Nephrin and Podocin Expression Around the Onset of Puromycin Aminonucleoside Nephrosis

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Abstract. Decreased expression levels of the glomerular slit membrane proteins, nephrin and podocin, have been reported after the onset of puromycin aminonucleoside (PA) nephrosis. We examined nephrin and podocin expressions prior to the onset of proteinuria of PA nephrosis to elucidate the proteinuria induction mechanism of PA. PA nephrosis was induced by a subcutaneous single injection of 120 mg kg⁻¹ PA. The mRNA levels of nephrin and podocin in whole kidney total RNA were quantified by the TaqMan real time PCR quantification system. The localization and levels of nephrin and podocin molecules were analyzed by immunofluorescence and Western blotting, respectively. Albuminuria and proteinuria were significant on days 3 and 4 in PA nephrosis rats. The protein levels of nephrin and podocin decreased significantly at day 3. The protein localization of nephrin and podocin changed at day 2 and day 1, respectively. The mRNA level of nephrin increased at day 2 and subsequently decreased at day 4. The podocin mRNA level did not change significantly. In conclusions, the protein level of nephrin and podocin decreased at the onset of albuminuria in the PA nephrosis. However, the first change induced by PA was the change of podocin localization from a linear pattern to a dot-like one prior to the onset of albuminuria.

Keywords: nephrin, podocin, nephrosis, puromycin aminonucleoside

Introduction

Recent studies have suggested that proteinuria is a predictive independent risk factor for the progression of renal disease (1). Therefore, the mechanism of proteinuria should be elucidated to prevent the progression of renal disease. Puromycin aminonucleoside (PA) was identified as an inducer of proteinuria in rats. PA nephrosis has been used as an experimental model of human nephrotic syndrome with minimal change (2). However, the detailed mechanism of proteinuria induction by PA has yet to be determined.

With respect to genes related to nephrosis, NPHS1 and NPHS2 were identified as mutated genes in the human congenital nephrotic syndrome of the Finnish type (3) and in patients with autosomal recessive steroid-resistant nephrotic syndrome (4), respectively. Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis in humans (5). Gene targeting studies also revealed that the CD2AP gene (6) and the Neph1 gene (7) were responsible for nephrosis in mice. The respective gene product NPHS1, termed nephrin, as well as the Neph1 molecule, is a transmembrane glycoprotein of the slit diaphragm in the glomeruli, belonging to the immunoglobulin-like molecules (8). The respective gene product NPHS2, named podocin, is an integral protein of the slit diaphragm belonging to the stomatin family, and it has been shown to interact with nephrin (9) and Neph1 (10), as well as CD2AP (11).

The disappearance of the nephrin (12, 13) and podocin (14, 15) proteins in PA nephrosis has been
demonstrated by immunofluorescence studies at the exacerbated phase of proteinuria on days 7–9 following PA administration. Using semi-quantitative RT-PCR, the mRNA level of nephrin on day 3 was demonstrated to decrease to 60% of the control following a single injection of 200 mg kg\(^{-1}\) (16) or 150 mg kg\(^{-1}\) (12) PA. However, the mRNA level of nephrin on day 9 was also demonstrated controversially to decrease to 80% of control following a single injection of 100 mg kg\(^{-1}\) PA (13). With regards to podocin in PA nephrosis, stable mRNA levels were demonstrated at the time that podocin protein levels decreased (14, 15). Therefore, there are discrepancies between the mRNA level and the protein level of podocin in PA nephrosis.

The aim of this study is to investigate the mRNA and protein levels of nephrin and podocin before the onset of proteinuria in PA nephrosis from day 1 to day 5 following PA administration. The daily profiles of the expressions of nephrin and podocin before the onset of proteinuria will contribute to the elucidation of the mechanism of nephrosis induced by PA.

Materials and Methods

Animals

Thirty male Sprague-Dawley strain rats weighing 150–180 g (Saitama Experimental Animal, Saitama) were fed with a basal diet in metabolic cages (TECNIPLAST, Buguggiate, Italy) at room temperature with a 12-h light/dark cycle. Water was given ad libitum.

For the induction of PA nephrosis, 120 mg kg\(^{-1}\) of PA (Sigma P7130; Sigma, St. Louis, MO, USA) was dissolved at a concentration of 24 mg ml\(^{-1}\) in saline and injected into 15 rats subcutaneously on the first day of the experiment (day 0). Fifteen control rats were injected with the same amount of saline subcutaneously. One to five days following PA administration, three saline-injected rats and three PA-injected rats were weighed and anesthetized with an intraperitoneal injection of 50 mg kg\(^{-1}\) pentobarbital, and their kidneys were excised each day. Left kidneys were frozen immediately in liquid nitrogen for extraction of total RNAs. Right kidneys were sliced, embedded in OCT compound, and frozen on liquid nitrogen to prepare frozen sections. Animal handling followed the ethical guidelines of the Kyorin University School of Medicine.

Protein content assay and SDS-PAGE of the urine

Protein in 10 \(\mu\)l of urine was precipitated with 10% trichloroacetate on ice for 30 min and then centrifuged for 15 min at 15,000 rpm. The precipitated urinary proteins were dissolved in 50 \(\mu\)l of distilled water and the protein concentration was determined by BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Urinary excretion of protein was determined from the urinary protein concentration and the volume of urine collected by the metabolic cage. For the quantification of urinary albumin, the precipitated urinary proteins, corresponding to 1/20,000 of daily excretion from three rats, were also separated by SDS-PAGE with 10% polyacrylamide gels and stained with Coomassie Brilliant Blue R-250. The density of 60 kD band was analyzed using NIH image 1.62.

TaqMan real time RT-PCR quantitative system

Total RNA in frozen left kidney was extracted using 10 ml of ISOGEN kit (Nippongene, Tokyo) and homogenized with a POLYTRON homogenizer (PT10/35; KINEMATICA AG, Littau-Lucerne, Switzerland). The crude total RNA was purified with RNase-free DNase using an RNeasy Mini kit (QIAGEN, Valencia, CA, USA). One microgram of the total RNA was used for generating cDNAs by reverse transcription with 2.5 \(\mu\)M random hexamers and TaqMan Reverse Transcription Reagents (Applied Biosystems Japan, Tokyo). For rat nephrin mRNA quantification, primers (rNEF-Q1: 5'-TAATGTTGCTCGGGCCACCCAG-3' and rNEF-Q1R: 5'-TGTGTGGTGTGTCAGAGCCCA-3') and TaqMan probe (rNEF-TAQ1: 5'-CCCTCTTCAAATGCACGCCACCC-3') were synthesized. For rat podocin mRNA quantification, primers (rPodo-Q1: 5'-GGCACAAAGACAGGCCGTAATTGCTTGTTGAGCCCA-3') and TaqMan probe (rPodo-TAQ1: 5'-ACTCAGAGGCGACTCTTCTTCAAATGCACCCAG-3') were synthesized. Real time quantitative PCR was performed from the 0.02 \(\mu\)g cDNA using TaqMan Universal Master Mix and ABI 7700 sequence detector according to the manufacturer’s instructions.

Indirect immunofluorescence and confocal microscopy

Frozen kidney slices, embedded in OCT compound, were cut into 4-\(\mu\)m-thick sections and immunostained with anti-rat podocin polyclonal antibody and anti-human nephrin polyclonal antibody. Anti-rat podocin polyclonal antibody was generated against a synthetic peptide corresponding to KAGRGNRGRARPDAGAE (amino acids 28–45 of rat podocin). A rabbit was immunized with peptide conjugated with the carrier protein, keyhole limpet hemocyanin (KLH). The peptide-specific IgG fraction was further immunoaffinity-purified. Rabbit polyclonal antibody against the intracellular part of human nephrin (UP3) (8) was used for the immunofluorescence studies of rat nephrin. The kidney sections were incubated with blocking buffer for 1 h at room temperature and then reacted with anti-rat
podocin antibody (1:500) or anti-human nephrin polyclonal antibody (1 μg/ml) in blocking buffer for 60 min at room temperature. After washing three times with PBS-Tween 20, the kidney sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1:200; Molecular Probes, Inc., Leiden, Netherlands) for 40 min at room temperature. Following a final washing with PBS, the kidney sections were mounted in 1 mg/ml p-phenylene-diamine in PBS/glycerol (1:1) to retard laser bleaching and examined by fluorescence microscopy using a confocal laser scanning microscope (GB 200; Olympus, Tokyo).

**Western blotting**

For Western blotting, three control rats and three PA-injected rats from days 1 to 5 after PA administration were anesthetized, and both of their kidneys were excised. For isolation of glomeruli, minced cortical kidney tissue was scraped on ice-cold sieves of 180-μm pores and sieved through sequential sieves of 180- and 105-μm pores with ice-cold saline. The glomeruli were isolated on the sieve of 65-μm pores. After adding 2 ml of PBS containing protease inhibitors (Complete; Roche Diagnostics KK, Tokyo) to the isolated glomeruli from one rat, the mixture was homogenized in a Potter-Elvehjem homogenizer on ice. The homogenate was centrifuged at 6,000 x g for 15 min at 4°C, and the resultant supernatant was centrifuged at 100,000 x g for 30 min at 4°C. The precipitate was resuspended with the PBS-containing protease inhibitors as a crude membrane fraction. Protein concentrations of samples were determined with a BCA protein assay kit (Pierce Biotechnology).

Western blotting analysis was performed as follows: 1-μg samples of the crude membrane fraction of glomeruli from the control rat and PAN rats were separated on a 10% polyacrylamide gel by the Laemmli method and then semi-dry blotted on a PVDF filter (Immobilon-P, Millipore Co., Bedford, MA, USA). The blotted filter was shaken for 1 h at room temperature in blocking solution (5% blocking agent of the ECL kit (Amersham Biosciences) in the TBS with 0.02% Tween 20) and then washed three times with washing solution (150 mM NaCl, 10 mM Tris-HCl, pH 7.8, 0.1% Tween 20). The blocked filter was stained overnight at 4°C using primary antibodies in the TBS-T solution containing 1% bovine serum albumin. The detection was performed according to the manufacturer’s instructions with the ECL kit (Amersham Biosciences). The intensity of the bands was analyzed using NIH image 1.62.

**Data analysis and statistical assessment**

The results from the representative experiments are expressed as the mean ± S.E.M. Statistical analyses were performed using the unpaired t-test.

**Results**

**Daily profile of body weight and urinary protein content**

Figure 1A illustrates the daily profile of body weight in control and PAN rats. The average body weight of PAN rats decreased to 15.8 ± 2.1 g at day 1 after PA administration (n = 15). However, the weight gain of PAN rats was almost the same as that of control rats.
from day 2. This body weight loss of PAN rats indicated an acute toxicity of PA that was apparent for only one day. The average body weight of PAN rats was significantly lower than that of control rats from day 1 to day 5 following PA administration ($P<0.01$, $n = 3 - 15$). Increased body weight due to nephrosis-induced edema was not observed during this experimental period.

Figure 1B indicates the daily profile of urinary protein in control and PAN rats. Male control rats excreted 26.4 ± 3.1 mg protein per day in their urine ($n = 15$). A single subcutaneous injection of 120 mg kg$^{-1}$ PA significantly induced massive proteinuria from day 4, as determined from urine collected from days 3 to 4 ($P = 0.002$, $n = 3$). Urinary protein excretion at day 4 and day 5 after PA administration was 188.2 ± 24.2 mg protein per day and 392.5 ± 13.4 mg protein per day, respectively ($n = 3$).

**Daily profile of urinary albumin**

Figure 2A shows Coomassie Brilliant Blue-stained urinary proteins separated by SDS-PAGE. The band observed at approximately 60 kD in the urine from the PAN rats was interpreted as urinary albumin. The intensity of the band was analyzed as the urinary albumin content by comparing it with the intensity of bovine serum albumin as a protein standard. The band of albumin was not detected in the urine of control rats nor in the PAN rats at day 1 after PA injection. However, the faint band of albumin could be detected in the urine at day 2 following PA injection. As shown in Fig. 2B, a single subcutaneous injection of 120 mg kg$^{-1}$ PA induced significant albuminuria at day 3, as determined from urine collected from days 2 to 3 ($P = 0.04$, $n = 3$). Urinary albumin excretion at day 3 and day 4 after PA administration was 4.1 ± 1.7 mg protein per day and 16.4 ± 5.9 mg protein per day, respectively ($n = 3$).

**Daily profile of the mRNA levels of nephrin and podocin in the kidney**

Figure 3 indicates the daily profile of nephrin and podocin mRNA level analyzed with TaqMan real time quantitative PCR. To prevent RNA degradation, each left whole kidney was frozen by liquid nitrogen immediately after excision. TaqMan signal from genomic DNA was eliminated by RNase-free-DNase treatment. To prevent interruption of cDNA elongation, reverse transcription was conducted with random primer. GAPDH mRNA level was used for the internal standard.

![Graph](image1.png)

**Fig. 2.** Daily profile of urinary albumin in PA nephrosis. A) A photograph of SDS-polyacrylamide electrophoresis, which separated urinary proteins from control and PA treated rats. Urinary proteins were precipitated with 10% trichloroacetic acid and applied 1/20,000 of daily amount. Urinary albumin of PA treated rat was detected as a band of 60-kD protein by staining with Coomassie brilliant blue R-250. There were no protein bands in the urine of control rats. B) Daily profile of urinary albumin in PA treated rats. Mean ± S.E.M., $n = 3$ *$P<0.05$ vs control.

![Graph](image2.png)

**Fig. 3.** Daily profile of nephrin and podocin mRNA levels in the kidneys of control and PA treated rats. Open and closed circles represent the amount of nephrin mRNA in 1 mg total RNA from the kidneys of control and PA treated rats, respectively. Open and closed squares represent the amount of podocin mRNA in 1 mg total RNA from the kidneys of control and PA treated rats, respectively. Mean ± S.E.M., $n = 3$ **$P<0.01$, *$P<0.05$ vs control.
Fig. 4. Daily profile of the localization of nephrin and podocin protein in the glomeruli of control and PA-treated rats. A) Daily profile of nephrin protein localization in the glomeruli of PA-treated rats (n = 2). Normal localization of nephrin in the glomeruli of control rat is indicated in the left-upper panel. All photographs were taken under the same condition for the laser confocal microscope and shown as the same size as indicated by the 40-μm calibration bar at the lower-left corner of the photograph of the control rat. B) Daily profile of podocin protein localization in the glomeruli of PA-treated rats (n = 2). Normal localization of podocin in the glomeruli of control rat is indicated in the left-upper panel. Higher magnifications are shown in the insets.
of sample cDNAs. Nephrin mRNA was calculated to be 44.0 ± 4.7 pg in 1 mg total RNA from the control rat kidney. In PAN rat kidney, nephrin mRNA level was not decreased, but rather was significantly enhanced on days 2 and 3 after PA injection (P = 0.003 and 0.026, respectively, n = 3). Nephrin mRNA level was normalized on day 4 after injection and decreased on day 5 (P = 0.01). Podocin mRNA was calculated to be 8.7 ± 0.5 pg in 1 mg total RNA from the control rat kidney. In PAN rat kidney, podocin mRNA level was not changed significantly from days 1 to 5 after PA administration.

**Daily profile of the protein localizations of nephrin and podocin at the glomeruli**

Figure 4A shows the daily profile of nephrin protein localization using indirect immunofluorescence confocal microscopy. The expression pattern of nephrin in PAN rats on day 1 was not different from that of control rats. The intensity of nephrin immunofluorescence started to decline even on day 2 after PA administration, and the decrease in intensity continued until day 5.

Figure 4B shows the daily profile of podocin protein localization using indirect immunofluorescence confocal microscopy. The intensity of podocin immunofluorescence had declined on day 3 after PA administration and the decrease in intensity continued until day 5. Moreover, the localization of podocin in PA nephrosis on day 1 was a dot-like pattern, which was different from the linear pattern of control rats as indicated in the insets.

**Daily profile of protein expression of nephrin and podocin in the glomeruli**

Figure 5 shows the daily profile of protein expression of nephrin and podocin in the crude membrane fraction of isolated glomeruli. Similar to Fig. 4, the intensities of the bands of nephrin and podocin decreased significantly at day 3, at which time albuminuria had begun to develop. A photograph of Western blotting using the glomerular crude membrane fraction from a control rat is shown as an inset. Anti-nephrin and anti-podocin antibodies were used as indicated below the photograph. Rat nephrin was detected as a 180-kD strong band, and as a 110-kD faint band, in agreement with previous reports (13). Rat podocin was detected as a single band of 48 kD, almost the same molecular weight as previously reported, 42 kD (14).

**Discussion**

When PA was administered subcutaneously at 120 mg kg⁻¹, body weight loss was observed at day 1 as an acute toxicity of PA, similar to previous reports (17). Since most of the administered PA was excreted within 8 h (18), the return of body weight gain to normal at day 2 represents recovery from the acute PA toxicity. Although the cause of body weight loss is unknown, the normal gain of body weight indicates that protein synthesis in PAN rats was intact from day 2. Urinary protein was not increased significantly until day 4, with a 3-day latency period as reported previously (19). Urinary albumin appeared at day 3, when rats had already recovered from the acute toxicity of PA.

The protein levels of nephrin and podocin demonstrated by Western blotting decreased at day 3, at the onset of albuminuria. The nephrin and podocin proteins demonstrated by immunofluorescence seemed to disappear at day 2 and at day 3, respectively. Although the disappearance of nephrin (12, 13) and podocin (14, 15) in PA nephrosis has already been reported, in this study, we have demonstrated that these disappearances occurred at approximately the same time as the onset of albuminuria.

We could not detect any significant decrease of the mRNA level of podocin in this study, similar to previous reports (14, 15). Therefore, the decrease of podocin content in the crude membrane of glomeruli was not caused by the decrease of its transcription. Since protein synthesis and the body weight gain of PAN rats were intact from day 2, translation mechanisms also seem to be intact in podocytes on day 3. Moreover, PA is not a protein synthesis inhibitor; therefore, the inhibition of
translation of podocin mRNA was not caused by residual PA in the glomeruli. Although podocin protein does not have a transmembrane domain in its structure, podocin exists in the membrane fraction by interacting with nephrin and Neph1. Therefore, decrease of nephrin protein content in the crude membrane fraction seems to be followed by the decrease of podocin content in it.

Although the podocin localization changed at day 1 from a linear pattern to a dot-like one, the nephrin localization on day 1 was the same as that of the control. Since nephrin is a transmembrane protein localized at the slit diaphragm, no change of nephrin localization on day 1 suggests that the structure of the slit diaphragm was intact on day 1. Thus, the change of podocin localization on day 1 suggests detachment of podocin from nephrin or the dislocation of another interacting protein such as Neph1. The detachment of podocin from nephrin may induce increased turnover of the nephrin molecule.

Although the decrease in nephrin mRNA level was demonstrated at 2 h after PA administration (13), this decrease seems to be an acute toxicity of PA, which has RNA synthesis inhibitor activity (20). The higher nephrin mRNA level on days 2 and 3 in our study is coincident with a previous study, which reported that the nephrin mRNA level increased up to twofold during 4 and 8 weeks of streptozotocin-induced diabetes in rats (21). This increase of nephrin mRNA level suggests an increase of nephrin synthesis to maintain nephrin content in glomeruli under conditions of high nephrin molecule turnover.

The mRNA level of nephrin at day 3 was reported to decrease to 60% of the control by a single injection of 200 mg kg⁻¹ (16) or 150 mg kg⁻¹ PA (12). Our study also demonstrated that nephrin mRNA levels began to decrease at day 3 and were significantly decreased at day 4. Therefore, PA also induced a decrease in transcription of the nephrin gene around the onset of proteinuria. However, the change in protein-protein interactions of podocin preceded the decrease in nephrin mRNA levels. Kawachi et al. demonstrated a slight decrease in nephrin mRNA levels to 80% of the control on day 9 (13) when proteinuria was sustained. Thus, the elevation of protein turnover of nephrin may be more important to sustained nephrosis than the decrease in nephrin mRNA levels.

On the other hand, the nephrin protein levels demonstrated by immunofluorescence did not change in human glomeruli with minimal change nephrotic syndrome versus control (22). Therefore, the PA nephrosis in rats may not be a close model of human nephrotic syndrome with minimal change. Since the function of Neph1 is thought to be similar to that of nephrin based on the similarity of their structures, the decrease of protein level of Neph1 may induce albuminuria. Thus, the protein level of Neph1 in minimal change nephrotic syndrome and in PA nephrosis must be investigated in the future. Moreover, to clarify the mechanism of nephrosis, gene expression profiles in nephrotic models must be analyzed using cDNA microarray methods (23).

In summary, the protein level of nephrin and podocin decreased at the onset of albuminuria in PA nephrosis. However, the first change induced by PA was the change of podocin localization from a linear pattern to a dot-like one prior to the onset of albuminuria.

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References


