Beneficial Effects of the Rho Kinase Inhibitor Y27632 in Murine Puromycin Aminonucleoside Nephrosis

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Key Words

Rho GTPases · Glomerulonephritis · Blood pressure · Rho kinase

Abstract

Background and Aims: Rho kinase (ROCK) inhibition reduces systemic blood pressure (BP) and decreases renal damage in animal models of kidney disease. The aim of this study was to determine if ROCK inhibition might have beneficial effects in glomerular disease processes that are independent of systemic BP. **Methods:** We investigated the effects of the ROCK inhibitor Y27632 and hydralazine in murine puromycin aminonucleoside (PAN) nephrosis. **Results:** Treatment with either Y27632 or hydralazine similarly reduced systolic BP compared to vehicle-treated controls. Seven days after treatment with PAN, albuminuria, proteinuria and effacement of podocyte foot processes were significantly reduced in Y27632- and hydralazine-treated mice compared to vehicle-treated animals. Treatment with PAN significantly reduced expression of the podocyte proteins nephrin and Neph1, and the loss of glomerular nephrin was attenuated by treatment with Y27632 but not by treatment with hydralazine. In cultured podocytes, PAN potently activated both Rho and ROCK, and PAN-induced ROCK activation was prevented by

Y27632. **Conclusions:** The ROCK inhibitor Y27632 attenuated glomerular nephrin loss in murine PAN nephrosis independent of its effects on systemic BP.

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Introduction

Accumulating evidence suggests that the small G protein Rho and its downstream effector Rho kinase (ROCK) may play important roles in kidney biology [1–8]. In this regard, ROCK inhibits myosin light chain phosphatase, which promotes phosphorylation of myosin light chains and smooth muscle contraction [1]. This vasoactive effect may alter renal hemodynamics by modulating the tone of both the glomerular afferent and efferent arterioles [1, 9]. ROCK inhibition also affects systemic hemodynamics [10, 11]. Uehata et al. [10] found that the ROCK inhibitor Y27632 potently reduced systemic blood pressure (BP) in multiple hypertensive rat models. In addition, Rho-associated signaling cascades may have injury-promoting effects that are independent of its vasoactive actions. Rho-dependent signaling cascades modulate cellular adhesion, migration, proliferation and apoptosis as well as participate in fibrotic responses [1, 12–14]. In cultured renal cells,

Rho stimulates production of the transforming growth factor- β (TGF- β)-regulated gene connective tissue growth factor (CTGF) [14]. Elevated levels of CTGF are found in fibrotic lesions and may promote fibrosis [14]. In the glomerulus, Rho is involved in reorganization of the podocyte cytoskeleton in response to mechanical stress [15]. In the kidney, these pleiotropic effects may contribute to renal injury in glomerular disease processes.

Several groups have investigated the role of Rho-associated signaling cascades in mediating kidney damage. For example, Kanda et al. [2] found that the ROCK inhibitor fasudil reduced proteinuria and histologic evidence of renal injury in subtotaly nephrectomized, spontaneously hypertensive rats. Other groups have reported the beneficial effects of ROCK inhibition in rodent models of unilateral ureteral obstruction [3, 4], aldosterone-induced renal injury [5], hypertensive kidney disease [6] and ischemia reperfusion injury [7]. Inhibition of Rho activity has also been shown to prevent mesangial cell proliferation induced by high glucose conditions [16]. Taken together, these data suggest that inhibiting Rho and its downstream effector pathways may be a useful strategy for treating kidney diseases.

Less information is available on the role of Rho in promoting renal injury in glomerular disease processes. In cell culture systems, however, Rho is a negative regulator of the arborized morphology characteristic of the podocyte [17, 18]. In addition, indirect evidence suggests that Rho-associated signaling cascades may contribute to glomerular injury in vivo [19, 20]. In this regard, Rho proteins are modified after translation by the addition of the isoprenoid moiety which is essential for membrane localization of Rho and, in turn, its biological activity [1]. Treatment with 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase inhibitors ('statins') suppresses isoprenoid biosynthesis and inhibits the biological actions of Rho [1]. While it is likely that statins have effects that are independent of Rho, treatment with 'statins' ameliorates glomerular injury in puromycin aminonucleoside (PAN) nephrosis [19] as well as the anti-Thy 1.1 model of mesangial proliferative glomerulonephritis [20]. These data are consistent with the notion that Rho-associated signaling cascades promote injury in glomerular diseases.

To determine if ROCK is an important mediator of glomerular injury, we investigated the effect of the ROCK inhibitor Y27632 on nephrosis induced by PAN using a previously described mouse model [21]. Because of the potent effects of Y27632 on systemic BP [10, 11], we used a hydralazine-treated group to control for the effects of Y27632 on systemic BP. We found that both Y27632 and

hydralazine decreased systolic BP (SBP), albuminuria and proteinuria to a similar extent as well as reduced podocyte foot process effacement. Only ROCK inhibition, however, also attenuated the loss of glomerular nephrin expression in PAN nephrosis. These data suggest that ROCK inhibition may have beneficial effects on glomerular disease processes that are independent of systemic BP.

Materials and Methods

PAN was obtained from Sigma-Aldrich (St. Louis, Mo., USA). The ROCK inhibitor Y27632 [22] was obtained from Calbiochem (La Jolla, Calif., USA). Hydralazine was obtained from the Duke University Medical Center Clinical Pharmacy and was prepared by American Regent, Inc. (Shirley, N.Y., USA).

Experimental Protocol

Nephrosis was induced by a single injection of PAN (500 mg/kg, s.c.) as previously reported [21]. This dosage is higher than used in rats [19] and was not given by traditional intraperitoneal injection based on the following observations: (1) 100 mg/kg body weight did not produce proteinuria in B6SJLF1/J mice, and (2) the large volume of 0.9% normal saline vehicle needed to dissolve PAN appeared to be better tolerated when given subcutaneously. In addition, the protocol was modified to exclude mice younger than 4 months of age. By studying mice 4 months of age or older, the mortality rate was reduced to approximately 10% compared to 20–30% observed in previous experiments [21]. Moreover, the older mice tolerated the PAN injections without difficulty, remaining active and maintaining their body weight throughout the study. For the experiments, urine was collected from 4- to 6-month-old mice on the day prior to PAN injection (day 0) and on days 2 and 7 after injection. Mice were treated with either the ROCK inhibitor Y27632 (1 mg/kg), hydralazine (2 mg/kg) or vehicle (saline) by once daily subcutaneous injection starting on day 1. As a control, another group of mice was treated with PAN vehicle (saline) and then daily with Y27632 vehicle (saline). The week prior to PAN treatment as well as the week after the PAN injection SBP was measured as described below. Following the experimental protocol, mice were sacrificed and kidneys were harvested for light microscopic examination, electron microscopic examination, and preparation of enriched glomerular preparations as described below. All animal care conformed to the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' and was approved by the Institutional Animal Care and Use Committee at Duke University Medical Center, Durham, N.C., USA.

Isolation of Mouse Glomeruli

Enriched glomerular preparations were prepared using previously described methods [21]. The glomerular pellet was either (1) used to prepare total cellular RNA, or (2) solubilized in 50 mM Tris-HCl, 150 mM sodium chloride, 2 mM EDTA, 0.2% SDS, 0.2% Triton X-100, pH 7.4, by sonication. RNA and protein samples were then frozen at -70°C . By light microscopy, the purity of the glomerular preparations ranged from 60 to 70%.

Expression of Glomerular mRNAs Using Real-Time Quantitative RT-PCR

Total cellular RNA was prepared from enriched glomerular preparations using the Trizol reagent (Gibco) according to the recommendations of the manufacturer. The reverse transcription (RT) reaction was performed with Superscript reverse transcriptase (Gibco) and oligo (dT) primers. Real-time quantitative PCR was performed using the ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems Division), and the universal SYBR Green PCR master Mix Kit (Perkin-Elmer Applied Biosystems Division) as previously described [21] with additional primers for Neph1: CCTCACGTGCAGAGCATTTA and CTCAATGAGCAGTTGGCTGA. Data points are the average of triplicate measurements. Data are presented as expression relative to the control group not given PAN by dividing individual values by the mean value of the control mice.

Immunoblotting of Neph1

Neph1 protein levels were evaluated with rabbit polyclonal antibodies [23] from Alpha Diagnostic (San Antonio, Tex., USA). Glomerular proteins (20 μ g) were separated on 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, Calif., USA) and transferred to polyvinylidene difluoride (PVDF) membranes according to the directions of the manufacturer. Immunoblotting was performed using previously described methods [21]. Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia, Buckinghamshire, UK). The immunoblots were converted into a digital format using an Epson Perfection scanner 1670 (Seiko Epson Corporation, Nagano, Japan) and then analyzed using ScanAnalysis 2.5 software (Biosoft, Ferguson, Mo., USA). Densitometric data were normalized to an actin loading control and are presented as expression relative to the control group not given PAN by dividing individual values by the mean value of the control mice.

Measurement of Urinary Albumin and Protein

Urine was collected in metabolic cages specifically designed for use in mice (Hatteras Instrument, Cary, N.C., USA). Total protein concentration was measured using the method of Bradford [24]. Albuminuria was evaluated using an enzyme immunoassay kit from Exocell (South Philadelphia, Pa., USA). Normal values for albuminuria or proteinuria were defined as excretion rates greater than the mean plus 2 standard deviations of the mean in control mice not given PAN.

Light Microscopy

A portion of kidney was removed, bisected and fixed in 10% buffered formalin and then stained with either hematoxylin and eosin or a periodic acid-Schiff stain. The light microscopic sections were evaluated by a pathologist (T.A.F.) blinded to the treatment group.

Transmission Electron Microscopy and Quantitation of Patent Slit Diaphragms

Small blocks of cortical tissue were fixed in an aqueous solution of 8% glutaraldehyde (Sigma-Aldrich). Analysis at the electron microscopic level was initially performed in a qualitative fashion and areas of interest selected in semi-thin sections for preparation of ultrathin sections for examination by a pathologist (D.N.H.) blinded to treatment group. Digital images (2 animals

from each group) were analyzed for density of patent slit diaphragms using AdobePhotoshopCS software (Adobe Systems, Inc.). Data were expressed as the number of patent slit diaphragms per micrometer of glomerular basement membrane length.

Blood Pressure Measurements

SBP was measured in conscious mice by the noninvasive computerized tail-cuff method after 2 weeks of training. The method has been validated previously and correlates with direct measurements of intra-arterial pressure [25]. After the training period, BP was recorded daily for 7 days prior to PAN injection and 7 days after the injecting PAN. Values for each mouse represent the average of the daily measurements.

Culture of SV40 Transformed Mouse Glomerular Epithelial Cells

The immortalized mouse podocyte cell line was a gift from Dr. Paul E. Klotman (Mount Sinai Medical Center, New York, N.Y., USA) and was maintained in culture as described previously [26]. For the experimental studies, cells were treated overnight with 50 μ g/ml PAN in the presence or absence of 10 μ M Y27632.

Measurement of Rho Activity

Rho activity was determined by immunoprecipitation of GTP-bound Rho using the Rho-binding region of Rhotekin [27] covalently linked to agarose beads (Upstate Biotechnology, Lake Placid, N.Y., USA) according to the directions of the manufacturer.

Rho protein levels in the cellular lysates and immunoprecipitates were determined by immunoblotting using a mouse monoclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif., USA). Proteins were separated on 4–12% Bis-Tris polyacrylamide gels (Invitrogen, Carlsbad, Calif., USA) using either 10 μ l of cellular lysate or 20 μ l of the immunoprecipitates. The proteins were then transferred to PVDF membranes according to the directions of the manufacturer. PVDF membranes were blocked for 1 h in tris-(hydroxymethyl)-aminomethane (Tris)-saline buffer (20 mM Tris-HCl, 137 mM NaCl, pH 7.6; TBS) with 0.2% Tween 20 (T-TBS) and 5% non-fat dry milk (BLOTTO). After blocking, the primary antibody was added at a dilution of 1:400 in BLOTTO. The blot was incubated at 4°C overnight with gentle rocking followed by 3 washes with T-TBS. The horseradish peroxidase-labeled secondary antibody (Amersham) was added at a dilution of 1:2,000 in BLOTTO. After rocking for 1 h at 4°C, the blot was washed once with T-TBS and twice with TBS. Proteins were detected by ECL according to the manufacturer's specifications (Amersham Pharmacia, Buckinghamshire, UK). The immunoblots were analyzed by densitometry as described above. For the analyses, the values of GTP-bound Rho (active Rho) were normalized by dividing by the values for total Rho protein in the cellular lysates.

Measurement of ROCK Activity

The activity of ROCK was assessed by determining the phosphorylation state of the ROCK substrate myosin phosphatase targeting subunit 1 (MYPT1) [8, 19] using a rabbit phospho-specific MYPT1 antibody (Upstate Biotechnology). After treatment with PAN as described above, cells were scraped into 700 μ l of ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.2% sodium dodecyl sulfate, 0.2% Triton X-100, 50 nM calyculin) and then sonicated. The insoluble material was removed by centrifugation

at 14,000 g and the supernatant was used for immunoblotting at -70°C . Immunoblotting was performed as described above using 25 μl of cellular lysate and the primary antibody at a dilution of 1:1,000. To evaluate protein loading, immunoblots were stripped according to the ECL directions (Amersham) and immunoblotting was performed as described above using a mouse monoclonal antibody to actin (Chemicon International, Temecula, Calif., USA) at a concentration of 1 $\mu\text{g/ml}$. The immunoblots were analyzed by densitometry as described above.

Statistical Analysis

Data are presented as the mean $\pm$ standard error of the mean (SEM). For comparison of continuous variables between two groups, statistical significance was assessed by a Student's *t* test using the InStat computer program (GraphPad Software, Inc.). For comparison of nominal variables, statistical significance was assessed by a Fisher exact test using the InStat computer program.

Results

Effect of the Treatment Regimens on Systemic BP

SBP was measured the week prior to PAN injection and during treatment with either Y27632, hydralazine or vehicle as described in Materials and Methods. A group that was not treated with PAN was also included as an additional control (see Materials and Methods). As shown in figure 1, BP tended to decline during the 2-week study period in both the control group and the vehicle-treated group, but this difference was not statistically significant. In contrast, BP was similarly and significantly reduced in the mice receiving hydralazine or Y27632 compared to either their baseline values, vehicle-treated mice or the control group. These data suggest that both Y27632 and hydralazine reduced systemic BP to a similar extent.

Effect of the Treatment Regimens on Albuminuria and Proteinuria

Albuminuria and proteinuria were assessed prior to treatment with PAN (day 0) and on days 2 and 7 after treatment with PAN. The control group not given PAN was also studied. As shown in figure 2A, albuminuria was similar in all groups on days 0 and 2. On day 7, however, albuminuria was significantly increased in the vehicle-treated group compared to control animals not given PAN. In contrast, albuminuria was significantly reduced in both the Y27632 group and hydralazine group compared to mice treated with vehicle. At the 7-day time point, 52% of the saline-treated mice had albuminuria compared to 7.6 and 14% of the mice treated with either Y27632 ($p = 0.006$) or hydralazine ($p = 0.021$), respectively. As shown in figure 2B, the treatment regimens had a similar effect on the pattern of proteinuria. On day 7, pro-

Fig. 1. Effect of Y27632 on systemic BP. Systolic BP (SBP) was not statistically different from baseline in control mice not given PAN and in mice treated with vehicle. SBP was significantly reduced by treatment with either hydralazine or Y27632. Fourteen control, 17 vehicle-, 13 Y27632- and 14 hydralazine-treated mice were studied. * $p < 0.005$ versus baseline and $p < 0.01$ versus either vehicle or control; † $p < 0.005$ versus baseline and $p < 0.01$ versus either vehicle or control.

teinuria was significantly increased in vehicle-treated mice compared to control mice. Treatment with both Y27632 and hydralazine significantly reduced proteinuria compared to the group treated with vehicle. At the 7-day time point, 67% of the saline-treated mice had proteinuria compared to 18 and 7.1% of the mice treated with either Y27632 ($p = 0.021$) or hydralazine ($p = 0.004$), respectively. These data suggest that treatment with either Y27632 or hydralazine has similar beneficial effects on albuminuria and proteinuria in murine PAN nephrosis.

Effect of the Treatment Regimens on Renal Histology

We first determined the effects of the treatment regimens on renal histology. At the light microscopic level, renal histology was normal in PAN-treated mice except for mild, tubular reactive changes in mice with heavy proteinuria and 1 vehicle-treated mouse that had mild glomerular hypercellularity (not shown). Electron microscopic findings are shown in figure 3. Compared to control mice not given PAN (fig. 3A), treatment with

Fig. 2. Effect of treatment on albuminuria and proteinuria. **A** Albuminuria was significantly increased in vehicle-treated mice compared to control mice not receiving PAN. Both Y27632 and hydralazine significantly reduced albuminuria compared to ve-

hicle-treated mice. **B** A similar pattern was observed for proteinuria. Fourteen control, 17 vehicle-, 13 Y27632- and 14 hydralazine-treated mice were studied. ** $p < 0.005$ versus control; †† $p < 0.005$ versus vehicle; † $p < 0.025$ versus vehicle.

Fig. 3. Renal histology. **A** Glomerular ultrastructure in a control mouse. **B, C** Glomerular ultrastructure in mice treated with PAN and either saline vehicle or Y27632, respectively. In vehicle-treated mice, large areas of foot process effacement were detected (arrows, **B**). In contrast, foot processes were better preserved in Y27632-treated mice, although focal areas of foot process fusion and effacement were detected (arrow, **C**). **D** The number of patent slit diaphragms per micrometer of basement membrane is shown. Treatment with both Y27632 and hydralazine significantly reduced foot process effacement. Five separate fields were analyzed for the density of patent slit diaphragms per mouse and 4 mice were studied per group. * $p < 0.01$ versus vehicle; ** $p < 0.025$ versus vehicle.

Fig. 4. Expression of podocyte proteins in PAN nephrosis. Podocyte protein mRNA levels were quantitated by quantitative RT-PCR and data are presented as expression relative to the control group not given PAN as described in Materials and Methods. Treatment with PAN significantly reduced both nephrin and Neph1 mRNA levels in all treatment groups compared to control animals. The decrease in nephrin mRNA levels was significantly attenuated in mice treated with Y27632 compared to mice treated with either hydralazine or saline vehicle. Podocin mRNA levels tended to be lower in mice treated with PAN, but this decrease was not statistically significant. Podocalyxin, α -actinin-4, CD2AP and synaptopodin mRNA levels were similar to control mice in all treatment groups. * $p < 0.001$ versus control; ** $p < 0.01$ versus control; † $p < 0.005$ versus Y27632.

PAN was associated with large areas of foot process effacement (fig. 3B). In contrast, podocyte foot processes were better preserved in mice treated with either Y27632 (fig. 3C) or hydralazine (not shown), although focal areas of foot process fusion and effacement were detected. Figure 3D shows the number of patent slit diaphragms per micrometer of glomerular basement membrane in each group. Treatment with both Y27632 and hydralazine significantly reduced foot process effacement compared to vehicle-treated animals.

Effect of the Treatment Regimens on Expression of Podocyte Proteins

Expression of podocyte proteins is altered in glomerular disease processes in animal models [19, 28–34] and

acquired proteinuric renal diseases in humans [35–37]. We, therefore, assessed mRNA levels of podocyte proteins by quantitative RT-PCR using primer pairs specific for podocalyxin, podocin, synaptopodin, α -actinin-4, CD2-associated protein (CD2AP), Neph1 and nephrin as previously described [21]. Data are presented as expression relative to the control group not given PAN. As shown in figure 4, treatment with PAN significantly reduced nephrin and Neph1 mRNA levels in all treatment groups compared to control animals. The decrease in nephrin mRNA levels, however, was significantly attenuated in mice treated with Y27632 compared to mice treated with either hydralazine or saline vehicle. Treatment with PAN tended to reduce podocin mRNA levels, but this decrease was not statistically significant and was not affected by either Y27632 or hydralazine. Podocalyxin, α -actinin-4, CD2AP and synaptopodin mRNA levels were similar to control mice in all treatment groups.

To determine if the treatment regimens affected nephrin protein levels, we assessed nephrin expression in enriched glomerular preparations by immunoblotting. As shown in figure 5A, treatment with PAN reduced nephrin protein levels in all treatment groups compared to control mice. In mice receiving Y27632, however, the decrease in nephrin protein levels was attenuated compared to mice treated with either hydralazine or saline vehicle. Quantitation of the immunoblot data is shown in figure 5B. Treatment with PAN significantly reduced nephrin protein levels in all groups compared to control mice. This decrease in nephrin protein levels was significantly attenuated by treatment with Y27632 compared to mice treated with either hydralazine or saline vehicle. These data suggest that treatment with Y27632 attenuates the loss of nephrin expression in murine PAN nephrosis.

Effect of PAN on Rho and ROCK Activation in Cultured Podocytes

Previous studies have suggested that both Rho and ROCK are activated by PAN in cultured podocytes [19, 38]. To determine if PAN stimulated Rho activity in an immortalized podocyte cell line, we treated podocytes with PAN as described in Materials and Methods and then measured the quantity of GTP-bound Rho (active Rho) in podocyte lysates. As shown in figure 6A and B, Rho activity was significantly increased by treatment with PAN. Rho protein levels in the cell lysates were not affected by the treatment regimens (fig. 6A, lower panel). To determine if ROCK activity was increased by PAN, we measured phosphorylation of the ROCK substrate myosin phosphatase targeting subunit 1 (MYPT1) [8, 19] in

Fig. 5. Nephrin protein levels in PAN nephrosis. **A** A representative immunoblot of nephrin proteins in enriched glomerular preparations from control mice not receiving PAN and in mice treated with PAN and either saline vehicle, Y27632 or hydralazine. **B** Quantitation of the immunoblot data. Treatment with PAN significantly reduced nephrin protein levels in all treatment groups compared to control mice, and this decrease in nephrin protein levels was significantly attenuated by treatment with Y27632 compared to mice treated with either hydralazine or saline vehicle. With more prolonged exposure, nephrin appeared as a doublet in control mice not receiving PAN. For quantitation of the nephrin immunoblots, 10 mice were studied in each group. * $p < 0.005$ versus control; † $p < 0.025$ versus Y27632.

podocyte lysates as described in Materials and Methods. As shown in figure 6C and D, treatment with PAN significantly increased ROCK activity as assessed by phosphorylation of MYPT1. In contrast, treatment with Y27632 prevented PAN-induced ROCK activation. Protein loading was similar in all lanes as assessed by actin levels in the cell lysates (fig. 6C, lower panel). Taken together, these data are consistent with the notion that PAN stimulates both Rho and ROCK activity in glomerular podocytes.

Discussion

Rho and its downstream effector ROCK have been implicated in diverse animal models of kidney diseases [1–8]. The present studies extend these observations to a mouse model of glomerular injury. We found that both the ROCK inhibitor Y27632 and hydralazine similarly reduced SBP, albuminuria, proteinuria and improved glo-

Fig. 6. PAN-induced Rho and ROCK activation in cultured glomerular podocytes. **A, B** Rho activation by PAN. **A** The upper blot is GTP-bound Rho and the lower blot is total Rho protein in the cell lysates. Treatment overnight with 50 $\mu\text{g/ml}$ PAN enhanced Rho activity. The total amount of Rho protein in the lysates was not changed by the treatment regimens. **C, D** The phosphorylation state of the ROCK substrate MYPT1 was determined to monitor ROCK activity. The upper blot is phosphorylated MYPT1 and, in the lower blot, the immunoblots were stripped and re-probed for actin to monitor protein loading. PAN increased ROCK activity and PAN-induced ROCK stimulation was inhibited by Y27632. Actin levels were similar in each lane indicating equivalent protein loading. Data are the results of 4 separate experiments. * $p < 0.01$ versus vehicle; ** $p < 0.01$ versus PAN.

merular ultrastructure in PAN nephrosis. Y27632, however, also attenuated the loss of glomerular nephrin expression at both the mRNA level and protein level compared to hydralazine-treated mice. This attenuation of glomerular nephrin loss may be important in glomerular disease processes because of its key role in maintaining the integrity of the glomerular filtration barrier [39–41]. In this regard, nephrin was initially identified by positional cloning as the pathogenic cause of Finnish nephropathy [42]. The importance of nephrin in glomerular biology is supported by the observation that nephrin null mice develop foot process effacement, loss of slit diaphragms and massive proteinuria [39–41]. Nephrin may also act synergistically with other podocyte proteins to modulate glomerular permselectivity [43]. For example, proteinuria can be induced experimentally in mice by treatment with antibodies to the extracellular domain of either nephrin or Neph1 [43]. When these antibodies are administered at lower, sub-nephritogenic dosages, the antibodies act synergistically to enhance protein excre-

tion [43]. Thus, multiple sub-proteinuric insults are capable of causing proteinuria. Conversely, a partial loss of glomerular nephrin expression may not result in proteinuria unless the glomerulus sustains additional damage. In this scenario, attenuation of glomerular nephrin loss may make the glomerulus more resistant to a loss of permselectivity, that is, to the development of proteinuria after additional glomerular injury. Treatments that enhance glomerular nephrin expression such as ROCK inhibition might, therefore, have beneficial effects in glomerular disease processes that are independent of other beneficial actions such as the BP-lowering effect of Y27632.

Although nephrin levels were increased in mice treated with Y27632 compared to mice treated with hydralazine, nephrin levels remained lower than control animals that did not receive PAN. There are several possible explanations for this finding. First, it is possible that the inability of Y27632 to restore nephrin levels to normal might have been the result of incomplete ROCK inhibition. In this regard, the parenteral dosage of Y27632 chosen for the experiments has been shown to effectively inhibit ROCK enzymatic activity in mice [44]. However, higher cumulative daily dosages of Y27632 have been given orally in rat models of kidney disease [3]. It is, therefore, possible that additional beneficial effects might be observed by increasing the daily dosage of Y27632. Alternatively, other signaling pathways may modulate nephrin protein levels. For example, downregulation of nephrin in animal models of diabetic nephropathy is prevented by deletion of protein kinase C- α (PKC- α) suggesting a role for PKC- α in the regulation of nephrin expression [28]. The nephrin promoter also contains specific peroxisome proliferator-responsive elements (PPREs) that can be activated by peroxisome proliferator-activated receptor- γ agonists such as pioglitazone [29]. These PPREs appear to regulate nephrin expression because pioglitazone reduces proteinuria and prevents nephrin downregulation in an immune model of progressive nephropathy, passive Heymann nephritis [29]. Lastly, several laboratories have demonstrated that nephrin may function as a signaling molecule and is phosphorylated by Src family kinases such as Yes, Fyn and Src [45–48]. This phosphorylation may be important in regulating cell surface expression of nephrin because, in the absence of Yes-dependent phosphorylation of tyrosine 1193, nephrin interacts with the adapter protein β -arrestin 2 and induces its endocytosis [48]. These data suggest that multiple signaling pathways likely play important and different roles in regulating cell surface expression of nephrin.

Neph1 levels were also decreased in murine PAN nephrosis but, unlike nephrin, expression of Neph1 mRNA was not affected by treatment with the ROCK inhibitor. The importance of Neph1 in maintaining the integrity of the glomerular filtration barrier was discovered when a retrovirus-mediated mutagenesis strategy disrupted the gene locus for Neph1 in embryonic stem cells [49]. Generation of Neph1 null mice from these embryonic stem cells resulted in severe proteinuria and death in the first few weeks after birth [49]. Both Neph1 and nephrin are located at the slit diaphragm [50, 51] and Neph1 interacts with nephrin through extracellular immunoglobulin-like domains [43, 51, 52]. As mentioned above, the importance of this interaction has been investigated in vivo by disrupting the nephrin-Neph1 interaction by injecting a mixture of anti-Neph1 and anti-nephrin antibodies into rats [43]. This antibody combination induced proteinuria with preserved foot processes, although injection of either antibody alone had no significant effect on urinary protein excretion [43]. These data suggest that, similar to nephrin, Neph1 is an important determinant of glomerular permselectivity. Additional studies will be necessary, however, to determine if Neph1 expression is altered in other proteinuric animal models or in human glomerular diseases.

In contrast to Neph1, expression of nephrin has been investigated in numerous glomerular disease processes [19, 28–37]. For example, nephrin levels are decreased in subtotaly nephrectomized rats [33], rats with either Heymann nephritis [32, 34] or PAN nephrosis [30, 31] and animal models of diabetic nephropathy [28]. In human biopsy specimens, nephrin is decreased in pediatric patients with proliferative glomerular lesions [35] as well as some pediatric patients with minimal change disease [36]. In Heymann nephritis, treatment with either angiotensin II (ANG2) receptor blockers (ARBs) or angiotensin-converting enzyme inhibitors (ACEIs) preserves glomerular nephrin expression [32]. Similarly, ACEI treatment prevents the reduction in glomerular nephrin expression in subtotaly nephrectomized rats [33]. It is, therefore, of interest that activation of type-1 ANG2 receptors has been linked to Rho activation [53]. Indeed, some of the beneficial effects of ACEIs and ANG receptor blockers in disease processes [32, 33, 54] may be mediated by decreasing ANG2-dependent Rho activation. Because ANG2 has been shown to play a pathogenetic role in PAN nephrosis [54], it is possible that some of the beneficial effects of ROCK inhibition in the present study are mediated by antagonizing downstream effectors of ANG2-dependent Rho activation.

In addition to type-1 ANG2 receptors, other G protein-coupled receptors have been shown to activate Rho, including receptors for endothelin, platelet-activating factor (PAF), cysteinyl-leukotrienes, and thromboxane [55–57]. Each of these receptors has been implicated in glomerular disease processes [21, 58–62]. Indeed, thromboxane, leukotrienes, platelet-activating factor and ANG2 are important mediators of renal injury in PAN nephrosis [54, 63]. Given the likelihood that multiple G protein-coupled receptor systems contribute to renal injury in glomerular diseases [21], we speculate that Rho-associated signaling may be a final common signaling pathway that promotes renal injury in kidney diseases. Targeted therapy that inhibits Rho and its downstream effectors are, therefore, a potentially attractive strategy for the development of therapeutic agents to treat chronic kidney diseases. These types of therapies may not only have beneficial effects on systemic BP but may also have beneficial effects in the kidney that are independent of their systemic actions.

Although most investigators have reported that ROCK inhibition attenuates injury in disease processes affecting the kidney [1–7], Fu et al. [64] found that ROCK1 null mice were not protected from renal fibrosis following unilateral ureteral ligation. The authors speculated that the lack of a beneficial effect might be the result of either participation of other ROCK isoforms in the fibrotic response or a critical role for other signaling cascades in disease pathogenesis [64]. Additional studies will be necessary to clarify these possibilities. Indeed, we had

planned to utilize our podocyte culture system to study the role of Rho GTPase and ROCK in regulating nephrin expression. Unfortunately, similar to the results of most [65], but not all [66] investigators, we have been unable to detect nephrin mRNA in cultured podocytes, although we are able to detect several other podocyte proteins including synaptopodin, podocalyxin and Wilms tumor-associated antigen (WT1) by RT-PCR. These data suggest that some characteristics of differentiated podocytes are lost in culture.

In summary, we found that treatment with either the ROCK inhibitor Y27632 or hydralazine lowered systemic BP and had beneficial effects on albuminuria, proteinuria and glomerular ultrastructure in a murine model of PAN nephrosis. Y27632 treatment also attenuated the loss of glomerular nephrin expression characteristic of this animal model [19, 30, 31]. We speculate that inhibition of Rho-dependent signaling cascades and, in turn, attenuation of glomerular nephrin loss may have beneficial effects in glomerular disease that are independent of changes in systemic BP.

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