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Attenuation of glomerular filtration barrier damage in adriamycin-induced nephropathic rats with bufalin: An antiproteinuric agent

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ABSTRACT

Proteinuria is an important risk factor for the progression and prognosis of chronic kidney disease. Bufalin, a cardiotonic steroid, has been shown to posses a variety of biological activities including cardiotonic, anaesthetic and antineoplastic activities, and regulate the immune response. This study investigated the effects of bufalin against proteinuria and glomerular filtration barrier damage in rats with adriamycin (ADR)-induced nephropathy. We compared the blood and urine biochemical indices and the histologic and ultrastructure of the glomerulus in ADR rats with and without the intervention of bufalin or prednisone. The transcription, expression and distribution of the podocyte-associated molecules were compared utilising RT-PCR, western blotting and immunohistochemical staining. We found that bufalin reduced the urinary protein excretion and optimised the lipidaemia of the ADR rats. Bufalin alleviated the removal of podocyte foot processes and attenuated the changes in nephrin, podocin and integrin-linked kinase (ILK) stainings in the glomerulus of the ADR rats. Bufalin notably decreased the expression of nephrin and ILK but inhibited the down-regulation of podocin in protein levels on the renal cortex of the ADR rats. Additionally, bufalin inhibited the up-regulation of podocin and ILK in mRNA levels but did not affect nephrin mRNA levels. These results suggest that bufalin could alleviate ADR-induced proteinuria by protecting the glomerular filtration barrier and may be a novel potential therapeutic agent for proteinuria-associated kidney disease.

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1. Introduction

Proteinuria is regarded as an isolated risk factor for the progression and prognosis of chronic kidney disease [1–3]. Proteinuric diseases are still formidable challenges in nephrology [4]. Glomerular filtration barrier defects, as manifested by proteinuria, are the primary pathologic feature of many primary glomerular diseases that eventually progress to end-stage renal failure [5]. The glomerular filtration barrier consists of the fenestrated endothelium, the glomerular basement membrane (GBM), and the slit diaphragm (SD) between the podocyte foot processes. Podocytes are highly differentiated polarised epithelial cells that cover the outermost layer of the glomerular filtration barrier. Podocytes play a major role in maintaining the integrity and permeability of the glomerular filtration barrier. The SD, a specialised cell–cell adhesion complex of podocytes, is an important structure of the glomerular filtration barrier and initiates cell signalling that protects podocyte function in response to injurious stimulations [6–8]. NPHS1 and NPHS2, the genes that encode the podocyte-associated proteins nephrin and podocin help maintain the integrity of SD and prevent proteinuria [9,10]. ILK is an intracellular serine/threonine protein kinase that interacts with the cytoplasmic domains of β-integrins and mediates the integrin signalling in diverse types of cells [11,12]. In ILK knockout mice, podocyte-specific ablation of ILK resulted in heavy albuminuria, glomerulosclerosis and kidney failure [13]. All of the above studies suggest that nephrin, podocin, and ILK play important roles in maintaining the structural and functional integrity of the glomerular filtration barrier and the development of proteinuria.

Glucocorticoids, such as prednisone, remain the mainstay of treatment for nephrotic syndrome including minimal change nephrotic syndrome (MCNS) and focal segmental glomerulosclerosis (FSGS). Immunosuppressants such as cyclophosphamide (CTX), have been administered to induce the remission of nephrotic syndrome. However, drug resistance, drug dependence and other side effects of glucocorticoids and immunosuppressants are formidable problems. Further studies have demonstrated that children with a steroid-resistant nephrotic syndrome (i.e., FSGS or MCNS) are at a high risk for developing resistance to immunosuppressive therapy [14]. Therefore, novel therapeutic strategies are necessary for a better clinical management of patients with nephrotic syndrome.

Bufalin ($C_{24}H_{34}O_4$), a cardiotonic steroid, is a major active component of the toad venom preparation *Chan Su* [15,16], which

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has a variety of biological activities, including cardiotonic, anaesthetic and antineoplastic activities, and regulates the immune responses [17–20]. Schoner and Scheiner-Bobis [16] demonstrated that bufalin activates various intracellular signalling pathways, presumably by a Na^+/K^+-ATPase-dependent mechanism. Recently, Ye et al. [21] found that bufalin inhibits tumour necrosis factor (TNF) signalling and could be used to treat inflammatory and autoimmune diseases. The most frequent glomerular disease in children is MCNS, which is characterised by repeated episodes of heavy proteinuria. Although the pathogenesis of MCNS is not known, this disease is known to be associated with inflammation or an immune reaction. A previous study found that *Chan Su* could protect the renal function in chronic renal failure [22] and reduce urinary protein excretion in the ADR rats. Because bufalin is a major component of *Chan Su*, we explored the role of bufalin in reducing proteinuria, protecting podocytes and restoring the glomerular filtration barrier in an ADR-induced MCNS rat model.

2. Materials and methods

2.1. Materials

Bufalin was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and prepared to 0.2 mg/ml by adding anhydrous alcohol and water for injection. The ADR (Pfizer Italia S.r.l, MI, Italy) was prepared to a 2.0-mg/ml concentration with normal saline. The prednisone (LiSheng Pharmaceutical Co., Beijing, China) was diluted to 1.0 mg/ml using anhydrous alcohol and distilled water. Podocin antibodies were also from Sigma Chemical Co. ILK antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa, CA, USA), and nephrin antibodies were purchased from Abcame Co. (Abcam, Cambridge, MA, USA).

2.2. Animals

Eighty male Sprague-Dawley (SD) rats (Shanghai Laboratory Animal Centre, Shanghai, China) that were 6–8 weeks of age and weighed 180–220 g were housed in individual metabolic cages in a temperature-controlled room with a 12-h light/dark cycle and free access to water and food. All animal experiments were conducted in accordance with our institutional guidelines for animal research. The rats were randomly divided into two groups at the beginning. To establish the MCNS model, 7.5 mg/kg of ADR was singly administered through the tail vein of the rats ($n = 65$). An equivalent volume of normal saline was given to the vehicle control group (group A, $n = 15$). Seven days after the injection, 24-h urine samples were collected, and 5 rats of each group were sacrificed. The remaining ADR rats were randomly divided into the following three groups with 20 rats in each group: the ADR group (group B); the ADR + bufalin group (group C), intraperitoneally injected with 0.1 mg/kg of bufalin (Fig. 1) daily; the ADR + prednisone group (group D), which was intragastrically given 6.0 mg/kg of prednisone daily. Rats in the ADR + bufalin group received an intragastric administration of equivalent volume distilled water, and rats in the ADR + prednisone group were injected intraperitoneally with an equivalent volume of normal saline once daily. Rats in group A and group B were injected intraperitoneally with normal saline and intragastrically administered an equivalent volume of distilled water every day. Two rats died in the group D and were excluded from the experiment. Each week, 24-h urine collections were performed for all groups. Rats from each group were sacrificed at Days 21 and 35 after administering ADR (group A, $n = 5$; group B, $n = 10$; group C, $n = 10$; group D, $n = 9$). In our study, the blood serum and renal cortex of all the sacrificed rats were collected and preserved.

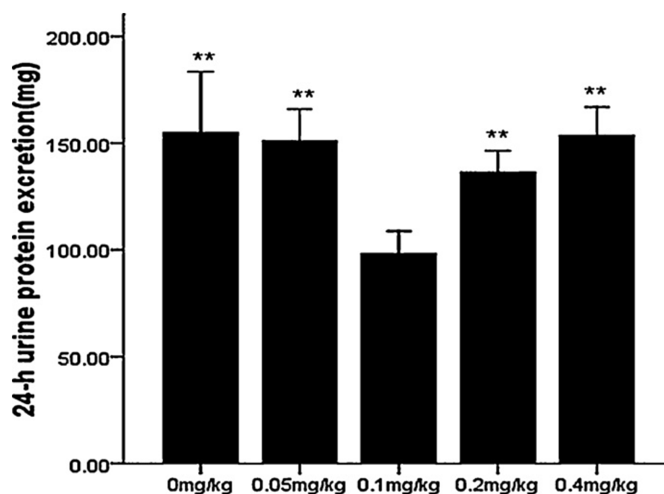

Fig. 1. Effects of bufalin at different doses on 24-h urine protein excretion. After 7 days of ADR administration, bufalin was injected intraperitoneally into five groups at doses of: 0 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.2 mg/kg and 0.4 mg/kg for 14 days. Values are the mean ± the standard error of the mean provided by a one-way ANOVA. $^{**}P < 0.01$ vs. the ADR rats given a bufalin dose of 0.1 mg/kg.

2.3. Measurement of biochemical indices

To measure the 24-h urine protein excretion, each animal was housed separately in a metabolic cage, and the daily urine volume was measured. The 24-h urine protein excretion was determined using a colorimetric assay. Serum albumin (Alb), creatinine (Cr), urea nitrogen (UN), total cholesterol (TC) and triglyceride (TG) levels were measured with a completely automatic biochemical analyser (HITACHI 7170).

2.4. Transmission electron microscopy

A small part of the renal cortex was fixed in 2.5% buffered glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin. Ultrathin sections (50 nm thick) were stained with uranyl acetate and lead citrate and examined in a transmission electron microscope (15,000×, JEM-1010, Feol Electron Inc., Japan) at 80 kV. Five fields in the transverse planes of each section were randomly selected.

2.5. Light microscopy and immunohistochemistry

The renal cortex was dissected and fixed with 4% buffered formaldehyde and embedded in paraffin. Sections (4 μm thick) were stained with periodic acid–Schiff (PAS) and hematoxylin–eosin (HE) using standard protocol, and observed under a light microscopy.

To observe immunostained podocin and ILK, the renal cortex samples were fixed with 4% paraformaldehyde and embedded in paraffin. The 4-μm-thick sections were deparaffinised and rehydrated. The sections were pretreated with 0.3% H_2O_2 in methanol for 15 min to quench the endogenous peroxide activity and boiled at 100 °C for 10 min in a 10% citrate buffer to unmask the antigens. Sections were incubated in primary antibodies (anti-ILK 1:150, anti-podocin 1:200) at 4 °C overnight and stained with HRP-labelled anti-mouse or anti-rabbit IgG (1:100). After the washes, DAB substrate was applied to the sections.

Immunofluorescence studies of nephrin proceeded essentially as described [23] using the primary antibody (anti-nephrin 1:100) and anti-rabbit IgG antibody (1:50).

2.6. RT-PCR

The renal cortex samples were flash-frozen in liquid nitrogen and stored at −80 °C. The total RNA (1 μg) extracted from renal cortex with TRIZOL (Gibco BRL, Gaithersburg, MD) was reverse-transcribed using Superscript Reverse Transcriptase to yield the respective cDNA. The efficiency of the RT-PCR was controlled by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification. Each sample mixture contained a standard PCR buffer, 10-mM dNTP, 2 U Taq polymerase, and 5 pM of each of the following primers: GAPDH, 5′-CAAGTTCAACGGCACAGTCAA-3′ and 5′-TGGTGAAGACGCCAGTAGACTC-3′; nephrin, 5′-GGCGTAGCTTAGGGAC-3′ and 5′-CCTAGCCGCCAATCAC-3′; podocin, 5′-CTAAGCAG TCTAGCTCATG-3′ and 5′-CAATCACCCGCACTTT-3′; ILK, 5′-ATGTGATGAATCGTGGGGAT -3′ and 5′-TTGAGCTTTGCCAGGAAGTT-3′. The PCR programme consisted of the following steps: 95 °C for 5 min; a denaturation step at 94 °C for 30 s; an annealing step at 52.3 °C (GAPDH), at 53.1 °C (nephrin), at 54.1 °C (podocin and ILK); and an extension step at 72 °C for 30 s, then 36 cycles for nephrin, podocin and ILK; 28 cycles for GAPDH; and a final extension at 72 °C for 7 min. The expected PCR product size was 149 bp for GAPDH, 231 bp for nephrin, 271 bp for podocin, 418 bp for ILK. The PCR products were subjected to a computer-assisted densitometry after electrophoresis on a 1% agarose gel and staining with ethidium bromide.

2.7. Western blotting

Small pieces of renal cortex were stored at −80 °C. Total proteins were extracted with RIPA buffer that contained 150 mM NaCl, 0.5% deoxycholate, 0.1% Triton X-100, 50 mM Tris–HCl (pH 7.0), 0.1% SDS, 1 mM EDTA and proteinase inhibitors. Protein concentrations were determined by the BCA method (Beyotime Institute of Biotechnology, China). The proteins were separated on a gel (5–12%) and blotted onto an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking the membranes with 5% nonfat dry milk in tris-buffered saline (TBS), the membranes were incubated overnight at 4 °C with primary antibodies for nephrin (1:5000), podocin (1:1000), ILK (1:500), β-actin (1:500), followed by a peroxidase-conjugated secondary antibody. Bound antibodies were visualised using enhanced chemiluminescence ECL. The detected proteins were standardised to β-actin or the respective total protein as appropriate.

2.8. Statistical analysis

The data were analyzed using SPSS 17.0. The differences among the four groups were tested using a one-way ANOVA, and the variance between two groups was compared using an SNK-q test. All values are expressed as the mean ± SD, and statistical significance was defined as $P < 0.05$.

3. Results

3.1. Effects of bufalin at different doses on 24-h urine protein excretion

In keeping with the prior literature regarding the effects of bufalin, at the beginning of our study, bufalin was injected intraperitoneally into five groups of the ADR rats at doses of 0 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.2 mg/kg and 0.4 mg/kg for 14 days (Fig. 1). The results showed a minimal 24-h urine protein excretion in the ADR rats that were given bufalin at a dose of 0.1 mg/kg. These results suggest that the dose of 0.05 mg/kg was too small to produce a marked effect on urine protein and that as the dose increases, the toxicity and side effects may become more severe. The 24-h urine protein excretion was greater in the ADR rats given bufalin at a dose of 0.2 mg/kg and 0.4 mg/kg than those given 0.1 mg/kg.

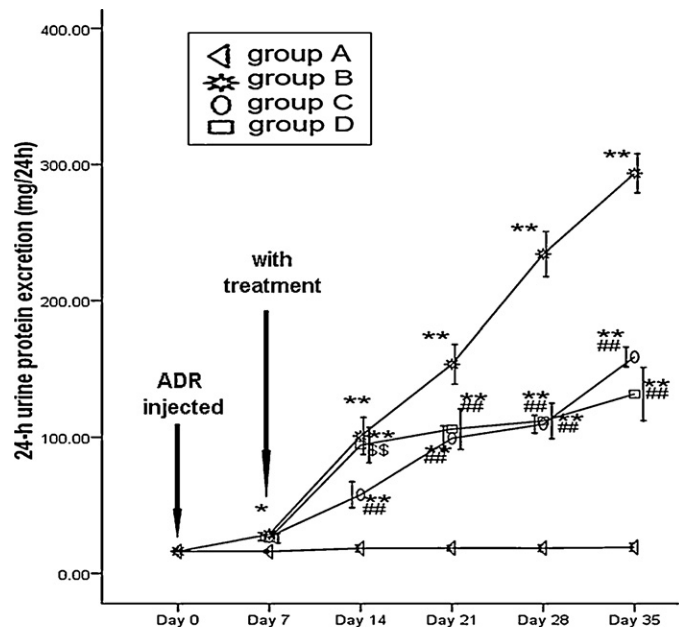

Fig. 2. Effects of bufalin or prednisone on 24-h urine protein excretion in ADR rats. The 24-h urine protein excretion was determined weekly after the administration of ADR. Group A: control group; Group B: ADR group; Group C: ADR + bufalin group; Group D: ADR + prednisone group. Values are the mean ± standard error of the mean that were provided by a one-way ANOVA. $^{*}P < 0.05$ vs. group A; $^{**}P < 0.01$ vs. group A; $^{\#}P < 0.05$ vs. group B; $^{\#\#}P < 0.01$ vs. group B; $^{\$}P < 0.05$ vs. group C; $^{\$\$}P < 0.01$ vs. group C.

3.2. Bufalin or prednisone effects on 24-h urine protein excretion

The 24-h urine protein excretion was determined weekly after the administration of ADR (Fig. 2). We found that the 24-h urine protein excretion in the ADR group increased 7–35 days after administering ADR compared to the control group (Day 7: $P < 0.05$; Day 14–35: $P < 0.01$). However, with bufalin or prednisone treatments, the 24-h urine protein excretion was remarkably decreased compared to untreated rats that had received ADR. It is interesting that the urine protein in the ADR + bufalin group started to decrease at Day 14, but it decreased in the ADR + prednisone group at Day 21. This finding indicates that bufalin could decrease the 24-h urine protein excretion earlier than prednisone. However, over time, the urine protein in the ADR + prednisone group increased more slowly than that of the ADR + bufalin group. The dose–response curve indicated that bufalin has a different mechanism from prednisone to alleviate the urine protein excretion, but we do not know the underlying mechanism at this time.

3.3. The effect of bufalin or prednisone on blood biochemical indices

The related blood biochemical indices (Table 1) were measured at Days 7, 21 and 35 after the administration of ADR. The biochemical parameters showed no difference between all groups at 7 and 21 days after the ADR injection. However, 35 days after giving ADR, the Alb level was lower, and the TC, TG and Cr levels were significantly higher in the ADR group than in the control group ($P < 0.05$). With the bufalin treatment, the levels of TC, TG and Cr were dramatically decreased, but the level of Alb had not changed compared to the ADR group (TC, $P < 0.05$; TG, $P < 0.05$; Cr, $P < 0.01$). With the

Table 1
Bufalin or prednisone effects on blood biochemical indices at Day 35.

	Group A (n = 5)	Group B (n = 10)	Group C (n = 10)	Group D (n = 9)
Alb (g/l)	35.34 ± 0.63	32.16 ± 1.94*	33.26 ± 0.92*	33.62 ± 2.12
TC (mmol/l)	1.56 ± 0.46	3.97 ± 1.13**	2.20 ± 0.76#	3.26 ± 1.62*,$
TG (mmol/l)	1.76 ± 0.58	4.54 ± 1.65**	2.69 ± 0.57#	3.31 ± 1.59
BUN (mmol/l)	8.69 ± 0.38	9.40 ± 0.88	8.54 ± 0.79	8.21 ± 0.72#
Cr (μmol/l)	60.58 ± 2.45	68.44 ± 2.57**	60.06 ± 2.54##	62.76 ± 3.29##

Values are the mean ± standard error of the mean, as tested by a one-way ANOVA. Group A: control group; Group B: ADR group; Group C: ADR + bufalin group; Group D: ADR + prednisone group.
* $P < 0.05$ vs. group A.
** $P < 0.01$ vs. group A.
# $P < 0.05$ vs. group B.
$P < 0.01$ vs. group B.
$ $P < 0.05$ vs. group C.

prednisone treatment, the Alb level showed no difference from the control group ($P > 0.05$), and the Cr level was lower than that in the ADR group ($P < 0.01$). These results suggest that bufalin might have an advantage in lipid metabolism but is not as good as prednisone at restoring the level of Alb in ADR-induced nephropathy.

3.4. Ultrastructural changes of podocyte foot processes

The foot processes in the control group remained intact and adhered to the GBM (Fig. 3A). The foot processes in the ADR group showed no remarkable alterations other than a somatic swelling of the podocytes at Day 7 compared with the control group (Fig. 3E). However, an increasing podocyte foot process effacement and fusion were observed from Day 21 (Fig. 3B) to Day 35 (Fig. 3F). We also noticed the thickening and segmental irregularity of the GBM in some areas of the glomeruli at Day 35 (Fig. 3F). The foot process effacement and fusion of podocytes were markedly improved with bufalin or prednisone treatments at Day 21 (bufalin treatment, Fig. 3C; prednisone treatment, Fig. 3D) and Day 35 (bufalin treatment, Fig. 3G; prednisone treatment, Fig. 3H). No marked changes in the GBM lesions were observed with bufalin or prednisone treatments at Day 35 compared with the control group.

3.5. Histology characteristics and the distribution of podocyte-associated molecules

The renal cortex of all rats in the four groups had no notable differences on the PAS or HE stains.

In normal rats, podocin (Fig. 4B:P1) staining was detected along the glomerular capillary loop as a fine, linear-like pattern, and nephrin (Fig. 4A:N1) staining was also a linear-like pattern but a little more dispersed than that of podocin. ILK (Fig. 4C:I1) localisation within the podocytes showed a linear-like pattern along the GBM. In the ADR rats, the linear distribution of the three molecules

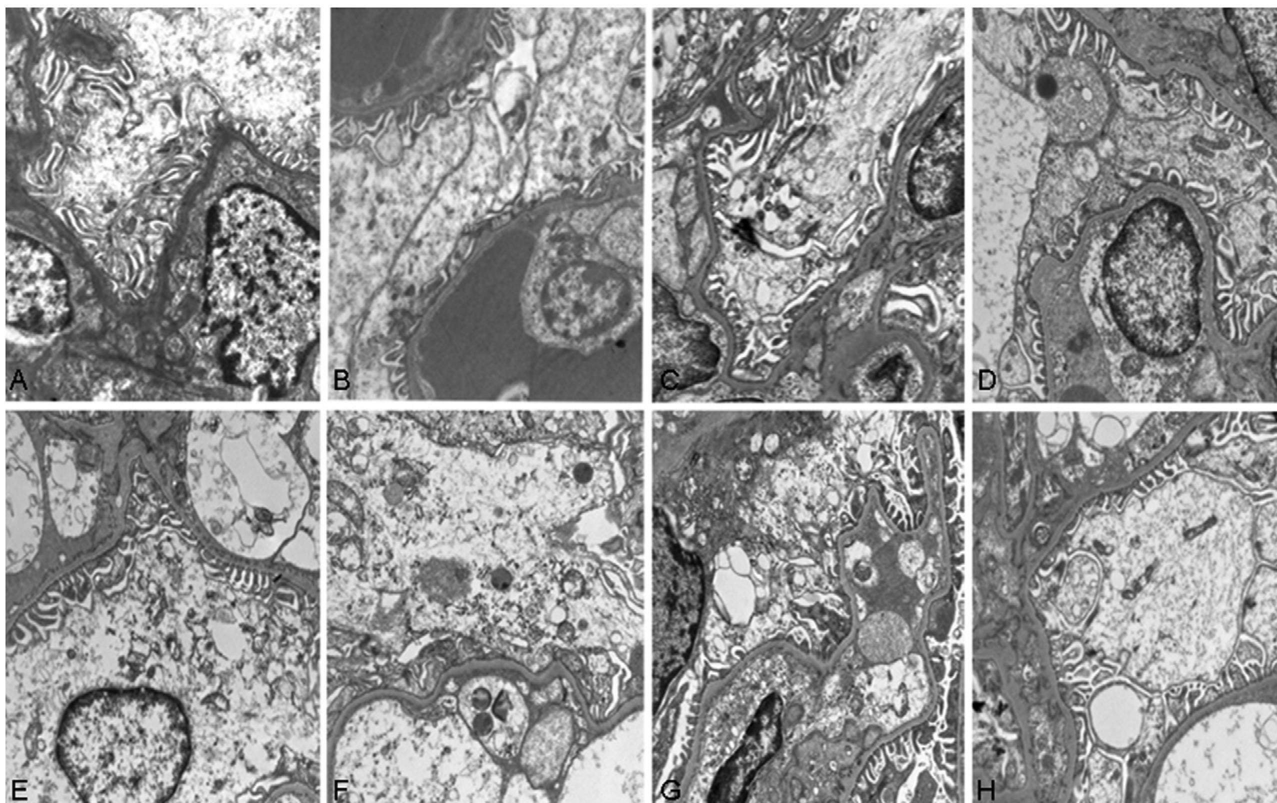

Fig. 3. Effect of bufalin or prednisone on podocyte foot processes. Seven days after ADR was injected, bufalin or prednisone was administered until Day 21 or Day 35. Representative transmission electron micrographs demonstrate the ultrastructural alterations. (A) The control group; (E, B and F) the ADR group at Days 7, 21, 35; (C and G) the ADR + bufalin group at Days 21, 35; (D and H) the ADR + prednisone group at Days 21, 35.

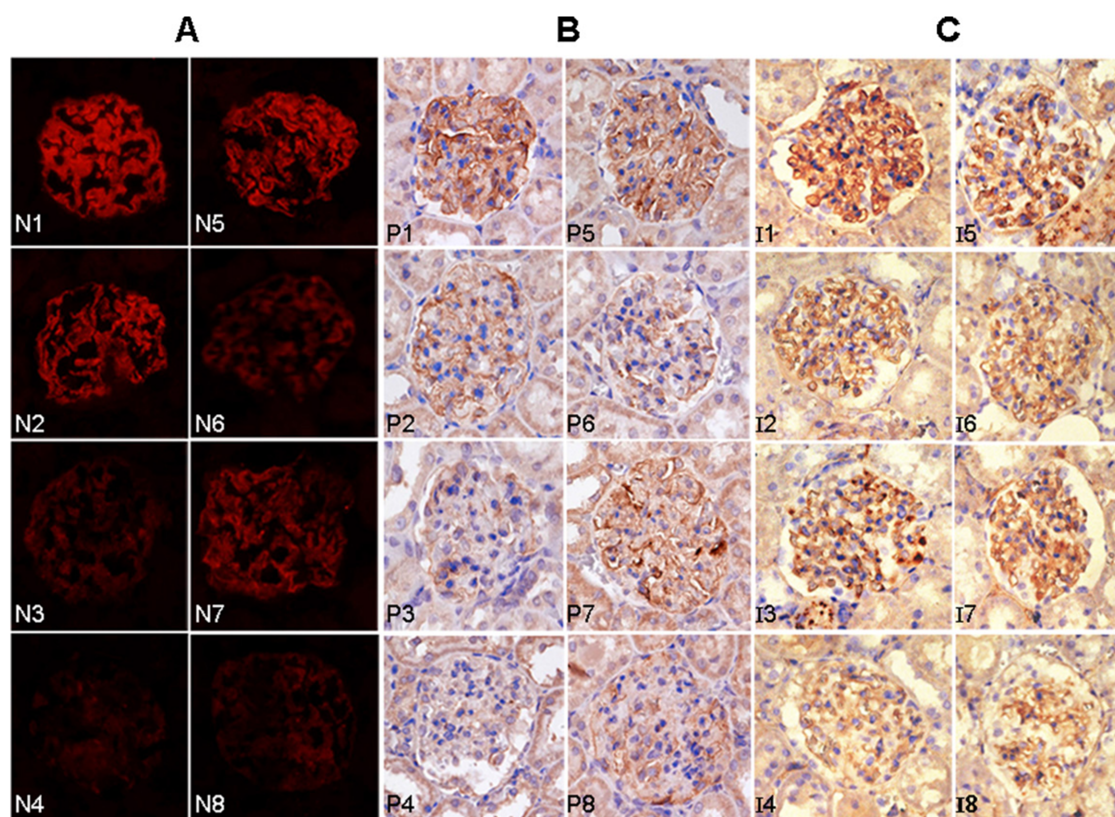

Fig. 4. The effects of bufalin or prednisone on the distribution of nephrin (A), podocin (B) and ILK (C). Seven days after ADR was injected, bufalin or prednisone was administered until Day 21 or Day 35. Representative immunohistochemistry of nephrin, podocin and ILK in the renal glomerulus: (1) the control group; (2–4) the ADR group at Days 7, 21 and 35; (5 and 6) the ADR + bufalin group at Days 21 and 35; (7 and 8) the ADR + prednisone group at Days 21 and 35.

was disrupted with the progression of the model (Fig. 4A:N2–N4; Fig. 4B:P2–P4; Fig. 4C:I2–I4). The staining showed a discontinuous, coarse, granular pattern in the ADR rats. Treatment with bufalin (Fig. 4A:N5 and N6; Fig. 4B:P5 and P6; Fig. 4C:I5 and I6) or prednisone (Fig. 4A:N7 and N8; Fig. 4B:P7 and P8; Fig. 4C:I7 and I8) alleviated the changes of the distribution of the above three molecules to a varying degree. Although the staining was not completely restored to the control pattern after treatment with bufalin or prednisone, the changes in the three molecular stainings were less severe than those in the ADR rats.

3.6. Effect of bufalin or prednisone on the gene expression of podocyte-associated molecules

In the ADR rats, the podocin mRNA at Days 21, and 35 and the ILK mRNA at Day 35 showed an up-regulation compared to the control rats. However, the mRNA expression of ILK at Day 21 and nephrin at Days 21 and 35 showed no significant difference compared to the control rats. With bufalin or prednisone treatment, both podocin and ILK mRNA showed a down-regulation compared to the untreated ADR rats at Days 21 and 35. However, nephrin mRNA showed no significant difference in bufalin or prednisone treatment. The above results are shown in Fig. 5. These results may suggest that bufalin and prednisone have parallel effects on the gene expression regulation of podocyte-associated molecules.

3.7. Effect of bufalin or prednisone on the protein expression of podocyte-associated molecules

Compared to the control rats, nephrin and ILK proteins were up-regulated at Day 21 and Day 35 in the ADR rats, but the protein expression of podocin was decreased at Days 21 and 35. Compared to the ADR rats, nephrin was down-regulated at Day 35, and ILK was markedly reduced at Day 21 with bufalin or prednisone treatment. Nephrin at Day 21 and ILK at Day 35 in the bufalin- or prednisone-treated rats showed no change compared to the ADR rats. The protein expression of podocin increased with the bufalin treatment at Day 21 and Day 35. However, with the prednisone treatment, the podocin protein increased only at Day 21 and displayed no change at Day 35 compared to the ADR rats. All of the above results are shown in Fig. 6. We observed that bufalin and prednisone have similar effects on the regulation of nephrin and ILK proteins expression but have some differences on the podocin protein expression. This finding indicates that bufalin could protect podocin protein better than prednisone.

4. Discussion

Our study is the first to reveal that bufalin alleviates proteinuria and attenuates the severity of foot process effacement and fusion in the ADR rats with comparable therapeutic efficacy to prednisone. A decrease in 24-h urine protein excretion occurred earlier with bufalin treatment (at Day 14) than with prednisone treatment (at Day 21) in the ADR rats. We suppose that bufalin starts to decrease the proteinuria earlier by protecting the glomerular filtration barrier from becoming damaged during the prophase, but the actual mechanism is unknown. Biochemically, compared with the ADR rats, the bufalin treatment in ADR rats reduced the serum Cr to the same degree as the prednisone treatment; however, bufalin treatment alone decreased the TC and TG elevation. The serum Alb level was not as optimised with bufalin treatment as with the prednisone treatment. These results indicate that bufalin might have an advantage in lipid metabolism but is not as good as prednisone in restoring the level of Alb in

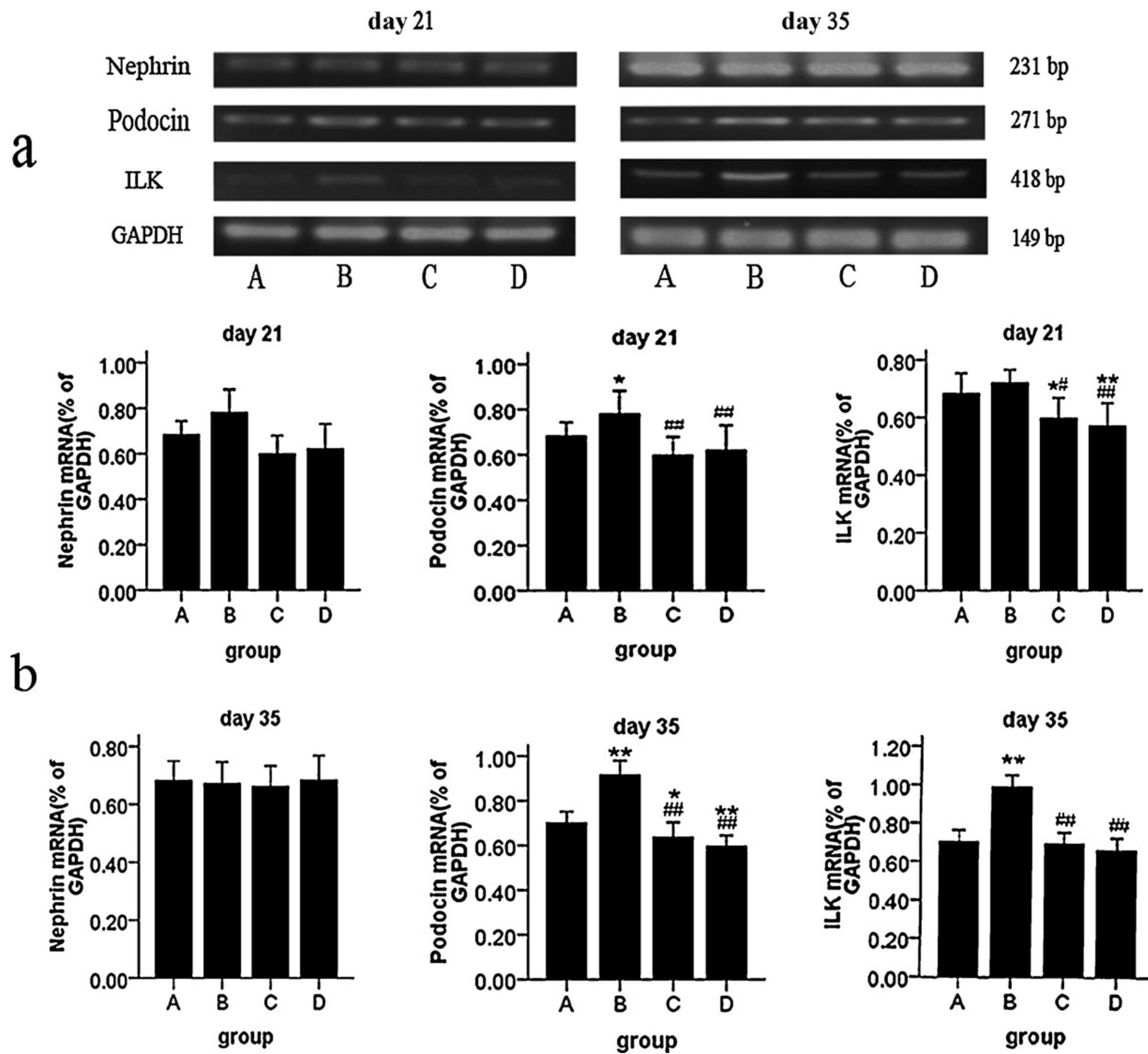

Fig. 5. Effects of bufalin or prednisone on the mRNA expression of nephrin, podocin and ILK. (a) Seven days after ADR was injected, bufalin or prednisone was administered until Day 21 or Day 35. The total RNA was extracted and analyzed by RT-PCR as described in Section 2. A: control group; B: ADR group; C: ADR + bufalin group; D: ADR + prednisone group. Similar results were obtained from repeated experiments. (b) Quantitative analyses were performed for nephrin, podocin and ILK by a densitometer. Each bar represents the mean ± S.D. ($n = 3$, $^{*}P < 0.05$ vs. group A; $^{**}P < 0.01$ vs. group A; $^{\#}P < 0.05$ vs. group B; $^{\#\#}$ $P < 0.01$ vs. group B).

ADR-induced nephropathy. If bufalin treatment were combined with prednisone in the ADR rats, the efficacy might be improved. Ultrastructurally, the foot process effacement and fusion of podocytes in the ADR rats were markedly improved with bufalin or prednisone treatment. The altered transcription, expression and redistribution of podocyte-associated molecules in the ADR rats were partially restored with bufalin or prednisone treatment. Hence, our data suggest that the protective effect of bufalin in ADR-induced proteinuria might be associated with the restoration of the expressional pattern of podocyte-associated proteins and the maintenance of podocyte foot process in the glomerular filtration barrier.

As a traditional agent for remission of overt proteinuria, glucocorticoids provide antiproteinuric effects with immunosuppression-dependent and independent mechanisms, by modulating the transcription of inflammation and immune reaction associated genes [24], and optimising the expression pattern of podocyte SD proteins, such as nephrin, podocin, CD2AP, and α-actinin-4 [25]. In this study, bufalin showed a similar effect to prednisone in the restoration of SD proteins and the maintenance of podocyte morphology.

Nephrin, a transmembrane protein that is a putative member of the immunoglobulin superfamily, appears to be at the heart of the working SD and is integral to podocyte functioning [26]. In addition, nephrin also transmits signals from the SD into the cytoplasma of podocytes. We observed that bufalin or prednisone could improve the distribution of nephrin in the glomerulus and reduce the protein expression of nephrin in the ADR rats without affecting the gene expression. This observation suggests that bufalin or prednisone could affect the regulation of the procession nephrin translation but does not affect the gene transcription procession. Furthermore, it is possible that the time points we observed were badly timed, which may explain why we did not catch the changes of nephrin mRNA. Some other drugs used to treat nephropathy also affect the genetic or nephrin protein expression. The results from experimental diabetic nephropathy and passive Heymann nephritis showed that ACE inhibitors can prevent the down-regulation of nephrin expression [27–30]. Suzuki et al. [31] reported that all-trans retinoic acid (ATRA) can regulate the expression of nephrin by enhancing the transcription of nephrin gene in rats with PAN. The differences in the change in gene or protein expression of nephrin between our results and others may be due to the different

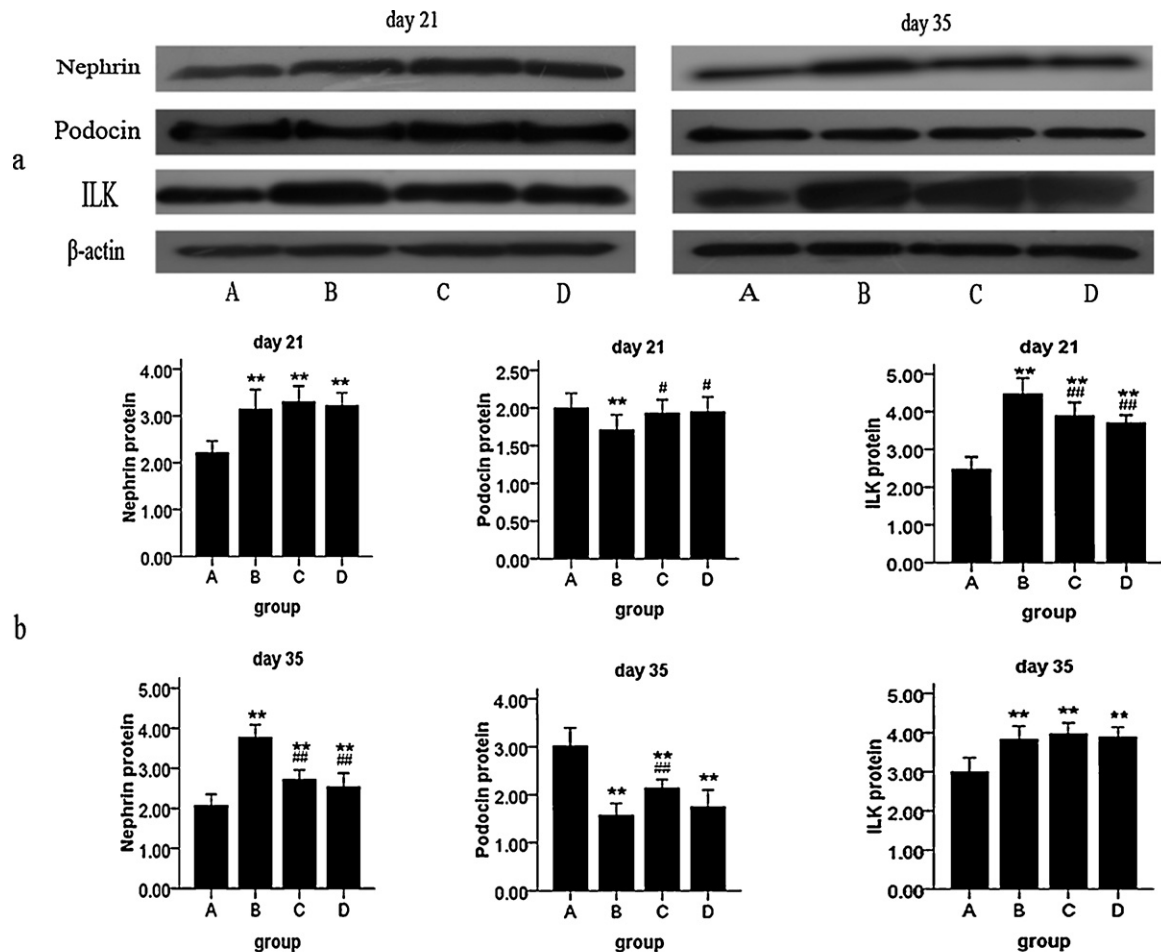

Fig. 6. Effect of bufalin or prednisone on the protein expression of nephrin, podocin and ILK. (a) Seven days after ADR was injected, bufalin or prednisone was administered until Day 21 or Day 35. The total renal cortex protein was extracted and a western blot analysis as described in Section 2. A: control group; B: ADR group; C: ADR + bufalin group; D: ADR + prednisone group. Similar results were obtained from repeated experiments. (b) Protein quantification results of nephrin, podocin and ILK (values were normalised to β-actin). Each bar represents the mean ± S.D. ($n = 3$, $^{*}P < 0.05$ vs. group A; $^{**}P < 0.01$ vs. group A; $^{\#}P < 0.05$ vs. group B; $^{\#\#}P < 0.01$ vs. group B).

animal model, different time points observed, a different quantitation method, or a combination of these [25].

Podocin is a membrane-associated protein of the band-7-stomatin family that interacts with the cytosolic tail of nephrin and connects nephrin signalling to the cytoskeleton [32]. Mutations in the NPHS2 gene cause an autosomal recessive nephrotic syndrome and have been linked to proteinuria in some populations [33]. Nakhoul et al. [34] demonstrated in ADR rats that treatment with the ACE inhibitor enalapril alone or in combination with losartan resulted in a significant preservation of podocin. In the present study, we observed that both bufalin and prednisone could regulate the gene and protein expression of podocin and improve its distribution in the glomerulus. The podocin protein was increased at Days 21 and 35 with the bufalin treatment, but it was increased only at Day 21 with the prednisone treatment, which implies that podocin might exhibit a more prominent role during the proteinuria intervention with bufalin. We also found that the podocin mRNA was increased but the protein was decreased in the ADR rats. The inconsistencies between mRNA and protein expression were detected in the preceding study. Koop et al. [35] also described the discordance between mRNA and proteins of nephrin and podocin. Although the precise mechanism of this discordance has not been clarified, it is suggested that these podocyte molecules seem to show a stereotypic reaction at both the mRNA and protein levels in proteinuric states [25].

ILK is an intracellular serine/threonine protein kinase that interacts with the cytoplasmic domains of β-integrins and has been reported to be implicated in the pathogenesis of proteinuria [36]. By interacting with nephrin and α-actinin-4, ILK builds a complex that is essential for the maintenance of the podocyte function and the glomerular filter integrity [13]. Dysregulation of ILK expression has been identified in the pathogenesis of a wide variety of chronic kidney diseases, including nephrotic syndrome and diabetic and obstructive nephropathy. ILK regulates podocyte cell matrix interaction, proliferation, and slit membrane gene expression in podocyte damage [37–40]. Kanasaki et al. [41] demonstrated a critical role for podocyte–GBM interactions mediated by β1 integrin and ILK in the normal assembly of the GBM and the cell-matrix integrin signalling. The cell–cell adhesion SD signallings are intrinsically coupled through an ILK-dependent mechanism in podocyte. We have shown herein that the expression of mRNA and the protein of ILK were increased and that the distribution of ILK changed in the ADR rats; these parameters were improved with bufalin or prednisone treatment. Bufalin could significantly decrease the mRNA levels at Days 21 and 35 and the protein expression at Day 21. Unfortunately, bufalin or prednisone did not decrease the protein expression of ILK at Day 35. We believe that bufalin or prednisone might protect the podocyte function and glomerular filter integrity through an ILK-dependent mechanism in the prophase, not in the anaphase. However, we conclude that bufalin might affect the

ILK-dependent signalling pathway to maintain the integrity of podocyte function.

In summary, our preliminary research illustrated that bufalin is a potential antiproteinuric agent because it protects the normal glomerular filtration barrier structure. The antiproteinuric effect of bufalin occurred earlier than prednisone and showed advantages in optimising the levels of serum TC and TG. However, bufalin does not maintain the Alb level as well as prednisone. The anti-proteinuric mechanism of bufalin is associated with its antagonism to ADR-induced expression and the altered distribution and transcription of nephrin, podocin and ILK; however, the underlying signalling still remains to be elucidated. As a novel potential therapeutic agent for the treatment of podocyte injury and proteinuria, bufalin may become an alternative choice for the treatment of steroid-dependent or resistant nephrotic syndrome. Further studies of its antiproteinuric mechanism are warranted.

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