Prostaglandin E-Prostanoid$_4$ Receptor Mediates Angiotensin II–Induced (Pro)Renin Receptor Expression in the Rat Renal Medulla

Fei Wang, Xiaohan Lu, Kexin Peng, Yaomin Du, Shu-Feng Zhou, Aihua Zhang, Tianxin Yang

**Abstract**—Angiotensin II (Ang II) stimulates (pro)renin receptor (PRR) expression in the renal collecting duct, triggering the local renin response in the distal nephron. Our recent study provided evidence for involvement of cyclooxygenase-2–prostaglandin E$_2$ pathway in Ang II–dependent stimulation of PRR expression in the collecting duct. Here, we tested the role of E-prostanoid (EP) subtypes acting downstream of cyclooxygenase-2 in this phenomenon. In primary rat inner medullary collecting duct cells, Ang II treatment for 12 hours induced a 1.8-fold increase in the full-length PRR protein expression. To assess the contribution of EP receptor, the cell was pretreated with specific EP receptor antagonists: SC-51382 (for EP$_1$), L-798106 (for EP$_3$), L-161982 (for EP$_4$), and ONO-AE3-208 (ONO, a structurally distinct EP$_4$ antagonist). The upregulation of PRR expression by Ang II was consistently abolished by L-161982 and ONO and partially suppressed by SC-51382 but was unaffected by L-798106. The PRR expression was also significantly elevated by the EP$_4$ agonist CAY10598 in the absence of Ang II. Sprague-Dawley rats were subsequently infused for 1 or 2 weeks with vehicle, Ang II alone, or in combination with ONO. Ang II infusion induced parallel increases in renal medullary PRR protein and renal medullary and urinary renin activity and total renin content, all of which were blunted by ONO. Both tail cuff plethysmography and telemetry demonstrated attenuation of Ang II hypertension by ONO. Overall, these results have established a crucial role of the EP$_4$ receptor in mediating the upregulation of renal medullary PRR expression and renin activity during Ang II hypertension. (Hypertension. 2014;64:369-377.)

**Key Word:** dinoprostone
activity in the CD during Ang II hypertension. The activation of renal medullary PRR may serve as an important mechanism triggering the local renin response that may participate in regulation of blood pressure and fluid metabolism during Ang II hypertension.

The biological action of prostaglandin E (PGE) is mediated by G protein–coupled E-prostanoid (EP) receptors designated EP₁, EP₂, EP₃, and EP₄. These 4 subtypes of EP receptor couple to distinct signaling pathways. Among the 4 EP subtypes, the EP₄ receptor plays a dominant role in regulation of renin release from the juxtaglomerular apparatus. We hypothesize that the EP₄ receptor may participate in Ang II–induced renin response in the renal medulla through an effect on PRR. To test this hypothesis, we used pharmacological inhibitors and activators of the EP₄ receptor to study their effect on renal medullary PRR expression and renin activity and hypertension development after Ang II treatment.

**Methods**

**Animals**

Male Sprague-Dawley rats (220–250 g, Charles River Laboratories, Wilmington, MA) were cage-housed and maintained in a temperature-controlled room with a 12:12-hour light–dark cycle, with free access to tap water and standard rat chow for 14 days. The animal protocols were approved by the Animal Care and Use Committee at Sun Yat-sen University, China. Rats randomly received sham operation, Ang II infusion (Human Ang II, Sigma, St. Louis, MO) via a subcutaneous osmotic minipump (Alzet model 2002, Alza, Palo Alto, CA) at a rate of 100 ng/min, or coadministered with ONO-AE3-208 (ONO; MedChemexpress LLC, Princeton, NJ) at 0.2 mg/kg per day for 14 days. Under isoflurane anesthesia, the minipump was subcutaneously implanted in the back of the neck area. At the end of the experiment, systolic blood pressure (SBP) was monitored by tail cuff plethysmography; the rats were placed in metabolic cages for 24-hour urine collections. At day 14, under isoflurane anesthesia, blood was withdrawn from vena cava and kidneys were harvested and cut into cortex and inner medulla. To validate the blood pressure results, telemetry was performed in a separate experiment to monitor daily mean arterial pressure (MAP) in rats infused with Ang II alone or in combination with ONO for 7 days at the same doses.

**Primary Cultures of Rat Inner Medullary Collecting Duct Cells**

Primary cultures enriched in inner medullary collecting duct (IMCD) cells were prepared from pathogen-free male Sprague-Dawley rats (40–100 g body wt) as previously described. After 24 hours of serum deprivation, the IMCD cells were pretreated for 1 hour with

![Figure 1](https://hyper.ahajournals.org/)

**Figure 1.** Effect of E-prostanoid (EP₄) antagonists on angiotensin II (Ang II)–induced (pro)renin receptor (PRR) protein expression in primary inner medullary collecting duct (IMCD) cells. The cells were pretreated for 1 hour with 2 structurally distinct EP₄ antagonists, L-16982 (A) and ONO-AE3-208 (ONO) (B), and then treated for 12 hours with 1 μmol/L Ang II. In a separate experiment, the cells were treated for 12 hours with ONO in the absence of Ang II (C). PRR protein expression was analyzed by immunoblotting. A, C, and E, Representative PRR immunoblot from 2 to 3 independent experiments. The full-length PRR protein was detected as a 43-kDa band. B, D, and F, Densitometric analysis of PRR protein and normalized by β-actin (n=6 per group). Data are mean±SE.
structurally distinct EP, antagonists, ONO at 1 μmol/L or L-161982 at 10 μmol/L (Cayman Chemical, Ann Arbor, MI), an EP, antagonist SC-51382 at 10 nmol/L (Cayman Chemical, Ann Arbor, MI), or an EP antagonist L-798106 at 10 μmol/L (Tocris Bioscience, United Kingdom), followed by Ang II treatment at 100 nmol/L or 1 μmol/L for various time periods. To study the effect of EP, agonism on PRR expression, the IMCD cells were exposed to an EP, agonist CAY10598 at 0.1 μmol/L (Cayman Chemical, Ann Arbor, MI) in the absence of Ang II. After these treatments, the cells were harvested for gene expression analysis or renin assay.

Sample Preparation for Renin Activity Assay
The blood samples were collected into tubes with 5.0 mmol/L EDTA, and plasma renin activity was assayed. Urine and cell culture medium were applied to molecular weight 10,000 cutoff centrifugal tubes (Amicon Ultra) to concentrate proteins higher than ≈30 kDa. The renal inner medulla and cortex were homogenized in 2.6 mmol/L EDTA, 3.4 mmol/L hydroxyquinoline, 5 mmol/L ammonium acetate, 200 μmol/L phenylmethanesulfonil fluoride, and 0.256 μmol/L dimercaprol. The homogenates were centrifuged at 4000 rpm at 4°C for 30 minutes, and the supernatant was collected.

Assay of Renin Activity
The samples were spiked with 1 μmol/L synthetic renin substrate tetradecapeptide (Sigma) for plasma, urine, and kidney tissues and with a final concentration of 1 μmol/L angiotensinogen for cell culture medium. After incubation at 37°C for 1 hour, the Ang I generation was assayed using an EIA kit according to the manufacturer’s instruction (S-1188 Angiotensin-I EIA kit from Bachem). To exclude the effect of peptidases, identical urine samples, renin substrate tetradecapeptide with the specific renin inhibitor WFML peptide (AnaSpec, Fremont, CA), were used as controls. The values were expressed as nanograms per milliliter per hour of generated Ang I. Trypsinization activates prorenin to renin.22 For trypsinization, samples incubated with trypsin from bovine pancreas (T1426 from sigma) in 37ºC for 18 hours, and the reaction was then terminated with soybean trypsin inhibitor (100 g/L) for 10 minutes on ice. Renin activity was determined in the native condition, active renin content with excessive angiotensinogen, and total renin content with excessive angiotensinogen plus trypsinization.

Immunoblotting
Renal tissues were lysed and subsequently sonicated in PBS that contained 1% Triton X-100, 250 μmol/L phenylmethanesulfonil fluoride, 2 mmol/L EDTA, and 5 mmol/L dithiothreitol (pH 7.5). Protein concentrations were determined by the use of Coomassie reagent. Forty micrograms of protein for each sample was denatured in boiling water for 10 minutes then separated by SDS-PAGE and transferred onto nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline, followed by incubation for 1 hour with rabbit anti-PRR antibody (Cat No. ab40790, Abcam). In Figures 1A, 2A, and 5, the membranes were stripped and reprobed with mouse anti–β-actin antibody (Cat No. A1979, Sigma). In Figures 1C, 1E, 2A, 2A, 6A, and 6D, independent anti–β-actin immunoblotting was performed because the stripping protocol did not yield optical results. After washing with Tris-buffered saline, blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized using enhanced chemiluminescence. The blots were quantified by using Imagepro-plus.

Quantitative Reverse Transcription Polymerase Chain Reaction
Total RNA isolation and reverse transcription were performed as previously described.23 Oligonucleotides were designed using Primer3 software (available at http://flypush.imgen.bcm.tmc.edu/primer/primer3_www.cgi). Primers for PRR were 5′-gggaagcttgtaggagaag-3′ (sense) and 5′-ggtcttcactaccatggagaagg-3′ (antisense); primers for GAPDH were 5′-ggttgtagggactttgggtgt-3′ (sense) and 5′-tctatgtagcttgagggcag-3′ (antisense).

Blood Pressure Measurement
SBP was measured by tail cuff plethysmography using a Visitech BP2000 Blood Pressure Analysis System (Apex, NC).24 All animals

Figure 2. Effect of E-prostanoid (EP), agonism on (pro)renin receptor (PRR) protein expression in primary inner medullary collecting duct cells. The cells were treated with the EP4 agonist CAY10598 at 0.1 μmol/L for 12 hours. PRR protein expression was analyzed by immunoblotting. A, Representative PRR immunoblot from 3 independent experiments. B, Densitometric analysis of PRR protein and normalized by β-actin (n=6–8 per group). Data are means±SE.

Figure 3. A, Effects of E-prostanoid (EP4) and EP1 antagonists on angiotensin II (Ang II)–induced (pro)renin receptor (PRR) protein expression in primary inner medullary collecting duct cells. The cells were pretreated for 1 hour with the EP1 antagonist SC-51382 and the EP4 antagonist L-798106 and then treated for 12 hours with 1 μmol/L Ang II. Shown is the densitometric result from PRR immunoblotting. B, Effects of EP antagonists on PRR mRNA expression during Ang II treatment. The cells were pretreated for 1 hour with SC-51382, ONO-AE3-208 (ONO), or L-798106 and then treated for 12 hours with 1 μmol/L Ang II. PRR mRNA was detected by quantitative reverse transcription polymerase chain reaction and normalized by GAPDH (n=6 per group). Data are means±SE.
were habituated to the blood pressure measurement device for 7 days. They all underwent 2 cycles of 20 measurements reordered per day for a minimum of 3 days. Telemetry measurement of MAP was performed as previously described. Daily MAP was recorded as mean values of 4-hour recordings from 5:00 pm to 9:00 pm.

Statistical Analysis
Data are summarized as means±SE. Statistical analysis was performed using ANOVA with the Bonferroni test for multiple comparisons or by unpaired Student t test for 2 comparisons. P<0.05 was considered statistically significant.

Results
In Vitro Investigation of the Role of the EP Receptor in Mediating Ang II–Induced PRR Expression in Primary Rat IMCD Cells
We attempted to examine the EP subtypes involved in regulation of PRR expression in primary rat IMCD cells after Ang II treatment. IMCD cells were isolated from the inner medulla of Sprague-Dawley rats and grown in 6-well plates. After reaching confluence, the cells were exposed to Ang II in the presence or absence of an EP antagonist. In our previous study, we observed that 1 μmol/L Ang II induced a peak stimulation of PRR protein expression at 12 hours. Accordingly, this experimental condition was used in the subsequent experiments to investigate the involvement of EP receptors. By immunoblotting, the full-length PRR was detected as a 43-kDa band. Exposure to 1 μmol/L Ang II for 12 hours elevated the total protein abundance of PRR (Figure 1). The increase of PRR protein abundance was abolished by an EP4 antagonist L-161982 at 10 μmol/L (Figure 1A and 1B). The same result was obtained by using a structurally distinct EP4 antagonist ONO at 1 μmol/L (Figure 1C and 1D). In a separate experiment, we found that the basal PRR protein expression was significantly suppressed by EP4 antagonism (Figure 1E and 1F). To validate EP4 regulation of PRR expression further, we examined the effect of the EP4 agonist CAY10598 on PRR expression in primary IMCD cells. After 12-hour exposure to 100 nmol/L CAY10598, PRR protein expression was significantly increased (Figure 2).

In separate experiments, we examined the involvement of EP1 and EP3 by using the respective EP antagonists. The EP1 antagonist SC-51382 at 10 nmol/L effectively attenuated Ang II–induced PRR protein expression, but the EP3 antagonist L-798106 at 10 μmol/L was without an effect. Figure 3A showed the densitometry results from these Western blots. Quantitative reverse transcription polymerase chain reaction was performed to examine PRR mRNA expression after the treatment with Ang II alone or in combination with an EP antagonist (for EP1, EP3, or EP4). However, we found no change in PRR mRNA in any of the experimental group (Figure 3B).

In light of the potential role of PRR in regulation of renin activity, we performed an assay for renin activity in the medium of primary IMCD cells exposed to 1 μmol/L Ang II alone or in combination with ONO. The assay was
performed using angiotensin-I ELISA kit. To validate specificity of this kit, we examined its cross-activity with Ang II. The cross-activity was undetectable for 1 or 100 μmol/L Ang II and 3/100,000 for 1 mmol/L Ang II, consistent with the report from the manufacturer (Peninsula Laboratories, LLC). As shown in Figure 4, 1 μmol/L Ang II treatment for 12 hours significantly increased medium renin activity, and this increase was almost completely abolished by ONO (Figure 4A). Similar results were obtained for active renin content (Figure 4B). By quantitative reverse transcription polymerase chain reaction, renin mRNA was altered in a similar fashion as renin activity and active renin content (Figure 4C).

Given the concern about the high concentration of Ang II used in the above-described experiments, we validated some of the major results from these experiments by using 100 μmol/L Ang II. Time course studies demonstrated that in response to 100 μmol/L Ang II treatment, PRR protein expression was induced at 3 hours and decreased rapidly thereafter.

Figure 6. The expression of (pro)renin receptor (PRR) in the renal inner medulla of rats treated with vehicle, angiotensin II (Ang II), or Ang II+ONO-AE3-208 (ONO). A, Representative PRR immunoblot from 3 independent experiments. B, Densitometric analysis of PRR protein. The PRR protein expression was normalized by β-actin (n=5 per group). C, Quantitative reverse transcription polymerase chain reaction analysis of PRR mRNA. The mRNA expression was normalized by GAPDH. D, Effect of ONO on baseline PRR protein expression in the rat renal inner medulla. The expression was determined by using immunoblotting analysis (n=5 per group). Data are mean±SE. CTR indicates control.

Effect of ONO on baseline PRR protein expression in the rat renal inner medulla. The expression was determined by using immunoblotting analysis (n=5 per group). Data are mean±SE.

In Vivo Investigation of the Role of the EP4 Receptor in Mediating Ang II–Induced PRR Expression in Rat Renal Inner Medulla

Sprague-Dawley rats were treated for 14 days with Ang II in combination with or without ONO. The end points included renal medullary expression of PRR expression, plasma, urinary, and tissue renin activity and content, as well as blood pressure. Fourteen-day Ang II infusion significantly increased PRR protein expression in the inner medulla as assessed by immunoblotting (4.2±0.4 versus 1.0±0.2; P<0.05; Figure 6A and 6B). EP4 antagonism with ONO completely abolished the upregulation of renal medullary PRR expression by Ang II (0.6±0.2 in the Ang II+ONO group; Figure 6A and 6B). In contrast, quantitative reverse transcription polymerase chain reaction detected no change in PRR mRNA expression in the inner medulla after Ang II treatment (Figure 6C). In a separate experiment, we examined the effect of ONO on the baseline PRR protein expression in the rat renal inner medulla. A 14-day ONO treatment induced a small but significant reduction of the baseline PRR expression in the inner medulla (0.84±0.15 versus 1.0±0.17; n=4; P<0.05; Figure 6D).

Plasma, the renal cortex, and the inner medulla were subjected to measurement of renin activity, active renin content, and total renin content. All renin parameters including renin activity and active and total renin content in plasma were significantly suppressed after Ang II infusion, which was unaffected by ONO treatment (Figure 7A–7C). A similar pattern of changes in these renin parameters was observed in the renal cortex (Figure 7D–7F). In a sharp contrast, these parameters were all elevated in urine after Ang II infusion, which was almost completely reversed by ONO treatment (Figure 8A–8C). These results were almost identical to those in the inner medulla (Figure 8D–8F). These results reflect the opposite responses of systemic and renal medullary renin system and also suggest that urinary renin is of renal medullary origin.

SBP was measured by using tail cuff plethysmography. SBP was significantly higher in the Ang II group than in the control group (182.3±14.5 in the Ang II group versus 116.2±6 mm Hg in the control group; P<0.05), and the increase in SBP was less in the Ang II+ONO group (143.5±7.0 mm Hg; Figure 9A). To validate this result, in a separate experiment, we compared MAP between the Ang II group and the Ang II+ONO group using telemetry. Again, the MAP was lower in the Ang II+ONO group than in the Ang II group (Figure 9B). In another separate experiment, we examined the effect of ONO on baseline MAP in rats. The baseline MAP was unaffected during a 7-day ONO treatment (106.7±6.4 mm Hg in the control group versus 107.5±5.6 mm Hg in the control group, n=5 per group; P>0.05). Together, these results suggest that the EP4 receptor mediates Ang II–induced hypertension but may play no role in the control of baseline blood pressure.
Discussion


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PGE2 is a major prostanoid produced in the kidney, particularly in the CD. As an autocrine/paracrine factor, PGE2 exerts a diverse range of action at the site of its production, affecting renal medullary blood flow and tubular sodium and water transport, as well as cell survival.26,27 The biological action of PGE2 is mediated by 4 distinct EP4 receptors (EP1–4). We for the first time demonstrated a dominant role of the EP4 receptor in mediating Ang II–induced PRR expression and renin activity in the renal medulla. The evidence for this conclusion is compelling. First, EP4 antagonists were highly efficient in inhibiting PRR expression in that they not only blocked Ang II–induced PRR expression but also remarkably reduced the basal expression. The use of structurally distinct EP4 antagonists has resolved the specificity issue related to the pharmacological approach. Second, the involvement of EP4 has also been demonstrated by EP4 agonism. Last, the observation with EP4 antagonist was initially made in vitro and was subsequently confirmed in vivo.

The detailed signaling pathway downstream of the EP4 receptor in the CD is not known. This EP subtype is presumed to signal through the Gs protein, which elevates intracellular cAMP. It seems reasonable to speculate that cAMP pathway may be involved in EP4-dependent stimulation of PRR expression during Ang II hypertension. Currently, the direct evidence supporting this notion is lacking. However, the cGMP–protein kinase G signaling pathway is shown to mediate the upregulation of PRR expression in IMCD cells exposed to salt depletion.28 Although the relationship between cAMP and cGMP is generally antagonistic to each other, the 2 mediators can couple together to simulate renin secretion in juxtaglomerular cells. The cooperation between the 2 signaling pathways in renin regulation is reflected by the fact that cGMP inhibits phosphodiesterases 3 that degrades cAMP. Future studies need to assess the relative importance of the 2 signaling pathways in regulation of PRR in the CD during Ang II hypertension.

Abundant in vitro evidence demonstrates that PRR binds renin and prorenin to increase their catalytic activity.14,29–32

Figure 7. Renin levels in plasma and renal cortex of rats treated with vehicle, angiotensin II (Ang II), or Ang II+ONO-AE3-208 (ONO). A, Plasma renin activity. B, Plasma active renin content. C, Plasma total renin content. D, Renal cortical renin activity. E, Renal cortical active renin content. F, Renal cortical total renin content (n=5 per group). Data are means±SE. CTR indicates control.
Accordingly, PRR is considered as a potential regulator of tissue RAS. However, solid in vivo evidence to support this notion is still lacking. Overexpression of human PRR in rats resulted in proteinuria and nephropathy but did not elevate blood pressure or renal Ang II levels.33,34 The lack of viable PRR null mice, systemic or tissue-specific, has made it difficult to convincingly prove PRR as a key player in RAS.35 In the present study, we observed that the decreased renal medullary PRR expression by EP4 antagonism was in parallel with the reduction of renal medullary renin levels and blood pressure. This observation represents indirect evidence supporting PRR being involved in renin regulation in the renal medulla during Ang II treatment.

The PGE2/EP4 pathway has an established role in the macula densa signal for release of renin. For example, infusion of PGE2 into the kidney stimulates renin secretion in various ex vivo and in vitro juxtaglomerular cell culture models,36,37 and this effect depends on cAMP.38 Deletion of EP4 receptors reduces renin stimulation by 70% after furosemide administration, whereas deletion of EP2 has no effect,39 supporting the dominant role of this receptor in renin regulation. Therefore, we cannot rule out the possibility that EP4 activation may directly regulate renin expression, activity, or release in the CD independent of PRR.

The EP1 receptor was originally described as a smooth muscle constrictor.17 This receptor generally signals through intracellular calcium and protein kinase C. In the present study, we found that EP1 antagonism effectively blocked Ang II–induced PRR expression, suggesting involvement of the EP1 receptor in the upregulation of PRR. It seems possible that the corporation between EP1 and EP4 subtypes may be required for the full PRR response during Ang II hypertension. Whether intracellular calcium and protein kinase C mediate the effect of EP1 activation is not known. Of note, like the EP1 receptor, the EP3 receptor also signals through intracellular calcium and protein kinase C and induces vasoconstriction.39,40 However, we found no effect of EP3 antagonism on PRR expression. This may be related to the complex signaling properties of the EP3 receptors via Gi (inhibition of cAMP formation), Gs (stimulation of cAMP formation), and Gq (stimulation of intracellular Ca2+ release), which may exert diverse influence on PRR expression.41 Of note, the present study is limited in that the functional role of the EP1 receptor in PRR regulation was not tested because of the lack of commercially available antagonist for this receptor subtype.

The PRR-mediated local renin response is expected to contribute to Ang II hypertension. cyclooxygenase-2 deficiency or inhibitors like refecoxib and nimesulide exhibit potent antihypertensive action in rodent models of Ang II hypertension.42–44 The present study has extended these observations by elucidating the EP1 receptor as the responsible

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Figure 8. Renin levels in urine and renal inner medulla of rats treated with vehicle, angiotensin II (Ang II), or Ang II+ONO-AE3-208 (ONO).
A, Urine renin activity. B, Urine active renin content. C, Urine total renin content. D, Cortical renin activity in the inner medulla. E, Active renin content in the inner medulla. F, Total renin content in the inner medulla (n=5 per group). Data are mean±SE. CTR indicates control.
We provide in vitro and in vivo evidence supporting the EP<sub>4</sub> receptor as a major regulator of PRR expression in the renal medulla during Ang II hypertension. These results provide new insight into the interaction between renal medullary PRR and PGE<sub>2</sub> in Ang II signaling. More importantly, the results suggest that EP antagonism may represent a novel intervention in management of hypertension and kidney disease by targeting PRR.

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**Disclosures**

None.

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