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Original Article

LCZ696 (Sacubitril/valsartan) ameliorates oxidative stress, inflammation, fibrosis and improves renal function beyond angiotensin receptor blockade in CKD

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Abstract: Progressive deterioration of kidney function in chronic kidney disease (CKD) is mediated by hypertension, oxidative stress, inflammation, and fibrosis. Renin-angiotensin blockade is commonly used to retard CKD progression. In addition, vasoactive peptides have been shown to reduce blood pressure and exert antioxidant, anti-inflammatory and anti-fibrotic effects. We hypothesized that administration of LCZ696 (sacubitril/valsartan) is more effective than valsartan alone in slowing progression of CKD. Male Sprague Dawley rats underwent sham surgery or 5/6 nephrectomy and after two weeks the CKD animals were randomized to no treatment, valsartan (30 mg/kg), or LCZ696 (60 mg/kg) daily by gavage. Serum, urine and kidney tissue analyses were performed after 8 weeks. The untreated CKD rats exhibited hypertension, proteinuria, tubular and glomerular damage, upregulation of pro-inflammatory, pro-oxidant and pro-fibrotic pathways; reduction in nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2) and its key target products. LCZ696 administration improved renal function and histology and attenuated most of the molecular markers of oxidative stress, inflammation and fibrosis. Furthermore, LCZ696 was more effective than valsartan therapy alone in delaying the progression of kidney disease. Future clinical trials are needed to determine the safety and efficacy of this agent in treatment of patients with CKD.

Keywords: Chronic kidney disease, natriuretic peptides, LCZ696, oxidative stress, inflammation, fibrosis

Introduction

Chronic kidney disease (CKD) is an irreversible and progressive condition marked by sustained kidney damage and a significant increase in the risk of cardiovascular (CV) and overall mortality. Patients with CKD have a significantly increased risk of mortality with CV disease being a major contributor to the high risk of death in this population [1]. It is also important to note that the prevalence of CKD in the United States and worldwide is increasing. For example, recent projections indicate that China may have a higher prevalence of treated end-stage renal disease (ESRD) than the United States in the near future [2, 3]. Therefore, CKD is a worldwide epidemic with significant healthcare, societal and economic implications. Unfortunately, treatment strategies aimed at preventing and slowing the progression of CKD are limited. Consequently, there is significant interest in developing novel therapeutic options for this disease complex.

Progressive deterioration of kidney function in CKD is mediated by a constellation of pathophysiologic processes including systemic and glomerular hypertension and hyperfiltration, oxidative stress, inflammation, and renal fibrosis [4, 5]. The CKD-related oxidative stress is caused by excessive reactive oxygen species (ROS) production, depletion of antioxidants and impairment of the antioxidant pathways. Significant upregulation of nicotinamide adenine dinucleotide phosphate-oxidase (NAD[P]H oxidase), increased production of oxygen free radicals, down-regulation of antioxidant enzymes and inhibition of the nuclear factor erythroid

2-related factor 2 (Nrf-2) pathway are some of the major features of CKD which work in concert to promote tissue oxidative stress [6]. In turn, oxidative stress causes inflammation and fibrosis through activation of the redox-sensitive pro-inflammatory signal transduction pathways and by direct toxic effects of reactive oxygen species (ROS) [7]. Inflammation can cause further oxidative stress creating a vicious cycle whereby each begets and amplifies the other leading to chronic damage and progressive renal injury. Furthermore, glomerular and systemic hypertension, oxidative stress, inflammation and fibrosis, not only play a major role in pathogenesis and progression of renal injury, they are also major contributors to other complications of CKD including atherosclerosis, CV disease and death [8-10]. Therefore, therapeutic strategies aimed at alleviating these pathogenic processes hold significant potential for improving outcomes in patients with CKD.

One promising therapeutic option which has shown significant potential utility in treatment of CKD relies on modulation of natriuretic peptides (NPs). Enhancement of NPs (mainly atrial and brain type natriuretic peptide [ANP and BNP respectively]) can target common underlying pathologic processes involved in progression of CKD including oxidative stress, inflammation, endothelin-1 dysfunction and fibrosis [11-18]. While many studies have evaluated delivery of NPs by infusion, the level of endogenous NPs can also be augmented via inhibition of neprilysin [also known as neutral endopeptidase (NEP)] which is the key enzyme responsible for their degradation [19]. Neprilysin is a membrane-bound metalloproteinase which is present in several tissues but is most abundant in the brush border of proximal renal tubular cell. Pharmacologic inhibition of neprilysin leads to increased levels of vasoactive peptides including NPs, which have been shown to be effective in prevention of renal injury and slowing progression of renal disease in the laboratory and clinical setting [20-23]. However, clinical utility of previous formulations of neprilysin inhibitors, which were combined with angiotensin converting enzyme inhibitors (ACEI), was limited due to reduce the breakdown of bradykinin production and subsequent risk of angioedema. More recently, a new drug has been developed which combines two active components: the angiotensin receptor blocker (ARB) valsartan (VAL) and the neprilysin inhibitor (NEPi) prodrug sacubitril in a 1:1 molar ratio in the form of a sodium salt complex. In the PARADIGM-HF trial, treatment with sacubitril/valsartan resulted in a reduction of heart failure (HF) hospitalizations and risk of CV death in patients with HF and reduced ejection fraction (HFrEF) when compared with enalapril. This medication, which is called LCZ696 (LCZ), has been shown to provide a survival benefit in patients with HF without increased risk of serious angioedema [24]. Furthermore, LCZ696 was found to have cardio and renoprotective effects in animal models and patients with heart failure [25-28].

Based on the above observations, we hypothesized that administration of LCZ in an animal model of CKD may delay progression of kidney disease. We used renal mass reduction via surgical removal of one and two third of the contralateral kidney (surgical 5/6 nephrectomy) to evaluate the impact of LCZ on progression of renal disease due to nephron mass reduction and hyperfiltration. In addition, we included a group of CKD animals treated with VAL alone to further delineate any potential benefit of LCZ beyond that conferred by renin-angiotensin-aldosterone system (RAAS) blockade.

Materials and methods

Study groups

Male Sprague-Dawley rats with an average body weight of 250 g (Charles River Labs, Raleigh, NC, USA) were used in this study. Animals were housed in a climate-controlled vivarium with 12 h day/night cycles with food and water *ad libitum*. The animals underwent sham surgery (n=6) or 5/6 nephrectomy and after two weeks the CKD animals were randomized in three groups: no treatment (CKD) (n=10), VAL 30 mg/kg (n=12) or LCZ 60 mg/kg (n=12). VAL or LCZ was dissolved in sesame oil for 30 minutes before each treatment and animals received respective drugs by oral gavage every day during the 8 weeks treatment period. Meanwhile, animals from the control and CKD groups received the same volume of sesame oil via oral gavage every day during the 8 weeks treatment period. At the beginning and the final week of the study, the animals were placed in metabolic cages for a 24 h urine collection and systolic blood pressure (SBP) was measured by

Table 1. General Data in the CTL, CKD, LCZ, and VAL groups

	CTL (N=6)	CKD (N=10)	LCZ (N=12)	VAL (N=12)
SBP (mmHg)	90.0±1.5[a,b,c]	143.1±2.1[a,d,e]	115.6±2.2[b,d]	123.1±3.1[c,e]
ΔBody Weight (g)	194.1±27.1[a,b,c]	143.3±35.0[a]	141.4±30.2[b]	131.5±28.7[c]
BUN (mg/dL)	8.6±0.2[a,b,c]	53.8±1.8[a,d]	45.2±1.7[b,d,f]	52.5±2.3[c,f]
Serum Creatinine (mg/dL)	0.34±0.02[a,b,c]	1.11±0.10[a,e]	0.81±0.06[b,d,f]	1.11±0.07[c,f]
24 h Urine Protein (mg/dL)	4.6±0.5[a]	20.5±2.3[a,d,e]	9.0±0.7[d]	9.1±1.2[e]
Urine Ptn/Cr Ratio (mg/g)	3.3±0.9[a,b,c]	24.7±1.4[a,d,e]	13.2±0.9[b,d]	13.7±1.5[c,e]
Kidney/Body Weight Ratio	0.003±0.0004[a]	0.005±0.0005[a,d,e]	0.003±0.0001[d]	0.003±0.0002[e]

(a) CTL vs. CKD: $P<0.05$; (b) CTL vs. LCZ: $P<0.05$; (c) CTL vs. VAL: $P<0.05$; (d) CKD vs. LCZ: $P<0.05$; (e) CKD vs. VAL: $P<0.05$; (f) VAL vs. LCZ: $P<0.05$.

tail plethysmography as described previously [29]. For surgical procedures and euthanasia, the animals were placed into a sealed anesthesia induction chamber under 5% isoflurane (Piramal Clinical Care, Bethlehem, PA, USA)/oxygen gaseous mixture to induce sedation and maintained at 2-4%. Before start of 5/6 nephrectomy surgical procedures, all animals received 0.05 mg/kg of buprenorphine (Reckitt Benckiser Pharmaceutical Inc., Richmond, VA, USA) for pain relief. Animals were euthanized by cardiac exsanguination and the kidney were immediately removed and processed for histological evaluation and Western blot analyses. Serum urea was measured by QuantiChrom™ Urea Assay Kit (BioAssay Systems, Hayward, CA, USA). Urinary creatinine was measured by QuantiChrom™ Creatinine Assay Kit (BioAssay Systems, Hayward, CA, USA). All kits were used according to manufactures instructions. Serum creatinine was measured by capillary electrophoresis using PA800 Plus Pharmaceutical Analysis System (Beckman Coulter) from George M O'Brian Kidney Research Core at University of Texas Southwestern Medical Center. This study was approved by the Ethical Committee of University of California Irvine and all methods were performed in accordance with the Guide for the Care and Use of Laboratory Animals defined by Ethical Committee of University of California Irvine.

Histologic analysis

The kidneys were removed after sacrifice and fixed with 10% formalin solution. Histologic sections were stained with hematoxylin and eosin (H&E), and Masson trichrome. Glomerulonephritis was graded on 0-4 scale and tubulointerstitial injury was graded on a 0-5 scale, as detailed previously [30, 31].

Western blot analyses

Cytoplasmic and nuclear extracts of the renal tissue were prepared as described previously [32]. The protein of interest in the cytoplasmic and/or nuclear fractions of the kidney tissue was measured by Western blot analysis as previously described [33, 34] using the following antibodies. Rabbit against rat nuclear factor-κB p65 (NF-κB p65) (Cat # ab16502), monocyte chemotactic protein 1 (MCP-1) (Cat # ab25124), inducible nitric oxide synthase (iNOS) (Cat # ab3526), NADPH-oxidase 4 (NOX4) (Cat # ab133303), $Gp91^{phox}$ (Cat # ab129068), nitrotyrosine (Cat # ab183391), Nrf-2 (Cat # ab31163), copper-zinc superoxide dismutase (Cu/Zn-SOD) (Cat # ab13498), cyclooxygenase-2 (COX-2) (Cat # 15191), heme oxygenase-1 (HO-1) (Cat # ab13248), catalase (Cat # ab676110), myeloperoxidase (MPO) (Cat # ab65871), endothelial nitric oxide synthase (eNOS) (Cat # ab95254), transforming growth factor-β (TGF-β) (Cat # ab92486), plasminogen activator inhibitor-1 (PAI-1) (Cat # ab66705), and α-smooth muscle actin (α-SM actin) (Cat # ab5694) were purchased from Abcam (Cambridge, MA). Antibody against glutathione peroxidase (GPX) (Cat # AF3798) was obtained from R&D Systems (Minneapolis, MN). Mouse antibodies against histone H3 (Cat # ab32356) and GAPDH (Cat # ab8245) from Abcam (Cambridge, MA) were used for measurements of housekeeping proteins for nuclear and cytosolic target proteins, respectively.

Statistical analysis

Data are presented as mean ± SEM. One-way ANOVA and multiple comparisons were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA). Tukey's post-test was

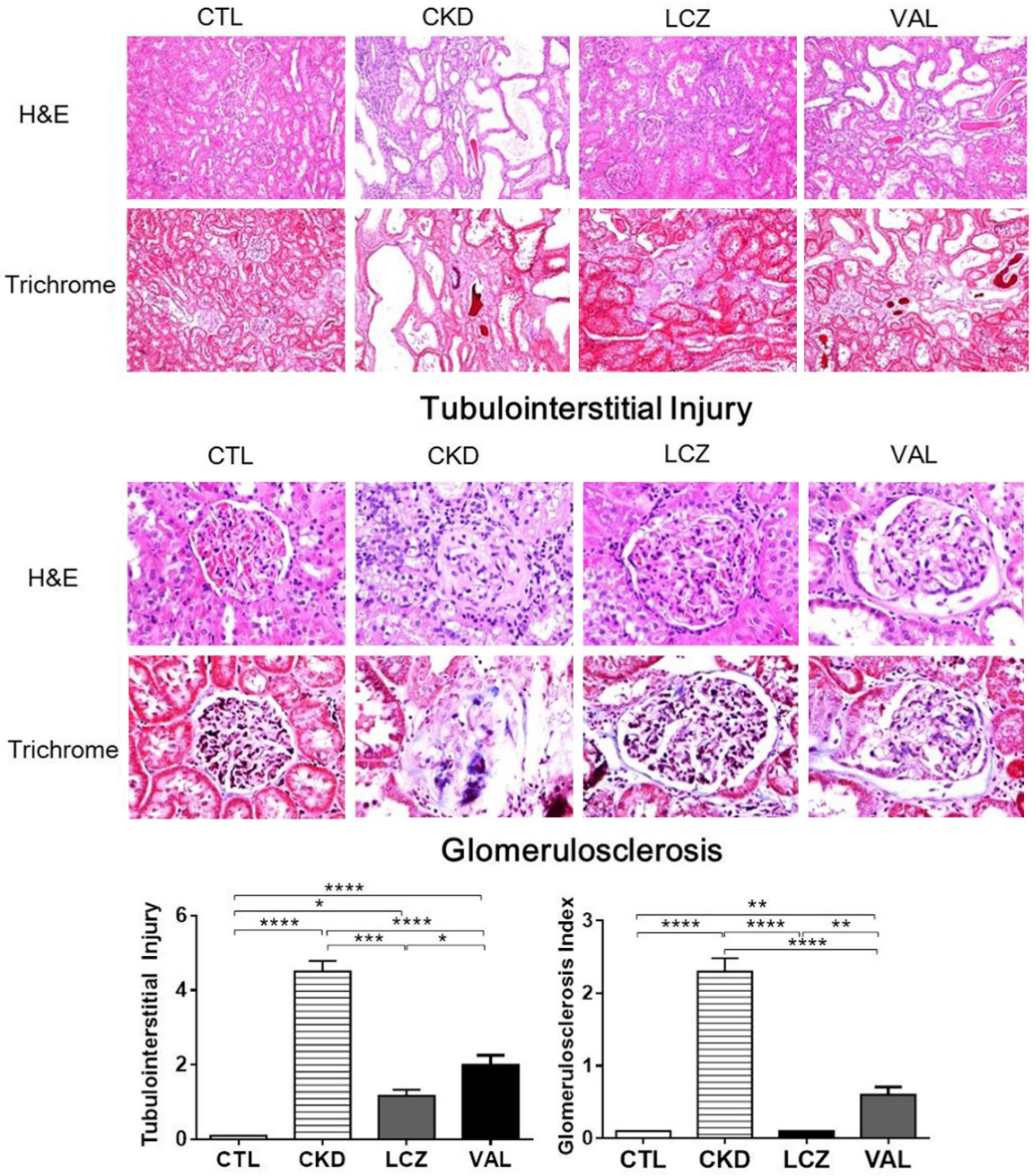

Figure 1. Representative photomicrographs of the H&E and Trichrome stained kidney sections in control (n=6), CKD (n=9), LCZ (n=12) and VAL (n=12) treated animals. Data depicting glomerulosclerosis index and tubulointerstitial injury in different groups. Data are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001.

used to determine differences between the groups. *P* values less than 0.05 were considered significant.

Results

General data

Data are shown in **Table 1**. As expected, the CKD animals had a modest rise in systolic arterial pressure when compared with the controls (**Table 1**). In addition, renal mass reduction resulted in significant hypertrophy of the remnant kidney. Both VAL and LCZ administration resulted in a significant reduction of SBP and reduction in remnant renal hypertrophy. Furthermore, urinary protein excretion which was markedly elevated in the untreated CKD animals was significantly lowered by VAL and LCZ

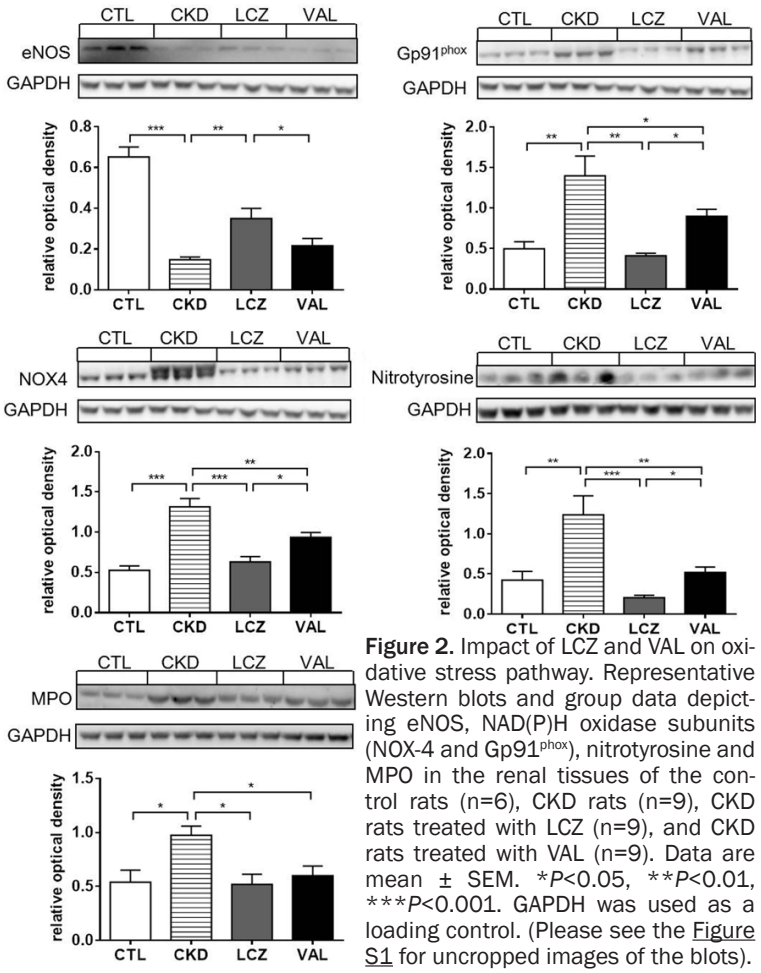

Figure 2. Impact of LCZ and VAL on oxidative stress pathway. Representative Western blots and group data depicting eNOS, NAD(P)H oxidase subunits (NOX-4 and Gp91^{phox}), nitrotyrosine and MPO in the renal tissues of the control rats (n=6), CKD rats (n=9), CKD rats treated with LCZ (n=9), and CKD rats treated with VAL (n=9). Data are mean ± SEM. *P<0.05, **P<0.01, ***P<0.001. GAPDH was used as a loading control. (Please see the Figure S1 for uncropped images of the blots).

treatments. Although the difference did not reach statistical significance, the mean arterial pressure in LCZ group (115.6±2.2 mmHg) was lower than that found in the VAL group (123.1±3.1 mmHg). There was no difference between VAL and LCZ in the degree of improvement in renal hypertrophy and proteinuria. As expected, the CKD animals had elevated plasma urea and creatinine concentrations and LCZ treatment resulted in a significant decrease in these parameters when compared with the untreated and VAL-treated groups. Though the untreated CKD animals gained significantly less body weight than the control, neither LCZ nor VAL treatment improved this parameter.

Histological findings

Representative photomicrographs of H&E and Masson's trichrome stained kidney sections are shown in **Figure 1**. The kidney tissues in untreated CKD rats showed severe tubulointerstitial injury and significant increases in glomerulosclerosis index. The severity of tubulointerstitial injury and glomerulosclerosis in VAL and LCZ treated animals was significantly reduced when compared to the untreated CKD group. The degree of im-provement in these indices was more substantial with LCZ treatment when compared with VAL alone.

Impact of LCZ and VAL on oxidative stress and inflammatory pathways

Data are shown in **Figures 2** and **3**.

The untreated CKD group showed a significant decrease in eNOS and accumulation of nitrotyrosine in kidney tissue, pointing to presence of oxidative stress and generation of reactive nitrogen species. This was accompanied by a significant upregulation of NOX-4 and gp91^{phox} subunits of NAD(P)H oxidase, iNOS, COX-2, and MPO pointing to increased production of reactive oxygen, nitrogen, and halogen species in the remnant kidney tissue. While VAL treatment reversed or markedly attenuated these abnormalities, these effects were significantly enhanced in CKD animals treated with LCZ for all of the markers mentioned except for iNOS and MPO. Furthermore, the remnant kidney showed a significant increase in nuclear translocation of p65, pointing to activation of NF-κB, the general transcription factor for numerous pro-inflammatory molecules including MCP-1 which was also significantly elevated. Although VAL attenuated NF-κB activation and lowered MCP-1, LCZ treatment improved these indices beyond the level observed by RAAS blockade alone.

Impact of LCZ and VAL on Nrf2 pathway

Data are shown in **Figure 4**.

The remnant kidney from the CKD animals showed a significant reduction in nuclear trans-

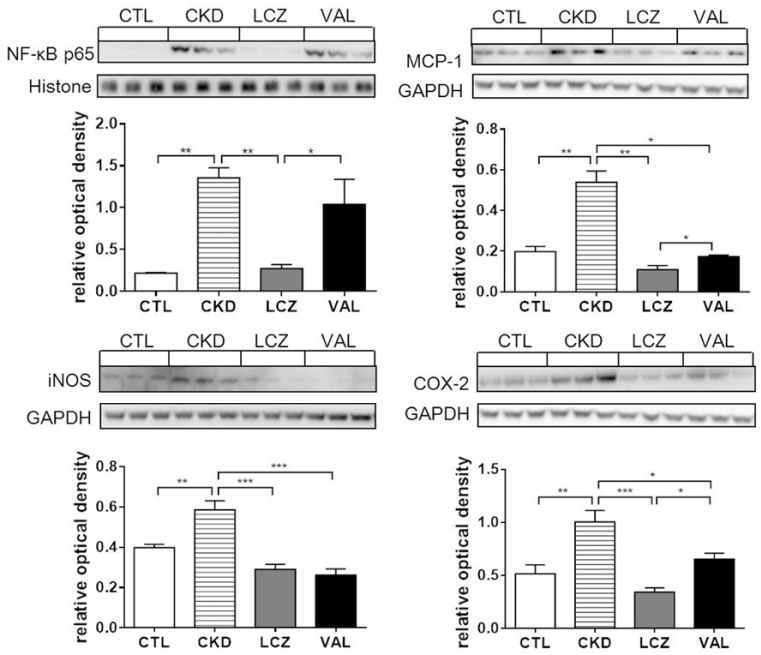

Figure 3. Impact of LCZ and VAL on inflammatory pathway. Representative Western blots and group data depicting nuclear content of p65 active subunit of NF-κB and protein abundance of MCP-1, iNOS and COX-2 in the renal tissues of the control rats (n=6), CKD rats (n=9), CKD rats treated with LCZ (n=9), and CKD rats treated with VAL (n=9). Data are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.Histone was used as a loading control for nuclear extracts. GAPDH was used as a loading control for cytoplasmic extracts. (Please see the Figure S2 for uncropped images of the blots).

location of Nrf2, significant increase in cytoplasmic Keap1 abundance and down-regulation of its key target gene products including Cu/Zn-SOD, catalase, GPX, and HO-1. These results are in line with our earlier studies [33, 34] and point to impairment of the Nrf2 pathway which makes a major contribution to the prevailing oxidative stress and inflammation in CKD. While administration of VAL alone was only effective in significantly improving Cu/Zn-SOD and HO-1 protein abundance, LCZ treatment resulted in a significant reduction of cytoplasmic Keap1 abundance, increased nuclear translocation of Nrf2 and increased abundance of its measured target protein products.

Impact of LCZ and VAL on fibrosis pathway

Data are shown in **Figure 5**.

As expected, activation of inflammatory and oxidative pathways in the kidneys of CKD animals was accompanied by a significant increase in renal protein abundance of PAI-1, TGF-β and α-SM actin, pointing to activation of fibrotic pathways. While administration of VAL attenuated the mentioned abnormalities, LCZ treatment significantly augmented the beneficial effects seen with VAL alone.

Discussion

In accordance with our previous studies, we found that renal mass reduction by 5/6 nephrectomy resulted in progressive proteinuria, glomerulosclerosis and tubulointerstitial injury. This was accompanied by a significant upregulation of inflammatory and oxidative pathways which are partly driven by impaired activity of Nrf2 system and reduction of its antioxidant and cytoprotective target proteins. In addition, there was a significant increase in pro-fibrotic proteins, likely due to increased inflammation and oxidative stress. It is well known that CKD is associated with significant oxidative stress, inflammation and fibrosis [5, 35]. Hence, strategies aimed at alleviating these pathologic processes may be effective in retarding the progression of CKD [6, 36].

One effective and well-studied approach in preventing chronic renal injury is blockade of RAAS [34, 37]. The salutary effects of RAAS blockade in slowing progression of CKD are not only caused by its antihypertensive and hemodynamic properties, but also by its ability to reduce oxidative stress, inflammation, and fibrosis [38]. Binding of angiotensin II to its receptor increases production of ROS via protein kinase C-mediated activation of NAD(P)H oxidase, leading to tissue damage, inflammation, and fibrosis [39, 40]. Therefore, RAAS blockade provides renoprotection by mitigating this pathologic cascade of events [41]. Hence, it is not surprising that in the current study treatment of CKD animals with VAL resulted in significant amelioration of oxidative stress, inflammation, fibrosis and ultimately tubulointerstitial disease and glomerulosclerosis.

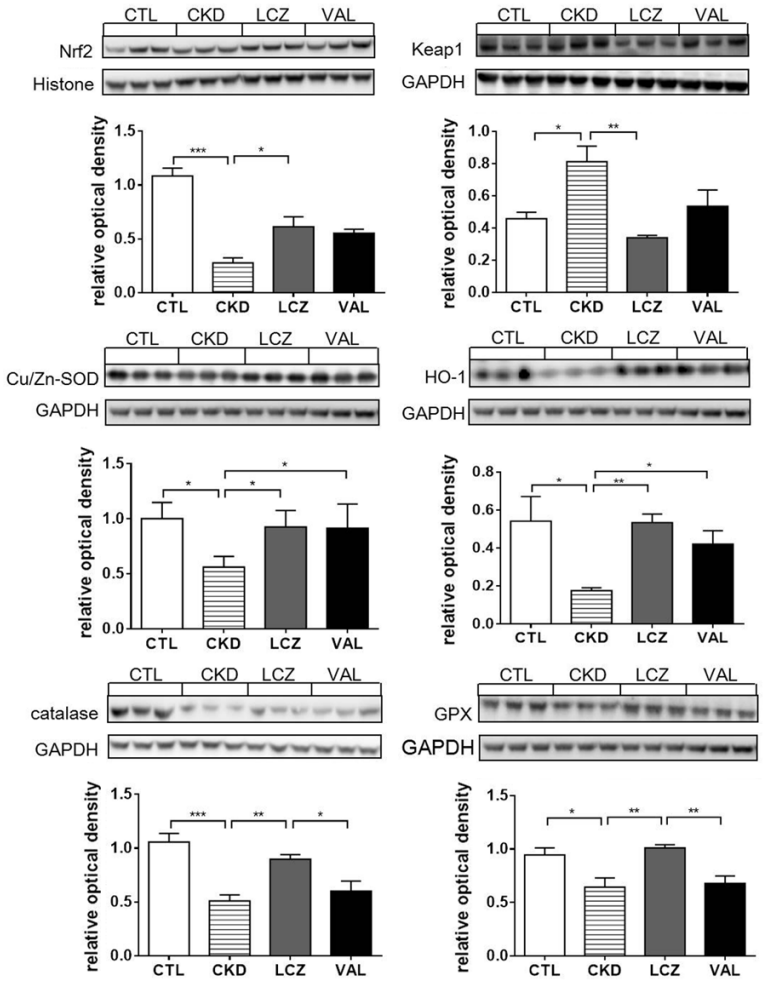

Figure 4. Impact of LCZ and VAL on Nrf2 pathway. Representative Western blots and group data depicting nuclear translocation of Nrf2 and protein abundances of its repressor Keap1 and downstream gene products, Cu/Zn-SOD, catalase, GPX and HO-1 in the renal tissues of the control rats (n=6), CKD rats (n=9), CKD rats treated with LCZ (n=9), and CKD rats treated with VAL (n=9). Data are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001. Histone was used as a loading control for nuclear extracts. GAPDH was used as a loading control for cytoplasmic extracts. (Please see the Figure S3 for uncropped images of the blots).

Another intriguing potential mechanism by which prevention of renal injury and progression of CKD may be achieved is through augmentation of NPs. NPs are a family of peptides that include atrial, brain and c-type NPs (ANP, BNP and CNP, respectively) which are formed as pre-pro-peptides and undergo cleavage to form active peptides. ANP and BNP have a wide range of CV and renal effects which include natriuresis, diuresis and regulation of blood pressure. These activities are mediated through regulation of renal sodium and water handling via inhibition of RAAS and antidiuretic hormone [19]. While some of the therapeutic effects of NPs are mediated via their inhibitory impact on the RAAS pathway, there is also abundant evidence that NPs (especially ANP and BNP) have antioxidant, anti-inflammatory and antifibrotic properties which may significantly contribute to their renoprotective properties [42]. For example, it has been shown that ANP inhibits the activation of the nuclear factor NF-κB and iNOS, thereby, decreases the production of reactive nitrogen, halogen and oxygen species and reduces generation and release of cytokine and chemokine [43-45]. Furthermore, ANP has been found to have an inhibitory effect on the production of inflammatory mediators in macrophages, endothelial cells and cardiomyocytes [46, 47]. In addition, renal ablation in transgenic mice overexpressing hepatic BNP has been shown to reduce renal fibrosis and decrease protein abundance of major proteins in the fibrotic pathway when compared to control animals and this effect was independent of blood pressure [48].

Therefore, it is not surprising that in CKD animals treated with LCZ there was a significant improvement in indices of oxidative stress, inflammation, fibrosis and Nrf2 system beyond that was seen with ARB therapy alone. This was accompanied by a significant improvement in markers of renal function including renal histology, serum urea and creatinine levels. These results are in line with previously published studies on the impact of neprilysin inhibition on progression of renal disease. In an animal model of CKD, Cao et al demonstrated that treatment with omapatrilat, a combination neprilysin and ACE inhibitor, was associated with significant improvements in blood pressure and proteinuria in addition to slowing the progression of glomerulosclerosis, tubulointerstitial fibrosis

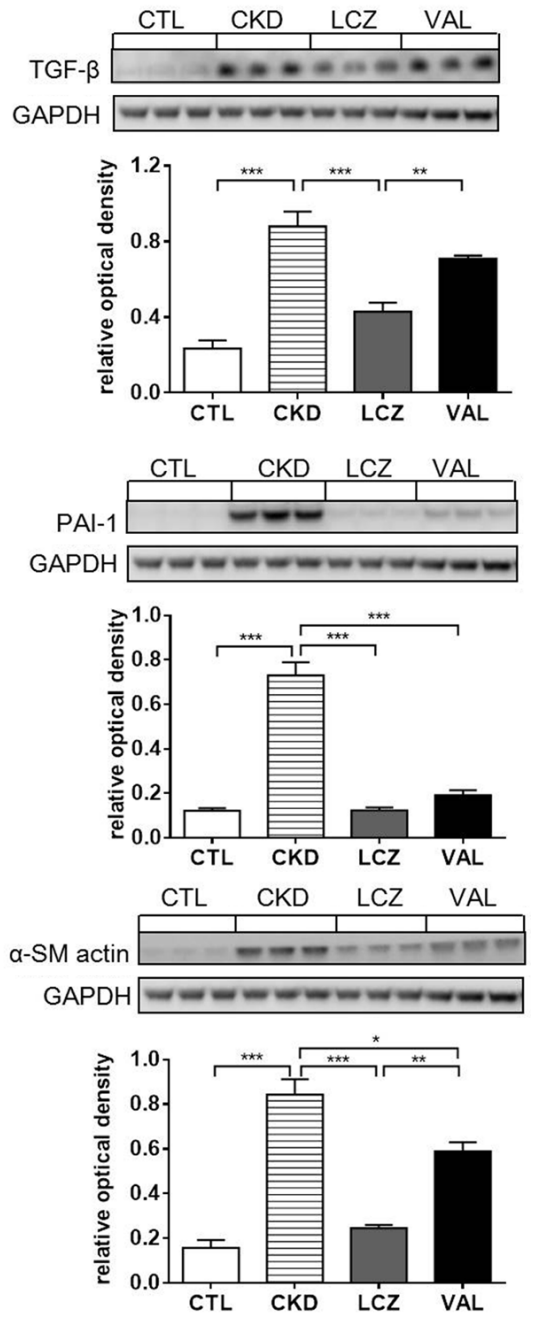

Figure 5. Impact of LCZ and VAL on fibrosis pathway. Representative Western blots and group data depicting TGF-β, α-SM actin, and PAI-1 abundance in the renal tissues of the control rats (n=6), CKD rats (n=9), CKD rats treated with LCZ (n=9), and CKD rats treated with VAL (n=9). Data are mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001. GAPDH was used as a loading control for cytoplasmic extracts. (Please see the Figure S4 for uncropped images of the blots).

and renal injury [49]. However, the degree of improvement was not beyond that of ACE inhibition alone. In a similar study, Taal et al reported that omapatrilat treatment led to greater reductions in SBP and glomerular capillary pressure and this was associated with a greater reduction in proteinuria, glomerulosclerosis and delayed progression of renal disease when compared with ACE inhibition alone [50]. In another study of nephron mass reduction and hypertension, Benigni et al compared the antihypertensive and renoprotective effects of omapatrilat plus enalapril compared to ACE inhibition alone. They also found that treatment with omapatrilat resulted in a significant reduction in proteinuria, glomerulosclerosis and tubulointerstitial fibrosis. In addition, there was decreased renal synthesis of endothelin-1 and increased nitric oxide production resulting in reduced renal vasoconstriction [20]. Furthermore, they found significantly increased tubular ANP release in animals treated with omapatrilat [20]. While there are similarities between those studies and the current report, there are significant differences between our study and the above-mentioned studies. First and foremost, the latter studies used a model of renal mass reduction which utilizes removal of one kidney and ligation of the blood supply to 2/3 of the contralateral kidney. This results in significant activation of RAAS and severe hypertension which can be a major contributor to progression of renal injury in this model. Therefore, while these investigations carefully attempted to account for this critical characteristic of the model, it is possible that treatment of hypertension and hemodynamic abnormalities played a major role in the findings of these previous studies. However, the model utilized in our study used surgical removal of renal tissue to induce renal mass reduction without renal artery ablation. While nephron mass reduction and hyperfiltration associated with this model is also accompanied by hypertension, the level of increase in blood pressure is not as severe as that observed in models relying on ligation of renal artery. However, it should also be noted that there was a modest but statistically insignificant decrease in the blood pressure of animals treated with LCZ when compared with VAL group. Hence partial contribution of blood pressure control to the beneficial effect of LCZ therapy cannot be excluded. Another important difference between the current work and previous studies is their use of omapatrilat whose clinical utility with ACE inhibition has been limited due to reduce the breakdown of bradykinin pro-

duction and risk of angioedema. It is for this reason that LCZ uses a different formulation of neprilysin inhibition together with an ARB. Clinical trials using this agent in treatment of heart failure have shown significant improvements in mortality without an unacceptable risk of angioedema. It is also important to note that in patients with HF and preserved ejection fraction, therapy with LCZ has been found to be associated with significantly greater preservation of eGFR when compared with VAL therapy alone [27]. However, the current study is the first study to our knowledge which assesses the efficacy of LCZ in slowing progression of renal disease in an animal model of CKD and provides some of the potential mechanisms responsible for the renoprotective properties of this medication.

While the findings of this study are novel, there are several limitations which should be mentioned. Firstly, these *in vivo* findings in a well-accepted animal model of CKD will need to be confirmed by clinical studies [51]. Secondly, longer duration of follow up and evaluation of different doses of LCZ in treatment of CKD should be undertaken in the future. Furthermore, although we did not find a significant difference between VAL and LCZ in regards to their impact on proteinuria, further evaluation of LCZ in an animal model of nephrotic syndrome is necessary to fully assess the effectiveness of this therapy in the setting of heavy proteinuria. In addition, while the blood pressure of CKD animals treated with LCZ was not significantly different from those treated with VAL, we cannot exclude the potential role of hemodynamic effects of LCZ as one of the mechanisms responsible for its renoprotective effects. This is because we used tail-cuff techniques to measure blood pressure in this study, a method which is not as accurate as telemetry or more invasive methodologies [52]. Therefore, use of tail-cuff blood pressure measurement techniques is a limitation of our study and future investigations will need to confirm our findings. However, it is also important to note that there are several studies which have used telemetry and found no difference in blood pressure measurements in animals treated with LCZ when compared to VAL therapy alone [28, 52, 53]. Finally, additional mechanistic studies are needed to further elucidate the molecular pathways by which neprilysin inhibition mediates antioxidant, anti-inflammatory and antifibrotic effects.

In conclusion, LCZ treatment of animals with surgical renal mass reduction resulted in a significant improvement of kidney function and amelioration of glomerulosclerosis, tubulointerstitial injury and fibrosis beyond that observed with ARB therapy alone. This was associated with and likely partly mediated by a significant reversal of upregulation of oxidative, inflammatory, profibrotic mediators and improvement of the Nrf2 antioxidant pathway in the remnant kidney.

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Disclosure of conflict of interest

None.

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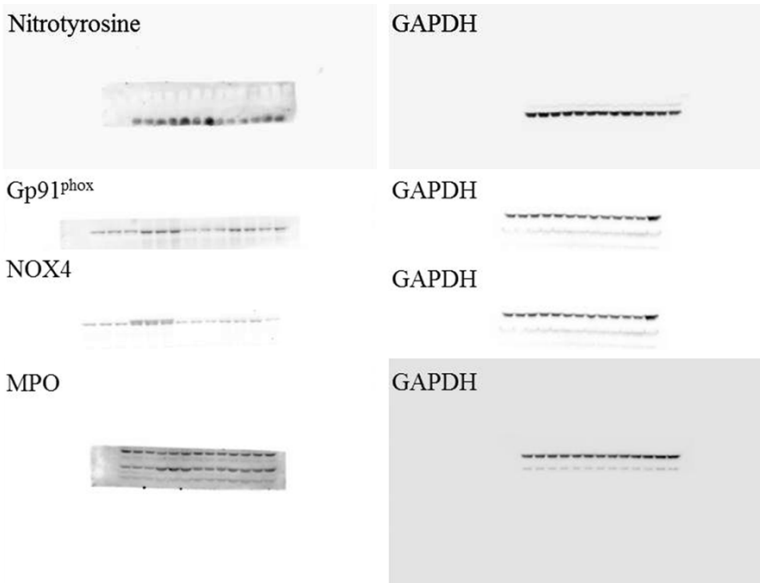

Figure S1. The uncropped western blot results of nitrotyrosine, the NAD(P)H oxidase subunits (NOX-4 and Gp91^{phox}), MPO and GAPDH in the renal tissues.

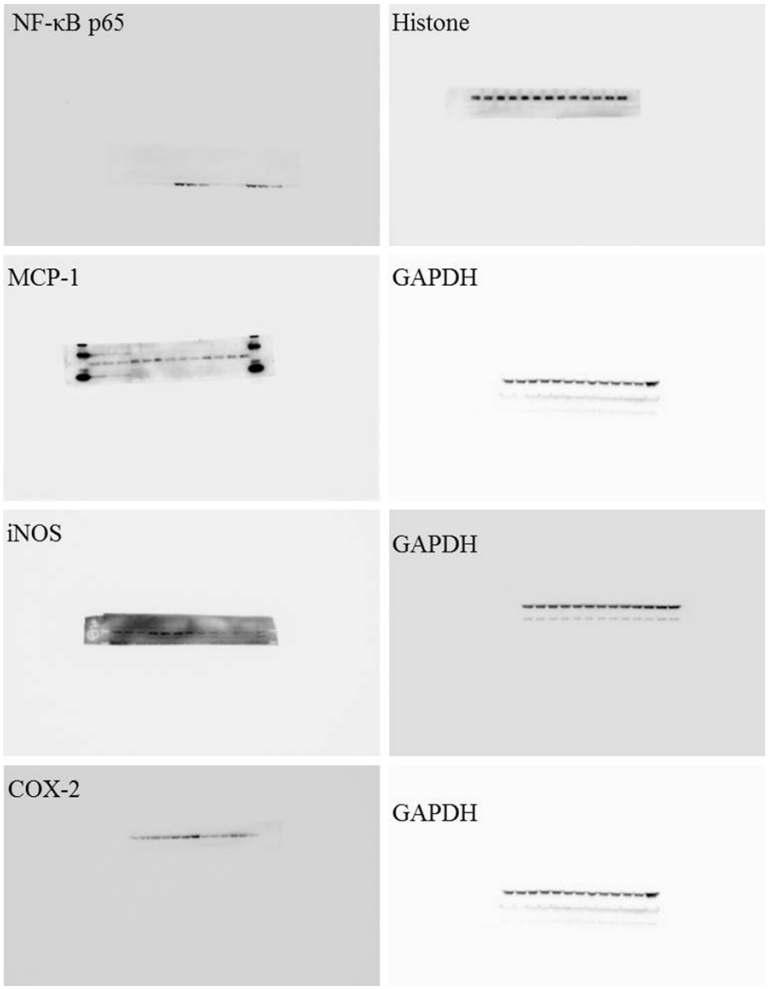

Figure S2. The uncropped western blot results of p65 active subunit of NF-κB and protein abundance of MCP-1, iNOS, COX-2, histone and GAPDH in the renal tissues.

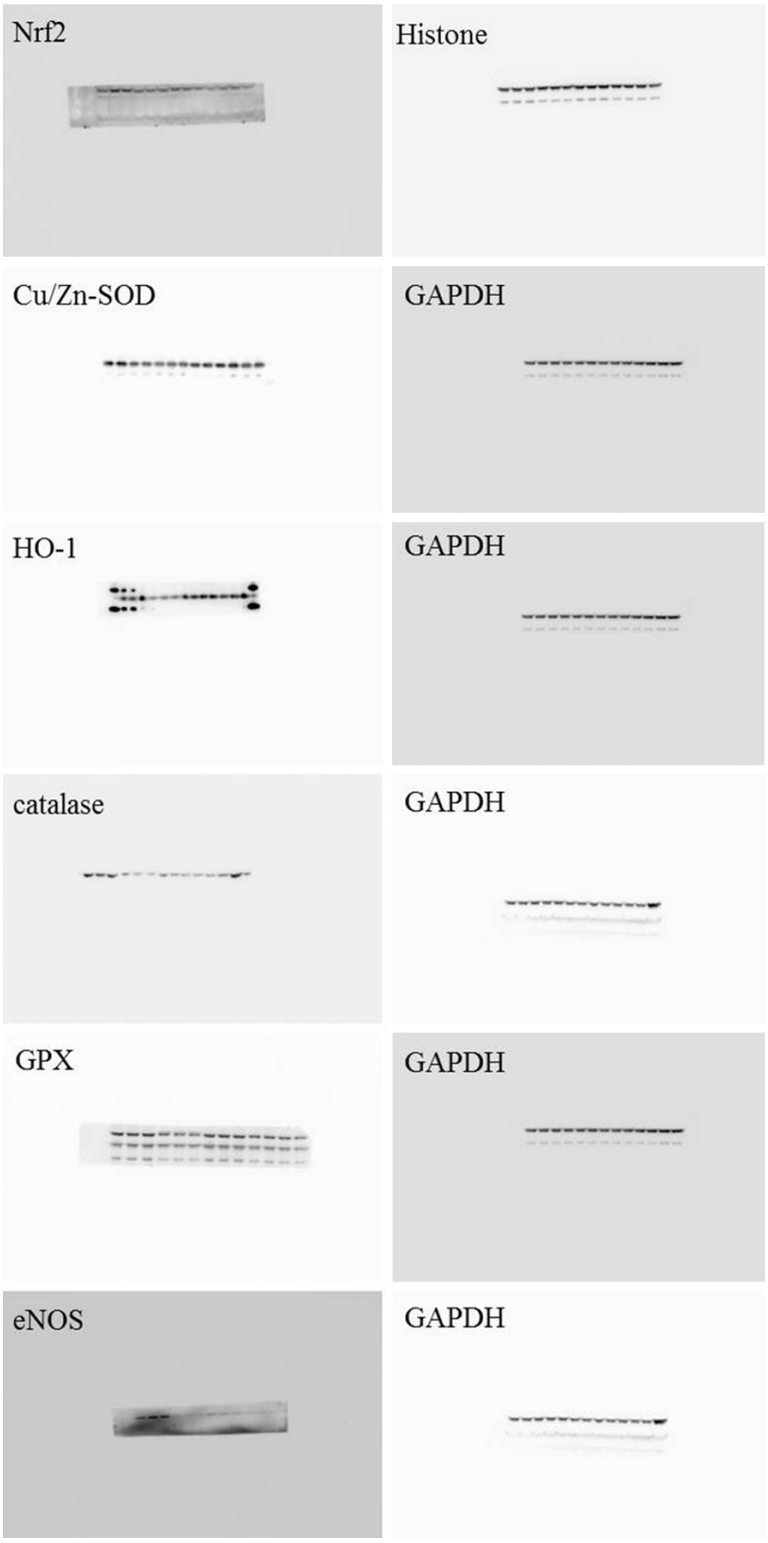

Figure S3. The uncropped western blot results of nuclear translocation of Nrf2 and protein abundances of its downstream gene products, Cu/Zn-SOD, catalase, GPX, HO-1, eNOS, histone and GAPDH in the renal tissues.

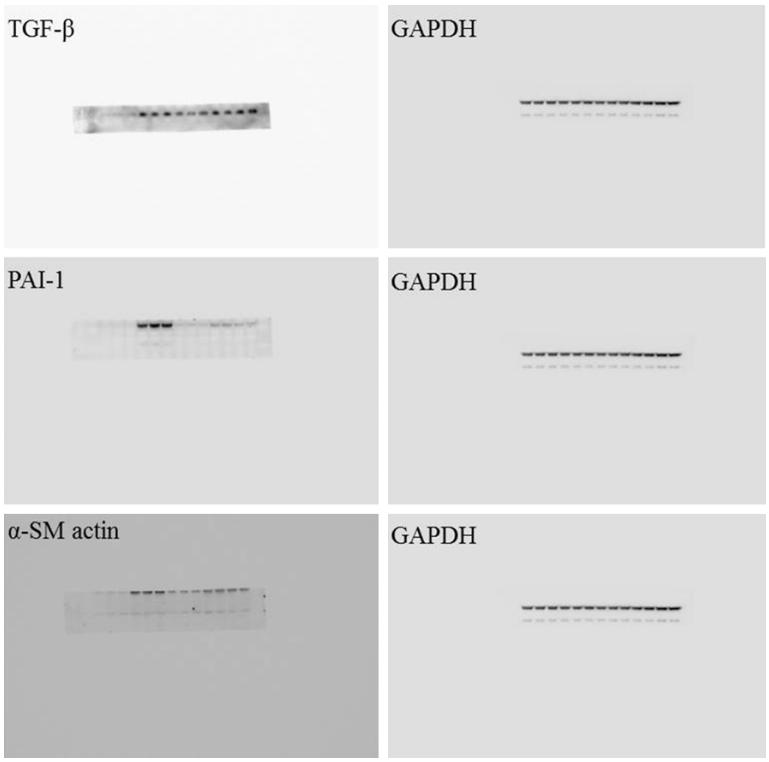

Figure S4. The uncropped western blot results of TGF-β, α-SM actin, PAI-1 and GAPDH abundance in the renal tissues.