Statins have been reported to confer renoprotection in several experimental models of renal disease through pleiotropic actions. The roles of statins in glomerular podocytes have not been explored. The objective of this study was to evaluate the effects of fluvastatin on podocyte and tubulointerstitial injury in puromycin aminonucleoside (PAN)-induced nephrosis. PAN induced massive proteinuria and serum creatinine elevation on day 7, which were significantly suppressed by fluvastatin. Immunofluorescence studies of podocyte-associated proteins nephrin and podocin revealed diminished and discontinuous staining patterns in rats with PAN nephrosis, indicating severe podocyte injury. Fluvastatin treatment dramatically mitigated the abnormal staining profiles. Reduction of nephrin expression by PAN and its reversal by fluvastatin were confirmed by quantitative analyses. By electron microscopy, effacement of foot processes was ameliorated in fluvastatin-treated rats. Fluvastatin also mitigated tubulointerstitial damage in PAN nephrosis, with the repression of PAN-induced NF-κB and activator protein-1 activation in the kidneys. In addition, expression of activated membrane-bound small GTPase RhoA was markedly increased in the glomeruli of PAN nephrosis, which was inhibited by fluvastatin treatment. In cultured podocytes, fluvastatin suppressed PAN-evoked activation of RhoA and actin cytoskeletal reorganization. Furthermore, fasudil, a specific Rho-kinase inhibitor, successfully ameliorated PAN-induced podocyte damage and proteinuria. In summary, fluvastatin alleviated podocyte and tubulointerstitial injury in PAN nephrosis. The beneficial effects of fluvastatin on podocytes can be attributable to direct modulation of excessive RhoA activity. Our data suggest a therapeutic role for statins in clinical conditions that are relevant to podocyte injury.

Fluvastatin Ameliorates Podocyte Injury in Proteinuric Rats via Modulation of Excessive Rho Signaling

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actions were evaluated further using cultured podocytes (21) and fasudil, a specific Rho-kinase inhibitor.

Materials and Methods
Animal Experimental Design
Male Sprague-Dawley rats (Tokyo Laboratory Animals Science, Tokyo, Japan) that weighed 150 to 170 g were fed a standard diet. All animal procedures conducted were in accordance with the guidelines for the care and use of laboratory animals approved by the University of Tokyo Graduate School of Medicine. Rats were divided into control, PAN nephrosis, and fluvastatin-treated PAN nephrosis groups. PAN nephrosis was induced by a single intravenous injection of PAN (10 mg/100 g body wt; Sigma, St. Louis, MO). Fluvastatin treatment (10 mg/kg per d orally) was started 5 d before PAN injection and continued throughout the experiment. The dose was selected because fluvastatin at a dose of 10 mg/kg per d does not affect serum total cholesterol level in Sprague-Dawley rats (22). In some experiments, rats were treated with fasudil (Asahi Kasei, Tokyo, Japan), a Rho-kinase inhibitor (23). Fasudil was administered orally at a dose of 30 mg/kg per d from 5 d before PAN injection and continued until the end of the experiment (23). On day 6, urine was collected for 24 h using a metabolic cage (n = 10 to 13 per group). On the following day, rats were killed under ether anesthesia. Kidneys were dissected, and glomeruli were isolated by the sieving method (24). The purity of glomeruli is >95% as assessed by light microscopy. Organs were frozen in liquid nitrogen or fixed in 4% paraformaldehyde solution.

Immunohistochemistry
Immunostaining was performed as described previously with some modifications (25). Briefly, cryosections (5 μm thick) were incubated with rabbit anti-rat podocin (1:500), mouse anti-rat mAb 5-1-6 (antibody against nephrin, 1:2000), mouse anti-rat ED-1 (Serotec, Oxford, UK; 1:500), or mouse anti-rat osteopontin (MPIIIB10, Developmental Hybridoma Studies Institute, Iowa City, IA; 1:500) overnight and subsequently with biotinylated anti-mouse or anti-rabbit IgG. Immunoreactivity was detected using an ABC kit (Vector Laboratories, Burlingame, CA) and a Metal enhanced DAB kit (Pierce, New York, NY). For nephrin and podocin, sections were reacted with streptavidin-FITC (PerkinElmer, Wellesley, MA; 1:500), and signals were detected using fluorescence microscopy under the fixed exposure condition. For semiquantitative evaluation of macrophage infiltration in the interstitium, the number of cells that expressed ED-1 was counted in 10 randomly selected high-power fields (×400, n = 4) by a blinded observer.

Western Blotting
Western blotting was performed as described previously (26). Briefly, samples were homogenized in a 6× volume of homogenization buffer H using a Teflon homogenizer. Homogenization buffer H was composed of 1% Triton X-100, 50 mM Hepes (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, and protease inhibitor (PI) cocktail (Roche, Basel, Switzerland). The lysates (20 μg) were separated on 7.5% polyacrylamide gels and transferred to nitrocellulose membrane, and immunoblotted with rabbit anti-nephrin (1:10,000) or anti–phospho-myosin phoshatase targeting subunit 1 (phospho-MYPT) antibody (Upstate, Waltham, MA; 1:5000). After incubation with peroxidase-conjugated anti-rabbit IgG, signals were visualized using ECL Western blotting detection system (Amersham, Piscataway, NJ).

RhoA activation was tested using membrane-bound RhoA (GTP-RhoA). Samples were homogenized in lysis buffer that contained 20 mM Tris (pH 7.4), 2 mM MgCl₂, 250 mM sucrose, and PI cocktail. Nuclei and unlysed cells were removed by low-speed centrifugation (500 × g), and the supernant was centrifuged at 100,000 × g for 30 min. The pellet was resuspended in buffer that contained 1% Triton X-100. Five or 10 μg of protein was separated on 12.5% polyacrylamide gel followed by immunoblotting using rabbit anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA; 1:200).

RNA Extraction and Real-Time Quantitative Reverse Transcription–PCR
Gene expression was determined by real-time quantitative reverse transcription–PCR (RT-PCR) according to the procedure described previously (25). TaqMan chemistry and Assay on demand primers and probe sets were used for the rat nephrin, monocyte chemoattractant protein-1 (MCP-1), osteopontin, vimentin, and β-actin.

Transmission Electron Microscopy
Small pieces of cortex were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde, dehydrated through graded ethanol and propylene oxide, and embedded in Epon 812 by standard procedures. Ultrathin sections were stained with uranyl acetate for 10 min and subsequently in Reynolds lead citrate for 2 min. The specimens were observed using HITACHI transmission electron microscope H-7000 (Tokyo, Japan).

Histology
For morphologic evaluations, paraffin sections (3 μm) were stained with periodic acid-Schiff (PAS) reagents. The PAS-stained kidney sections of PAN nephrosis rats or fluvastatin-treated PAN nephrosis rats (n = 10 animals/group) were analyzed semiquantitatively for tubulointerstitial injury, as described previously (27). Tubulointerstitial injury was defined as tubular cast formation, sloughing of tubular epithelial cells, tubular atrophy, or thickening of tubular basement membrane. Ten cortical fields (>20 objective) of each kidney were scored on a scale of 0 to 4, according to the following criteria: 0, no tubulointerstitial injury; 1, <25% of the tubulointerstitium injured; 2, 25 to 50% of the tubulointerstitium injured; 3, 51 to 75% of the tubulointerstitium injured; 4, >75% of the tubulointerstitium injured. The areas of the injured tubulointerstitium were calculated digitally using an image analysis program (ImageJ).

Electrophoretic Mobility Shift Assay
Nuclear extracts were prepared by the method of Schreiber et al. (28) with some modifications. Briefly, frozen samples were homogenized in Tris-buffered saline and centrifuged for 1 min at 8000 × g at 4°C. After removal of the supernatant, the pellet was suspended in buffer A (10 mM Hepes [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol) and centrifuged for 1 min at 15,000 × g at 4°C for washing. Then, the pellet was resuspended in buffer A, homogenized, and chilled on ice for 15 min. NP-40 (0.625%) was added to the sample, which was vortexed and centrifuged at 15,000 × g for 5 min at 4°C. The resultant pellet was resuspended in buffer B (20 mM Hepes [pH 7.9], 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol), incubated on ice for 15 min, and centrifuged at 15,000 × g for 5 min at 4°C, and the supernatant was used as nuclear protein. For the electrophoretic mobility shift assay (EMSA), the double-stranded oligonucleotide that contained an AP-1 or NF-kB consensus-binding sequence (Promega, Madison, WI) was end-labeled with [α-32P]dATP using T4 polynucleotid kinase. Nuclear protein (10 μg) was incubated with 50,000 cpm of 32P-labeled double-stranded oligonucleotide that contained an AP-1 or NF-kB consensus-binding sequence in binding buffer (Promega) for 30 min. Supershift EMSA was performed by the addition of 1 μl of antiserum against the AP-1 and Rel/NF-kB family proteins.
(c-fos, c-jun, p65, p50, p52, RelB, and C-Rel; Santa Cruz Biotechnology) for 30 min before incubation with the labeled oligonucleotide. Protein-DNA complexes were electrophoresed through 4% polyacrylamide gel. Autoradiogram was analyzed by Cyclone Storage Phosphor System (PerkinElmer). Specificity of the complexes was confirmed by use of excess (25-fold) unlabeled oligonucleotide to compete with labeled probes that bind to nuclear proteins.

Cell Culture
A clonal cell line of conditionally immortalized murine podocytes was used (21). Podocytes were grown and induced to differentiate as described previously (25). Treatment consisted of the addition of PAN (5 μg/ml) and PAN with fluvastatin (1 μM). In experiments with fluvastatin, the cells were preincubated with fluvastatin for 24 h before PAN exposure.

F-Actin Staining
Podocytes were incubated in the presence or absence of PAN for 48 h. Fluvastatin was added 24 h before PAN exposure. F-actin was stained according to the procedures described previously (25). In some experiments, podocytes were co-treated with mevalonate (200 μM), farnesyl pyrophosphate (5 μM), or geranylgeranyl pyrophosphate (GGPP; 5 μM).

Statistical Analyses
Data are expressed as mean ± SEM. Statistical analyses were performed by ANOVA and subsequent Tukey simultaneous multiple comparison. P < 0.05 was considered to be statistically significant.

Results
Urinary Protein and Serum Creatinine Levels
PAN administration caused heavy proteinuria and loss of renal function, as reflected in the serum creatinine elevation (Table 1). Treatment with fluvastatin resulted in a significant decrease in the urinary protein excretion compared with PAN nephrosis rats. Serum creatinine elevation was reduced by 66% in fluvastatin-treated rats (P < 0.01).

Effects of Fluvastatin on Podocytes in PAN Nephrosis Rats
By light microscopy, glomerular change was unremarkable in PAN nephrosis rats (Figure 1A). Previous studies suggested that the podocyte-specific molecules nephrin and podocin are severely decreased and their distributions are altered in this model. Thus, we analyzed the effects of fluvastatin on the podocyte markers. In immunofluorescence study, nephrin and podocin were detected linearly along the capillary loop in the control rat kidneys (Figure 1B, left). The staining intensities were strikingly diminished in PAN nephrosis with discontinuous pattern (Figure 1B, middle). In fluvastatin-treated animals, nephrin and podocin expressions retained the linear pattern seen in the controls (Figure 1B, right). We quantitatively analyzed the nephrin expression by Western blotting (Figure 1C). Fluvastatin treatment significantly ameliorated the decreased expression of nephrin in PAN nephrosis rats (P < 0.05).

Nephrin gene expression is reported to decrease just after PAN injection in this model (13). To analyze the gene expression quantitatively, we applied real-time RT-PCR 12 h after PAN administration. The amount of nephrin mRNA was significantly decreased in the glomeruli of PAN-treated rats (52% versus control), which was almost completely prevented by fluvastatin (Figure 1D).

We further examined the integrity of podocyte foot processes by transmission electron microscopy. As reported previously (29), foot processes were effaced extensively in PAN nephrosis rats (Figure 1E, top). In fluvastatin-treated rats (Figure 1E, bottom), the foot process structure was protected compared with PAN nephrosis rats, although effaced processes were noted in some areas.

Effects of Fluvastatin on Tubulointerstitial Injury
We next focused on tubulointerstitial injury. Representative photomicrographs of PAS-stained sections are demonstrated in Figure 2A. PAN administration caused tubular dilation, atrophy, and intratubular cast formation (Figure 2A, b). The damage was markedly alleviated in the kidneys of fluvastatin-treated rats (Figure 2A, c). Semiquantitative histologic analysis revealed that fluvastatin treatment significantly reduced the tubulointerstitial damage in comparison with PAN nephrosis rats (0.88 ± 0.09 versus 2.09 ± 0.11; P < 0.01). Severity of the tubulointerstitial injury in PAN nephrosis is reported to correlate with the degree of proteinuria (15). To address the association between these two parameters in our model, we plotted tubulointerstitial injury scores against the levels of urinary protein (Figure 2B). In PAN nephrosis rats, heavy proteinuria was accompanied by severe tubulointerstitial injury (Figure 2B, ◇). In contrast, fluvastatin-treated rats showed marked attenuation of tubulointerstitial damage in the presence of moderate proteinuria (Figure 2B, ○). We also evaluated macrophage in-

Table 1. Urinary protein, serum creatinine, and serum cholesterola

<table>
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<th>Control</th>
<th>PAN</th>
<th>PAN + FV</th>
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<tbody>
<tr>
<td>Urinary protein (mg/d)</td>
<td>6.4 ± 0.84</td>
<td>432.0 ± 25.1b</td>
<td>292.6 ± 40.1c</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.23 ± 0.01</td>
<td>0.52 ± 0.03b</td>
<td>0.33 ± 0.05c</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl; day 7)</td>
<td>84 ± 2</td>
<td>367 ± 71b</td>
<td>238 ± 37c</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl; 12 h)</td>
<td>88 ± 5</td>
<td>113 ± 7</td>
<td>105 ± 6</td>
</tr>
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</table>

aData are mean ± SEM. P values calculated by ANOVA and subsequent Tukey simultaneous multiple comparison. Control, control rats that received saline injection; PAN, puromycin aminonucleoside (PAN) nephrosis rats without treatment; PAN + FV, PAN nephrosis rats with fluvastatin treatment.

bP < 0.01 versus control.

cP < 0.01 versus PAN.
filtration by immunohistochemistry for ED-1 (Figure 2C). There were large numbers of infiltrating macrophages in the interstitial areas in PAN nephrosis rats, which were significantly suppressed by fluvastatin (Figure 2D).

To validate the histologic and immunohistochemical analyses described above, we performed real-time RT-PCR using three different markers: Osteopontin, MCP-1, and vimentin in kidney tissues (16,27). The increased mRNA expressions in PAN nephrosis were significantly ameliorated by fluvastatin treatment (Figure 3, A through C). We confirmed by immuno-
Transcription Factors NF-κB and AP-1 Are Activated in the Whole Kidney but not in Glomeruli in PAN Nephrosis Rats

NF-κB and AP-1 are considered to be responsible for tubulointerstitial inflammation (30,31) and possible targets for statins (10). In addition, activation of NF-κB by podocytes may contribute to proteinuria in experimental nephritis (32). Accordingly, we performed EMSA to address the NF-κB and AP-1 activation in the kidneys and the glomeruli of PAN nephrosis rats. In the whole kidney samples, the DNA-binding activity of NF-κB (Figure 4A) and AP-1 (Figure 4B) was significantly augmented in PAN nephrosis rats. Fluvastatin reduced the NF-κB activation by 31%, although this reduction did not reach statistical significance (0.05 < P < 0.1). AP-1 activation was significantly inhibited by fluvastatin treatment (P < 0.01). In contrast, the binding activity of NF-κB and AP-1 in the glomeruli did not differ among these three groups (Figure 5). This result suggests that the alternative mechanisms other than inactivation of proinflammatory transcription factors could be...
responsible for the favorable effects of fluvastatin on podocytes in this model.

Fluvastatin Inhibits PAN-Induced RhoA Activation

Small GTPase Rho regulates cytoskeletal organization in podocytes (33,34), and its inappropriate activation may cause disruption of foot process architecture (35). Therefore, we next analyzed the expression of activated membrane-bound RhoA and phospho-MYPT, a marker for Rho-kinase activity, by Western blotting. As shown in Figure 6A, we found that membrane-associated RhoA was increased by 2.4-fold in the kidneys of PAN nephrosis rats (P < 0.01), which was significantly reduced by fluvastatin (P < 0.05). Overexpression of membrane RhoA was also demonstrated in the glomeruli (Figure 6B), and the expression tended to be lower in fluvastatin-treated rats (versus PAN; 0.05 < P < 0.1). Phospho-MYPT was also upregulated in the whole kidney (Figure 6C) and in the glomeruli (Figure 6D) of PAN nephrosis rats, which was significantly suppressed by fluvastatin (P < 0.05).

In Vitro Studies

Next, we investigated the involvement of Rho in podocyte damage and the protective effect of fluvastatin using murine podocyte cell line (21). We analyzed the membrane-bound RhoA expression and actin cytoskeletal organization in podocytes that were treated with PAN (5 μg/ml) in the presence or absence of fluvastatin (1 μM). As shown in Figure 7A, PAN treatment increased the expression of membrane-bound RhoA by 2.2-fold (P < 0.05). Co-treatment with fluvastatin significantly reversed the PAN-induced upregulation (P < 0.05). F-actin staining revealed that PAN treatment altered the pattern of actin fibers in podocytes, which was inhibited in the presence of fluvastatin (Figure 7B, a through c). We further investigated the mechanism of the statin-mediated inhibition of actin reorganization. The effect of fluvastatin was prevented by the co-addition of mevalonate (Figure 7B, d) or GGPP (Figure 7B, f) but not by the addition of farnesyl pyrophosphate (Figure 7B, e). These results indicate that the depletion of GGPP and modulation of RhoA activity are critical to the effect of fluvastatin.

A Specific Rho-Kinase Inhibitor Ameliorates Proteinuria and Podocyte Damage in PAN Nephrosis Rats

Finally, to confirm that the protective effect of fluvastatin on podocytes in PAN nephrosis was mediated through modulation of Rho, we examined the effects of fasudil, a specific Rho-kinase inhibitor, on PAN nephrosis. As shown in Figure 8A, fasudil administration significantly reduced proteinuria in PAN nephrosis rats on day 7 (225 ± 41 mg/d in fasudil-treated PAN nephrosis rats versus 399 ± 57 mg/d in PAN nephrosis rats without treatment; P < 0.05). Moreover, Western blotting of glomerular protein revealed that the decreased expression of nephrin in PAN nephrosis was alleviated by fasudil treatment.
Immunofluorescence study demonstrated that the staining pattern for nephrin resembled that seen in the controls (Figure 8C). These results corroborate the hypothesis that fluvastatin has protective effects on podocytes by modulating overactivity of Rho signaling in PAN nephrosis.

**Discussion**

Our study demonstrates that fluvastatin reduces urinary protein excretion and retains the expressions of nephrin and podocin in PAN nephrosis rats. The foot process structure of podocytes is partially protected by fluvastatin. Fluvastatin also ameliorates PAN-induced acute tubulointerstitial nephritis in parallel with the reductions in tubular expressions of chemokines and macrophage infiltration. Rho signaling is enhanced in the glomeruli of PAN-injected rats, which is suppressed by fluvastatin treatment. In cultured podocytes, fluvastatin inhibits PAN-evoked RhoA activation and reorganization of actin cytoskeleton through depletion of GGPP. Furthermore, a specific Rho-kinase inhibitor ameliorates proteinuria and podocyte damage in PAN nephrosis rats. These results suggest that fluvastatin alleviates podocyte damage in PAN-induced nephrosis by modulating overactivation of Rho.

Several lines of evidence indicate that statins reduce proteinuria and urinary podocyte loss in patients with kidney diseases (18–20). So far, very few studies investigated the salutary effects of statins on podocyte function and their potential molecular mechanisms. Although it has been shown that statins suppress glomerular injury in several experimental models (12,36), these studies focused on mesangial cells as the direct cellular target of statins. Two groups previously explored the effects of statins in nephrotic rats (37,38), and the results are conflicting. Drukker et al. (37) showed that lovastatin without pretreatment failed to attenuate albuminuria in PAN-treated rats. We consider that pretreatment is necessary in this acute nephrosis model because the HMG-CoA reductase inhibition should be sufficient at the moment of acute insult. Conversely, Park et al. (38) indicated that pretreatment with lovastatin reduced PAN-induced albuminuria on day 10 after injection, which is compatible with our finding on day 7. They found significant increases in glomerular MCP-1 expression and macrophage infiltration in PAN nephrosis on day 10, which was reduced by lovastatin. It should be noted, however, that these changes were not observed on day 5, when podocyte damage and proteinuria were already evident. Thus, infiltration of macrophages does not seem to be involved in the induction of foot process effacement. In fact, they attributed MCP-1 induction to mesangial cells and did not analyze podocyte function. We demonstrated that the reduced expressions of nephrin and podocin, the slit diaphragm–associated proteins and molecular markers for podocyte injury (13,14,39), were restored by fluvastatin treatment in proteinuric rats. Our in vitro study using cultured murine podocytes provided evidence for the direct actions of fluvastatin in podocytes. Taken together, fluvastatin is considered to have protective effects against podocyte injury in this model.

Another prominent finding of our study is that PAN administration resulted in the activation of the small GTPase RhoA and phosphorylation of MYPT, a target for Rho-kinase, in the glomeruli, which had not been described in this model. We also demonstrated that PAN enhanced RhoA stimulation in cul-
tured podocytes. Furthermore, we showed that fasudil, a specific Rho-kinase inhibitor, successfully reduced proteinuria and podocyte damage in PAN nephrosis. These results indicate that RhoA overactivation in podocytes mediates PAN-induced podocyte injury. Accumulating evidence suggests that a well-developed actin cytoskeletal structure plays a critical role in the maintenance of podocyte foot process architecture and filtration barrier function (34). Pathogenic stimuli such as PAN cause actin cytoskeletal reorganization (40,41), which is proposed to be the common pathway leading to foot process effacement in podocytes (42). Because small GTPase Rho regulates cytoskeletal organization in podocytes (8,33), we could speculate that overactivation of Rho that was observed in PAN nephrosis causes cytoskeletal rearrangement in podocytes and consequently impairs permselectivity. Indeed, one report by Togawa et al. (35) suggested the pathophysiological significance of Rho signaling in podocytes: They reported that knockout mice that lack Rho GDP dissociation inhibitor-\(\gamma\)9251, an intrinsic inhibitory regulator of Rho activity, show massive proteinuria and loss of foot process architecture. In this respect, one should bear in mind that one of the well-documented pleiotropic effects of statins is assigned to inactivation of small GTPase Rho (43). Because HMG-CoA reductase is the rate-limiting enzyme of the mevalonate pathway, statins reduce the synthesis of GGPP, which is necessary for the membrane localization and function of RhoA. We observed that fluvastatin corrected the PAN-evoked alteration of actin fibers in parallel with RhoA inactivation, which was reversed by the addition of mevalonate as well as GGPP. Thus, our observations indicate that fluvastatin prevents cytoskeletal alteration by inactivating RhoA through isoprenoids depletion. Similarly, fluvastatin is considered to ameliorate podocyte injury and proteinuria in PAN nephrosis rats via modulation of uncontrolled Rho activation in podocytes.

In addition to the influences on podocytes, fluvastatin ameliorated acute tubulointerstitial damage during the course of nephrotic syndrome in PAN-treated rats, as reflected by the reduction of ED-1-positive cells, scores for tubulointerstitial injury, and serum creatinine decrement (15). It is generally

![Figure 6](image_url)

Figure 6. Activation of RhoA and Rho-kinase in PAN nephrosis rats is suppressed by fluvastatin. Membrane-bound RhoA (active form) and phosphorylated-myosin phosphatase target subunit (phospho-MYPT) were detected by Western blotting (\(n = 3\) or 4 animals/group). (A and B) Expression of membrane-bound RhoA in the kidneys (A) and the glomeruli (B). Protein extracted from Ctrl is indicated in the left two lanes, protein from PAN is indicated in the middle two lanes, and protein from PAN + FV is indicated in the right two lanes. Each lane represents 10 \(\mu g\) of membrane fraction protein. (C and D) Expression of phosphorylated-MYPT in the kidneys (C) and the glomeruli (D). Each lane represents 20 \(\mu g\) of protein.

![Figure 7](image_url)

Figure 7. Fluvastatin inhibits PAN-induced RhoA activation and actin cytoskeletal reorganization in cultured podocytes. (A) Membrane-bound RhoA expression in cultured podocytes at 24 h after exposure to PAN. Protein extracted from Ctrl is indicated in the left two lanes, protein from PAN is indicated in the middle two lanes, and protein from PAN + FV is indicated in the right two lanes. Each lane represents 5 \(\mu g\) of membrane fraction protein (\(n = 3\)). (B) F-actin distribution of podocytes after incubation for 48 h with vehicle alone (a), PAN (b), PAN + FV (c), and PAN + FV in the presence of mevalonate (d), farnesyl pyrophosphate (e), or geranylgeranyl pyrophosphate (f). Similar results were obtained in three independent experiments. Bar = 50 \(\mu m\).
accepted that proteinuria itself is the main cause of tubulointerstitial nephritis in proteinuric nephropathies, including PAN nephrosis (44,45). Filtered proteins are reabsorbed by tubular epithelial cells, which triggers the activation of NF-κB, induction of proinflammatory chemokines and cytokines, recruitment of macrophages, and epithelial to mesenchymal transition. In our study, fluvastatin significantly inhibited tubular upregulation of chemotactic cytokine MCP-1 and osteopontin, a secreted acidic glycoprotein with potent chemoattractant effects, as well as vimentin, a marker for epithelial to mesenchymal transition. We also found that activation of NF-κB and AP-1, key transcription factors that regulate inflammation, was involved in PAN nephrosis, which was never analyzed previously in this model, and that their activation was suppressed by fluvastatin. Thus, our observations are in agreement with the proposed mechanisms of proteinuria-evoked tubulointerstitial damage. On the basis of this viewpoint, reduction of protein overload to tubules through amelioration of podocyte injury can be considered as the central mechanism by which fluvastatin limited the tubulointerstitial injury.

It seems, however, that the improvement of tubulointerstitial damage cannot be explained solely by this mechanism of proteinuria-associated nephritis, because macrophage infiltration and tubulointerstitial damage were largely prevented by fluvastatin, whereas reduction of proteinuria remained partial. As reported previously, severity of the tubulointerstitial injury in PAN nephrosis correlates with the degree of proteinuria (15). Consistent with the previous work, a plot of tubulointerstitial injury score versus urinary protein excretion indicated that heavy proteinuria was accompanied by severe tubulointerstitial injury in PAN nephrosis rats. In contrast, fluvastatin-treated rats showed marked attenuation of tubulointerstitial damage in the presence of moderate proteinuria. This could reflect the direct actions of fluvastatin on tubulointerstitial damage, in addition to the consequence of amelioration of podocyte damage and proteinuria. The candidate mechanism for this effect could be anti-inflammatory actions via suppressed inflammatory cytokine induction and inhibition of transcription factors NF-κB and AP-1 (43,46). However, these transcription factors do not seem to be involved in the podocyte damage in this model, because these are not activated in the glomeruli.

In this study, urinary protein excretion was significantly reduced but still present in fluvastatin-treated rats. There may be two reasons for the persistent proteinuria. First, podocyte damage that was induced by PAN was not fully inhibited by fluvastatin. The quantitative analysis of nephrin protein expression by Western blotting showed that although the decreased expression of nephrin was alleviated, fluvastatin could not afford complete protection when compared with the controls. Consistent with the finding, the foot process structure was partially protected in fluvastatin-treated rats, with effaced processes noted in some areas by electron microscopy. Thus, one reason for the proteinuria in fluvastatin-treated rats is that fluvastatin does not fully protect podocytes in PAN nephrosis rats.

The persistent proteinuria in fluvastatin-treated rats could also be explained by the reduction in the intracellular trafficking of filtered proteins in tubules. Recently, Sidaway et al. (47) reported that statins can inhibit receptor-mediated endocytosis of protein by blocking isoprenoid metabolism. Thus, statins could increase protein excretion of tubular origin by blocking protein uptake in the tubules (48).

Elevation of total cholesterol in PAN nephrosis was significantly reduced by fluvastatin on day 7. However, we do not think that the renoprotective effects of fluvastatin were related to the correction of hyperlipidemia, because mRNA expression of nephrin was significantly ameliorated by fluvastatin treatment 12 h after PAN injection, when serum cholesterol levels were not different among three groups (Table 1). Therefore, we assume that the decreased cholesterol level on day 7 probably reflects the reversal of nephrosis rather than the lipid-lowering action of fluvastatin.

Conclusion
Fluvastatin ameliorated PAN-induced podocyte injury and acute tubulointerstitial damage. Overactivation of Rho may cause derangement of actin cytoskeleton in podocyte foot process and contribute to impaired permselectivity in PAN nephrosis. The beneficial effects of fluvastatin can be attributable to direct modulation of excessive RhoA activity.

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