Angiotensin Subtype-2 Receptors Inhibit Renin Biosynthesis and Angiotensin II Formation

Helmy M. Siragy, Chun Xue, Peter Abadir, Robert M. Carey

Abstract—Renin is regulated by angiotensin subtype 1 (AT$_1$) receptor, but it is unknown whether angiotensin subtype 2 (AT$_2$) receptor contributes to this regulation. We hypothesized that AT$_2$ receptors inhibit angiotensin II (Ang II) through inhibition of renin biosynthesis. We monitored changes in renal Ang II, renin mRNA and protein expression, and plasma renin concentration (PRC) in response to renal cortical administration of the AT$_1$ receptor blocker valsartan or the AT$_2$ receptor blocker PD 123319 (PD) in conscious rats administered low sodium intake (LS). Renal interstitial Ang II increased by 47-fold in response to LS and increased further in response to valsartan or PD by 67-fold and 61-fold from normal sodium diet (NS) and by $\approx$41% and 29% from LS, respectively. Renin mRNA increased 63% during LS, and either valsartan or PD increased it further by 600% and 250% from NS and 538% and 187% from LS, respectively. Similarly, renal renin content and PRC increased in response to LS and increased further in response to combined LS and valsartan or PD administration. Immunostaining for renal renin protein demonstrated an increase in its expression in juxtaglomerular and tubular cells during LS and increased further during AT$_1$ or AT$_2$ receptor blockade. These data demonstrate for the first time to our knowledge that AT$_2$ receptors regulate the renin-angiotensin system activity via inhibition of renin synthesis. (Hypertension. 2005;45:1-5.)

Key Words: receptors, angiotensin II ■ renin ■ kidney ■ angiotensin

Angiotensin II (Ang II) is the major effector hormone of the renin-angiotensin system (RAS). Most of the known physiological functions and pathologic effects associated with Ang II are mediated by the angiotensin subtype 1 (AT$_1$) receptor, including renin inhibition. In contrast, the functions of the angiotensin subtype 2 (AT$_2$) receptor are still being elucidated. The AT$_2$ receptor is expressed abundantly during fetal development and declines after birth. In mammalian adults, the AT$_2$ receptor had been reported in multiple organs, including the kidney. Currently, the AT$_2$ receptor has several described functions related to inhibition of cell growth, promotion of cell differentiation, and stimulation of apoptosis. Unlike the well-known effect of AT$_1$ receptor on renin production, the influence of the AT$_2$ receptor on renal renin biosynthesis and Ang II production is not known.

In this study, we tested the hypothesis that the AT$_2$ receptor inhibits renal renin synthesis and Ang II production. We used multiple techniques including renal interstitial microdialysis, real-time quantitative reverse-transcription polymerase chain reaction, immunohistochemistry, and enzyme-linked immunoassays for monitoring changes in renal Ang II, renin mRNA, renin protein expression, and plasma renin concentration to investigate whether intrarenal AT$_2$ receptors regulate renin production and Ang II formation. We monitored renal interstitial levels of Ang II in conscious rats during sodium restriction, a condition known to increase AT$_2$ receptor expression, and during AT$_1$ and AT$_2$ receptor blockade. A distinct advantage of the microdialysis technique is the ability to monitor renal Ang II levels in conscious rats without undesirable hemodynamic changes. In this study, we demonstrate for the first time to our knowledge that AT$_2$ receptors regulate the activity of the RAS through inhibition of renin biosynthesis in young rats.

Methods

Renal Microdialysis Technique

For the determination of renal interstitial fluid (RIF) Ang II, we constructed a microdialysis probe as previously described. Unlike the well-known effect of AT$_1$ receptor on renin production, the influence of the AT$_2$ receptor on renal renin biosynthesis and Ang II production is not known.

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Animal Preparation

The experiments were approved by the University of Virginia Animal Research Committee and conducted in accordance with institutional guidelines. Experiments were conducted in 4-week-old female Sprague–Dawley rats (n=8 in each group; Harlan Teklad, Madison, Wis). For collecting RIF, the microdialysis probe was inserted in the kidney as previously described. For infusions into the renal cortical interstitial space, a 10-cm-long polyethylene tube was used.
Effects of Sodium Depletion and Angiotensin AT1 or AT2 Receptor Blockade on RIF Ang II

In this study, rats (n = 8 each group) were placed in metabolic cages. Baseline 24-hour urine collections were obtained for calculation of urinary sodium excretion, and RIF samples were obtained for Ang II while rats were consuming a normal sodium diet (0.28% NaCl; BioServe Biotechnologies, Frenchtown, NJ). Then, rats were placed on a low-sodium diet (0.05% NaCl) for 8 days. At day 7, we monitored 24-hour urinary sodium excretion (Uu,Na). While the rats continued to consume the low-sodium diet (day 8), RIF Ang II levels were monitored during intrarenal cortical interstitial administration (5 µL/min for 8 hours) in random order of D5W vehicle (n = 8), PD 123319 (n = 8; Parke-Davis-Warner Lambert Co, Ann Arbor, Mich), a specific AT1 receptor antagonist,22,23 or the AT2 receptor blocker, valsartan (n = 8; Novartis, East Hanover). PD was infused at 10 µg/kg per minute for 8 hours (a dose that remains highly specific for AT2 receptor), whereas the dose for valsartan was 10 mg/kg per 8 hours.

Renal Renin mRNA, Protein Expression, Renin Concentration and Ang II, and Plasma Renin Concentration in Response to AT1 or AT2 Receptor Blockade

At the end of each protocol, animals were euthanized and blood was collected in tubes containing EDTA for measurement of plasma renin concentration. The infused kidneys were removed, placed on ice, and divided into sections for RNA extraction, total renin and Ang II measurements, and renin immunostaining.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

The kidney tissue was weighed promptly and homogenized on ice, and the total renal RNA was extracted using RNaseasy Kit (Qiagen, Gmbh, Hilden, Germany). Quality of RNA is confirmed by ethidium bromide staining in 2% agarose gel. Single-stranded cDNA is synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, Calif). Gene-specific renin primers are designed using the Gene Bank. The exon–intron boundaries are determined using the University of California Santa Cruz Genome Bioinformatics Site (http://genome.ucsc.edu). The left primer is 5' gaggcatgtccactcagct 3', and right primer is 5' cctcctcacaacaagct 3'. The corresponding cDNA product size is 124 bp. Amplification products are verified by melting curves. Quantitative real-time polymerase chain reaction is performed using iCycler (Bio-Rad, Hercules, Calif) and threshold cycle number is determined using iCycler software version 3.0 (Bio-Rad). Reactions are performed in triplicate, and threshold cycle numbers are averaged. Nontemplate control was used as a negative control. Samples are calculated with normalization to actin. Fold down-expression or up-expression is calculated according to the formula 2^(-ΔΔCt), where ΔCt = Ct (reference gene observed in the test sample) - Ct (reference gene observed in the control sample), and R̄t is the threshold cycle number for the experimental gene observed in the test sample. Renin Immunohistochemistry

Kidney tissues were fixed in Bouin solution and embedded in paraffin. Six-micrometer sections were stained for renin using a previously characterized polyclonal goat anti-renin antibody24 (a gift from Dr Tadashi Inagami at the Vanderbilt University), avidin-biotin-peroxidase (Vectastain ABC kit; Vector Laboratories, Burlingame, Calif), and methods described previously.25 Negative controls included omission of primary and secondary antibodies.

The aforementioned studies were repeated in another group of animals during normal sodium intake. Renal renin mRNA, renin protein expression, protein expression and total renin concentration were monitored in response to AT1 or AT2 receptor blockade.

Analytical Methods

Urinary sodium levels were measured by a NOVA analyzer (NOVA Biomedical; Waltham, Mass). RIF samples for Ang II underwent sample extraction followed by RIA.19 Total renin renin26 and Ang II content26,27 were measured according to previously published methods.

Statistical Analysis

Comparisons among different treatment groups were examined by ANOVA, including a repeated measure term, using the general linear models procedure of the Statistical Analysis System (SAS Software, SAS Institute, Cary, NC). Data are expressed as mean ± SE. P < 0.05 was considered statistically significant.

Results

Systolic Blood Pressure Responses to Low Sodium Intake Alone and Combined With Intrarenal Administration of Valsartan of PD

During normal sodium intake, urinary sodium excretion was 590 ± 34 µmol/d and decreased to 35 ± 7 µmol/d (P < 0.0001) after 7 days of low sodium intake. Systolic blood pressure (n = 8 each group) in rats on normal sodium intake was 108 ± 1 mm Hg, and did not change significantly during low-sodium diet alone or combined with intrarenal cortical administration of valsartan or PD.

RIF Ang II Responses to Low Sodium Intake Alone and Combined With Intrarenal Administration of Valsartan or PD

RIF Ang II levels (Figure 1A) during normal sodium intake were 2 ± 0.2 fmol/mL (n = 8) and increased during dietary sodium restriction to 96 ± 1.5 fmol/mL (P < 0.0001). Intrarenal cortical administration of valsartan or PD (n = 8 each treatment) during low sodium intake caused a further increase in RIF Ang II to 136 ± 2 and 124 ± 2 fmol/mL, respectively (P < 0.00001 from normal sodium and P < 0.0001 from low sodium intake).

Total Renal Ang II Content in Response to Low Sodium Intake Alone and Combined With Intrarenal Administration of Valsartan or PD

During normal sodium intake (n = 8), total renal Ang II content was 164 ± 16 fmol/g kidney weight (Figure 1B). Low sodium intake (n = 8) increased renal Ang II content to 338 ± 49 fmol/g kidney weight (P < 0.0001). Intrarenal cortical...

(PE 60; Becton Dickinson, Sparks, Md) was inserted into the right renal cortex as previously described.21 The interstitial infusate rate was determined from preliminary studies and is optimum at 5 µL/min (a rate that ensures distribution of the infused substance to the entire renal cortex without dislodging the catheter from the kidney). To demonstrate renal distribution of intravenously infused substances, using fluorescein microscopy we were able to localize fluorescence-labeled Ang II in the renal cortex. Rats were housed under controlled conditions (temperature: 21 ± 1°C; humidity: 60 ± 10%; lighting 8 to 20 hours) and allowed 7 days to recover and to acclimatize to the laboratory. Experiments were started at the same time (8:00 AM) each day to avoid any diurnal variation of the measured substances. During the first and last 30 minutes of each study, systolic arterial pressure was measured every 10 minutes in the rat tail (Rat Tail Manometer-Tachometer System, Natsume model KN-210; Peninsula Laboratories, Inc, Belmont, Calif) and the recorded values were averaged for each study.

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**Renal Renin Immunostaining in Response to Low Sodium Intake Alone and Combined With Intrarenal Administration of Valsartan or PD**

There was no renin staining when IgG preimmune was used as control (Figure 3A and 3F). During normal sodium intake (Figure 3B and 3G), renin immunostaining was very low, localized mainly in the juxtaglomerular apparatus, and did not change in response to valsartan or PD treatment (data not shown). Low sodium intake (Figure 3C and 3H) caused significant increase in renin immunostaining in the glomeruli and juxtaglomerular apparatus. Combined low salt intake with valsartan (Figure 3D and 3I) or PD (Figure 3E and 3J) caused further increase in renin staining in glomeruli, juxtaglomerular apparatus, and tubules compared with normal (Figure 3B and 3G) and low salt (Figure 3C and 3H) intake alone.

**Plasma Renin Concentration in Response to Low Sodium Intake Alone and Combined With Intrarenal Administration of Valsartan or PD**

During normal sodium intake, plasma renin concentration levels (Figure 4) were 10±1.1 ng/mL and increased in response to low sodium intake to 25±4.2 ng/mL (P<0.01). Plasma renin concentration increased further to 62±12.2 and 48±11.4 ng/mL during combined low sodium intake and valsartan or PD treatment, respectively (P<0.0001 from normal sodium intake and P<0.01 from low sodium intake).

**Discussion**

This study demonstrates, for the first time to our knowledge, the regulation of the RAS by the AT₂ receptor. We hypothesized that AT₂ receptor decreases generation of Ang II through reduction in renin synthesis. Our data support this hypothesis based on the observed increase in renal renin mRNA, renin protein, and Ang II during AT₂ receptor...
blockade. The renin and Ang II response to the AT2 receptor blockade was similar to the response observed during valsartan treatment and suggests that both AT1 and AT2 receptors have an inhibitory effect on the activity of the RAS. This result was unexpected because previous studies demonstrated opposite effects for these 2 receptors. AT3 receptor inhibition of renin synthesis and secretion is in agreement with its presumed vasodilator and cardiovascular protective effects.15,17 It is highly unlikely that the observed results are caused by the interaction of PD with the AT1 receptor because the infusion rate used was small, one-fifth of previously reported doses,15–17 and did not produce any changes in blood pressure. Based on the affinity studies, PD at the dose used in this study is specific for the AT2 receptor and does not interact with the AT1 receptor.29

All the components of the RAS are present within the kidney and local renal production of Ang II has been demonstrated.20 Renin is the rate-limiting enzyme in the synthesis of Ang II.30 In addition to juxtaglomerular cells, renin is produced by mesangial16 and tubulartubular cells. Angiotensinogen and angiotensin-converting enzyme are generated within the kidney.30 AT1 and AT2 receptors are also located within the kidney.28 In particular, AT2 receptors are present in juxtaglomerular cells and have been shown to inhibit prorenin processing.33

Previously published data demonstrated a short negative feedback loop of the AT1 receptor to suppress renin and Ang II production.1,18,19 A regulatory role for the AT2 receptor in modulating RAS activity has not been described previously. A murine strain with disruption of the AT2 receptor gene displays slightly elevated baseline blood pressure, exaggerated vasopressor response to Ang II, increased prostaglandin E2 (PGE2), and reduced NO and cGMP production.16 In those studies, systemic or renal tissue renin or Ang II levels were not evaluated. In a renal vascular hypertension rat model,15 AT2 receptor blockade caused further increase in blood pressure. Taken together, current and previous data suggest that the AT2 receptor regulates the RAS either directly or through multiple mechanisms involving NO, cGMP, or PGE2.

The increase in Ang II in parallel to the increase in renin mRNA, renin protein, and plasma renin concentration suggests that the AT2 receptor regulates the RAS mainly through increased production of renin. The current study cannot differentiate whether the increase in the activity of the RAS is directly linked to decreased AT2 receptor activity or is directly linked to reduction in its mediator NO and cGMP. The influence of NO is controversial, with studies showing stimulation,34,35 inhibition,16 or no direct influence37–39 on renin production. Cyclic GMP has been shown to inhibit renin expression and secretion.40 Activation of cGMP-dependent protein kinases by cGMP decreased renin secretion from the isolated perfused rat kidney, isolated renal juxtaglomerular cells, and kidney slices.40–42 PGE2 enhances renin production through stimulation of EP3 and EP4 receptors. Absence of AT2 receptor activity increases renal PGE2 levels through enhancing the activity of AT1 receptor13,16 or decreasing the metabolism of PGE2 to 6-ketoPGF1α.44 Thus, our previous finding of AT2 receptor mediation of NO,14 cGMP,13 and PGE213,16 production supports conclusion that this receptor regulation of renin production, at least in young animals.

Absence of PD effects on renin synthesis and secretion during normal sodium intake, a condition associated with low AT2 receptor expression,5 strengthens the argument for its involvement in renin regulation. Previous studies demonstrated that AT1 and AT2 receptors counterbalance each other.28 The current study demonstrates a rare instance in which both of these receptors share a similar function.

**Perspectives**

This study introduces the novel concept of AT2 receptor regulation of the RAS activity. Development of an AT2...
receptor agonist could help provide a pharmacological tool for further RAS inhibition, in addition to angiotensin-converting enzyme and AT1 receptor blockers, to manage cardiovascular disease.

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References

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