Murine recombinant angiotensin-converting enzyme 2 attenuates kidney injury in experimental Alport syndrome

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Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase in the renin-angiotensin system that catalyzes the breakdown of angiotensin II to angiotensin 1-7. We have reported that ACE2 expression in the kidney is reduced in experimental Alport syndrome but the impact of this finding on disease progression has not been studied. Accordingly, we evaluated effects of murine recombinant ACE2 treatment in *Col4a3* knockout mice, a model of Alport syndrome characterized by proteinuria and progressive renal injury. Murine recombinant ACE2 (0.5 mg/kg/day) was administered from four to seven weeks of age via osmotic mini-pump. Pathological changes were attenuated by murine recombinant ACE2 treatment which ameliorated kidney fibrosis as shown by decreased expression of COL1α1 mRNA, less accumulation of extracellular matrix proteins, and inhibition of transforming growth factor-β signaling. Further, increases in proinflammatory cytokine expression, macrophage infiltration, inflammatory signaling pathway activation, and heme oxygenase-1 levels in *Col4a3* knockout mice were also reduced by murine recombinant ACE2 treatment. Lastly, murine recombinant ACE2 influenced the turnover of renal ACE2, as it suppressed the expression of tumor necrosis factor-α converting enzyme, a negative regulator of ACE2. Thus, treatment with exogenous ACE2 alters angiotensin peptide metabolism in the kidneys of *Col4a3* knockout mice and attenuates the progression of Alport syndrome nephropathy.

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Kidney fibrosis, associated with renal failure, is known to be the common final stage of progressive renal disease. Alport syndrome (AS) is a hereditary nephropathy characterized by progressive kidney fibrosis.[1–3] Studies in Alport mice suggest that angiotensin inhibition not only has antiproteinuric effects but also suppresses cytokine and collagen production as well as tubulointerstitial fibrosis and inflammation.[4] Inhibitors of the renin-angiotensin system (RAS), including angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers, have been demonstrated to slow kidney disease progression in both experimental and clinical AS.[3,5–8]

Recently we have demonstrated that the RAS activation–associated increase in angiotensin II (Ang II) and decrease in angiotensin-(1–7) (Ang-[1–7]) were caused by decreased intrarenal angiotensin-converting enzyme 2 (ACE2) expression and activity and may play an important pathogenic role in AS.[9] Ang II has been shown to trigger cleavage of ACE2 by tumor necrosis factor-α (TNFα)–converting enzyme (TACE) and its shedding as a soluble form from the membrane in the myocardium.[10] In the AS model, the mechanism of decreased ACE2 expression has not been established, and TACE may play a role in this process.

Loss of ACE2 is associated with age-dependent development of glomerulosclerosis and albuminuria,[11] and exacerbation of diabetic kidney injury in mice.[12] We have recently reported that mice with experimental AS exhibit a loss of ACE2 in the brush border of proximal tubules in association with a rise in intrarenal Ang II and a decrease in Ang-(1–7) levels. We also observed that administration of recombinant ACE2 attenuated these changes in peptide levels.[9] Taken together, these studies suggest that treatment with ACE2 may limit kidney injury in AS. Thus, we treated *Col4a3*$^{-/-}$ mice with murine recombinant ACE2 (mrACE2) after weaning from 4 to 7 weeks of age, before mortality became a significant confounding factor, and studied the effects of mrACE2

on the development of kidney fibrosis, inflammation, and oxidative stress.

RESULTS

Treatment with mrACE2 attenuated morphological changes in experimental AS

At 7 weeks of age, *Col4a3*$^{-/-}$ mice had lower body weights but higher kidney-to-body weight ratios and 24-hour urinary outputs than wild-type mice (Table 1). These parameters were not significantly different between mrACE2- and saline-treated *Col4a3*$^{-/-}$ mice. Plasma ACE2 protein levels decreased in *Col4a3*$^{-/-}$ mice compared with wild-type mice; however, mrACE2 administration increased plasma ACE2 protein (Supplementary Table S1). In a separate set of experiments performed in wild-type mice, we found that treatment with mrACE2 increased plasma ACE2 activity (Supplementary Figure S1). In the 3 groups of mice, there were no significant differences in urinary ACE2 protein levels ($P = 0.15$) (Supplementary Table S1). ACE2 mRNA and protein were decreased in kidneys of *Col4a3*$^{-/-}$ mice (Figure 1a, c, and d). TACE protein was increased and there was a trend toward increased TACE mRNA in kidneys of *Col4a3*$^{-/-}$ mice (Figure 1b, c, and e). ACE2 and TACE activity levels complemented mRNA and protein measurements (Figure 1f and g, Supplementary Figure S2). Accordingly, we have previously shown[9] that treatment with mrACE2 was able to attenuate the increase in Ang II and induce a corresponding elevation of Ang-(1–7) levels in the kidneys of *Col4a3*$^{-/-}$ mice (Supplementary Table S2), and now we show that treatment with mrACE2 partially reversed the changes in renal levels of ACE2 and TACE.

Studies have suggested that increasing ACE2 expression or activity has important effects on organ function. For example, Yamazato and colleagues showed that overexpression of ACE2 in the brain lowered blood pressure in spontaneously hypertensive rats.[13] Therefore, we sought to assess the effect of mrACE2 administration on kidney function and morphology. Heart rate and mean arterial pressure were significantly elevated in *Col4a3*$^{-/-}$ mice compared with wild-type controls (Figure 2a–d). Although the differences did not reach statistical significance, there was a numerical decrease in blood pressure after treatment with mrACE2, suggesting that mrACE2 may have an antihypertensive effect in this model.

Table 1 | Whole animal data

	WT + saline	KO + saline	KO + mrACE2
Body weight (g)	21.23 ± 0.54	18.45 ± 0.70[a]	19.03 ± 0.49[a]
Kidney weight (g)	0.162 ± 0.005	0.166 ± 0.005	0.171 ± 0.008
KW (g)/BW (kg)	7.62 ± 0.09	9.04 ± 0.27[a]	8.97 ± 0.22[a]
Urine output (ml)	0.58 ± 0.16	2.94 ± 0.20[a]	2.25 ± 0.27[a]

BW, body weight; KO, *Col4a3*$^{-/-}$; KW, kidney weight; KW/BW, kidney weight–to–body weight ratio; mrACE2, murine recombinant angiotensin-converting enzyme 2; WT, wild type.
BW and KW were recorded at the time of killing at 7 weeks of age. Urine output was measured for 24 hours the day before killing. For all groups, $n = 8$. Values are presented as mean ± SE.
[a]$P < 0.05$ compared with WT + saline.

Tissue sections were stained with periodic acid–Schiff (PAS) and assessed for glomerular injury (Figure 2e and f). Interestingly, the extent of glomerular involvement was quite variable in *Col4a3*$^{-/-}$ mice with or without mrACE2 treatment. Damage to the tubular compartment could also be seen from PAS-stained sections, and levels of urinary neutrophil gelatinase-associated lipocalin (NGAL) were used to quantify tubular injury. The urinary NGAL excretion rate was significantly higher in *Col4a3*$^{-/-}$ mice, but was reduced by mrACE2 treatment (Figure 2g). The urinary albumin excretion rate was profoundly elevated in *Col4a3*$^{-/-}$ mice and was significantly reduced by mrACE2 treatment (Figure 2h). As expected, values for plasma creatinine tended to increase in *Col4a3*$^{-/-}$ mice, although there was considerable variability in this measurement. Although urinary albumin excretion was reduced by mrACE2 treatment, mean values for plasma creatinine and blood urea nitrogen did not decline (Supplementary Table S3). To further assess the effects of mrACE2 on kidney function, creatinine clearance was calculated (Figure 2i). Treatment with recombinant ACE2 led to a numerical improvement in creatinine clearance that failed to reach statistical significance ($P = 0.07$).

Treatment with mrACE2 ameliorated kidney fibrosis in experimental AS

Kidney cortical expression of collagen type I alpha 1, transforming growth factor beta 1 (TGF-β1), alpha smooth muscle actin (αSMA), and fibronectin was quantified by quantitative polymerase chain reaction. The mRNA levels of these fibrotic markers were higher in *Col4a3*$^{-/-}$ mice than in wild-type mice but showed a decreasing trend after mrACE2 treatment (Figure 3). In agreement with mRNA expression data, picrosirius red staining revealed increased collagen deposition in glomeruli and the tubulointerstitium of *Col4a3*$^{-/-}$ mice, which was attenuated by mrACE2 treatment (Figure 4a and b). Immunohistochemical staining and Western blot for αSMA, a myofibroblast marker, also showed increased positivity of this profibrotic protein in kidney tissue of *Col4a3*$^{-/-}$ mice (Figure 4a, c, and d). Treatment with mrACE2 reduced the level of αSMA in *Col4a3*$^{-/-}$ mice.

We also investigated the effect of mrACE2 on TGF-β signaling in *Col4a3*$^{-/-}$ mice. There were no differences in TGF-β1 tissue levels between the 3 groups of mice, but there was evidence of TGF-β pathway activation in *Col4a3*$^{-/-}$ mice as indicated by higher TGF-β1 levels in urine (Figure 5a–c). Phosphorylation of downstream signal mediators SMAD2 and SMAD3, and expression of SMAD4, were also increased in kidneys of *Col4a3*$^{-/-}$ mice (Figure 5d–g). Recent work has shown that TGF-β can also exert its functions through other noncanonical signal transduction pathways, including the RhoA and mitogen-activated protein kinase (MAPK) pathway.[14,15] We, therefore, investigated extracellular signal–regulated kinase (ERK) and found a trend toward increased activation of ERK in *Col4a3*$^{-/-}$ mice (Figure 5d and h). With mrACE2 administration, levels of urinary TGF-β1, SMAD4,