Angiotensin II Stimulates Renin in Inner Medullary Collecting Duct Cells via Protein Kinase C and Independent of Epithelial Sodium Channel and Mineralocorticoid Receptor Activity

Alexis A. Gonzalez, Liu Liu, Lucienne S. Lara, Dale M. Seth, L. Gabriel Navar, Minolfa C. Prieto

Abstract—Collecting duct (CD) renin is stimulated by angiotensin (Ang) II, providing a pathway for Ang I generation and further conversion to Ang II. Ang II stimulates the epithelial sodium channel via the Ang II type 1 receptor and increases mineralocorticoid receptor activity attributed to increased aldosterone release. Our objective was to determine whether CD renin augmentation is mediated directly by Ang II type 1 receptor or via the epithelial sodium channel and mineralocorticoid receptor. In vivo studies examined the effects of epithelial sodium channel blockade (amiloride; 5 mg/kg per day) on CD renin expression and urinary renin content in Ang II–infused rats (80 ng/min, 2 weeks). Ang II infusion increased systolic blood pressure, medullary renin mRNA, urinary renin content, and intrarenal Ang II levels. Amiloride cotreatment did not alter these responses despite a reduction in the rate of progression of systolic blood pressure. In primary cultures of inner medullary CD cells, renin mRNA and (pro)renin protein levels increased with Ang II (100 nmol/L), and candesartan (Ang II type 1 receptor antagonist) prevented this effect. Aldosterone (10⁻¹⁰ to 10⁻⁷ mol/L) with or without amiloride did not modify the upregulation of renin mRNA in Ang II–treated cells. However, inhibition of protein kinase C with calphostin C prevented the Ang II–mediated increases in renin mRNA and (pro)renin protein levels. Furthermore, protein kinase C activation with phorbol 12-myristate 13-acetate increased renin expression to the same extent as Ang II. These data indicate that an Ang II type 1 receptor–mediated increase in CD renin is induced directly by Ang II via the protein kinase C pathway and that this regulation is independent of mineralocorticoid receptor activation or epithelial sodium channel activity. (Hypertension. 2011;57[part 2]:00-00.)

Key Words: angiotensin II–dependent hypertension ■ collecting duct renin ■ renin gene expression ■ protein kinase C ■ cell signaling pathway

Regulation of renin expression in the kidney juxtaglomerular (JG) apparatus has been widely studied; however, the mechanisms regulating expression of renin in collecting ducts (CDs) and its augmentation in animal models of hypertension remain unclear. Increased renin synthesis is mediated by the angiotensin II (Ang II) type 1 receptor (AT₁R) independent of changes in blood pressure. In vitro studies also suggest that CD renin synthesis and secretion can be directly increased by Ang II; however, the mechanisms involved are poorly understood.

Ang II is present at high levels in the kidneys of hypertensive animals, and an important fraction of this Ang II is generated by de novo synthesis as a consequence of augmented angiotensinogen expression in proximal tubule cells, leading to augmented angiotensinogen excretion in urine. Along with the expression of angiotensin-converting enzyme in the distal nephron, increases in CD renin expression and activity may have a key role in the intrarenal Ang II content, contributing to additional Ang II generation in distal nephron segments.

Ang II, generated by de novo synthesis as a consequence of augmented angiotensinogen expression in proximal tubule cells, leading to augmented angiotensinogen excretion in urine. Along with the expression of angiotensin-converting enzyme in the distal nephron, increases in CD renin expression and activity may have a key role in the intrarenal Ang II content, contributing to additional Ang II generation in distal nephron segments.

AT₁R activation suppresses renin synthesis in JG cells via protein kinase C (PKC) and Ca²⁺; however, in CD cells, the signaling pathways responsible for Ang II–mediated upregulation of CD renin are unclear. In vivo and in vitro evidence suggest that the possible mechanisms could be different from the mechanisms described in JG cells. AT₁Rs are expressed in CD principal cells and activate the epithelial sodium channel (ENaC), stimulating sodium reabsorption. In Ang II–dependent hypertension, high Ang II plasma levels lead to increased levels of plasma aldosterone and activation of mineralocorticoid receptor (MR) with a corresponding increase in Na⁺ reabsorption through ENaC in principal CD cells. Thus, increased CD renin in the Ang II infusion model could be attributed to enhanced MR activity or ENaC stimulation.

To address these possibilities, we compared CD renin mRNA levels primarily in renal inner medullary tissues and the urinary renin content (URC) in Ang II–infused rats with or without cotreatment with the ENaC blocker, amiloride. To further evaluate the effects of Ang II, aldosterone, and ENaC...
blockade on renin mRNA and protein levels, we used rat cultures of inner medullary CD (IMCD) cells. Because PKC mediates some of the effects of AT,R activation, we further evaluated the effects of PKC inhibition in Ang II–treated IMCD cells and the effect of direct activation of PKC on renin expression.

Materials and Methods

Experimental Animals and Sample Collections

All of the experimental protocols were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Tulane Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (150 to 175 g) were cage housed and maintained in a temperature-controlled room with 12-hour light/dark cycles and free access to tap water and standard rat chow. Ang II was infused at a rate of 80 ng/min via osmotic minipump for 14 days, as described previously7:8; amiloride (Sigma Chemical Co) was administered daily by oral gavage at a dose of 5 mg/kg per day7,17,16 according to the described half-life of this compound.18 Sham-operated rats were used as controls. After a training period of 1 week before treatments, the rats were euthanized by conscious decapitation, and trunk blood samples were collected for determination of serum creatinine, plasma sodium concentration, and creatinine levels. Rats were euthanized by conscious decapitation, and trunk blood samples were collected for determination of serum creatinine, plasma renin activity (PRA), and plasma Ang II concentration, as described previously.7

Urinary Renin Content

Active renin content in the urine was measured in the absence of trypsin using a modified protocol of PRA (GammaCoat PRA125I radioimmunoassay kit, Diasorin Inc) according to instructions of the manufacturer. Briefly, 500 μL of urine from 24-hour collection were spiked with 1 nmol of synthetic renin tetradecapeptide for 30 minutes in a 37°C water bath to measure the amount of Ang I generation by radioimmunoassay. To exclude the effect of peptidases, replicates were spiked with the specific renin inhibitor WFML peptide (AnaSpec) as a control. Values are expressed as international enzyme units per day, and each enzyme unit is defined as microgram of renin per milliunit of renin activity.

Quantitative Real-Time RT-PCR

Total RNA was isolated from microdissected inner medullary rat tissues. Twenty nanograms of total RNA were used to amplify renin gene (Ren1c) by quantitative RT-PCR using primer and probe sequences described previously.4 Primers for γ-ENaC amplification were 5′-ACTCCCTTGGGCTAGGTTA-3′ (sense); 5′-TGTCATTTACCTGCTTCT-3′ (antisense); and 5′-6-FAM-TGATCAAAGAAGTGGTTGCTGCC-BHQ1-3′ (fluorogenic probe). Data were normalized against β-actin mRNA levels using the following primers: 5′-ATCGATGTTGTTAGTGGCTCA-3′ (sense); 5′-GATTTACCTGCTTAGAGGC-3′ (antisense); and 5′-6-HEX-TCTATGCCAACACATGGCTGTCGTT-BHQ2-3′ (fluorogenic probe).

Western Blot Analysis

A rabbit polyclonal antirenin IgG (sc-H-105, Santa Cruz Biotechnology, Inc) mapping to an internal region of (pro)renin/renin (amino acids 116 to 220) of human renin was used at a 1:200 dilution followed by a Donkey antirabbit IgG IRDye 800 CW (Li-cor Biosciences) at a 1:30 000 dilution. Densitometric analyses were done by normalization against β-actin (Santa Cruz Biotechnology). Recombinant human renin and (pro)renin (Lee BioSolutions) were used as positive standards.

Primary Cultures of Rat IMCD Cells

To assess CD (pro)renin/renin expression, IMCD cell cultures from 6 to 8 independent experiments were prepared as described previously19 using inner medullary tissues to avoid contribution from JG renin component. After kidney excision, inner medullary tissues were digested in 10 mL of DMEM-Ham F-12, 20 mg of collagenase B, 7 mg of hyaluronidase, 80 mmol/L of urea, and 130 mmol/L of NaCl and incubated at 37°C under continuous agitation for 90 minutes. After centrifugation, the pellet was washed in prewarmed culture medium without enzymes (DMEM-Ham F-12, 80 mmol/L of urea, 130 mmol/L of NaCl, 10 mmol/L of HEPES, 2 mmol/L of L-glutamine, penicillin-streptomycin [10 000 U/mL], 50 mmol/L of hydrocortisone, 5 μM 3,3,5-triiodothyronine, 1 mmol/L of sodium selenite, and 5 mg/L of transferrin, without FBS [pH 7.4; 640 mosmol/kg of H2O]). The resulting IMCD cell suspension was seeded in 3-mm petri dishes and incubated with treatments described in the Results section. Ang II, aldosterone, candesartan, amiloride, calphostin C, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co.

Immunofluorescence

For immunofluorescence experiments, 50% to 60% subconfluent IMCD cells cultured in chamber slides (Nalge Nunc) were fixed in cold methanol for 20 minutes, blocked with PBS-Tween (0.1%) plus BSA (3%) for 1 hour, stained with rabbit anti-aquaporin-2 (Calbiochem) or antirenin (sc H-105, Santa Cruz Biotechnology Inc) at a 1:200 dilution and detected with Alexa Fluor 488 conjugated to antirabbit IgG (Invitrogen Life Science, Co). Samples were counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen). Negative controls were obtained by omission of the specific primary antibody.

Calcium Measurements in Primary Cultures of Rat IMCD Cells

Changes in free intracellular Ca2+ were measured in subconfluent IMCD cells plated on glass coverslips incubated with the membrane-permeant acetoxymethyl ester form of Fura-2 (2 μmol/L; Molecular Probes) following the protocol described previously.20 A ratio of 340:380 nm was recorded at a rate of 1 per second and analyzed with the Photon Technology International software at basal levels and after incubation with Ang II (10−8 and 10−7 mol/L).

Statistical Analyses

Results are expressed as mean±SEM. Data were evaluated by the Grubb test followed when appropriate by paired and unpaired Student t test or by 1-way ANOVA with Tukey post test. For mRNA and protein data, control levels were defined as 100%. Significance was defined as P<0.05. The Student t test was used to evaluate changes in plasma sodium, creatinine clearance, or fractional sodium excretion among the groups. Amiloride treatment slightly but significantly reduced the rate of systolic blood pressure progression with Ang II infusion (R2: 0.65±0.06 for Ang II–infused rats plus amiloride versus 0.83±0.02 for Ang II infused rats; P<0.05; Figure 1A). As expected, Ang II infusion suppressed PRA, and amiloride treatment did not alter this effect (Table). To evaluate
whether ENaC blockade influences the response to chronic Ang II infusions, we measured Ang II levels in the cortex and medulla. Ang II contents were augmented in both groups compared with sham-operated rats in cortex (606±71 and 605±21 versus 247±43 fmol/g; P<0.05) and medulla (2066±155 and 1897±214 versus 645±36 fmol/g; P<0.05; Figure 1B).

Table. Physiological Parameters at Day 14

<table>
<thead>
<tr>
<th>Variable</th>
<th>Sham</th>
<th>Ang II</th>
<th>Ang II + Amiloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>318±2</td>
<td>269±4†</td>
<td>275±7†</td>
</tr>
<tr>
<td>Plasma Na⁺, mEq/L</td>
<td>142±1</td>
<td>142±1</td>
<td>144±2</td>
</tr>
<tr>
<td>Na⁺ excretion, mEq/24 h</td>
<td>2.6±0.1</td>
<td>2.8±0.2</td>
<td>3.1±0.1*</td>
</tr>
<tr>
<td>Fractional Na⁺ excretion, %</td>
<td>1.0±0.2</td>
<td>0.9±0.2</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td>Creatinine clearance, mL/min</td>
<td>0.52±0.12</td>
<td>0.59±0.10</td>
<td>0.60±0.12</td>
</tr>
<tr>
<td>per 100 g of body weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urinary volume, 24 h</td>
<td>12±2</td>
<td>37±4*</td>
<td>40±2†</td>
</tr>
<tr>
<td>PRA, ng of Ang I per mL per hour</td>
<td>9.60±1.21</td>
<td>0.19±0.06*</td>
<td>0.05±0.03*</td>
</tr>
<tr>
<td>Plasma Ang II, fmol/mL</td>
<td>34.6±5.9</td>
<td>169.8±52.9†</td>
<td>98.0±17.8*</td>
</tr>
</tbody>
</table>

Values are mean±SEM. No differences were observed between Ang II and Ang II+amiloride groups for the variables analyzed (n=10).

*P<0.05 vs sham-operated rats.
†P<0.001 vs sham-operated rats.

Effect of ENaC Blockade on Ang II–Mediated Stimulation of Renin mRNA Levels and URC

Medullary renin mRNA levels were significantly augmented in Ang II–infused rats compared with sham-operated rats (411±144% versus 100±48%; P<0.05), and this augmentation was not affected by amiloride treatment (384±136% versus 100±48%; P<0.05; Figure 2A). As shown in Figure 2B, URC was increased both in Ang II–infused and Ang II–infused rats treated with amiloride compared with sham (4.4±0.9 and 4.2±0.8 versus 1.5±0.6×10⁻⁶ enzymatic units per day, respectively; P<0.05). Thus, ENaC blockade did not alter the augmentation of CD renin in Ang II–infused rats.

In Vitro Studies

Methanol-fixed IMCD cells from primary cultures showed specific AQP-2 immunolabeling in the plasma membrane of principal cells (Figure 3A). Likewise, these principal cells immunoexpressed renin/(pro)renin in a granular pattern (Figure 3B). Functional expression of AT₁R in the plasma membrane was evaluated by intracellular Ca²⁺ measurements in response to Ang II (10⁻⁷ and 10⁻⁸ mol/L). As shown in Figure 3C, Ang II at 10⁻⁸ mol/L increased the ratio 340:380 nm, indicating increases in intracellular Ca²⁺. By immunoblotting and using recombinant human renin and (pro)renin as positive controls, it was apparent that most of the protein levels detected in IMCD cell extracts were (pro)renin (47 kDa). Posttranscriptional processing such as glycosylation of endogenous (pro)renin in the rat may explain differences in molecular weight compared with recombinant human (pro)renin (45 kDa). Ang II treatment (10⁻⁷ mol/L) increased (pro)renin protein levels (Figure 3D and 4C).
Effects of MR Activation and ENaC Blockade on Renin mRNA Expression in IMCD Cells Treated With Ang II

The efficacy of aldosterone treatment in IMCD cells was assessed from α-ENaC mRNA expression at different concentrations (10^{-10} to 10^{-7} mol/L). Compared with vehicle-treated cells, aldosterone treatment (6 hours) significantly increased α-ENaC expression in IMCD cells at concentrations of 10^{-7} mol/L (143±11% versus 100±9%; P<0.05) and 10^{-6} mol/L (136±4% versus 100±9%; P<0.05; Figure 3E); however, aldosterone (10^{-10} to 10^{-7} mol/L) did not stimulate renin mRNA levels (Figure 3F). Nevertheless, Ang II treatment (10^{-7} mol/L) increased renin mRNA expression compared with vehicle-treated cells (524±96% versus 100±19%; P<0.05), and this effect was blocked by candesartan (126±36% versus 100±19%; P value not significant), showing that this effect is mediated by AT_{1}R activation. In Ang II–dependent hypertension, high circulating levels of aldosterone and Ang II stimulate α-ENaC expression and ENaC activity, respectively; thus, we evaluated the effects of Ang II plus aldosterone in IMCD cells with or without amiloride. A dose of amiloride 10 times higher than IC_{50} (∼10^{-7} mol/L) was used. This concentration also avoids nonspecific inhibition of other transporters, observed in the range of 10^{-3} mol/L. Both groups showed a significant increase in renin mRNA as compared with vehicle-treated cells (Ang II plus aldosterone: 436±102%; P<0.05; Ang II plus aldosterone plus amiloride: 529±115%, P<0.05), but no differences between both groups or versus the Ang II group were detected. In addition, no effects were observed with amiloride alone (108±26% versus 100±19%; P value not significant).

Increased Renin mRNA Expression Is Mediated by AT_{1}R via PKC Pathway

Because PKC mediates some of the effects of AT_{1}R, we tested the effect of PMA on renin expression in IMCD cells. After 6 hours, renin mRNA levels were increased by PMA (10^{-6} mol/L) compared with vehicle-treated cells (586±80% versus 100±19%; P<0.05). We next tested the ability of the PKC inhibitor calphostin C to block the stimulatory effect of Ang II. As shown in Figure 4A, calphostin C (10^{-6} mol/L) blocked the effect of Ang II (10^{-7} mol/L) on renin mRNA expression (112±65%, P value not significant; Figure 4A). As shown in Figure 4B, immunofluorescence experiments confirmed the results observed by quantitative RT-PCR. Figure 4C shows a (pro)renin protein dose-response curve after 8 hours of Ang II treatment. To confirm the data observed by renin quantitative RT-PCR, we evaluated the changes in the (pro)renin protein levels in IMCD cells according to treatments. A representative Western blot is displayed in Figure 4D. Percentage versus control (100±2%) were as follows: (1) Ang II, 137±8%, P<0.05; (2) Ang II+calphostin C: 83±10%, P value not significant from vehicle alone; and (3) PMA: 167±20%, P<0.05.

Discussion

Previous studies have shown that chronic Ang II infusion stimulates CD renin synthesis independent of blood pressure. Novel findings from this study demonstrate that augmentation of renin expression is mediated directly by AT_{1}R via a PKC pathway and that this effect is independent of ENaC and MR activation. In vivo data show that ENaC
Renin mRNA levels were analyzed after 6 hours of incubation

Figure 4. Renin mRNA and protein levels in IMCD cells. A, Renin mRNA levels were analyzed after 6 hours of incubation (n=8). B, Renin immunofluorescence in subconfluent primary cultures of IMCD cells after 8 hours of treatment. Negative

densitometric analyses were done from 6 independent experiments according to the ratio between (pro)renin and β-actin bands. Control levels were defined as 100%. *P<0.05.
for CD renin regulation. In JG cells, Ang II suppresses renin secretion and basal levels of renin mRNA, and these effects can be blunted by PKC inhibition. Using mouse renin 1c promoter luciferase gene constructs expressed in As4.1 cells, Klar et al showed 2 important regulatory sequences for Ca\(^{2+}\)-mediated inhibition of renin gene transcription in the promoter region that were independent of PKC. However, in the present study using cultured IMCD cells expressing functional AT\(_1\)R, PKC activation with PMA increased renin mRNA and (pro)renin protein levels, suggesting that the downstream pathway in the control of CD renin expression is mediated by PKC. Calphostin C inhibits the classic (α) and the novel (δ, ε, and η) but not the atypical (ζ) PKC isozymes described in the rat IMCD cells. Treatment with PMA specifically activates classic and novel PKCs, suggesting that in this pathway the atypical PKC isoform is not involved. The combination of in vivo and in vitro data also demonstrates that AT\(_1\)R is the main regulator of CD renin/(pro)renin synthesis and secretion, independent of MR activation or ENaC activity.

**Perspectives**

Although the mechanisms for renin regulation in JG cells are well described, the present study provides evidence for a different mechanism in the regulation of renin synthesis in the CD. Our study shows that AT\(_1\)R activation in CD cells leads to increases in renin mRNA and renin excretion, in vivo, independent of ENaC activity and MR activation. Activation of AT\(_1\)R increases intracellular Ca\(^{2+}\) levels in IMCD cells suggesting that Ca\(^{2+}\) is not a negative modulator of renin synthesis in CD cells. Furthermore, in vitro studies showed that activation of PKC increases CD renin mRNA and (pro)renin protein levels, whereas PKC inhibition completely blocked the Ang II-mediated stimulation of CD renin synthesis. Collectively the results provide further evidence for an important role of augmented CD renin to contribute to increased intrarenal Ang II generation in Ang II–dependent hypertension.

**Sources of Funding**

This work was supported by the National Institutes of Health through the Institutional Development Award Program of the National Center for Research Resources (P20RR-017659), HL26371, American Heart Association (09BGIA2280440), and Eunice Kennedy Shriver Eunice Kennedy Shriver National Institute of Child Health and Human Development (K12HD043451). A.A.G. is a recipient of a Comisión Nacional de Investigación Científica y Tecnológica postdoctoral fellowship from Chile. L.S.L. is a recipient of a CAPES Postdoctoral Fellowship from Brazil.

**Disclosures**

None.

**References**

Angiotensin II Stimulates Renin in Inner Medullary Collecting Duct Cells via Protein Kinase C and Independent of Epithelial Sodium Channel and Mineralocorticoid Receptor Activity
Alexis A. Gonzalez, Liu Liu, Lucienne S. Lara, Dale M. Seth, L. Gabriel Navar and Minolfa C. Prieto

Hypertension, published online January 31, 2011;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/early/2011/01/31/HYPERTENSIONAHA.110.165902

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/