COX-2 mediates angiotensin II-induced (pro)renin receptor expression in the rat renal medulla

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1Institute of Hypertension, Sun Yat-sen University School of Medicine, Guangzhou, China; 2Department of Nephrology, The First Affiliated Hospital, Sun Yat-sen University, Guangzhou, China; 3Internal Medicine, University of Utah and Veterans Affairs Medical Center, Salt Lake City, Utah; and 4Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, Florida

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Wang F, Lu X, Peng K, Zhou L, Li C, Wang W, Yu X, Kohan DE, Zhu SF, Yang T. COX-2 mediates angiotensin II-induced (pro)renin receptor expression in the rat renal medulla. Am J Physiol Renal Physiol 307: F25–F32, 2014. First published April 16, 2014; doi:10.1152/ajprenal.00548.2013.—(Pro)renin receptor (PRR) is predominantly expressed in the distal nephron where it is activated by angiotensin II (ANG II), resulting in increased renin activity in the renal medulla thereby amplifying the de novo generation and action of local ANG II. The goal of the present study was to test the role of cyclooxygenase-2 (COX-2) in mediating ANG II-induced PRR expression in the rat medulla in vitro and in vivo. Exposure of primary rat inner medullary collecting duct cells to ANG II induced sequential increases in COX-2 and PRR protein expression. When the cells were pretreated with a COX-2 inhibitor NS-398, ANG II-induced upregulation of PRR protein expression was almost completely abolished, in parallel with the changes in medium active renin content. The inhibitory effect of NS-398 on the PRR expression was reversed by adding exogenous PGE₂. A 14-day ANG II infusion elevated renal medullary PRR expression and active and total renin content in parallel with increased urinary renin, all of which were remarkably suppressed by the COX-2 inhibitor celecoxib. In contrast, plasma and renal cortical active and total renin content were suppressed by ANG II treatment, an effect that was unaffected by COX-2 inhibition. Systolic blood pressure was elevated with ANG II infusion, which was attenuated by the COX-2 inhibition. Overall, the results obtained from in vitro and in vivo studies established a crucial role of COX-2 in mediating upregulation of renal medullary PRR expression and renin content during ANG II hypertension.

(Pro)renin receptor; renin activity; cyclooxygenase-2; inner medullary collecting duct; prostaglandin E₂

THE RENIN-ANGIOTENSIN SYSTEM (RAS) plays a pivotal role in the regulation of blood pressure, cardiovascular function, renal hemodynamics, and tubular sodium reabsorption (8). Compared with the well-recognized role of systemic RAS in regulation of blood pressure and in the pathogenesis of hypertension, in recent years, there has been increasing appreciation of the potential role of local RAS found in a variety of tissues including the brain, heart, adrenal glands, vasculature, and the kidney (17, 18). The existence of intrarenal RAS is highlighted by the discovery of renin expression in the connecting tubules and cortical and medullary collecting ducts (CDs) (12, 24) and angiotensinogen expression in the proximal tubule (11), the two key elements of paracrine tubular RAS. A striking feature of the local RAS is that the expression of renin in the distal nephron is positively regulated by ANG II. In this regard, chronic ANG II infusion elevates medullary renin mRNA, urinary renin content, and intrarenal ANG II levels (21). This effect appears to be direct since in primary cultures of inner medullary CD cells, renin mRNA and (pro)renin protein levels are increased with ANG II treatment (21). The potential role of local ANG II generation is suggested by the observation that luminal ANG II stimulates amiloride-sensitive sodium transport in the initial collecting tubule of cortical nephrons (26). It seems likely that the local renin response to increased circulating ANG II levels may contribute to ANG II hypertension through the augmented ANG II generation and action in the distal nephron.

(Pro)renin receptor (PRR) (2) is a newly discovered component of the RAS, being capable of binding renin and prorenin with almost equal affinity to increase their catalytic activity (19). In addition to its role in enhancing prorenin enzymatic activity toward angiotensin I (ANG I) generation, PRR binding triggers intracellular signaling cascades such as the activation of MAP kinases ERK1 and ERK2 (19). Within the kidney, by immunostaining and in situ hybridization, PRR expression is predominantly expressed in the intercalated cells of the CD (1). Chronic infusion of ANG II in rats increased renal PRR transcript levels and augmented the PRR activity in renal medullary tissues, which may contribute to increased renin activity in the CD during ANG II hypertension (7). The activation of renal medullary PRR is considered as a key component of the local renin response that may participate in regulation of blood pressure and fluid metabolism during ANG II hypertension. However, the mechanism of how ANG II stimulates PRR expression in the CD is unknown.

Prostanoids including PGE₂, PGF₂α, PGD₂, PGL₂, and thromboxane A2 are derivatives of arachidonic acid through the activity of constitutive isoform COX-1 or the inducible isoform COX-2 (29). Prostanoids participate in blood pressure control by acting on the kidneys, blood vessels, endocrine organs, and brain. Despite the initial characterization as inducible cyclooxygenase, COX-2 has been recognized as an important regulator of renin secretion, renal function, and blood pressure (4, 9). In particular, at basal condition, COX-2 is abundantly expressed in the renal medulla where its expression is elevated by salt loading (5, 30). Renal medullary COX-2 plays an important role in regulation of renal medullary blood flow or tubular sodium transport (22). In the hypertension model induced by chronic ANG II infusion, COX-2 deficiency

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AngII expression was analyzed by immunoblotting and qRT-PCR. The full-length PRR protein was detected as a 43-kDa band. Cells were exposed to 1 μM ANG II at the indicated time periods and PRR expression was analyzed by immunoblotting and qRT-PCR. A: representative immunoblot of PRR. The full-length PRR protein was detected as a 43-kDa band. B: densitometric analysis of PRR protein. N = 3–6 per group. *P < 0.05. C: qRT-PCR analysis of PRR mRNA. The expression was normalized by GAPDH. N = 6 per group. Data are means ± SE.

METHODS

Animals. Male Sprague-Dawley (SD) rats (220–250 g; Charles River Laboratories, Wilmington, MA) were cage-housed and maintained in a temperature-controlled room with a 12:12-h 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 either sham operation, ANG II infusion (Human ANG II, Sigma, St. Louis, MO) via a subcutaneous osmotic minipump (Alzet model 1002, Alza, Palo Alto, CA) at a rate of 100 ng/min, or coadministered with celecoxib mixed with chow (30 mg·kg⁻¹·day⁻¹) for 14 days. At the end of the experiment, systolic blood pressure (SBP) was monitored by tail-cuff plethysmography (IITC, Woodland Hills, CA) after a 1-wk training period; the rats were placed in metabolic cages for 24-h urine collections. At day 14, rats were killed and blood and kidneys were harvested. Trunk blood was collected for measuring plasma renin activity (PRA) levels. After decapsulation, the kidneys were weighed, and cut into cortex and inner medulla.

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 SD rats (40–100 g body wt) as previously described (6). The IMCD cells were pretreated for 1 h with NS-398 (10 μM) or PGE₂ (1 μM) for 1 h, followed by ANG II treatment at 100 nM or 1 μM for various time periods. After the treatment, 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 PRA was assayed. Urine and cell culture medium were applied to MW 10,000 cut-off centrifugal tubes (Amicon Ultra) to concentrate proteins higher than ~30 kDa. The renal inner medulla and cortex were homogenized in 2.6 mM EDTA, 3.4 mM hydroxyquinoline, 5 mM ammonium acetate, 200 μM phenylmethanesulfonyl fluoride (PMSF), and 0.256 μM dimeracrol. The homogenates were centrifuged at 4,000 rpm at 4°C for 30 min and the supernatant was collected.

Assay of renin content. For measurement of active renin content, the samples were spiked with 1 μM synthetic renin substrate tetracapeptide (RST; Sigma) for plasma, urine, and kidney tissues and with 1 μM angiotensinogen for cell culture medium. Following incubation or inhibitors like refecoxib and nimesulide exhibit potent antihypertensive action (14, 23, 27). The goal of the present study is to examine whether COX-2-derived products regulate PRR expression in the renal medulla, which will provide new insight into the intrarenal mechanism of ANG II hypertension.

Fig. 1. Effect of ANG II on (pro)renin receptor (PRR) mRNA and protein expression in primary rat inner medullary collecting duct (IMCD) cells. The cells were exposed to 1 μM ANG II at the indicated time periods and PRR expression was analyzed by immunoblotting and qRT-PCR. A: representative immunoblot of PRR. The full-length PRR protein was detected as a 43-kDa band. B: densitometric analysis of PRR protein. N = 3–6 per group. *P < 0.05. C: qRT-PCR analysis of PRR mRNA. The expression was normalized by GAPDH. N = 6 per group. Data are means ± SE.

Fig. 2. Effect of ANG II on cycloxygenase (COX)-2 expression in primary rat IMCD cells. The cells were exposed to 1 μM ANG II at the indicated time periods and COX-2 expression was analyzed by immunoblotting and qRT-PCR. A: representative immunoblot of COX-2. B: densitometric analysis of COX-2 mRNA. N = 3–6 per group. C: qRT-PCR analysis of COX-2 mRNA. *P < 0.05. D: representative immunoblot of COX-1. In this experiment, the IMCD cells were treated for 12 h with vehicle or 1 μM ANG II. The expression was normalized by GAPDH. N = 6 per group. Data are means ± SE.
at 37°C for 1 h, 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-RST with the specific renin inhibitor WFM3 peptide (AnaSpec, Fremont, CA) were used as controls. The values were expressed as nanograms per milliliter per hour of generated ANG I. For measurement of total renin content, trypsinization is performed to activate prorenin to renin (25). The samples were incubated with trypsin from bovine pancreas (T1426 from Sigma) in 37°C for 18 h and the reaction was then terminated with Soybean Trypsin Inhibitor (100 g/l) for 10 min on ice.

Immunoblotting. Renal tissues were lysed and subsequently sonicated in PBS that contained 1% Triton X-100, 250 μM PMSF, 2 mM EDTA, and 5 mM dithiothreitol (pH 7.5). Protein concentrations were determined by the use of Coomassie reagent. Forty micrograms of protein for each sample were denatured in boiling water for 10 min, then separated by SDS-PAGE, and transferred onto nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline (TBS), followed by incubation for 1 h with anti-PRR antibody (cat. no.: ab40790, Abcam) (10) or anti-COX-2 antibody (cat. no.: 62600, Cayman Chemicals). After being washed with TBS, blots were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody and visualized using enhanced chemiluminescence.

qRT-PCR. Total RNA isolation and reverse transcription were performed as previously described (20). Oligonucleotides were designed using Primer3 software (available at http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers for PRR were 5′-ggttgtagggactttggccag-3′ (sense) and 5′-gtcttcactaccatggagaagg-3′ (antisense); primers for GAPDH were

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\begin{align*}
5′-\text{gtctcactacatgagaagg-3′ (sense)} & \quad \text{and} \quad 5′-\text{tcattgatcctgagcagcg-3′ (antisense).} \\
& \\
\end{align*}
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Blood pressure measurement. SBP was measured by a tail-cuff method using a Visitech BP2000 Blood Pressure Analysis System (Apex, NC) (13). All animals were habituated to the blood pressure measurement device for 7 days. They all underwent two cycles of 20 measurements reordered per day for a minimum of 3 days.

Statistical analysis. Data are summarized as means ± SE. Statistical analysis was performed using one-way ANOVA with the Bonferroni test for multiple comparisons or by unpaired Student’s t-test for two comparisons. P < 0.05 was considered statistically significant.

RESULTS

In vitro investigation of the role of COX-2 in mediating ANG II-induced PRR expression in primary rat IMCD cells. Renal medullary PRR expression or activity was shown to be regulated during ANG II hypertension in rats. To examine the cellular mechanism of this phenomenon, we performed in vitro studies to examine the direct effect of ANG II on PRR expression in primary rat IMCD cells and further test whether this effect was mediated by COX-2. Pilot experiments demonstrated that ANG II in the range of 100 nM-1 μM dose-dependently stimulated PRR expression. The 1 μM was chosen for subsequent studies since this concentration gave rise to the maximal effect. As seen in Fig. 1A, immunoblotting detected a 43-kDa full-length PRR protein but was unable to detect the soluble form of this protein. Following ANG II treatment, PRR protein abundance was significantly increased at 12 h, but not

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\begin{align*}
\text{PRR density} & \quad \text{expressed as nanograms per milliliter per hour of generated ANG I.} \\
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\end{align*}
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Fig. 3. Effect of NS-398 on PRR expression in primary IMCD cells at baseline or after ANG II treatment. The cells were exposed to 1 μM ANG II for 12 h and PRR protein expression was analyzed by immunoblotting (A, B, and C). In a separate experiment, the cells were exposed to vehicle or NS-398 for 12 h and PRR was analyzed by the same method (D, E, and F). A, D: representative immunoblot of PRR. B, E: densitometric analysis of PRR protein. N = 6 per group. C, F: qRT-PCR analysis of PRR mRNA. D: expression was normalized by GAPDH. N = 6 per group. Data are means ± SE.
In vivo investigation of the role of COX-2 in mediating ANG II-induced PRR expression in rat renal inner medulla. SD rats were treated for 14 days with ANG II in combination with or without celecoxib. The endpoints included renal medullary expression of PRR and COX-2 expression, plasma, urinary, and tissue renin activity, as well as blood pressure. Fourteen-day ANG II infusion significantly increased PRR protein expression in the renal inner medulla as assessed by immunoblotting (1.4 ± 0.35 vs. 1.0 ± 0.2, P < 0.05; Fig. 6, A and B). However, qRT-PCR detected no change in PRR mRNA expression in the inner medulla (Fig. 6C), suggesting posttranscriptional regulation. Renal medullary COX-2 protein and mRNA expression were examined by the similar methods as for PRR. The ANG II treatment induced parallel increases in COX-2 protein (1.5 ± 0.2 vs. 1.0 ± 0.16, P < 0.05; Fig. 7, A and B) and mRNA in the inner medulla (2.7 ± 0.22 vs. 1.0 ± 0.15, P < 0.05; Fig. 6C). Inhibition of COX-2 with celecoxib completely abolished the upregulation of renal medullary PRR expression by ANG II (0.69 ± 0.19 in ANG II + celecoxib vs. 1.4 ± 0.35 in ANG II, P < 0.05; Fig. 6, A and B). The abundance of PRR protein in the ANG II + celecoxib group was even much lower than that in the control group, suggesting dependence of basal PRR expression on COX-2 as well.

Plasma, urine, the renal cortex, and the inner medulla were subjected to measurement of renin in the absence and presence of trypsin to reflect active and total renin content, respectively. ANG II infusion resulted in distinct changes in renin levels in plasma, urine, and the kidney regions, with suppression of renin levels in plasma and the renal cortex but augmentation of renin levels in the renal inner medulla and urine (Fig. 8), reflecting the opposite responses of systemic and renal medullary renin system. These results also support the origin of urinary renin from the renal medulla but not the circulation or

![Image](https://via.placeholder.com/150)

**Fig. 4.** Effect of NS-398 on ANG II-induced renin content in IMCD cells. The cells were treated for 12 h in the presence or absence of 10 μM NS-398 and active renin content in the medium was determined. N = 3 per group. Data are means ± SE.

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**Fig. 5.** Effect of NS-398 in combination with PGE2 on ANG II-induced PRR expression in the IMCD cells. The cells were treated for 12 h with 1 μM ANG II in the absence or presence of 10 μM NS-398 or in combination with 1 μM PGE2. A: representative immunoblot of PRR. B: densitometric analysis of PRR protein. N = 6 per group. C: qRT-PCR analysis of PRR mRNA. The expression was normalized by GAPDH. N = 6 per group. Data are means ± SE.
renal cortex. The increases in renal medullary active and total renin content in response to ANG II infusion were completely abolished by celecoxib, whereas the renal cortical renin levels were unaffected by this treatment (Fig. 8). These results support a dominant role of COX-2 in regulation of the local ANG II-induced renin response in the renal medulla but not in the renal cortex.

SBP was measured by using tail-cuff plethysmography. SBP was significantly higher in the ANG II group than in the control group (174.9 ± 7.0 in the ANG II group vs. 116.4 ± 3.9 mmHg in the control group, \( P < 0.05 \)) and the increase in SBP was less in the ANG II + celecoxib group (151.8 ± 4.8 mmHg, \( P < 0.05 \) vs. ANG II; Fig. 9).

DISCUSSION

The goal of the present study was to test the role of COX-2 in mediating the stimulation of PRR in the CD in response to ANG II treatment. Both in vitro and in vivo data from the present study demonstrated that ANG II treatment elevated PRR protein expression without an effect on the transcript levels. This consistent finding provides a clue for possible posttranscriptional regulation of PRR expression. The posttranscriptional mechanism may involve changes at the levels of protein translation, degradation, or cleavage. To our knowledge, there are no previous studies documenting posttranscriptional regulation of PRR in general and this regulation in the renal medulla in particular. Our results generally agree with the observation by Gonzalez et al. (7) that the activity of PRR in the renal medulla is stimulated during ANG II hypertension but with a few differences. For example, this study reported increased soluble form but decreased full-length form of PRR in the renal medulla of ANG II-treated rats. However, we found that ANG II consistently increased the full-length form of PRR protein in the rat inner medulla in vivo and also in primary IMCD cells in vitro. The reason for this discrepancy is likely related to different sources of anti-PRR antibody, which may be raised against different regions of the antigen leading to differences in recognition of full-length and cleaved PRR protein. This issue has been documented elsewhere in the literature. In Gonzalez’s study, the PRR transcript levels were significantly elevated in both cortical and medullary regions.
after ANG II treatment contrasting with the lack of alteration of the transcript levels in our study. Importantly, in extension of the previous study by Gonzalez et al., we performed an in vitro study to examine the direct effect of ANG II on PRR expression in primary rat IMCD cells. Our study is the first to demonstrate a direct stimulatory effect of ANG II on PRR protein but not mRNA expression in primary IMCD cells, which parallels the increase of medium renin activity.

The most novel finding of the present study was the demonstration that COX-2 functions as a mediator of ANG II-induced elevation of PRR expression in the inner medulla. In response to ANG II treatment, the increases in COX-2 and PRR protein expressions were first observed at 4 and 12 h, respectively. This sequence of events supports COX-2 as a mediator acting upstream of PRR in ANG II-elicited signaling cascades. Both COX-2 inhibitors, NS-398 and celecoxib, completely abolished the upregulation of PRR expression in cultured IMCD cells and the renal inner medulla, respectively, indicating the dominant role of COX-2-derived products in regulation of PRR expression at least in the setting of ANG II treatment. Strikingly, PRR expression in the ANG II/celecoxib group was much lower than in the control group, suggesting that the basal expression of renal medullary PRR is also under the control of COX-2. Additionally, in vitro studies showed that addition of exogenous PGE$_2$ completely reversed the inhibitory effect of NS-398 on PRR expression, supporting PGE$_2$
as the major COX-2-derived product contributable to the ANG II stimulation of PRR expression in IMCD cells. PGE₂ is generated by prostaglandin E synthase (PGES) that exists in three major isoforms: membrane-associated PGES (mPGES)-1, mPGES-2, and cytosolic PGES (cPGES) (16, 28). The biologic action of PGE₂ is mediated by G protein-coupled E-prostanoid receptors designated EP₁, EP₂, EP₃, and EP₄ (3). Future studies are needed to define a specific PGES and EP subtype in regulation of PRR expression in the distal nephron.

PRR likely plays an important role in augmentation of renin activity through direct binding to renin or prorenin. Therefore, the changes in PRR expression are expected to lead to corresponding changes in renin activity. Indeed, we found that in primary cultures of IMCD cells renin activity was elevated by ANG II and suppressed by NS-398, and similarly, in SD rats, renal medullary and urinary renin levels were increased with ANG II but remarkably reduced by celecoxib. In both cases, the changes in renal medullary renin agree completely with those in renal medullary PRR expression. On a sharp contrast, renal cortical and plasma renin activity were suppressed by ANG II, which was unaffected by celecoxib. These results support that concept that COX-2-mediated activation of PRR may act within the renal medulla to control the local renin activity without affecting the systemic RAS.

We found that celecoxib significantly attenuated the hypertension induced by 14-day ANG II infusion. This result substantiates the previous observation that in the ANG II hypertension model COX-2 deficiency or inhibitors like refecoxib and nimesulide exhibit potent antihypertensive action (14, 23, 27). It seems reasonable to speculate that COX-2-mediated activation of renal medullary PRR expression may be, at least in part, contributed to ANG II hypertension through enhancement of sodium reabsorption in the distal nephron. Contrary to its prohypertensive action in ANG II hypertension, however, renal medullary COX-2 exerts antihypertensive action in rodent models of salt-sensitive hypertension. Renal medullary COX-2 expression is increased in response to chronic salt loading and intramedullary delivery of NS-398 induces salt-sensitive hypertension in rats (30, 31). Therefore, renal medullary COX-2 can exert both positive and negative influence on hypertension depending on the etiologies. Salt depletion was shown to stimulate renal PRR expression (15) but the effect of chronic salt loading remains uninvestigated. These results suggest that the renin PRR-mediated mechanism may be only operative during ANG II- but not salt-sensitive hypertension.

In summary, the present study examined the role of COX-2 in ANG II-induced PRR expression in the renal medulla. In primary rat IMCD cells, ANG II induced COX-2 expression, followed by increased PRR expression; the increases in PRR expression along with renin activity were suppressed by COX-2 inhibition. Consistent with these results, in a rat model of ANG II hypertension, renal medullary PRR expression and renin levels were elevated in parallel, both of which were remarkably suppressed by COX-2 inhibition, accompanied by a reduction of blood pressure. Together, these results demonstrate an essential role of COX-2-derived products in mediating PRR-dependent activation of local renin activity in the renal medulla, a novel mechanism likely contributable to the pathogenesis of ANG II hypertension.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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