Aldosterone-induced brain MAPK signaling and sympathetic excitation are angiotensin II type-1 receptor dependent

Zhi-Hua Zhang,1* Yang Yu,1* Shun-Guang Wei,1 and Robert B. Felder1,2
1Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa; and 2Medical Service, Department of Veterans Affairs Medical Center, Iowa City, Iowa

Submitted 29 August 2011; accepted in final form 7 November 2011

Zhang ZH, Yu Y, Wei SG, Felder RB. Aldosterone-induced brain MAPK signaling and sympathetic excitation are angiotensin II type-1 receptor dependent. Am J Physiol Heart Circ Physiol 302: H742–H751, 2012. First published November 11, 2011; doi:10.1152/ajpheart.00856.2011.—Angiotensin II (ANG II)-induced mitogen-activated protein kinase (MAPK) signaling upregulates angiotensin II type-1 receptors (AT1R) in hypothalamic paraventricular nucleus (PVN) and contributes to AT1R-mediated sympathetic excitation in heart failure. Aldosterone has similar effects to increase AT1R expression in the PVN and sympathetic drive. The present study was undertaken to determine whether aldosterone also activates the sympathetic nervous system via MAPK signaling and, if so, whether its effect is independent of ANG II and AT1R. In anesthetized rats, a 4-h intravenous infusion of aldosterone induced increases (P < 0.05) in phosphorylated (p-) p44/42 MAPK in PVN, PVN neuronal excitation, renal sympathetic nerve activity (RSNA), mean blood pressure (MBP), and heart rate (HR). Intracerebroventricular or bilateral PVN microinjection of the p44/42 MAPK inhibitor PD-98059 reduced the aldosterone-induced RSNA, HR, and MBP responses. Intracerebroventricular pretreatment (5 days earlier) with pooled small interfering RNAs targeting p44/42 MAPK reduced total and p-p44/42 MAPK, aldosterone-induced c-Fos expression in the PVN, and the aldosterone-induced increases in RSNA, HR, and MBP. Intracerebroventricular infusion of either the mineralocorticoid receptor antagonist RU-28318 or the AT1R antagonist losartan blocked aldosterone-induced phosphorylation of p44/42 MAPK and prevented the increases in RSNA, HR, and MBP. These data suggest that aldosterone-induced sympathetic excitation depends upon that AT1R-induced MAPK signaling in the brain. The short time course of this interaction suggests a nongenomic mechanism, perhaps via an aldosterone-induced transactivation of the AT1R as described in peripheral tissues.

extracellular related kinase 1/2; mineralocorticoid receptor; hypothalamic paraventricular nucleus; blood pressure

ACCUMULATING CLINICAL AND experimental data have aroused increasing appreciation for the role of aldosterone and mineralocorticoid receptors (MR) in cardiovascular disease. Recent animal studies have demonstrated a strong relationship between brain MR and increased sympathetic drive, an important predictor of adverse outcomes in heart failure and hypertension. Activation of brain MR has long been associated with augmented sympathetic drive in hypertension (9, 10, 15, 29) and heart failure (3, 4, 14), although the underlying mechanisms are still under intensive investigation.

We have demonstrated that brain mitogen-activated protein kinase (MAPK) signaling plays a critical role in mediating renin-angiotensin system influences on sympathetic nerve activity. In a rat model of heart failure, phosphorylated MAPK increases in the hypothalamic paraventricular nucleus (PVN) and the subfornical organ, important cardiovascular regions of the brain (28). In these heart failure rats, acute inhibition of brain p44/42 MAPK (also known as ERK1/2) activity dramatically reduces mean arterial pressure, heart rate, and renal sympathetic nerve activity (27). Blood-borne angiotensin II (ANG II), which is increased in heart failure and contributes to sympathetic activation, is one factor that we know can increase MAPK activity in the brain (26). In the present study, we hypothesized that blood-borne aldosterone, which activates MAPK signaling in peripheral tissues, might also activate brain MAPK to increase sympathetic drive.

Cross-talk between ANG II and aldosterone signaling is now well described (19, 23, 29). ANG II type-1 receptors (AT1R) contribute to aldosterone actions in peripheral tissues and in the brain (19, 23, 29). We recently demonstrated (37) that aldosterone upregulates AT1R expression in the PVN, and others have demonstrated a dependence of aldosterone-induced hypertension on brain AT1R (29).

In the present study in anesthetized rats, we examined the effect of an acute intravenous infusion of aldosterone on the expression of p44/42 MAPK and p38 MAPK in the PVN and the relationships among MAPK signaling, AT1R, and aldosterone-driven sympathetic and cardiovascular responses.

METHODS

Male Sprague-Dawley rats (300–350 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). Rats were housed in a temperature- and light-controlled University of Iowa Animal Care Facility. All experimental protocols used in this study were performed in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. The experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa and the Research and Development Committee of the Iowa City Department of Veterans Affairs Medical Center.

Surgical Preparations

General. Rats were anesthetized with urethane (1.5 g/kg ip). Supplemental doses of urethane (0.1–0.3 g/kg ip or iv) were given when spontaneous increases in blood pressure (BP), heart rate (HR), or respiratory rate were observed during experiments. The depth of anesthesia was monitored by testing nociceptive reflex responses. The left femoral vein was cannulated with PE-20 tubing for the administration of drugs. The left femoral artery was cannulated with PE-50 tubing filled with heparinized saline (50 U/ml) and connected to a pressure transducer to record BP, using a Hewlett-Packard 7754A chart recorder (HP Medical Products Group, Andover, MA). Core temperature was maintained at 37 ± 0.3°C with a temperature controller (model K-100; Baxter Healthcare, Valencia, CA).

* Z.-H. Zhang and Y. Yu contributed equally to this work.

Address for reprint requests and other correspondence: R. B. Felder, Univ. of Iowa College of Medicine, E318-GH, 200 Hawkins Drive, Iowa City, IA 52242 (e-mail: robert-felder@uiowa.edu).
Access for central administration of antagonists, inhibitors, and small interfering RNA. The head was fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). One or two small craniotomies were performed to expose the brain for intracerebroventricular injections into the right lateral cerebral ventricle or for bilateral microinjection into the PVN.

For intracerebroventricular administration of drugs or small interfering (si)RNA (34, 37), a 23-gauge stainless steel guide cannula was positioned in the right lateral cerebral ventricle using the following stereotaxic coordinates: -1.0 mm from bregma, 1.5 mm lateral to midline, and 3.5 mm ventral to dura (22). A 29-gauge stainless steel cannula was advanced beyond the tip of the guide cannula for drug delivery. At the conclusion of each experiment, pontamine sky blue (2%, 100 nl) was injected to validate the position of the intracerebroventricular cannula.

For bilateral PVN microinjections (35, 36), two 29-gauge guide cannulas were placed 1.8 mm posterior to bregma, one 0.4 mm lateral to midline and the other 1.9 mm lateral to midline but angled 10° toward midline. Both cannula tips were advanced to a final position 6.6 mm ventral to dura, 1 mm above the PVN (22). To make the injections, a 35-gauge (128 μm outer diameter; 51.2 μm inner diameter) stainless-steel injection cannula was advanced 1.0 mm beyond the tip of each guide cannula. At the end of study, pontamine sky blue (2%, 100 nl) was injected at the same locations for histological verification of the microinjection sites.

Placement of recording electrodes. A renal sympathetic nerve was isolated and prepared for recording as previously described (25, 36, 37). In brief, the left kidney was exposed through a flank incision. A branch of the renal nerve was dissected free from surrounding tissue and placed on bipolar silver wire recording electrodes. When an optimal signal-to-noise ratio was established, the electrode and the renal nerve were covered with silicon sealant (World Precision Instruments, Sarasota, FL). The electrodes were sutured to the back muscles. The recording session began at least an hour after completion of the surgical preparation.

Electrophysiological and Hemodynamic Recordings

Renal sympathetic nerve activity (RSNA) was recorded using methods previously described (23, 33, 34). Renal nerve activity was amplified (model P511; Grass Instruments, Quincy, MA) and displayed on an oscilloscope (TDS 3014; Tektronix, Beaverton, OR). The noise level was determined at the end of experiment after ganglionic blockade with hexamethonium (30 mg/kg iv). The net value of RSNA was calculated by subtracting the background noise from the actual recorded value during the experiment. The BP signal and the rectified and integrated RSNA voltage were fed into an online data acquisition system consisting of a Cambridge Electronics Design (CED, Cambridge, UK) 1401 Plus computer interface coupled with a Dell Pentium personal computer. Mean blood pressure (MBP) and HR were derived from the arterial pressure tracing. MBP, HR, and RSNA were averaged over a 3-min interval every 30 min. A 3-min baseline was used as control. Because of the baseline variations from animal to animal, the data are presented as changes from baseline instead of absolute values. Windowed RSNA (spikes/s) and integrated RSNA (mV) are shown in the figures, but only the integrated RSNA was used for statistical analysis. Changes in integrated RSNA were calculated as a percent change from the baseline activity.

Immunohistochemical and Immunofluorescent Studies

Brains were removed and kept in fixative for 24 h and then transferred to 30% sucrose in 0.1 M PBS overnight. The forebrain region containing PVN was sliced into 16-μm coronal sections with a cryostat. Sections were mounted on the slides and stored at −80°C for later immunofluorescent or immunohistochemical studies.

Immunofluorescent staining was used to examine the phosphorylated (p-) p44/p42 MAPK and p38 MAPK expression. The sections were incubated with the primary antibodies, the rabbit monoclonal antibody to p-p44/p42 MAPK (Thr202/Tyr204; no. 4377; 1:250) or p-p38 (Thr180/Tyr182; 1:100; no. 9211; Cell Signaling Technology, Danvers, MA), followed by secondary antibodies (Alex Fluor 488 goat anti-rabbit IgG; A-11070; 1:200; Invitrogen, Carlsbad, CA). Some sections were further incubated with mouse monoclonal antibody to NeuN (no. MAB 377; 1:200; Millipore, Billerica, MA) to identify PVN neurons. Immunofluorescent staining was visualized with a confocal laser-scanning microscope (Zeiss LSM 510;Carl Zeiss, Thornwood, NY).

c-Fos activity was detected using the avidin-biotin-peroxidase complex (ABC kit, PK-6101; Vector Laboratories, Burlingame, CA), technique, as previously described (17, 31, 32, 37). The sections were incubated for 24 h at 4°C with a rabbit anti-rat polyclonal anti-Fos antibody (K-25, 1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA), followed by a secondary antibody (1:5,000; biotinylated anti-rabbit IgG, PK-6101; Vector Laboratories) for 1 h at room temperature. The c-Fos-positive neurons were colored with a DAB kit (SK-4100; Vector Laboratories). In each animal, c-Fos-positive neurons within a standardized window superimposed over dorsal parvocellular, medial parvocellular, ventrolateral parvocellular, and posterior magnocellular subregions of PVN were counted manually, as previously described (32, 37). Counts from two representative 16-μm transverse sections approximately −1.80 mm from bregma were averaged to obtain a single value for data analysis. Data were represented as number of positive cells per 104 μm².

Western Blot

MAPK protein for p-p44/42 and p-p38, total p44/42 and p38, and β-actin protein levels were measured with a Western blotting technique as previously described (17, 31, 33), using polyclonal primary antibodies to p-p44/42 (Thr202/Tyr204; no. 4377; 1:250) or p-p38 (Thr180/Tyr182; 1:250; no. 9211), total p44/42 (no. 4695; 1:1,000), or p38 (no. 9212; 1:1,000) and β-actin (no. 4970; 1:1,000; Cell Signaling Technology, Danvers, MA). The density of the bands was quantified using National Institutes of Health Image-J analysis software.

Drugs Administered

Aldosterone, the AT1R blocker losartan, the p44/42 MAPK inhibitor PD-98059, the p38 MAPK inhibitor SB-203580, the MR antagonist RU-28318, hexamethonium bromide, and urethane were purchased from Sigma (St. Louis, MO). Losartan was dissolved in artificial cerebrospinal fluid (aCSF), PD-98059, RU-28318, and SB-203580 were first dissolved in DMSO and then diluted in aCSF to make a 1% final DMSO concentration. The intracerebroventricular or PVN vehicle (Veh) for PD-98059, RU-28318, and SB-203580 was first dissolved in DMSO and then diluted in aCSF to make a 1% final DMSO concentration. The intracerebroventricular or PVN vehicle (Veh) for PD-98059, RU-28318, and SB-203580 was asCSF containing 1% DMSO. Other drugs were dissolved in saline for intravenous injections. The aldosterone dose was determined in preliminary studies demonstrating that a 4-h intravenous infusion caused small increases in BP, HR, and RSNA. The doses of losartan, the MAPK inhibitors, and the MR antagonist were derived from previous studies from our laboratory (26, 37, 38) and others (2) and were optimized in preliminary experiments.

Pooled small interfering (si)RNAs (SMARTpool siRNA) targeting p44/42 that were specifically designed for in vivo use in animal models and a nontargeting control siRNA were obtained from Thermo Fisher Scientific (Lafayette, CO). JetSI 10 mM, a cationic amphiphile designed for in vivo use in animal models and a nontargeting control siRNA were obtained from Polyplus-transfection, (New York, NY). The doses for injection were optimized in preliminary studies based on the manufacturer’s instructions.
Experimental Protocols

All rats were anesthetized and underwent a continuous intravenous 4-h infusion of aldosterone (30 μg/0.3 ml/h) or 0.9% NaCl (0.3 ml/h), alone or in conjunction with one of the following treatments:

- The MR antagonist RU-28318 (10 μg/10 μl·1·h⁻¹), the p44/42 MAPK inhibitor PD-98059 (5 μg/10 μl·1·h⁻¹), or vehicle (Veh, aCSF containing 1% DMSO) was infused intracerebroventricularly continuously for 4 h starting at the beginning of the intravenous aldosterone infusion. The same doses of PD-98059 or Veh alone were infused as controls.
- The p44/42 MAPK inhibitor PD-98059 (0.5 g in 0.2 μl) or Veh was microinjected bilaterally into PVN at the beginning of the intravenous aldosterone infusion. The same dose of PD-98059 or Veh alone was microinjected bilaterally in PVN as control.
- The AT1R blocker losartan (10 g in 2 μl) or Veh was injected intracerebroventricularly at the beginning of the intravenous aldosterone infusion. The same dose of losartan or Veh alone was administered intracerebroventricularly as control.
- A SMART pool siRNA targeting p44/42 MAPK (1 μg in 20 μl in 10 mM JetSi/h), or a nontargeting control siRNA, was injected intracerebroventricularly 5 days before the intravenous aldosterone infusion.

One group of rats was euthanized and transcardially perfused with 4% paraformaldehyde in 0.1M PBS to collect brain tissue for immunohistochemical and immunofluorescent studies to assess effects on c-Fos and phosphorylated MAPK expression in PVN.

A second group of rats underwent continuous electrophysiological recordings to assess effects on RSNA, MBP, and HR. Brain tissues from some rats in this group were subsequently collected for molecular analysis.

Statistics

All group data are presented as means ± SE. Statistical significance among multiple comparisons was determined by two-way repeated-measures ANOVA followed by a post hoc Tukey’s test. For other unpaired data, a Student’s t-test was used for comparison between groups. P < 0.05 was considered to indicate statistical significance.

RESULTS

Sympathetic and Cardiovascular Responses to Intravenous Infusion of Aldosterone

Intravenous infusion of aldosterone elicited significant (P < 0.05, vs. Veh) peak increases of integrated RSNA (23.2 ± 4.8% from baseline), MBP (9.2 ± 2.1 mmHg from baseline), and HR (29.0 ± 6.5 beat/min from baseline; Fig. 1). These responses occurred gradually and simultaneously after the infusion was started, reached significance ~60 min, and were maintained during the infusion. Aldosterone-induced cardiovascular and sympathetic responses were completely prevented with concomitant intracerebroventricular infusion of mineralocorticoid receptor antagonist RU-28318 (Fig. 1). Intravenous infusion of the same dose of RU-28318 had no effects on aldosterone-induced responses (data not shown). Intravenous infusion of the same volume of 0.9% NaCl and intracerebroventricular infusion of RU-28318 alone had no effects on MBP or HR (Table 1) or on RSNA. No significant differences between groups in baseline values of MBP or HR were observed (Table 1).

Effects of Intravenous Aldosterone on MAPK Expression in Hypothalamic PVN

Intravenous infusion of aldosterone induced significant increases of p-p44/42 MAPK and p-p38 MAPK activity in the PVN region as shown by immunofluorescent staining and confirmed by Western blot analysis (Fig. 2).
Table 1. Values for HR and MBP at baseline, 2 and 4 hours

<table>
<thead>
<tr>
<th>Groups</th>
<th>HR, beats/min</th>
<th>MBP, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>baseline</td>
<td>2 h</td>
</tr>
<tr>
<td>IV Aldo + ICV Veh (n = 6)</td>
<td>291 ± 7</td>
<td>308 ± 8</td>
</tr>
<tr>
<td>IV Aldo + ICV RU-28318 (n = 7)</td>
<td>301 ± 8</td>
<td>399 ± 7</td>
</tr>
<tr>
<td>IV Veh + ICV Veh (n = 5)</td>
<td>295 ± 8</td>
<td>294 ± 7</td>
</tr>
<tr>
<td>IV Veh + ICV RU-28318 (n = 5)</td>
<td>293 ± 7</td>
<td>295 ± 8</td>
</tr>
<tr>
<td>IV Aldo + ICV PD-98059 (n = 7)</td>
<td>302 ± 8</td>
<td>305 ± 7</td>
</tr>
<tr>
<td>IV Veh + ICV PD-98059 (n = 5)</td>
<td>293 ± 7</td>
<td>287 ± 8</td>
</tr>
<tr>
<td>IV Aldo + ICV SB-203580 (n = 6)</td>
<td>299 ± 7</td>
<td>308 ± 7</td>
</tr>
<tr>
<td>IV Veh + ICV SB-203580 (n = 5)</td>
<td>301 ± 8</td>
<td>298 ± 7</td>
</tr>
<tr>
<td>IV Aldo + PVN Veh (n = 5)</td>
<td>294 ± 6</td>
<td>311 ± 8</td>
</tr>
<tr>
<td>IV Aldo + PVN PD-98059 (n = 7)</td>
<td>297 ± 7</td>
<td>299 ± 8</td>
</tr>
<tr>
<td>IV Veh + PVN PD-98059 (n = 5)</td>
<td>301 ± 8</td>
<td>297 ± 7</td>
</tr>
<tr>
<td>IV Aldo (n = 6)</td>
<td>288 ± 8</td>
<td>306 ± 7</td>
</tr>
<tr>
<td>IV Aldo + ICV control siRNA (n = 5)</td>
<td>296 ± 8</td>
<td>316 ± 7</td>
</tr>
<tr>
<td>IV Aldo + ICV p44/42 siRNA (n = 7)</td>
<td>299 ± 7</td>
<td>309 ± 6</td>
</tr>
<tr>
<td>IV Aldo + ICV losartan (n = 7)</td>
<td>297 ± 8</td>
<td>306 ± 7</td>
</tr>
<tr>
<td>IV Veh + ICV losartan (n = 5)</td>
<td>293 ± 8</td>
<td>301 ± 9</td>
</tr>
</tbody>
</table>

Values are means ± SE. HR, heart rate; MBP, mean blood pressure; PVN, paraventricular nucleus; Aldo, aldosterone; Veh, vehicle; IV, intravenous; ICV, intracerebroventricular.

d-p44/42 (Fig. 2A) and p-p38 (Fig. 2B) MAPK immunoreactivities were largely confined to the PVN, with some scattered activity in other regions, at this stereotaxic level. Concomitant intracerebroventricular infusion of the mineralocorticoid receptor antagonist RU-28318 prevented aldosterone-induced p-p44/42 MAPK expression (Fig. 2C) and reduced p-p38 MAPK expression (Fig. 2D) in PVN, as measured by Western blot.

**Effects of Centrally Administered MAPK Inhibitors on Aldosterone-Induced Sympathetic Excitation**

The intracerebroventricular infusion of p44/42 MAPK inhibitor PD-98059 prevented the RSNA, MBP, and HR responses to intravenous aldosterone; the intracerebroventricular p38 MAPK inhibitor SB-203580 reduced the cardiovascular responses (HR and MBP), but not RSNA (Fig. 3). Intracere-

![Fig. 2. Increased expression of phosphorylated (p-)44/42 MAPK and p-p38 MAPK in hypothalamic paraventricular nucleus (PVN) region after IV infusion of Aldo. A and B: representative immunofluorescent images showing p-p44/42 MAPK and p-p38 MAPK expression (green). Left, Veh-treated; middle, Aldo-treated; right, high-power view taken from the regions indicated by the red rectangles. Full expanse of PVN (unilateral, third ventricle to the left) is shown at left and middle. C and D: representative Western blots and group data for total and phosphorylated p44/42 and p38 MAPK protein expression in PVN of rats treated with IV Aldo infusion, with or without concomitant ICV infusion of the mineralocorticoid receptor antagonist RU-28318. Values are expressed as means ± SE of the ratio of p-p44/42 or p-p38 to total p44/42 or p38 MAPK (n = 5 for each group). *P < 0.05 vs. IV Veh + ICV Veh; †P < 0.05 vs. IV Aldo + ICV Veh.**
broventricular infusion of PD-98059, SB-203580 or vehicle
alone had no significant effects on MBP or HR (Table 1) or
on RSNA.

Bilateral PVN microinjection of the p44/42 MAPK inhibitor
PD-98059 significantly reduced the RSNA response to aldo-
sterone and inhibited the cardiovascular responses (MBP and
HR) (Fig. 4). There were no significant differences in baseline
MBP or HR values between groups (Table 1).

**Effect of Intracerebroventricular p44/42 MAPK siRNA on
Aldosterone-Induced p-p44/42 Expression in PVN, PVN
Neuronal Excitation, and Sympathetic Drive**

Intracerebroventricular pretreatment with siRNA for p44/42
MAPK significantly reduced aldosterone-induced expression of
p-p44/42 MAPK in the PVN, as suggested by immunofluores-
cent staining (Fig. 5A) and confirmed by Western blot (Fig. 5, B

---

![Image](http://example.com/image.png)

Fig. 3. Representative recordings (A) and grouped data (B) showing the effects of IV infusion of Aldo (30 µg/h), with and without concomitant continuous ICV
infusion of the p44/42 MAPK inhibitor PD-98059, the p38 MAPK inhibitor SB-203580, or Veh on HR, RSNA, BP, and MBP. Arrows indicate start point of
IV Aldo and ICV MAPK inhibitors or Veh. IV Aldo + ICV Veh and IV Veh + ICV Veh values are the same as in Fig. 1. Values are expressed as means ±
SE (n = 5–7 for each group). *P < 0.05 vs. IV Veh + ICV Veh; †P < 0.05 vs. IV Aldo + ICV Veh.

![Image](http://example.com/image.png)

Fig. 4. Representative recordings (A) and grouped data (B) showing the effects of continuous IV infusion of Aldo (30 µg/h), with and without bilateral PVN
microinjection of PD-98059 or Veh on HR, RSNA, BP, and MBP. Arrow indicates start point of infusion and microinjections. Values are expressed as mean ±
SE (n = 5–7 for each group). *P < 0.05 vs. IV Veh + PVN Veh; †P < 0.05, IV Aldo + PVN PD-98059 vs. IV Aldo + PVN Veh.
Double staining with the neuronal marker NeuN revealed that p-p44/42 MAPK expression was mainly localized in PVN neurons (Fig. 5A, right). At the same stereotaxic level, p-p44/42 expression outside the PVN was not affected by the aldosterone infusion. Western blot showed no effect of the aldosterone infusion or the control siRNA treatment on total (i.e., nonphosphorylated) p44/42 (Fig. 5C).

The aldosterone infusion increased c-Fos activity in all subnuclei of the PVN (Fig. 6, A and B). At the same stereotaxic level, regions outside PVN had only scattered c-Fos expression, unaffected by aldosterone infusion. Pretreatment with the p44/42 MAPK siRNA significantly reduced aldosterone-induced c-Fos expression in parvocellular regions of PVN but not in magnocellular PVN, compared with control siRNA group (Fig. 6B).

Pretreatment with the p44/42 MAPK siRNA significantly diminished the sympatho-excitatory responses (preventing RSNA and MAP responses, reducing HR response) to intravenous aldosterone, compared with the control siRNA (Fig. 7). Baseline MBP and HR were not significantly different in rats pretreated with p44/42 MAPK siRNA or with control siRNA (Table 1).

**Effect of Intracerebroventricular Losartan on Aldosterone-Induced p-p44/42 Expression in PVN and Sympathetic Excitation**

Intracerebroventricular injection of the AT1R blocker losartan significantly diminished the aldosterone-induced p-p44/42 expression in PVN area (Fig. 8). Intracerebroventricular injection of losartan also prevented the aldosterone-induced increases in RSNA and MBP and reduced the increases in HR (Fig. 9). Intracerebroventricular injection of losartan or vehicle alone had no significant effects on MBP or HR (Table 1) or on RSNA.

**DISCUSSION**

The present study in normal rats demonstrated that intravenous infusion of aldosterone increased phosphorylated MAPK expression and neuronal excitation in hypothalamic PVN and
activated the sympathetic nervous system. Concomitant intracerebroventricular infusion of the mineralocorticoid antagonist RU-28318 prevented the aldosterone-induced phosphorylation of p44/42 MAPK, reduced the aldosterone-induced phosphorylation of p38 MAPK, and prevented the aldosterone-induced sympathetic and cardiovascular responses. Intracerebroventricular or bilateral PVN microinjection of a p44/42 MAPK inhibitor, or intracerebroventricular pretreatment with a p44/42 MAPK siRNA in a dose that reduced the expression of phosphorylated p44/42 MAPK in PVN and the excitation of PVN neurons, also substantially reduced the cardiovascular and sympathetic responses to aldosterone. Of particular interest is the finding that intracerebroventricular losartan prevented the aldosterone-induced phosphorylation of p44/42 MAPK in

Fig. 6. A: representative transverse sections of PVN region (3rd ventricle to the left) from rats treated with IV Aldo infusion after ICV pretreatment with p44/42 siRNA or control siRNA, showing c-Fos immunoreactivity as an indicator of neuronal excitation. Dark dots indicate c-Fos positive neurons. B: quantification of c-Fos positive neurons in 4 different regions of the PVN from each experimental group. Values are expressed as means ± SE (n = 4 for each group). *P < 0.05 vs. artificial cerebrospinal fluid (Veh). †P < 0.05, Aldo + p44/42 siRNA vs. Aldo + control siRNA. pm, Posterior magnocellular; vlp, ventrolateral parvocellular; mp, medial parvocellular; dp, dorsal parvocellular.

Fig. 7. Representative recordings (A) and grouped data (B) showing the effects of continuous IV infusion of Aldo (30 µg/h), in rats pretreated with ICV p44/42 siRNA or control siRNA on HR, RSNA, BP, and MBP. Arrows indicate start point of IV infusion of Aldo. Recording segments are 200 s in duration, separated by an interval of ~60 min. Values are expressed as means ± SE (n = 5–7 for each group). *P < 0.05 vs. IV Veh. †P < 0.05, Aldo + p44/42 siRNA vs. Aldo + control siRNA.
PVN and the aldosterone-induced sympatho-excitation. These results indicate that 1) blood-borne aldosterone acts on brain MR to initiate MAPK signaling in the PVN, 2) p44/42 MAPK signaling in PVN is required for aldosterone-induced activation of PVN neurons and the full expression of the sympathetic response, and 3) aldosterone-induced activation of MAPK signaling and sympathetic excitation is dependent at least in part on activation of AT1R in the brain.

Chronic systemic or brain infusion of aldosterone produces hypertension that can be prevented by inhibiting or silencing of brain MR (9, 11, 15, 29, 37). In an experimental model of ischemia-induced heart failure (5), inhibiting brain MR has been shown to reduce renal sympathetic nerve activity. However, the molecular mechanisms underlying aldosterone’s central effects remain largely unknown.

Aldosterone has both genomic effects, mediated by activation of traditional MR, and rapid nongenomic actions that are independent of transcription (6, 13, 18). The complex mechanisms underlying these effects are beyond the scope of this study and this discussion. However, one mechanism that has emerged as important in both nongenomic (8, 13, 18, 20) and genomic (12) actions of aldosterone in peripheral organs is phosphorylation of ERK1/2, i.e., p44/42 MAPK. To the best of our knowledge, the present study is the first to demonstrate that aldosterone upregulates brain MAPK signaling and that p44/42 MAPK signaling is critical to aldosterone-induced sympathetic excitation. The very early onset of these responses argues strongly in favor of a nongenomic mechanism, despite the effectiveness of the MR antagonist.

Previous work from our laboratory (26, 38) has demonstrated that ANG II and LPS activate MAPK signaling in the PVN. In those studies, p44/42 MAPK played a role in upregulating the PVN expression of mRNA for AT1R, which has been associated with increased sympathetic nerve activity in experimental models of heart failure and hypertension, and p38 MAPK mediated the sympathetic response to centrally administered LPS, which stimulates proinflammatory cytokine production. In the present study, systemically administered aldo-

![Fig. 8. Representative Western blots and group data for total and phosphorylated p44/42 MAPK protein expression in PVN of rats treated with IV Aldo infusion, with or without ICV injection of losartan or Veh. Values are expressed as means ± SE of the ratio of p-p44/42 to total p44/42 MAPK (n = 5–6 for each group). *P < 0.05 vs. IV Veh + ICV Veh. †P < 0.05 IV Veh + ICV vs. IV Aldo + ICV Veh.](image)

![Fig. 9. Representative recordings (A) and grouped data (B) showing the effects of IV infusion of Aldo (30 µg/h), with and without ICV injection of the angiotensin II type 1 receptor (AT1R) blocker losartan or Veh on HR, RSNA, BP, and MBP. Arrows indicate start point of IV Aldo and ICV injection of losartan or Veh. IV Aldo + ICV Veh and IV Veh + ICV Veh values are the same as in Fig. 1. Values are expressed as means ± SE (n = 5–7 for each group). *P < 0.05, vs. IV Veh + ICV Veh. †P < 0.05 vs. IV Aldo + ICV Veh.](image)
sterone increased the expression of both p-p44/42 MAPK and p-p38 MAPK in the PVN, but the p44/42 MAPK inhibitor was more effective than the p38 MAPK inhibitor in reducing the sympathetic and hemodynamic responses to aldosterone. In a previous study in rats with heart failure (27), in which blood-borne ANG II, aldosterone, and the proinflammatory cytokines are all increased, an acute intracerebroventricular injection of a p44/42 MAPK inhibitor substantially reduced sympathetic activity but a p38 MAPK inhibitor had little effect. Taken together, these results suggest that ANG II and aldosterone share a common molecular mechanism, phosphorylation of p44/42 MAPK, that is necessary for activation the sympathetic nervous system. Notably, MAPK signaling is redox dependent (24), and ANG II, aldosterone, and the proinflammatory cytokines can all stimulate NADPH oxidase dependent production of superoxide (1, 7, 21). Downstream MAPK signaling may account at least in part for the central interactions that have been described among these central mediators (16, 29, 38).

The present study provides new mechanistic insights into the cross-talk that has been described between ANG II and aldosterone (19, 23). Recent work has identified a nongenomic mechanism for rapidly occurring aldosterone-ANG II interactions in vascular tissues: aldosterone activation of transglutaminases that can induce AT1R dimerization (23, 30). Although further study is necessary to determine what role this mechanism may play in the central nervous system actions of aldosterone, it seems reasonable to speculate that transactivation of the AT1R may account for the observed dependence of early aldosterone effects on AT1R activity. Alternatively, a nongenomic effect of aldosterone on angiotensinergic neurons innervating the PVN might result in AT1R-mediated MAPK expression.

Finally, it is worth noting that the ability of systemically administered aldosterone to induce MAPK activity in the PVN and augment sympathetic drive within a 4-h interval implies a direct effect of blood-borne aldosterone on brain tissue. While the present study did not address the specific central nervous system target(s) of aldosterone, i.e., inside or outside the blood brain barrier, the observed effects occurred far too early to be explained by de novo synthesis of aldosterone within brain tissue.

Limitations of the Study

The present study focused on events in the PVN, because of its known role as a source of sympathetic drive in cardiovascular disease states like heart failure and hypertension, in which circulating aldosterone levels are increased. In the sections of hypothalamus we examined, phosphorylated MAPK expression appeared to be limited to the PVN, and interventions confined to the PVN reduced the sympathetic response to aldosterone. However, we cannot exclude the possibility that MAPK signaling may be augmented in other cardiovascular related nuclei, e.g., the rostral ventrolateral medulla or subfornical organ. Accordingly, the partial inhibitory responses to MAPK inhibitors and siRNA that we observed may have been dose related, or may have reflected unblocked MAPK activity in other sites.

Perspectives

The dramatic increase in brain MAPK expression by the several humoral factors known to contribute to sympathetic activation in hypertension and heart failure suggests that it may be an ideal target for therapeutic intervention. Selective inhibition of p44/42 MAPK activity may effectively reduce the central effects of the renin-angiotensin-aldosterone system, a principle source of sympathetic activation in heart failure. Inhibiting p38 activity may provide additional benefit by reducing the effects of central inflammation. Inhibiting downstream mechanisms that are common to several excitatory mediators, rather than blocking the specific receptors for each of them, may be a more effective strategy for reducing sympathetic activation in pathophysiological states. However, such an approach will require further development of ways to selectively deliver therapeutic agents to the central nervous system.

GRANTS

This material is based on work supported in part by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development, and by National Heart, Lung, and Blood Institute Grants R01-HL-073986 (to R. B. Felder) and R01-HL-096671 (to R. B. Felder) from the National Heart, Lung, and Blood Institute. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


