ORIGINAL RESEARCH

A local renal renin–angiotensin system activation via renal uptake of prorenin and angiotensinogen in diabetic rats

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Abstract: The mechanism of activation of local renal renin–angiotensin system (RAS) has not been clarified in diabetes mellitus (DM). We hypothesized that the local renal RAS will be activated via increased glomerular filtration and tubular uptake of prorenin and angiotensinogen in diabetic kidney with microalbuminuria. Streptozotocin (STZ)-induced DM and control rats were injected with human prorenin and subsequently with human angiotensinogen. Human prorenin uptake was increased in podocytes, proximal tubules, macula densa, and cortical collecting ducts of DM rats where prorenin receptor (PRR) was expressed. Co-immunoprecipitation of kidney homogenates in DM rats revealed binding of human prorenin to the PRR and to megalin. The renal uptake of human angiotensinogen was increased in DM rats at the same nephron sites as prorenin. Angiotensin-converting enzyme was increased in podocytes, but decreased in the proximal tubules in DM rats, which may have contributed to unchanged renal levels of angiotensin despite increased angiotensinogen. The systolic blood pressure increased more after the injection of 20 µg of angiotensinogen in DM rats than in controls, accompanied by an increased uptake of human angiotensinogen in the vascular endothelium. In conclusion, endocytic uptake of prorenin and angiotensinogen in the kidney and vasculature in DM rats was contributed to increased tissue RAS and their pressor response to angiotensinogen.

Keywords: renal renin–angiotensin system, prorenin, angiotensinogen, diabetic nephropathy, microalbuminuria

Introduction

The renin–angiotensin system (RAS) was originally considered to be a circulating system that regulated sodium and fluid balance and hemodynamics. However, more recent studies have detected a local RAS in the kidneys that was independent of the systemic RAS.[1–4] Whereas the systemic RAS activity was reported to be normal or suppressed in diabetes mellitus (DM),[5–7] most studies have reported that the local renal RAS was upregulated,[5,8–11] but the mechanisms underlying these findings have not been clarified. This is important because RAS inhibition is a mainstay of treatment for proteinuric diabetic nephropathy.

Activation of the classical circulating RAS is initiated by the release of renin and prorenin from juxtaglomerular cells of the kidney.[12,13] Circulating prorenin can be extracted by organs, including the kidney and converted locally to renin by binding to the prorenin receptor (PRR).[13]

The messenger RNA (mRNA) and protein of all of the components of the RAS have been detected in the kidney where they might function as an endogenous renal RAS.[3,8,14,15] However, since cells expressing angiotensinogen mRNA may not produce

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angiotensinogen protein,[16] it is possible that components of the renal tissue RAS are derived from distant organs, filtered by the glomerulus, and reabsorbed by the renal tubules. Indeed, although the mRNA for angiotensinogen was readily detected in proximal tubular cells of mice, nearly all of the proximal tubular angiotensinogen protein originated in the liver.[17]

An impaired glomerular filtration barrier in diabetic nephropathy will increase the filtration of some RAS components whose molecular sizes are smaller than albumin (68 kDa), including prorenin (47 kDa) and angiotensinogen (52 kDa). Whereas, megalin mediates the endocytosis of angiotensinogen by the proximal tubule,[16] the PRR is expressed on podocytes, intercalated cells of the cortical collecting duct (CCD), and vascular smooth muscle cells and could mediate prorenin endocytosis.[18]

In the present study, we examined the hypothesis that circulating human angiotensinogen and human prorenin are extracted by the kidney and the vascular endothelium in the diabetic rat and contribute to a local renal and vessel wall RAS and to a pressor system.

Materials and methods
Animals and experimental designs
Male Sprague Dawley rats (Charles River Laboratories, Shizuoka, Japan) weighing 130–150 g were housed in a temperature- and humidity-controlled room with access to tap water and standard animal chow. Rats were allocated as controls (n=6) or diabetes (n=7) group. DM was induced by an intravenous (IV) injection of streptozotocin (STZ) (60 mg/kg body weight in citrate buffer) from the tail vein. After 4 weeks, diabetes and microalbuminuria were confirmed by measurement of the urinary glucose and protein by dipstick. Animals were anesthetized with pentobarbital (50 mg/kg body weight) prior to invasive procedures. All procedures were conducted in accordance with the Guide for Animal Experimentation of the Faculty of Medicine, The University of Tokyo (Tokyo, Japan) and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. This study was approved by the Medical Experimental Animal Ethics Committee of the University of Tokyo (approval number P10-079), and for this study and the use of human prorenin and angiogtensinogen we also followed the principles outlined in the Declaration of Helsinki.

Administration of human prorenin and angiotensinogen
Rats were anesthetized for IV injections of 20 µg recombinant human prorenin (Innovative Research Inc., Novi, MI, USA,

dissolved in 0.2 mL saline) through a jugular vein catheter. Subsequently, incremental doses of recombinant human angiotensinogen (Calbiochem, San Diego, CA, USA) were injected at 5-minute intervals (0.02–20 µg/0.2 mL saline). Blood pressure (BP) was recorded from a catheter in the abdominal aorta. Both kidneys were removed 15 minutes after completion of the IV injections. The right kidney was used for renal tissue angiotensin (Ang II) measurement and Western blot analyses, and the left kidney was fixed with periodate–lysine–paraformaldehyde solution, embedded in wax and used for the immunostaining for human prorenin, angiotensinogen, and rat PRR.

Measurement of the renal tissue Ang II and the urinary protein, creatinine, and glucose levels
The renal tissue Ang II concentration was measured by radioimmunoassay and related to the protein content of the kidney homogenate.[19] Urine samples were collected in metabolic cages. Protein and creatinine concentrations were measured by spectrophotometry.[20] Blood glucose concentration was measured by Glutest ProR (Sanwa Kagaku Kenkyusho, Nagoya, Japan) using tail vein blood under the condition with free access to food.

Immunohistochemistry and immunoelectron microscopy for prorenin, the PRR, angiotensinogen, and angiotensin-converting enzyme
Light microscopic immunohistochemistry was performed as described.[21,22] Kidney sections (2 µm) were incubated with rabbit polyclonal antibodies against human prorenin (1:100 dilution; Serotech, Oxford, UK), PRR (1:100 dilution; Abcam, Cambridge, UK), or human angiotensinogen (1:100 dilution; Abcam) overnight, and thereafter with a horseradish peroxidase (HRP)-conjugated antirabbit IgG secondary antibody (Dako, Glostrup, Denmark) at a 1:50 dilution for 1 hour. HRP signals were developed by diaminobenzidine (DAB, Dojindo Laboratories, Kumamoto, Japan). The immunoreactivity for angiotensin-converting enzyme (ACE) stained with mouse monoclonal antibody for ACE (1:100 dilution, Chemicon International, Temecula, CA, USA) and HRP-conjugated antimouse IgG secondary antibody (Dako) was semiquantified with scores: 0, no; 1, weak; 2, moderate; 3, strong staining counting 100 glomeruli and proximal tubules in each group. To detect the subcellular localization of human prorenin, pre-embedding immunoelectron microscopy was performed as described previously.[23] Tissue sections

(50 μm) cut by a vibratome (Dosaka EM, Kyoto, Japan) were incubated overnight with a rabbit antibody against human prorenin (1:100 dilution) followed by an HRP-conjugated antirabbit IgG secondary antibody (1:50 dilution). HRP signals were developed by DAB, postfixed with 2% osmium solution, and embedded in epoxy resin. Ultrathin sections were examined by a transmission electron microscope (Hitachi H-7000, Tokyo, Japan).

Western blot analyses of kidney homogenates or isolated glomeruli for human prorenin, angiotensinogen, and PRR

Homogenates of whole kidneys were obtained and glomerular and tubular fractions were separated by graded sieving as described previously.[21,24] Homogenate proteins (20 μg) were loaded on 4/20% gels, separated by electrophoresis, the protein bands transferred to polyvinylidene difluoride membranes (Millipore Co, Billerica, MA, USA), incubated with rabbit antihuman prorenin antibody (1:250 dilution), rabbit anti-PRR antibody (1:500 dilution), or rabbit antihuman angiotensinogen antibody (1:500 dilution) as the primary antibody, and with HRP-conjugated antirabbit immunoglobulin (Dako) as the secondary antibody (1:1,000 dilution). The blots were detected by DAB with nickel chloride. As loading control, the membranes were incubated with anti-β-actin antibody (Abcam, Tokyo, Japan), and processed as mentioned earlier. The area and density of bands for each protein were measured using a NIH image software program. The specificity of the antibody for human angiotensinogen was confirmed by Western blot analysis using increasing amount (10, 100, 1,000 ng) of rat angiotensinogen and human angiotensinogen on the membrane.

Immunoprecipitation of human prorenin extracted from glomerular proteins with antibodies against PRR and megalin

Kidney homogenates from rats injected with human prorenin and angiotensinogen were incubated with protein A-sepharose for 30 minutes to remove nonspecific precipitates, centrifuged at 5,000× g for 1 minute and the supernatant was incubated for 2 hours at 4°C with the addition of either a rabbit anti-PRR antibody (1:500 dilution) or a rabbit antimegalin antibody and 10 μL of protein A-sepharose. After five repeated washes by resuspension in 4°C lysis buffer and centrifugation at 2,000× g for 2 minutes, 20 μL of sample buffer was added to the precipitates which were boiled for 2 minutes and centrifuged at 15,000× g for 5 minutes. The supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and a Western blot analysis was performed with rabbit antihuman prorenin antibody at a 1:200 dilution, followed by incubation with an HRP-conjugated secondary antibody at a 1:1,000 dilution and DAB staining.

Statistical analyses

All data were shown as mean ± standard error of the mean. The mean values were compared between two groups using the unpaired Student's t-test. The BP response to the increasing doses of angiotensinogen was analyzed by a repeated-measures analysis of variance. P-values <0.05 were considered to be significant.

Results

STZ-induced diabetic rats had increased blood glucose (427±86 vs 183±41 mg/dL, DM vs control, $P<0.002$) and proteinuria (6.5±1.9 vs 1.7±0.5 mg/d/100 g body weight, $P<0.05$), but their systolic and diastolic BPs were similar (systolic BP: 84±12 vs 85±9 mmHg, ns; diastolic BP: 55±11 vs 53±15 mmHg, ns).

Changes in the systolic and diastolic BP after the administration of human angiotensinogen and human prorenin in control and STZ-induced diabetic rats

Continuous BP recording in anesthetized DM rats revealed that an IV injection of human prorenin and human angiotensinogen to these rats dose-dependently increased both systolic and diastolic BP (Figure 1). In contrast, similar injections into normal rats did not produce a significant change from baseline.

The uptake of exogenous human prorenin is enhanced in diabetic rats

Immunostaining for human prorenin was increased in the podocytes, proximal tubules, macula densa, and CCDs of DM rats (Figure 2A–D). This was confirmed by Western blot for human prorenin in whole kidney homogenates, which showed almost twice as much human renal prorenin uptake in DM rat kidneys (Figure 2E and F). The expression of prorenin was increased in both glomerular and tubular fractions (Figure 2A–D). There was more human prorenin in glomeruli and tubules from DM rats (Figure 2G). Pre-embedding immunoelectron microscopy localized the human prorenin in DM rats to the vesicles of podocytes and proximal tubular epithelial cells, but this was not obvious in controls (Figure 3). These results indicated that human prorenin molecules entered the urinary space where they were taken up by renal
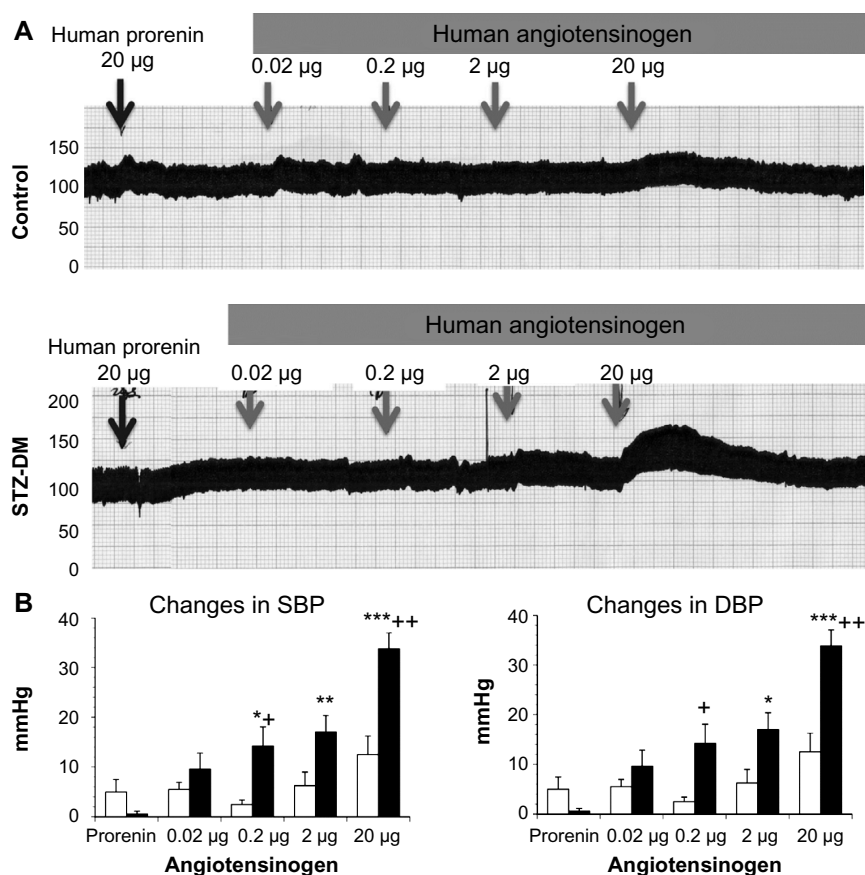
Figure 1 Changes in the blood pressure of control and STZ-induced DM rats after the administration of human prorenin and human angiotensinogen.
Notes: (**A**) Records of arterial blood pressure in a control and diabetic rat injected with human prorenin and angiotensinogen. (**B**) Graphs showing the variations in the SBP and DBP in control and diabetic rats. A significant increase in blood pressure was observed only in the diabetic rats. White bars indicate control and black bars indicate diabetic rats. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs changes after prorenin injection for each group, +$P<0.05$, ++$P<0.01$ vs the control rats at the same dose. N=4 in each group.
Abbreviations: DBP, diastolic blood pressure; DM, diabetes mellitus; SBP, systolic blood pressure; STZ, streptozotocin.

cells, especially podocytes and tubular epithelial cells. This uptake was enhanced in DM rats.

Enhanced endocytosis of prorenin mediated by the PRR and megalin in diabetic rats

Megalin and PRR are expressed by podocytes and renal tubular epithelial cells. An immunoprecipitation analysis identified PRR in podocytes and cells of the macula densa and CCDs, and, to a lesser extent, of the proximal tubules. However, the proximal tubular immunoreactivity appeared to be increased in DM rats (Figure 4A–D). A Western blot analysis for PRR in kidney homogenates confirmed that the renal PRR expression was increased almost threefold in DM rats (Figure 4E and F). An immunoprecipitation study of human prorenin using antibodies for megalin or the PRR demonstrated that human prorenin was bound to both megalin and the PRR. The amount of bound prorenin was increased in DM rats (Figure 5). Thus, endocytic uptake

of human prorenin by the kidney may be mediated by both the PRR and megalin, both of which were enhanced in DM rats.

Uptake of human angiotensinogen

Following its IV injection, immunoreactive human angiotensinogen was detected in podocytes, proximal tubules, macula densa, and collecting ducts, which was a similar distribution to that found for human prorenin and the PRR. The immunoreactive staining for angiotensinogen appeared increased in DM rats, notably in the podocytes (Figure 6A and B) and the vascular endothelial cells (Figure 6C and D). Indeed, there was an increase in 52 kDa human angiotensinogen detected by a Western blot of glomerular fraction isolated from DM rats, whereas it was a slight increase in the tubular fraction (Figure 6E–G). The specificity of the antibody was confirmed by Western blot demonstrating a reaction with human angiotensinogen in a dose-dependent manner, but faintly with rat angiotensinogen (Figure 6E).
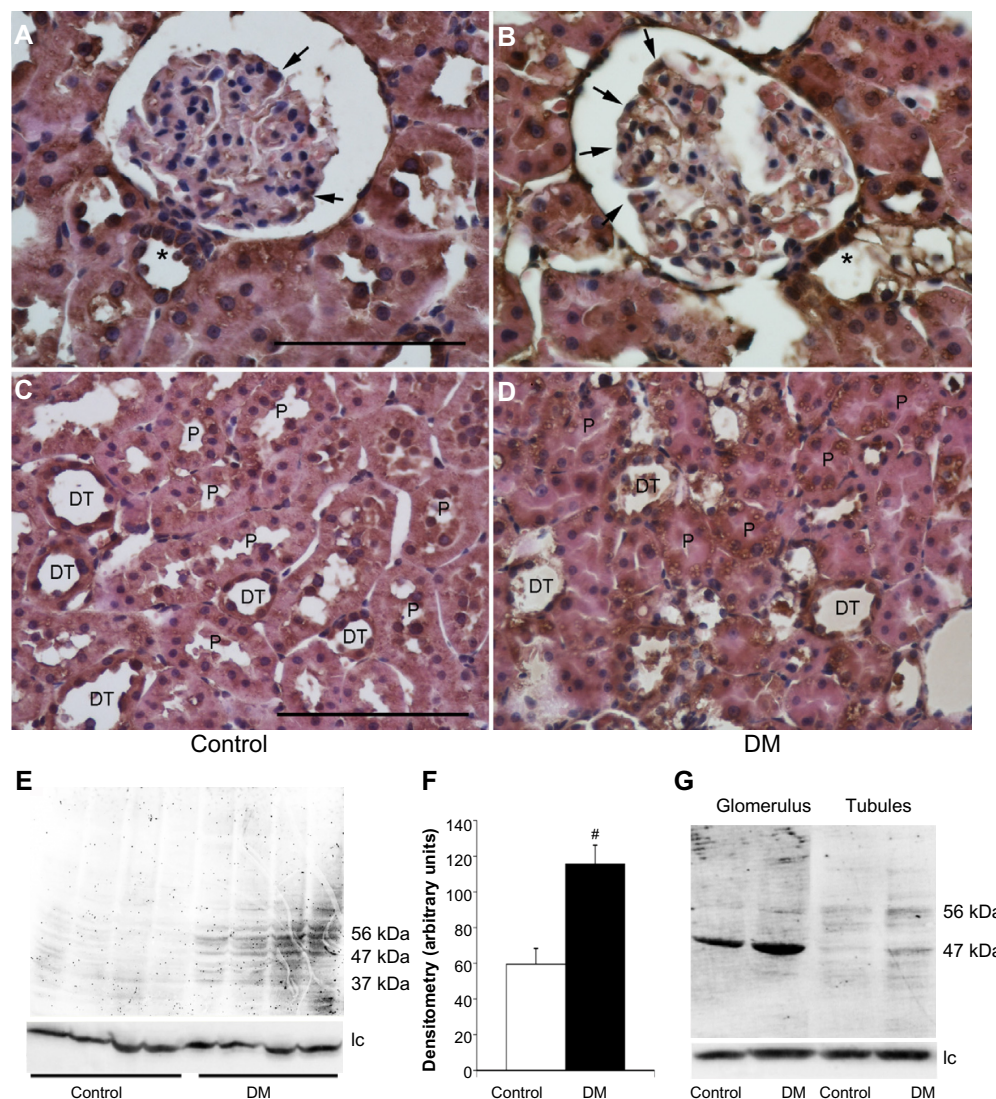
Figure 2 The uptake of exogenous human prorenin is enhanced in diabetic rats.
Notes: Immunostaining for human prorenin (brown color staining in **A–D**) in podocytes (arrows), proximal tubules (P), macula densa (asterisks), and distal nephrons (DT) including cortical collecting ducts, appeared enhanced in diabetic rats (**B** and **D**) compared with controls (**A** and **C**). Bar: 100 μm. A Western blot analysis for human prorenin demonstrating an increased uptake of human prorenin in the whole kidney homogenates (**E** and **F**) and also in glomerular and tubular fractions in diabetic rats (**G**). #$P<0.01$ vs control.
Abbreviations: DM, streptozotocin-induced diabetes mellitus rats; lc, loading control.

Renal Ang II concentration and ACE type 1 expression

Renal concentrations of Ang II were not significantly different between DM and control rats (6.1±1.6 in DM vs 5.7±1.0 pg/mg protein in control, ns). An immunohistochemical study detected ACE type 1 (ACE-1; Figure 7) mainly in the proximal tubules, especially in the brush border membranes of control kidneys. Its expression in DM rats was absent in proximal tubules but increased in glomeruli.

Discussion

The main new findings in this study are that exogenous human prorenin and human angiotensinogen were filtrated and taken up by renal cells, including podocytes, proximal tubular cells, macula densa cells, and CCD cells. This process was enhanced in diabetic rats. Renal uptake was mediated by binding to the PRR and megalin.

The origin of renal angiotensinogen remains controversial. The findings of enhanced mRNA for angiotensinogen in the proximal tubule cells of diabetic rats[25,26] certainly suggested that it originated predominantly from the proximal tubule.[27,28] This is important since renal angiotensinogen excretion has been used as a marker of intrarenal Ang II and early kidney injury in conditions that include DM.[8,29] However, there have been conflicting conclusions concerning the origin of renal angiotensinogen and the role of
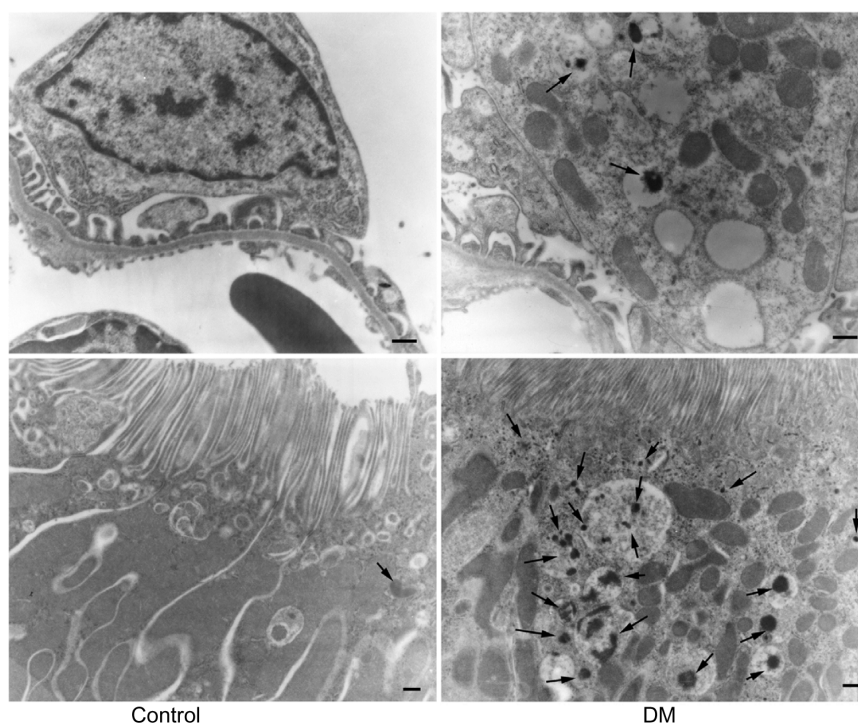
Figure 3 Pre-embedding electron microscopy for human prorenin.
Notes: Black label for human prorenin was detected in podocyte vesicles and proximal tubules in diabetic rats 40 minutes after its first intravenous injection (arrows), but was barely detected in controls. Bar: 500 nm.
Abbreviation: DM, diabetes mellitus rats.

renal tubular angiotensinogen synthesis.[16,17,30–32] Thus, Pohl et al[16] reported abundant endocytosis of angiotensinogen by megalin in the S1 segment of proximal tubules of mice, whereas angiotensinogen mRNA was localized to the S2/S3 segments where angiotensinogen protein expression was low. Furthermore, inhibition of endocytosis by colchicine decreased renal angiotensinogen levels.[33] Matsusaka et al[17] reported that angiotensinogen was expressed in the kidneys of kidney-specific angiotensinogen gene knockout mice but was absent from the kidneys of liver-specific angiotensinogen knockout mice. They concluded that renal angiotensinogen derives predominantly from angiotensinogen secreted into the bloodstream by the liver and extracted by the kidneys.[17]

Our study is the first to show directly that exogenous angiotensinogen is taken up in the kidney preferentially in diabetic rats with microalbuminuria. This is consistent with increased glomerular filtration of angiotensinogen since its excretion parallels that of albumin.[27,28] Indeed, the estimated glomerular sieving coefficients of prorenin and angiotensinogen, judged from their molecular size, are approximately tenfold higher than that of albumin.[34,35] Moreover, the injected human angiotensinogen was detected in glomerular podocytes and proximal tubules and its uptake

into glomerular podocytes was increased in diabetic rats, suggesting increased filtration of angiotensinogen associated with albuminuria.

A second important finding from our study was that circulating human prorenin also was taken up in excess by diabetic kidneys and was localized to the podocytes, proximal tubules, and distal nephron, including the CCD where PRR is expressed. Immunoprecipitation studies indicated that prorenin was bound to, and taken up by both PRR and megalin consistent with prior reports that prorenin uptake and activation occurs in vascular endothelial cells.[36,37] Thus, an enhanced uptake of filtered angiotensinogen and prorenin by podocytes and renal tubules contributed to an enhanced RAS in diabetic kidneys.

ACE-1 expression was reduced in the brush border membrane of the proximal tubule of diabetic rats, but was increased in glomeruli and vascular endothelial cells, consistent with previous reports.[5,38] A limited expression of renal tubular ACE-1 may underlie the maintained renal Ang II in DM rats despite enhanced uptake of prorenin and angiotensinogen. There are conflicting reports of renal Ang II levels in diabetes,[7,11,39] which may reflect the degree of filtration of angiotensinogen as well as salt intake. An increased uptake of exogenous angiotensinogen
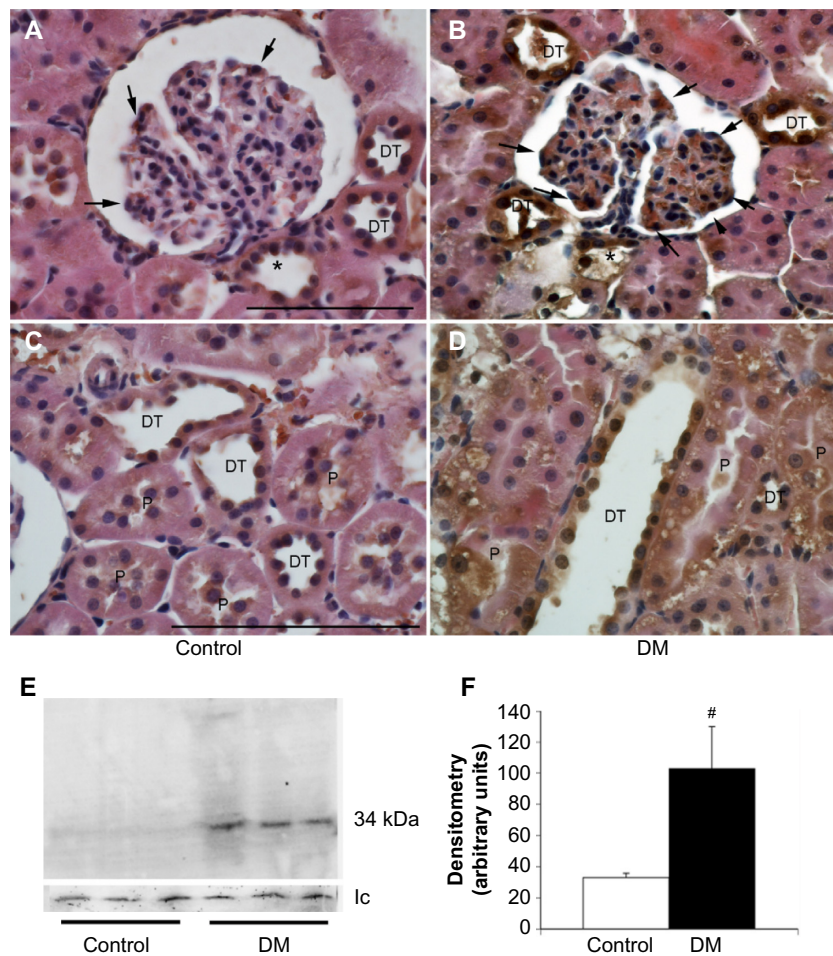
Figure 4 The expression of the prorenin receptor in normal and diabetic rats.
Notes: Immunohistochemical analysis of the prorenin receptor in podocytes (arrows), proximal tubules (P), macula densa (asterisks), and distal nephron including cortical collecting ducts (DT), appeared increased in diabetic rats (**B** and **D**) compared with controls (**A** and **C**). This was confirmed by the results of a Western blot analysis (**E** and **F**). $^{#}P<0.05$ vs control. Bar: 100 μm.
Abbreviations: DM, diabetes mellitus rats; lc, loading control.
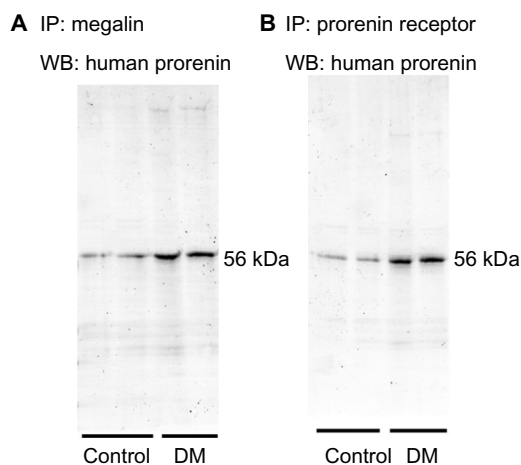
Figure 5 Immunoprecipitation of human prorenin by antibodies for megalin (**A**) or the prorenin receptor (**B**).
Note: The level of renal human prorenin bound to both megalin and the prorenin receptor was increased in diabetic rats compared with controls.
Abbreviations: DM, diabetes mellitus rats; IP, immunoprecipitation; WB, Western blot.

by the vascular endothelial cells in DM rats may have contributed to the enhanced BP responses of these rats to injected human angiotensinogen and to hypertension in DM.

The enhanced renal uptake of prorenin evident in podocyte, proximal tubule, macula densa, and CCDs of DM rats mediated as an up-regulated PRR at their sites could have two consequences. First, increased circulating prorenin in DM[40] combined with increased filtration in proteinuric states may enhance tubular renin activity since binding of prorenin to the PRR generates active renin. Second, the PRR activates the mitogen-activated protein kinase-extracellular signal-regulated kinase 1/2 pathway that increases the expression of profibrotic molecules such as transforming growth factor β1, plasminogen activator inhibitor-1, fibronectin, and collagen, and also induces mesangial proliferation.[41–43] Thus, an enhanced plasma level and filtration of prorenin combined
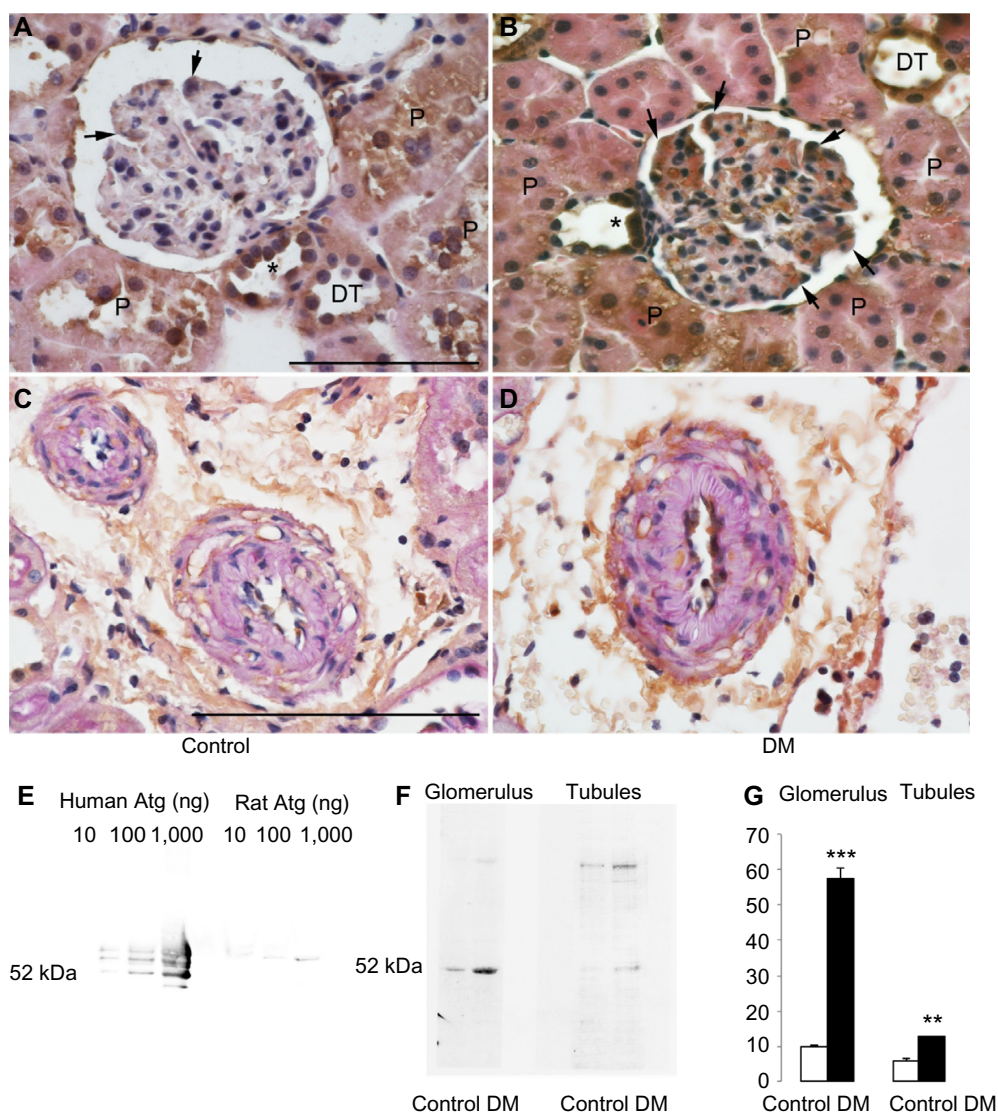
Figure 6 Human angiotensinogen uptake in normal and diabetic rats.

Notes: Immunohistochemical analysis for human angiotensinogen expression (brown color staining in **A–D**) in podocytes (arrows), proximal tubules (P), *macula densa and the distal nephron (DT) including collecting ducts and endothelial cells of renal small artery (**C** and **D**) appeared increased in diabetic rats, especially in podocytes. Western blot for human angiotensinogen demonstrated that the specificity of the antibody which reacted with human angiotensinogen in a dose-dependent manner, but only weakly with rat angiotensinogen (**E**). Western blot analysis showed increased human angiotensinogen uptake in the glomerular fraction of DM rats, but slightly increased in the tubular fraction of DM rats compared with those of control (**F** and **G**). **$P<0.001$, ***$P<0.0001$ vs control. Bar: 100 μm.

Abbreviations: Atg, angiotensinogen; DM, diabetes mellitus rats.

with an enhanced expression of the PRR may promote renal damage in DM, and PRR blockade was shown to improve DM nephropathy.[44]

Although microalbuminuria is an established risk factor for renal disease progression and cardiovascular disease, the mechanism is unknown.[45,46] Approximately 3 g of albumin are filtered daily by the glomeruli and reabsorbed by the tubules.[34] Albumin endocytosis is enhanced in the podocytes and proximal tubules in proteinuric conditions.[47] We now demonstrate enhanced endocytosis of prorenin and angiotensinogen in the kidneys and the vascular endothelium of DM rats with albuminuria. Thus, the true culprit molecules

that mediate progression of CKD and the development of CVD during microalbuminuria may be angiotensinogen and prorenin whose glomerular filtration, tubular uptake, and renal excretion is proportional to microalbuminuria.[17,27,28,30] This scenario would identify albumin excretion as a marker for renal filtration and uptake of these RAS components.

Conclusion

Endocytosis of exogenous prorenin and/or angiotensinogen molecules was enhanced in the podocytes, renal tubules, and vascular endothelial cells in diabetic rats, implying that the glomerular filtration and uptake of these molecules by
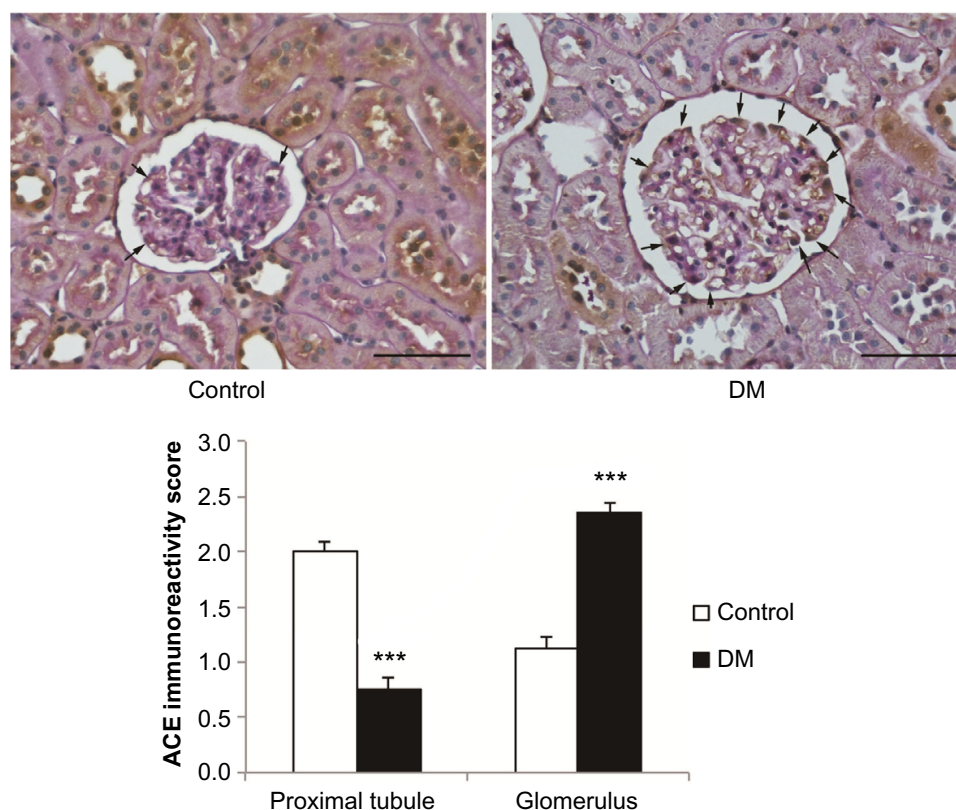
Control   DM

Figure 7 The expression of ACE in normal and diabetic rats.
Notes: The semiquantification of immunoreactivity for ACE indicated reduced brush border membrane of proximal tubules but enhanced podocytes of diabetic rats (arrows). ***$P < 0.0001$. Bar: 100 μm.
Abbreviations: ACE, angiotensin-converting enzyme; DM, diabetes mellitus rats.

renal cells and the blood vessels may activate a local RAS in diabetes.

Acknowledgments

Author contributions

AT and SK performed animal studies and all authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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