

## ORIGINAL ARTICLE

# Loss of ACE2 accelerates time-dependent glomerular and tubulointerstitial damage in streptozotocin-induced diabetic mice

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As angiotensin-converting enzyme-2 (ACE2) was identified as a negative regulator of the renin–angiotensin system, there have been many reports concerning its role in several tissues, including the kidney. However, the role of ACE2 during the development of diabetic nephropathy remains undetermined, as previous reports did not necessarily support a protective role against renal injury. Thus, we performed detailed observations of kidneys in ACE2-knockout (ACE2-KO) mice at early (4 weeks) and advanced (18 weeks) stages of diabetes. ACE2-KO and wild-type C57BL/6 mice were rendered diabetic by intraperitoneal injection of streptozotocin. Diabetic ACE2-KO mice showed earlier onset and more severe progression of albuminuria than those did wild-type mice. The elevation of serum creatinine and urea nitrogen levels at 18 weeks of diabetes was more prominent in ACE2-KO mice. Periodic acid-Schiff-stained cross-section of diabetic ACE2-KO mice showed a more severe time-dependent increase in glomerular/tubulointerstitial damage than did that of wild-type mice, confirmed by the immunostaining of  $\alpha$ -smooth muscle actin, collagen IV and F4-80 antigen. Glomeruli of diabetic ACE2-KO mice showed earlier and more severe decrease in the expression of nephrin, whose degradation is involved in the onset of albuminuria, and more potent increase of vascular endothelial growth factor expression. In addition, treatment with AT1 receptor blocker olmesartan significantly, but not totally, ameliorated the functional and morphological deterioration of diabetic nephropathy in ACE2-KO mice. These results suggest that ACE2 might continuously protect from both glomerular and tubulointerstitial injury during the development of diabetic nephropathy. The renal-protective effect of ACE2 might involve more than just suppressing angiotensin II-mediated AT1 receptor signaling.

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**Keywords:** ACE2; diabetic nephropathy; renin–angiotensin system

## INTRODUCTION

The renin–angiotensin system (RAS) has a key role in the development of many renal diseases and blocking this system is an established therapeutic target for preserving kidney function in several chronic kidney diseases.<sup>1–5</sup> In particular, use of an angiotensin-converting enzyme (ACE) inhibitor or AT1 subtype angiotensin II receptor blocker (ARB) in diabetic nephropathy, currently one of the most common reasons for chronic dialysis, reduced the progression of kidney dysfunction in several clinical trials.<sup>1–3</sup> ACE inhibitors or ARBs in diabetic nephropathy contributes to renal protection not only by reducing renal artery pressure but also by blocking angiotensin II (Ang II)-induced cellular signaling that is increased in such conditions and damages several types of kidney cells. Thus, to understand the pathophysiology of diabetic nephropathy, it is important to comprehend the mechanism regulating Ang II signaling.

Angiotensin-converting enzyme-2 (ACE2) is a membrane-bound carboxymonopeptidase containing a single enzymatic site. It cleaves

Ang II at a single residue to generate angiotensin 1–7 (Ang 1–7).<sup>6,7</sup> The expression of ACE2 is observed in many tissues, including the heart, aorta, lung and kidney. ACE2 is thought to function as a negative regulator of the RAS by degrading Ang II and producing Ang 1–7 to counterbalance the Ang II-forming activity of ACE.

In the kidney, ACE2 is reported to be localized in renal tubules and glomeruli,<sup>8–11</sup> and several studies showed that the expression of ACE2 is reduced during the development of diabetic nephropathy.<sup>10,12,13</sup> To investigate the functional significance of ACE2 in diabetic nephropathy, several *in vivo* studies were performed; some administered ACE2 inhibitor, others carried a targeted disruption of the ACE2 gene to diabetic mice. In the reports of Soler *et al.*<sup>14</sup> and Ye *et al.*,<sup>8</sup> diabetic mice treated by specific ACE2 inhibitor MLN-4760 showed increased albuminuria and glomerular/tubular injury compared with untreated diabetic mice. As for the studies using ACE2-knockout (ACE2-KO) mice, Wong *et al.*<sup>15</sup> used ACE2-KO mice bred with diabetic AKITA mice, and Tikellis *et al.*<sup>16</sup> generated diabetic ACE2-KO mice by

injecting the mice with streptozotocin to assess the influence of ACE2 on pathology and renal function in diabetic nephropathy. Although they showed similar results in terms of increased albumin excretion ratio with deletion of ACE2, there were differences between the two studies with regard to pathological changes after induction of diabetic nephropathy. In the report of Wong *et al.*, deletion of ACE2 resulted in enhanced glomerular hypertrophy and interstitial fibrosis at 3 months of age, whereas renal hypertrophy and expression of fibrotic markers were attenuated in diabetic ACE2-KO mice of Tikellis *et al.* at 10 weeks of diabetes. In addition, the influence of ACE2 on sequential changes in renal pathophysiology during development of diabetic nephropathy remains unknown, as renal pathophysiological assessment was performed at one time point in each study. Thus, their findings raised the possibility that a role of ACE2 in diabetic nephropathy might vary according to the duration or severity of diabetes. Therefore, we compared renal pathophysiological changes of streptozotocin-administered ACE2-KO mice with those of wild-type mice at the early (4 weeks) and advanced (18 weeks) stages of diabetes, to investigate a sequential role of ACE2 during development of diabetic nephropathy. Also, we used ARB to investigate whether diabetic ACE2-KO and wild-type mice exhibited any difference in renal phenotype in the absence of AT1 signaling.

## METHODS

### Animal model

The creation of the ACE2-deficient mice was previously described.<sup>17</sup> All animals were housed at the Institute of Laboratory Animals at Osaka University. These animals were studied in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were placed on a protocol approved by the Animal Research Committee of the Osaka University.

### Experimental protocol

Male ACE2-KO ( $-/-$ ) and wild-type ( $+/+$ ) littermates were bred from ACE2 $+/y$  male and ACE2 $-/+$  female mice with a C57BL/6N background. Six- to eight-week-old ACE2-KO and wild-type littermates were rendered diabetic after 5 daily intraperitoneal injections of streptozotocin at a dose of 55 mg kg $^{-1}$  in 10 mM citrate buffer.<sup>18</sup> Mice with blood glucose levels of  $>350$  mg per 100 ml 7 days after the last injection were included in this study. The mice had free access to water and standard mouse chow. Treatment with AT1 receptor blocker Olmesartan (Sankyo Corporation, Tokyo, Japan) to some mice was started a week before the injection of streptozotocin and continued during the follow-up period. Olmesartan was administered in drinking water to obtain a dose of 4 mg kg $^{-1}$  day $^{-1}$ .<sup>19–21</sup>

Four or 18 weeks after induction of diabetes, the animals were anesthetized by an intraperitoneal injection of pentobarbital sodium. Blood was collected from the inferior vena cava and then centrifuged to obtain serum or was stored as plasma, with addition of phenylmethylsulfonyl fluoride and EDTA, for subsequent analyses. Kidneys were dissected and immediately snap-frozen (for protein extraction) or stored in 4% neutral buffered formalin (for histological studies) or immediately homogenized (for determination of Ang II concentrations). We performed these experiments 4 weeks after induction of diabetes on the basis of previous reports in which the early phase of urinary albumin excretion was detected in streptozotocin-induced diabetic C57BL/6 mice at this time point.<sup>22</sup> The 18-week period was also based on previous reports showing that diabetic C57BL/6 mice begin to show morphological and functional kidney changes 16 to 20 weeks after induction of diabetes.<sup>18,23</sup>

### Physiological and biochemical analysis

Glucose levels in fresh tail vein blood were measured every week during the first 4 weeks, and then at 8, 12 and 18 weeks after the induction of diabetes in non-fasted mice by Glucose Vision (Aventir Biotech, LLC, Carlsbad, CA, USA). Serum creatinine, urea nitrogen and sodium concentrations were measured at the Nissei Bilis Corporation (Osaka, Japan).

To assess urine albumin excretion, animals were placed in metabolic cages for 24-h urine collection. Albumin concentrations were measured using the ELISA kit Albuwell M Assay Kit (Exocel, Philadelphia, PA, USA) according to the manufacturer's instructions. Blood pressure was measured using a non-invasive tail-cuff method in conscious mice.

### Renal pathology

Formalin-fixed kidneys were embedded in paraffin. Then 2  $\mu$ m sections were stained with periodic acid-Schiff. Digital images were obtained from microscopy, and the degree of renal lesions was assessed in a blinded manner using previously described semiquantitative methods with minor modifications.<sup>18,24</sup> As an evaluation of glomerular injury, all glomeruli in each kidney section were graded on periodic acid-Schiff-stained sections according to the severity of the glomerular damage, which included mesangial matrix expansion and/or hyalinosis with focal adhesions, capillary dilation, and true glomerular occlusion and sclerosis: grade 0, intact glomerulus; grade 1, glomerular injury involving  $<25\%$  of the glomerulus; grade 2, 25–50%; grade 3, 50–75%; grade 4, severe damage or sclerosis involving  $>75\%$  of the glomerulus. For tubulointerstitial injury, 20 microscopic fields ( $\times 200$ ) from the cortex in each kidney section were randomly selected. Tubular lesions were graded according to the severity of tubular dilations and/or atrophy: grade 0, no tubular injury; grade 1,  $<25\%$  of the field involved; grade 2, 25–50%; grade 3, 50–75%; grade 4,  $>75\%$ .

These indices of glomerular/tubulointerstitial injuries were calculated by averaging the grades assigned to the glomeruli/tubulointerstitial fields.

### Immunohistochemistry

The primary antibodies for immunostaining were as follows: anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, Dako Japan, Kyoto, Japan), anti-collagen type IV (Southern Biotechnology Associates, Birmingham, AL, USA), anti-nephrin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-vascular endothelial growth factor (VEGF, EMD Chemicals, Darmstadt, Germany) and anti-mouse tissue macrophage antibody (anti-F4/80 antibody, Serotec, Oxford, UK). In brief, sections were dewaxed and hydrated, and antigen retrieval was performed by proteinase K purchased from Dako Japan. Endogenous peroxidase activity was blocked by Dako REAL Peroxidase Blocking Solution, and then the sections were incubated with the primary antibodies for 12 h at 4°C. As the secondary antibody, ENVISION+ mouse (Dako Japan) was applied in a humid atmosphere for 30 min. For the chromogen development, DAB+ reagent (Dako Japan) was applied for 10 min. After staining, the sections were counterstained with Mayer's hematoxylin.

The assessment was as follows: F4/80-positive macrophages were counted in 20 randomly selected microscopic fields ( $\times 400$ ) per animal and expressed as the mean number of cells per field. For assessment of glomerular nephrin staining, which was generally too weak to assess by computer software, a semiquantitative analysis was performed on the basis of a pathologist-established score, as previously described.<sup>8</sup> Briefly, 1, no staining; 2, weak staining; and 3, strong staining. Then, the percentage of glomeruli with strong staining was calculated for each animal. As for other immunostaining, the percent positive staining area was calculated within 20 randomly selected glomerular tufts or cortical tubulointerstitial areas by computer image analysis using Image J software (<http://rsb.info.nih.gov/ij/>). All sections were examined by three different blinded observers.

### Plasma and kidney Ang II concentration

After blood collection from the inferior vena cava and adequate perfusion with heparinized saline, the isolated kidney was homogenized on ice in 0.9% saline/0.1 mol/l HCl containing 0.1 mol/l aprotinin. The total protein content of an aliquot of the homogenate was determined using the Bradford protein assay. Peptide extraction was performed as previously described.<sup>17</sup> Ang II concentration was measured by radioimmunoassay using two antibodies specific for Ang II (SRL Corporation, Tokyo, Japan) and described as pg ml $^{-1}$  (plasma) or pg mg $^{-1}$  protein (kidney). This antibody has cross-reactivity with angiotensin I and III of  $<0.1$  and 21%, respectively.

### Statistical analysis

Data were analyzed with Stat View version 4.51 and are presented as the mean  $\pm$  s.e.m. Statistical analysis was performed using the two-tailed Student's *t*-test and analysis of variance where applicable. Multiple group comparisons were carried out by analysis of variance with Fisher's *post hoc* comparison. A value of *P* less than 0.05 was considered statistically significant.

## RESULTS

### Physiological and biochemical markers of diabetic mice

At first, we bred nondiabetic ACE2-KO mice and wild-type littermates (6 weeks of age at the beginning of the study) for 18 weeks. Those nondiabetic ACE2-KO mice had similar body weight, blood pressure, kidney weight and other metabolic markers, including albuminuria, compared with wild type both at baseline and 18 weeks later (Supplementary Table). Eighteen weeks after administration of streptozotocin, blood sugar levels of ACE2-KO and wild-type mice were equally elevated (Table 1). Neither diabetic ACE2-KO nor wild-type mice showed body weight loss compared with age-matched nondiabetic mice. Diabetes was associated with an increase in serum creatinine and urea nitrogen levels in both strains, but a significantly larger elevation was observed in ACE2-KO mice. Mean blood pressure was not changed after the onset of diabetes in either mouse strain and kidney weight increased similarly in both strains. Heart weight was not affected by ACE2 deletion or the onset of diabetes.

Streptozotocin-induced diabetes was accompanied by time-dependent development of albuminuria in both ACE2-KO and wild-type mice. However, onset and elevation of albuminuria occurred earlier in ACE2-KO mice, and was more severe than in wild-type mice (4 weeks after induction of diabetes, ACE2-KO=48.9  $\pm$  12.6  $\mu$ g per 100 ml *vs.* wild type=24.8  $\pm$  11.7  $\mu$ g per 100 ml; *P*<0.05). The enhanced excretion of albuminuria in ACE-KO mice was observed up to 18 weeks after induction of diabetes, the end of the follow-up period (18 weeks after induction of diabetes, ACE2-KO=87.7  $\pm$  21.9  $\mu$ g per 100 ml *vs.* wild type=49.3  $\pm$  16.3  $\mu$ g per 100 ml; *P*<0.05) (Figure 1a).

### Plasma and kidney Ang II concentrations in ACE-KO and wild-type mice

Nondiabetic ACE2-KO mice had higher kidney Ang II concentrations than nondiabetic wild-type mice (Table 2), which was consistent with a previous report by Crackower *et al.*<sup>25</sup> Four weeks after the onset of diabetes, tissue Ang II was increased both in the kidneys of ACE2-KO and wild-type mice, and the difference between the two groups

disappeared (*P*=0.32). In contrast, there was no difference in plasma Ang II concentrations between ACE2-KO and wild-type mice, before or after diabetes induction.

### Glomerular and tubulointerstitial injury in diabetic mice

The kidneys of nondiabetic ACE2-KO and wild-type mice at baseline and 18 weeks later showed no structural abnormalities under light microscopy (Supplementary Figure). Four weeks after diabetes induction, ACE2-KO mice showed mild glomerular injury including glomerular hypertrophy, mesangial matrix expansion and mesangial hypercellularity. Eighteen weeks after streptozotocin injection, these changes were significantly more severe in ACE2-KO mice than in age-matched wild-type mice. Some glomeruli in 18-week diabetic ACE2-KO mice showed more progressive changes, including glomerulosclerosis and atrophy, reflected as an increased semiquantitative glomerular injury index (Figure 1).

Similarly, tubulointerstitial injuries were also accelerated in diabetic ACE2-KO mice. Although both ACE2-KO and wild-type mice showed almost normal tubulointerstitium under light microscopy at baseline, diabetic ACE2-KO mice had increased number of dilated or atrophied tubules compared with age-matched wild-type mice (Figure 1).

### Localization and expression of proteins indicative of kidney injury in diabetic mice

Immunohistochemical studies were performed to characterize kidney injury in the diabetic state using antibodies against proteins well known to be upregulated in diabetic nephropathy. There was a greater time-dependent increase in glomerular  $\alpha$ -smooth muscle actin and glomerular/interstitial collagen IV in diabetic ACE2-KO mice than in wild-type mice, confirming accelerated diabetic glomerular and tubulointerstitial injury in ACE2-KO mice (Figure 2).

To evaluate macrophage infiltration as an important aspect of tubulointerstitial injury, F4/80 immunostaining was performed. Similar to the case of  $\alpha$ -SMA and collagen IV, there was a time-dependent increase in the number of interstitial macrophages and the change was earlier and greater in diabetic ACE2-KO mice than in wild-type mice (Figure 3).

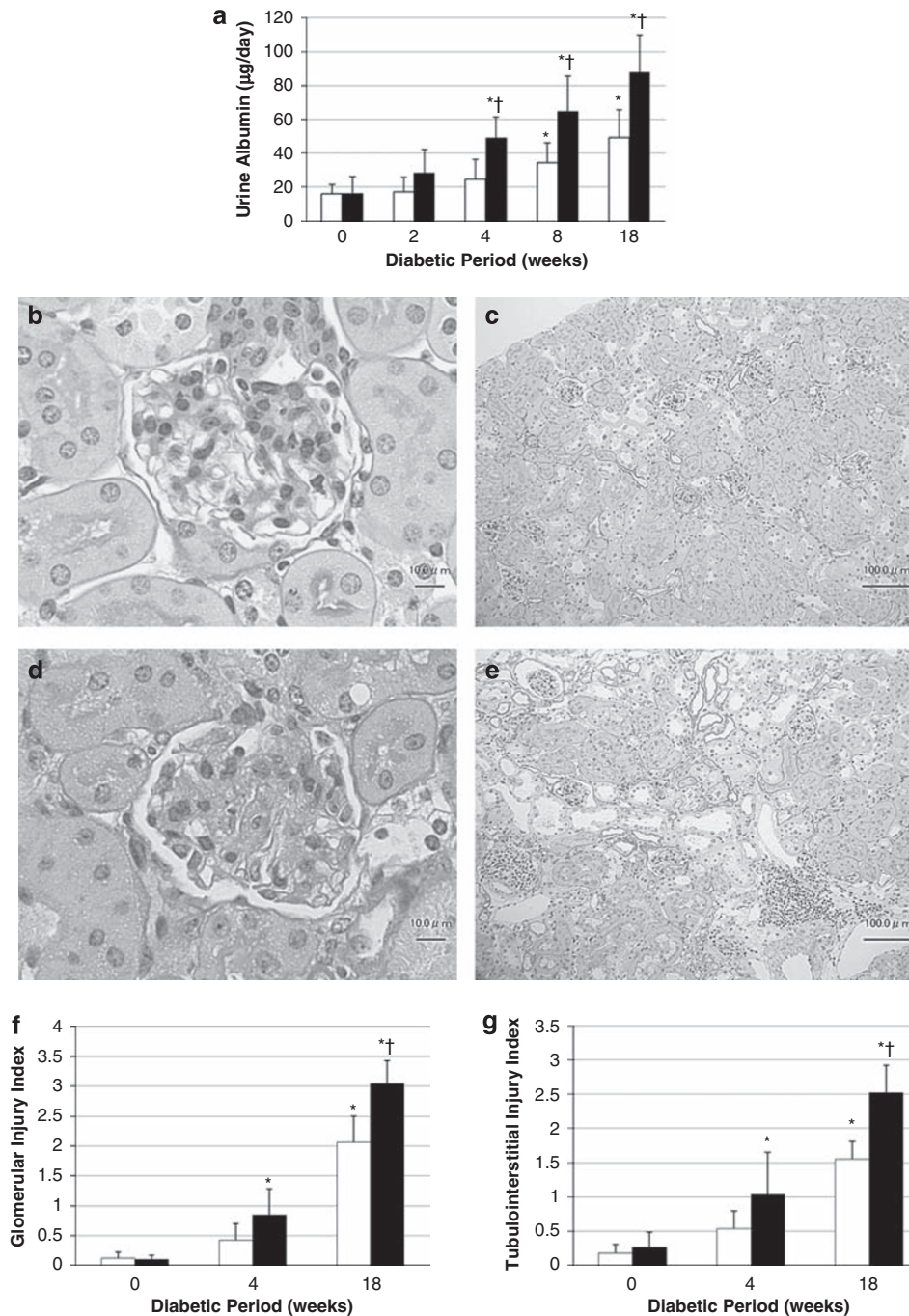
We also performed immunohistochemistry to find other factors involved in accelerated functional and morphological abnormalities in diabetic ACE2-KO mice. Glomeruli of early diabetic mice showed decreased expression of nephrin, comparable with previous reports.<sup>26–28</sup> In addition, the change was greater in diabetic ACE2-KO mice than in

**Table 1 Physiological and biochemical parameters**

	Nondiabetic wildtype (n=10)	Nondiabetic ACE2-KO (n=10)	18 Weeks diabetic wildtype (n=10)	18 Weeks diabetic ACE2-KO (n=10)
Body weight (g)	32 $\pm$ 2.9	34 $\pm$ 1.9	28 $\pm$ 5.4	30 $\pm$ 4.7
Mean blood pressure (mm Hg)	77 $\pm$ 12.4	84 $\pm$ 6.72	76 $\pm$ 11.3	88 $\pm$ 8.31
Blood glucose (mmol l <sup>-1</sup> )	14.2 $\pm$ 1.38	12.4 $\pm$ 2.62	24.0 $\pm$ 3.85*	25.2 $\pm$ 4.81*
Serum creatinine ( $\mu$ mol l <sup>-1</sup> )	16 $\pm$ 1.24	13 $\pm$ 2.75	23 $\pm$ 0.97	36 $\pm$ 5.8* <sup>†</sup>
Serum urea nitrogen (mmol l <sup>-1</sup> )	9.7 $\pm$ 0.47	10 $\pm$ 0.93	13 $\pm$ 0.41*	19 $\pm$ 1.3* <sup>†</sup>
Serum Na (mmol l <sup>-1</sup> )	142 $\pm$ 2.0	139 $\pm$ 3.8	144 $\pm$ 11	145 $\pm$ 9.1
Kidney weight (mg)	178 $\pm$ 11.6	166 $\pm$ 21.0	234 $\pm$ 24.1*	242 $\pm$ 36.7*
Heart weight (mg)	137 $\pm$ 8.50	125 $\pm$ 16.0	126 $\pm$ 19.5	133 $\pm$ 25.3

Abbreviation: ACE2-KO, angiotensin-converting enzyme-2-knockout.

All nondiabetic and diabetic mice were age-matched. Data are presented as mean  $\pm$  s.e.m. \**P*<0.05 *vs.* genotype-matched nondiabetic group, and <sup>†</sup>*P*<0.05 *vs.* diabetic wildtype.



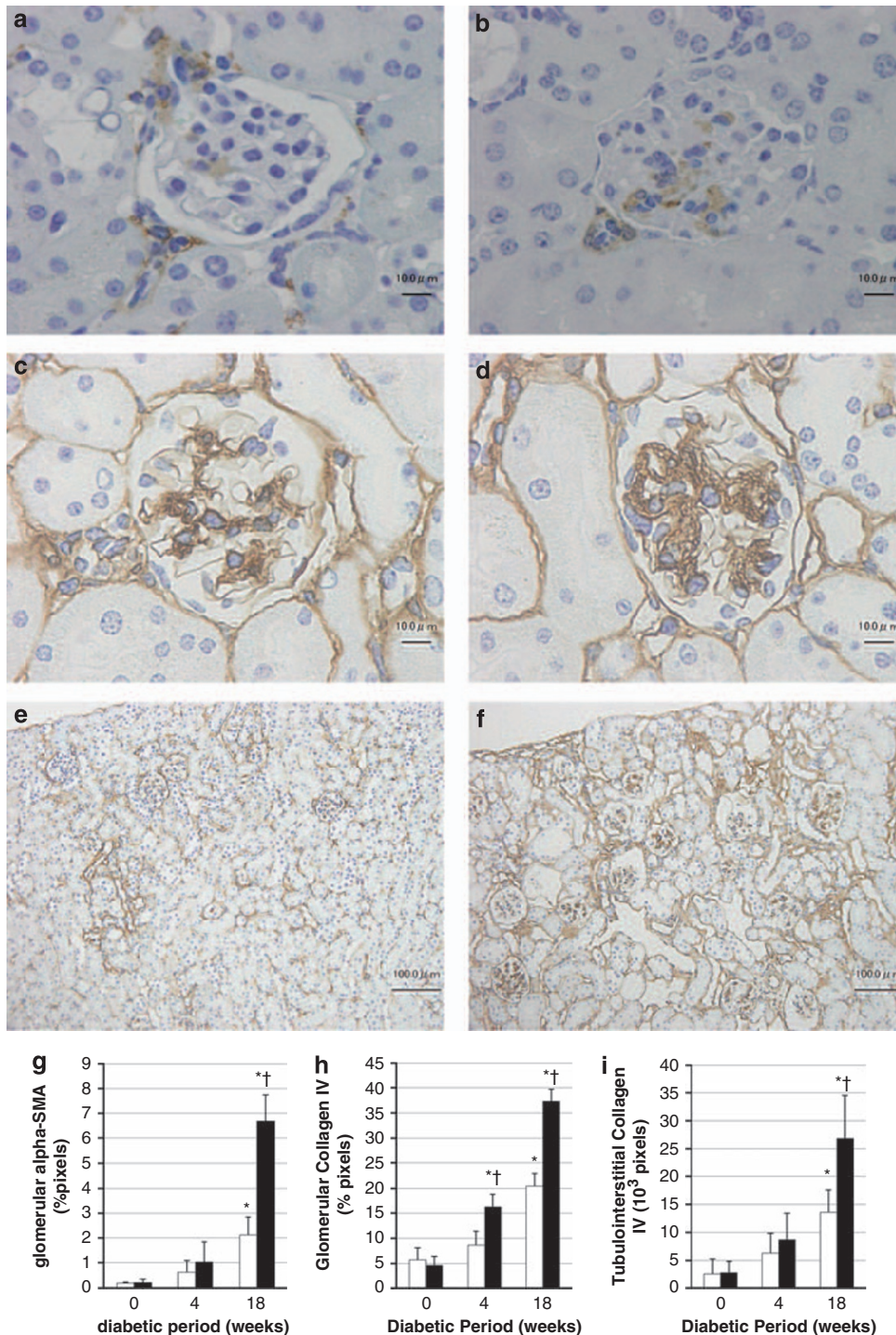
**Figure 1** (a) Daily urinary albumin excretion of ACE2-KO and wild-type mice at various time points after induction of diabetes determined by ELISA. (b–e) Representative photomicrographs of glomeruli and tubulointerstitium in PAS-stained kidney sections from wild-type (b, c) and ACE2-KO mice (d, e) 18 weeks after induction of diabetes. Original magnifications  $\times 800$  and  $\times 200$ . (f, g) Glomerular and tubulointerstitial injury index by semiquantitative analysis. White bars=wildtype ( $n=10$ ); black bars=ACE2-KO ( $n=10$ ). Data are presented as mean  $\pm$  s.e.m. \* $P < 0.05$  compared with a genotype-matched baseline group, and  $\dagger P < 0.05$  compared with age-matched wildtype.

**Table 2 Tissue/plasma Angiotensin (Ang) II concentration**

	Nondiabetic wildtype ( $n=6$ )	Nondiabetic ACE2-KO ( $n=6$ )	4 Weeks diabetic wildtype ( $n=6$ )	4 Weeks diabetic ACE2-KO ( $n=6$ )	18 Weeks diabetic wildtype ( $n=6$ )	18 Weeks diabetic ACE2-KO ( $n=6$ )
Plasma Ang II ( $\text{pg ml}^{-1}$ )	$53.8 \pm 9.1$	$47.3 \pm 13.2$	$64 \pm 19.8$	$51.5 \pm 20.1$	$64.3 \pm 24.1$	$49.8 \pm 17.6$
Kidney Ang II ( $\text{pg per mg protein}$ )	$6.09 \pm 0.3$	$20.1 \pm 4.7^{\ddagger}$	$33.2 \pm 5.9^*$	$29.2 \pm 5.1^*$	$26.5 \pm 8.7^*$	$37 \pm 6.2^*$

Abbreviation: ACE2-KO, angiotensin-converting enzyme-2-knockout.

Data are presented as mean  $\pm$  s.e.m. \* $P < 0.05$  vs. genotype-matched nondiabetic group, and  $\ddagger P < 0.05$  vs. nondiabetic wildtype.

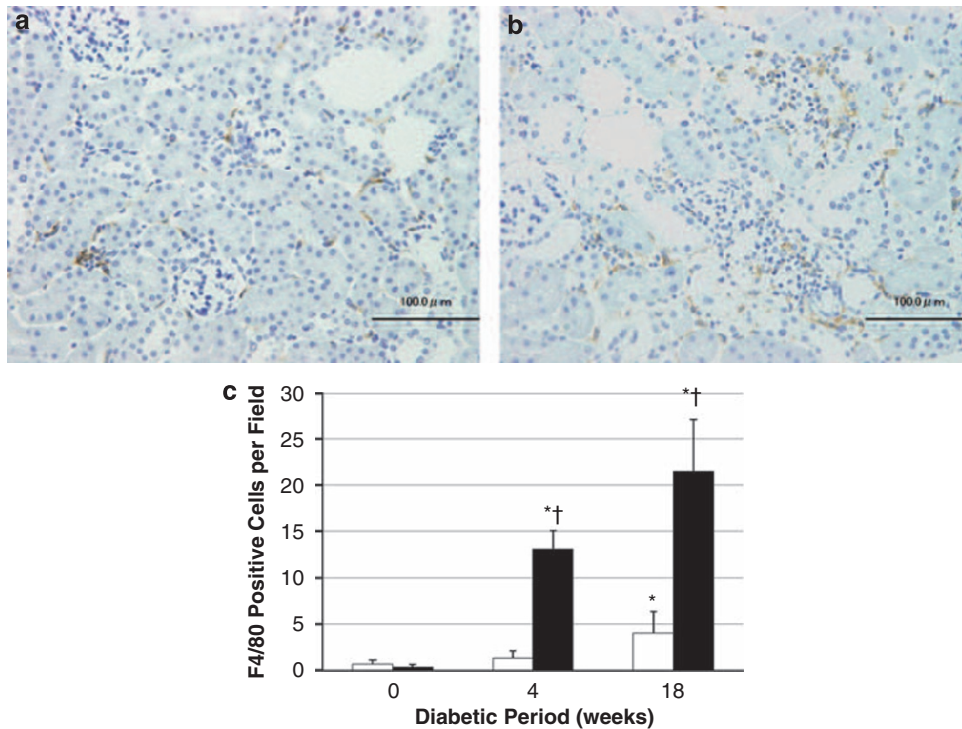


**Figure 2** (a–f) Representative photomicrographs of immunohistochemical staining for glomerular  $\alpha$ -SMA (a, b) and for glomerular/tubulointerstitial collagen IV (c–f) in the kidneys of wild-type (a) and ACE2-KO mice (b) with 18 weeks of diabetes. Original magnification  $\times 800$  (a–d) and  $\times 200$  (e, f). (g–i) Quantification. White bars=wildtype ( $n=6$ ); black bars=ACE2-KO ( $n=6$ ). Data are presented as mean  $\pm$  s.e.m. \* $P < 0.05$  compared with a genotype-matched baseline group, and  $\dagger P < 0.05$  compared with age-matched wildtype.

diabetic wild-type mice (Figure 4a–f, m). VEGF staining in the glomeruli showed a reverse pattern from the staining of nephrin; both types of diabetic mice showed a time-dependent increase in the expression of VEGF, which was more prominent in ACE2-KO mice than in wild-type mice (Figures 4g–l, n).

#### The effect of AT1 receptor blocker on nephropathy of diabetic ACE2-KO mice

Treatment with Olmesartan did not affect body weight, blood glucose and blood pressure, but significantly decreased albuminuria, serum markers and glomerular/tubulointerstitial injury in both types of



**Figure 3** (a, b) Representative photomicrographs of F4/80 immunostained kidney sections from wild-type (a) and ACE2-KO mice (b) 18 weeks after induction of diabetes. Original magnification  $\times 400$ . (c) Quantification. White bars=wildtype ( $n=6$ ); black bars=ACE2-KO ( $n=6$ ). Data are presented as mean  $\pm$  s.e.m. \* $P < 0.05$  compared with a genotype-matched baseline group, and † $P < 0.05$  compared with age-matched wildtype.

mice, although there remained significant differences between the genotypes (Figure 5, Table 3).

## DISCUSSION

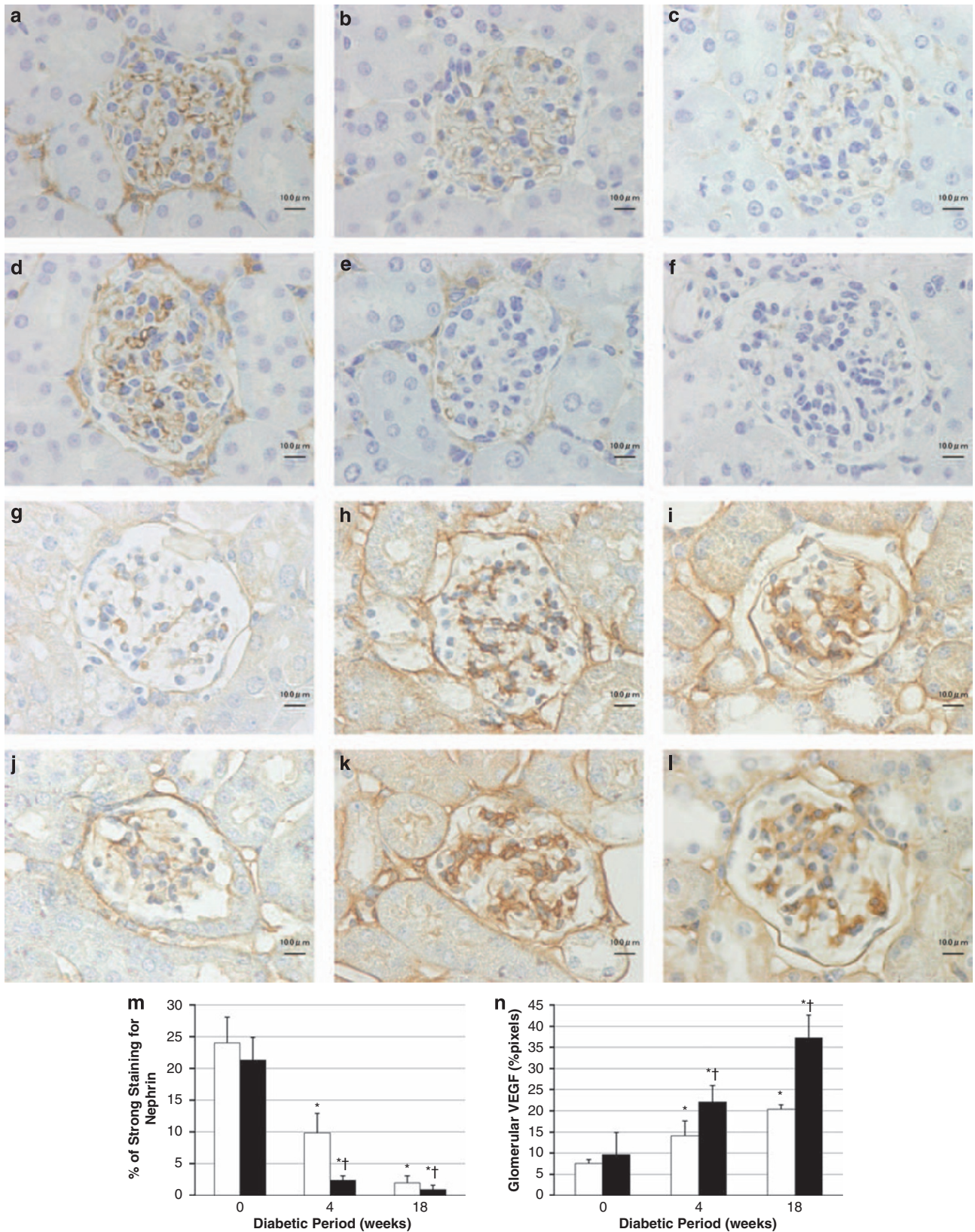
In our study, ACE2 disruption in streptozotocin-induced diabetes resulted in an earlier onset of albuminuria and accelerated progression of glomerulosclerosis, tubular injury, macrophage infiltration, and interstitial fibrosis with increased serum blood urea nitrogen and creatinine. These findings imply that ACE2 might continuously protect from glomerular and tubulointerstitial injury during development of diabetic nephropathy.

Our findings of diabetic ACE2-KO mice are comparable with those of Wong *et al.*<sup>15</sup> who used ACE2-KO mice crossed with Akita mice in models of type I diabetes, and showed more severe albuminuria and increased glomerular volume, mesangial matrix expansion and glomerular basement membrane thickness at 3 months of age. However, results of the recent report by Tikellis *et al.*<sup>16</sup> somewhat differed from our findings, where renal hypertrophy and fibrogenesis represented by renal mass increment and expression of PCNA, fibronectin and osteopontin were significantly attenuated by ACE2 disruption, but streptozotocin-induced diabetic ACE2-KO mice showed increased albuminuria 10 weeks after induction of diabetes in accordance with our study. These discrepancies might be due to differences in the source of ACE2-KO mice. Indeed, the ACE2-KO mice used in the previous study had a different cardiac phenotype from ours and other researchers, as reviewed in detail elsewhere.<sup>29</sup> At any rate, the different findings from the previous report led us to observe detailed functional and morphological changes in diabetic ACE2-KO mice over time to clarify that our findings resulted purely from a disruption of the gene.

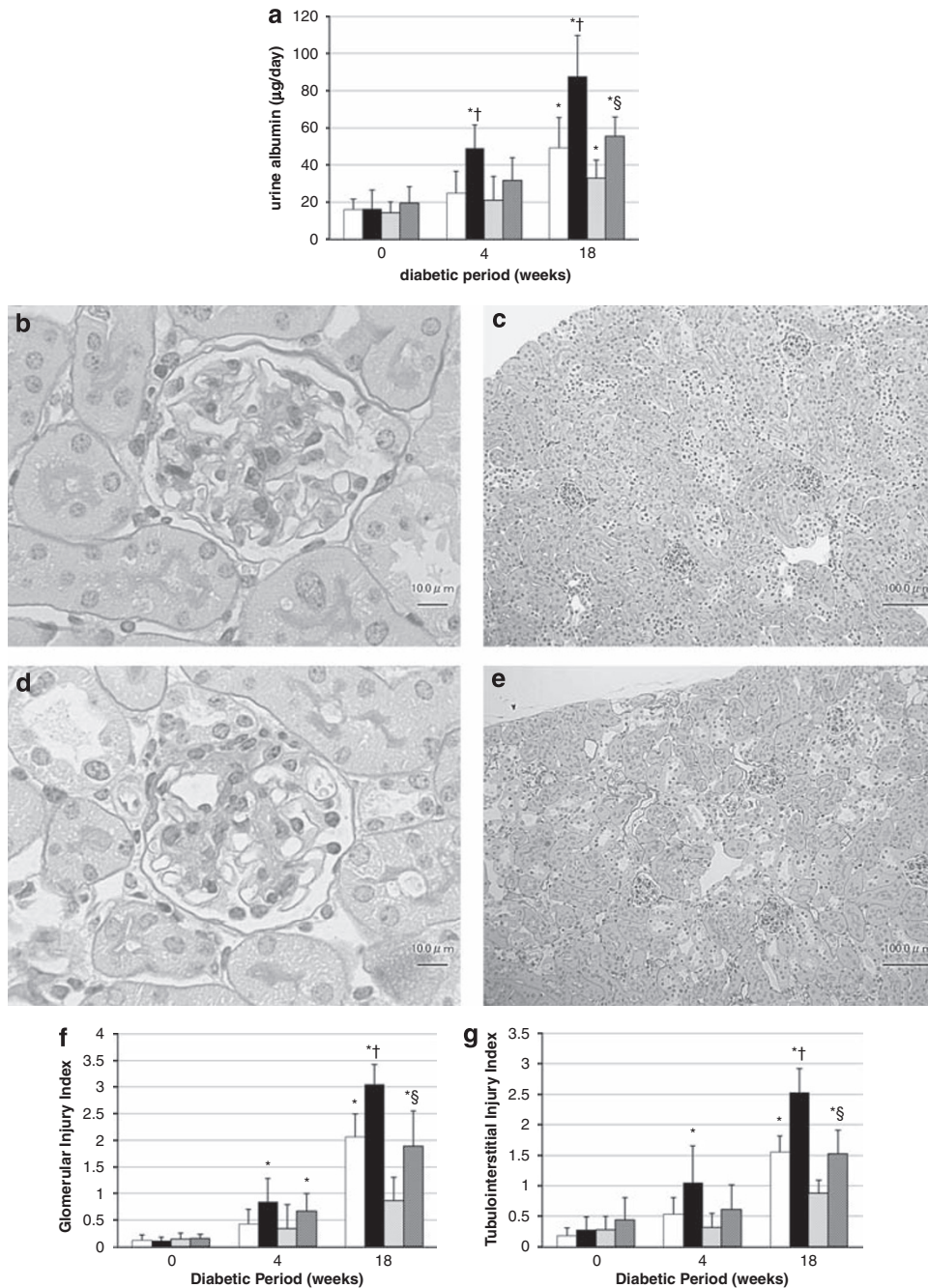
In immunohistological analysis, disruption of ACE2 enhanced the reduction of renal nephrin protein expression induced by diabetes. Glomerular epithelial cells (podocytes), as well as basement membrane and endothelial cells, are the main components of the filtration barrier. It is known that nephrin, a transmembrane protein in the immunoglobulin superfamily, is predominantly expressed in podocytes. Mutation of the nephrin gene is associated with severe congenital nephritic syndrome.<sup>30,31</sup> In diabetic nephropathy, renal nephrin mRNA and protein expression are downregulated.<sup>28,32</sup> Mechanism of nephrin downregulation in the diabetic milieu is unknown, but some reports suggest involvement of the RAS.<sup>26–28</sup> In this experiment, disruption of ACE2 might induce negative regulation of nephrin expression, leading to early onset of albuminuria.

We also assessed VEGF expression. VEGF is an important signaling molecule in both vasculogenesis and angiogenesis. It involves in excess filtration and development of microalbuminuria in diabetes by increasing vascular permeability.<sup>33–35</sup> In our study, diabetic ACE2-KO mice showed a prominent increase in glomerular VEGF expression, suggesting the involvement of RAS in the regulation of VEGF expression, in accordance with past reports.<sup>36,37</sup>

In some previous reports, ARB inhibited increase of albuminuria in diabetic mice with genetical disruption or pharmacological inhibition of ACE2, comparable with vehicle-treated diabetic control mice.<sup>8,15</sup> Our results showed that treatment with Olmesartan significantly ameliorated albuminuria as well as serum markers and renal histological change both in diabetic ACE2-KO and wild-type mice. As the difference in renal function between ACE2-KO and wild-type mice did not completely vanish by inhibition of Ang II signaling with Olmesartan, it is postulated that mechanism besides elevated Ang II might be involved in the renal phenotype of diabetic ACE2-KO mice. ACE2



**Figure 4** (a–f) Representative photomicrographs of immunohistochemical staining for nephrin in wild-type (a–c) and ACE2-KO mice (d–f); baseline (a, d), 4 weeks (b, e) and 18 weeks (c, f) after induction of diabetes. Original magnification  $\times 800$ . (g–l) Representative photomicrographs of immunohistochemical staining for VEGF in wild-type (g–i) and ACE2-KO mice (j–l); baseline (g, j), 4 weeks (h, k) and 18 weeks (i, l) after induction of diabetes. Original magnification  $\times 800$ . (m, n) Quantification. White bars=wildtype ( $n=6$ ); black bars=ACE2-KO ( $n=6$ ). Data are presented as mean  $\pm$  s.e.m. \* $P < 0.05$  compared with genotype-matched baseline group, and  $\dagger P < 0.05$  compared with age-matched wildtype.



**Figure 5** (a) Daily urinary albumin excretion of ACE2-KO and wild-type mice with or without administration of AT1 receptor blocker Olmesartan. (b–e) Representative photomicrographs of glomeruli and tubulointerstitium in PAS-stained kidney sections from ARB-treated wild-type (b, c) and ACE2-KO mice (d, e) at 18 weeks of diabetes. Original magnifications  $\times 800$  and  $\times 200$ . (f, g) Glomerular and tubulointerstitial injury index. White bars=wildtype; black bars=ACE2-KO; hatched bars=wild type+Olmesartan; slashed bars=ACE2-KO+ Olmesartan ( $n=10$  at each group). Data are presented as mean  $\pm$  s.e.m. \* $P<0.05$  compared with genotype-matched baseline group, † $P<0.05$  compared with age-matched wildtype, and § $P<0.05$  vs. Olmesartan-treated diabetic wildtype.

cleaves Ang II to produce Ang 1–7. Several physiological activities of Ang 1–7 are exerted through the Mas receptor, as previously reported.<sup>38</sup> In the kidney, Mas is expressed mainly in tubules in the renal cortex.<sup>39</sup> Some reports imply that Ang 1–7 has renoprotective effects. It was reported that Ang 1–7 attenuated sodium reabsorption caused by Ang II infusion,<sup>40</sup> and that intrarenal Ang 1–7 blockade decreased the glomerular filtration rate, renal plasma flow, and sodium excretion in 1K1C hypertension, as well as salt-depleted SD

and transgenic rats.<sup>41</sup> These data suggest that Ang 1–7 exerts counter-regulatory actions in response to the activation of the RAS. Thus, lack of Ang 1–7 might contribute to the phenotype of our diabetic ACE2-KO mice. However, determining the contribution of decreased Ang 1–7 to renal damage is difficult in our model; studies in Mas receptor KO mice might be useful in elucidating any renal-protective effects.

In the nondiabetic state, ACE2-KO mice showed higher kidney Ang II concentrations than did wild-type mice, consistent with Crackower



**Table 3 Physiological and biochemical parameters of ARB-treated or untreated 18 weeks diabetic mice and nondiabetic control**

	Nondiabetic wildtype (n=10)	Nondiabetic ACE2-KO (n=10)	18 Weeks diabetic wildtype (n=10)	18 Weeks diabetic ACE2-KO (n=10)	18 Weeks diabetic wildtype treated with Olmesartan (n=10)	18 Weeks diabetic ACE2-KO treated with Olmesartan (n=10)
Body weight (g)	32 ± 2.9	34 ± 1.9	28 ± 5.4	30 ± 4.7	31 ± 5.3	31 ± 3.6
Mean blood pressure (mm Hg)	77 ± 12.4	84 ± 6.72	76 ± 11.3	88 ± 8.31	64 ± 19.8	70 ± 12.4
Blood glucose (mmol l <sup>-1</sup> )	14.2 ± 1.38	12.4 ± 2.62	24.0 ± 3.85*	25.2 ± 4.81*	28.2 ± 5.72*	21.9 ± 6.37*
Serum creatinine (μmol l <sup>-1</sup> )	16 ± 1.24	13 ± 2.75	23 ± 0.97	36 ± 5.8* <sup>†</sup>	20 ± 3.72	27 ± 4.31* <sup>§</sup>
Serum urea nitrogen (mmol l <sup>-1</sup> )	9.7 ± 0.47	10 ± 0.93	13 ± 0.41*	19 ± 1.3* <sup>†</sup>	8 ± 1.75	15 ± 2.29* <sup>§</sup>
Serum Na (mmol l <sup>-1</sup> )	142 ± 2.0	139 ± 3.8	144 ± 11	145 ± 9.1	143 ± 7.5	141 ± 7.7
Kidney weight (mg)	178 ± 11.6	166 ± 21.0	234 ± 24.1*	242 ± 36.7*	176 ± 30	203 ± 42.5
Heart weight (mg)	137 ± 8.50	125 ± 16.0	126 ± 19.5	133 ± 25.3	118 ± 10.8	124 ± 19.3

Abbreviations: ACE2-KO, angiotensin-converting enzyme-2-knockout; ARB, angiotensin II receptor blocker.

All groups of mice were age-matched. Data are presented as mean ± s.e.m. \**P*<0.05 vs. genotype-matched nondiabetic group, <sup>†</sup>*P*<0.05 vs. untreated diabetic wildtype, and <sup>§</sup>*P*<0.05 vs. ARB-treated diabetic wildtype.

*et al.*<sup>25</sup> However, after the administration of streptozotocin, kidney Ang II concentrations increased in both ACE2-KO and wild-type mice, but no significant difference was observed between the groups. These findings are consistent with Wong *et al.*<sup>15</sup> who measured tissue Ang II concentrations in whole kidney. ACE2-KO mice showed similar levels of tissue Ang II to wild-type mice in the diabetic state. As previously discussed, whole kidney Ang II concentrations might not reflect local Ang II activity, because of the complex kinetics of Ang II production and distribution in the kidney.<sup>15,42</sup> However, further investigations will be required to determine the influence of ACE2 on Ang II distribution in kidney.

In conclusion, ACE2 deletion caused accelerated renal damage in the development of diabetes, including not only glomerulopathy accompanied by albuminuria but also tubulointerstitial injury and eventual renal dysfunction. In addition to reducing Ang II, yielding Ang 1–7 might be involved in ACE2-mediated renal protection.

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