Prorenin Receptor Blockade Inhibits Development of Glomerulosclerosis in Diabetic Angiotensin II Type 1a Receptor–Deficient Mice

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Blockade of the renin-angiotensin system slows the progression of diabetic nephropathy but fails to abolish the development of end-stage nephropathy of diabetes. The prorenin-to-active renin ratio significantly increases in diabetes, and prorenin binding to its receptor in diabetic animal kidney induces the nephropathy without its conventional proteolytic activation, suggesting that angiotensin II (AngII) may not be the decisive factor causing the nephropathy. For identification of an AngII-independent mechanism, diabetes was induced in wild-type mice and AngII type 1a receptor gene–deficient mice by streptozotocin treatment, and their development and progression of diabetic nephropathy were assessed. In addition, prolonged inhibition of angiotensin-converting enzyme and prolonged prorenin receptor blockade were compared for their efficacy in preventing the nephropathy that occurred in diabetic AngII type 1a receptor gene–deficient mice. Only the prorenin receptor blockade with a short peptide of prorenin practically abolished the increased mitogen-activated protein kinase (MAPK) activation and nephropathy despite unaltered increase in AngII in diabetic kidney. These results indicate that the MAPK activation signal leads to the diabetic nephropathy but not other renin-angiotensin system–activated mechanisms in the glomeruli. It is not only AngII but also intraglomerular activation of MAPK by the receptor-associated prorenin that plays a pivotal role in diabetic nephropathy.

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Diabetic nephropathy is the leading cause of ESRD and accounts for 30 to 40% of patients who are on renal replacement therapy in industrial nations. Clinical studies have reported that blockade of the renin-angiotensin system (RAS) slows the progression of diabetic nephropathy (1,2), and prescriptions for RAS inhibitors worldwide have increased steadily in number and variety. Nevertheless, the number of patients who have diabetes with ESRD increases unabated. The inefficiency of RAS inhibitors suggests that possible RAS-independent mechanisms of diabetic nephropathy and their blockade may achieve complete inhibition of the development and progression of the disease.

We recently found that the binding of prorenin to an intrinsic prorenin-binding receptor plays a pivotal role in the development of diabetic nephropathy (3) by a mechanism that involves the receptor-associated prorenin system (RAPS), and inhibition of the RAPS completely prevented the development of diabetic nephropathy (3). In addition, Nguyen *et al.* and we (3,4) have provided *in vitro* evidence suggesting that the RAPS may consist of two major pathways: (*1*) Conversion to an active form of prorenin by a conformational change in receptor-bound prorenin instead of proteolytic cleavage of the prosegment of prorenin and (*2*) stimulation of tyrosine phosphorylation as a result of activation of the prorenin receptor by its ligand (prorenin), leading to activation of the extracellular signal–related protein kinases (Erk 1 and 2) that presumably are induced by prorenin's binding to the receptor (4,5). Therefore, a RAPS mechanism may contribute to both a RAS-dependent and a RAS-independent mechanism of diabetic nephropathy.

We hypothesized that diabetic nephropathy develops not only through the RAS-dependent mechanism but also mainly through a RAS-independent mechanism involving the RAPS for a long-term renal complication. To test this hypothesis, we induced diabetes by streptozotocin in angiotensin II (AngII) type 1a receptor (AT_{1a}) gene–deficient (ATKO) mice and compared RAS inhibition and a "handle region" decoy peptide (HRP), a blocker of RAPS, for their efficacy in preventing diabetic nephropathy with proteinuria (3) and phosphorylation of all three members of the mitogen-activated protein kinase

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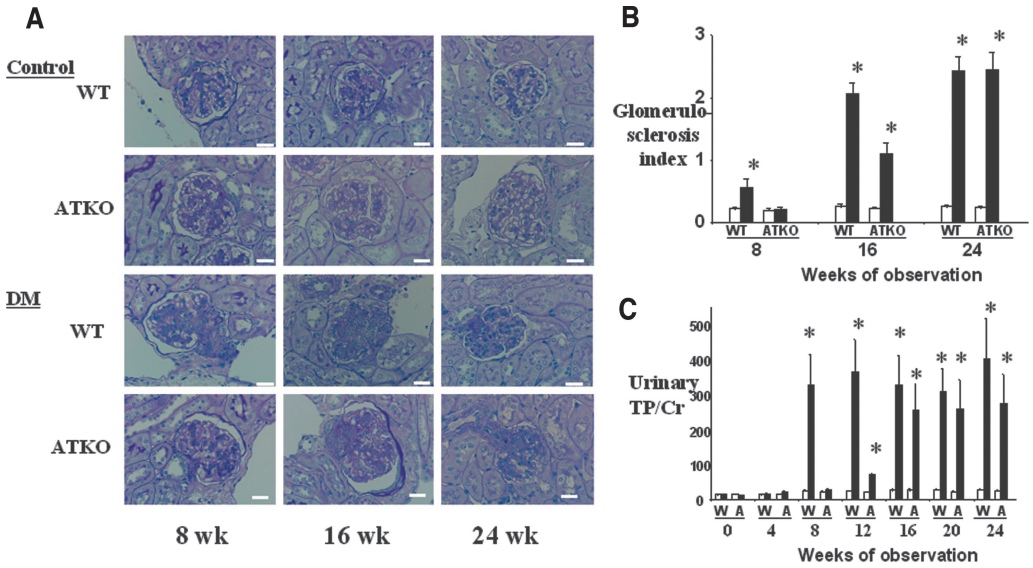

Figure 1. Renal morphology and urinary protein excretion in wild type (WT or W) mice (□; $n = 8$), diabetic WT mice (■; $n = 8$), angiotensin II (AngII) type 1a receptor gene–deficient (ATKO or A) mice (□; $n = 8$), and diabetic ATKO mice (■; $n = 8$) during the 24-wk observation period. (A) The photomicrographs of the periodic acid-Schiff (PAS)-stained kidney sections show the development and progression of diabetic glomerulosclerosis at 8 to 24 wk after streptozotocin and in both the WT and ATKO mice. Bars = 25 μm. (B) The graph shows the onset of increase in glomerulosclerosis index at 8 wk in the diabetic WT mice and significantly above normal value of the index at 16 wk and later in the diabetic ATKO mice. *$P < 0.05$, diabetic mice *versus* control mice. (C) The graph shows a progressive increase in urinary protein excretion in both the diabetic WT mice and diabetic ATKO mice.

(MAPK) family, including Erk 1 and 2, p38, and c-Jun NH2-terminal kinase (Jnk) in the kidneys. We present evidence that a novel RAS-independent mechanism exists.

Materials and Methods

Animals

We maintained male ATKO mice (6) and wild-type C57BL/6J (WT) mice at 23°C in a temperature-controlled room with a 12:12-h light/dark cycle. The mice had free access to water and standard rat chow (0.4% NaCl; CE-2; Nihon Clea, Tokyo, Japan). The Keio University Animal Care and Use Committee approved all experimental protocols. At 4 wk of age, we divided the mice into four groups: WT group, diabetic WT group, ATKO group, and diabetic ATKO group. Mice were made diabetic by intraperitoneal injection of 100 mg/kg streptozotocin (Wako, Osaka, Japan) in 10 mM citrate buffer twice, on day 0 and day 2, and control mice received 10 mM citrate buffer alone. The diabetic mice were divided further into four groups: An untreated group and groups that were treated with an angiotensin-converting enzyme (ACE) inhibitor imidapril alone, HRP alone, or both. Every 28 d, we replaced the subcutaneously implanted osmotic minipump (model

Table 1. Changes in systolic blood pressure[a]

	Weeks after Treatment		
	0	12	24
WT	131 ± 1	132 ± 1	137 ± 4
Diabetic WT	127 ± 2	133 ± 1	139 ± 3
Diabetic WT with HRP	128 ± 1	131 ± 1	138 ± 1
Diabetic WT with ACEi	127 ± 2	127 ± 2[b]	129 ± 2[b]
Diabetic WT with HRP + ACEi	127 ± 2	126 ± 2[b]	127 ± 2[b]
ATKO	123 ± 3[c]	126 ± 3[c]	124 ± 4[c]
Diabetic ATKO	122 ± 2[c]	125 ± 2[c]	127 ± 2[c]
Diabetic ATKO with HRP	122 ± 2[c]	125 ± 2[c]	127 ± 2[c]
Diabetic ATKO with ACEi	123 ± 2	126 ± 3	127 ± 3
Diabetic ATKO with HRP + ACEi	123 ± 2	125 ± 3	128 ± 3

[a]Data are mean ± SEM. ACEi, angiotensin-converting enzyme inhibitor; ATKO, angiotensin II (Ang II) type 1a receptor gene–deficient; HRP, "handle region" peptide; WT, wild-type.
[b]$P < 0.05$ *versus* untreated diabetes.
[c]$P < 0.05$ for ATKO mice *versus* WT mice.

2004 for 28-d use; Alzet, Cupertino, CA) that contained saline or HRP (0.1 mg/kg), and we decapitated six mice at 8, 16, and 24 wk of observation to obtain the blood and kidneys of each animal. In our preliminary study, an osmotic minipump with NH_2-MTRLSAE-COOH (positions 30 to 36 of prorenin prosegment; 0.1 mg/kg; $n = 4$) also was implanted in diabetic mice as a placebo control of HRP. The placebo control peptide, however, did not inhibit the development of proteinuria or glomerulosclerosis in diabetic rats. The 4-wk-old diabetic ATKO mice were treated with the ACE inhibitor imidapril hydrochloride at 0.015% in their drinking water for 24 wk. The diabetic mice drank >5 ml of water every day and thus ingested at least 15 mg/kg per d imidapril. We measured the systolic arterial BP of the mice by tail-cuff plethysmography at 0, 12, and 24 wk after the first day of streptozotocin injection, collected 24-h urine in a metabolic cage, and determined urinary protein excretion and creatinine with a Micro TP test kit (Wako, Osaka, Japan) and a Creatinine HA test kit (Wako), respectively. Blood was obtained from the tail vein, and glucose was analyzed with a Glucose C test kit (Wako).

Morphologic and Immunohistochemical Evaluation

Part of the kidney that was removed from each animal was fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4), and paraffin sections were stained by the periodic acid-Schiff method. The total area of sclerosis within each glomerular tuft was determined by the semiquantitative scoring system proposed by El Nahas *et al.* (7). A glomerulosclerosis index was derived for each animal by examination of 100 glomeruli at ×400 magnification. The severity of glomerulosclerosis was expressed on an arbitrary scale from 0 to 4: Grade 0, normal glomeruli; grade 1, presence of mesangial expansion/thickening of the basement membrane; grade 2, mild/moderate segmental hyalinosis/sclerosis involving <50% of the glomerular tuft; grade 3, diffuse glomerular hyalinosis/sclerosis involving >50% of the tuft; and grade 4, diffuse glomerulosclerosis with total tuft obliteration and collapse. The resulting index in each animal was expressed as a mean of all scores obtained.

For immunohistochemical staining, deparaffinized sections were pretreated with proteinase K. The sections then were boiled in citrate buffer with microwaves to unmask antigenic sites, and endogenous biotin was blocked with a Biotin Blocking System (X0590; DAKO Corp., Carpinteria, CA). The sections then were immersed in 0.3% H_2O_2 in methanol to inhibit endogenous peroxidase and precoated with 1% nonfat milk in PBS to block nonspecific binding. For immunohistochemical staining of the exposed active center of renin, goat polyclonal

Table 2. Changes in components of the circulating RAS

	Weeks after Treatment		
	8	16	24
Plasma renin activity (ng/ml per h)			
WT	1.92 ± 0.21	2.02 ± 0.32	2.08 ± 0.30
Diabetic WT	1.20 ± 0.16[b]	1.33 ± 0.16[b]	1.27 ± 0.16[b]
Diabetic WT treated with HRP	1.32 ± 0.17[b]	1.43 ± 0.18[b]	1.35 ± 0.15[b]
ATKO	3.37 ± 0.85[c]	3.53 ± 0.64[c]	2.57 ± 0.33[c]
Diabetic ATKO	1.85 ± 0.11[b,c]	2.00 ± 0.11[b,c]	1.62 ± 0.29[b]
Diabetic ATKO treated with HRP	1.80 ± 0.16[b,c]	1.82 ± 0.17[b,c]	1.52 ± 0.25[b]
Plasma prorenin concentration (ng/ml per h)			
WT	3.50 ± 0.59	4.03 ± 0.43	4.17 ± 0.53
Diabetic WT	5.63 ± 0.98[b]	5.24 ± 0.58[b]	5.83 ± 0.50[b]
Diabetic WT treated with HRP	6.40 ± 0.72[b]	5.45 ± 0.70[b]	6.01 ± 0.43[b]
ATKO	5.07 ± 0.71[c]	6.07 ± 0.58	5.13 ± 0.67
Diabetic ATKO	7.57 ± 0.95[b,c]	8.05 ± 0.53[b,c]	6.78 ± 0.41[b]
Diabetic ATKO treated with HRP	7.37 ± 0.76[b]	7.72 ± 0.77[b]	6.53 ± 0.48[b]
Plasm angiotensin I level (fmol/ml)			
WT	121 ± 16	80 ± 16	82 ± 18
Diabetic WT	126 ± 8	73 ± 15	72 ± 11
Diabetic WT treated with HRP	129 ± 9	88 ± 14	87 ± 15
ATKO	278 ± 35[c]	138 ± 18[c]	148 ± 32[c]
Diabetic ATKO	253 ± 36[c]	138 ± 18[c]	148 ± 32[c]
Diabetic ATKO treated with HRP	263 ± 47[c]	145 ± 15[c]	129 ± 19[c]
Plasma angiotensin II level (fmol/ml)			
WT	69 ± 9	79 ± 9	131 ± 19
Diabetic WT	67 ± 10	63 ± 9	105 ± 16
Diabetic WT treated with HRP	63 ± 8	71 ± 9	124 ± 14
ATKO	417 ± 113[c]	252 ± 19[c]	319 ± 32[c]
Diabetic ATKO	398 ± 86[c]	254 ± 19[c]	270 ± 21[c]
Diabetic ATKO treated with HRP	433 ± 132[c]	256 ± 23[c]	266 ± 33[c]

[a]Data are mean ± SEM. RAS, renin-angiotensin system.
[b]$P < 0.05$ for diabetes *versus* control.
[c]$P < 0.05$ for ATKO mice *versus* WT mice.

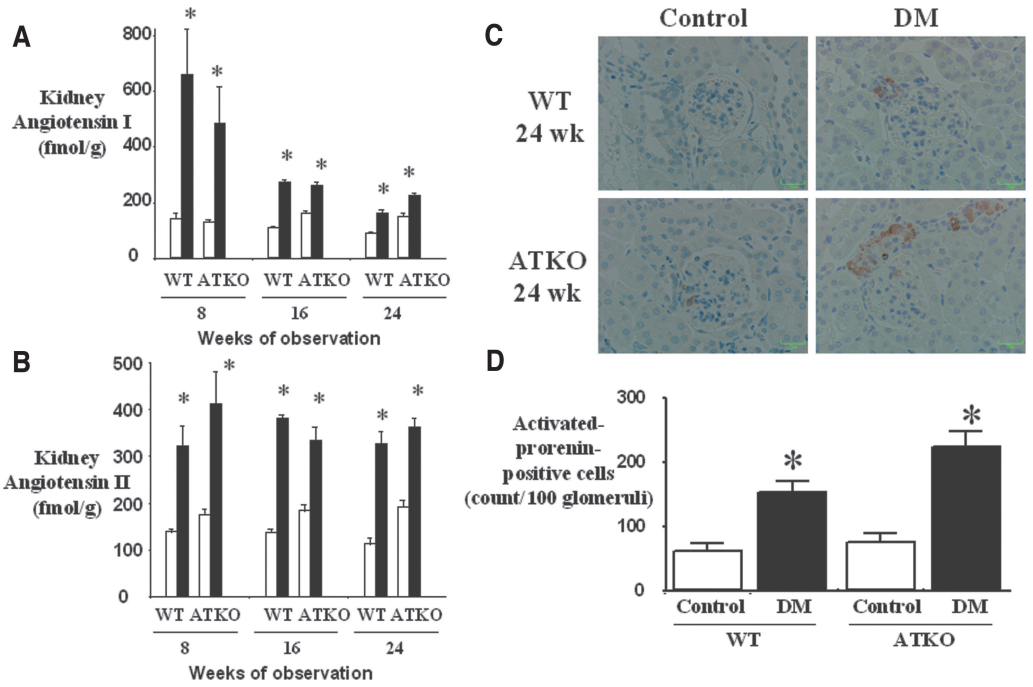

Figure 2. Changes in components of the kidney renin-Ang system (RAS) in WT mice (□; $n = 8$), diabetic WT mice (■; $n = 8$), ATKO mice (■; $n = 8$), and diabetic ATKO mice (■; $n = 8$) during the 24-wk observation period. (A) The graph shows an increased kidney AngI level in both the diabetic WT mice and diabetic ATKO mice. (B) The graph shows an increased kidney AngII level in both the diabetic WT mice and diabetic ATKO mice. (C) Immunohistochemistry of the active center of mouse prorenin was detected with antibodies to the active center of rat renin. The photomicrographs show increased immunoreactivity to the active center of prorenin in the juxtaglomerular area of the kidneys from diabetic WT and diabetic ATKO mice. Bars = 25 μm. (D) Quantitative analysis of nonproteolytically activated prorenin in the juxtaglomerular area. The graph shows an increase in nonproteolytically activated prorenin in both the diabetic WT and diabetic ATKO mice. *$P < 0.05$, diabetic mice *versus* control mice.

antibody to rat renin that cross-reacts with nonproteolytically activated mouse prorenin but not with natural mouse prorenin (1:10,000) (8–10) was used. The antibody was applied to the sections as the primary antibody, and the sections were incubated with a biotin-conjugated anti-goat IgG as the secondary antibody. The antibody reaction was visualized with a Vectastain ABC Standard Kit (Vector Laboratories) and an AEC Standard Kit (DAKO) according to the manufacturers' instructions. We quantitatively determined the immunoreactivity of nonproteolytically activated prorenin by counting the number of juxtaglomerular cells in which the signal intensity of the reaction products was visible. The final overall score was calculated as the mean of the value in 100 glomeruli per group of mice. For immunohistochemical stainings of phosphor-Erk, phospho-p38, and phospho-Jnk, the monoclonal mouse anti–phospho-44/42 MAPK (E10) antibody that cross-reacts with rat phosphorylated Erk 1 and 2 (1:800; Cell Signaling Technology, Beverly, MA), the monoclonal mouse anti–phospho-p38 (D8) antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA), and the monoclonal mouse anti–phospho-Jnk (G7) antibody (1:400; Santa Cruz Biotechnology), respectively, were applied to the sections as the primary antibodies. The immunoreactive phospho-MAPK–positive area in each glomerulus was determined at ×200 magnification with a Mac SCOPE (version 2.5; Mitani Corp., Fukui, Japan) and expressed as a percentage of the whole cross-sectional area of the glomerulus.

Measurement of Renin and Angiotensin Peptides

Immediately after decapitation, a 3-ml blood specimen was collected into a tube that contained 30 μl of EDTA (500 mM), 15 μl of enalaprilat (1 mM), and 30 μl of o-phenanthrolene (24.8 mg/ml) and pepstatin (0.2 mM), and a plasma sample was obtained by centrifugation. The plasma renin activity was determined with a RIA coated-bead kit (Dinabott Radioisotope Institute, Tokyo, Japan). The plasma prorenin level was calculated by subtracting plasma renin activity from total plasma renin activity (inactive + active), as described previously (11). For measurement of the total kidney renin content, part of cortex of the removed kidney was weighed; placed in 5 ml of buffer that contained 2.6 mM EDTA, 1.6 mM dimercaprol, 3.4 mM 8-hydroxyquinoline sulfate, 0.2 mM PMSF, and 5 mM ammonium acetate; homogenized in a chilled glass homogenizer; and centrifuged. The renin activity of the supernatant was determined as described previously (12). For determination of kidney AngI and AngII content, half of the removed kidney was weighed, placed in ice-cold methanol (10% wt/vol), homogenized with a chilled glass homogenizer, and centrifuged. The supernatant then was dried and reconstituted in 4 ml of 50 mM sodium phosphate buffer that contained 1% albumin. Plasma and reconstituted kidney samples were extracted with a Bond-Elut column (Analytichem, Harbor City, CA), and the eluents were evaporated to dryness and reconstituted in angiotensin peptide assay diluent. AngI and AngII content was determined quantitatively by RIA with rabbit anti-AngI antiserum and rabbit anti-AngII antiserum (Arnel, New York, NY) as previously reported (13).

Real-Time Quantitative Reverse Transcription–PCR Analysis

We extracted total RNA from part of cortex of the kidney that was removed from each animal with an RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan) and performed a real-time quantitative reverse transcrip-

Table 3. Changes in components of the kidney RAS[a]

	Weeks after Treatment		
	8	16	24
Renal renin content (μg/h per g)			
WT	36.8 ± 4.0	29.6 ± 4.1	17.4 ± 1.7
Diabetic WT	20.4 ± 0.7[b]	17.9 ± 1.0[b]	16.1 ± 1.6
Diabetic WT treated with HRP	19.5 ± 2.4[b]	18.0 ± 2.4[b]	18.8 ± 1.9
ATKO	68.7 ± 7.6[c]	53.5 ± 6.8[c]	27.7 ± 1.4[c]
Diabetic ATKO	28.0 ± 1.7[b,c]	24.6 ± 1.2[b,c]	18.9 ± 1.1[b]
Diabetic ATKO treated with HRP	26.8 ± 2.0[b,c]	23.1 ± 2.4[b,c]	19.5 ± 2.2[b]
Renal renin mRNA (ratio to GAPDH mRNA)			
WT	1.42 ± 0.19	0.33 ± 0.06	0.06 ± 0.01
Diabetic WT	0.23 ± 0.07[b]	0.07 ± 0.01[b]	0.05 ± 0.01
Diabetic WT treated with HRP	0.15 ± 0.01[b]	0.09 ± 0.01[b]	0.06 ± 0.01
ATKO	2.25 ± 0.32[c]	1.34 ± 0.22[c]	0.82 ± 0.09[c]
Diabetic ATKO	0.90 ± 0.08[b,c]	0.28 ± 0.11[b,c]	0.20 ± 0.04[b,c]
Diabetic ATKO treated with HRP	1.10 ± 0.14[b,c]	0.25 ± 0.10[b,c]	0.19 ± 0.10[b,c]
Renal angiotensinogen mRNA (ratio to GAPDH mRNA)			
WT	3.82 ± 0.28	2.98 ± 0.15	2.44 ± 0.27
Diabetic WT	3.79 ± 0.37	3.26 ± 0.46	2.66 ± 0.48
Diabetic WT treated with HRP	4.17 ± 0.87	3.24 ± 0.43	2.71 ± 0.47
ATKO	4.19 ± 0.37	3.04 ± 0.31	2.53 ± 0.15
Diabetic ATKO	4.33 ± 0.36	3.28 ± 0.45	2.63 ± 0.22
Diabetic ATKO treated with HRP	4.32 ± 0.38	3.12 ± 0.37	2.45 ± 0.17
Renal angiotensin-converting enzyme mRNA (ratio to GAPDH mRNA)			
WT	1.36 ± 0.06	1.47 ± 0.21	1.00 ± 0.10
Diabetic WT	1.36 ± 0.06	1.40 ± 0.22	0.99 ± 0.04
Diabetic WT treated with HRP	1.22 ± 0.10	1.38 ± 0.15	0.97 ± 0.10
ATKO	1.85 ± 0.06[c]	1.11 ± 0.11	0.77 ± 0.06
Diabetic ATKO	1.70 ± 0.22[c]	1.13 ± 0.12	0.78 ± 0.06
Diabetic ATKO treated with HRP	1.64 ± 0.17[c]	1.18 ± 0.06	0.81 ± 0.10
Renal cathepsin B mRNA (ratio to GAPDH mRNA)			
WT	1.33 ± 0.07	1.42 ± 0.05	1.37 ± 0.05
Diabetic WT	1.28 ± 0.07	0.89 ± 0.04[b]	0.89 ± 0.06[b]
Diabetic WT treated with HRP	1.25 ± 0.04	0.89 ± 0.04[b]	0.92 ± 0.05[b]
ATKO	1.47 ± 0.06	1.38 ± 0.03	1.36 ± 0.03
Diabetic ATKO	1.36 ± 0.06	0.92 ± 0.03[b]	0.91 ± 0.03[b]
Diabetic ATKO treated with HRP	1.40 ± 0.05	0.93 ± 0.05[b]	0.88 ± 0.03[b]

[a]Data are mean ± SEM. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[b]$P < 0.05$ for diabetes *versus* control.
[c]$P < 0.05$ for ATKO mice *versus* WT mice.

tion–PCR with the TaqMan One-Step RT-PCR Master Mix Reagents Kit with an ABI Prism 7700 HT Detection System (Applied Biosystems, Foster City, CA) and probes and primers for the mouse genes encoding *Ren1*-renin (forward, 5′-CCGAGCTGCCCCTGATC-3′; reverse, 5′-GGGAAAGCCCATGCCTAGAA-3′; probe, 5′-CTTTCATGCTGGCCAAGTTTGACGG-3′), ACE (forward, 5′-CCTTGCCGTTGTAGAAGTCCC-3′; reverse, 5′-AAGCCCACCGATGGAAGG-3′; probe, 5′-AGGTGGTGTGCCATCCCTCAGCC-3′), angiotensinogen (forward, 5′-TTGTCTAGGTTGGCGCTGAAG-3′; reverse, 5′-AGATGCAGAAGATGGCCT-3′; probe, 5′-ACACAGAAGCAAATGCACAGATCGGAGA-3′), cathepsin B (forward, 5′-ACTTTGGGTACACTTCCTACAGCG-3′; reverse, 5′-TCATGCTTGTATACTCCTGATTTGTAAG-3′; probe, 5′-CAGTGGAGGGTGCCTTCACTGTGTTTTC-3′), and glyceraldehyde-3-phosphate dehydrogenase (forward, 5′-TTCACCACCATGGAGAAGGC-3′; reverse, 5′-GGCATGGACTGTGGTCATGA-3′; probe, 5′-TGCATCCTGCACCAACTGCTTAG-3′), as described previously (14,15).

Preparation of Mouse Ren1-Prorenin "Handle" Peptide

To cover the "handle region" (positions 11 to 15 [16]), we designed an octapeptide, NH2-IPLKKMPS-COOH (positions 11 to 18), as an HRP of mouse *Ren1*-prorenin and purified it by HPLC on a C-18 reverse-phase column. The mass of the product was 913.4, similar to the theoretical mass value of 913.2. We constructed COS-7 cells that express mouse prorenin receptor (accession no. AB192471 in DDBJ) and showed the binding of mouse recombinant Ren-1-prorenin and its activation to a

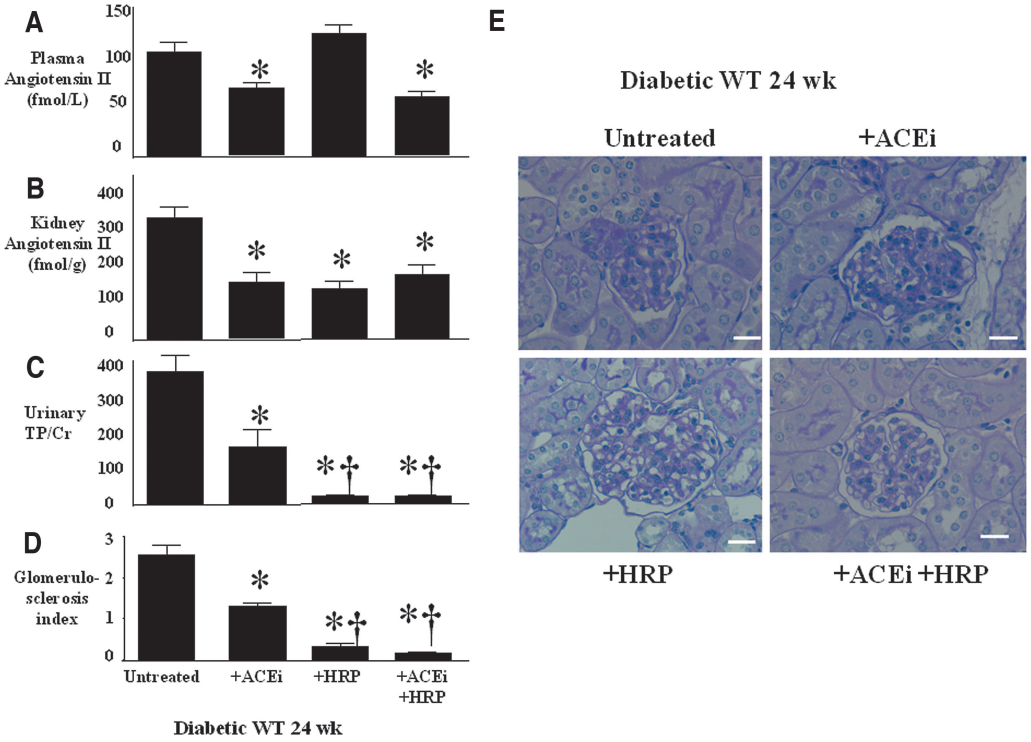

Figure 3. Plasma and kidney AngII levels, urinary protein excretion, and renal morphology of untreated diabetic WT mice ($n = 6$) and diabetic WT mice treated with the angiotensin-converting enzyme (ACE) inhibitor (ACEi) imidapril alone ($n = 6$), "handle region" decoy peptide alone (HRP; $n = 6$), and both (ACEi+HRP; $n = 6$) at 24 wk of observation. (A) The ACEi significantly decreased the plasma AngII level. (B) Both HRP and ACEi significantly decreased the kidney AngII level. (C) Both HRP and the ACEi significantly decreased urinary protein excretion, but the decrease with HRP was greater than that with ACEi. (D) Both HRP and the ACEi significantly decreased the glomerulosclerosis index, but the decrease with HRP was greater than that with ACEi. (E) The photomicrographs of the PAS-stained kidney sections show the diabetic glomerulosclerosis in the untreated diabetic WT mice at 24 wk of observation. Clear glomerulosclerosis developed in the diabetic WT mice, and its severity was attenuated by 24 wk of treatment with ACEi. However, HRP significantly inhibited the development of diabetic glomerulosclerosis in the absence or presence of ACEi. Bars = 25 μm. *$P < 0.05$ *versus* untreated diabetic ATKO mice;. $^{\dagger}P < 0.05$ *versus* diabetic WT mice that were treated with ACEi alone.

renin activity level of 30 ± 3% ($n = 4$) of the maximal level attainable by trypsin activation (2800 ng/ml per h AngI). The activation was practically abolished by 1 μM mouse HRP (11P to 18P) that was used as a decoy to block the binding of the HRP region to the receptor. However, another prosegment peptide (20P to 30P and 30P to 38P) with an amino acid sequence outside the handle region failed to inhibit the activation, indicating that the inhibitory action of HRP on prorenin activation is specific to the handle region. These results indicate that mouse HRP inhibits specific binding of muse *Ren1*-prorenin to the receptor protein and provide a cell model that supports the contention that mouse HRP significantly inhibits activation of mouse *Ren1*-prorenin by competing out binding of the prorenin receptor to mouse "handle region" of mouse prorenin.

Statistical Analyses

Within-group statistical comparisons were made by one-way ANOVA for repeated measures followed by the Newman-Keuls *post hoc* test. Differences between two groups were evaluated by two-way ANOVA for repeated measures combined with the Newman-Keuls *post hoc* test. $P < 0.05$ was considered significant. Data are reported as means ± SEM.

Results

Development of Diabetic Nephropathy in ATKO Mice

We detected in diabetic WT mice a few sclerotic glomeruli in the kidney at 8 wk, which were exacerbated at 16 and 24 wk (Figure 1, A and B). AT_1 deficiency delayed the onset and progression of glomerular sclerosis but did not prevent it at 24 wk in terms of glomerular sclerosis index or visual inspection of periodic acid-Schiff–stained photomicrograph. Like renal morphology, urine protein levels reached the plateau level 325 ± 90 mg/creatinine (cre) in 8 wk in diabetic WT mice (Figure 1C), whereas in diabetic ATKO mice, the onset of proteinuria was considerably delayed, reaching the plateau value (252 ± 76 ng/cre) in 16 wk. The plateau levels remained unchanged thereafter in both groups. These results showed that type 1a AngII receptor deletion merely delayed the onset of glomerulosclerosis and proteinuria but failed to prevent full development of glomerular damage over a long term.

Blood glucose in diabetic animals with or without HRP infusion rose to the plateau levels 450 ± 22 to approximately 469 ± 26 mg/dl in 4 wk and remained at this level through the

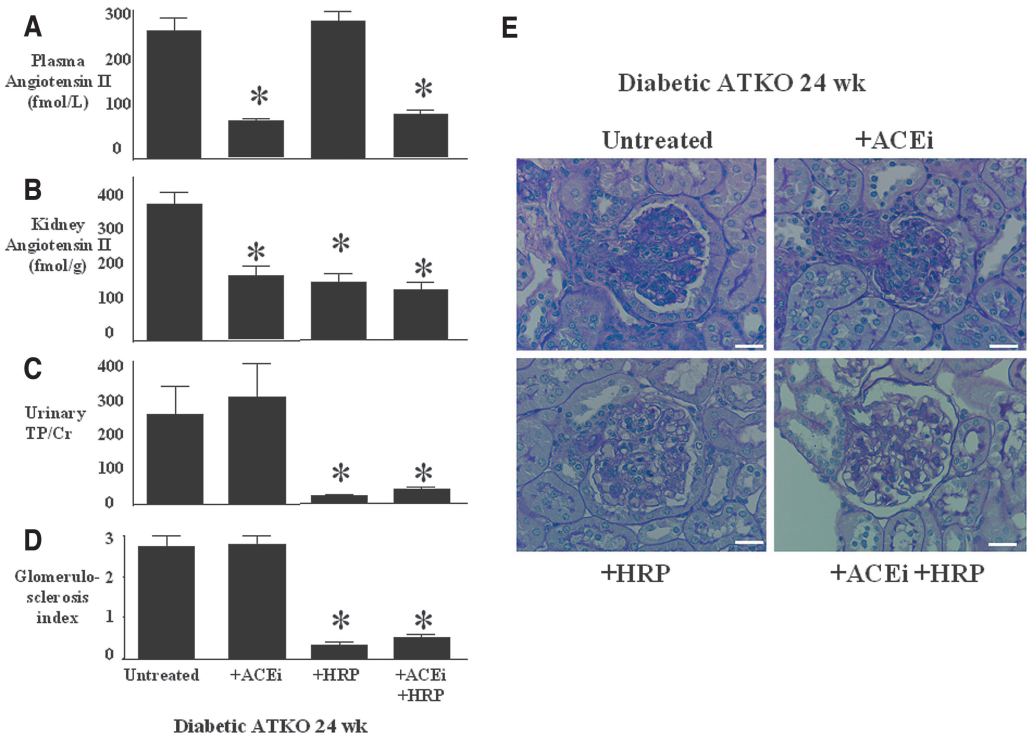

Figure 4. Plasma and kidney AngII levels, urinary protein excretion, and renal morphology of untreated diabetic ATKO mice ($n = 6$) and diabetic ATKO mice treated with the ACEi imidapril alone ($n = 8$), "handle region" decoy peptide alone (HRP; $n = 6$), and both (ACEi+HRP; $n = 6$) at 24 wk of observation. (A) The ACEi significantly decreased the plasma AngII level. (B) Both HRP and ACEi significantly decreased the kidney AngII level. (C) HRP but not the ACEi significantly decreased urinary protein excretion. (D) HRP but not the ACEi significantly decreased the glomerulosclerosis index. (E) The photomicrographs of the PAS-stained kidney sections show the diabetic glomerulosclerosis in the untreated diabetic ATKO mice at 24 wk of observation. Clear diabetic glomerulosclerosis developed in the diabetic ATKO mice despite 24 wk of treatment with ACEi, but HRP significantly inhibited the development of diabetic glomerulosclerosis in the absence or presence of ACEi. Bars = 25 μm. *$P < 0.05$ *versus* untreated diabetic ATKO mice.

24th week, whereas in nondiabetic WT and ATKO mice, the blood glucose remained normal (123 ± 6 and 118 ± 6 mg/dl, respectively). As shown in Table 1, systolic BP was lower in the diabetic and nondiabetic ATKO mice than in the diabetic and nondiabetic WT mice. As shown in Table 2, plasma renin activity was significantly lower in the diabetic mice than in the nondiabetic mice throughout the diabetic period. The plasma prorenin level was significantly higher in diabetic mice than in nondiabetic mice throughout the 24-wk observation period. The plasma AngI and AngII levels of the diabetic and nondiabetic mice were similar throughout 24 wk but were significantly higher in the ATKO mice than in the WT mice.

In marked contrast to plasma RAS components, diabetic kidney contents of AngI and AngII are markedly elevated over 8 to 24 wk after streptozotocin administration (Figure 2, A and B), suggesting the possible causative roles of elevated renal AngII in diabetic proteinuria, as suggested by Onozato *et al.* (17). As shown in Table 3, renal levels of total renin protein (renin + prorenin) and renin mRNA in diabetic WT or diabetic ATKO mice were lower than in nondiabetic mice throughout the 24-wk studies, but they were always higher in the ATKO mice than in WT mice. There was not a marked difference in kidney ACE or kidney angiotensinogen mRNA levels. Cathepsin B mRNA level was significantly lower in the diabetic mice than in the nondiabetic mice at 16 and 24 wk. This is interesting because cathepsin B is considered to mediate the proteolytic activation of prorenin to active renin. To estimate the kidney levels of nonproteolytically activated prorenin in diabetic mice, we immunohistochemically probed the kidneys that were collected from mice that had been diabetic for 24 wk. The antibody directed to the active center of mouse renin that was used in our study binds to activated prorenin. Activated prorenin immunoreactivity was significantly increased in the juxtaglomerular area of the diabetic WT and ATKO mice (Figure 2, C and D). Because HRP treatment inhibited the activated prorenin immunoreactivity (data not shown), the kidneys of diabetic mice contain an increased level of nonproteolytically activated prorenin but not proteolytically activated renin.

Effects of ACE Inhibitors and HRP Decoy Peptide on Diabetic Nephropathy

Long-term administration of the ACE inhibitor imidapril caused significantly decreased systolic BP and plasma and kidney AngII levels in the 24-wk diabetic WT mice (Table 1, Figure 3, A and B), and it attenuated both the increase in urinary protein excretion and the glomerulosclerosis index of

the 24-wk diabetic WT mice (Figure 3, C through E). HRP infusion completely suppressed the increases in urinary protein excretion and glomerulosclerosis index in untreated diabetic WT mice and diabetic WT mice that were treated with imidapril, although HRP did not affect blood glucose or BP.

In the 24-wk diabetic ATKO mice, long-term administration of the ACE inhibitor imidapril did not alter systolic BP in the presence or absence of HRP infusion (Table 1) but significantly decreased the plasma and kidney AngII levels to those similar to or lower than those of the nondiabetic ATKO mice (Figure 4, A and B), but it did not attenuate the increase in urinary protein excretion or prevent the morphologic change (Figure 4, C and E). In addition, the glomerulosclerosis index of the 24-wk diabetic ATKO mice that were treated with imidapril was similar to that of the untreated diabetic ATKO mice (Figure 4D). It is interesting that HRP infusion completely suppressed the increases in urinary protein excretion and glomerular sclerosis index in untreated diabetic ATKO mice and diabetic ATKO mice that were treated with imidapril, although HRP did not affect blood glucose or BP (Table 1).

As shown in Tables 1 and 2, HRP administration did not affect systolic BP or the components of circulating RAS in either the diabetic WT or diabetic ATKO mice. As shown in Table 3, however, HRP infusion completely prevented the increases in kidney AngI levels and kidney AngII levels in the diabetic WT and diabetic ATKO mice. HRP administration did not alter any other components of the kidney RAS in either the diabetic WT or diabetic ATKO mice, although renal levels of total renin content and renin mRNA were significantly greater in ATKO mice than in WT mice.

Phosphorylation of MAPK in the Diabetic Kidneys

At 24 wk of observation, the immunoreactivity of phospho-Erk1/2 increased in the glomeruli of the kidneys from diabetic WT and diabetic ATKO mice but did not change in the glomeruli of the kidneys from control WT or control ATKO mice (Figure 5). The increased immunoreactivity of phospho-Erk1/2 in diabetic WT mice was attenuated by long-term administration of the ACE inhibitor imidapril and completely inhibited by HRP treatment. The increased immunoreactivity of phospho-Erk1/2 in diabetic ATKO mice was inhibited by HRP treatment

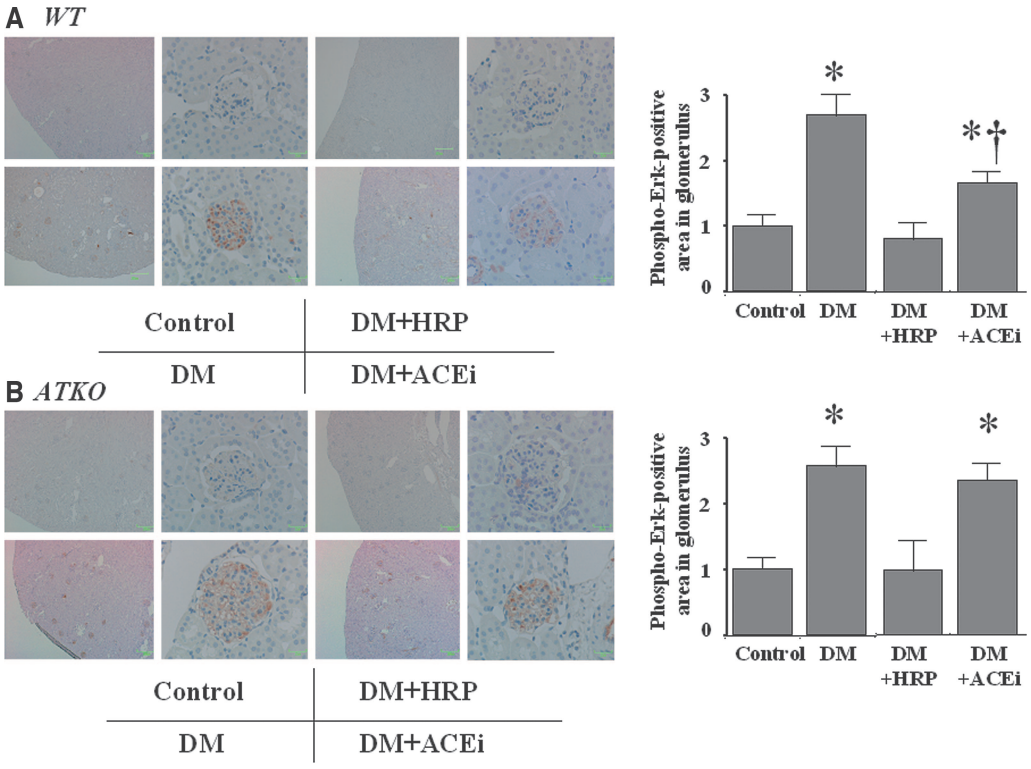

Figure 5. Immunohistochemistry of phospho-extracellular signal–related protein kinases 1 and 2 (Erk1/2) in the mouse kidneys at 24 wk of observation. (A) Immunohistochemistry and quantitative analysis (fold *versus* control mice) in control WT mice ($n = 4$), untreated diabetic WT mice ($n = 5$), and diabetic WT mice treated with HRP ($n = 5$) and the ACEi imidapril ($n = 5$). The graphs show increased immunoreactivity to phospho-Erk1/2 in the glomeruli of the kidneys from diabetic WT mice. The ACEi attenuated the increase of immunoreactivity of phospho-Erk1/2 in diabetic WT mice, but HRP completely inhibited it. Bars = 200 (left) and 25 μm (right). (B) Immunohistochemistry and quantitative analysis (fold *versus* control mice) in control ATKO mice ($n = 6$), untreated diabetic ATKO mice ($n = 6$), and diabetic ATKO mice treated with HRP ($n = 6$) and ACEi ($n = 6$). The graphs show increased immunoreactivity to phospho-Erk1/2 in the glomeruli of the kidneys from diabetic ATKO mice. HRP inhibited the increase of immunoreactivity of phospho-Erk1/2 in diabetic ATKO mice, but ACEi did not affect it. Bars = 200 (left) and 25 μm (right). $^*P < 0.05$ *versus* control mice; $^\dagger P < 0.05$ *versus* untreated diabetic mice.

but was not by long-term administration of the ACE inhibitor imidapril. The immunoreactivity of phospho-p38 increased in the glomeruli of the kidneys from the 24-wk diabetic mice compared with the 24-wk control mice (Figure 6). HRP treatment completely inhibited the increase in immunoreactivity of phospho-p38 in both diabetic WT mice and diabetic ATKO mice, but a long-term administration of the ACE inhibitor imidapril attenuated the increased immunoreactivity of phospho-p38 in diabetic WT mice and did not affect it in diabetic ATKO mice. Figure 7 showed the increased immunoreactivity of phospho-Jnk in the glomeruli of the kidneys from the 24-wk diabetic WT and diabetic ATKO mice but not in the glomeruli of the kidneys from the 24-wk control WT or control ATKO mice. HRP treatment completely inhibited the increased immunoreactivity of phospho-Jnk in both diabetic WT and diabetic ATKO mice, but a long-term administration of the ACE inhibitor imidapril attenuated the increased immunoreactivity of phospho-Jnk in diabetic WT mice and failed to decrease it in diabetic ATKO mice. The immunoreactivities of total Erk1/2, total p38, and total Jnk were similar in all groups of WT and ATKO mice (data not shown).

Discussion

Renal glomerulosclerosis is one of the most prevalent and serious consequences of diabetes. Clinical and experimental observation of amelioration of proteinuria by inhibitors of the RAS led investigators to the view that AngII is responsible for the renal end-organ damage in diabetes. Our studies produced two lines of credible evidence that AngII does not play decisive roles in eliciting diabetic proteinuria and nephropathy. We found that AT_{1a} deficiency merely delays the onset of proteinuria and glomerular sclerosis in streptozotocin-induced diabetes (Figure 1). Also, long-term treatment of the diabetic WT or ATKO mice with ACE inhibitor failed to prevent the diabetic nephropathy completely (Figures 3 and 4).

Although plasma AngI and AngII were higher in the ATKO mice than in WT mice (Table 2), diabetes lowered plasma AngII in both ATKO and WT mice. However, kidney AngI and AngII levels were markedly elevated in diabetic animals with or without AT_{1a} receptors (Figure 2). In the kidney of diabetic animals, there was no increase in other components of the kidney RAS (Table 3). These results suggested that enhanced kidney RAS activity may have contributed to the development

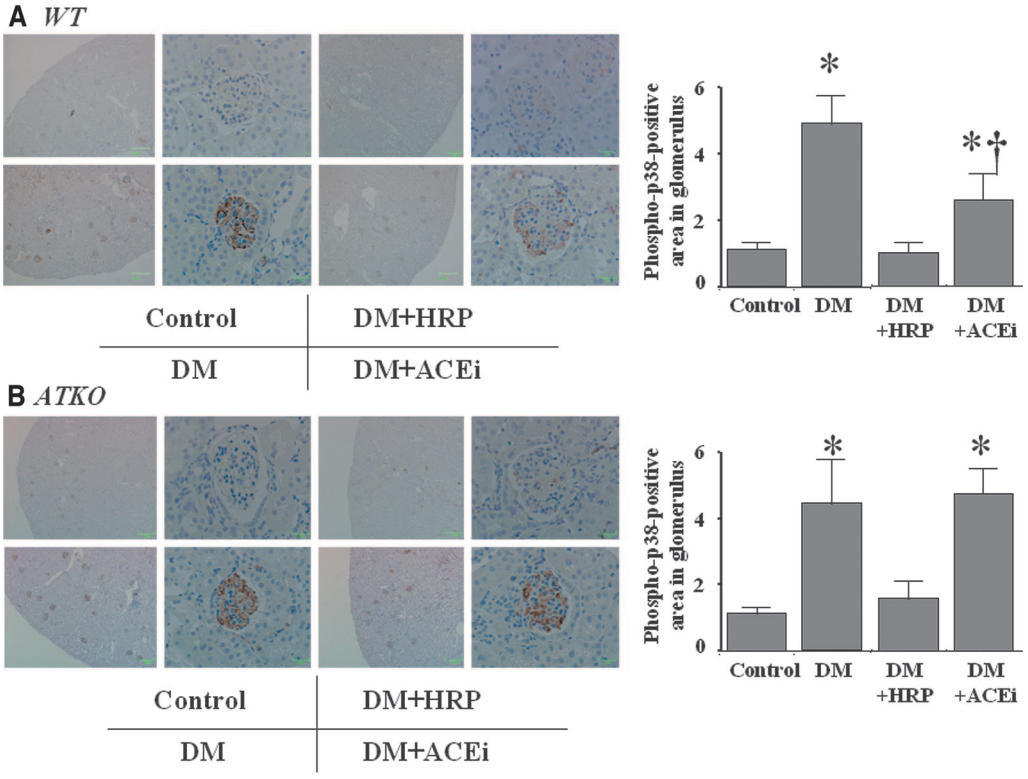

Figure 6. Immunohistochemistry of phospho-p38 in the mouse kidneys at 24 wk of observation. (A) Immunohistochemistry and quantitative analysis (fold *versus* control mice) in control WT mice ($n = 4$), untreated diabetic WT mice ($n = 5$), and diabetic WT mice treated with HRP ($n = 5$) and the ACEi imidapril ($n = 5$). The graphs show increased immunoreactivity to phospho-p38 in the glomeruli of the kidneys from diabetic WT mice. The ACEi attenuated the increase of immunoreactivity of phospho-p38 in diabetic WT mice, but HRP completely inhibited it. Bars = 200 (left) and 25 μm (right). (B) Immunohistochemistry and quantitative analysis (fold *versus* control mice) in control ATKO mice ($n = 6$), untreated diabetic ATKO mice ($n = 6$), and diabetic ATKO mice treated with HRP ($n = 6$) and ACEi ($n = 6$). The graphs show increased immunoreactivity to phospho-p38 in the glomeruli of the kidneys from diabetic ATKO mice. HRP inhibited the increase of immunoreactivity of phospho-p38 in diabetic ATKO mice, but ACEi did not affect it. Bars = 200 (left) and 25 μm (right). $^{*}P < 0.05$ *versus* control mice; $^{\dagger}P < 0.05$ *versus* untreated diabetic mice.

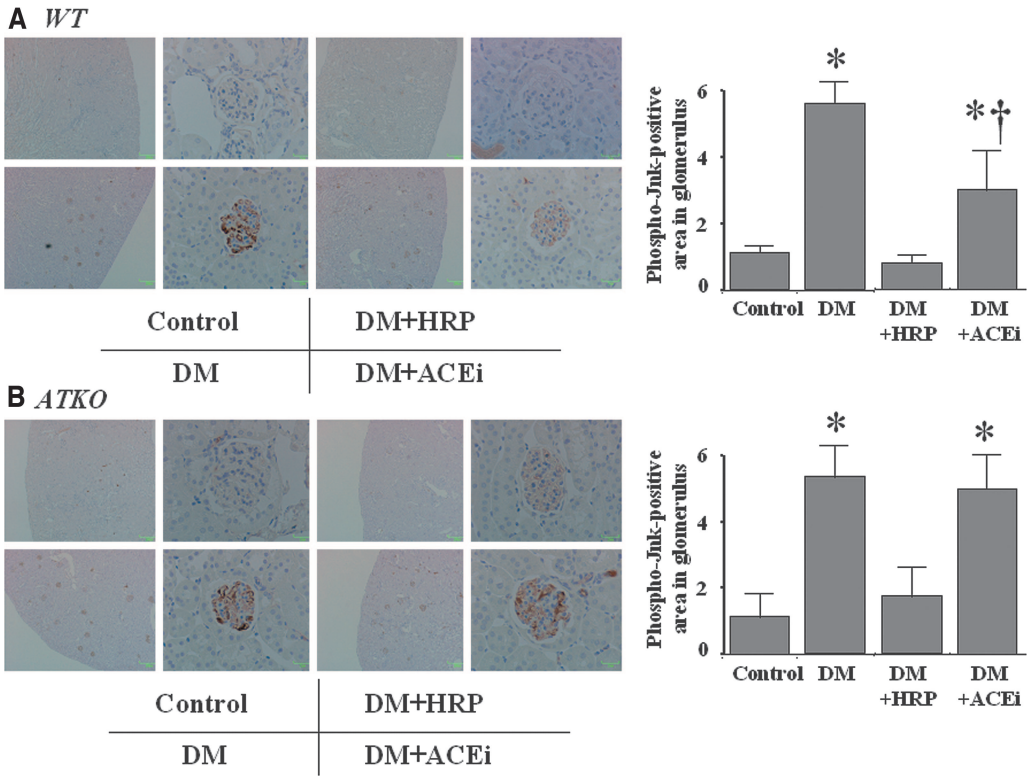

Figure 7. Immunohistochemistry of phospho-Jnk in the mouse kidneys at 24 wk of observation. (A) Immunohistochemistry and quantitative analysis (fold *versus* control mice) in control WT mice ($n = 4$), untreated diabetic WT mice ($n = 5$), and diabetic WT mice treated with HRP ($n = 5$) and the ACEi imidapril ($n = 5$). The graphs show increased immunoreactivity to phospho-Jnk in the glomeruli of the kidneys from diabetic WT mice. The ACEi attenuated the increase of immunoreactivity of phospho-Jnk in diabetic WT mice, but HRP completely inhibited it. Bars = 200 (left) and 25 μm (right). (B) Immunohistochemistry and quantitative analysis (fold *versus* control mice) in control ATKO mice ($n = 6$), untreated diabetic ATKO mice ($n = 6$), and diabetic ATKO mice treated with HRP ($n = 6$) and ACEi ($n = 6$). The graphs show increased immunoreactivity to phospho-Jnk in the glomeruli of the kidneys from diabetic ATKO mice. HRP inhibited the increase of immunoreactivity of phospho-Jnk in diabetic ATKO mice, but ACEi did not affect it. Bars = 200 (left) and 25 μm (right). $^{*}P < 0.05$ *versus* control mice; $^{\dagger}P < 0.05$ *versus* untreated diabetic mice.

of nephropathy in both groups of diabetic mice despite the absence of AT_{1a} in the kidneys of ATKO mice (6). To investigate whether the increased kidney AngII damaged the organ through angiotensin receptors other than AT_{1a} as suggested by a recent study (18), we treated the diabetic ATKO mice with the ACE inhibitor imidapril at doses high enough to reduce the plasma and kidney levels of AngII; however, imidapril failed to prevent the development of diabetic nephropathy (Figure 4). Previous studies have demonstrated that ACE inhibitors and AngII receptor blockers significantly diminish but do not completely inhibit urinary protein excretion in rats with streptozotocin-induced diabetes (17,19). The above findings indicate that although kidney RAS plays an important role in the development and progression of diabetic nephropathy, the ultimate development of this type of end-organ damage involves an AngII-independent mechanism.

When a prorenin binding protein (prorenin receptor) interacts with prorenin through the "handle region" in its prosegment, the prorenin molecule undergoes a conformational change to an enzymatically active state (16). This prorenin binding and activation are called RAPS, and they can be blocked by HRP's acting as a decoy that effectively inhibits prorenin binding to its receptor *in vitro* and *in vivo* (3). In this study, we obtained *in vitro* evidence of mouse *Ren1*-prorenin binding to the prorenin receptor and of inhibition of the binding by mouse HRP decoy peptide in COS-7 cells that express the prorenin receptor protein. Although the ACE inhibitor imidapril attenuated but did not completely inhibit the development of glomerulosclerosis in the diabetic WT mice, chronic infusion of mouse HRP with subcutaneous minipumps completely prevented the increase in urinary protein excretion and the development of glomerulosclerosis in both diabetic WT mice and diabetic ATKO mice. Urinary protein excretion and renal morphology of the diabetic mice that were treated with HRP were similar to those of the control mice (Figures 3 and 4). In addition, HRP administration significantly prevented the increase in nonproteolytically activated prorenin that was detected by immunoassay in the juxtaglomerular area, suggesting an increase in the complex formation between prorenin and its receptor protein in diabetic kidneys (data not shown). It therefore is possible that binding of prorenin to its receptor not only exerts a nonproteolytic activation of prorenin, leading to generation of AngI and AngII, but also stimulates postreceptor pathways beyond the receptor activation. Recent studies pro-

vided *in vitro* evidence that the prorenin receptor–prorenin complex is capable of activating Erk independent of RAS activation (4,5). Because Erk is activated in glomeruli from diabetic animals and in mesangial cells that are cultured under high-glucose conditions (20,21) and contributes to mesangial cell hypertrophy (22), the intracellular signal transduction of prorenin receptor may play an important role in the development of diabetic nephropathy. In addition, other subgroups of the MAPK family, p38 and Jnk, are, respectively, reported to be activated in glomeruli from diabetic animals (23) and contribute to mesangial cell hypertrophy (22). Our study provided novel *in vivo* evidence that phospho-Erk, phospho-p38, and phospho-Jnk increased in the kidneys of diabetic ATKO mice; these increases were inhibited by HRP but not by ACE inhibitors. These findings suggest that activation of the MAPK family is evoked by signals from the prorenin-bound receptor by AngII-independent mechanisms and is responsible for the development of the diabetic organ damage in diabetic ATKO mice.

Our study has provided a credible explanation for the increased complex formation between prorenin and its receptor in diabetes. In agreement with previous *in vitro* and *in vivo* studies (3,24,25), kidney cathepsin B mRNA levels were found to decrease significantly during the development of diabetic nephropathy (Table 3), suggesting that the decreased prorenin processing can increase the ratio of prorenin to renin in diabetic mouse kidneys and as a result increase complex formation between prorenin and its receptor. In fact, immunohistochemical staining of the active center of renin significantly increased in the kidneys of both the diabetic WT and diabetic ATKO mice (Figure 2, C and D), although the total renin content of the diabetic mouse kidneys was similar to or less than the control mouse kidneys (Table 3). Because the inhibition of prorenin binding to its receptor by HRP almost completely prevented the increase in immunohistochemical staining of the exposed active center of renin in the kidneys of diabetic mice (3), the increased staining observed indicates that the activated prorenin retains a covalently linked prosegment that contains the "handle region." Therefore, the observed activation of kidney prorenin in diabetes does not seem to be due to proteolytic activation of prorenin. Alternatively, nonproteolytic prorenin activators, such as a prorenin receptor (4), or their affinity to prorenin might be increased in diabetes.

The kidneys of 3-wk-old angiotensinogen-deficient mice were found to develop glomerulosclerosis similar to that in the kidneys of the diabetic mice in this study, even though neither the angiotensinogen-deficient mice nor the mice in this study were hypertensive (26). These results suggest that glomerulosclerosis can develop *via* a mechanism independent of hemodynamic change or action of Ang peptides in the kidneys and plasma. Because the angiotensinogen-deficient mice had increased prorenin/renin levels in the juxtaglomerular area (26), like the diabetic mice in our study, the increased prorenin/renin levels also may be involved in the mechanism of the development of glomerulosclerosis in angiotensinogen-deficient mice. Therefore, the RAPS may play an important role in the RAS-independent mechanism of the development of glomerulosclerosis.

It is widely known that AT_{1a} activation by AngII activates all three members of MAPK family, including Erk1/2, p38, and Jnk in a variety of cells (27) and in cultured human mesangial cells (28). However, phospho-Erk, phospho-p38, and phospho-Jnk increased significantly in the kidneys not only of diabetic WT mice but also of ATKO mice. ACE inhibition or the AT_{1a} deletion failed to prevent completely the activation of these major subgroups of the MAPK family, and HRP infusion significantly inhibited the increased levels of phospho-Erk, phospho-p38, and phospho-Jnk in the kidneys of both diabetic WT and diabetic ATKO mice. These results indicate that the MAPK activation in diabetic nephropathy may be due at least in part to the direct activation of prorenin receptor, which is independent of the AngII-activated AT_{1a} signal pathway. The details of such a signaling pathway of the direct action of the prorenin receptor may involve a novel mechanism, and further studies are needed to clarify this.

The ATKO mice that were used in our study still have AngII type 1b receptors and type 2 receptors. However, because the ACE inhibitor imidapril did not affect the development of proteinuria or glomerulosclerosis in the diabetic ATKO mice, AngII type 1b receptors or type 2 receptors may not be involved in the development of nephropathy in diabetic mice. In addition, our study determined urinary excretion of total protein and could not distinguish nonspecific proteinuria from urinary albumin excretion in diabetic mice. Therefore, there was the possibility that albuminuria might not show the same results as the total protein data.

Conclusions

Diabetic nephropathy that developed in ATKO mice with streptozotocin-induced diabetes was not normalized by an ACE inhibitor at doses high enough to suppress AngII levels in their plasma and kidneys, suggesting a contribution by an AngII-independent mechanism to the development of diabetic nephropathy. This study clearly demonstrated that inhibition of the RAPS completely prevented the development of diabetic nephropathy and the activation of the renal MAPK family in diabetic ATKO mice, suggesting that the RAPS significantly contributes to the AngII-independent mechanism of the development of diabetic nephropathy.

Acknowledgments

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