Diversities of Podocyte Molecular Changes Induced by Different Antiproteinuria Drugs

YAN XING, JIE DING,[1] QINGFENG FAN, AND NA GUAN

Department of Pediatrics, Peking University First Hospital, Beijing, People's Republic of China

Nephrin, podocin, CD2AP, and α-actinin-4 are important podocyte proteins that help maintain the integrity of the slit diaphragm and prevent proteinuria. Studies have shown that angiotensin-converting enzyme inhibitors, glucocorticoids, and all-*trans* retinoic acid (ATRA) have antiproteinuric effects. However, it is still unclear whether these drugs, with different pharmacological mechanisms, lead to a reduction in proteinuria by changing the expression and distribution of these important podocyte proteins. In this study, changes in the expression and distribution of nephrin, podocin, CD2AP, and α-actinin-4 were dynamically detected in Adriamycin-induced nephrotic (ADR) rats treated with three different drugs: lisinopril, prednisone, and ATRA. Nephropathy was induced by an intravenous injection of Adriamycin. After Adriamycin injection, rats received lisinopril, prednisone, and ATRA treatment, respectively. Renal tissues were collected at Days 3, 7, 14, and 28. The distribution and the expression of messenger RNA and protein of nephrin, podocin, CD2AP, and α-actinin-4 were detected by indirect immunofluorescence, real-time polymerase chain reaction, and Western blotting, respectively. With the intervention of lisinopril, prednisone, and ATRA, changes in the expression of nephrin, podocin, and CD2AP were diverse, which was different from that detected in ADR rats. After lisinopril and prednisone intervention, podocin exhibited prominent earlier changes compared with those of nephrin and CD2AP, whereas CD2AP showed more prominent changes after ATRA intervention. There was no change in the expression of α-actinin-4 molecule. In summary, we conclude that the antiproteinuric effects of lisinopril, prednisone, and ATRA were achieved by changes in the expression and distribution of the important podocyte molecules nephrin, podocin, CD2AP, and α-actinin-4. The pattern in the change of podocyte molecules after lisinopril and prednisone intervention was similar, but the pattern in the change of podocyte molecules after ATRA intervention was different from that of lisinopril or prednisone intervention. Exp Biol Med 231:585–593, 2006

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[1] To whom correspondence should be addressed at Department of Pediatrics, Peking University First Hospital, No. 1, Xi An Men Da Jie, Beijing 100034, People's Republic of China. E-mail: djnc_5855@126.com; jieding@public.bta.net.cn

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Introduction

Proteinuria is the most important clinical manifestation of nephrotic syndrome; it is also an important risk factor for the progression of renal diseases. The glomerular filtration barrier is composed of three layers: a fenestrated endothelium, the glomerular basement membrane, and the slit diaphragm (SD) between the podocyte foot processes. Recently, some novel podocyte proteins (nephrin, podocin, CD2AP, and α-actinin-4) have been identified. These discoveries have given new insight into the pathophysiology and mechanism of proteinuria and the regulation of the glomerular filtration barrier (1–4). Further studies showed that nephrin, podocin, and CD2AP are the three very important SD-associated proteins, and form the SD complex with other podocyte proteins such as P-cadherin, FAT, and NEPH1–3 (5, 6). α-Actinin-4, an actin-filament cross-linking protein, is important for the integrity of the podocyte cytoskeleton, and directly or indirectly interacts with the SD complex components (7, 8). Some studies have also demonstrated that nephrin, podocin, CD2AP, and α-actinin-4 were all involved in the development of proteinuria of acquired and experimental nephrotic syndrome (9–13), which suggests that the four podocyte molecules play a crucial role in maintaining the structural and functional integrity of glomerular SD, and in the occurrence and development of proteinuria.

The data from clinical studies and animal experiments have demonstrated that many drugs have antiproteinuric effects, such as angiotensin-converting enzyme (ACE) inhibitors, glucocorticoids, all-*trans* retinoic acid (ATRA), and so on. However, the mechanism of action of these drugs is unclear. The present study aimed to investigate the changes of four podocyte proteins in expression and distribution as a result of three alternative pharmacological interventions (ACE inhibitors, glucocorticoids, and ATRA).

Materials and Methods

Animal Model. All animal experiments were approved by the Medical Ethical Committee of Experiment Animal (Peking University First Hospital, Beijing, China).

Male Sprague-Dawley rats (Chinese Academy of Sciences, Beijing, China) weighing 150–180 g were acclimated for 3 days in our animal quarters prior to use. Because the intervention drugs should be administrated in two different ways, orally or intraperitoneally (ip), the entire experiment was designed into two parts and carried out sequentially.

In the first part of the experiment, 96 rats were randomly divided into four groups: the control group 1 (Group 1), the Adriamycin-nephropathy (ADR) group 1 (Group 2), the lisinopril-treated group (Group 3), and the prednisone-treated group (Group 4) (n = 24 rats/group). Nephropathy was induced by a single injection of Adriamycin (6.5 mg/kg body wt; Pharmacia & Upjohn; Bridgewater, NJ) through the tail vein. The rats in Group 1 were injected with normal saline. From the second day after Adriamycin injection until the day of sacrifice, the rats in Groups 3 and 4 were administered lisinopril (10 mg/kg body wt; AstraZeneca UK Ltd., Luton, UK) and prednisone (6.25 mg/kg body wt; LiSheng Pharmaceutical Co. Ltd., Beijing, China) by gastric gavage once a day, respectively. Distilled water (vehicle) was administered by gastric gavage to the rats in Groups 1 and 2. The experiment was terminated 28 days after Adriamycin injection.

In the second part of the experiment, 72 rats were randomly divided into three groups: the control group 2 (Group 5), the Adriamycin-nephropathy (ADR) group 2 (Group 6), and the ATRA-treated group (Group 7) (n = 24 rats/group). Nephropathy was also induced by a single tail intravenous injection of Adriamycin (6.5 mg/kg body wt). The rats in Group 5 received a single tail intravenous injection of normal saline. From the second day after Adriamycin injection until the day of sacrifice, ATRA (5 mg/kg body wt; Sigma Chemical Co., Ltd., St. Louis, MO) was administered ip once a day to the rats in Group 7. The rats in Groups 5 and 6 received only corn oil and 5% dimethyl sulfoxide (DMSO) (vehicle). The experiment was terminated 28 days after Adriamycin injection. Rat groups and treatments for the entire experiment are shown in Table 1.

Urine Collection and Tissue Preparation. At Days 3, 7, 14, and 28 after Adriamycin injection, 24-hr urine was collected in metabolic cages for measuring urinary protein with an automatic biochemical analyzer (7170A; Hitachi, Tokyo, Japan). Rats (n = 6/group) were anesthetized and sacrificed by ip injection of pentobarbital (50 mg/kg body wt), and the kidneys were removed. A part of the renal cortex was fixed in 3% glutaraldehyde for transmission electron microscopy and embedded in OCT compound for immunofluorescence, respectively. The remaining renal cortex was stored at −70°C for RNA isolation and protein extraction.

Antibodies. The following primary antibodies were used: rabbit polyclonal antibody against the entire extracellular domain of human nephrin (a generous gift from Prof. Karl Tryggvason), rabbit anti-human podocin antibody p35 raised against the C-terminal part of human podocin (a kind gift from Prof. Corinne Antignac), rabbit polyclonal antibody against a recombinant protein corresponding to amino acids 350–639 mapping at the C-terminus of CD2AP of human origin (sc-9137; Santa Cruz Technology, Santa Cruz, CA), mouse monoclonal antibody against an epitope in the N-terminal region of human α-actinin (MAB1682; Chemicon, Temecula, CA).

Table 1. Groups and Treatments

Group	Treatments
1	Normal saline + distilled water (2 ml/day by gastric gavage)
2	Adriamycin + distilled water (2 ml/day by gastric gavage)
3	Adriamycin + lisinopril (10 mg/kg/day by gastric gavage)
4	Adriamycin + prednisone (6.25 mg/kg/day by gastric gavage)
5	Normal saline + corn oil and 5% DMSO (0.5 ml/day intraperitoneally)
6	Adriamycin + corn oil and 5% DMSO (0.5 ml/day intraperitoneally)
7	Adriamycin + ATRA (5 mg/kg/day intraperitoneally)

Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit or mouse IgG (Santa Cruz) was used as the secondary antibody for immunolabeling.

Indirect Immunofluorescence. Cryosections 5 μm in thickness were cut and stored at −20°C until use. The immunolabeling was performed as follows: the slides were fixed in ice-cold acetone for 10 mins, subsequently permeabilized with 0.5% Triton X-100 for 15 mins, and then blocked with 10% goat serum for 30 mins. The slides were incubated overnight with the primary antibody at 4°C (rabbit anti-nephrin, 1:50; rabbit anti-podocin, 1:300; rabbit anti-CD2AP, 1:20; mouse anti-α-actinin, 1:100) and thereafter washed in phosphate-buffered saline (PBS). The slides were then incubated with the FITC-conjugated anti-rabbit or mouse IgG antibody (1:50) for 45 mins and covered with 15% Mowiol and 50% glycerol in PBS. Images were obtained by confocal laser-scanning microscopy using a Bio-Rad Radiance 2100 TM confocal system attached to a Nikon TE 300 microscope, and processed with Adobe Photoshop 7.0 software. Ten glomeruli photographs from each antibody staining per rat were randomly obtained with a digital camera at ×400 magnification and analyzed by a person who was blinded to the treatment groups.

Reverse Transcription Reaction and Real-Time Polymerase Chain Reaction. Total RNA of every specimen was extracted from renal cortex with Trizol Reagent (Life Technologies, Paisley, Scotland, UK) according to the manufacturer's instructions. RNA concentration and quality were assessed spectrophotometrically at wavelengths of 260 and 280 nm. Total RNA (4 μg) was used for complementary DNA (cDNA) synthesis in the presence of random primers and Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) was used according

Table 2. Primer Sequence, Annealing Temperature, and Product Size for Real-Time PCR

Gene name	Primer sequence	Annealing temperature (°C)	Product size[a]
NPHS1	Forward: 5′-ATGGGCGCTAAGAGAGTCAC-3′ Reverse: 5′-CGCAGTCAGGTTTTCAGACA-3′	60	171
NPHS2	Forward: 5′-TCTTGTCCTCTCCTCCCTGA-3′ Reverse: 5′-AGACGGAGGTCAACCTTGTG-3′	60	195
CD2AP	Forward: 5′-GCTGGTGGAAAGGTGAACTG-3′ Reverse: 5′-CATCTCTGTCTTCCGCCTTC-3′	60	192
ACTN4	Forward: 5′-GCTGAAGTTCAACCGGATCAT-3′ Reverse: 5′-GCTCGCTCCGAAGTTCCTC-3′	60	187
GAPDH	Forward: 5′-AAACCCATCACCATCTTCCA-3′ Reverse: 5′-GTGGTTCACACCCATCACAA-3′	60	198

[a] Base pairs.

to the following sequence: 70°C for 5 mins, 37°C for 60 mins, and 75°C for 15 mins. Then the 1.6-μl cDNA was mixed with 2 μl of 10×SYBR Green polymerase chain reaction (PCR) buffer (PE Biosystems, Warrington, UK), 1.2 μl of 25 m*M* $MgCl_2$, 1.6 μl of 2.5 nmol/μl dNTPs, 0.8 μl of 5 pmol/l forward and reverse primers, and 0.2 μl of 5 U/μl *Taq* Gold DNA polymerase (PE Biosystems) for real-time PCR. The sequences of the primers and product sizes are shown in Table 2. Real-time PCR was performed using the GeneAmp 5700 sequence detector and software (PE Biosystems). All samples for each gene were run in duplicate. Amplification cycles were 95°C for 10 mins, followed by 35 cycles at 95°C for 45 secs, 60°C for 45 secs, and 72°C for 1 min. The quantity of expression of nephrin, podocin, CD2AP, and α-actinin-4 messenger RNA (mRNA) was corrected by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression in the same specimen.

Western Blotting. The renal cortex was lysed with RIPA buffer (consisting of 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Triton X-100, 150 m*M* NaCl, 10 m*M* EDTA, and 25 m*M* Tris·HCl pH 7.2) with protease inhibitors (1 m*M* phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pepstatin [Sigma]) using an ultrasonic homogenizer on ice. Lysates were centrifuged at 12,000 *g* for 10 mins, and the supernatants containing the protein were collected. Seventy-five micrograms of total protein were subjected to SDS-polyacrylamide gel electrophoresis with 8% acrylamide gel and transferred to a nitrocellulose membrane (Amersham Life Science, Piscataway, NJ) at 100 mA for 2 hrs. After being blocked with 5% low-fat milk powder, the membranes were incubated with antinephrin, podocin, CD2AP, α-actinin, or GAPDH antibodies at appropriate dilutions. Subsequently, the membranes were rinsed in Tris-buffered saline with 0.02% Tween-20 and incubated with horseradish peroxidase-conjugated anti-rabbit or mouse IgG antibody (Santa Cruz) at 1:4000 dilution. After a final washing, the membranes were developed using an enhanced chemiluminescence reagent (Santa Cruz), and the specific protein bands were scanned and quantitated in relation to the GAPDH (36 kDa). Mouse monoclonal antibody against GAPDH from rabbit muscle (MAB 374; Chemicon) was used in this study.

Transmission Electron Microscopy. The renal cortex was immediately fixed in 3% glutaraldehyde in cacodylate buffer for 2 hrs, postfixed in 1% osmium tetroxide for 1 hr, dehydrated in graded ethanol, washed in acetone, and embedded in Epon 812. Ultrathin sections for ultrastructural examination were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (JEM 100 CX-II; JEOL Inc., Tokyo, Japan). Kidney specimens of rats at Days 14 and 28 from each group were further measured for the average foot-process width (*Wp*). Fifteen micrographs at a magnification of ×15,000 were randomly taken from each specimen. On each photograph, the entire curved length of the peripheral capillary basement membrane (BM) was measured using a computer-based morphometric system. The numbers of the slits overlying the measured curved length of the capillary BM were counted and the *Wp* was calculated using the following formula (14, 15): $Wp = \pi/4 \times \Sigma BML/\Sigma Slits$. $\Sigma Slits$ is the number of slits counted on the photograph from each rat; ΣBML is the total peripheral capillary BM length for that rat; π/4 corrects for presumed random variation in the angle of section relative to the long axis of the podocyte.

Statistical Analyses. All values are expressed as mean ± SD. The statistical significance was evaluated by one-way ANOVA. Statistical significance was defined as $P < 0.05$. Statistical analyses were performed by SPSS version 12.0.

Results

Twenty-Four Hour Urinary Protein. The 24-hr urinary protein of ADR rats was increased at Day 14 (15.24 ± 5.35 mg/24 hrs in Group 1 vs. 82.25 ± 33.32 mg/24 hrs in Group 2, $P < 0.01$; 10.57 ± 2.58 mg/24 hrs in Group 5 vs. 150.18 ± 23.56 mg/24 hrs in Group 6, $P < 0.01$) and the increment persisted up to Day 28 (15.17 ± 6.52 mg/24

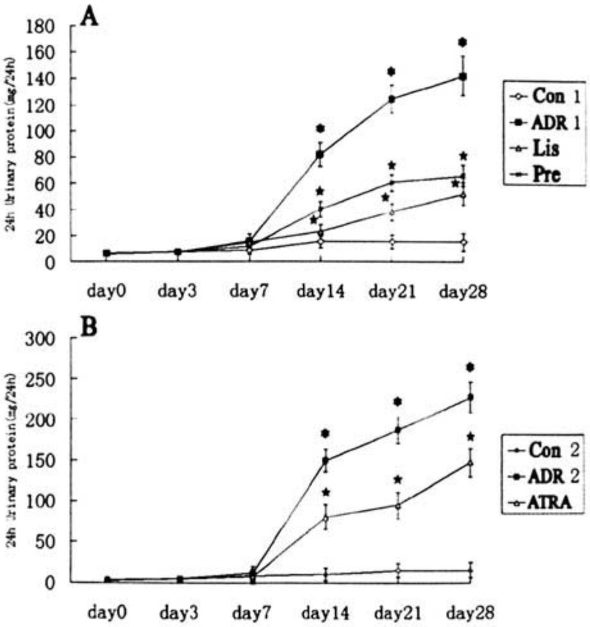

Figure 1. (A) The 24-hr urinary protein of ADR rats in the first part of the experiment was increased, and lisinopril and prednisone treatment prevented an increase in urinary protein. Con 1, Group 1 rats; ADR 1, Group 2 rats treated with Adriamycin; Lis, Group 3 rats treated with Adriamycin and lisinopril; Pre, Group 4 rats treated with Adriamycin and prednisone. Data are expressed as mean ± SD. ∗, $P < 0.01$ versus Con 1; ⋆, $P < 0.01$ versus ADR 1. (B) The 24-hr urinary protein of ADR rats in the second part of the experiment was also increased, and ATRA treatment prevented an increase in urinary protein. Con 2, Group 5 rats; ADR 2, Group 6 rats treated with Adriamycin; ATRA, Group 7 rats treated with Adriamycin and ATRA. Data are expressed as mean ± SD. ∗, $P < 0.01$ versus Con 2; ⋆, $P < 0.01$ versus ADR 2.

hrs in Group 1 vs. 142.78 ± 45.19 mg/24 hrs in Group 2, $P < 0.01$; 16.31 ± 4.45 mg/24 hrs in Group 5 vs. 228.81 ± 59.75 mg/24 hrs in Group 6, $P < 0.01$). This result was consistent in two parts of the experiments. In ADR rats treated with lisinopril, prednisone, or ATRA, the 24-hr urinary protein levels were all reduced at Days 14 and 28 (Day 14, 22.73 ± 7.73 mg/24 hrs in Group 3; 40.12 ± 19.02 mg/24 hrs in Group 4; 80.92 ± 15.23 mg/24 hrs in Group 7, $P < 0.01$; Day 28, 52.30 ± 19.95 mg/24 hrs in Group 3; 66.29 ± 23.13 mg/24 hrs in Group 4; 148.85 ± 36.23 mg/24 hrs in Group 7, $P < 0.01$), compared with the untreated ADR rats. Results are shown in Figure 1.

Ultrastructural Change. As is shown in Figure 2, in ADR rats at Day 14, the normal arrangement of interdigitating foot processes was lost and the foot processes broadened; the podocyte appeared to swell, and cell bodies of podocytes showed intracytoplasmic vesicles and inclusions. At Day 28, the widespread fusion and effacement of foot processes appeared; podocyte alterations were more extensive than at Day 14 and were characterized by numerous intracytoplasmic vesicles and inclusions. All changes observed were limited to podocyte and glomerular basement membrane, endothelial, and mesangial cells did not show marked changes via electron microscopy, which is similar to human minimal change nephrotic syndrome. The *Wp* of ADR rats was greater than that of control rats (1733.97 ± 227.57 nm vs. 336.14 ± 12.86 nm, $P < 0.01$; and 3060.01 ± 562.79 nm vs. 338.34 ± 13.76 nm, $P < 0.01$ for ADR and control rats at Days 14 and 28, respectively). The *Wp*s were all significantly decreased compared with those of ADR rats after treatment with lisinopril, prednisone, and ATRA (Table 3).

Distribution of Nephrin, Podocin, CD2AP, and α-Actinin. In control rats, podocin staining was detected as a fine, linear-like pattern along the glomerular capillary loop; nephrin staining was also a linear-like pattern but a little more dispersed than that of podocin; CD2AP showed a linear-like staining pattern; and α-actinin staining was detected as a dotted, linear-like pattern.

In ADR rats, the staining patterns of nephrin, podocin, CD2AP, and α-actinin started to change at Day 7 and the changes were aggravated with time. The nephrin and podocin staining changed to a discontinuous, coarse granular pattern. CD2AP staining showed an intense granular pattern in part of the glomerular capillary wall, and the staining of α-actinin became uneven throughout the glomerulus, and a continuous linear pattern was observed in part of the glomerular capillary wall.

Treatment with lisinopril, prednisone, or ATRA all evidently prevented a change in nephrin, podocin, CD2AP, and α-actinin staining to a different degree from Day 7. Although staining did not fully restore to the normal pattern after treatment with lisinopril, prednisone, or ATRA, the

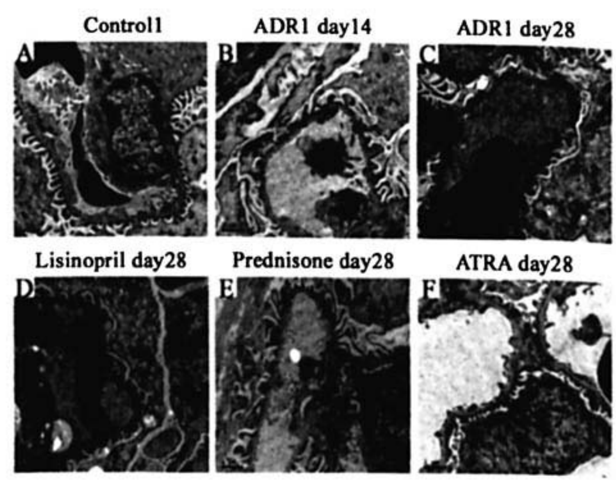

Figure 2. Morphology change in the podocyte foot process (transmission electron microscopy, ×15,000). (A) The foot processes in control rats were tall and narrow. (B) At Day 14 after Adriamycin injection, the foot processes broadened. (C) At Day 28 after Adriamycin injection, the fusion and effacement of foot processes was observed. (D) The fusion and effacement of foot processes was less serious in lisinopril-treated rats than that in ADR rats at Day 28. (E) Prednisone treatment alleviated the severity of fusion and effacement of foot processes compared with ADR rats at Day 28. (F) With ATRA treatment, the severity of fusion and effacement of foot process was reduced at Day 28. Con 1, Group 1 rats; ADR 1, Group 2 rats treated with Adriamycin; Lis, Group 3 rats treated with Adriamycin and lisinopril; Pre, Group 4 rats treated with Adriamycin and prednisone; ATRA, Group 7 rats treated with Adriamycin and ATRA. Bar, 1 μm.

Table 3. Average Foot-Process Width[a]

	Foot-process width			Foot-process width	
Day	ADR 1	Lis	Pre	ADR 2	ATRA
14	1733.97 ± 227.57	548.65 ± 24.16*	638.35 ± 56.56*	1630.93 ± 258.89	744.28 ± 68.07*
28	3060.01 ± 562.79	472.96 ± 18.85*	498.54 ± 40.52*	3763.00 ± 1026.08	527.62 ± 7.60*

[a] Measurements are expressed in nanometers; data are mean ± SD. ADR 1, Group 2 rats treated with Adriamycin; Lis, Group 3 rats treated with Adriamycin and lisinopril; Pre, Group 4 rats treated with Adriamycin and prednisone; ADR 2, Group 6 rats treated with Adriamycin; ATRA, Group 7 rats treated with Adriamycin and ATRA.
*$P < 0.01$ versus ADR 1 or ADR 2.

changes in these molecular stainings were less serious than those in ADR rats (Fig. 3).

Messenger RNA Expression of Nephrin, Podocin, CD2AP, and α-Actinin-4. In ADR rats, nephrin mRNA showed an up-regulation at Day 7 (0.79 ± 0.37 in Group 1 vs. 1.35 ± 0.29 in Group 2, $P < 0.05$; 0.46 ± 0.05 in Group 5 vs. 1.24 ± 0.23 in Group 6, $P < 0.05$), and returned to normal at Days 14 and 28; podocin and CD2AP mRNA increased consistently from Day 3 and became level at Day 14 (podocin, 2.41 ± 0.84 in Group 1 vs. 3.35 ± 0.47 in Group 2, $P < 0.05$; 0.39 ± 0.13 in Group 5 vs. 0.93 ± 0.42 in Group 6, $P < 0.05$) and Day 28 (podocin, 3.44 ± 0.64 in Group 1 vs. 5.08 ± 1.75 in Group 2, $P < 0.05$; 0.92 ± 0.53 in Group 5 vs. 2.11 ± 1.13 in Group 6, $P < 0.05$; CD2AP, 1.47 ± 0.67 in Group 1 vs. 2.55 ± 0.95 in Group 2, $P < 0.05$; 0.61 ± 0.12 in Group 5 vs. 1.48 ± 0.62 in Group 6, $P < 0.05$), respectively; and the mRNA expression of α-actinin-4 showed no change.

Podocin mRNA was evidently higher in lisinopril-treated rats than that in ADR rats at Day 7, but it was prominently lower at Day 28. The mRNA expression of nephrin, CD2AP, and α-actinin-4 showed no significant difference between the Lis group and the ADR 1 group.

Compared with ADR rats, podocin mRNA was evidently reduced with prednisone treatment at Day 7, and the decrease persisted until Day 28 (2.45 ± 0.69 in Group 4 vs. 5.08 ± 1.75 in Group 2, $P < 0.05$). CD2AP mRNA was elevated at Day 14 (1.97 ± 0.40 in Group 4 vs. 1.42 ± 0.37 in Group 2, $P < 0.05$) and was lower at Day 28 (1.22 ± 0.32 in Group 4 vs. 2.55 ± 0.95 in Group 2, $P < 0.05$) with prednisone treatment. Nephrin and α-actinin-4 mRNA showed no change in prednisone-treated rats.

In ATRA-treated rats, podocin mRNA was decreased at Day 14 (0.52 ± 0.19 in Group 7 vs. 0.93 ± 0.42 in Group 6, $P < 0.05$) and Day 28 (1.12 ± 0.50 in Group 7 vs. 2.11 ± 1.13 in Group 6, $P < 0.05$), CD2AP mRNA was

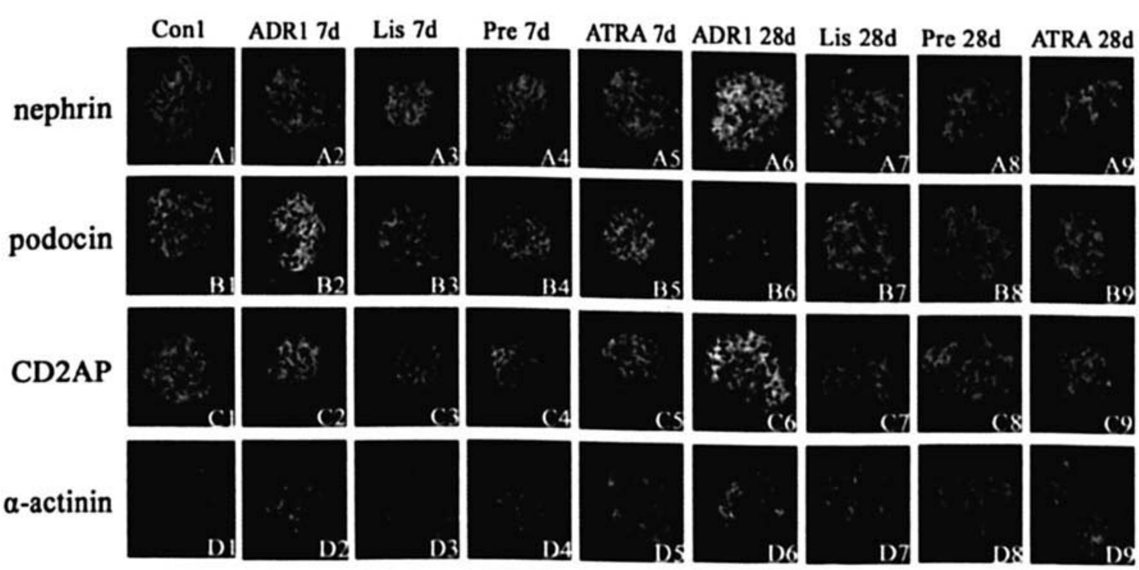

Figure 3. Immunofluorescence staining of nephrin, podocin, CD2AP, and α-actinin. A1–A9, nephrin staining. B1–B9, podocin staining. C1–C9, CD2AP staining. D1–D9, α-actinin staining. In control rats, nephrin and podocin staining showed a continuous, linear-like pattern along the glomerular capillary loop; CD2AP showed a linear-like staining; and α-actinin showed a dotted, linear-like pattern along the glomerular capillary loop. In ADR rats, the discontinuous, coarse granular staining pattern of nephrin and podocin was revealed from Day 7 to Day 28; CD2AP staining showed an intense granular pattern in part of the glomerular capillary wall, and the staining of α-actinin became uneven throughout the glomerulus and a continuous linear pattern was observed in part of the glomerular capillary wall. Treatment with lisinopril, prednisone, and ATRA prevented an alteration in the distribution of nephrin, podocin, CD2AP, and α-actinin. Con 1, Group 1 rats; ADR 1, Group 2 rats treated with Adriamycin; Lis, Group 3 rats treated with Adriamycin and lisinopril; Pre, Group 4 rats treated with Adriamycin and prednisone; ATRA, Group 7 rats treated with adriamycin and ATRA. Bar, 30 μm.

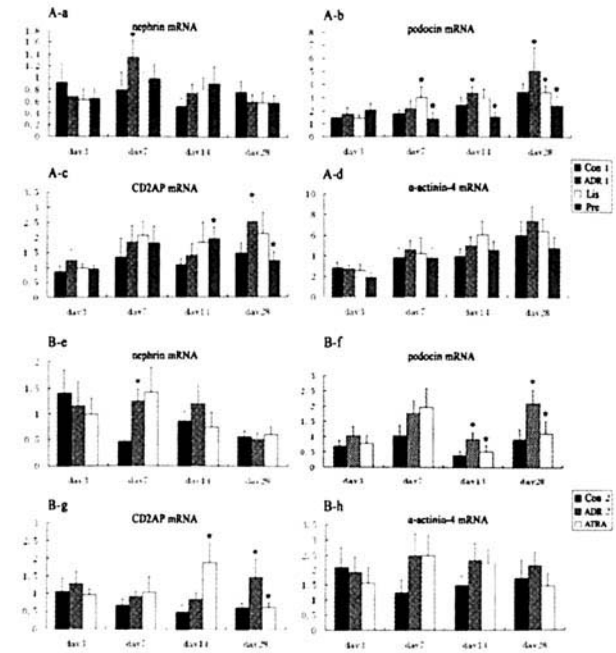

Figure 4. The mRNA expression of nephrin, podocin, CD2AP, and α-actinin-4 detected by real-time PCR. (A) The mRNA expression level in the first part of experiment. (a) Nephrin mRNA showed a significant increase at Day 7 after Adriamycin injection, and lisinopril and prednisone treatment did not affect the expression of nephrin mRNA. (b) Podocin mRNA was elevated in ADR rats at Days 14 and 28. It was elevated at Day 7 and decreased at Day 28 with lisinopril treatment, and declined in Pre rats from Day 7 to Day 28. (c) CD2AP mRNA was up-regulated in ADR rats at Day 28 and showed no change with lisinopril treatment. It was elevated at Day 14 but decreased at Day 28 in Pre rats. (d) α-Actinin-4 mRNA. There was no difference among four groups. Con 1, Group 1 rats; ADR 1, Group 2 rats treated with Adriamycin; Lis, Group 3 rats treated with Adriamycin and lisinopril; Pre, Group 4 rats treated with Adriamycin and prednisone. Data are expressed as mean ± SD. *, $P < 0.05$ versus Con 1; ★, $P < 0.05$ versus ADR 1. (B) Messenger RNA expression levels in the second part of experiment. Compared with Con 2 rats, the results of ADR 2 rats were consistent with those of ADR 1 rats in the first part of the experiment. (e) Nephrin mRNA. ATRA treatment did not affect nephrin mRNA. (f) Podocin mRNA was decreased at Days 14 and 28 in ATRA rats. (g) CD2AP mRNA was elevated at Day 14 but decreased at Day 28 in ATRA rats. (h) α-actinin-4 mRNA. ATRA did not affect α-actinin-4 mRNA. Con 2, Group 5 rats; ADR 2, Group 6 rats treated with Adriamycin; ATRA, Group 7 rats treated with Adriamycin and ATRA. Data are expressed as mean ± SD. *, $P < 0.05$ versus Con 2; ★, $P < 0.05$ versus ADR 2.

evidently up-regulated at Day 14 but down-regulated at Day 28, and the mRNA expression of nephrin and α-actinin-4 did not change compared with that of ADR rats (Fig. 4).

Protein Expression of Nephrin, Podocin, CD2AP, and α-Actinin. In ADR rats, compared with control rats, the protein expression of nephrin started to increase at Day 7 and was elevated until Day 28 (1.84 ± 0.41 in Group 1 vs. 5.79 ± 3.31 in Group 2, $P < 0.05$; 2.58 ± 0.65 in Group 5 vs. 6.73 ± 3.48 in Group 6, $P < 0.05$); podocin protein was dramatically up-regulated at Day 7, and thereafter recovered again, but was down-regulated at Day 28 (2.34 ± 0.85 in Group 1 vs. 1.03 ± 0.23 in Group 2, $P < 0.05$; 2.94 ± 0.84 in Group 5 vs. 1.43 ± 0.73 in Group 6, $P < 0.05$); CD2AP protein prominently increased at Days 14 and 28; α-actinin protein showed no change.

Compared with ADR rats, nephrin protein was reduced at Day 28 (2.78 ± 1.80 in Group 3, 2.94 ± 1.66 in Group 4 vs. 5.79 ± 3.31 in Group 2, $P < 0.05$), and podocin protein at Day 7 and CD2AP at Days 14 and 28 was also markedly decreased with lisinopril and prednisone treatment. Whereas podocin at Day 28 was elevated with lisinopril treatment (1.99 ± 0.54 in Group 3 vs. 1.03 ± 0.23 in Group 2, $P < 0.05$), it was not affected by prednisone treatment compared with ADR rats.

Compared with ADR rats, nephrin protein was down-regulated at Day 28 (3.01 ± 1.63 in Group 7 vs. 6.73 ± 3.48 in Group 6, $P < 0.05$) and CD2AP was also decreased at Day 14 with ATRA treatment. However, podocin was not affected by ATRA treatment. α-Actinin protein showed no change with lisinopril, prednisone, or ATRA treatment. The protein expression change of these podocyte molecules is shown in Figures 5 and 6.

Discussion

In this study, lisinopril, prednisone, and ATRA all reduced proteinuria and attenuated the severity of foot process fusion and effacement in ADR rats. Moreover, our study is the first to reveal that the expression and distribution of the podocyte molecules nephrin, podocin, and CD2AP, and the distribution of α-actinin-4, all changed with the intervention of lisinopril, prednisone, and ATRA. However, the changes in total protein level and the pattern of distribution at different time points in ADR rats resulted from three alternative pharmacological interventions by ACE inhibitors, glucocorticoids, and ATRA. Compared with ADR rats, lisinopril intervention significantly prevented podocin protein from increasing at Day 7 and decreasing at Day 28, CD2AP protein from increasing at Days 14 and 28, and nephrin protein from increasing at Day 28. Meanwhile, the changes in the distribution of nephrin, podocin, and CD2AP were also corrected with lisinopril treatment. The pattern of the changes in podocyte molecules observed with prednisone intervention was similar to that observed with lisinopril intervention, whereas the pattern of the changes in podocyte molecules after ATRA intervention was different from that of lisinopril as well as prednisone. ATRA treatment markedly prevented CD2AP protein from increasing at Days 14 and 28, and nephrin protein from increasing at Day 28, and did not affect the expression level of podocin protein. Although changes in the expression and distribution of nephrin, podocin, and CD2AP were diverse with the intervention of three antiproteinuria drugs in ADR rats, these results evidently indicate that the antiproteinuric effects of different drugs all involve the novel podocyte molecules nephrin, podocin, CD2AP, and α-actinin-4.

Previous studies by different groups have revealed the individual pharmacological mechanism of the antiprotein-

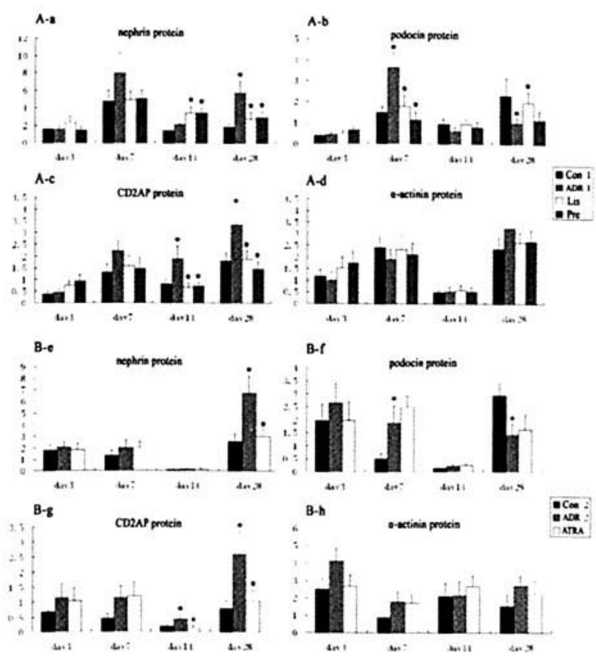

Figure 5. Protein quantification results of nephrin, podocin, CD2AP, and α-actinin (values were corrected by GAPDH). (A) Protein expression levels in the first part of the experiment. (a) Nephrin protein showed an increase at Day 28 after Adriamycin injection. It was elevated at Day 14 and reduced at Day 28 in Lis rats and Pre rats. (b) Podocin protein was up-regulated at Day 7 and down-regulated at Day 28 after Adriamycin injection. It was reduced at Day 7 and up-regulated at Day 28 in Lis rats, and decreased at Day 7 in Pre rats. (c) CD2AP protein was up-regulated at Days 14 and 28 after Adriamycin injection. Lisinopril and prednisone treatment prevented an increase in CD2AP protein at Days 14 and 28. (d) α-Actinin-4 protein showed no change. Con 1, Group 1 rats; ADR 1, Group 2 rats treated with Adriamycin; Lis, Group 3 rats treated with Adriamycin and lisinopril; Pre, Group 4 rats treated with Adriamycin and prednisone. Data are expressed as mean ± SD. *, $P < 0.05$ versus Con 1; ★, $P < 0.05$ versus ADR 1. (B) Protein expression levels in the second part of the experiment. Compared with Con 2 rats, the results of ADR 2 rats were consistent with those of ADR 1 rats in the first part of the experiment. (e) Nephrin protein was reduced at Day 28 in ATRA rats. (f) ATRA did not affect podocin protein. (g) CD2AP protein was reduced at Days 14 and 28 with ATRA treatment. (h) ATRA did not affect α-actinin-4 protein. Con 2, Group 5 rats; ADR 2, Group 6 rats treated with Adriamycin; ATRA, Group 7 rats treated with Adriamycin and ATRA. Data are expressed as mean ± SD. *, $P < 0.05$ versus Con 2; ★, $P < 0.05$ versus ADR 2.

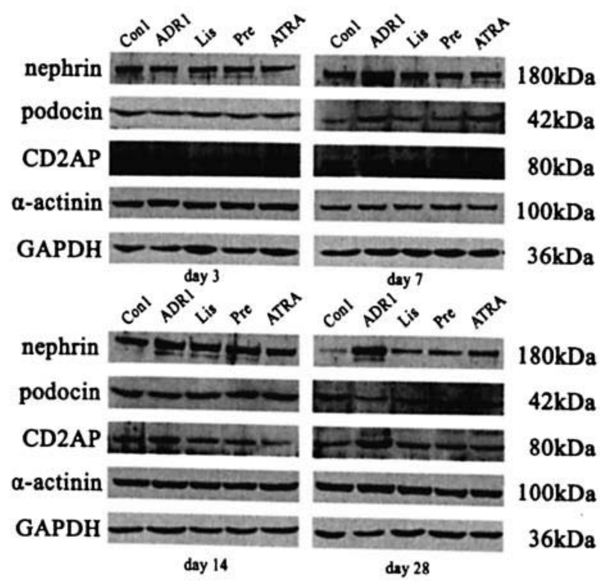

Figure 6. Western blotting analysis of nephrin, podocin, CD2AP, and α-actinin. Con 1, Group 1 rats; ADR 1, Group 2 rats treated with Adriamycin; Lis, Group 3 rats treated with Adriamycin and lisinopril; Pre, Group 4 rats treated with Adriamycin and prednisone; ATRA, Group 7 rats treated with Adriamycin and ATRA.

uric effects for ACE inhibitors, glucocorticoids, and ATRA. ACE inhibitors achieved a potent nephroprotective effect by the inhibition of angiotensin II production (16). ACE inhibitors prevented the conversion of angiotensinogen *via* angiotensin I to the main effector molecule angiotensin II, by inhibiting ACE (17). Glucocorticoids have their most profound effect by modifying the immunological or inflammatory mechanisms responsible for glomerular injury. Glucocorticoids act by binding to a specific receptor that, upon activation, translocates to the nucleus and either activates or inhibits gene expression (18). ATRA exerts a wide variety of biologic effects, of which anti-inflammatory and antiproliferative action are two of the most basic effects. ATRA induces the expression of several target genes *via* the receptors for retinoids. When ATRA binds to its receptor, the complex activates retinoic acid response elements, and then causes the expression of target genes (19). Studies by other groups of researchers have shown that ATRA could reduce proteinuria in puromycin aminonucleoside nephrosis (PAN) and experimental glomerulonephritis in rats (20, 21). In addition, several studies in recent years also indicated that three antiproteinuria drugs all can directly act on podocyte or podocyte proteins, and demonstrated that podocyte has the functional receptors for angiotensin II, glucocorticoids, and ATRA (20, 22, 23). Liebau *et al.* (24) demonstrated functional expression of key components of the renin angiotensin system in differentiated human podocytes, including ACE Type 1. Thus, ACE inhibitors can directly inhibit the activity of ACE in podocytes and further interfere with the local effect of angiotensin II at the level of the podocyte. Dexamethasone can decrease the high permeability of murine podocyte induced by Adriamycin and alter the expression of heat shock protein 27 (HSP27), alphaB-crystallin, and ciliary neurotrophic factor (CNTF) in cultured podocyte (25, 26). ATRA can induce podocyte differentiation and alter nephrin and podocin expression *in vitro* (27). Thus, our findings, combined with results *in vitro* mentioned above, suggest that podocytes may constitute an important and common molecular target for the three antiproteinuria drugs and that the important podocyte molecules nephrin, podocin, CD2AP, and α-actinin-4 also play the key role in the intervention of proteinuria with ACE inhibitors, glucocorticoids, and ATRA.

Our study also revealed that changes in the expression of nephrin, podocin, and CD2AP were not pa each other during the intervention with three diffe After the intervention of lisinopril or prednisone, in the expression of podocin occurred before that of nephrin and CD2AP. Previously, we also found that the knockdown of podocin mRNA could induce a change in the expression and distribution of nephrin in cultured murine podocytes (28). In addition, ADR rats treated with lisinopril or prednisone presented a remarkable increase in podocin protein at Day 7 compared with that of ADR rats without treatment, which occurred before the significant reduction of proteinuria at Day 14. In other words, the change in podocin expression was previous to the reduction of proteinuria. Our results suggest that podocin might exhibit a more prominent role than nephrin and CD2AP molecules during the intervention of proteinuria with lisinopril and prednisone. Nevertheless, with the intervention of ATRA the change in the expression of CD2AP, instead of podocin, was most evident. The significant decrease in CD2AP protein took place simultaneously with the dramatic reduction of proteinuria at Day 14 with ATRA intervention, which implies that CD2AP might exhibit a more prominent role during the proteinuria intervention with ATRA. Recently, studies by other groups also investigated the effect of ACE inhibitors and ATRA on the podocyte molecules in experimental nephrotic syndrome. Results from several other studies on experimental diabetic nephropathy and passive Heymann nephritis showed that ACE inhibitor can prevent the down-regulation of nephrin expression (29–32). Nakhoul *et al.* (33) revealed that the antiproteinuric effects of the ACE inhibitor enalapril was associated with the preservation of nephrin and podocin expression levels, and podocin distribution did not restore with enalapril treatment in ADR rats. However, our results showed that not only podocin expression, but also its distribution, were both preserved with lisinopril intervention in ADR rats. Suzuki *et al.* (20) reported ATRA can regulate the expression of nephrin by enhancing the transcription of nephrin gene in rats with PAN. Our results showed that ATRA markedly prevented the increase of nephrin protein at Day 28 in ADR rats. The differences in the change in expression or distribution of podocyte molecules between our results and those of others may result from the different animal model, different time points observed, different quantitation method, or a combination of these.

In our study, the mRNA and protein expression of α-actinin-4 did not change either in ADR rats or in ADR rats treated with different drugs.

The change in distribution of α-actinin-4 was evidently prevented after the intervention of three antiproteinuria drugs. Previous studies by other researchers revealed that α-actinin-4 connected the actin cytoskeleton with the SD complex in the podocyte (34). Thus, it is implied that the preservation of normal distribution in α-actinin-4 caused by drug intervention in ADR rats might reserve the connection between cytoskeleton and SD complex and further maintain the structural and functional integrity of the SD complex.

In addition, the discordance between mRNA and protein expression of nephrin, podocin, and CD2AP was detected in the present study. Koop *et al.* (9) and Luimula *et al.* (12) have also described the inconsistencies between mRNA and protein of nephrin, podocin, and α-actinin-4. Although the precise mechanism of this discordance has not been clarified, it suggests that these podocyte molecules seem to show a stereotypic reaction at both the mRNA and protein levels in proteinuric states.

In summary, we conclude that the antiproteinuric effects of lisinopril, prednisone, and ATRA were achieved by altering the expression and distribution of novel podocyte molecules nephrin, podocin, CD2AP, and α-actinin-4. The pattern of the change in podocyte molecules after the intervention of lisinopril or prednisone was very similar. Podocin exhibited a more prominent role than nephrin, CD2AP, or α-actinin-4. The pattern of the change in podocyte molecules after ATRA intervention was different than that with lisinopril or prednisone intervention. CD2AP exhibited a more prominent role than other podocyte molecules in ADR rats treated with ATRA.

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