minute, intracerebroventricular infusion of kynurenic acid was performed at the infusion rate used on each side of the PVN as well as the combined dose from both sides (0.14 or 0.28 µg/min for 10 minutes).

Kynurenic Acid Followed by Candesartan in the PVN

Candesartan was infused in the PVN of rats with subcutaneous Ang II at 150 or 500 ng/kg per minute. Kynurenic acid was infused in the PVN at the peak BP response to candesartan.

Kynurenic Acid Followed by Candesartan in the PVN

Candesartan was infused in the PVN at the peak BP response to kynurenic acid in rats with subcutaneous Ang II at 500 ng/kg per minute.

Intracerebroventricular Infusion of Eplerenone Followed by Kynurenic Acid and Candesartan in the PVN

Three experimental protocols were used in 3 groups of rats. Arterial cannulations were performed in the morning of day 12 to 13 of subcutaneous infusion. Timing of the BP recording varied according to the experimental protocols.

Intracerebroventricular Infusion of Eplerenone

A 1-hour intracerebroventricular infusion of eplerenone was performed in the afternoon (4:00–6:00 pm) after arterial cannulation in rats with subcutaneous Ang II at 500 ng/kg per minute. BP and HR were recorded at baseline and 1, ≥16, and ≥24 hours after start of eplerenone infusion. In a subgroup of rats with subcutaneous infusion of saline (vehicle), intracerebroventricular infusion of eplerenone was also performed in the afternoon, and BP and HR were recorded at baseline and 1 and ≥16 hours after start of eplerenone infusion.

Intracerebroventricular Infusion of Eplerenone With Kynurenic Acid and Candesartan in the PVN

To evaluate whether glutamate and AT₁-receptor blockade in the PVN further reduced BP after central MR blockade, a 1-hour intracerebroventricular infusion of eplerenone or vehicle was performed in the afternoon after arterial cannulation in rats with subcutaneous Ang II at 500 ng/kg per minute. The following morning (≥16 hours after the eplerenone infusion), infusion of candesartan was performed in the PVN, and BP and HR were recorded. Kynurenic acid was then infused into the PVN at the peak BP response to candesartan.

Intravenous Infusion of Eplerenone

To assess whether the BP effects from intracerebroventricular infusion of eplerenone result from its action in the brain, a 1-hour intravenous infusion of eplerenone was performed in the afternoon after arterial cannulation in rats with subcutaneous Ang II at 500 ng/kg per minute. BP and HR were recorded at baseline, and 1 and ≥16 hours after start of eplerenone infusion. The infusion dose, volume, and rate were the same as those used for intracerebroventricular infusions.

Statistical Analysis

See the online-only Data Supplement.

Results

Body weight was similar in rats after subcutaneous vehicle or Ang II for 2 weeks (322±13 g versus 320±10 and 316±3 g; Ang II 150 and 500 ng/kg per minute). Water intake was larger (P<0.05) at days 11 to 12 of subcutaneous Ang II compared with vehicle (36±2 and 44±2 mL/d; Ang II 150 and 500 ng/kg per minute versus 27±2 mL/d; n= 6, 12, 6).

BP and HR Responses to Subcutaneous Ang II

Resting MAP increased by 10 to 15 and 50 to 60 mm Hg after subcutaneous Ang II at 150 and 500 ng/kg per minute for 2 weeks (Table S1). HR was similar in rats infused with vehicle or Ang II (Table S1).
Kynurenate in the PVN or Intracerebroventricular Infusion of Rats With Subcutaneous Ang II

Bilateral infusion of vehicle in the PVN did not change MAP or HR (Figure 1). In contrast, kynurenate in the PVN rapidly decreased MAP and HR of rats with subcutaneous Ang II at 150 (Figure 1) or 500 ng/kg per minute (Figures 1 and 2). In rats with subcutaneous Ang II at 500 ng/kg per minute, MAP decreased \((P<0.05)\) by 20±2 mm Hg within the first 2 to 4 minutes and peak decreases by 60 mm Hg occurred 10 to 12 minutes after start of kynurenate (Figure 2). Kynurenate in the PVN fully reversed the increase in BP from subcutaneous Ang II (150 ng/kg per minute, from 125±4 to 108±4 mm Hg; 500 ng/kg per minute, from 170±2 to 114±8 mm Hg). Decreases in HR by 30 to 45 bpm were significantly different from vehicle according to the peak responses (Figure 1) or area under the curve (Figure 2).

In rats with positional failure of the PVN guide cannula, bilateral infusion of vehicle or kynurenate at tissue sites (most from 0.3–1 mm) outside the PVN caused nonsignificant changes in MAP and HR (vehicle with Ang II at 150 and 500 ng/kg per minute combined [MAP, ±1 mm Hg; HR, ±11; n=5]; kynurenate with Ang II at 150 and 500 ng/kg per minute combined [MAP, −1±1 mm Hg; HR, ±7, n=5]).

In rats with subcutaneous Ang II at 500 ng/kg per minute, intracerebroventricular infusion of kynurenate at the dose used in the PVN did not lower BP after 2 to 4 minutes, and decreased MAP by only 14±3 mm Hg after 4 to 6 minutes, with peak decreases in MAP by 30 to 35 mm Hg and in HR by 20 to 25 bpm at ±10 minutes (Figure 3). Intracerebroventricular infusion of kynurenate at 0.28 μg/min only partially reversed the Ang II–induced decrease in BP (from 181±3 to 148±4 mm Hg).

Candesartan Followed by Kynurenate in PVN

Candesartan decreased BP, but increased HR in rats with subcutaneous Ang II (Figures 1 and 2). In rats with subcutaneous Ang II at 500 ng/kg per minute, MAP began to decrease within the first 5 minutes and peak decreases occurred 25 to 30 minutes after start of infusion (Figures 1 and 2). HR increased within the first 5 minutes and peak increases by 30 to 40 bpm occurred 20 to 25 minutes after start of infusion (Figure 2). Candesartan in the PVN fully reversed the increase in BP from subcutaneous Ang II (150 ng/kg per minute, from 133±3 to 117±4 mm Hg; 500 ng/kg per minute, from 175±11 to 110±6 mm Hg). Candesartan infused outside the PVN did not cause significant changes in MAP and HR (candesartan with Ang II at 150 and 500 ng/kg per minute combined [MAP, 2±2 mm Hg; HR, 1±7; n=5]). At the peak BP response to candesartan, kynurenate in the PVN caused minor (nonsignificant) decreases in MAP and HR (Figures 1 and 2).

Kynurenate Followed by Candesartan in PVN

Bilateral infusion of kynurenate in PVN caused the same decreases in MAP and HR when it was infused alone or after vehicle (see above) in the PVN of rats with subcutaneous Ang II at 500 ng/kg per minute (−56±10 versus −59±6 mm Hg; −47±11 versus −43±12 bpm). At the peak BP response to kynurenate, bilateral infusion of candesartan did not further change MAP, but did increase HR (Figures 1 and 2).

Intracerebroventricular or Intravenous Infusion of Eplerenone

Intracerebroventricular infusion of eplerenone for 1 hour did not change MAP or HR at 1 or 16 hours after start of its infusion in rats with subcutaneous vehicle (Figures 4 and 5). In rats with subcutaneous Ang II at 500 ng/kg per minute, intracerebroventricular infusion did not change MAP or HR after 1 or 16 hours (Figure 4). Intracerebroventricular eplerenone also did not change MAP at 1 hour after start of its infusion but decreased MAP markedly at 16 hours and less at 24 hours (Figure 4). After 16 hours, eplerenone fully reversed the increase in BP from subcutaneous Ang II (before eplerenone with subcutaneous angiotensin II (Ang II) at 150 or 500 ng/kg per minute for 2 weeks. The second blocker was infused at the peak blood pressure response to the first blocker. Values are mean±SEM. \(*P<0.05\) vs vehicle with same Ang II (150 or 500 ng/kg per minute), a \(P<0.001\) vs same treatment without prior infusion.

Intracerebroventricular Infusion of Eplerenone Followed by Kynurenate and Candesartan in PVN

After intracerebroventricular vehicle, bilateral infusion of candesartan in the PVN fully reversed the increase in BP...
from subcutaneous Ang II at 500 ng/kg per minute (before candesartan, 181±7 mm Hg; after candesartan, 117±5 mm Hg; Figure 6). Intracerebroventricular eplerenone also fully reversed the increase in BP from subcutaneous Ang II (before eplerenone, 177±6 mm Hg; after eplerenone, 110±9 mm Hg).

After eplerenone, candesartan in the PVN did not further change MAP (Figures 6 and S2). In contrast, candesartan caused the same increase in HR when it was infused after intracerebroventricular infusion of vehicle or eplerenone (Figures 6 and S2).

After intracerebroventricular vehicle or eplerenone, and candesartan in the PVN, infusion of kynurenate in the PVN caused only minor (nonsignificant) further decreases in MAP and HR (Figures 6 and S2).

**Discussion**

The current study shows that bilateral infusion of candesartan or kynurenate in the PVN fully reverses the increase in BP from a chronic increase in circulating Ang II in Wistar rats. Infusion of kynurenate after candesartan or candesartan after kynurenate does not further lower BP. A 1-hour intracerebroventricular infusion of eplerenone does not change BP at 1 hour but at 16 hours fully reverses the increase in BP from circulating Ang II. After eplerenone, candesartan and kynurenate in the PVN do not further decrease BP. These findings suggest that increased MR activation in the brain activates a slow neuromodulatory pathway that leads to increased AT1 and glutamate receptor-dependent signaling in the PVN, thereby contributing to the maintenance of hypertension from a chronic increase in circulating Ang II.

**Candesartan and Kynurenate in the PVN of Rats With Subcutaneous Ang II**

Consistent with previous studies, subcutaneous infusion of Ang II caused a dose-related increase in BP. Water intake was also higher in Ang II hypertensive rats, likely from the effects of circulating Ang II on AT1 receptors in circumventricular organs of the lamina terminalis (LT) that lack a blood–brain barrier, such as the subfornical organ (SFO).

In contrast to absent effects in normal rats, infusion of candesartan or kynurenate in the PVN lowered BP in rats after subcutaneous Ang II for 2 weeks. These findings are consistent with other models of chronic sympathetic hyperactivity and indicate that increased glutamate and AT1-receptor activation in the PVN contributes to the maintenance of elevated BP from a chronic increase in circulating Ang II. Angiotensinergic and glutamatergic sympathoexcitatory pathways between the LT and PVN likely contribute to these effects of circulating Ang II. Regions in the circumventricular organs of the LT, that is, SFO and organum vasculosum of the LT known to project to parvocellular subdivisions of the PVN are densely filled with Ang II–containing neurons.

Connections between the SFO and PVN seem to use Ang II...
as a neurotransmitter because stimulation of neurons in the SFO by Ang II increased Ang II release in the PVN.25 Microinjection of Ang II in the SFO increased BP, and this effect of Ang II was prevented by an AT1-receptor blocker in the PVN.26 These findings suggest that increased AT1-receptor stimulation in, for example, the SFO from a rise in circulating Ang II, increases Ang II release in the PVN, thereby increasing local AT1-receptor activation. A glutamate receptor blocker in the PVN also abolished the sympathetic and pressor responses from Ang II in the SFO.27 Ang II inhibits GABA release onto presympathetic neurons of the PVN,16,17 enhancing the sympathoexcitatory effects of tonic glutamatergic input to presympathetic neurons.28 The sympathetic and pressor responses to a GABA_A-receptor blocker in the PVN were no longer present after 1 week intravenous infusion of Ang II,6 indicating that a chronic increase in circulating Ang II decreases GABAergic inhibition in the PVN, presumably by inhibiting local GABA release. Ang II also increases glutamate release from glutamatergic interneurons in the PVN,14 likely presynaptic to presympathetic neurons.15 In the current study, both kynurenate and candesartan fully reversed the increase in BP from circulating Ang II, and kynurenate after candesartan or candesartan after kynurenate did not further lower BP. The effects of increased AT1-receptor activation in the PVN are, therefore, likely mediated by local glutamate receptor activation, presumably by inhibition of GABA release or by increased glutamate release via glutamatergic interneurons.

Consistent with findings in Dahl S rats on high-salt diet,13 spontaneously hypertensive rats,11 or water-deprived rats,12 HR decreased in response to kynurenate but increased after candesartan in Ang II hypertensive rats. Considering that presympathetic neurons of the PVN innervate discrete subsets of tissues throughout the thoracic region nonuniformly,29 in contrast to BP regulation, unique sympathetic pathways regulating HR are likely affected after blockade of AT1 versus glutamate receptors in the PVN.

**Intracerebroventricular Infusion of Eplerenone**

Consistent with previous studies using an MR blocker in the central nervous system (CNS),30,31 intracerebroventricular infusion of eplerenone caused no change in BP and HR in rats with subcutaneous infusion of vehicle. These findings suggest that in sodium-replete conditions MR activation, and presumably the activity of the aldosterone–MR neuromodulatory pathway, is low and does not contribute to the maintenance of resting BP and HR. In contrast, 1-hour intracerebroventricular infusion of eplerenone fully reversed the increase in BP from subcutaneous Ang II. Intraavenous infusion of eplerenone at the dose used centrally did not change BP in Ang II hypertensive rats, indicating that the BP effect from intracerebroventricular eplerenone results from its specific action in the CNS. Intracerebroventricular infusion of an AS inhibitor,2 MR blocker,3,27 or EO-binding Fab fragments2 largely prevent increase in BP from subcutaneous infusion of Ang II. Altogether, these findings suggest that the aldosterone–MR–EO pathway in the brain plays a critical role in the development as well as maintenance of hypertension from a chronic...
PVN. In addition, Ang II increases mRNA expression of and both magnocellular and parvocellular subdivisions of the antigen-like immunoreactivity in the SFO, supraoptic nucleus, Ang II. Chronic subcutaneous Ang II increases Fos-related maintenance of hypertension in rats with elevated circulating dependent signaling in the PVN, thereby contributing to the increase in BP from subcutaneous Ang II, candesartan and heart rate (HR) from bilateral infusion of candesartan or kynurene after candesartan in the paraventricular nucleus (PVN) after intracerebroventricular infusion of vehicle or eplerenone for 1 hour and subcutaneous angiotensin II at 500 ng/kg per minute for 2 weeks. Infusion of the first blocker in the PVN was performed 16 hours after start of intracerebroventricular infusion of vehicle or eplerenone. The second blocker was infused 20 to 30 minutes after start of the first blocker. Peak responses are indicated at the top right corner. Values are mean±SEM.

In conclusion, our results indicate that activation of glutamate and AT1 receptors in the PVN contributes to the maintenance of BP in hypertensive rats with chronically elevated circulating Ang II. Increased MR activation in the brain activates a slow neuromodulatory pathway that maintains enhanced angiotensinergic and glutamatergic signaling in the CNS.

Intracerebroventricular Infusion of Eplerenone With Candesartan and Kynurene in PVN After intracerebroventricular infusion of eplerenone reversed the increase in BP from high-salt diet was reversed by intracerebroventricular injection of the Fab fragments at 18 but not yet at 4 hours postinjection in Dahl S rats, and by intracerebroventricular injection of an MR blocker at 8 hours postinjection in spontaneously hypertensive rats. MR effects in the PVN seem, therefore, to reflect delayed genomic effects rather than rapid nongenomic MR effects. Reversal of genomic effects of MR activation takes time, and time is also needed to reverse activation of downstream signaling mechanisms associated with the slow aldosterone–MR–EO pathway in the CNS.

CYP11B2 (AS) in the supraoptic nucleus and aldosterone content in the hypothalamus. Intracerebroventricular infusion of an AS inhibitor prevents increase in hypothalamic aldosterone, and both MR blocker and AS inhibitor markedly attenuate Fos-related antigen expression in magnocellular and parvocellular subdivisions of the PVN but not in the SFO and supraoptic nucleus. One may speculate that a chronic increase in circulating Ang II activates neurons in the SFO, leading to increased production of aldosterone in the supraoptic nucleus and increased release in the PVN. In the PVN, MR stimulation, likely via EO release, can increase local AT1-receptor activation.

Limitations
One may consider that the BP-lowering effect of kynurene and candesartan infused in the PVN of Ang II hypertensive rats resulted from unwanted drug action at other brain regions as a consequence of reflux up/around the, chronically implanted, guide canulas toward the ventricles. However, Evans Blue infused in the PVN did not appear in the ventricles, and if leakage into the ventricular system played a relevant role, one would also expect BP decreases by infusions in brain tissue outside the PVN, which did not occur. Moreover, direct intracerebroventricular infusion of kynurene at the same infusion rate used in the PVN caused ≈2 minutes delayed response and only a partial fall in BP compared with its direct infusion in the PVN. If leakage of drug infused in the PVN into the cerebrospinal fluid played a major role, one would expect the opposite pattern. However, considering that nuclei in the LT play an important role in initiating the central responses to circulating Ang II, one would expect that intracerebroventricular infusion of kynurene at the PVN dose would cause some blockade in the LT, and some decrease in BP.

In conclusion, our results indicate that activation of glutamate and AT1 receptors in the PVN contributes to the maintenance of BP in hypertensive rats with chronically elevated circulating Ang II. Increased MR activation in the brain activates a slow neuromodulatory pathway that maintains enhanced angiotensinergic and glutamatergic signaling in the CNS.

Perspectives
Plasma Ang II increases in chronic heart failure and in several forms of hypertension. Our previous study showed that an increase in circulating Ang II activates an aldosterone–MR–EO pathway in the brain leading to sympathetic hyperactivity and hypertension. The current study shows that this slow neuromodulatory pathway maintains the elevated BP from circulating Ang II by enhancing AT1, and glutamate receptor-dependent signaling in the PVN. Central blockade of steps in this neuromodulatory pathway also largely prevents sympathetic hyperactivity in chronic heart failure rats. Glutamate and AT1-receptor blockers in the PVN also decrease sympathetic nerve activity in chronic heart failure rats, suggesting that postmyocardial infarction, an increase in plasma Ang II activates an aldosterone–EO pathway in the brain, leading to enhanced angiotensinergic and glutamatergic signaling in the PVN, and sympathetic hyperactivity.
Further studies are needed to assess how Ang II activates this aldosterone–EO pathway.

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Disclosures

None.

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**Novelty and Significance**

**What Is New?**

- Consistent with other hypertension models, the increase in blood pressure from a chronic increase in plasma angiotensin II (Ang II) can be fully reversed by blockade of AT1 or glutamate receptors in the paraventricular nucleus.
- Intracerebroventricular infusion of an mineralocorticoid receptor blocker also fully reverses the blood pressure increase.
- After mineralocorticoid receptor blockade, AT1 or glutamate receptor blockers in the paraventricular nucleus do not further decrease blood pressure.

**What Is Relevant?**

- Plasma Ang II increases in chronic heart failure and in several forms of hypertension.
- An aldosterone–mineralocorticoid receptor neuromodulatory pathway in the brain plays a critical role in both the development and maintenance of Ang II hypertension.
- This pathway maintains the elevated blood pressure from plasma Ang II by enhancing AT1 and glutamate receptor-dependent signaling in the paraventricular nucleus.

**Summary**

Increased mineralocorticoid receptor activation in the brain by plasma Ang II activates a neuromodulatory pathway that maintains enhanced AT1 and glutamate receptor-dependent signaling in the paraventricular nucleus, and thereby the hypertension from a chronic increase in circulating Ang II.
Central Mineralocorticoid Receptors and the Role of Angiotensin II and Glutamate in the Paraventricular Nucleus of Rats With Angiotensin II–Induced Hypertension
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Central Mineralocorticoid Receptors and the Role of Ang II and Glutamate in the PVN of Rats with Ang II-Induced Hypertension

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Short title: Ang II and glutamate in PVN of hypertensive rats
Methods

Animals and Diets
Six to 7 weeks old male Wistar rats were obtained from Charles River Breeding Laboratories, Montreal, Quebec, Canada. The rats were housed in a climatized room on a 12-h light/dark cycle at constant room temperature and humidity, and provided with a standard commercial rat chow (120 µmol Na⁺/gram) and tap water ad libitum. Animals were acclimatized for 1 week prior to surgeries. All experiments were carried out according to the guidelines of the Canadian Council on Animal Care, which conform to National Institutes of Health guidelines and were approved by the University of Ottawa Animal Care and Use Committee.

Surgical Procedures
For all surgeries, rats were anesthetized with 2% isoflurane in oxygen. For major surgeries (icv and PVN cannulation) 0.1 mg/kg of slow release Buprenorphine was administered sc 40-60 minutes before the start of procedure which is sufficient for 3 days. For minor surgeries (arterial cannulation, sc minipump implantation) one sc injection (0.04 mg/kg) of regular Buprenorphine was administered pre-surgery.

Osmotic minipumps (Alzet®, model 2002) filled with Ang II at 150 or 500 ng/kg/min⁴ were implanted subcutaneously for a 2 week infusion. In sham groups, rats were implanted with pumps filled with saline. One week after sc pump implantation, intra-cerebral cannulations of the lateral ventricle and PVN were performed. A guide cannula (23 gauge) was positioned bilaterally 0.5 mm above the PVN according to the rat atlas of Paxinos and Watson², 1.8 mm posterior to the bregma, 0.4 mm lateral to the sagittal suture, and 7.9 mm ventral from the surface of the skull³. For intracerebroventricular infusions, a guide cannula was positioned above the left lateral cerebral ventricle at 0.4 mm posterior to the bregma, 1.4 mm lateral to the sagittal suture, and 3.5 mm ventral to the dura⁴. Guide cannulas were secured to the skull with two jeweler’s screws and acrylic cement and closed with stainless steel obturators. On day 12-13 of sc infusion, the left femoral artery was cannulated with polyethylene-50/10 tubing filled with heparin (1,000 U/ml in 0.9% NaCl). For experiments with intravenous (iv) infusions, the left femoral vein was also cannulated. Arterial and venous catheters were tunneled subcutaneously and externalized through a small puncture in the skin on the back of the neck. The rats were kept in a quiet room over night and the next morning BP was measured without any premedication.

Vehicles and Drugs
Artificial cerebrospinal fluid (121 NaCl, 3.4 KCl, 1.2 MgCl₂, 0.6 NaH₂PO₄, 29 NaHCO₃, and 3.4 glucose mmol/L, pH 7.4, and osmolarity 296 mosmol/kg H₂O) was used as vehicle for eplerenone (acetonitrile and aCSF: 2:98 ratio) and for candesartan and kynurenate (dimethyl sulfoxide and aCSF: 2:98 ratio). Icv infusion of eplerenone was performed at 20 µg/3.8 µL for 1 min, followed by 0.83 µg/3.8 µL for 60 min. This dose (70 µg total) was based on our preliminary studies assessing doses of eplerenone needed to reverse the hypertension from sc infusion of Ang II at 500 ng/kg/min (data not shown). Infusion of candesartan (0.5 µg/300 nL/min) or kynurenate (0.14 µg/300 nL/min) was performed bilaterally in the PVN (5 or 1.4 µg total on each side) for 10 min. These doses were adapted from our previous study showing that the hypertension from high salt diet in Dahl S rats was reversed by candesartan or kynurenate in

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the PVN\(^3\). All compounds were purchased from Sigma or received as gifts (see acknowledgements).

**Statistical Analysis**
Values are expressed as means ± SE. For comparisons of the area under the curve (AUC) or peak responses among treatments and rat strains, a one or two-way ANOVA was used followed by a Student-Newman-Keuls post hoc multiple comparison. For testing peak changes from baseline, paired \( t \)-tests were used. To test the time-course of changes from baseline, a one-way ANOVA with repeated measures followed by Dunnett’s test was used. The level of significance was set at \( p < 0.05 \).

**Statistical Analyses for the data in Figures 1-6 in Manuscript**

Fig. 1. By one-way ANOVA, responses in MAP and HR were significantly different between treatments in rats with sc infusion of Ang II at 150 ng/kg/min (MAP: \( F=23.8 \); HR: \( F=14.0 \)); [vehicle vs. kynurenate (MAP and HR: \( P<0.05 \)); vehicle vs. candesartan (MAP: \( P<0.05 \)); vehicle vs. kynurenate after candesartan (HR: \( P<0.05 \)) and Ang II at 500 ng/kg/min (MAP: \( F=36.9 \); HR: \( F= 25.0 \)); [vehicle vs. kynurenate or candesartan (MAP: \( P<0.001 \); HR: \( P<0.05 \)); vehicle vs. candesartan after kynurenate (HR: \( P<0.05 \)); kynurenate or candesartan vs. same treatment without prior infusion (MAP: \( P<0.001 \))].

Fig. 2. By one-way ANOVA of the area under the curve, MAP and HR responses were significantly different between treatments (MAP: \( F=56.9 \); HR: \( F= 45.4 \)); [vehicle vs. candesartan or kynurenate (MAP and HR: \( P<0.05 \)); candesartan vs. candesartan after kynurenate or kynurenate vs. kynurenate after candesartan (MAP: \( P<0.05 \)). By one-way ANOVA with repeated measures, MAP and HR responses to kynurenate were significantly different from vehicle from 2-4 min for MAP and from 8-10 min for HR. Candesartan was significantly different from vehicle from 2-4 min for MAP and from 8-10 min for HR.

Fig. 3. By one-way ANOVA with repeated measures both doses of kynurenate decreased MAP (\( F=22.4 \) and 23.1) and HR (\( F=34.9 \) and 24.9), all \( p<0.001 \). MAP and HR responses to kynurenate were significant different from baseline from 4-6 min for MAP and from 6-8 min for HR.

Fig. 4. By two-way ANOVA, responses in MAP were significantly different between treatments, time points and the interaction between treatments and time points (\( F= 41.7, 43.5, 43.9 \)); [icv eplerenone with sc Ang II after 1 h vs. 16h (\( P<0.001 \)); icv eplerenone with sc Ang II after 16 h vs. all other treatments after 1 or 16 h (\( P<0.001 \))]. By one-way ANOVA, responses in MAP from icv infusion of eplerenone with sc infusion of Ang II were significantly different between time points after eplerenone infusion (\( F=48.6 \); [1 vs. 24 h after eplerenone (\( P<0.05 \)); 16 vs. 24 h after eplerenone (\( P<0.001 \))].

Fig. 5. By two-way ANOVA, MAP was significantly different between treatments, time points and the interaction between treatments and time points (\( F= 17.0, 41.1, 19.2 \)); [before eplerenone: icv infusion of eplerenone with sc vehicle vs. others (\( P<0.001 \)); 16 h after eplerenone: icv infusion of eplerenone with sc vehicle or Ang II vs. iv infusion of eplerenone with sc Ang II (\( P<0.001 \)); before vs. 16 h after icv infusion of eplerenone with sc Ang II (\( P<0.001 \))].
Fig. 6. By one-way ANOVA of the area under the curve, MAP and HR responses were significantly different between treatments (MAP: $F=28.3$; HR: $F=10.5$); [candesartan in the PVN after icv vehicle vs. others (MAP: $P<0.001$); candesartan in the PVN after icv vehicle or eplerenone vs. kynurenate after candesartan in the PVN after icv vehicle or eplerenone (HR: $P<0.05$). By one-way ANOVA with repeated measures, MAP responses to candesartan in the PVN after icv vehicle were significantly different from others from 4-6 min. For HR, responses to candesartan in the PVN after icv vehicle or eplerenone were significantly different from others from 8-10 min. For peak response data, by one-way ANOVA, MAP and HR responses were significantly different among treatments (MAP: $F=31.7$; HR: $F=17.1$); [candesartan in the PVN after icv vehicle vs. others (MAP: $P<0.001$); candesartan in the PVN after icv vehicle or eplerenone vs. kynurenate after candesartan in the PVN after icv vehicle or eplerenone (HR: $P<0.01$).
References


Table S1: Baseline MAP and HR levels prior to the first treatment in experimental groups.

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>n</th>
<th>MAP (mmHg)</th>
<th>HR (bpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without icv or iv infusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle, kynurenate in PVN, sc Ang II (150 ng/kg/min)</td>
<td>5</td>
<td>131±2*</td>
<td>430±12</td>
</tr>
<tr>
<td>Vehicle, kynurenate in PVN, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>170±1*</td>
<td>456±25</td>
</tr>
<tr>
<td>Candesartan, kynurenate in the PVN, sc Ang II (150 ng/kg/min)</td>
<td>4</td>
<td>133±3*</td>
<td>462±18</td>
</tr>
<tr>
<td>Candesartan, kynurenate in the PVN, sc Ang II (500 ng/kg/min)</td>
<td>5</td>
<td>175±11*</td>
<td>436±6</td>
</tr>
<tr>
<td>Kynurenate, candesartan in the PVN, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>172±4*</td>
<td>455±10</td>
</tr>
<tr>
<td><strong>With icv or iv infusion</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Icv eplerenone, sc vehicle</td>
<td>4</td>
<td>120±6</td>
<td>441±11</td>
</tr>
<tr>
<td>Icv eplerenone, sc Ang II (500 ng/kg/min)</td>
<td>5</td>
<td>180±6*</td>
<td>425±22</td>
</tr>
<tr>
<td>Iv eplerenone, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>172±3*</td>
<td>463±23</td>
</tr>
<tr>
<td>Icv veh., cand., kyn. in PVN, sc Ang II (500 ng/kg/min)</td>
<td>4</td>
<td>180±6*</td>
<td>447±14</td>
</tr>
<tr>
<td>Icv epler., cand., kyn. in PVN, sc Ang II (500 ng/kg/min)</td>
<td>5</td>
<td>177±6*</td>
<td>439±30</td>
</tr>
</tbody>
</table>

Values are mean ± SEM; * p<0.05 vs. sc vehicle, a p<0.05 vs. groups with sc Ang II (150 ng/kg/min)
Figure S1

Schematic representation of infusion sites inside the PVN, plotted according to the location of dye circles from bilateral injections of 100 nL Evans Blue dye. Infusions into the PVN were performed after sc infusion of Ang II at 500 ng/kg/min for 2 weeks. Symbols connected by broken lines represent bilateral infusion sites from different experimental groups inside the PVN. f indicates fornix; 3V, third ventricle.
Resting MAP and HR after bilateral infusion of candesartan or kynurenate after candesartan in the PVN following icv infusion of vehicle or eplerenone for 1 hour and sc Ang II at 500 ng/kg/min for 2 weeks. Infusion of the first blocker in the PVN was performed 16 h after start of icv infusion of vehicle or eplerenone. The second blocker was infused 20-30 min after start of the first blocker. Values are mean ± SEM.

By two-way ANOVA, MAP was significantly different between treatments, time points and the interaction between treatments and time points (F= 17.1, 13.2, 11.9); [16 h after icv vehicle and before candesartan vs. all others (P<0.001)]. * p<0.001 vs. others.