Angiotensin II regulates brain (pro)renin receptor expression through activation of cAMP response element-binding protein

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Abstract

We reported that brain (pro)renin receptor (PRR) expression levels are elevated in deoxycorticosterone acetate (DOCA)-salt–induced hypertension; however, the underlying mechanism remained unknown. To address whether angiotensin (Ang II) type 1 receptor (AT\textsubscript{1}R) signaling is involved in this regulation, we implanted a DOCA pellet and supplying 0.9% saline as the drinking solution to C57BL/6J mice; SHAM pellet-implanted mice provided regular drinking water served as controls. Concurrently, mice were intracerebroventricularly (ICV) infused with the AT\textsubscript{1}R blocker losartan, angiotensin converting-enzyme inhibitor captopril, or artificial cerebrospinal fluid for 3 weeks. ICV infusion of losartan or captopril attenuated DOCA-salt–induced PRR mRNA elevation in the paraventricular nucleus of the hypothalamus, suggesting a role for Ang II/AT\textsubscript{1}R signaling in regulating PRR expression during DOCA-salt hypertension. To test which Ang II/AT\textsubscript{1}R downstream transcription factors were involved in PRR regulation, we treated Neuro-2A cells with Ang II with or without CREB (cAMP response element-binding protein) or AP-1 (activator protein-1) inhibitors, or CREB siRNA. CREB and AP-1 inhibitors as well as CREB knockdown abolished Ang II-induced increases in PRR levels. Ang II also induced PRR up-regulation in primary cultured neurons. Chromatin immunoprecipitation assays revealed that Ang II treatment increased CREB binding to the endogenous PRR promoter in both cultured neurons and hypothalamic tissues of DOCA-salt hypertensive mice. This increase in CREB activity was reversed by AT\textsubscript{1}R blockade. Collectively, these findings indicate that Ang II acts via AT\textsubscript{1}R to up-regulate PRR expression both in cultured cells and in DOCA-salt hypertensive mice by increasing CREB binding to the PRR promoter.
Introduction

The brain renin-angiotensin system (RAS) plays an essential role in neurogenic hypertension (4, 21). The (pro)renin receptor (PRR) is a newly discovered component of the RAS that is highly expressed in the brain (16, 26). Binding of renin to the PRR increases the catalytic activity of renin by approximately 4- to 5-fold. Prorenin also gains enzymatic activity by binding to the PRR (24). Binding of renin or prorenin to the PRR also directly initiates an intracellular signaling pathway independent of angiotensin II (Ang II) that increases the synthesis of profibrotic molecules, including plasminogen activator inhibitor-1, fibronectin, collagen, and transforming growth factor-β (10, 25, 26).

We and others have reported that the PRR is up-regulated in the central nervous system (CNS) in different hypertensive models, including spontaneously hypertensive rats (SHR) (27) and human renin-angiotensinogen (RA) double-transgenic hypertensive mice (16). Knocking down PRR in the brain through viral-mediated introduction of small hairpin (inhibitory) RNA against the PRR (PRR-shRNA) attenuates hypertension in both SHR and RA mice. We also recently reported that PRR expression levels are elevated throughout the brain during deoxycorticosterone acetate (DOCA)-salt–induced hypertension (17), and neuron-specific PRR deletion prevents the development of DOCA-salt hypertension. Taken together, these reports suggest that the PRR plays a pivotal role in the central regulation of blood pressure. However, it is still not clear how the PRR is up-regulated in hypertension.

A common mechanism among these hypertensive animal models is activation of the brain RAS and increased Ang II formation. Here, we report that Ang II acts through the
type 1 angiotensin receptor (AT₁R) to increase PRR expression in the CNS during DOCA-salt hypertension. We also demonstrate that cAMP response element-binding protein (CREB) acts downstream of Ang II/AT₁R signaling to mediate the increase in PRR expression in response to DOCA-salt by binding to sites identified in the promoter region of the PRR gene (Atp6ip2).

**Methods**

**Animals**

All animal procedures were approved by the Institutional Animal Care and Use Committee of Colorado State University and were performed in accordance with the National Institutes of Health Guide to the Care and Use of Experimental Animals. Wild-type C57BL/6J mice were from the Jackson Laboratory (Bar Harbor, ME).

**Cell culture and Ang II treatment**

Mouse Neuro-2A cells (American Type Culture Collection, Manassas, VA) were plated at a density of $1 \times 10^5$ cells/ml in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Corporation, Gaithersburg, MD) containing 10% fetal bovine serum (FBS) (25). A consistent passage (P3–P6 from the initially cryopreserved cells) of Neuro-2A cells was used throughout. In Ang II stimulation studies, cells were starved by incubating overnight in serum-free medium followed by treatment with Ang II (100 nM) for different durations (see Results), with or without the AT₁R blocker losartan (10 μM; Sigma-Aldrich, St. Louis, MO), the activator protein-1 (AP-1) inhibitor SR-11302 (5) (10 μM; Santa Cruz Biotechnology, Dallas, TX), the CREB-CBP interaction inhibitor CAS92-78-4 (1, 15) (10 μM; EMD Millipore, Billerica, MA), or the nuclear factor-kappaB (NF-κB)
inhibitor parthenolide (10 µM; EMD Millipore) for 2 hours. All inhibitors were added 30 minutes before Ang II stimulation. After treatment, cells were harvested and used for real-time reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and chromatin immunoprecipitation (ChIP) assays. N-values represent the number of separate experiments; within an experiment, triplicate determinations were performed for each group.

DOCA-salt hypertension model and intracerebroventricular infusion

C57BL/6 mice were anesthetized by isoflurane inhalation (3% for induction and 1.5% for maintenance) and then subcutaneously implanted with a 50-mg pellet of DOCA (17) or a SHAM pellet. After recovery from anesthesia, animals were housed singly in standard forced-air shoebox cages. Control animals were maintained on standard chow and provided ad libitum access to tap water. DOCA animals were maintained on standard chow and provided ad libitum access to a 0.15 M (0.9%) sodium chloride solution.

In protocol 1, losartan (3 mg/kg/d), captopril (3 mg/kg/d), or artificial cerebrospinal fluid (aCSF) was intracerebroventricularly (ICV) infused into the lateral cerebroventricle (0.11 μl/h) concurrently with DOCA-salt treatment using implanted osmotic minipumps (Alzet, Cupertino, CA) connected to the ICV cannula (coordinates: 0.3 mm posterior and 1 mm lateral to bregma and a depth of 3 mm) (17). Blood pressure in captopril- and aCSF-treated groups was continuously recorded during DOCA-salt treatment using radio telemetry. At the end of the protocol, brains were harvested and immediately frozen in OCT (optimum cutting temperature) compound on dry ice. Paraventricular nuclei (PVN) were micro-punched in a cryostat using a brain punch kit (0.75 mm
diameter with a depth of 1 mm; Stoelting Co., Wood Dale, IL) (11), and used for PRR mRNA measurement.

In protocol 2, mice were ICV infused with losartan (3 mg/kg/d), the CREB-CBP interaction inhibitor CAS92-78-4 (100 μM, 0.11 μl/h), or aCSF concurrently with DOCA-salt or SHAM treatment. For ChIP assays, the hypothalamus was dissected; for PRR mRNA measurements, the PVN was micro-punched as described above.

**Primary neuron culture**

Neurons were isolated and cultured from postnatal day 0 (P0) to P2 C57Bl/6 mouse pups as previously described (23), with slight modifications. Briefly, pups were anesthetized with isoflurane and euthanized by decapitation. Whole brains were rapidly dissected under a stereomicroscope (Olympus, Lake Success, NY) in isolation medium consisting of Neurobasal Medium (Life Technologies, Carlsbad, CA) supplemented with 0.002 mg/ml penicillin/streptomycin and 0.004 mg/ml neomycin. Tissue was digested with dispase (1.5 U/ml; Sigma-Aldrich) and stirred with fresh dispase medium for 10 minutes; the medium was collected, and the process was repeated for a total of four extractions. Extracted cell supernatants were kept on ice and pooled at the end of tissue digestions. Cells were seeded on poly-D-lysine-coated 25-mm coverslips or 6-well culture plates at a density of $3 \times 10^5$ cells per coverslip or well. Neuronal cultures were grown/maintained in Neurobasal Media supplemented with 2% B-27 (Life Technologies) and 5 mM Glutamax-1 (Life Technologies) at 37°C in a humidified 5% CO₂ environment for at least 1 week prior to further experiments. N-values represent the number of
separate experiments; within an experiment, triplicate determinations were performed for each group.

**Immunostaining of primary cultured neurons**

Primary neurons on glass coverslips were fixed with methanol for 20 minutes at -20°C. After fixation, coverslips were washed three times for 5 minutes each in 1X Tris-buffered saline (TBS), then incubated in blocking buffer (1% donkey/goat serum in 1X TBS) for 1 hour at room temperature. Rabbit anti-MAP2 primary antibody (1:500 in blocking buffer; Abcam, Cambridge, UK) was then added to coverslips and incubated overnight at 4°C. On the next day, coverslips were washed three times for 5 minutes each in 1X TBS before incubating with anti-rabbit Alexa Fluor 555-conjugated secondary antibody (1:500; Life Technologies) at room temperature for 1 hour. Coverslips were washed three more times for 5 minutes each in 1X TBS and then mounted on glass slides with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector, Burlingame, CA) and stored at 4°C until used for imaging.

**siRNA-mediated CREB knockdown**

Neuro-2A cells were plated in 6-well culture plates and cultured for 24 hours. For transfection, the culture medium was removed and cells were washed with 2 ml of siRNA Transfection Medium (Santa Cruz Biotechnology, Dallas, TX). Cells were then incubated with 0.8 ml of transfection mixture composed of CREB siRNA (Santa Cruz Biotechnology) and siRNA Transfection Reagent (Santa Cruz Biotechnology) for 6 hours at 37°C in a CO₂ incubator. Normal growth medium (1 ml) was then added to the wells and cells were cultured for an additional 24 hours. Thereafter, the medium was
replaced with fresh normal growth medium, and cells were cultured for 48 hours before further experiments. Scrambled siRNA from the same vendor was used as a negative control. CREB knockdown efficiency was evaluated by assessing total CREB and phosphorylated CREB levels by Western blotting (see below).

**RNA isolation and real-time RT-PCR**

Total RNA from brain tissue or cell cultures was isolated using a standard RNA extraction procedure (RNeasy mini kit; Qiagen Technologies, Hilden, Germany) and quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA). cDNA was synthesized from 200 ng total RNA using a reverse transcription kit (Applied Biosystems, Foster City, CA). Specific primers for mouse PRR (16) were designed using PrimerQuest Software (Integrated DNA Technologies, Coralville, IA). Real-time RT-PCR was performed on an Mx3000P System (Stratagene, La Jolla, CA) using the SYBR green qPCR master mix (USB Corporation, Cleveland, OH) according to the manufacturers’ instructions. Cycling conditions were 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds, and 72°C for 30 seconds. Relative expression was calculated according to the 2-ΔΔCT method (20) using MxPro software. Values were expressed as a ratio relative to control groups. The expression levels of targeted mRNAs were normalized to the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

**Western blot analysis**

Brain tissues and cell cultures were harvested in lysis buffer (Thermo Scientific) containing a protease inhibitor cocktail (Sigma-Aldrich) and homogenized with a glass
pestle. The lysate was centrifuged at 13,000 rpm for 10 minutes, and the supernatant was transferred to a clean tube. Protein concentration was measured using a BCA assay kit (Thermo Scientific). Lysate samples containing equal amounts of protein (25 μg) were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (Invitrogen, Carlsbad, CA), heated at 70°C for 10 minutes, and electrophoresed on a 4–20% Tris-Glycine gel (Invitrogen). After transferring proteins to nitrocellulose membranes using an iBlot Gel Transfer Device (Invitrogen), membranes were blocked by incubating with 5% non-fat milk in phosphate-buffered saline containing 0.1% Tween-20 (PBS-T) for 2 hours at room temperature, and then incubated overnight at 4°C with an antibody against phosphorylated CREB (P-CREB, phosphorylated at Ser133, 1:1000; Cell Signaling Technology, Danvers, MA), total CREB (T-CREB, 1:1000; Cell Signaling Technology), phosphorylated c-Jun (P-c-Jun, phosphorylated at Ser63, 1:500; Santa Cruz Biotechnology), total c-Jun (T-c-Jun, 1:500; Santa Cruz Biotechnology), or PRR (1:500) (16). Membranes were then incubated with peroxidase-conjugated goat anti-rabbit IgG antibody (1:10000; Jackson Immunoresearch Laboratories, West Grove, PA) for 1 hour at room temperature. Immunoreactive protein bands were detected with a UVP Bio-imaging System. After stripping by incubating with Western Blot Stripping Buffer (Thermo Scientific) for 15 minutes at room temperature, blots were incubated with rabbit anti-mouse β-actin antibody (1:1000; Sigma-Aldrich) for 1 hour at room temperature, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (1:10000) for 1 hour at room temperature. Proteins in blots were again visualized using the UVP Bio-imaging System. Target proteins and β-actin were quantified by a separate investigator, blinded to group-
identifying information, using NIH Image J software. The expression levels of targeted proteins were normalized to the expression levels of β-actin protein.

**ChIP assay**

Hypothalami from mice that had received 3-week SHAM or DOCA-salt treatment (with ICV infusion of aCSF or losartan) and Neuro-2A cells (with or without Ang II treatment) were fixed with 1% formaldehyde. ChIP assays were subsequently performed according to the manufacturer's instructions (EMD Millipore). Briefly, chromatin was isolated and sheared by sonicating nine times for 10 seconds each. The sheared chromatin was immunoprecipitated with anti-P-CREB (5 μg/IP) or anti-P-c-Jun (5 μg/IP) antibody overnight at 4°C. Normal mouse IgG (2 μg/IP) was used as a negative control, and anti-RNA polymerase II (2 μg/IP) antibody was used as a positive control. Non-precipitated chromatin served as input. After eluting protein/DNA complexes, DNA was purified by removing the antibodies. Input, immunoprecipitate, and negative and positive control fractions were subsequently analyzed by real-time PCR using SYBR green qPCR master mix (USB Corporation). Primer pairs designed to amplify appropriate fragments of the promoter region of the PRR gene are listed in Figure 4A and Table 1. Relative expression was calculated according to the 2-ΔΔCT method (20) using MxPro software. The binding activity of targeted transcription factors was normalized to input.

**Statistical analysis**

Data are expressed as means ± SEM. Data were analyzed by one-way or two-way analysis of variance (ANOVA), as appropriate, with Bonferroni post hoc tests to compare replicate means. Statistical comparisons were performed using Prism5.
Results

DOCA-salt increases brain PRR expression in an Ang II-dependent manner

We recently reported that PRR levels are significantly up-regulated during DOCA-salt–induced hypertension, and showed that chronic ICV infusion of losartan prevents the development of DOCA-salt hypertension (17). To address whether Ang II acts through AT1R to participate in the up-regulation of PRR in the PVN of the hypothalamus during DOCA-salt hypertension, we first assessed PRR mRNA levels in the PVN following DOCA-salt treatment. PRR mRNA levels trended higher on day 1 (fold change relative to control: 1.18 ± 0.02) after DOCA-salt treatment and were significantly elevated on day 3 (1.49 ± 0.11), day 7 (1.83 ± 0.13), and day 21 (2.56 ± 0.2) (Figure 1A). Chronic ICV infusion of losartan or captopril for 21 days prevented the DOCA-salt–induced up-regulation of PRR mRNA (Figure 1B) without affecting PRR mRNA levels in SHAM animals, confirming the involvement of Ang II/AT1R signaling. Consistent with this, ICV infusion of captopril prevented the development of DOCA-salt hypertension (Figure 1C), recorded by telemetry. PRR protein levels were also significantly elevated after 21 days of DOCA-salt treatment (Figure 1D).

Involvement of AP-1 and CREB in Ang II-induced PRR up-regulation in Neuro-2A cells

To gain insight into the molecular mechanism by which Ang II regulates PRR expression, we performed a transcription factor prediction search (TFSEARCH ver.1.3).
This analysis revealed the presence of binding sites for CREB, AP-1, and NF-κB in the PRR promoter region. We first used cultured Neuro-2A cells to examine whether these transcription factors are indeed involved in the regulation of PRR expression by Ang II. As also reported previously by our laboratory (16), Ang II significantly increased PRR expression in Neuro-2A cells compared to controls; this effect was blocked by losartan (Figure 2A). We then tested which of these Ang II/AT₁R downstream transcription factors were involved in PRR regulation. The CREB inhibitor CAS92-78-4 prevented Ang II-induced PRR mRNA up-regulation in Neuro-2A cells (Figure 2B) without affecting PRR mRNA levels in the control group. Similarly, the AP-1 inhibitor SR-11302 also prevented Ang II-induced PRR mRNA up-regulation (Figure 2C) and, like CAS92-78-4, had no effect on PRR expression in control cells. In contrast, NF-κB inhibition (parthenolide) had no effect on PRR expression in either control or Ang II-treated cells (Figure 2D).

**CREB knockdown prevents Ang II-induced PRR up-regulation in Neuro-2A cells**

Figure 3A shows a representative Western blot and quantification for P-CREB, T-CREB, and β-actin in Neuro-2A cells after transfection with scrambled siRNA or CREB siRNA. CREB siRNA decreased both P-CREB and T-CREB levels compared with scrambled siRNA. Ang II induced a time-dependent increase in PRR protein levels, with a significant increase observed 4 hours after stimulation (Figure 3B). CREB siRNA did not change PRR protein levels in the controls. Importantly, CREB knockdown prevented Ang II-induced (4 hours) PRR protein up-regulation (Figure 3C). Similarly, CREB knockdown prevented Ang II-induced PRR mRNA up-regulation (Figure 4C).
Ang II increases PRR expression in primary cultured neurons

The Neuro-2A cell line is derived from a mouse neuroblastoma obtained from the hypothalamic brain region. To confirm Ang II effects on PRR regulation in primary neurons, we isolated and cultured mouse brain neurons. Figure 4A1 shows a merged differential interference contrast (DIC, grey) image and DAPI (cyan)-counterstained image of primary neurons; Figure 4A2 shows an image of immunostaining with the neuronal marker MAP2 (red); and Figure 4A3 shows a merged image of MAP2, DAPI, and DIC (64X magnification). Ang II increased PRR mRNA levels as early as 1 hour after stimulation (Figure 4B) and increased PRR protein expression starting 4 hours after stimulation (Figure 4C).

Ang II induces a time-dependent increase in CREB and c-Jun phosphorylation in Neuro-2A cells

Figure 5A shows a representative Western blot for P-CREB (phosphorylated at Ser133), T-CREB, and β-actin in Neuro-2A cells at different time points after stimulation with Ang II. Densitometric quantification of blots showed that Ang II significantly increased CREB phosphorylation at Ser133 in a time-dependent manner, with the maximal increase observed 60 minutes after Ang II treatment (Figure 5B). Losartan prevented the Ang II-induced increase in CREB phosphorylation, restoring P-CREB to control levels. Ang II had no effect on T-CREB expression within 2 hours of incubation (Figure 5C). Figure 5D shows a representative Western blot for P-c-Jun (phosphorylated at Ser63), T-c-Jun, and β-actin at different time points after Ang II stimulation. Quantification of blots showed that Ang II increased c-Jun phosphorylation in a time-
dependent manner, with the maximal increase observed 60 minutes after Ang II stimulation (Figure 5E). As was the case for P-CREB, losartan treatment completely prevented the Ang II-induced increase in c-Jun phosphorylation. Ang II had no effect on T-c-Jun expression within 2 hours of incubation (Figure 5F).

Ang II increases CREB binding to the endogenous PRR promoter in Neuro-2A cells

Six pairs of primers targeting the PRR promoter were designed, covering a 1042-base-pair (bp) region upstream of the starting codon (Figure 6A). Oligonucleotide sequences are presented in Table 1. To detect P-CREB and P-c-Jun binding to different regions of the endogenous PRR promoter in response to Ang II treatment in Neuro-2A cells, we performed ChIP assays. P-CREB binding activity in the -270/-39 bp promoter region, detected by primer 6, was increased in Ang II-treated cells compared to controls (Figure 6B). A CREB-binding sequence (TGACGTCG) that is highly conserved compared with the consensus CRE sequence (TGACGTCA) was identified at -212/-205 within this region. No increased P-c-Jun binding at the PRR promoter was detected, although decreased binding activity was detected by primers 1 and 2 following Ang II treatment (Figure 6C).

Increases in CREB phosphorylation and CREB binding to the endogenous PRR promoter in the hypothalamus of DOCA-salt hypertensive mice

Figure 7A shows a representative Western blot for P-CREB, T-CREB, and β-actin in the mouse hypothalamus in SHAM, SHAM with ICV infusion of losartan, DOCA-salt with ICV infusion of aCSF, and DOCA-salt with ICV infusion of losartan conditions. Twenty-

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one days of DOCA-salt treatment significantly increased CREB phosphorylation in the hypothalamus. Chronic ICV infusion of losartan prevented the DOCA-salt–induced increase in CREB phosphorylation (Figure 7B). However, neither DOCA-salt nor chronic ICV infusion of losartan affected T-CREB levels (Figure 7C). Furthermore, P-CREB binding to the PRR promoter detected by primer 6 was significantly increased following 3 weeks of DOCA-salt treatment (Figure 8A). Notably, ICV infusion of losartan completely prevented the DOCA-salt–induced increase in P-CREB binding to the endogenous PRR promoter in the hypothalamus. Importantly, ICV infusion of the CREB inhibitor CAS92-78-4 completely prevented DOCA-salt–induced PRR mRNA up-regulation in the PVN (Figure 8B).

Discussion

The PRR plays a pivotal role in brain Ang II formation and the central regulation of blood pressure (17, 18). However, our understanding of the factors that influence the expression of this receptor in the CNS is still evolving. In the present study, we sought to identify Ang II signaling pathways involved in regulating PRR expression. We found that Ang II up-regulated PRR expression in neuronal cells and primary cultured neurons, and showed that pharmacological intervention of AP-1 and CREB signaling pathways blocked Ang II-induced PRR expression. Furthermore, we showed that AT1R or CREB blockade prevented DOCA-salt–induced PRR up-regulation in the brain and identified the functional binding sites of CREB on the promoter region of the PRR. Finally, we demonstrated that CREB binding to the endogenous PRR promoter was elevated in the hypothalamus of DOCA-salt hypertensive mice and this increase was reversed by AT1R blockade.
The first important finding in this study is that Ang II increased PRR expression in Neuro-2A cells, an effect that was blocked by losartan. Ang II is the major bioactive member of the RAS, and over-activity of the RAS in the CNS is a hallmark of several disease states characterized by sympathoexcitation, such as hypertension. Ang II is known to activate numerous transcription factors, including AP-1 (2) and CREB (6, 7). It has been reported that Ang II increases phosphorylation of c-Fos and c-Jun, two components of AP-1, in the rostral ventrolateral medulla, a major sympathetic control center of the brain (19). Phosphorylation of CREB is important for the regulation of gene expression and hypertrophy of vascular smooth muscle cells by Ang II (6). Ang II-induced CREB phosphorylation is mediated by at least three distinct pathways (6): (i) an extracellular signal-regulated protein kinase (ERK) pathway that can be blocked by the ERK inhibitor PD98059, (ii) a p38 mitogen-activated protein kinase (MAPK) pathway that can be blocked by the P38 inhibitor SB203580, and (iii) a protein kinase A (PKA)-dependent pathway that can be blocked by the PKA inhibitor H89. Phosphorylation of CREB at Ser133 results in recruitment of the transcriptional co-activator, CREB-binding protein (CBP), which is essential for transcriptional activation (3). Our results are in agreement with previous reports that Ang II activates CREB through phosphorylation at Ser133 and induces AP-1 activity through phosphorylation of c-Jun, as well as with reports that losartan blocks Ang II-induced CREB and c-Jun phosphorylation (2, 7). A new finding in this study is that inhibition of CREB-CBP prevents Ang II-induced PRR up-regulation in cultured Neuro-2A cells. We also found that CREB phosphorylation and PRR expression were increased in the mouse hypothalamus during DOCA-salt hypertension, and this increase was prevented by ICV infusion of losartan, suggesting a
role for Ang II in CREB phosphorylation and PRR up-regulation in this hypertensive
model.

The fact that inhibition of CREB-CBP or AP-1 prevented Ang II-induced PRR up-
regulation suggested that these transcription factors might directly regulate PRR
transcription through binding to elements in the PRR promoter. ChIP assays performed
in vitro to identify potential binding sites on the PRR promoter revealed that CREB
binding activity increase in the -270/-39 region of the PRR promoter after Ang II
treatment. Surprisingly, these assays showed no increase, but rather a small decrease,
in c-Jun binding to the PRR promoter. The physiological significance of this decrease in
P-c-Jun binding is not clear at this time, but it is an issue that we plan to address at
some point in the future. Our data showed that although Ang II phosphorylates c-Jun,
P-c-Jun binding to the PRR promoter was not elevated, suggesting that at least within
the region of the PRR promoter tested, AP-1 does not contribute to Ang II-induced up-
regulation of PRR expression. Instead, a reduction in P-c-Jun binding might be a
compensatory mechanism for PRR up-regulation induced by other mechanisms, such
as the CREB pathway. It is also possible that other P-c-Jun-binding sites lying outside
the tested 1042-bp upstream region of the PRR promoter may participate in PRR
regulation, a question we plan to investigate in our future study. In addition, increasing
evidence suggests that transcription factors function collectively in a regulated network
to ultimately affect cell signaling (7, 14), providing a possible alternative explanation for
decrease in P-c-Jun binding on PRR promoter regions we tested as well as the effect of
AP-1 inhibitor on PRR mRNA levels. For example, crosstalk between CREB and
c-Fos/c-Jun confers both gain and loss of function in the fine-tuning of regulatory events
involved in transcription (22). In our case, AP-1 inhibition might interrupt CREB binding to the PRR promoter in response to Ang II and, in turn, prevent the Ang II-induced increase in PRR expression. In the hypothalamus of DOCA-salt hypertensive mice, CREB binding activity at the -270/-39 region of the endogenous PRR promoter was markedly increased, and ICV infusion of losartan or CREB inhibitor prevented this increase, suggesting that the CREB mechanism observed in vitro also operates in vivo.

We previously reported that PRR expression was only increased 1.2- to 1.5-fold, depending on brain area, in human RA double-transgenic mice (16). In this mouse model, the major phenotype is chronic hypertension accompanied by high plasma and tissue Ang II levels, suggesting that the increase in PRR expression may be caused by high levels of Ang II. It has also previously been shown that PRR expression is increased 1.3- to 1.7-fold in different brain regions in the SHR model (27). Although the authors of this latter study did not elucidate the mechanism of PRR up-regulation, hyperactivity of the brain RAS is known to play a critical role in the development of hypertension in the SHR model (8) and might contribute to PRR regulation. In our in vitro study, Ang II induced a 1.4-fold increase in PRR mRNA levels and about a 2-fold increase in PRR protein levels; it also increased CREB binding activity by 1.6-fold. Interestingly, DOCA-salt induced a 2.6-fold increase in PRR expression and about a 4-fold increase in CREB binding activity. The fact that ICV infusion of losartan prevented both DOCA-salt–induced PRR up-regulation and increased CREB binding activity suggests the importance of Ang II/AT₁R signaling in the regulation of PRR in DOCA-salt hypertension, but begs the question: why is the increase in PRR expression and CREB binding much more dramatic in the DOCA-salt hypertension model than in a pure
Ang II-induced hypertension model? A recent study showed that, during DOCA-salt hypertension, histone deacetylase (HDAC) activity is increased in association with up-regulation of cardiac hypertrophic markers (13). HDAC catalyzes the removal of acetyl groups from histone residues and plays a key role in epigenetic regulation of histone and non-histone proteins. It has also been shown that HDAC inhibition suppresses the expression of inflammatory cytokines during DOCA-salt hypertension (12), suggesting a role for HDAC in regulating the expression of inflammatory cytokines. Collectively, these reports suggest the possibility that other mechanisms (e.g., epigenetic regulation) could facilitate the recruitment of additional transcription factors, such as CREB, to the PRR promoter region and thus induce higher levels of PRR expression. Additional studies will be needed to confirm this supposition.

Interestingly, a previous report showed that renal PRR expression is also up-regulated in the streptozotocin-induced diabetic rat model, and that AT1R blockade inhibits this up-regulation (28), suggesting a role for Ang II in up-regulation of renal PRR during diabetes. Huang et al. reported that sodium depletion significantly up-regulates PRR mRNA and protein expression in the rat kidney (9). Although the authors did not demonstrate a direct relationship between Ang II and PRR up-regulation in this model, it is well known that sodium depletion increases renal renin synthesis (29) and activates the systemic RAS. In the present study, we found that Ang II directly increased PRR expression in the CNS through increased CREB binding to the PRR promoter. Activation of prorenin by the PRR is one of the major mechanisms for producing Ang II in the brain (17), suggesting the existence of a positive feedback system for PRR up-regulation in the CNS.
In summary, our study indicates that Ang II acts via AT\textsubscript{1}R to up-regulate PRR expression in both cultured cells and DOCA-salt hypertension by increasing CREB binding to the PRR promoter. The positive feedback between Ang II and the PRR may be a key contributor to the development of neurogenic hypertension.

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Disclosure

None.

References


Figure legends

Figure 1. DOCA-salt treatment increases brain PRR expression in an Ang II-dependent manner. (A) PRR mRNA levels in the PVN of the hypothalamus of mice in the SHAM (control) group and in mice treated with DOCA-salt for 1 day (DOCA D1), 3 days (DOCA D3), 7 days (DOCA D7), or 21 days (DOCA d21) (n = 5–6 PVNs/group; *P < 0.05 vs. SHAM). (B) PRR mRNA levels in the PVN of mice treated with SHAM; SHAM + ICV infusion of losartan; or DOCA-salt with ICV infusion of aCSF, losartan, or captopril for 21 days (n = 5–6 PVNs/group; *P < 0.05 vs. SHAM, #P < 0.05 vs. DOCA + ICV aCSF D21). (C) Mean arterial pressure (MAP) recorded by radio telemetry in mice treated with DOCA-salt and ICV-infused aCSF or captopril. Arrow indicates the initiation of DOCA-salt treatment and ICV infusion (n = 5 mice/group; *P < 0.05 vs. DOCA + ICV aCSF). (D) Representative Western blots for PRR and β-actin, and quantification of PRR protein expression in the hypothalamus of SHAM mice and mice treated with DOCA-salt for 21 days (n = 4 hypothalami/group; *P < 0.05 vs. SHAM).

Figure 2. Inhibition of AT₁R, AP-1, or CREB prevents Ang II-induced increases in PRR mRNA levels in Neuro-2A cells. (A) PRR mRNA levels in Neuro-2A cells incubated with or without (control) Ang II (100 nM, 1 hour) in the presence or absence of losartan (10 μM) for 2 hours (n = 4 in control and Ang II groups; n = 3 in control + losartan group and Ang II + losartan group). (B) PRR mRNA levels in control, control + CREB inhibitor (CAS92-78-4; 10 μM), Ang II (100 nM, 1 hour), and Ang II + CAS92-78-4 (n = 3) groups. (C) PRR mRNA levels in control, control + AP-1 inhibitor (SR11302; 10 μM), Ang II (100 nM, 1 hour), and Ang II + SR11302 (n = 3) groups. (D) PRR mRNA levels in control, control + NF-κB inhibitor (parthenolide; 10 μM), Ang II (100 nM, 1 hour),
and Ang II + parthenoloid (n = 3) groups. N-values represent the number of separate experiments; within an experiment, triplicate determinations were performed for each group (*P < 0.05 vs. control, #P < 0.05 vs. Ang II).

**Figure 3. CREB knockdown prevents Ang II-induced PRR up-regulation.** (A) Representative Western blots for P-CREB, T-CREB and β-actin, and quantification of P-CREB and T-CREB protein expression in Neuro-2A cells transfected with scrambled siRNA or CREB siRNA (n = 3; *P < 0.05 vs. scrambled siRNA). (B) Representative Western blots for PRR and β-actin, and quantification of PRR protein expression in Neuro-2A cells treated with Ang II (100 nM) for 2, 4, or 6 hours (n = 3; *P < 0.05 vs. control). (C) Representative Western blots for PRR and β-actin, and quantification of PRR protein expression in Neuro-2A cells transfected with or without CREB siRNA in the presence and absence of Ang II (100 nM) stimulation for 4 hours (n = 3; *P < 0.05 vs. control, #P < 0.05 vs. Ang II). (D) PRR mRNA levels in control, control + CREB siRNA, Ang II (100 nM, 1 hour), and Ang II + CREB siRNA (n = 4) groups (*P < 0.05 vs. control, #P < 0.05 vs. Ang II). N-values represent the number of separate experiments; within an experiment, duplicate (for protein) or triplicate (for mRNA) determinations were performed for each group.

**Figure 4. Ang II increases PRR expression in primary cultured neurons.** (A) DIC (grey) imaging and DAPI (cyan) counterstaining of primary neurons (A1); immunostaining with the neuronal marker MAP2 (red) (A2); and merged image of MAP2, DAPI, and DIC (64X magnification) (A3). (B) PRR mRNA levels in controls and animals treated with Ang II (100 nM) for 1, 2, or 4 hours (n = 3; *P < 0.05 vs. control). (C) Representative Western blots for PRR and β-actin, and quantification of PRR protein
expression in primary cultured neurons treated with Ang II (100 nM) for 2, 4, or 6 hours (n = 3; *P < 0.05 vs. control). N-values represent the number of separate experiments; within an experiment, duplicate (for protein) or triplicate (for mRNA) determinations were performed for each group.

**Figure 5. Ang II increases CREB and c-Jun phosphorylation in Neuro-2A cells.** (A) Representative Western blots for P-CREB, T-CREB, and β-actin in Neuro-2A cells at different time points after treatment with Ang II (100 nM) or Ang II + losartan for 60 minutes. (B) Quantification of P-CREB following Ang II treatment. (C) Quantification of T-CREB following Ang II treatment. (D) Representative Western blots for P-c-Jun, T-c-Jun, and β-actin at different time points after treatment with Ang II or Ang II + losartan for 60 minutes. (E) Quantification of P-c-Jun following Ang II treatment. (F) Quantification of T-c-Jun following Ang II treatment (n = 3; *P < 0.05 vs. control, #P < 0.05 vs. Ang II treatment). N-values represent the number of separate experiments; within an experiment, duplicate determinations were performed for each group.

**Figure 6. ChIP assays showing Ang II-induced increases in CREB binding to the endogenous PRR promoter in Neuro-2A cells.** (A) Design of primers targeting the PRR promoter, covering a 1042-bp region upstream of the starting codon. (B) ChIP assay results showing relative enrichment for P-CREB binding to the PRR promoter in control and Ang II-treated cells. (C) Relative enrichment for P-c-Jun binding to the PRR promoter in control and Ang II-treated cells (n = 3; *P < 0.05 vs. control). N-values represent the number of separate experiments; within an experiment, triplicate determinations were performed for each group.
Figure 7. CREB phosphorylation is increased in the hypothalamus of DOCA-salt hypertensive mice. (A) Representative Western blots for P-CREB, T-CREB, and β-actin in the hypothalamus of mice in SHAM, SHAM + ICV losartan, DOCA-salt, and DOCA-salt + ICV-infused losartan (3 mg/kg/day) groups after 21 days of treatment. (B) Quantification of P-CREB following treatment with DOCA-salt, with or without ICV infusion of losartan. (C) Quantification of T-CREB following treatment with DOCA-salt, with or without ICV infusion of losartan (n = 4–5 hypothalami/group; *P < 0.05 vs. SHAM, #P < 0.05 vs. DOCA + ICV aCSF).

Figure 8. An Ang II/AT1R-dependent increase in CREB binding to the endogenous PRR promoter is responsible for PRR up-regulation during DOCA-salt hypertension. (A) ChIP assay showing relative enrichment for P-CREB binding to the PRR promoter in the hypothalamus of mice in SHAM, SHAM + ICV losartan, DOCA-salt, and DOCA-salt + ICV-infused losartan (3 mg/kg/day) groups after 21 days of treatment (n = 5 hypothalami/group; *P < 0.05 vs. control, #P < 0.05 vs. DOCA + ICV aCSF). (B) PRR mRNA levels in the PVN of mice in SHAM, SHAM + ICV infusion of losartan, and DOCA-salt with ICV infusion of aCSF or losartan groups after 21 days of treatment (n = 4–5 PVNs/group; *P < 0.05 vs. SHAM, #P < 0.05 vs. DOCA + ICV aCSF).
Table 1. Oligonucleotides employed in RT-PCR and ChIP assays

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<th>Oligonucleotides</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>Mouse PRR forward</td>
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<tr>
<td>Mouse PRR reverse</td>
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**A**

Relative Enrichment

P-CREBChip

**B**

PRR mRNA Expression

SHAM

SHAM + ICV CAS92-78-4

DOCA + ICV aCSF

DOCA + ICV CAS92-78-4

PRR/GAPDH