The Collecting Duct Is the Major Source of Prorenin in Diabetes

Jung J. Kang, Ildikó Toma, Arnold Sipos, Elliott J. Meer, Sarah L. Vargas, János Peti-Peterdi

Abstract—In addition to the juxtaglomerular apparatus, renin is also synthesized in renal tubular epithelium, including the collecting duct (CD). Angiotensin (Ang) II differentially regulates the synthesis of juxtaglomerular (inhibition) and CD (stimulation) renin. Because diabetes mellitus, a disease with high intrarenal renin-Ang system and Ang II activity, is characterized by high prorenin levels, we hypothesized that the CD is the major source of prorenin in diabetes. Renin granular content was visualized using in vivo multiphoton microscopy of the kidney in diabetic Munich-Wistar rats. Diabetes caused a 3.5-fold increase in CD renin, in contrast to less pronounced juxtaglomerular changes. Ang II type 1 receptor blockade with Olmesartan reduced CD renin to control levels but significantly increased juxtaglomerular renin. Using a fluorogenic renin assay, the prorenin component of CD renin content was measured by assessing the difference in enzymatic activity of medullary homogenates before and after trypsin activation of prorenin. Trypsinization caused no change in control renin activity but a 5-fold increase in diabetes. Studies on a CD cell line (M1) showed a 22-fold increase in renin activity after trypsinization and a further 35-fold increase with Ang II treatment. Therefore, prorenin significantly contributes to baseline CD renin. Diabetes, possibly via Ang II, greatly stimulates CD prorenin and causes hyperplasia of renin-producing connecting segments. These novel findings suggest that, in a rat model of diabetes, prorenin content and release from the CD may be more important than the juxtaglomerular apparatus in contrast to the existing paradigm. (Hypertension. 2008;51:1597-1604.)

Key Words: prorenin ■ renin ■ collecting duct ■ diabetes ■ hypertension ■ quinacrine

The renin-angiotensin system (RAS) is one of the most significant physiological mechanisms of blood pressure regulation, and its dysfunction causes many pathologies. Renin release is the rate-limiting step of RAS activation, and according to the existing paradigm, renin and its biosynthetic precursor prorenin are mainly produced in the kidney by granular cells of the juxtaglomerular apparatus (JGA) in the terminal afferent arteriole. Recent work, however, has established that renin transcripts and protein are also present in renal tubular segments, including the collecting duct (CD). The 2 predominantly studied renal sources of (pro)renin (a term denoting both renin and prorenin) are granular cells of the JGA and principal cells of the CD. Because principal cells of the CD synthesize renin de novo and are far more abundant than the limited population of juxtaglomerular cells, the CD provides a potentially significant supply of (pro)renin. Angiotensin II (Ang II), the potent effector of RAS, oppositely regulates renin synthesis at these intrarenal locations: Ang II feedback inhibits JGA renin but stimulates CD renin. Therefore, CD renin appears to be more relevant to high-Ang II states.

The body of evidence has revealed expanded capabilities of RAS, revealing its complexity compared with the traditional model, in particular, in diabetes mellitus. Although direct data from experimental animals have failed to show significantly elevated intrarenal Ang II levels in diabetes, experimental and clinical studies showing the efficacy of RAS inhibitors as treatment reveal the activation and importance of some RAS components. Although studies have not shown the expected correlation between renin and RAS activity, diabetes has been firmly associated with vastly increased prorenin levels. Prorenin may, in turn, be proteolytically (enzymatically) or nonproteolytically (by a receptor) activated into active renin. Thus, prorenin may serve as an overlooked and largely undetected source of Ang II.

Our laboratory recently developed an experimental approach to quantitatively visualize kidney functions in vivo in the intact kidney and in vitro in the JGA, including real-time imaging of renin granular content, release, and tissue activity. This approach applies multiphoton confocal fluorescence microscopy, an ideal technique for deep optical sectioning of living tissues. Quinacrine has been used extensively to stain acidic vesicular organelles, including lysosomes, that are much smaller (in the nanometer range). Colocalization of quinacrine fluorescence with JGA renin by immunohisto-
chemistry validates the use of this dye to label JGA renin granules. In the present studies, we visualized quinacrine-stained granules in the JGA and CD as an indication of both renin and prorenin contents in control and diabetic conditions. Because Ang II differentially regulates JGA and CD (pro)renin, we hypothesized that the CD could be the major source of prorenin in diabetes. Investigations have shown increased synthesis of other RAS components, like angiotensinogen, in Ang II–rich environments. Therefore, we studied whether Ang II was also responsible for augmenting CD renin. Ang II upregulation of CD prorenin could offer an explanation for the RAS paradox in diabetes: high prorenin levels and RAS activity despite JGA renin suppression.

Methods

In Vivo Multiphoton Fluorescence Imaging of the Kidney

A Leica TCS SP2 confocal microscope system (Leica Microsystems) was used to image renal tissues in vivo as described. Briefly, Munich-Wistar-Fromter rats (200 g, Harlan, Madison, Wis) were used because of their superficial glomeruli. Diabetes was induced by a single streptozotocin injection (50 mg/kg IP). Other groups received vehicle injections. Experimental groups received Olmesartan (Sankyo) at 5 mg/d in chow for 4 weeks. For imaging, the animals were anesthetized by thiobutabarbital (inactin, 130 mg/kg IP). The left femoral vein and artery were cannulated for dye infusion and blood pressure measurements. The left kidney was exteriorized through a small dorsal incision, and the animal was placed on an inverted microscope as described. Core body temperature was maintained throughout procedures using a homeothermic table. All of the animal protocols were approved by the institutional animal care and use committee at the University of Southern California. Chemicals, if not indicated, were purchased from Sigma.

Fluorescent Probes

A 70-kDa dextran-rhodamine B conjugate (Invitrogen), quinacrine, and a fluorescence resonance energy transfer–based fluorogenic renin substrate (AnaSpec) were used to label the circulating plasma, renin content, and activity as described previously.

Semiquantitative Analysis of Renin Content

In Vivo

The quinacrine-positive length of the afferent arteriole was used as an index of JGA renin content. An index of CD quinacrine labeling was analyzed as the ratio of quinacrine fluorescence intensity in principal cells to intercalated cells. Principal cells were identified by characteristic bulging of their apical poles into the concentrated, highly fluorescent CD lumen.

Sample Collection

Kidneys were harvested after 4 weeks of diabetes. One kidney was fixed in formalin for immunohistochemistry, as described. Polyclonal rabbit antibody against rat (pro)renin was provided by Dr Tadashi Inagami (Vanderbilt University, Nashville, Tenn) and used on paraffin-embedded rat sections or M1 cells. Polyclonal rabbit antibody against proliferating cell nuclear antigen (PCNA) was from Santa Cruz Biotechnology, Inc. Samples were incubated overnight at 4°C, with 1:250 dilution of renin or 1:100 PCNA antibodies, 1:500 Alexa 594-conjugated secondary antibody (Invitrogen), and Vectashield mounting medium containing 4',6-diamidino-2-phenylindole for nuclear labeling (Vector Laboratories).

The other kidney was dissected into the cortex and medulla. CD renin was analyzed by homogenizing medullary samples and extracting protein in protease inhibitor (BD Biosciences) at 1:50 in homogenization buffer (20 mmol/L of Tris HCl and 1 mmol/L of EGTA; pH 7.0) and was stored at −80°C until use.

Western blots against rat renin (1:5000, Fitzgerald) and β-actin (1:1000, Abcam) were performed with 40 μg of protein as described previously. Blots were visualized with an Odyssey Infrared Imaging System (LI-COR Biosciences).

Trypsinization Protocol

Prorenin was evaluated as the difference in renin activity before (active renin) and after trypsinization (total renin). Medullary samples were harvested as described above. M1 (mixed phenotype CD cell line; American Type Culture Collection) cells were lysed with 1:50 BaculoGold protease inhibitor (BD Biosciences) in cell lytic, and supernatant was protein collected. Trypsinization activates prorenin to renin. Samples were trypsinized (50 g/L) for 60 minutes on ice, and reaction was stopped with Soybean Trypsin Inhibitor (100 g/L) for 10 minutes on ice.

Spectrofluorometry to Measure Renin Activity

Renin activity was evaluated by cuvette-based fluorometry (Quantamaster-8, Photon Technology, Inc) using a fluorescence resonance energy transfer–based renin substrate assay, as described previously. Substrate was present in excess, so the initial reaction rate (within 50 seconds of adding a 40-μg sample) estimated renin activity, using Felix software (PTI). Trypsin in the presence of trypsin inhibitor did not interact with the renin substrate. To control for specificity, the renin active site inhibitor Aliskiren (Novartis; 50 μmol/L) was incubated with sample for 5 minutes at 25°C.

Cell Culture

M1 cells were from American Type Culture Collection. Using previously validated primers, RT-PCR showed de novo renin synthesis, confirmed by sequencing. The Ang II concentration of 100 nmol/L was drawn from studies showing intratubular levels exceeding plasma levels 1000-fold in disease. M1 cells were treated with Ang II twice daily for 5 days, lysed, and protein extracted for renin activity assays.

Statistics

Data are expressed as means±SEs. Statistical significance was tested using ANOVA. Significance was accepted at P<0.05.

Results

JGA and CD Granular Content Visualized In Vivo

Multiphoton imaging visualized quinacrine-stained granules in the intact kidney with subcellular resolution. Figure 1A shows renin granules (green) lining the terminal afferent arteriole at the JGA. CD staining was low in control animals (Figure 1B), but we unexpectedly observed significant quinacrine fluorescence in the CD in diabetes. Closer inspection showed quinacrine-labeled granules at both apical (Figure 1C) and basolateral poles of principal cells (Figure 1D), having access to the tubular lumen or systemic circulation via the directly adjacent peritubular capillaries. This observation suggested significant CD renin synthesis and inspired the following investigations using quantitative and more specific techniques.

Effects of Ang II Type 1 Receptor Blockade

Diabetes is characterized by an activated RAS and is an ideal model to study the effects of RAS components at the JGA and CD. Quinacrine fluorescence in the JGA and CD were visualized and quantified as an index of both renin and prorenin content in control and diabetic conditions. Figure 2A shows JGA renin in early diabetes. Interestingly, quinacrine
labeling was vastly increased in the CD in diabetes (Figure 2B), with overflow into the tubular lumen, as well as the basolateral surface, toward the interstitium and peritubular capillaries. To distinguish the effects of Ang II at each region, Ang II binding was prevented by the Ang II type 1 receptor blocker (ARB), olmesartan. ARB treatment unmasked the differential regulation of granular content by Ang II at these 2 sites. The ARB blocked Ang II–mediated negative feedback at the JGA, permitting uninhibited, high renin production at the JGA and CD under control, diabetic, ARB-treated control, and ARB-treated diabetic conditions. A, Quinacrine staining was vastly increased in the CD in diabetes (Figure 3B) in contrast, olmesartan reduced quinacrine labeling in the CD, reflecting a decrease in granule number. AA indicates afferent arteriole; EA, efferent arteriole; G, glomerulus. Bar=20 μm (A, C, and D).

Figure 1. Visualization of quinacrine-labeled granules (green) in the JGA and CD in vivo. Control (A and B) and diabetic kidney (C and D). A dextran-rhodamine B conjugate (70-kDa) labeled the plasma or the concentrated CD lumen (C) red. A, Quinacrine identified JGA renin granules in the terminal afferent arteriole (AA). The efferent arteriole (EA) is seen leaving the glomerulus (G), opposite the AA. Note the absence (B) or abundance (C) of quinacrine staining in the bulging apical aspects of CD principal cells of the cells, close to peritubular capillaries (PTC). Bars=15 μm (B), 20 μm (A, C, and D).

Figure 2. Effects of AT1 receptor blockade. Untreated (A and B) and treated (C and D) diabetic conditions. A, Quinacrine staining (green) showed slightly elevated JGA renin in early diabetes. B, Quinacrine labeling was vastly increased in the CD. C, Olmesartan treatment increased quinacrine staining (a reflection of increased renin granular content) in the JGA. D, In contrast, olmesartan reduced quinacrine labeling in the CD, reflecting a 5-fold increase in renin activity after trypsinization confirmed that diabetes was a high prorenin-low renin condition (Figure 3D). Because ARB treatment silenced quinacrine staining in the CD, we expected ARBs to reduce plasma prorenin if the CD is indeed the major source of prorenin in diabetes. Plasma prorenin in untreated diabetic subjects (806±240 U/s) was 4-fold higher than in ARB-treated diabetic rats (230±59 U/s), and the ratio of plasma prorenin:renin also significantly decreased with ARB treatment (Figure 3D).

Immunolocalization of Increased CD (Pro)renin in Diabetes

As suggested by in vivo quinacrine labeling, renin expression in the CD was confirmed by labeling rat kidney sections with a (pro)renin antibody (Figure 4A). Figure 4B shows that CD (pro)renin expression was substantially elevated in diabetes, and both granular structures (similar to quinacrine labeling in vivo) and diffuse intracellular labeling (likely prorenin) were observed. Note the presence of renin-negative intercalated cells adjacent to renin-rich principal cells, which are packed with granular contents at apical and basolateral poles. Because the renin antibodies detect both prorenin and renin, immunohistochemistry alone cannot distinguish which form contributes more substantially to the increased total renin content.

Increased CD Prorenin in Diabetes: Effects of Trypsinization

The pretrypsinization activity measures endogenous active renin, and the posttrypsinization activity reflects the activity
of both native renin and activated prorenin. Because JGA renin contaminates the cortex, medullary homogenates (JGA free) were used to study CD renin.\(^4\) Western blot validated trypsinization as an effective method for prorenin activation. Using antibodies against (pro)renin, we observed an upper band of \(\approx 50\) kDa pretrypsinization and a 47-kDa lower band posttrypsinization, consistent with the literature.\(^20\) Figure 5A shows that trypsinization reduced the intensity of the higher prorenin band and increased the intensity of the lower renin band. Total prorenin content in diabetic animals (upper 50-kDa band, lanes 9 to 12) was significantly increased compared with control animals (lanes 1 to 4). Trypsinization produced even more renin in diabetes (lower 47-kDa band, lanes 13 to 16) compared with control animals (lanes 5 to 8).

Figure 3. Summary of the effects of olmesartan on JGA and CD quinacrine labeling in vivo (A and B) and plasma prorenin (C and D). A, ARB treatment increased the quinacrine-positive length of the afferent arteriole (AA) in both control and diabetic animals (\(n=9\) each; \(P<0.001\)). B, Quinacrine staining in the CD increased 3-fold in diabetes and decreased to below control levels with ARB treatment in control and diabetic animals (\(n=9\) each; \(P<0.001\)). Values are means±SEs of 9 measurements per experimental group; 4 animals per group. C, In vitro renin assays of plasma samples from the same rats. Serum prorenin levels were high in diabetes but reduced by ARB treatment. D, Fold increase in renin activity after trypsinization using the samples shown in C (\(n=6\) each; \(P<0.05\)).

**Effects of Ang II on Prorenin Synthesis by a CD Cell Line**

To further study the renal source of prorenin, the M1 CD cell line was used. Figure 6A shows that control and Ang II–treated M1 cells produce renin de novo, confirmed by RT-PCR. Ang II feedings were conducted across multiple concentrations and time courses (data not shown), with optimal effects at 100 nM and 5 days of treatment. Olmesartan was used as a control for the integrity of the Ang II, and it blocked the effects of Ang II and reduced renin to control levels (data not shown). Immunocytochemistry confirmed that, compared with control (Figure 6B), renin is increased in Ang II–treated cells (Figure 6C). Interestingly, there is a reticular pattern of staining in Ang II–treated cells (Figure 6C), revealing prorenin accumulation in the protein synthetic machinery of the endoplasmic reticulum and the Golgi apparatus. The number and size of renin granules, as well as cytoplasmic staining, increased in Ang II–treated cells, indicating increased synthesis of both renin and prorenin. Figure 6D shows that control and Ang II–treated M1 cells contain endogenous renin activity (\(n=4\) each). M1 renin activity increased 23±2-fold with trypsinization, showing significant baseline prorenin content in the cell line (Figure 6E). Ang II promoted the accumulation of prorenin, causing a 35±5-fold increase in renin activity posttrypsinization (Figure 6E; \(n=4\); \(P<0.01\)), signifying the direct upregulation of CD (pro)renin content by Ang II.

**Figure 4.** Immunohistochemical verification of renin granules in the CD. Both prorenin and renin were detected by a rat (pro)renin-specific antibody (red, Alexa 594). Nuclei were stained with 4’,6-diamidino-2-phenylindole (blue). A, (Pro)renin is expressed in principal cells of the CD under control conditions. B, (Pro)renin expression is substantially elevated in diabetes, and labeling is present both in granular structures and in a diffuse, intracellular pattern. Bar=20 \(\mu\)m.

**Figure 5.** Immunoblot analysis of prorenin and renin in diabetic and control kidney medullary homogenates. A, Western blot using (pro)renin-specific antibody (red, Alexa 594). Nuclei were stained with 4’,6-diamidino-2-phenylindole (blue). Total prorenin content in diabetic animals (upper 47-kDa band, lanes 1 to 4) was significantly increased compared with control animals (lanes 5 to 8). B, Quinacrine staining in the CD increased 3-fold in diabetes and decreased to below control levels with ARB treatment. C, In vitro renin assays of plasma samples from the same rats. Serum prorenin levels were high in diabetes but reduced by ARB treatment. D, Fold increase in renin activity after trypsinization using the samples shown in C (\(n=6\) each; \(P<0.05\)).
Connecting Segment Proliferation in Diabetes

The connecting segment (CNT) is a transitional region of variable length connecting the distal tubule and CD, and it contains the same mixed phenotype of cells as the CD. CNT is the major site of (pro)renin synthesis.\(^5\) CNT-CD proliferation has been reported in diseases like lithium-induced nephrogenic diabetes insipidus.\(^21\) We discovered CNT proliferation in the cortex of animals with diabetes mellitus. Compared with control animals (Figure 7A), an abundance of renin-positive principal cells was found next to renin-negative intercalated cells in diabetes (Figure 7B). CNT has overtaken the field and changed the population of the cortex, which is normally predominantly composed of glomeruli and proximal tubules (Figure 7A). Immunohistochemistry for PCNA has been used to confirm active mitosis of cells in the proximal tubules (Figure 7A). Immunohistochemistry for PCNA staining showed CNT hyperplasia in the cortex.

Discussion

Conventional knowledge assigns juxtaglomerular renin the role of gatekeeper for RAS and its end product, Ang II, with a critical negative feedback function as an internal control of its activity. However, recent findings have prompted a re-examination of our standard understanding of the RAS. This study provides quantitative, functional, and in vivo visual analysis of (pro)renin in the rat kidney. Quinacrine staining of the JGA and CD was visualized in vivo using multiphoton microscopy in control and diabetic conditions (Figures 1 and 2), and the data suggested differential regulation of JGA and CD (pro)renin by Ang II. In addition, the present studies provided further information about the distal RAS,\(^4,5\) namely, that immunoreactive CD renin was actually predominantly the precursor prorenin. In diabetes, CD (pro)renin content was greatly upregulated, and trypsinization differentiated prorenin from renin. Treatment of the CD cell line M1 with Ang II further suggested that the CD may be the source, and Ang II may be the stimulus, to explain the high prorenin levels and persistent RAS activity in diabetes despite JGA renin suppression.\(^7,9\)

De novo renin synthesis in the CD and its upregulation by Ang II infusion are established.\(^5\) Renin enzymatic activity assays on medullary homogenates isolated CD renin from contamination by JGA renin. In control animals, trypsinization of the same samples. Preincubation of medullary homogenates (whether or not) with aliskiren (renin inhibitor) reduced renin activity, confirming assay specificity for enzymatically active renin. C, There was no statistically significant increase in renin activity after trypsinization in control kidney homogenates. There was a 5-fold increase in diabetic animals (n=4 animals; \(^*P<0.001\)).
in the endoplasmic reticulum, with posttranslational processing into prorenin. Prorenin is then transported to the Golgi apparatus and sorted to small clear vesicles for constitutive secretion or targeted to large dense protease-containing vesicles for processing into mature renin that is secreted in a regulated fashion. Excessive stimulation of prorenin synthesis backs up processing, evidenced by visual cues like increased endoplasmic reticulum. For example, in type 2 diabetes, persistent stimulation of insulin secretion increases prorenin synthesis, producing more renin within large granules, as well as a reticular pattern of staining, indicating the accumulation of prorenin in the protein production machinery. Nuclei (blue) were stained with 4',6-diamidino-2-phenylindole. D and E, Renin activity posttrypsinization increased 22-fold in M1 cell lysates. Ang II treatment increased M1 cell renin activity 35-fold posttrypsinization (n=7 each; \( *P<0.05 \)).

The onset of hyperglycemia in diabetes elicits a pressor response involving a rise in mean arterial pressure and RAS activation. Because the JGA is the primary source of active renin, JGA renin is the gatekeeper responsible for early Ang II production. However, Ang II then has negative feedback on the JGA. Although Ang II suppresses JGA renin, it activates CD (pro)renin production. CD prorenin may then be released to cause pathological actions at the (pro)renin receptor or may be cleaved to serve as a source of renin in the face of JGA renin suppression. Our work supports other studies assigning the kidneys as the primary source of renin.

**Figure 7.** CNT proliferation in diabetes. A, The normal architecture of the kidney cortex is composed mainly of glomeruli (G), proximal tubule (PT), and various other nephron segments, including the renin-containing CNT. Red indicates renin; blue, nuclei. B, There is CNT hyperplasia in diabetes. Renin-rich (red) principal cells and renin-negative intercalated cells (white arrows) overtake the region. C, Representative image of control rat section negative for PCNA (red), a marker of cell turnover and hyperplasia. D, Diabetic rat section shows positive labeling for PCNA (red). Bar=20 \( \mu m \).
prorenin in the rat species,25 but work in humans suggests that there are also significant, nonrenal sources of prorenin.26

(Pro)renin in the CD may enter the tubular lumen (Figure 1B) to access local RAS in the distal nephron,3 culminating in intratubular Ang II generation. The significance of a paracrine RAS in the distal nephron has been confirmed by studies showing ENaC stimulation by both Ang I and Ang II in the CD lumen.27,28 Renin granules also appear to be localized to the basolateral aspect of principal cells, with access to interstitial RAS components, the systemic circulation via peritubular capillaries, and the recently localized (pro)renin receptor.29 (Pro)renin receptor binding increases the catalytic efficiency of Ang I generation 4-fold and activates mitogen-activated protein kinases, illustrating both RAS-dependent and -independent effects for (pro)renin.30

Although prorenin is a proenzyme, recent evidence questions its ostensible inactivity. Prorenin may be cleaved into active renin and regulate RAS, or it can exert RAS-independent effects through the (pro)renin receptor.11,30 Thus, distal prorenin may be an important constituent in the management of Ang II–dependent and diabetes-associated hypertension. In this regard, the present studies identified a thus far overlooked potential benefit of ARBs: suppression of CD (pro)renin synthesis (Figure 2B and 2D). Also, we discovered proliferation of the CNT in diabetes mellitus (Figure 7). Although Ang II stimulates prorenin on the individual cell level, proliferation of the CNT has larger-scale effects by increasing the entire population of the prorenin-producing principal cells. This further supports our hypothesis that the major source of plasma prorenin in diabetes is the CD.

Perspectives

The increased incidence and prevalence of hypertension reflect shortcomings in our management of this epidemic. The majority of cases have unknown etiology, but the eventual onset of hypertension in diabetic subjects suggests a relationship between the 2 pathologies. Despite their comorbidity and hypotheses about their interaction, the mechanism by which diabetes causes hypertension remains unresolved. Diabetes is associated with RAS activation despite paradoxically low plasma renin levels.7–9

The present studies show that Ang II can renew CD prorenin, providing substrate for amplification of a hypertensive signal, and serving as a potential target for disease management. The (pro)renin receptor has been identified and localized to vascular smooth muscle cells in the heart and kidney, glomerular mesangial cells, and collecting tubular cells of the kidney.29 The proximity of prorenin to the (pro)renin receptor establishes a volatile setting for persistent RAS activation and Ang II production. Elevated plasma prorenin correlated with and predicted diabetic microvascular complications and microalbuminuria.31 Tissue RAS activation and prorenin levels also contribute to the development and progression of end-organ damage like cardiac hypertrophy and fibrosis, vascular damage, diabetic glomerulosclerosis, and nephropathy.32 We postulate that CD (pro)renin, which is stimulated by Ang II, may be a critical site to target in the treatment of diabetic hypertension and nephropathy.

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Disclosures

None.

References


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