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Renal (pro)renin receptor contributes to development of diabetic kidney disease through TGFβ1-CTGF signaling cascade

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SUMMARY

1. Transforming growth factor β1 (TGFβ1) and connective tissue growth factor (CTGF) are expressed in renal glomeruli and contribute to development of diabetic nephropathy. Recently we demonstrated that (pro)renin receptor (PRR) is upregulated in the kidneys of streptozocin (STZ)-induced diabetes rat model. We hypothesized that in the presence of hyperglycemia, increased renal PRR expression contributes to enhanced TGFβ1-CTGF signaling activity, leading to development of diabetic kidney disease.
2. *In vivo* and *in vitro* studies were conducted in Sprague-Dawley rats and rat mesangial cells (RMCs). PRR blockade was achieved *in vivo* by treating STZ induced diabetes rats with the handle region peptide (HRP) of prorenin and *in vitro* by HRP or PRR siRNA in RMCs. Angiotensin AT1 receptor blockade was achieved by *Valsartan* treatment.
3. Results showed that expression of PRR, TGFβ1 and CTGF were upregulated in diabetic kidneys and RMCs exposed to high glucose. Glucose exposure also induced PRR phosphorylation, a process that was inhibited by HRP, *Valsartan* or PRR siRNA. HRP and *Valsartan* significantly attenuated renal TGFβ1 and CTGF expression in diabetic animals and high glucose treated RMCs. Similar results were observed in high glucose exposed RMCs in response to PRR siRNA. TGFβ receptor blockade decreased CTGF expression in RMCs. Combined administration of *Valsartan* and PRR siRNA demonstrated further reduction of TGFβ1 and CTGF expression in RMCs.
4. In conclusion, PRR contributes to kidney disease in diabetes through enhanced TGFβ1-CTGF signaling cascade.

Keywords

(pro)renin receptor; Transforming growth factor β1; Connective tissue growth factor; hyperglycemia; kidney; mesangial cells

INTRODUCTION

Previous studies demonstrated that activation of renin-angiotensin-system (RAS) contributes to increased activity of the transforming growth factor β (TGFβ)- connective tissue growth factor (CTGF) axis in renal diseases (1–12). Similarly, TGFβ and CTGF expressions and their intracellular signals are increased in diabetes (13–19). CTGF gene contains a novel

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DISCLOSURE

Authors have nothing to disclose.

TGF β response element initiates promoter region (20) and is thought to be a downstream mediator of TGF β effects (21,22). Thus, TGF β -CTGF axis activation contributes to development of kidney disease in diabetes.

(Pro)renin receptor (PRR), a new member of RAS, is a 350 amino acid protein with a single transmembrane domain that binds and activates prorenin (23,24). The role of PRR in the development of kidney disease is not established yet. Our previous studies showed that PRR is upregulated in kidneys of diabetic rats and renal mesangial cells (RMCs) exposed to high glucose concentration (25–28). PRR blockade attenuated diabetic proteinuria (27,29–30) and also inhibited cytokines production (26,27). In this study, we hypothesized that hyperglycemia induced PRR expression enhances TGF β 1-CTGF signaling pathway leading to the development of kidney disease. The influence of PRR function or expression on TGF β 1-CTGF axis was evaluated *in vivo* and *in vitro* by utilization of the PRR blocker, handle region peptide (HRP) of prorenin, the angiotensin AT1 receptor blocker *Valsartan* and PRR small interference RNA (siRNA) in RMCs exposed to high glucose.

MATERIALS AND METHODS

In vivo studies of handle region peptide (HRP) of prorenin and *Valsartan* in diabetic rats

The University of Virginia Animal Care and Use Committee approved all protocols. Kidneys tissues from previously studied rats (27) were used in this study. In brief, male Sprague-Dawley rats (Charles River Laboratories; Wilmington, MA) weighing 230 to 260g were divided randomly into five groups: normoglycemic control group ($n = 8$), diabetes group (DM; $n = 10$), DM treated with HRP group (DM + HRP; $n = 10$), DM treated with the angiotensin subtype-1 receptor (AT1R) blocker *Valsartan* group (DM + Val; $n = 10$), and DM treated with both HRP and *Valsartan* group (DM + HRP + Val; $n = 9$). Diabetes was induced by intraperitoneal administration of 65 mg/kg of streptozotocin (STZ; Sigma-Aldrich, Saint Louis, MO). Normoglycemic control rats were treated with an equal volume of vehicle (0.9% NaCl). The decapeptide NH₃-RILLKKMPSV-COOH (HRP) was dissolved in saline and administered at 0.2 mg/kg. The angiotensin AT1 receptor antagonist *Valsartan* (Novartis, East Hanover, NJ, USA) was used at 2 mg/kg/day, a dose that does not affect blood pressure in rats (31). Both HRP and *Valsartan* were administered directly into the left renal cortex interstitium for 14 days via osmotic minipump (Alzet, Cupertino CA) as previously described (32). Controls and non-treated diabetic rats were implanted with a sham osmotic minipump containing 0.9% NaCl. At the end of experiments, kidneys were harvested for protein and total RNA extraction.

High glucose exposure, HRP treatment and receptors blockade by *Valsartan* and SD208 in cultured rat mesangial cells

Rat mesangial cells (RMCs) were obtained from the American Type Culture Collection (ATCC, Manassas VA) and cultured according to ATCC recommended protocol. The glucose exposure was conducted by culturing cells in high glucose medium (30mM D-glucose for experimental groups and 30mM L-glucose for controls) for 2 weeks as detailed previously (26,28).

Cells were serum starved for 12 hrs at the end of the 2 weeks glucose exposure, and then various treatment maneuvers were applied. With continuation of cell starvation, different cell groups were treated with HRP (1 μ M), *Valsartan* (1 μ M) or TGF β receptor inhibitor SD-208 (500 nM, EMD Biosciences, Inc., La Jolla CA) for 12 hrs, then cultured supernatants were collected and cells were harvested for preparation of whole cell lysate and total RNA extraction.

Other groups of control and high glucose treated cells were used for PRR siRNA as discussed below.

(Pro)renin receptor knockdown by siRNA interference

As previously described (26), single-stranded 19-nt RNA duplexes targeting rat ATP6AP2 mRNA (GeneBank Access No. XM_217592.4) (5'-CCUACAACCUUGCGUAUAA-3') for silencing of PRR were purchased from Dharmacon Research Inc, (Dharmacon, Boulder CO). RMCs plated in six-well culture dishes were transfected with siRNA duplexes against rat PRR mRNA by using siPORT NeoFX transfect reagent (Ambion, Austin TX) following the manufacturer's suggested protocol. Our previous studies indicated that 100 nM siRNA duplex resulted in a maximal suppression of PRR mRNA for 48 hrs and of PRR protein expression for 72hrs (26). Cells for total RNA extraction were harvested at 48 hrs-post-transfection. Cells for whole cell lysate preparation and culture supernatants for ELISA assays were collected at the 72 hrs-post-transfection respectively. For combined PRR knockdown and AT1 receptor blockade, *Valsartan* was applied to PRR siRNA treated cells for 12 hrs prior to cell harvesting.

Assessing genes expressions of PRR, TGFβ1 and CTGF

Determinations of genes expressions were done as previously described (25–28). Quantitative real-time RT-PCR was used to validate mRNA changes in the genes expressions. Briefly RNA was extracted from kidneys and cultured cells with the RNeasy total RNA isolation kit (Qiagen, Valencia CA). The RNA integrity was assessed by 2% formaldehyde agarose gel electrophoresis. Expression levels of PRR, TGFβ1 and CTGF mRNA were measured by real-time RT-PCR iCycler according to the manufacturer's instructions (Bio-Rad, Hercules CA). Single-stranded cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad, Hercules CA). PCR was performed with iQTM SYBR green supermix (Bio-Rad, Hercules CA) according to the manufacturer's instructions. Primers sequences are as followed. PRR forward: 5'-TGG CCT ATA CCA GGA GAT CG-3'; PRR reverse: 5'-AAT AGG TTG CCC ACA GCA AG-3'; TGFβ1 forward: 5'-ATA CGC CTG AGT GGC TGT CT-3'; TGFβ1 reverse: 5'-TGG GAC TGA TCC CAT TGA TT-3'; CTGF forward 5'-TAG CAA GAG CTG GGT GTG TG-3'; CTGF reverse 5'-TTC ACT TGC CAC AAG CTG TC-3'; β-actin forward: 5'-AGC CAT GTA CGT AGC CAT CC-3'; β-actin reverse: 5'-ACC CTC ATA GAT GGG CAC AG-3'. 18S rRNA forward: 5'-CGA AAG CAT TTG CCA AGA AT-3'; 18S rRNA reverse: 5'-AGT CGG CAT CGT TTA TGG TC-3'. Reactions were performed in triplicate, and threshold cycle numbers were averaged. Nontemplate control was used as negative control. Samples were calculated with normalization to β-actin mRNA or 18S rRNA.

Whole cell lysates were extracted from kidneys and cultured cells with lysis buffer as detailed in previous studies (25–28). Total 10 – 50 μg of cell lysate was loaded for each sample, separated by SDS-PAGE and electro-transferred to polyvinylidene fluoride membrane (Immun-Blot 0.2 μm, Bio-Rad, Hercules CA). The following primary antibodies against ATP6AP2 (Abcam, Cambridge MA), TGFβ1 (Santa Cruz Biotechnology, Santa Cruz CA) and CTGF (Novus Biologicals, Littleton CO) were employed in this study. Blots were treated with Restore Western Blot Stripping Buffer according to manufacturer's recommendation (Pierce Biotechnology, Rockford IL) and followed by re-probing with anti-β-actin antibody (Sigma, St. Louis MO). The bands densitometry analysis was performed by ImageMasterTM TotalLab Version 2.0 (Amersham Pharmacia BioTech, Piscataway NJ). The bands densities of target protein were normalized to the corresponding density of β-actin. The arbitrary unit of bands densities is presented as the expression levels.

Detection of PRR phosphorylation by co-immunoprecipitation

Cultured cells were washed with ice-cold PBS and cell lysate was prepared in lysis buffer (50mM Tris-HCl, pH8.0, 150mM NaCl, 1% IGEPAL CA-630, 50mM NaF, 2mM sodium orthovanadate, 1mM PMSF and 1X protease inhibitor cocktail) and separated by centrifugation at 14,000 rpm for 20 min at 4°C. Cell lysate was incubated for 2 hrs with polyclonal antibody to ATP6AP2 linked to antibody coupling gel (Pierce Biotechnology, Rockford IL) according to manufacturer's instructions. The Elutes were mixed with loading buffer containing 200mM DTT or 12.5% β -mercaptoethanol, boiled for 10 min and separated on 4–20% Tris-HCl Criterion precast gel. Blots were incubated with mouse monoclonal anti-phosphoserine antibody (Sigma, St. Luis MO) for further Western immunoblot detection as described above.

Enzyme-linked immuno-sorbent assay of rat TGF β 1 and CTGF

Parallel experiments in RMCs with the same design as described above were conducted simultaneously to determine the total cell number in each sample. The total cell number was determined by quantitative measurement of RMCs release of lactate dehydrogenase (LDH) by using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Madison WI). Cells in serial dilution (0; 5,000; 10,000; 20,000 cells) (for plotting standard curve) and those in experiments were lysed to prepare cell lysates. LDH level in lysates was colorimetrically assayed at 490nm. The total cell number (1×10^6 cells) was the average of triplicates and used for the normalization of TGF β 1 and CTGF samples.

Rat TGF β 1 levels in culture supernatants of RMCs were analyzed by TGF β 1 Quantikine ELISA kit (R & D Systems, Minneapolis MN). The protocol was performed according to the manufacturer's instructions. The sensitivity of the TGF β 1 is in the range of 1.7 – 15.4 pg/ml. The intra- and inter-assay are 3.4% and 8.4%, respectively.

Rat CTGF levels in culture supernatants of RMCs were analyzed by CTGF ELISA development kit according to manufacturer's instructions (PeproTech Inc., Rocky Hill, NJ). Quantitative measurement of natural CTGF in this kit is within the range 63–4000 pg/ml. The intra- and inter-assay are 5% and 10%, respectively.

Statistical analysis

The data analysis was performed using *STATISTICA* v. 5.0 (StatSoft Inc., Tulsa, OK, USA). Results are expressed as mean + SE. Comparisons among different treatment groups were evaluated by analysis of variance (ANOVA) with repeated measures, and Bonferroni correction method as a *post hoc* test. The p value of <0.05 is defined as statistically significant.

RESULTS

TGF β 1 and CTGF expressions in kidneys of diabetic rats

Renal expression of TGF β 1 and CTGF mRNAs and proteins were significantly increased in diabetic rats (Figure 1). HRP or *Valsartan* significantly attenuated the expressions of TGF β 1 and CTGF mRNAs and proteins (Figure 1). Combined treatment of HRP and *Valsartan* caused reduction in mRNA and protein expressions of TGF β 1 and CTGF and similar to individual treatment with HRP or *Valsartan*.

Expression of PRR, TGF β 1 and CTGF in cultured RMCs

High glucose upregulated PRR mRNA and protein expression while PRR blockade with HRP did not influence PRR expression in control and high glucose-treated RMCs (Figure 2A and B).

TGF β 1 and CTGF mRNAs and proteins were significantly increased in high glucose treated RMCs (Figure 2C–F). HRP attenuated the expression of TGF β 1 and CTGF mRNA and protein in high glucose treated RMCs (Figure 2C–F).

Effects of high glucose, HRP, *Valsartan* and PRR siRNA on serine-phosphorylation of PRR in RMCs

Short term (180 min) high glucose exposure induced a rapid PRR phosphorylation at serine residues within 30 min in RMCs and lasted for 180 min (Figure 3).

The rapid PRR phosphorylation induced by one hour of high glucose exposure was decreased by *Valsartan* while HRP did not have significant effects (Figure 4A).

Long term high glucose exposure also induced the increase of PRR phosphorylation that was observed at 14 days of this treatment. Both HRP and *Valsartan* decreased PRR phosphorylation when evaluated at the end of study in controls and high glucose treated RMCs (Figure 4B).

Similarly, PRR siRNA significantly decreased PRR phosphorylation in control and high glucose treated RMCs (Figure 4C).

Effects of individual and combined administration of PRR siRNA and *Valsartan* on TGF β 1 and CTGF expressions in RMCs

High glucose treatment caused significant increase in TGF β 1 and CTGF mRNA and protein expressions (Figure 5A–D). PRR siRNA or *Valsartan* (Figure 5A–D) attenuated high glucose induced expression of TGF β 1 and CTGF mRNA and protein. Combined treatment of PRR siRNA and *Valsartan* led to further reduction in TGF β 1 and CTGF expressions compared to each individual treatment.

Effect of TGF β receptor blockade on CTGF expression in RMCs

At basal condition, TGF β receptor inhibitor SD208 significantly inhibited CTGF mRNA and protein expression (Figure 6). High glucose increased CTGF mRNA and protein expression in RMCs (Figure 6). SD208 treatment significantly attenuated high glucose induced increase in CTGF (Figure 6).

DISCUSSION

Our studies showed that TGF β 1 and CTGF expressions were upregulated in kidneys of diabetic rats and in renal mesangial cells treated with high glucose. CTGF is a downstream mediator of TGF β (19–22). In the present study we demonstrated that TGF β receptor blockade with SD208 significantly inhibited CTGF expression in normo- and hyperglycemic environments. These data confirm that TGF β 1-CTGF signaling cascade exists in mesangial cell model and is enhanced in the presence of high glucose level. Furthermore, we demonstrated that enhanced TGF β 1-CTGF axis induced by hyperglycemia was efficiently inhibited by PRR blockade with HRP both in the kidneys of diabetic rats and cultured RMCs exposed to high glucose concentration. A similar inhibitory effect was observed *in vitro* by PRR siRNA. These results confirmed the involvement of this receptor in the upregulation of TGF β 1-CTGF cascade in response to hyperglycemia.

Current study confirmed our previous observation of increased PRR expression in response to hyperglycemia (25,26,28). The rapid PRR phosphorylation (30 min) at serine residues in response to high glucose exposure suggests the proximity of PRR activation prior to upregulation of TGF β 1 and CTGF. This rapid PRR phosphorylation was inhibited by *Valsartan* but not HRP. However, PRR phosphorylation was inhibited by prolonged HRP, *Valsartan* and PRR siRNA treatments in response to longer period of high glucose exposure. The reason for requiring longer duration of HRP treatment to inhibit PRR phosphorylation is not clear at present time and could be related to this drug pharmacokinetics or mechanisms of action. The similarity in the responses to PRR siRNA, *Valsartan* and HRP suggest that TGF β -CTGF axis activation occurred via PRR signaling pathway

Recently, we demonstrated a reduction of albuminuria in diabetic rats with PRR blockade (27), suggesting involvement of this receptor in development of diabetic nephropathy. These results are in agreement with previous studies demonstrating reduction in development of diabetic nephropathy with PRR blockade (33). Moreover, PRR blockade was shown to attenuate the progression of proteinuria and glomerulosclerosis (34), cardiac fibrosis (35), inflammation (27,36) and angiogenesis (36). In contrast, other studies reported the lack of effects of PRR blockade with HRP in high renin animal models (37,38). In our studies, we confirmed that HRP attenuated PRR phosphorylation and decreased the expression of TGF β 1 and CTGF in the kidney of diabetic rats and in high glucose treated RMCs. Similarly, PRR siRNA attenuated high glucose induced increase of PRR phosphorylation and TGF β 1 and CTGF expression. These results suggest that HRP effects were mediated by PRR. However, in contrast to HRP, PRR siRNA inhibited PRR mRNA and protein expression. This difference between HRP and PRR siRNA on PRR expression could explain the potentiating effect of the latter when combined with AT1R blockade.

In the present study, AT1R blockade with *Valsartan*, PRR blockade with HRP or inhibition of PRR expression with siRNA attenuated the increase of TGF β 1 and CTGF expression associated with high glucose both *in vivo* and *in vitro*. Combined treatment of *Valsartan* and PRR siRNA caused further reduction in TGF β 1 and CTGF expression, suggesting complementary effects between AT1R and PRR on TGF β 1 and CTGF expression. These results suggest that AT1R and PRR may independently influence TGF β 1-CTGF axis. It is also possible that PRR increases TGF β 1 and CTGF expression via enhancing angiotensin II formation and stimulation of AT1R. Therefore, based on this study, we cannot totally exclude the possibility that non-angiotensin II-dependent mechanism(s) or cross talk with other signaling pathways may contribute to PRR effects on TGF β -CTGF expression in presence of hyperglycemia. Other mechanisms could be involved and mediate the pathologic effects of PRR in the kidney. Recently, a possible link between PRR and vacuolar H⁺-ATPase was suggested in the kidney (39). Similarly, essential roles of PRR in the assembly and function of vacuolar H⁺-ATPase were elucidated in the heart (40). It also has been suggested that PRR functions as an adaptor protein between Wnt receptor and the vascular H⁺-ATPase (41). Since Wnt signaling pathway promotes renal fibrosis, glomerulosclerosis and proteinuria (42,43), it is possible that PRR may act through PRR-H⁺-ATPase-Wnt signaling pathway.

In conclusion, PRR contributes to kidney disease in diabetes through enhanced TGF β 1-CTGF signaling cascade.

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References

1. Border WA, Noble NA. Interactions of transforming growth factor-beta and angiotensin II in renal fibrosis. *Hypertension*. 1998; 31:181–8. [PubMed: 9453300]
2. Wolf G. Link between angiotensin II and TGF-beta in the kidney. *Miner Electrolyte Metab*. 1998; 24:174–80. [PubMed: 9525702]
3. Mezzano SA, Ruiz-Ortega M, Egido J. Angiotensin II and renal fibrosis. *Hypertension*. 2001; 38:635–8. [PubMed: 11566946]
4. Gaedeke J, Noble NA, Border WA. Angiotensin II and progressive renal insufficiency. *Curr Hypertens Rep*. 2002; 4:403–7. [PubMed: 12217260]
5. Wolf G, Mueller E, Stahl RA, Ziyadeh FN. Angiotensin II-induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-beta. *J Clin Invest*. 1993; 92:1366–72. [PubMed: 7690779]
6. Wolf G, Ziyadeh FN, Zahner G, Stahl RA. Angiotensin II-stimulated expression of transforming growth factor beta in renal proximal tubular cells: attenuation after stable transfection with the c-mas oncogene. *Kidney Int*. 1995; 48:1818–27. [PubMed: 8587241]
7. Zhou Y, Poczek MH, Berecek KH, Murphy-Ullrich JE. Thrombospondin 1 mediates angiotensin II induction of TGF-beta activation by cardiac and renal cells under both high and low glucose conditions. *Biochem Biophys Res Commun*. 2006; 339:633–41. [PubMed: 16310163]
8. Naito T, Masaki T, Nikolic-Paterson DJ, Tanji C, Yorioka N, Kohno N. Angiotensin II induces thrombospondin-1 production in human mesangial cells via p38 MAPK and JNK: a mechanism for activation of latent TGF-beta1. *Am J Physiol Renal Physiol*. 2004; 286:F278–87. [PubMed: 14583433]
9. Kagami S, Border WA, Miller DE, Noble NA. Angiotensin II stimulates extracellular matrix protein synthesis through induction of transforming growth factor-beta expression in rat glomerular mesangial cells. *J Clin Invest*. 1994; 93:2431–7. [PubMed: 8200978]
10. Ruiz-Ortega M, Lorenzo O, Egido J. Angiotensin III up-regulates genes involved in kidney damage in mesangial cells and renal interstitial fibroblasts. *Kidney Int Suppl*. 1998; 68:S41–5. [PubMed: 9839282]
11. Huang Y, Wongamorntham S, Kasting J, et al. Renin increases mesangial cell transforming growth factor-beta1 and matrix proteins through receptor-mediated, angiotensin II-independent mechanisms. *Kidney Int*. 2006; 69:105–13. [PubMed: 16374430]
12. Ma J, Weisberg A, Griffin JP, Vaughan DE, Fogo AB, Brown NJ. Plasminogen activator inhibitor-1 deficiency protects against aldosterone-induced glomerular injury. *Kidney Int*. 2006; 69:1064–72. [PubMed: 16528256]
13. Chen S, Hong SW, Iglesias-de la Cruz MC, Isono M, Casaretto A, Ziyadeh FN. The key role of the transforming growth factor-beta system in the pathogenesis of diabetic nephropathy. *Ren Fail*. 2001; 23:471–81. [PubMed: 11499562]
14. Gupta S, Clarkson MR, Duggan J, Brady HR. Connective tissue growth factor: potential role in glomerulosclerosis and tubulointerstitial fibrosis. *Kidney Int*. 2000; 58:1389–99. [PubMed: 11012874]
15. Murphy M, Godson C, Cannon S, et al. Suppression subtractive hybridization identifies high glucose levels as a stimulus for expression of connective tissue growth factor and other genes in human mesangial cells. *J Biol Chem*. 1999; 274:5830–4. [PubMed: 10026205]
16. Wahab NA, Yevdokimova N, Weston BS, et al. Role of connective tissue growth factor in the pathogenesis of diabetic nephropathy. *Biochem J*. 2001; 359:77–87. [PubMed: 11563971]
17. Sharma K, Ziyadeh FN. Renal hypertrophy is associated with upregulation of TGF-beta 1 gene expression in diabetic BB rat and NOD mouse. *Am J Physiol*. 1994; 267:F1094–01. [PubMed: 7810696]
18. Border WA, Noble NA. Evidence that TGF-beta should be a therapeutic target in diabetic nephropathy. *Kidney Int*. 1998; 54:1390–1. [PubMed: 9773681]
19. Riser BL, Denichilo M, Cortes P, et al. Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *J Am Soc Nephrol*. 2000; 11:25–38. [PubMed: 10616837]

20. Grotendorst GR, Okochi H, Hayashi N. A novel transforming growth factor beta response element controls the expression of the connective tissue growth factor gene. *Cell Growth Differ.* 1996; 7:469–80. [PubMed: 9052988]
21. Grotendorst GR. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev.* 1997; 8:171–9. [PubMed: 9462483]
22. Yokoi H, Sugawara A, Mukoyama M, et al. Role of connective tissue growth factor in profibrotic action of transforming growth factor-beta: a potential target for preventing renal fibrosis. *Am J Kidney Dis.* 2001; 38:S134–8. [PubMed: 11576939]
23. Nguyen G, Delarue F, Berrou J, Rondeau E, Sraer JD. Specific receptor binding of renin on human mesangial cells in culture increases plasminogen activator inhibitor-1 antigen. *Kidney Int.* 1996; 50:1897–903. [PubMed: 8943472]
24. Nguyen G, Delarue F, Burckle C, Bouzahir L, Giller T, Sraer JD. Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. *J Clin Invest.* 2002; 109:1417–27. [PubMed: 12045255]
25. Siragy HM, Huang J. Renal (pro)renin receptor upregulation in diabetic rats through enhanced angiotensin AT1 receptor and NADPH oxidase activity. *Exp Physiol.* 2008; 93:709–14. [PubMed: 18192338]
26. Huang J, Siragy HM. Glucose promotes the production of interleukine-1beta and cyclooxygenase-2 in mesangial cells via enhanced (Pro)renin receptor expression. *Endocrinology.* 2009; 150:5557–65. [PubMed: 19861503]
27. Matavelli LC, Huang J, Siragy HM. (Pro)renin receptor contributes to diabetic nephropathy by enhancing renal inflammation. *Clin Exp Pharmacol Physiol.* 2010; 37:277–82. [PubMed: 19769609]
28. Huang J, Siragy HM. Regulation of (pro)renin receptor expression by glucose-induced mitogen-activated protein kinase, nuclear factor-kappaB, and activator protein-1 signaling pathways. *Endocrinology.* 2010; 151:3317–25. [PubMed: 20444941]
29. Ichihara A, Hayashi M, Kaneshiro Y, et al. Inhibition of diabetic nephropathy by a decoy peptide corresponding to the “handle” region for nonproteolytic activation of prorenin. *J Clin Invest.* 2004; 114:1128–35. [PubMed: 15489960]
30. Kaneshiro Y, Ichihara A, Sakoda M, et al. Slowly progressive, angiotensin II-independent glomerulosclerosis in human (pro)renin receptor-transgenic rats. *J Am Soc Nephrol.* 2007; 18:1789–95. [PubMed: 17494887]
31. Yagi S, Morita T, Katayama S. Combined treatment with an AT1 receptor blocker and angiotensin converting enzyme inhibitor has an additive effect on inhibiting neointima formation via improvement of nitric oxide production and suppression of oxidative stress. *Hypertens Res.* 2004; 27:129–35. [PubMed: 15005276]
32. Jin XH, McGrath HE, Gildea JJ, Siragy HM, Felder RA, Carey RM. Renal interstitial guanosine cyclic 3', 5'-monophosphate mediates pressure-natriuresis via protein kinase G. *Hypertension.* 2004; 43:1133–9. [PubMed: 15007031]
33. Takahashi H, Ichihara A, Kaneshiro Y, et al. Regression of nephropathy developed in diabetes by (Pro)renin receptor blockade. *J Am Soc Nephrol.* 2007; 18:2054–61. [PubMed: 17538189]
34. Ichihara A, Kaneshiro Y, Takemitsu T, et al. Contribution of nonproteolytically activated prorenin in glomeruli to hypertensive renal damage. *J Am Soc Nephrol.* 2006; 17:2495–503. [PubMed: 16885412]
35. Ichihara A, Kaneshiro Y, Takemitsu T, et al. Nonproteolytic activation of prorenin contributes to development of cardiac fibrosis in genetic hypertension. *Hypertension.* 2006; 47:894–900. [PubMed: 16585419]
36. Wilkinson-Berka JL, Heine R, Tan G, et al. RILLKKMPSV influences the vasculature, neurons and glia, and (pro)renin receptor expression in the retina. *Hypertension.* 2010; 55:1454–60. [PubMed: 20368504]
37. Feldt S, Maschke U, Dechend R, Luft FC, Muller DN. The putative (pro)renin receptor blocker HRP fails to prevent (pro)renin signaling. *J Am Soc Nephrol.* 2008; 19:743–8. [PubMed: 18235083]

38. Muller DN, Klanke B, Feldt S, et al. (Pro)renin receptor peptide inhibitor “handle-region” peptide does not affect hypertensive nephrosclerosis in Goldblatt rats. *Hypertension*. 2008; 51:676–81. [PubMed: 18212268]
39. Advani A, Kelly DJ, Cox AJ, et al. The (Pro)renin receptor: site-specific and functional linkage to the vacuolar H⁺-ATPase in the kidney. *Hypertension*. 2009; 54(2):261–9. [PubMed: 19546380]
40. Kinouchi K, Ichihara A, Sano M, et al. The (pro)renin receptor/ATP6AP2 is essential for vacuolar H⁺-ATPase assembly in murine cardiomyocytes. *Circ Res*. 2010; 107:30–4. [PubMed: 20570919]
41. Cruciat CM, Ohkawara B, Acebron SP, et al. Requirement of prorenin receptor and vacuolar H⁺-ATPase-mediated acidification for Wnt signaling. *Science*. 2010; 327:459–63. [PubMed: 20093472]
42. He W, Dai C, Li Y, Zeng G, Monga SP, Liu Y. Wnt/beta-catenin signaling promotes renal interstitial fibrosis. *J Am Soc Nephrol*. 2009; 20:765–76. [PubMed: 19297557]
43. Dai C, Stolz DB, Kiss LP, Monga SP, Holzman LB, Liu Y. Wnt/beta-catenin signaling promotes podocyte dysfunction and albuminuria. *J Am Soc Nephrol*. 2009; 20:1997–2008. [PubMed: 19628668]

Figure 1.

Effects of the (pro)renin receptor (PRR) blocker, HRP and the angiotensin AT1 receptor blocker, Valsartan on the expression of TGFβ1 (A and B) and CTGF (C and D) in kidneys of diabetic rats. Control: Normoglycemic rats; DM: Diabetic rats; Val: Valsartan. Asterisk * and ** represent p<0.05 and p<0.01 (number of nominal multiple comparisons k = 4).

Figure 2.

Effects of the (pro)renin receptor (PRR) blocker, HRP on the expression of PRR (A and B), TGFβ1 (C and D) and CTGF (E and F) in cultured RMCs. Control: 30 mM L-glucose; Glucose or Glu-: 30 mM D-glucose. Asterisk * represents p<0.01 (number of nominal multiple comparisons k = 3).

Figure 3.

Immunoprecipitation (IP) analysis of the effects of high glucose on (pro)renin receptor (PRR) phosphorylation at serine residues in rat mesangial cells. Control: 30 mM L-glucose; Glucose: 30 mM D-glucose. IP antibody: mouse-anti-phosphoserine; Western blotting antibody: Rabbit-anti-PRR.

Figure 4.

Effects of HRP, *Valsartan* and PRR siRNA on (pro)renin receptor (PRR) phosphorylation at serine residues in rat mesangial cells. (A) Effect of high glucose, HRP and *Valsartan* for 1 hr treatment in RMCs after 12hrs serum starvation. HRP and *Valsartan* pretreatment was conducted 30 min prior to terminating serum free culture. Cells were exposed to L- or D-glucose, HRP and *Valsartan* for 1 hr; (B) Effect of long term treatment with high glucose, HRP or *Valsartan*. RMCs were treated with L- or D-glucose for 14 days. Serum starvation combining L- or D-glucose alone or combined with HRP or *Valsartan* was conducted 12 hrs prior to the end of the experiment; (C) Effect of PRR siRNA. Control: 30 mM L-glucose; Glucose: 30 mM D-glucose. IP antibody: mouse-anti-phosphoserine; Western blotting antibody: Rabbit-anti-PRR. Final concentration of HRP, *Valsartan* and PRR siRNA are 1 μ M, 1 μ M and 100nM respectively.

Figure 5.

Effect of (pro)renin receptor (PRR) siRNA and angiotensin AT1 receptor blockade with valsartan on the expression of TGFβ1 (A and B) and CTGF (C and D) in rat mesangial cells. Glucose (-): 30mM L-glucose; Glucose (+): 30 mM D-glucose; Final concentration for PRR siRNA and Valsartan were 100 nM and 1μM respectively. Asterisk * and ** represent $p < 0.05$ and $p < 0.01$ (number of nominal multiple comparisons $k = 4$).

Figure 6. Effect of TGF β receptor blockade with SD-208 on CTGF expression in rat mesangial cells. Final concentration of TGF receptor inhibitor SD-208 was 500 nM. Control: 30 mM L-glucose; Glucose: 30 mM D-glucose. Asterisk * represents $p < 0.05$ (number of nominal multiple comparisons $k = 3$).