Original Paper

Angiotensin-Converting Enzyme Inhibitor (ACEI)-Mediated Amelioration in Renal Fibrosis Involves Suppression of Mast Cell Degranulation

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Key Words

Renal fibrosis • Mast cell • Degranulation

Abstract

Background/Aims: The mechanism by which angiotensin-converting enzyme inhibitors (ACEIs) attenuate renal fibrosis has not been fully uncovered. **Methods:** Renal fibrosis in rats was triggered by unilateral ureteral obstruction (UUO) and treated with Enalapril. **Results:** Enalapril attenuated renal fibrosis, as evidenced by the fibrosis scores (1.07 ± 0.73 versus 2.18 ± 0.75 for 200 mg/ml Enalapril versus control, $p < 0.01$) of Enalapril-treated UUO rats compared to mock-treated UUO rats. The amelioration was mast cell dependent, as Enalapril exhibited no effects on mast cell-deficient $\text{Kit}^{\text{wsh/wsh}}$ mice developing renal fibrosis. We detected lower levels of transforming growth factor β (TGF- β) and alpha-smooth muscle actin (α -SMA, a fibroblast activation marker) in the kidney tissue of Enalapril-treated UUO rats relative to the control UUO rats. Enalapril-treated UUO rats exhibited far fewer mast cells infiltrating per area in the kidney tissue than the control UUO rats (8.00 ± 0.65 versus 29.00 ± 0.57 , $p < 0.01$). Electron microscopy images revealed that mast cell degranulation was inhibited by Enalapril treatment. Further, IgE-mediated passive cutaneous anaphylaxis demonstrated that Enalapril blocked mast cell degranulation *in vivo*. **Conclusion:** Enalapril attenuated renal fibrosis in UUO rats, possibly by a mechanism involving the suppression of mast cell degranulation.

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Introduction

Regardless of the initial causes, patients with chronic kidney diseases commonly undergo renal fibrosis prior to end-stage renal failure [1]. Renal fibrosis, including tubulointerstitial fibrosis and glomerulosclerosis, is characterized by the excessive accumulation and deposition of extracellular matrix components [2, 3]. Most of our knowledge on renal fibrosis derives from studies in experimental animal models [4, 5]. Rats subjected to unilateral ureteral obstruction (UUO), in which one of the ureters is ligated to obstruct urine evacuation from the kidney, are often used as a renal fibrosis model. Interstitial fibrosis has been observed in the operated kidney as early as 3 days after surgery [6-8]. Renal fibrosis involves a complex network of cellular interactions, cytokine/chemokine production, and signaling pathways [2] of which dysregulations may cause fibroblast activation [9, 10], epithelia to mesenchymal transition (EMT) [11], monocyte/macrophage infiltration [12], and cellular apoptosis [13], eventually leading to histological alterations in the kidney tissue. In this process, transforming growth factor beta (TGF- β) plays a significant role as an inflammatory mediator [14-16].

Mast cells are among the major components of infiltrating leukocytes in kidney tissue undergoing fibrosis [17]. A number of studies have reported the correlation between mast cells and renal fibrosis in both animal models and human diseases [17-19]. Nevertheless, the pathophysiological role of mast cells in renal fibrosis is not yet conclusive, as both aggravating and protective roles of mast cells in renal fibrosis have been reported even by studies using similar mast cell-deficient animal models [17, 20, 21]. Nonetheless, substantial evidence supports that mast cells promote fibrosis in renal diseases. First, mast cells have been reported to produce TGF- β and fibroblast growth factor (FGF), which trigger the activation and proliferation of fibroblasts [22, 23]. Apart from secreting profibrotic cytokines, the mast cell-specific proteases tryptase and chymase may be involved in tissue remodeling and fibrosis [24, 25]. Based on these findings, mast cells are suggested as a therapeutic target for renal fibrosis [26]. Reportedly, stabilizing mast cells with drugs in a rat UUO model attenuated the development of tubulointerstitial fibrosis [19].

Recently, studies have shown that mast cells can express and secrete active renin and that the ensuing angiotensin II in the renin-angiotensin system (RAS) may trigger excessive collagen deposition and changes in vascular resistance to accelerate renal fibrosis [19, 27]. This finding provides a rationale for using angiotensin-converting enzyme inhibitors (ACEIs) to block the conversion from angiotensin I to angiotensin II to attenuate renal fibrosis [28]. Accordingly, preliminary results from rat UUO models support the efficacy of ACEIs in ameliorating renal fibrosis [29].

Although ACEIs appear to be promising for the treatment of renal fibrosis, the mechanism by which ACEIs reduce renal fibrosis has not been fully revealed. In this study, we observed the effects of ACEI on TGF- β , fibroblast activation, and mast cells in a rat UUO model.

Materials and Methods

Animal models

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal-related protocols in this study were conducted with approval from the Medical Ethics Committee of Tianjin Medical University. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Six- to 8-week-old male Sprague-Dawley rats weighing 180~200 g were purchased from the Beijing Experimental Animal Center (Beijing, China). Kit^{wsh/wsh} mice were kind gifts from Professor Zhi Yao at Tianjin Medical University. All animals were housed in the animal core facility of the institution under standard environmental conditions. Rats and mice had *ad libitum* access to standard chow and drinking water. Rats were randomly assigned to four groups (n=15 for each group, total N=60): the sham-operated group, UUO group, UUO group administered Enalapril at 100 mg/ml in drinking water, and UUO group administered

Enalapril at 200 mg/ml in drinking water. UUO was performed as previously described [4] and maintained for two weeks. Enalapril and placebo were administered daily for the treatment group and control group, respectively, following UUO surgery. Two weeks after surgery, the rats were euthanized, and the operated kidneys were excised for analysis.

IgE-dependent passive cutaneous anaphylaxis (PCA) was induced in the ear pinna. Briefly, mice under anesthesia and analgesia were sensitized passively to IgE by the intradermal injection of 20 ng DNP-specific IgE antibody (clone SPE-7; Sigma-Aldrich, St. Louis, MO, USA) in 20 μ l phosphate buffer saline (PBS) and challenged 24 h later by intravenous injection with 200 μ g DNP (Sigma-Aldrich) in 100 μ l PBS. Ear swelling was measured immediately before antigen challenge and at 30-min intervals after antigen challenge for 6 h using a micrometer (Mitutoyo Digimatic Micrometer 0–25 mm).

Measure of UUN, UCR, BUN, and SCR and fibrosis scoring

Blood and urine samples were collected to measure urea urine nitrogen (UUN), urea creatine (UCR), blood urine nitrogen (BUN), and serum creatine (SCR), as previously described [30]. The extent of renal fibrosis was scored as defined elsewhere [19].

Immunohistochemistry

Kidney tissue was fixed in 4% paraformaldehyde and then embedded in paraffin wax to make 4-micrometer thick sections. The kidney sections were deparaffinized with xylene and rehydrated using graded ethanol followed by distilled water. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and sections were heated in 0.1% sodium citrate buffer (pH 6.0) for antigen retrieval. Anti- α -SMA antibody (ab5694, Abcam, MA, USA), anti-TGF- β antibody (ab66043), and anti-mast cell tryptase antibody (ab2378) were diluted at 1:100 for primary antibody incubation. Peroxidase-conjugated secondary antibodies were used for the color-producing reaction.

RNA extraction and quantitative real time PCR

Total RNA extraction from kidney tissue was conducted using Trizol reagent (Invitrogen, USA) in compliance with the manufacturer's instructions. The total RNA was used for cDNA synthesis with the Super-Script II Kit (Qiagen, Germany). Real-time PCR was performed following a standard protocol on the ABI 7900HT System (Applied Biosystems, USA). The relative expression levels of TGF- β and α -SMA were normalized to the internal control (β -actin). The primers for TGF- β and α -SMA cDNA amplification are listed below:

The forward primer for TGF- β : 5'-AGCGCATCGAAGCCATCCGTG-3'

The reverse primer for TGF- β : 5'-CACTGCTTCCCGAATGTCTGACGT-3'

The forward primer for α -SMA: 5'-GCGGGCATCCACGAAACCAC-3'

The reverse primer for α -SMA: 5'-TGACAGGCCAGGGCTAGAAGGGTA-3'

Western blot analysis

Kidney tissue was extracted in RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with proteinase and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO, USA). Protein samples (15 μ g per load) were resolved by 10% SDS-PAGE and then electro-transferred onto the nitrocellulose membrane (Millipore, USA). The blots were blocked in TBS buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl) with 5% milk and 0.02% Tween-20 at room temperature for one hour. Primary antibody incubation was performed overnight at 4 °C. HRP-conjugated secondary antibody (Jackson Immunoresearch laboratory, USA) incubation was conducted at room temperature for one hour. The blots were developed with Western Lightning-Enhanced Chemiluminescence Substrate (Perkin Elmer) and exposed to X-ray films. Specific antibodies against TGF- β , α -SMA, chymase (ab186417), and β -actin (ab8227) were obtained from Abcam (MA, USA).

Electron microscopy

Electron microscopic observation of mast cells followed previously established protocols [31]. Briefly, kidney tissues were fixed with 3% glutaraldehyde in 0.1 mM phosphate buffer (pH 7.4). Ultrathin sections were placed onto a copper grid, stained with uranyl acetate and lead citrate, and observed under a transmission

Table 1. Comparison of UUN, UCR, BUN, and SCR of the different rat groups

Group	N	UUN (mmol/L)	UCR (μ mol/L)	BUN (mmol/L)	SCR (μ mol/L)
Sham	15	0.88 $\pm$ 0.62	2567.00 $\pm$ 422.47	7.11 $\pm$ 1.79	31.00 $\pm$ 1.67
UUO	15*	1.34 $\pm$ 0.22	3652.75 $\pm$ 188.26	16.30 $\pm$ 0.47	33.96 $\pm$ 1.22
UUO+ACEI double dosage	15*▲	0.56 $\pm$ 0.89	2788.00 $\pm$ 178.98	7.25 $\pm$ 0.47	30.47 $\pm$ 4.48
UUO+ACEI single dosage	15*★	0.71 $\pm$ 0.30	3020.00 $\pm$ 172.90	8.12 $\pm$ 0.22	31.59 $\pm$ 2.10

The values were presented as mean $\pm$ standard deviation. *, $p < 0.01$ compared with the Sham group by ANOVA; ▲, $p < 0.01$ and ★, $p < 0.05$ compared with the UUO group by ANOVA.

electron microscope (Hitachi H-600, Japan). Mast cell granules were discerned by the morphological features at a magnification of 10,000 $\times$.

Statistical analysis

Three repeats were performed for each experiment. ANOVA was performed to examine the differences among multiple (≥ 3) groups. The statistical significance level was set at $p < 0.05$.

Results

ACEI attenuated renal fibrosis in UUO rats

Fibrosis scoring showed that the UUO rats had obvious fibrosis in the operated kidneys compared with the sham-operated rats in which the kidneys showed almost no fibrosis (Figure 1, $p < 0.01$ ANOVA). Fibrosis scores reflected that UUO rats receiving Enalapril in the drinking water exhibited significant amelioration in renal fibrosis relative to the mock-treated UUO rats. UUO rats administered 200 mg/ml Enalapril in drinking water exhibited considerably reduced renal fibrosis compared with both the mock-treated UUO rats ($p < 0.01$, ANOVA) and the UUO rats treated with 100 mg/ml Enalapril ($p < 0.05$, ANOVA).

The blood and urea parameters of the different groups also indicated that Enalapril attenuated renal fibrosis in UUO rats (Table 1). The mock-treated UUO rats showed much higher levels of UUN, UCR, and BUN and a slightly higher level of SCR than the sham-operated rats ($p < 0.01$, ANOVA). The levels of UUN, UCR, BUN, and SCR in the UUO rats under treatment with both 100 mg/ml and 200 mg/ml Enalapril decreased significantly compared with the mock-treated UUO rats, with a greater decline under the treatment of 200 mg/ml Enalapril (ANOVA, $p < 0.05$).

TGF- β and α -SMA decreased in the operated kidney in UUO rats administered ACEI

It has demonstrated that the up-regulation of TGF- β and α -SMA is associated with renal fibrosis [32, 33]. To explore the mechanism by which Enalapril attenuates renal fibrosis, kidney tissues from control UUO rats and Enalapril-treated UUO rats were incubated with specific antibodies recognizing TGF- β / α -SMA. The results showed that the staining of both

Fig. 1. The mean fibrosis scores of rats under different treatments. The extent of fibrosis under the treatment of sham operation, UUO, UUO receiving a single dose of Enalapril (100 mg/ml), and UUO receiving a double dose of Enalapril (200 mg/ml) were scored. Each treatment started with 15 rats. “*” and “**” represent $p < 0.05$ and 0.01 by ANOVA, respectively.

Table 2. Comparison of IOD/area of TGF- β , α -SMA, and tryptase of the different rat groups

Group	N	TGF- β	α -SMA	tryptase
Sham	15	0.99 $\pm$ 0.57	1.11 $\pm$ 0.10	0.87 $\pm$ 0.33
UUO	15*	3.14 $\pm$ 0.44	3.41 $\pm$ 0.095	4.14 $\pm$ 0.20
UUO+ACEI double dosage	15* $\blacktriangle$	1.68 $\pm$ 0.53	1.66 $\pm$ 0.14	1.78 $\pm$ 0.26
UUO+ACEI single dosage	15**	2.18 $\pm$ 0.19	2.24 $\pm$ 0.06	2.31 $\pm$ 0.05

The values were presented as mean $\pm$ standard deviation. *, $p < 0.01$ compared with the Sham group by ANOVA; $\blacktriangle$, $p < 0.01$ and $\star$, $p < 0.05$ compared with the UUO group by ANOVA.

Fig. 2. The levels of TGF- β and α -SMA were reduced in response to Enalapril treatment. TGF- β (A-A') and α -SMA (B-B') in kidney tissue were immunostained. Arrowheads indicate the high levels of TGF- β and α -SMA in the kidney tissue of UUO rats (A and B) compared with Enalapril-treated UUO rats (A' and B'). Panels C and D show RT-PCR (C) and Western blot (D) results, respectively. "1", "2", and "3" indicate the samples from control UUO rats, UUO rats receiving Enalapril 200 mg/ml, and sham-operated rats, respectively.

TGF- β and α -SMA decreased appreciably in UUO rats under Enalapril treatment compared with the control UUO rats (Figure 2, A-B'). Statistic analysis further confirmed the significant difference in the index of density per area (IOD/area) of TGF- β / α -SMA between the UUO rats treated with Enalapril and the mock-treated UUO rats (Table 2, $p < 0.05$). In accordance with the immunohistochemical results, semi-quantitative RT-PCR and Western blot results showed that both the mRNA and protein levels of TGF- β and α -SMA in the operated kidney of UUO rats under Enalapril treatment decreased significantly compared with the control UUO rats (Figure 2, C, D). Further, quantitative RT-PCR analysis indicated that the levels of TGF- β and α -SMA were significantly raised in the operated kidney in UUO rats compared with the sham-operated rats, whereas Enalapril treatment resulted in the down-regulation of TGF- β and α -SMA in an apparently dose-dependent way (Figure 3).

ACEI attenuated renal fibrosis via mast cell modulation

To demonstrate that Enalapril exerts its effects through specifically modulating mast cells, we treated Kit^{wsh/wsh} mice (mast cell-deficient phenotype) developing UUO with Enalapril. We observed that the Kit^{wsh/wsh} mice developed renal fibrosis upon UUO operation. However,

Enalapril failed to attenuate the process as it did in wild-type UUO rats (Figure 4). Strikingly, Enalapril showed efficacy on Kit^{wsh/wsh} mice undergoing renal fibrosis with the reconstitution of mast cells from wild-type mice (Figure 4), strongly implying the direct modulation of mast cells by the drug.

Mast cell infiltration and degranulation were suppressed in UUO rats treated with ACEI

It has been shown that infiltrating mast cells are inflammation mediators during renal fibrosis and are associated with the progression of fibrosis in human diseases [27]. In this study, toluidine blue staining showed that the kidney tissue of UUO rats had more infiltrating mast cells than in the sham-operated rats, and Enalapril treatment reduced mast cell infiltration in the kidney tissue of UUO rats (Figure 5, B-B'' and Figure 6). Moreover, the number of mast cells per area in UUO rats was inversely correlated with the dosage of Enalapril (Figure 6). Previous studies suggested the profibrotic role of mast cell-specific proteases in renal fibrosis [25, 34, 35]. In this study,

Fig. 3. The relative levels of TGF-β and α-SMA in the kidney tissue of different rat groups, determined by qRT-PCR. Rats were randomly divided into four groups, namely sham-operated, UUO, UUO treated with 100 mg/ml Enalapril, and UUO treated with 200 mg/ml Enalapril.

Fig. 4. Evaluation of renal fibrosis in Kit^{wsh/wsh} mice under different treatments. Rats were randomly assigned to each group (n=15), and the treatment for each column in the graph was as indicated. Enalapril was given to mice in drinking water at 100 mg/ml and 200 mg/ml for single and double dose treatment, respectively. "*" and "**" represent p<0.05 and 0.01 by ANOVA, respectively.

Fig. 5. Enalapril attenuated renal fibrosis via regulating mast cell degranulation. (A–A'') The kidney tissues of control UUO rats and UUO rats receiving Enalapril were analyzed by transmission electron microscopy. Mast cells in the presence of Enalapril showed granules with larger volume and lower density than the control. The degranulation of mast cells in UUO rats is indicated by black arrowheads in A and A'. The cell membrane of mast cells was intact under the Enalapril treatment (A' and A''), and degranulation was hardly seen. Images in the middle panel (B–B'') show the representative results of toluidine blue staining. In control UUO rats, mast cells accumulated around the renal glomerulus, as well as in fibrosis areas (indicated by arrowheads in B). A single glomerulus is shown in B'' and B''' with mast cells indicated by red arrowheads. The bottom panel images (C–C'') show immunostaining of mast cell tryptase. A single glomerulus is shown in C'' and C'''. Red arrowheads point to the tryptase staining.

we found that tryptase-positive mast cells were significantly reduced in the kidney tissue of UUO rats treated with Enalapril compared with the control UUO rats (Figure 5, C–C''). Chymase is another major component of mast cell-specific proteases; however, the expression of chymase seemingly did not change in response to Enalapril treatment (Figure 7). By electron microscopy, we clearly saw the differences in granule morphology between the mast cells in mock-treated UUO rats and in Enalapril-treated UUO rats. The images showed that

Fig. 6. The number of mast cells per area in UUO rats receiving Enalapril decreased compared with control UUO rats. The number of infiltrating mast cells per area in kidney tissue of rats subjected to sham operation, UUO, UUO plus 100 mg/ml Enalapril, and UUO plus 200 mg/ml Enalapril were statistically analyzed. “*” and “**” represent $p < 0.05$ and 0.01 by ANOVA, respectively.

Fig. 7. Chymase expression was not altered in the presence of Enalapril. The lane 1, 2, and 3 represent samples from UUO rats, UUO rats receiving 200 mg/ml Enalapril, and UUO rats with 100 mg/ml Enalapril, respectively.

the granules of mast cells in UUO rats receiving Enalapril were much larger and less dense than in mock-treated UUO rats (Figure 5, A-A’). We observed the breakdown of the mast cell membrane and release of granules in mock-treated UUO rats (Figure 5, A and A’, black arrows). Under Enalapril treatment, the cell membrane integrity seemed well preserved, and very few granules were released (Figure 5, A’ and A’’, red arrows)

To further prove that Enalapril blocked mast cell degranulation *in vivo*, IgE-dependent passive cutaneous anaphylaxis (PCA) was performed. Mice were sensitized by IgE antibody, followed by antigen

challenge. We measured the ear thickness of mice subjected to PCA. The results showed that Enalapril significantly suppressed the ear swelling in mice (Figure 8), suggesting that the drug directly affected mast cell degranulation.

Discussion

Renal fibrosis is commonly seen in chronic kidney diseases [2]. Over-production of TGF- β , fibroblast activation (α -SMA), and infiltrating mast cells are hallmarks of this process and are proposed as targets for interfering with the progression of renal fibrosis in patients [36]. A number of studies have investigated the efficacy of ACEIs in preventing fibrosis in various injury and diseases models [28]. Among these studies, some have demonstrated that ACEIs

Fig. 8. Enalapril suppressed IgE-dependent passive cutaneous anaphylaxis. Ear thickness was measured immediately before antigen challenge and at 30 min intervals after antigen challenge for 6 hours.

ameliorated renal fibrosis in animal models, although they were not completely effective [29, 30]. To further explore the potential of ACEIs in treating renal fibrosis, first, we must fully understand the mechanism by which ACEI attenuates renal fibrosis. Here, we took advantage of the widely used renal fibrosis model in rats (UUO) to study how key profibrotic factors including TGF- β , fibroblast activation (α -SMA), and mast cells would be impacted by ACEIs.

Our results showed that levels of TGF- β and α -SMA were significantly decreased at both the protein and mRNA levels in the kidney tissue of UUO rats treated with Enalapril compared with the control UUO rats (Figure 2), which was consistent with the observations in previous studies [36, 37].

By using Kit^{wsh/wsh} mice developing UUO, we demonstrated that the efficacy of Enalapril on renal fibrosis was mast cell dependent. This finding highlights a direct modulation of mast cells by Enalapril in the UUO model. Thus, we focused on a mechanistic study of mast cells in the subsequent experiments. We observed fewer infiltrating mast cells and tryptase-positive mast cells in the kidney of UUO rats treated with Enalapril compared with the control UUO rats (Figure 5 and 6). Mast cell-specific proteases play a role in tissue remodeling during renal fibrosis [25]. Accordingly, the decrease in tryptase-positive mast cells under Enalapril treatment suggested less severe renal fibrosis in UUO rats. However, we did not see a decrease in chymase expression in the presence of Enalapril (Figure 7). The different impact on tryptase and chymase by Enalapril will be an interesting question for future studies.

Moreover, we found that Enalapril inhibited the degranulation of mast cells that infiltrated into the kidney tissue, as evidenced by the electron microscopy images showing seemingly intact cell membrane and granules within mast cells in the kidney tissue of UUO rats under Enalapril treatment (Figure 5). Further, IgE-dependent passive cutaneous anaphylaxis was shown to be attenuated by Enalapril. As ear swelling was dependent on mast cell degranulation in the experimental model, this result confirmed that Enalapril blocked mast cell degranulation *in vivo*.

In a proposed model, renin released from mast cell granules activates the renin-angiotensin system (RAS), resulting in fibroblast activation. The activated fibroblasts may increase the production of inflammatory mediator TGF- β , which aggravates the renal fibrosis [19]. Within the framework of this model, Enalapril blocks the conversion from angiotensin I to angiotensin II, which consequently inhibits the activation of RAS and reduces TGF- β production. Here, our data suggest that the suppression of mast cell degranulation might reflect a negative feedback regulation of the mast cell-RAS axis in the presence of Enalapril. We infer that as Enalapril blocks the conversion of angiotensin, a feedback signal may modulate mast cells to undergo less degranulation and renin release to further deactivate mast cell-RAS axis at the very beginning.

Conclusion

The results in this study demonstrate that angiotensin-converting enzyme inhibitors can attenuate renal fibrosis in a rat UUO model by regulating the pro-inflammatory cytokine TGF- β , fibroblast activation (α -SMA), mast cell infiltration, and possibly, mast cell degranulation. Our findings give new insights into the mechanism by which ACEIs reduce renal fibrosis in chronic kidney diseases.

Disclosure Statement

The authors have no conflict of interest to declare.

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