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Protective Effects of Eplerenone on Podocyte Injury in Adriamycin Nephropathy Rats*

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Summary: To investigate the protective effects of eplerenone on adriamycin-induced renal injury and the possible mechanisms involved, 36 male Sprague-Dawley rats were randomly divided into control group, adriamycin nephropathy (AN) group and eplerenone-treated group (100 $mg \cdot kg^{-1} \cdot d^{-1}$ eplerenone). Blood pressure, 24-h urinary protein, serum potassium, sodium and creatinine were measured 28 days after adriamycin injection (a single tail intravenous injection of 6.5 mg/kg adriamycin). The morphological changes of renal tissues were observed by light and electron microscopy. Immunohistochemistry and Western blotting were performed to examine the expression of TGF-β_1 and desmin in renal cortex. The results showed that 28 days after adriamycin injection, there were no significant changes in the level of serum potassium, sodium, creatinine concentrations and blood pressure values in the rats of the three groups. Meanwhile, the 24-h proteinuria excretion in the AN group was significantly higher than that in the control group (P<0.01), but that in the eplerenone-treated group was substantially reduced when compared with that in the AN group (P<0.05). Mild mesangial cell proliferation and matrix expansion, diffuse deformation and confluence of foot processes in podocytes were found in the AN group. By contrast, rats in the eplerenone-treated group exhibited obvious attenuation of these morphological lesions. The protein expression of TGF-β_1 and desmin in the AN group was markedly up-regulated in contrast to that in the control group (P<0.01), whereas that in the eplerenone-treated group was much lower than in the AN group (P<0.05). It was concluded that eplerenone may ameliorate the proteinuria and the development of pathological alteration in adriamycin-induced nephropathy presumably via the inhibition of cytokine release, and restore the morphology of podocytes independent of its blood pressure-lowing effects.
Key words: eplerenone; adriamycin; nephropathy; podocyte; TGF-β_1; desmin

Proteinuria, a common sign of chronic kidney disease (CKD), is also an independent risk factor of the progression of CKD. It usually results from the damage of the glomerular basement membrane (GBM). Podocytes, together with the GBM and the fenestrated endothelial cells, constitute the glomerular filtration barrier which prevents the leakage of protein. Recently, mounting evidence has shown that aldosterone causes progressive renal injuries in CKD and aldosterone antagonism attenuates podocyte injury and reduces the excretion of proteinuria[1]. However, the protective mechanisms of aldosterone antagonists in podocyte injury have not been fully understood. In the present study, rat models of adriamycin nephropathy, a classical experimental model of podocyte injury, were established, and the effect of eplerenone, a selective aldosterone receptor antagonist, on the adriamycin nephropathy was examined as well as the underlying mechanism.

1 MATERIALS AND METHODS

1.1 Materials

Adriamycin was purchased from Pharmacia Corporation (Switzerland). Eplerenone was a product of Pfizer Corporation (USA). RPMI 1640 medium and fetal bovine serum (FBS) were bought from Gibco Laboratory (USA). Collagen Ⅰ was from Sigma-Aldrich Corporation (USA). Recombinant mouse interferon-γ was procured from Peprotech Company (USA). Rabbit polyclonal antibodies against TGF-β_1, goat polyclonal antibodies against desmin, goat anti-rabbit and rabbit anti-goat horseradish peroxidase-conjugated IgG were provided by Santa Cruz Biotechnology Incorporation (USA). SP immunostaining kit and DAB kit were obtained from Beijing Zhong Shan Biotechnology Corporation (China).

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1.2 Model Establishment and Grouping

Male Sprague-Dawley rats at age of 6 to 8 weeks (obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China), weighing 200 to 220 g, were used for adriamycin nephropathy induction. All animals (n=36) were fed on a standard laboratory diet and had free access to tap water. They were randomly divided into three groups (n=12 in each): Control group, AN group and eplerenone-treated group. The rats in the AN group and eplerenone-treated group were given a single tail intravenous injection of adriamycin (6.5 mg/kg). And those in the eplerenone-treated group were then administered a daily dose of eplerenone (100 $mg \cdot kg^{-1} \cdot d^{-1}$) by gastric gavage for 28 days. Correspondingly, rats in the control group and AN group were daily treated with the same amount of distilled water as controls. On the day 28, animals were sacrificed after measurement of systolic blood pressure and collection of 24-h urine. Blood was taken from abdominal aorta of all rats. Serum was then separated by centrifugation and kept at –80°C pending analysis of biochemical parameters. The left kidney was rapidly removed and fixed in 10% PBS-buffered formalin and 2.5% glutaraldehyde for later light microscopic and electron microscopic evaluation of renal histology. The slices were processed by conventional pathologic methods for further histopathological examinations.

1.3 Detection of Biochemical Parameter, Blood Pressure and 24-h Urinary Protein Excretion

Serum potassium, sodium and creatinine concentrations were determined by using an autoanalyzer (Beckman CX3 Delta, USA). Systolic blood pressure was measured in quiet and conscious rats by the tail-cuff method with automated sphygmomanometer (Nanjing DeSci Biotech Ltd. Co, China) before and 28 days after adriamycin injection. All rats were placed in metabolism coops at day 27. The samples of 24-h urine were collected at day 28. Excretion of 24-h urinary protein was determined by Coomassie brilliant blue method. All final results were obtained by averaging three valid readings.

1.4 Preparation of Kidney Tissue Samples

One fourth cortex tissue of left kidney was fixed in 10% paraformaldehyde and then embedded in paraffin after some processing. Fixed sections of the left kidney were cut and stained with hematoxylin-eosin (HE) and PAS. Two cortex tissue samples (at a volume of 1 mm^3) were immersed in 2.5% glutaraldehyde for later electron microscopy. Other cortex tissue samples were stored at –80°C for total protein extraction.

1.5 Immunohistochemistry

Immunohistochemistrical technique (SP method) was used to detect the interstitial expression of TGF-β_1 and desmin. The staining procedure was performed by following the instruction manual of the kit. Fifteen high power fields were randomly selected and the absorbance of positive signals was detected by using MAPIS-500 High Acuity Color Pathologic Diagram-writing Analyzing System (Champion Image Engineering Company of Tongji Medical College, China). The average percentage of the TGF-β_1-positive and demin-positive areas was calculated for semi-quantitative assessment.

1.6 Western Blotting

The protein of all samples was isolated by a specific protein extraction kit according to the manufacturer's instructions. After assessment of the total protein concentrations by a BCA protein assay kit, all protein samples were separated by 10% SDS-PAGE under denaturing conditions and transferred onto nitrocellulose membranes which were later blocked with 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST) buffer for an hour at room temperature. After incubated with rabbit polyclonal antibodies against TGF-β_1 and goat polyclonal antibodies against demin overnight at 4°C, the blots were probed with rabbit anti-goat and goat anti-rabbit horseradish peroxidase-conjugated IgG for 1–2 h. Bands were detected using chemiluminescence and analyzed by an Electrophoresis Image Analysis System. For semi-quantification, mouse β-actin protein was included in every reaction serving as an internal control.

1.7 Statistical Analysis

All data were presented as $\bar{x} \pm s$. Comparisons among groups were made using one-way analysis of variance (ANOVA). The relationship among the excretion of 24-h urinary protein, percentage of TGF-β_1-positive and demin-positive areas was determined by Pearson correlation analysis. Statistical analysis was performed by using SPSS 12.0 software. P<0.05 was considered to be significantly different.

2 RESULTS

2.1 Biochemical Parameters and Blood Pressure

The serum potassium and sodium levels, creatinine concentration and blood pressure showed no significant changes at 28 days in all the three groups (P>0.05). Excretion of 24-h urinary protein at day 28 after adriamycin nephropathy induction was increased significantly in the AN group as compared with that in the control group (P<0.01), whereas that in the eplerenone-treated group was much lower than in the AN group (P<0.05, fig. 1).

2.2 Pathological Changes in Podocytes

Tweenty-eight days after adriamycin injection, electron microscopy displayed podocytes swelling, foot process effacement, cytoplasmic vacuolization, mat-like condensations of microfilaments, and detachment of foot process from GBM in the AN group. The glomeruli showed no obvious thickening of the GBM, but in some glomeruli, mild diffuse or focal mesangial expansion was found. By contrast, rats in the eplerenone-treated group exhibited obvious attenuation of these morphological lesions (fig. 2).

HE and PAS staining showed podocytes swelling, capillary congestion, some mild mesangial cell proliferation and subsequent mesangium expansion at 28 days in the AN group, which were conspicuously improved in the eplerenone-treated group (fig 3).

2.3 Expression of TGF-β_1 and Desmin

Immunohistochemistry revealed that 28 days after adriamycin injection, TGF-β_1 and desmin were found to strongly express in the glomeruli in the AN group, significantly different from that in the control group which only possessed weak TGF-β1 and desmin expressions in some glomeruli. Whereas the expression of TGF-β_1 and desmin in the eplerenone-treated group was significantly lower than in the AN group (P<0.05, fig. 4). Western blot results were in line with those of immunohistochemistry,

indicating that eplerenone administration could reverse the high expression of TGF-β_1 and desmin caused by adriamycin injection (fig. 5).

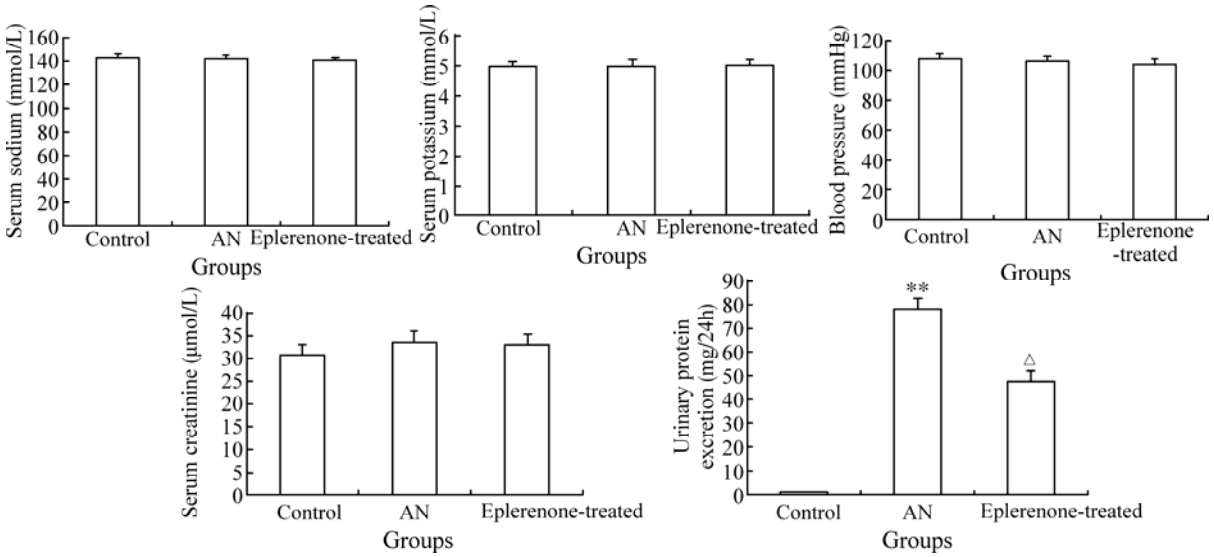

Fig. 1 Changes of blood biochemical parameters, blood pressure and the excretion of 24-h urinary protein among the 3 groups
$^{**}P<0.01$ as compared with control group; $^{\triangle}P<0.05$ as compared with the AN group

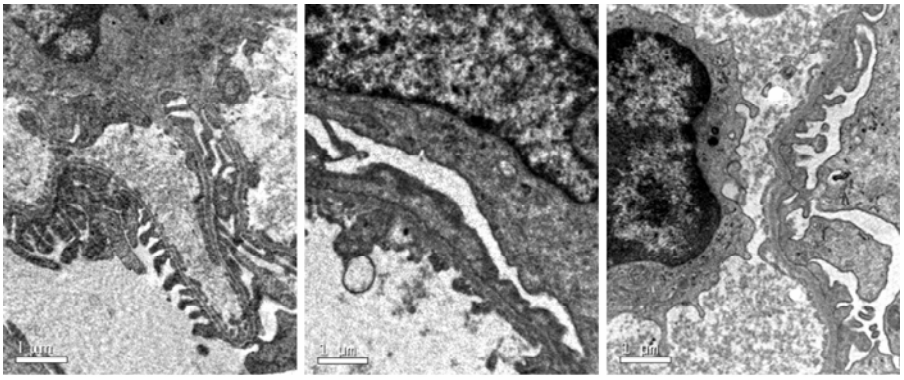

Fig. 2 Pathological changes in podocytes by electron microscopy

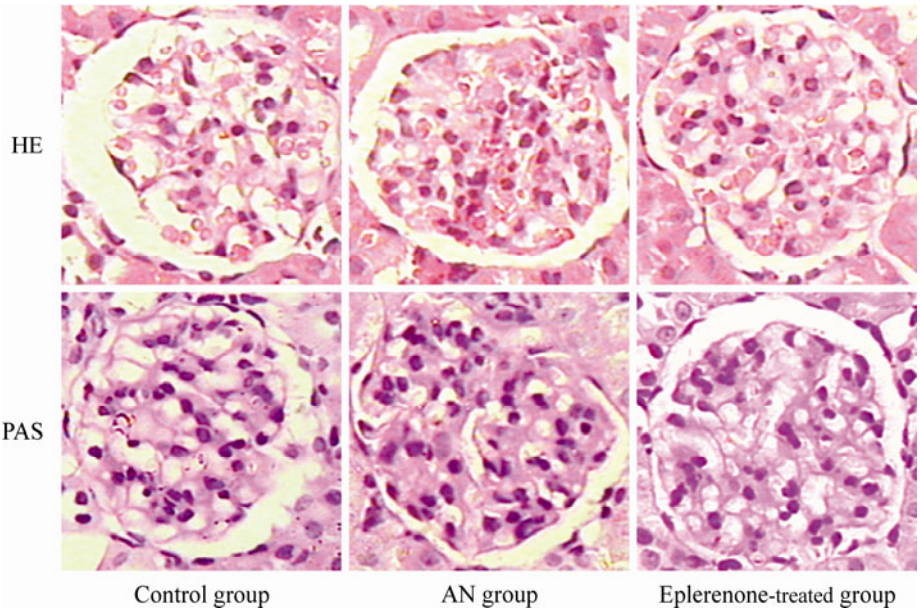

Fig. 3 HE staining and PAS staining results (×400)

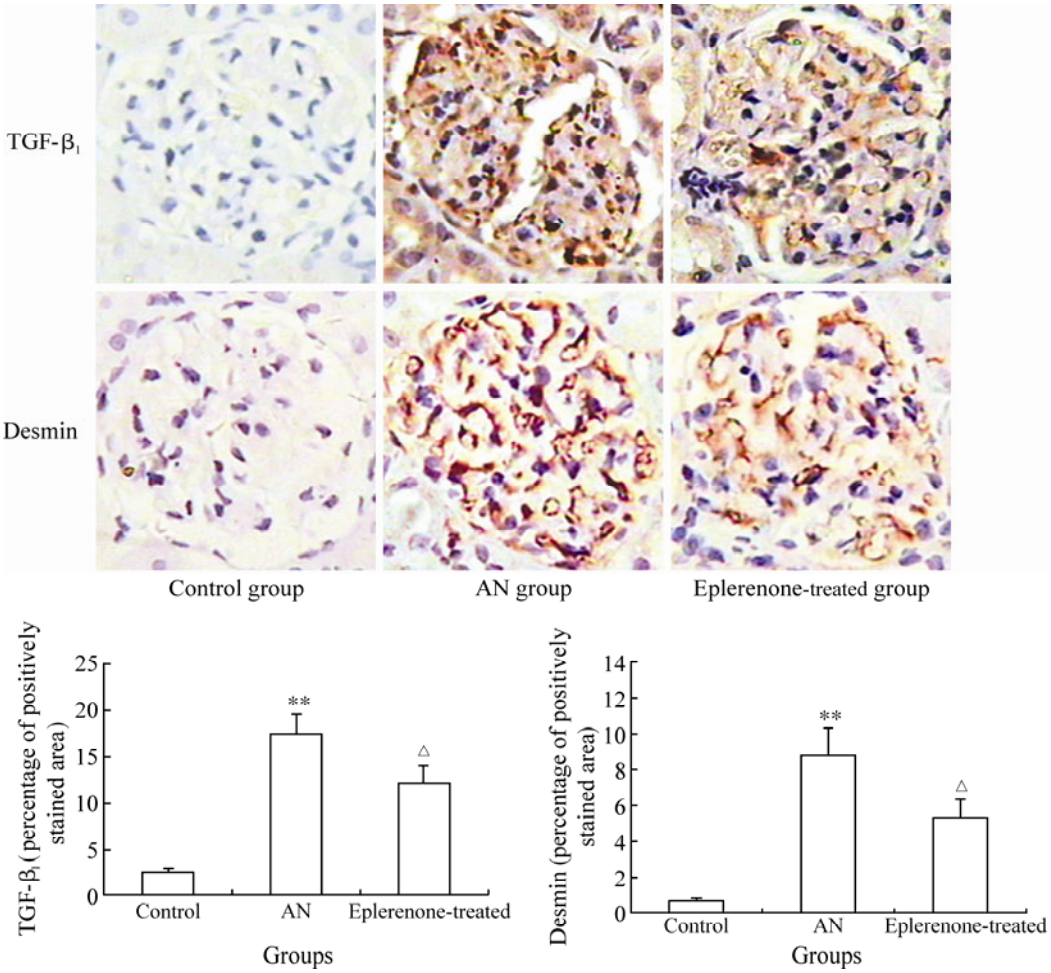

Fig. 4 The expression of TGF–β_1 and desmin in glomeruli (DAB, ×400)
$^{**}P$<0.01 as compared with the control group; $^{\triangle}P$<0.05 as compared with the AN group

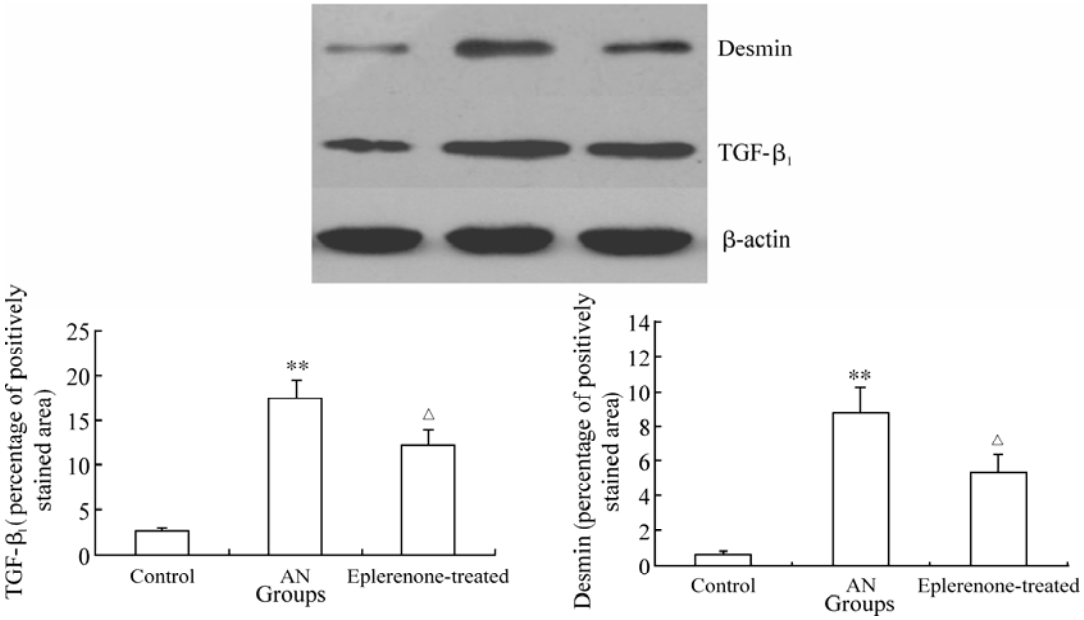

Fig. 5 Western blot analysis of the expression of TGF–β_1 and desmin in glomeruli
$^{**}P$ <0.01 as compared with the control group; $^{\triangle}P$<0.05 as compared with the AN group

2.4 Correlation Analysis Result

Correlation analysis revealed that there was a significant positive correlation between the excretion of 24-h urinary protein and the expression of TGF-β1 or

desmin (r_1=0.62, r_2=0.58, P<0.01). Meanwhile, a significant positive correlation was also found between the expression of TGF-β1 and desmin (r_1= 0.56, P<0.01).

3 DISCUSSION

The renin-angiotensin-aldosterone system (RAAS) actively participates in the derangement of renal function since the early stages of CKD. Although angiotensin-converting enzyme inhibitors (ACEI) and angiotensin type 1 receptor antagonists (ARBs) suppress the renin-angiotensin system, these agents do not adequately control plasma aldosterone levels. Substantial clinical and experimental evidence suggested that not only circulating aldosterone but also local aldosterone plays significant roles in the pathogenesis of CKD. Under pathologic conditions, the overproduction of aldosterone secreted by glomerular mesangial cells and renal tubular cells contribute to chronic renal damage by inducing inflammation, endothelial dysfunction, glomerular sclerosis, and tubular damage. Numerous studies found that eplerenone, a potent selective MR antagonist with less adverse reaction than spironolactone, significantly ameliorated glomerular and/or tubulointerstitial injury, reduced proteinuria in several animal models of nephropathy and also provided beneficial effects in patients with CKD independent of hemodynamic changes[2]. However, the molecular mechanisms underlying the action of eplerenone remained undetermined. In the current study, we have demonstrated that eplerenone effectively decreased the excretion of 24-h urinary protein, downregulated the expression of TGF-β_1 and desmin (podocyte injury marker) and ameliorated the pathological changes in adriamycin nephropathy. Furthermore, these effects were independent of blood pressure-lowing effects.

In animal models of type 1 and 2 diabetic nephropathy, eplerenone significantly reduced the excretion of 24-h urinary protein and ameliorated the pathological changes, such as glomerular hypertrophy, mesangial matrix expansion, tubulointerstitial injury, etc. These effects were independent of blood pressure and blood glucose alteration[3]. In addition, eplerenone also retarded the renal injury independent of hemodynamic changes in rat cyclosporine nephropathy model, DOCA-salt hypertensive model and spontaneously hypertensive model[4–6]. In the current study, it was observed that treatment with 100 mg/kg of eplerenone had little influence on the level of serum potassium, sodium concentrations and blood pressure, but significantly decreased the excretion of 24-h urinary protein and inhibited the foot process effacement and focal glomerular mesangial matrix expansion, which suggested that eplerenone could ameliorate renal injury independent of blood pressure level. With the dosage noted in our experiment, eplerenone didn't affect internal electrolyte balance and blood pressure, which is possibly related to the blockage of local action of aldosterone on kidney.

TGF-β_1 is a multifunctional cytokine related to many biological activities, such as cell growth, differentiation, extracellular matrix formation, tissue repair, and also inflammatory responses. TGF-β_1 mediates podocyte injury induced by high glucose, high protein and angiotensin Ⅱ[7–9]. Aldosterone induces macrophage infiltration, PAI-1 expression, TGF-beta1 expression presumably via MR in diabetic nephropathy models[10]. Juknevicius *et al* showed aldosterone infusion for 3 days in normal rats caused an increase in TGF-β_1 excretion without changes in systolic blood pressure[11]. These data showed that TGF-β_1 might participate in aldosterone-induced renal injury through multiple pathways. Our finding showed 28 days after injection of adriamycin, the expression of TGF-β_1 in renal cortex and glomeruli was dramatically increased as compared with that in the control group, while that in eplerenone-treated group was significantly lower than in the AN group. Correlation analysis revealed there was a significant positive correlation between the expression of TGF-β_1 and the excretion of 24-h urinary protein, suggesting that eplerenone could improve podocyte damage and retard the progression of proteinuria and glomerulosclerosis by suppressing the overproduction of cytokines in the early stage of kidney diseases.

Desmin expressed predominantly in mesangial cells and weakly in podocytes in a normal state. It has often been suggested to be a sensitive marker for podocyte injury and its upregulation has been described in various glomerular diseases[12]. Our finding showed 28 days after injection of adriamycin, the expression of desmin in glomeruli was substantially increased as compared with that in the control group, while that in eplerenone-treated group was significantly down- regulated when compared with that in the AN group. Correlation analysis revealed there was a significant positive correlation between the expression of desmin and the excretion of 24-h urinary protein or the expression of TGF-β_1, which indicated that eplerenone could inhibit the expression of TGF-β_1 and prevent podocytes from injury, consequently reducing the following excretion of urinary protein to prevent the progression of renal diseases.

In the current study, we demonstrated that eplerenone effectively attenuated the podocyte injury and decreased the excretion of urinary protein possibly via the inhibition of cytokine release and therefore prevented the progression of the nephropathy. Furthermore, these effects were independent of blood pressure.

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