Role of p38 Mitogen-Activated Protein Kinase Activation in Podocyte Injury and Proteinuria in Experimental Nephrotic Syndrome

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Podocytes play an important role in maintaining normal glomerular function and structure, and podocyte injury leads to proteinuria and glomerulosclerosis. The family of mitogen-activated protein kinases (MAPK; extracellular signal-regulated kinase [ERK], c-Jun N-terminal kinase, and p38) may be implicated in the progression of various glomerulopathies, but the role of MAPK in podocyte injury remains elusive. This study examined phosphorylation of p38 MAPK in clinical glomerulopathies with podocyte injury, as well as in rat puromycin aminonucleoside (PAN) nephropathy and mouse adriamycin (ADR) nephropathy. The effect of treatment with FR167653, an inhibitor of p38 MAPK, was also investigated in rodent models. In human podocyte injury diseases, the increased phosphorylation of p38 MAPK was observed at podocytes. In PAN and ADR nephropathy, the phosphorylation of p38 MAPK and ERK was marked but transient, preceding overt proteinuria. Pretreatment with FR167653 (day −2 to day 14, subcutaneously) to PAN or ADR nephropathy completely inhibited p38 MAPK activation and attenuated ERK phosphorylation, with complete suppression of proteinuria. Electron microscopy and immunohistochemistry for nephrin and connexin43 revealed that podocyte injury was markedly ameliorated by FR167653. Furthermore, early treatment with FR167653 effectively prevented glomerulosclerosis and renal dysfunction in the chronic phase of ADR nephropathy. In cultured podocytes, PAN or oxidative stress induced the phosphorylation of p38 MAPK along with actin reorganization, and FR167653 inhibited such changes. These findings indicate that the activation of MAPK is necessary for podocyte injury, suggesting that p38 MAPK and, possibly, ERK should become a potential target for therapeutic intervention in proteinuric glomerulopathies.

in producing proinflammatory cytokines (11), mediating cell survival or apoptosis (12), and regulating the stability of cytoskeleton (13). These notions have prompted us to investigate the role of p38 MAPK in podocyte injury.

In this study, we investigated the alteration of p38 MAPK in disease states and reveal the activation of p38 MAPK at podocytes in clinical and experimental nephrotic syndromes, specifically from the preproteinuric state in the latter. Furthermore, we demonstrate that the treatment with FR167653, an inhibitor of p38 MAPK, in PAN nephropathy and ADR nephropathy completely abolishes proteinuria and prevents podocyte injury, suggesting that p38 MAPK activation is a common upstream mechanism necessary for podocyte injury in proteinuric glomerulopathies.

Materials and Methods

Antibodies

Primary antibodies used for Western blotting and immunohistochemical studies were rabbit anti-p38 MAPK, rabbit anti–phospho-p38 MAPK, rabbit anti-ERK, rabbit anti–phospho-ERK (Cell Signaling Technology, Boston, MA), rabbit anti-JNK, mouse anti–phospho-JNK (Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-nephrin (14), rabbit anti-nephrin (8), rabbit anti-connexin43 (Sigma, St. Louis, MO), mouse anti-synaptopodin (Progen, Heidelberg, Germany), and rabbit anti–TGF-β1 (Santa Cruz Biotechnology) antibodies.

Human Tissue Samples

Tissue samples were obtained at diagnostic renal biopsy performed at Kyoto University Hospital. We investigated the samples from patients who had MCNS, MN, and FSGS and were manifesting nephrotic-range proteinuria. As control human samples, we used nontumor tissues of the kidney from patients who had renal cell carcinoma and underwent nephrectomy or biopsy samples from the patients with minor glomerular abnormalities. The study was conducted under informed consent and was approved by the ethics committee on human research of Kyoto University Graduate School of Medicine.

Animal Experiments

All animal experiments were conducted in accordance with our institutional guidelines for animal research. For inducing rat PAN nephropathy, male Wistar-Kyoto rats that weighed 180 to 200 g received an intravenous injection of 50 mg/kg body wt PAN (Sigma) diluted in 0.9% saline. For inducing mouse ADR nephropathy, male Wistar-Kyoto rats that weighed 180 to 200 g received an intravenous injection of 50 mg/kg body wt PAN (Sigma) diluted in 0.9% saline. Control animals received vehicle only. Administration of a dose of 33 mg/kg body wt diluted in 0.5% methylcellulose (Sigma) daily from day −2 to day 14. In another series of experiments, daily FR167653 injection was started 10 min or 4 h after induction of the disease and continued for 2 wk (from day 0 to day 14) in these models. Control animals received an injection of methylcellulose alone.

Animals were fed a standard diet and given water ad libitum. We maintained these animals under alternating 12-h cycles of light and dark. Animals were killed on determined days after induction of the disease. Kidneys were harvested immediately for histologic and Western blot analyses.

Measurement of Blood and Urine Samples

Blood samples were obtained at the time of killing, and serum creatinine levels were measured using a kit (Wako, Osaka, Japan). For urine measurements, each animal was housed separately in a metabolic cage (Shinano Manufacturing, Tokyo, Japan) and daily urine volume was measured. Urinary albumin excretion was assayed with a rat or murine albumin ELISA kit (Exocell, Philadelphia, PA) (17).

Renal Histology and Electron Microscopy

Kidney sections were fixed with 4% buffered formaldehyde and embedded in paraffin as described (17,18). One-micrometer-thick sections were stained with periodic acid-Schiff and examined by light microscopy. Occurrence of the sclerotic glomeruli per section was assessed by counting the total number of glomeruli in a representative section from each sample. The procedure was performed by two investigators without knowledge of the origin of the slides, and the mean values were calculated.

For electron microscopy, small blocks of kidneys were fixed in 2.5% buffered glutaraldehyde, postfixed in 2% osmium tetroxide, dehydrated in graded ethanol, and embedded in epoxy resin (19). Ultrathin sections (0.1 μm thick) were stained with uranyl acetate and lead citrate and examined in an electron microscope (H-7600; Hitachi, Tokyo, Japan) at 75 kV.

Immunohistochemistry

For immunohistochemistry of phospho–p38 MAPK, kidney samples were fixed with 4% paraformaldehyde and embedded in paraffin. Three-micrometer sections were deparaffinized and rehydrated. Sections were treated with 0.3% H2O2 in methanol for 15 min to quench endogenous peroxide activity and were boiled at 100°C for 10 min in 10% citrate buffer to unmask antigens. Sections were incubated with anti–phospho–p38 MAPK at 4°C overnight and visualized using Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin.

For immunofluorescence studies of nephrin and TGF-β1, 3-μm cryostat sections fixed with acetone were incubated for 1 h at 22°C with primary antibodies and stained with FITC-labeled anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). For double staining of connexin43 and synaptopodin, sections were incubated with mixed primary antibodies and stained with FITC-labeled anti-rabbit IgG and TRITC-labeled anti-mouse IgG (Jackson ImmunoResearch). Slides were examined with a confocal laser microscope (LSM5Pascal; Carl Zeiss, Munich, Germany) (19).

Western Blot Analysis

Glomeruli were isolated from the animals by graded sieving method (20). Western blot analysis was performed as described (17,18). In brief, isolated glomeruli or cells were lysed on ice in RIPA buffer that contained 10 μg/ml aprotinin, 2 mM dithiothreitol, 2 mM sodium orthovanadate, and 1 mM PMSF. Lysates were centrifuged at 15,000 rpm, and supernatants (50 μg protein/lane) were separated by 12.5% SDS-PAGE and transferred onto Immobilon filter (Millipore, Bedford, MA). After incubation with primary antibodies, immunoblots were developed with horseradish peroxidase–conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL) and a chemiluminescence kit (ECL plus; Amersham).

Cell Culture

A conditionally immortalized mouse podocyte cell line was provided by Dr. Peter Mundel (Albert Einstein College of Medicine, New York, NY) (21) and cultured as described (20). In brief, the cells were main-
tained at 33°C on dishes that were coated with collagen I (Koken, Tokyo, Japan), in RPMI 1640 medium (Nihonseiyaku, Tokyo, Japan) that contained 10% FBS (Sanko Junyaku, Tokyo, Japan) with 10 U/ml IFN-γ (Life Technologies BRL, Grand Island, NY). For differentiating podocytes, cells were cultured at 37°C with 5% FBS without IFN-γ for 2 wk. For experiment, cells were made quiescent in medium that contained 0.5% BSA (Sigma) for 24 h, pretreated with 10 μM FR167653 or vehicle 1 h before stimulation with 50 μg/ml PAN or 0.5 mM H2O2, and harvested at determined time after stimulation.

For analyzing actin cytoskeleton of cultured podocytes, cells were cultured on collagen I–coated coverslips in six-well dishes. For observation of F-actin filament, cells were fixed with 4% paraformaldehyde, incubated with 0.1% Triton X-100 for 10 min, and stained with 1 μM FITC-conjugated phalloidin (Sigma) for 30 min in darkness (21). Cover glasses were mounted, and the slides were examined by fluorescence microscopy.

**Statistical Analyses**

Data are expressed as means ± SEM. Statistical analysis was performed using ANOVA followed by Scheffe test. *P* < 0.05 was considered statistically significant.

**Results**

**Enhanced Phosphorylation of p38 MAPK in Human and Rodent Podocyte Injury Diseases**

In normal human kidneys, only a faint staining for phosphorylated p38 MAPK was observed in the glomeruli, mostly in the epithelial cells (Figure 1A). In proteinuric disorders, however, enhanced phosphorylation of p38 MAPK was detected at podocytes, as well as in parietal epithelial cells, as demonstrated in the biopsy specimens from the patients with MCNS (Figure 1B), MN (Figure 1C), and FSGS (Figure 1D). The mean occurrence of phospho–p38 MAPK–positive cells among nucleated cells per glomerulus (excluding parietal epithelial cells) was 5.2 ± 0.6, 11.1 ± 2.8, 18.2 ± 4.4, and 14.7 ± 4.8% in the samples from control kidney, MCNS, MN, and FSGS, respectively (*n* = 3 each).

We next examined rodent models of nephrotic syndrome. We found a marked increase of phosphorylated p38 MAPK in the isolated glomeruli of PAN nephropathy as well as ADR nephropathy on day 1 and day 3 (Figure 2, A and B), when urinary albumin excretion was still within normal range (see Figure 3, C and D). Enhanced phospho–p38 MAPK was confined mostly to podocytes (Figure 2, C and D). The mean occurrence of phospho–p38 MAPK–positive cells per glomerulus was 19.3 ± 1.9 and 16.7 ± 2.9% on day 1 in PAN and ADR nephropathy, respectively (*n* = 4 each). Thereafter, the levels of phospho–p38 MAPK got decreased and returned near the basal levels on day 7. We also examined the activation of other members of the MAPK family in these models of nephrotic syndrome. Phosphorylation of ERK (ERK-1 and -2) was increased in the glomeruli of PAN nephropathy on day 1 and day 3 and decreased on day 7, at a time course similar to that of p38 MAPK but with less pronounced activation (Figure 2E). Phosphorylation of JNK (JNK-1 and -2) was observed in the control glomeruli and unaltered during the course (Figure 2E). Essentially identical results for ERK and JNK changes were obtained in ADR nephropathy (data not shown). These findings indicate that p38 MAPK is markedly but transiently activated at podocytes before the appearance of overt proteinuria in rodent models of podocyte injury disease.

**Figure 1.** Phosphorylation of p38 mitogen-activated protein kinase (MAPK) in human glomerulopathies with podocyte injury. (A) In normal glomerulus, a weak staining for phospho–p38 MAPK was observed (arrowhead in inset). Enhanced phosphorylation of p38 MAPK was detected at nuclei of podocytes (arrowheads in insets) in the biopsy specimens from minimal-change disease (B), membranous nephropathy (C), and focal segmental glomerulosclerosis (D). Magnification, ×400; inset ×1000.
Urinary Albumin Excretion in PAN and ADR Nephropathy

In rats with PAN nephropathy, no overt proteinuria was observed until day 4. Urinary albumin excretion showed a significant increase on day 7, peaked at approximately day 14, and returned to almost normal range by day 28 (Figure 3C). In mice with ADR nephropathy, urinary albumin excretion was normal until day 4, was significantly increased on day 7, reached to the peak level at approximately day 14, and remained significantly elevated through day 28 (Figure 3D). Such time courses were compatible with those in previous reports (6,7).

Effect of p38 MAPK Inhibitor on Proteinuria in PAN and ADR Nephropathy

Because the phosphorylation of p38 MAPK at podocytes preceded the onset of overt proteinuria, we hypothesized that the activation of p38 MAPK would critically be relevant to the appearance of proteinuria in these models of podocyte injury disease. We therefore investigated the effect of administration of FR167653, a specific inhibitor of p38 MAPK (15,16), to PAN and ADR nephropathy models on their renal outcomes. FR167653 has been reported to selectively inhibit p38\(\alpha\) MAPK activity in vitro, without affecting the activities of other kinases,
including p38; ERK-1; JNK-2; protein kinases A, C, and G; and epidermal growth factor receptor (EGFR) kinase, as well as exerting no inhibitory effect on cyclooxygenase-1 or -2 activities, even at a dose 2 orders of magnitude higher than that for p38 MAPK inhibition (15).

In the glomeruli isolated from the animals that were pretreated with FR167653 from day 2, enhanced phosphorylation of p38 MAPK was effectively abolished to the control levels (Figure 3, A and B), indicating that the dose administered was sufficient to inhibit the activation of p38 MAPK at glomeruli in vivo. In this condition, we observed substantial inhibitory effects on the enhanced phosphorylation of ERK by administration of FR167653 in both models (Figure 2E, right), but the effects were less pronounced as compared with the effect on p38 MAPK; FR167653 did not affect JNK phosphorylation (data not shown). By daily subcutaneous administration of this compound to rats with PAN nephropathy for 17 d, urinary albumin excretion was almost completely suppressed to the control level (Figure 3C). Furthermore, in mice with ADR nephropathy, the administration of FR167653 for the same period completely abrogated the increase of urinary albumin excretion as well (Figure 3D). Thus, the blockade of p38 MAPK activation with FR167653 potently inhibited proteinuria in both models of podocyte injury disease, irrespective of initiating insults.

We next examined the effect of FR167653 administration after induction of the disease. When the compound was administered 4 h after induction of the diseases, FR167653 failed to suppress significantly the enhanced phosphorylation of p38 MAPK observed on day 1 (data not shown) in both models or subsequent increase in urinary albumin excretion (PAN day 14: 149.4 ± 26.8 versus 129.4 ± 18.8 mg/d; ADR day 14: 21.8 ± 2.6 versus 14.3 ± 2.1 mg/d; ADR day 28: 21.4 ± 1.5 versus 15.9 ± 3.8 mg/d; vehicle versus FR167653 treatment, respectively, n = 4). When the compound was injected 10 min after the induction of ADR nephropathy, half of the animals exhibited significant inhibition of phospho–p38 MAPK activation and effective suppression of the proteinuria (n = 3 each).

**Effect of p38 MAPK Inhibitor on Podocyte Injury**

We next investigated the morphologic changes with or without inhibition of p38 MAPK. In electron microscopic analysis, the glomeruli with PAN nephropathy 2 wk after onset showed foot process effacement, with occasional vacuolation of podocytes (Figure 4A, middle). By contrast, glomeruli from FR167653-treated rats with PAN nephropathy revealed that foot processes were almost intact as in the control (Figure 4A). Likewise, glomeruli from mice with ADR nephropathy showed
marked foot process effacement, and the treatment with FR167653 prevented such changes (Figure 4B).

Nephrin, a product of the gene mutated in congenital nephrotic syndrome (22), is decreased and its distribution is altered upon proteinuric conditions in human nephrotic syndrome (23) and in animal models (8). We therefore investigated the expression and distribution pattern of nephrin in experimental animals. In Western blot and immunofluorescence analyses, rats with PAN nephropathy exhibited the decreased expression of nephrin on day 14, although FR167653-treated animals showed no reduction in nephrin expression (Figure 5, A and C). By immunofluorescence study, normal glomeruli showed a linear staining pattern for nephrin along the capillary wall, whereas glomeruli with PAN nephropathy showed a coarse granular pattern for nephrin. FR167653 treatment retained a normal linear pattern. (B) Immunofluorescence study for connexin43 (green) merged with synaptopodin (red). Podocytes in the control glomerulus rarely expressed connexin43. PAN nephropathy showed increased expression of connexin43 at podocytes. FR167653 treatment decreased its expression. (C) Western blotting for nephrin and connexin43 in glomeruli of PAN nephropathy with or without FR167653. Nephrin was decreased in PAN nephropathy, whereas FR167653 treatment showed no reduction in nephrin expression. By contrast, connexin43 was increased in PAN nephropathy, whereas FR167653 treatment decreased connexin43 expression to the control level.

Connexin43 is a major gap junction protein that is expressed most abundantly among the connexin family in the kidney (24). Its expression is upregulated at podocytes upon injury (24) and therefore postulated as a marker for podocyte injury (19,24). We observed increased connexin43 in glomeruli with PAN nephropathy by Western blot and immunofluorescence analyses (Figure 5A). Rats that had PAN nephropathy and were treated with FR167653, however, retained such a linear pattern as seen in normal glomeruli (Figure 5A).

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Figure 6. Effects of FR167653 administration on renal histology and function in mice with ADR nephropathy. (A) In ADR nephropathy, urinary albumin excretion persisted until day 56 (■). FR167653 treatment in these mice prevented overt albuminuria throughout the course (■), whose levels were similar to the control (□). Mean ± SEM. *P < 0.05 versus control; #P < 0.05 versus FR167653 treatment; n = 4 to 7. (B) ADR nephropathy showed massive protein casts with marked tubular atrophy and degeneration. Administration of FR167653 revealed almost normal feature. (C) ADR nephropathy showed focal glomerulosclerosis, whereas FR167653 treatment revealed few sclerotic glomeruli. (D) Immunofluorescence study for TGF-β1. Some nonsclerotic glomeruli of ADR nephropathy exhibited an enhanced expression of TGF-β1, probably in the mesangial area. No glomeruli of FR167653-treated mice expressed TGF-β1. (E) The occurrence of sclerotic glomeruli on day 56 out of total glomeruli per section showed a markedly higher rate in ADR nephropathy than in control and FR167653 treatment. Mean ± SEM; n = 4. (F) The serum creatinine level on day 56 was significantly elevated in ADR nephropathy, but it remained normal with FR167653 treatment. Mean ± SEM; n = 4. Magnification, ×100 in B; ×400 in C, periodic acid-Schiff stain on day 56.
ADR nephropathy exhibited an enhanced glomerular expression of TGF-β1 (Figure 6D). Serum creatinine levels were also significantly elevated in ADR nephropathy (Figure 6F). Administration of FR167653 to mice with ADR nephropathy during the first 2 wk, however, maintained normal albumin excretion throughout the observation period (urinary albumin, 0.12 ± 0.07 mg/d on day 56; Figure 6A). Moreover, the treatment resulted in almost normal renal histology (Figure 6, B, C, and E), no glomerular augmentation of TGF-β1 (Figure 6D), and normal renal function (Figure 6F). Thus, FR167653 prevented the development of glomerulosclerosis and chronic renal dysfunction.

**Effect of p38 MAPK Inhibitor on Actin Reorganization in Cultured Mouse Podocytes**

Next we examined the effect of p38 MAPK activation on morphologic changes of podocytes using cultured mouse podocytes. Treatment of podocytes with PAN caused the phosphorylation of p38 MAPK (Figure 7A, left) and facilitated actin reorganization (Figure 7B). Pretreatment with FR167653 abolished the phosphorylation of p38 MAPK (Figure 7A) and prevented actin reorganization (Figure 7B). Likewise, the stimulation of podocytes with H₂O₂ as oxidative stress that activates p38 MAPK (10) increased phospho-p38 MAPK (Figure 7A, right) together with actin reorganization (Figure 7C). Pretreatment with FR167653 prevented these changes as well (Figure 7, A and C). Thus, the blockade of p38 MAPK activation inhibited actin reorganization.

**Discussion**

In this study, we investigated the significance of p38 MAPK in podocyte injury in clinical as well as experimental nephropathies and revealed that p38 MAPK activation at podocytes plays a crucial role for its pathophysiology. We showed an enhanced phosphorylation of p38 MAPK in glomeruli of clinical nephrotic syndrome. Furthermore, we demonstrated an essential role of p38 MAPK activation in rodent models of podocyte injury disease. In PAN nephropathy and ADR nephropathy, enhanced phosphorylation of p38 MAPK in the glomeruli was found during the first week of insult preceding overt proteinuria, and inhibition of p38 MAPK by FR167653

![Figure 7](image-url)
resulted in marked suppression of podocyte injury and proteinuria. Moreover, the treatment with FR167653 during an early phase effectively prevented the progression of renal histologic changes in the chronic phase of ADR nephropathy. These findings strongly suggest that p38 MAPK activation should be functionally relevant to the pathogenesis of podocyte injury, serving as an upstream event necessary for proteinuria.

We observed an enhanced phosphorylation of p38 MAPK in human nephritic states mainly in podocytes (Figure 1). It has already been reported that p38 MAPK activation in podocytes and tubules is found in various human nonproliferative as well as proliferative glomerulonephritis and correlates well with the degree of proteinuria and glomerular and/or interstitial injury (25). We also observed an enhanced p38 MAPK phosphorylation in podocytes of rodent models of nephrotic syndrome, especially during an early phase of the disease (Figure 2). It should be noted that there seems to be discrepancy between clinical nephropathies with sustained p38 MAPK phosphorylation and these rodent models with only transient activation of p38 MAPK. One explanation could be the difference in the cause and time course of podocyte injury. In experimental nephropathies, activation of p38 MAPK is elicited by a single burst of noxious stimulus, i.e., injection of PAN or ADR, but in human glomerulopathies, p38 MAPK activation may be prolonged because of continuous stimuli such as circulating immune complexes. Although circumstantial, this might be reflected in different time courses of proteinuria between these models and clinical conditions. However, the pathogenic role for prolonged p38 MAPK activation in causing proteinuria and podocyte injury seen in clinical nephrotic syndrome should await further clarification.

In our study, there might be the time dissociation between p38 MAPK phosphorylation that occurred very early in these models and the appearance of overt proteinuria that occurred several days later (Figures 2 and 3). It is well conceivable that the activation of p38 MAPK in podocytes should trigger several intracellular signaling cascades, giving rise to serial reactions of the genes and proteins that alter cytoskeleton integrity or barrier function and finally lead to overt proteinuria. Proteinuria may ensue well behind (e.g., months later) the transient activation and subsidence of triggering events, as shown in the ischemia/reperfusion injury model (26). In addition, a causal relationship between the only brief activation of p38 MAPK and the morphologic changes occurring several days later has been demonstrated in other cell types (27,28). Although the pretreatment by FR167653 in this study abrogated both the p38 MAPK activation and proteinuria, delayed FR167653 administration resulted in much less effect on proteinuria in parallel with the less efficient p38 MAPK inhibition (approximately 50% inhibition on both parameters when administered just after induction). These data may also suggest a close association between p38 MAPK activation and proteinuria.

Specificity of the inhibitor FR167653 for p38 MAPK is another issue to be addressed. Because this compound has been shown to selectively inhibit p38α MAPK without affecting ERK-1, JNK-2, or cyclooxygenase-1 and -2 in vitro (15), it is reasonable to assume that the results obtained here would be due to the specific inhibition of p38 MAPK (Figure 3A). The in vivo specificity of this compound, however, is not fully characterized. It is interesting that we found that FR167653 treatment attenuated ERK phosphorylation substantially, although not completely (Figure 2E), along with the complete inhibition of p38 MAPK activation. It is not clear at present whether such attenuation was direct and somehow contributed to the beneficial effects of this compound. Alternatively, ERK may have been downregulated secondary to p38 MAPK inhibition, as shown in thrombin-stimulated ERK activation in endothelium (29). Nevertheless, we cannot exclude the possibility that the beneficial effects of FR167653 exerted in this study could be due to either p38 MAPK or ERK pathway inhibition or both.

Activation of p38 MAPK is observed in embryonic kidneys and is required for renal development in rats (30). Although MAPK are thought to be largely inactive in adult kidneys at normal conditions, the activation of p38 MAPK can be detected in diseased kidneys from experimental models (16,31,32) and various human nephropathies (25,32,33). The role of p38 MAPK activation has been investigated so far by pharmacologic blockade in several experimental models. For example, p38 MAPK inhibition reduced proteinuria in anti-GBM glomerulonephritis (16) and ameliorated renal ischemia/reperfusion injury (34). In addition, blockade of p38 MAPK diminished angiotensin II-mediated renal damage, reducing mesangial matrix expansion (35). These reports have suggested that p38 MAPK should play important roles in cell proliferation or inflammatory responses in the mesangium and tubulointerstitium. In one report using complement-mediated cell injury, inhibition of p38 MAPK 7 d after induction of the disease failed to reverse and rather augmented cytotoxicity, suggesting that the activated p38 MAPK in podocytes may be cytoprotective during a later phase of this model (36). These studies, however, have not addressed the involvement of p38 MAPK in the pathogenesis of podocytopathy. In our study, we first demonstrate the pathogenic role of p38 MAPK activation in podocyte injury and proteinuria in vivo and effective prevention of the disease by an early inhibition of this activation. Furthermore, we reveal that the reduced expression of nephrin was effectively reversed by the treatment (Figure 5). Because nephrin expression largely correlates inversely with the degree of proteinuria (8,23), this perhaps may be an indirect consequence of inhibited proteinuria. Further study is needed to explore the relationship between nephrin expression and p38 MAPK activation.

In podocytes, several reports already investigated the role of p38 MAPK using conditionally immortalized mouse podocyte cultures. p38 MAPK mediated TGF-β-induced apoptosis in podocytes (37). Because podocyte loss is a key event leading to glomerulosclerosis (38), p38 MAPK activation may facilitate glomerulosclerosis. High glucose exposure to podocytes stimulated p38 MAPK phosphorylation and c-Jun(NF-κB) collagen expression via the 12-lipoxygenase-dependent pathway (39). Furthermore, mechanical stretch induced the upregulation of cyclooxygenase-2 and prostaglandin EP4 receptor in a p38 MAPK–dependent manner (40), which may facilitate actin depolymerization (40). In addition, high glucose to podocytes stimulates the expression of vascular endothelial growth factor...
(41), which in turn induces \( \alpha 3(IV) \) collagen production, acting downstream of TGF-\( \beta 1 \) (42). Of note, in mesangial cells, vascular endothelial growth factor is induced by TGF-\( \beta 1 \) via the p38 MAPK–dependent pathway and stimulates collagen and fibronectin expression (43). Whether such pathways are critically involved in the current beneficial effects exerted by p38 MAPK inhibition in \textit{vivo} is not clear and requires further investigation.

How does p38 MAPK activation affect actin cytoskeleton and induce podocyte injury? By using cultured podocytes, we demonstrated a close association between p38 MAPK activation and actin reorganization induced by PAN or oxidative stress \textit{in vitro}, and such changes were effectively abrogated by p38 MAPK inhibition (Figure 7). Therefore, one of the possibilities is that p38 MAPK activation may depolymerize actin filament through the molecule(s) that modulates actin polymerization. Several specific substrates for p38 MAPK that may be physiologically relevant have been identified. These include transcriptional factors ATF-2, CHOP, Elk-1, MEF2A, MEF2C, and Max, which can be phosphorylated and activated by p38 MAPK (10,44). p38 MAPK can also activate MAPK–activated protein kinases 2 and 3, which in turn phosphorylate small heat-shock protein 27 (hsp27) (10,13). Hsp27 is importantly involved in actin filament dynamics regulated by p38 MAPK (13), and the phosphorylated level of hsp27 is critically relevant to the morphologic changes and actin depolymerization in podocytes (45). Actin filament is a major constituent of foot processes, and depolymerization of actin filaments leads to foot process effacement (46). Because actin filaments interact with components of slit diaphragm and with integrins (46), depolymerized actin filaments may facilitate the loss of adhesive interactions, leading to disruption of slit diaphragm and detachment from GBM. Although p38 MAPK activation can induce actin reorganization \textit{in vitro}, their molecular link is not demonstrated \textit{in vivo}, and which molecular mechanisms are in fact involved in our study is unclear. Because the cell line of cultured podocytes used in this study does not form interdigitated foot processes or slit diaphragm (45), it will be required to establish the culture system reproducing the \textit{in vivo} podocyte phenotypes to answer these questions.

Podocyte dysfunction leads to progressive renal insufficiency. First, podocyte damage causes proteinuria. Sustained proteinuria gives rise to tubulointerstitial injury, eventually leading to renal failure (47). Second, podocyte injury impairs mesangial structure and function. In anti-Thy-1 glomerulonephritis, a reversible self-limiting model in itself, preceding minor podocyte injury with PAN pretreatment results in irreversible mesangial alteration (48). We showed recently that cysteine-rich protein 61 (Cyr61), a potent angiogenic protein that belongs to the CCN family of matrix-associated secreted protein family, is expressed in podocytes and upregulated in anti-Thy-1 glomerulonephritis (20). Cyr61 inhibits mesangial cell migration, suggesting that Cyr61 may play a modulatory role in limiting mesangial activation (20). Thus, podocytes may secrete various humoral factors that regulate mesangial structure and function, and their reduction could result in impaired mesangial function such as mesangial proliferation and matrix expansion. Third, podocyte loss or detachment from the GBM leads to glomerulosclerosis (38). In human diabetic nephropathy and IgA nephropathy, decreased podocyte number correlates significantly with poor prognosis (49,50). These studies suggest that podocyte injury is critical not only in podocyte-specific diseases such as MCNS and FSGS but also in podocyte-nonspecific diseases such as IgA nephropathy.

In conclusion, our study reveals that the activation of p38 MAPK is crucial for podocyte injury in experimental nephrotic syndrome, suggesting that p38 MAPK activation is a common upstream mechanism necessary for podocyte injury in various proteinuric glomerulopathies. Although we need to be cautious in interpreting these results and extrapolating them to clinical situations, our study opens up a possibility that p38 MAPK and, possibly, ERK could become potential targets for therapeutic intervention in proteinuric glomerulopathies.

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