Inhibition of p38 Mitogen-Activated Protein Kinase Augments Progression of Remnant Kidney Model by Activating the ERK Pathway

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p38, a mitogen-activated protein kinase, is a major intracellular signaling molecule involved in inflammation. To test the hypothesis that p38 mediates renal disease progression, we administered a novel p38 α inhibitor, NPC31169, to rats with remnant kidneys (RKs). RK rats showed increased p38 activation at 9 weeks (by p38 kinase assay), which was blocked by the inhibitor. In contrast to our expectation, treatment with the NPC31169 resulted in worse renal function, more proteinuria, and more severe glomerulosclerosis and tubulointerstitial injury. p38 inhibition resulted in marked cell proliferation in RK rats, with more proliferating tubular cells, myofibroblasts, and macrophages. In contrast, p38 suppression resulted in less tubular cell apoptosis. Interestingly, Western blot demonstrated increased ERK1/2 phosphorylation in p38-treated rats. No histological changes were observed in p38 inhibited sham-operated rats. Our findings indicate that, whereas blocking p38 usually shows benefit in inflammatory disease, in this model p38 inhibition resulted in accelerated renal progression. We conclude that blocking p38-dependent inflammation may have resulted in enhanced proliferation and increased ERK1/2 activation, and thereby explains the worse renal lesions observed. (*Am J Pathol* 2004, 164:477–485)

Mitogen-activated protein kinases (MAPK) are important mediators involved in the intracellular network of interacting proteins that transduce extracellular stimuli to intracellular responses.¹ A member of MAPK, p38, is a ubiquitous, conserved protein kinase.² Although its precise function remains controversial, p38 MAPK is particularly involved in the inflammatory process, and its activation is required for regulation of transcriptional activation of inflammatory cytokine genes including interleukin-1 β and tumor necrosis factor- α .³

Regardless of initial insults, chronic renal disease tends to progress through a process mediated by inflammation and fibrosis. To date, a number of cytokines and growth factors have been shown to be involved in this process.^{4–6} Given that p38 is primarily involved in the regulation of cytokine expression, suppression of the p38 pathway by using a specific inhibitor can be a potential candidate for treatment of inflammatory renal diseases.⁷ However, our understanding of the roles of intracellular mediators contributing to gene transcription, regulation of cellular growth, or apoptosis in the kidney remains incomplete.

The effect of p38 inhibition on disease progression has been tested in several animal models by using some types of p38-specific inhibitors. Treatment with SB239063 or SB203580, the most widely distributed p38 inhibitor, reduces acute inflammatory cell infiltration in animal models of lung fibrosis and colitis.^{8,9} Other p38 MAPK inhibitors have also been shown to block inflammation in experimental models.^{10–12} In the kidney, Stambe and colleagues¹³ reported that the p38 inhibitor, NPC 31145, blocked early neutrophil and platelet infiltration in the model of anti-glomerular basement membrane nephritis and this was associated with less proteinuria and preserved renal function. Using a lupus model in mice, Iwata and colleagues¹⁴ also reported that chronic administration of the p38 MAPK inhibitor, FR 167653, could inhibit the autoimmune response leading to a reduction in renal injury with preservation of renal function.

In the current study, we used a new p38 MAPK inhibitor, NPC31169, to treat rats with slowly progressive renal disease mediated by subtotal renal ablation. This is a model of renal injury characterized by low-level cytokine expression and progressive glomerulosclerosis and interstitial fibrosis.¹⁵ We report the surprising result that inhibition of p38 MAPK in this model was associated with activation of a different MAPK, ERK, and that this was associated with significant worsening of the renal lesion.

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Materials and Methods

Four groups of male Sprague-Dawley rats (250 to 280 g) were studied: group I, sham-operated ($n = 6$); group II, sham-operated + NPC31169 ($n = 6$); group III, remnant kidney (RK) ($n = 7$); and group IV, RK+NPC31169 ($n = 7$).

NPC31169 (Scios, Inc., San Francisco, CA) is an orally administered selective p38 α inhibitor that does not block phosphorylation of p38 α but inhibits the ability of phosphorylated-p38 α (p-p38 α) to phosphorylate its downstream targets such as activated transcription factor-2 (ATF-2). Similar to NPC31145,¹³ it is selective for p38 α and has minimal activity against other MAPKs.

The RK model was performed by resecting the right kidney with surgical extirpation of the upper and lower thirds of the left kidney.¹⁶ The sham operation consisted of a laparotomy with manipulation of the renal pedicles. One week after the operation, rats were matched for body weight and blood urea nitrogen (BUN) levels, and then randomized to receive or not receive NPC31169 in their diet. Animals were fed using a powdered diet (LabDiets no. 5001, PMI: Nutrition International) with special diet feeder (Rodent Powdered Diet Feeder; Britz-Heidbrink, Inc., Wheatland, WY) and water *ad libitum*. The drug NPC31169 (100 mg/body weight kg/day) was mixed in the diet using a coffee grinder for 1 minute to ensure homogeneity. The diet for control groups was also processed in the same manner without the drug added. The food consumption was assessed in each animal every 5 days, and the diet was supplemented accordingly to maintain pair feeding. The drug levels in the plasma were monitored at weeks 3 and 9. Animals were pair-fed to ensure equal food intake. Animals had systolic blood pressures and body weights measured weekly. On weeks 0, 3, 6, and 9, rats were placed overnight in metabolic cages for urine collection for urinary protein determination. At 9 weeks rats were sacrificed for blood and histological studies. The animal procedures were approved by the Animal Care Committee at Baylor College of Medicine, Houston, TX.

Renal Function and Blood Pressure

Twenty-four-hour urinary protein excretion was measured using the Bio-Rad protein assay (Bio-Rad, Richmond, CA). BUN levels were determined using a commercial kit (Sigma Diagnostics, St. Louis, MO). Blood pressure was measured in conscious, restrained rats with a tail cuff sphygmomanometer (IITC model 229; IITC Inc., Woodland Hills, CA). All rats were preconditioned with the blood pressure equipment for 1 week before the study.

Immunohistochemical Analysis

Tissues were fixed in methyl Carnoy's or 4% formalin solution, paraffin-embedded, sectioned (4 μ m), and stained with periodic acid-Schiff reagent or by immunoperoxidase as reported previously.¹⁶

Primary antibodies (Abs) include rabbit polyclonal anti-p38 (Santa Cruz, Santa Cruz, CA), mouse monoclonal

α -SM-1 (Sigma) for detection of α -smooth muscle cell actin (α -SMA)-positive cells, and mouse monoclonal anti-ED-1 (Serotec, Indianapolis, IN) for monocytes/macrophages.

For labeling with Abs, the tissue sections were first deparaffinized and treated for 30 minutes with 3% H₂O₂. All of the sections were then first incubated overnight at 4°C with primary Abs, and then for 30 minutes with biotinylated anti-rabbit IgG or goat anti-mouse IgG (1:100; DAKO, Glostrup, Denmark) and visualized using H₂O₂ containing diaminobenzidine buffer. Controls included omitting the primary antibody and substitution of the primary antibody with preimmune rabbit or mouse IgG.

To examine whether there is any evidence of smooth muscle cell or monocyte-macrophage proliferation, double immunostaining was performed with Abs to the proliferating cell nuclear antigen (PCNA) (Cappel, Aurora, OH) and α -SMA or ED-1 Ab. Sections were incubated with the PCNA Ab overnight at 4°C, followed sequentially by biotinylated horse anti-mouse IgG serum, peroxidase-conjugated avidin D, and color development with diaminobenzidine with nickel chloride. After incubation in 3% H₂O₂ for 8 minutes to eliminate any remaining peroxidase activity, sections were incubated with α -SM-1 or ED-1 overnight at 4°C, followed by biotinylated horse anti-mouse IgG for 30 minutes at room temperature. After incubation in alkaline phosphatase-streptavidin (Vector, Burlingame, CA), color was developed using AP-RED substrate kit (Zymed, San Francisco, CA).

Identification of Apoptosis

Apoptotic cells were identified based on the presence of fragmented nuclear DNA in histological sections labeled using TdT-FragEL DNA fragmentation detection kit (Oncogene, San Diego, CA). Deparaffinized 4- μ m-thick formalin-fixed sections were incubated with proteinase K (2 mg/ml) for 15 minutes at room temperature. After blocking endogenous peroxidase by immersion in 3% H₂O₂ in distilled water, sections were incubated with TdT buffer (1 mol/L sodium cacodylate, 0.15 mol/L Tris, 1.5 mg/ml bovine serum albumin, 3.75 mmol/L CoCl₂) at room temperature for 30 minutes. Sections were then incubated with TdT-labeling reaction mixture consisting of TdT-labeling reaction mix (labeled and unlabeled deoxynucleotides at a ratio optimized for DNA fragment end labeling with TdT) and TdT enzyme (0.5 mol/L EDTA, pH 8.0), and incubated for 1.5 hours at 37°C. The reaction was terminated by stop solution (0.5 mol/L EDTA). The labeled nuclei were visualized with peroxidase-streptavidin conjugate and H₂O₂-containing diaminobenzidine. Negative controls were generated by substituting distilled H₂O for the TdT in the reaction mixture during the labeling step according to the kit instruction.

Quantification of Morphological Data

All quantification was performed in a blinded manner. The glomerulosclerosis and tubulointerstitial injury were semiquantitatively assessed in periodic acid-Schiff-

stained sections of each biopsy using 40 randomly selected glomeruli or 40 fields in the cortical area under $\times 400$ magnification.¹⁶

For each glomerulus, the following system was used: 0, 0% glomerulosclerosis; 1, 25% glomerulosclerosis (one quarter of the glomerulus); 2, 50% glomerulosclerosis (one half of the glomerulus); 3, 75% glomerulosclerosis (three quarters of the glomerulus); and 4, 100% glomerulosclerosis (global glomerulosclerosis). Interstitial injury was graded as: 0, no fibrosis; 1, slightly increased interstitial fibrosis; 2, more severe interstitial fibrosis; and 3, marked interstitial fibrosis. Tubular atrophy was graded as: 0, normal tubules; 1, slightly atrophic tubules; 2, more severe atrophic tubules; and 3, marked atrophic tubules.

The number of p-p38-positive cells, PCNA-positive cells, apoptotic cells, ED-1-positive cells, ED-1- and PCNA-positive cells, and α -SMA- and PCNA-positive cells were calculated in 40 glomeruli or 40 fields of the cortical area under $\times 400$ magnification in each sample.¹⁷ ED-1-positive cells in the cortical area include all of the positive cells infiltrated within the tubular epithelium and adjacent interstitium, and the number of ED-1 cells were expressed per 100 tubules to exclude the effects of tubular dilatation or atrophy.

α -SMA-positive staining in the glomerulus or tubulointerstitium was measured using computer image analysis (Optimas 6.2; Media Cybernetics, Silver Springs, MD) and expressed as percentage of total glomerulus or tubulointerstitium containing α -SMA staining, respectively.

Western Blot

Whole cortical tissues were sonicated in cell lysis buffer (20 mmol/L, pH 7.5, Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L glycerolphosphate, 1 mmol/L Na_3VO_4 , 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 mmol/L phenylmethyl sulfonyl fluoride) (Cell Signaling, Beverly, MA) on ice, and the cell lysates were centrifuged at 4°C for 20 minutes at $16,000 \times g$. The protein concentration of supernatant was measured by the Bio-Rad protein assay (Bio-Rad) and adjusted by adding the lysis buffer accordingly to equalize the concentration. Then 4 $\times$ NuPage lithium dodecyl sulfate (LDS) sample buffer was added (Invitrogen, Carlsbad, CA) and heated at 75°C for 10 minutes. Each sample (60 μg protein) was loaded per lane on 10% NuPage Bis-Tris gel (Invitrogen) and transferred to membranes (Hybond, Amersham Pharmacia Biotech Limited, UK). Subsequently, membranes were blocked in 5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 hour. Membranes were incubated at 4°C overnight with Abs against phosphorylated ERK or total ERK (Cell Signaling). The primary Abs were detected using the horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling) and visualized by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). The intensity of the identified bands was quantified by densitometry analysis using NIH image software.

MAPK Assay

MAP kinase assays were performed using p38 MAPK assay and SAPK/JNK assay kits purchased from Cell Signaling.⁹ The protein concentration of supernatant was measured as described above and adjusted by adding the lysis buffer accordingly to equalize the concentration. For p38 kinase, soluble lysates containing 200 μg (protein) of sample were incubated with 20 μl of immobilized phospho-p38 MAP kinase (Thr180/Tyr182) monoclonal Ab with gentle rocking overnight at 4°C. Immune complexes were washed twice with lysis buffer and kinase buffer (25 mmol/L, pH 7.5, Tris, 5 mmol/L β -glycerolphosphate, 2 mmol/L dithiothreitol, 0.1 mmol/L Na_3VO_4 , 10 mmol/L MgCl_2) and resuspended with kinase buffer containing 200 $\mu\text{mol}/\text{L}$ ATP and 2 μg ATF-2 fusion protein. The reaction was initiated by incubation at 30°C for 30 minutes. Then, 3 $\times$ sodium dodecyl sulfate sample buffer was added to terminate the reaction, and samples were subjected to 10% NuPage Bis-Tris gel. Proteins were then analyzed by Western blot analysis, as describe above, with a polyclonal anti-phospho ATF-2 Ab. For JNK, soluble lysates (250 μg protein) were incubated with 20 μl of c-Jun fusion protein beads with gentle rocking overnight at 4°C. After washing, immune complexes were resuspended with kinase buffer containing 100 $\mu\text{mol}/\text{L}$ ATP. The reaction was initiated by incubation at 30°C for 30 minutes, and terminated by addition of 3 $\times$ sodium dodecyl sulfate sample buffer. Samples were analyzed by Western blot with a polyclonal anti c-Jun Ab. The intensity of the identified bands was also quantified by densitometry analysis.

NPC31169 Kinase Assays

NPC31169 was developed by Scios Inc. This drug does not affect phosphorylation of p38 α but it inhibits the ability of p-p38 α to phosphorylate its downstream targets such as ATF-2. Its specificity for p38 α inhibition was determined against a variety of enzymes in kinase assays. Individual kinases were isolated from cell lysates by immunoprecipitation with specific antibodies bound to Sepharose beads. Kinase assays were performed as described previously.¹³ Briefly, kinases were incubated with their specific target substrates together with 0.1 mmol/L [γ ³²P]ATP and 10 mmol/L magnesium acetate in the presence of increasing concentrations of NPC31169 for 10 minutes at 30°C. Protein was precipitated on filter paper and washed three times in 50 mmol/L phosphoric acid to remove ATP, and radioactivity was counted. The concentration of NPC31169 required to inhibit kinase activity by 50% was recorded as an IC_{50} value. NPC31169 inhibits kinase activity of p38 α with an IC_{50} of 0.014 $\mu\text{mol}/\text{L}$. The specificity of the compound for p38 α was more than 14-fold greater than for the closely related kinase isoform p38 β . There was no significant activity of NPC31169 against any of the other kinases investigated (Table 1).

Table 1. Specificity of p38 Inhibition by NPC31169 *in Vitro*

Kinase	IC ₅₀ (μmol/L)*
p38α	0.014
p38β	0.209
p38γ	>50
ERK2	>50
JNK-1	>50
TGFβ-R1	>50
TGFβ-R2	>50
PKC	>50
PKA	>50
cdc2	>50
EGFR	>50
PKD	>50
CaMKII	>50
p59fyn	>50
p56lck	>50
HER2	>50
MAPKAP-2	>50

*Concentration of NPC31169 (μmol/L) resulting in a 50% reduction in the activity of kinase enzymes (IC₅₀) as determined by *in vitro* phosphorylation of various substrates.

Statistical Analysis

All data are presented as mean ± SD. Two groups were compared by nonparametric Mann-Whitney test. Multiple comparisons were performed by using analysis of variance with Bonferroni's adjustment.

Results

In this study, we examined the effect of 8 weeks of treatment with a novel p38α MAPK inhibitor (NPC31169) in the RK model. Control groups included rats with RK and rats that underwent sham surgery. The treatment was started at week 1, thus allowing animals to be matched for renal function (BUN levels) and body weight before treatment (Table 2). Baseline weight of RK was indirectly measured by subtracting the resected left kidney weight from the weight of the right whole kidney. The baseline RK/body weight ratio was equivalent between the two groups (Table 2), indicating that the RK procedure was of equivalent severity.

p38 MAPK Is Activated in the RK Model

We first determined if p38 MAPK is activated in the RK model by immunostaining for activated (phosphorylated) p38 (p-p38). In sham-operated rats p-p38 was minimally

Table 2. Baseline Parameters before Treatment in RK and RK + NPC31169 Rats

	RK (n = 5)	RK + NPC31169 (n = 7)
BW (g)	286 ± 24	286.8 ± 24
RKW (g)	0.58 ± 0.08	0.58 ± 0.06
RKW (g)/BW (Kg)	2.03 ± 0.2	2.04 ± 0.2
BUN (mg/dl)	53.2 ± 5.2	55.2 ± 5.1

Data are expressed as mean ± SD. RK; Remnant kidney; BW, body weight; RKW, remnant kidney weight. No significance difference was seen between RK and RK + NPC31169 groups before treatment.

Figure 1. Involvement of p38 MAPK in RK model. Expression of p-p38 in glomerulus and tubules from sham (A, C) and RK rats (B, D) with quantification in glomeruli (E) and tubules (F). p-p38 is occasionally expressed in the nuclei of podocytes (arrowheads) and tubular cells (asterisks). Data are expressed as mean ± SD. *, *P* < 0.001 compared with sham. Original magnifications: ×600 (A, B); ×200 (C, D).

expressed in glomeruli and in the tubulointerstitium (Figure 1, A and C). In contrast, RK rats examined at week 9 demonstrated staining of p-p38 in occasional nuclei of podocytes in affected glomeruli (Figure 1, B and E). p-p38-positive cells were also found in dilated tubules of RK rats (Figure 1, D and F). These findings demonstrate that the p38 MAPK pathway is activated in the RK model.

Effect of p38 Inhibition in the RK Model

General Tolerability of Treatment with NPC31169

To determine the role of p38 in the RK model, rats were administered the p38α MAPK inhibitor, NPC31169. NPC31169 was tolerated well in the sham and RK rats. Specifically, rats behaved normally and there was no difference in weight gain between sham and sham+NPC31169 groups, and between the RK and RK+NPC31169 groups (Figure 2A). However, rats in the RK groups did show less weight gain than sham-operated rats, as expected for the severity of their renal failure. In addition, two rats from the control RK group died during the first 10 days related to the surgery and initial blood draw.

Blood Pressure and Renal Function

Blood pressure increased progressively in the RK rats and was significantly higher in RK versus sham-operated

Figure 2. Weight, blood pressure, and renal function. Data are expressed as mean $\pm$ SD. □, Sham; ■, sham+NPC31169; ○, RK; ●, RK+NPC31169. #, $P < 0.001$ compared to sham and sham+NPC31169; *, $P < 0.001$ compared to RK.

rats at 9 weeks (Figure 2B). However, there was no difference between RK and RK-NPC31169 groups at any time point. Sham-operated NPC31169-treated control rats showed normal renal function at all time points. In contrast, RK rats showed increasing proteinuria and worse renal function (assessed by BUN measurements). Treatment with the NPC31169 resulted in worse proteinuria and higher BUN levels at the later time points compared to RK alone (Figure 2, C and D).

Effect on Renal Hypertrophy

In the control rats with RK, a twofold increase in the kidney/body weight ratio was observed at 9 weeks compared to baseline, consistent with the known response of the rat kidney to undergo hypertrophy after the RK procedure (Figure 3, A and C). Interestingly, RK+NPC31169 rats showed a significantly greater increase in kidney/body weight ratio compared to RK rats (Figure 3, B and C). NPC31169 did not cause any changes to kidney/body weight ratio in sham-operated rats (data not shown).

Effect on Renal Histology

Sham-operated and NPC31169-treated rats had normal renal histology at 9 weeks (Table 3; Figure 4, A and B). In contrast, RK rats developed significant focal segmental glomerulosclerosis and interstitial fibrosis at 9 weeks (Table 3; Figure 4, C and D). RK rats treated with NPC31169 showed more severe tubular dilation universally throughout the renal cortex (Table 3; Figure 4, E and F). Both the glomerulosclerosis score and the severity of the interstitial fibrosis were greater in the RK+NPC31169 group than in RK rats.

RK rats also had a marked increase in infiltrating macrophages and α -SMA-positive myofibroblasts compared to sham-operated rats (Table 4). The infiltration of ED-1-positive monocytes/macrophages was more prominent in glomeruli and tubulointerstitium of RK+NPC31169 rats

Figure 3. Renal hypertrophy in RK and RK+NPC31169 rats. Kidneys at 9 weeks from RK (A) and RK+NPC31169 rats (B). Although there was no difference at day 0, RK+NPC31169 rats demonstrated greater renal hypertrophy compared to the RK group (C). Data are expressed as mean $\pm$ SD. ○, RK; ●, RK+NPC31169. *, $P < 0.001$ compared to RK. Scale bar, 2.5 mm.

compared to RK alone (Table 4; Figure 5, A and B). Furthermore, many of the ED-1-positive cells were in a proliferative state as evidenced by co-staining with PCNA (Figure 5E), suggesting that they were in an activated state. The total number of α -SMA-positive cells, a hallmark of fibrosis, was also increased in RK+NPC31169 rats particularly in severely damaged lesions while it was not so obvious in RK rats, and the number of α -SMA+PCNA-positive cells was also increased (Table 4 and Figure 5; C, D, and F).

The RK model was also associated with both increased proliferating glomerular and tubular cells and with an increase in apoptotic cells (Table 3). There were significantly more proliferating glomerular and tubular cells in the RK+NPC31169 rats compared to RK rats (Table 3; Figure 6, A and B). In contrast, apoptotic cells were more common in RK rats compared to the RK+NPC31169 group and this was particularly evident in the tubulointerstitial compartment (Table 3; Figure 6, C and D).

Effect of p38 Inhibition on p38 Kinase Activity

NPC31169 does not prevent phosphorylation of p38 but rather blocks its kinase activity and hence inhibits downstream targets such as the phosphorylation of ATF-2. Consistent with this mode of action, expression of p-p38 was demonstrated in the RK+NPC31169 group

Table 3. Effect of NC31169 on Glomerular and Tubulointerstitial Injury in RK Rats

	Sham (n = 6)	Sham + NPC31169 (n = 6)	RK (n = 5)	RK + NPC31169 (n = 7)
Glomerulosclerosis score (0–4)	0.015 ± 0.02	0.01 ± 0.01	1.31 ± 0.35*	2.20 ± 0.40* [‡]
Interstitial injury score (0–3)	0.008 ± 0.01	0.01 ± 0.02	0.74 ± 0.21*	2.1 ± 0.28* [‡]
Tubular atrophy score (0–3)	0.004 ± 0.01	0.01 ± 0.01	0.48 ± 0.13*	0.19 ± 0.16
PCNA-positive cells				
G (/cross section)	0.20 ± 0.05	0.18 ± 0.04	0.83 ± 0.30	3.90 ± 1.1* [‡]
T (/100 tubules)	7.34 ± 1.6	7.22 ± 2.17	32.2 ± 11.2*	86.9 ± 31* [‡]
Apoptotic cells				
G (/cross section)	0.02 ± 0.12	0.06 ± 0.09	0.19 ± 0.15*	0.15 ± 0.14*
T (/100 tubules)	0.33 ± 0.2	0.25 ± 0.28	73.0 ± 31.5* [‡]	17.5 ± 4.56 [‡]

Data are expressed as mean ± SD. G, Glomeruli; T, tubulointerstitium.

**P* < 0.001, [‡]*P* < 0.05 compared to sham and sham + NPC31169. [‡]*P* < 0.001 compared to RK or RK + NPC31169. No significance difference was seen between sham and sham + NPC31169 groups for any parameter.

and no difference was found in the number of the positive cells compared to RK rats (data not shown).

To assess p38 kinase activity, we performed Western blotting on renal tissue extracts for p-ATF-2. As shown in Figure 7A, control RK rats showed an up-regulation of p-ATF-2 compared to sham groups, consistent with activation of p38 in the RK model. NPC31169-treated rats showed minimal p-ATF-2 expression consistent with effective inhibition of the p38 MAPK pathway. Blood levels of NPC31169(N) were; sham+N 1126 ± 475 nmol/L, RK+N 1217 ± 654 nmol/L at 3 weeks, and sham+N

599 ± 105 nmol/L, RK+N 491 ± 194 nmol/L at 9 weeks. The levels were approximately two to four times the *in vitro* concentration needed to inhibit p38 (data from Scios, Inc).

Inhibition of p38 MAPK Results in Increased ERK1/2 MAPK Expression

The observation that inhibition of p38 MAPK was associated with worse renal injury in the RK rat suggests that alternative MAPK pathways may have been activated. To examine this possibility, renal cortical tissue was assayed by Western blot for phosphorylated members of the ERK1/2. Control RK rats showed increased ERK1/2 activity compared to sham-operated rats, but the activation was much greater in RK+NPC31169-treated rats (Figure 7B). In contrast neither sham control nor sham NPC31169-treated rats showed any evidence for ERK1/2 activation.

A mild activation of the JNK/SAPK pathway, as assessed by c-Jun phosphorylation, was also observed in RK rats (Figure 7C). Although c-Jun activation appeared greater in RK groups compared to the sham-operated rats, densitometric analysis demonstrated no significant difference between groups.

Discussion

The present work was performed to elucidate the role of p38 in the kidney in a slowly progressive model of renal failure. At 9 weeks RK rats showed significant glomerulosclerosis and tubulointerstitial fibrosis with evidence of p38 MAPK activation as reflected by increased expression of p-p38 in glomeruli and the tubulointerstitium (by immunostaining), and by increased cortical p-ATF-2 expression (by MAPK assay). Treatment with an orally active p38 MAPK inhibitor could block the activation of p38 MAPK as assessed by blotting for the downstream target, p-ATF-2. However, rather unexpectedly, RK rats treated with the inhibitor (NPC31169) had a worse outcome in terms of renal function (BUN and proteinuria) with more renal hypertrophy and greater glomerulosclerosis and tubulointerstitial fibrosis. Furthermore, by immunohistochemistry there were more proliferating glomerular and

Figure 4. Histological changes in RK and RK+NPC31169 rats. Glomerular and tubulointerstitial changes from sham+NPC31169 (A, B), RK (C, D), and RK+NPC31169 rats (E, F). Administration of NPC31169 alone caused no alterations in either the glomeruli (A) or tubulointerstitium (B) in the kidneys of sham rats. Although segmental glomerulosclerosis and adhesion to Bowman's capsule was observed in many glomeruli of the RK group (C, arrows), the lesions were more conspicuous in RK+NPC31169 rats (E and F, arrows). Some glomeruli in the RK+NPC31169 group were enlarged showing global sclerosis (E, arrow). Marked tubular dilatation was noted in RK+NPC31169 rats (F, asterisks) compared to RK rats (D) but no significant difference was seen in tubular atrophy between two groups. Original magnifications: ×200 (A, C, E); ×50 (B, D, F).

Table 4. Effect of NPC31169 on Macrophage (ED-1+) and Myofibroblast (α -SMA+) Expression in RK Rats

	Sham (n = 6)	Sham + NPC31169 (n = 6)	RK (n = 5)	RK + NPC31169 (n = 7)
ED-1-positive cells				
G (/cross section)	0.05 $\pm$ 0.03	0.06 $\pm$ 0.02	8.0 $\pm$ 1.3*	16.8 $\pm$ 1.9*†
T (/100 tubules)	1.56 $\pm$ 0.61	1.37 $\pm$ 0.57	108.6 $\pm$ 23.2*	189.4 $\pm$ 22.3*†
α -SMA-positive area				
G (%)	0.05 $\pm$ 0.005	0.07 $\pm$ 0.03	0.86 $\pm$ 0.29*	2.0 $\pm$ 0.61*†
T (%)	0.02 $\pm$ 0.01	0.02 $\pm$ 0.01	0.97 $\pm$ 0.42*	1.99 $\pm$ 0.57*†
ED-1 + PCNA-positive cells				
G (/cross section)	0.03 $\pm$ 0.02	0.02 $\pm$ 0.01	0.32 $\pm$ 0.02	1.09 $\pm$ 0.31*†
T (/100 tubules)	0.5 $\pm$ 0.11	0.43 $\pm$ 0.13	3.73 $\pm$ 1.4	16.8 $\pm$ 6.3*†
α -SMA + PCNA-positive cells				
G (/cross section)	0.03 $\pm$ 0.04	0.01 $\pm$ 0.02	0.27 $\pm$ 0.21	0.94 $\pm$ 0.5*†
T (/100 tubules)	0.32 $\pm$ 0.18	0.37 $\pm$ 0.21	3.7 $\pm$ 0.89	12.3 $\pm$ 5.0*†

Data are expressed as mean $\pm$ SD. G, Glomeruli; T, tubulointerstitium.

* $P < 0.001$ compared to sham and sham + NPC31169; † $P < 0.001$ compared to RK. No significance difference was seen between sham and sham + NPC31169 groups for any parameter.

tubular cells, more myofibroblasts, and more macrophages. In contrast, the number of apoptotic cells was decreased in the RK rats receiving the p38 inhibitor. The mechanism for the worsening for the renal disease was suggested when the activities of other MAPK were assessed. NPC31169-treated rats had a marked up-regulation of ERK1/2 but not JNK compared to RK alone. Because ERK1/2 is known to be strongly involved in proliferation-associated pathways,^{1,18,19} these studies are consistent with the hypothesis that blockade of p38 in this specific model of renal disease resulted in a com-

pensatory activation of ERK1/2 that caused exacerbation of the renal lesion.

Most studies of p38 MAP kinase inhibition have found salutary effects on models of acute inflammatory diseases such as acute colitis and lung fibrosis.^{8,9} Furthermore, p38 inhibition has also been found to be protective in the early neutrophil-mediated injury in the anti-glomerular basement membrane model¹³ and in a model of lupus nephritis in mice.¹⁴ However, there is evidence that inhibition of p38 may lead to activation of the ERK1/2 pathway under certain conditions. For example, the p38 MAPK inhibitor, SB203580, can induce differentiation of various types of cultured cells by stimulating ERK activation.^{20–23} It is possible that p38 inhibition might result in accumulation of upstream activators of p38, which might lead to crosstalk with the ERK pathway. However, it is important to note that we did not observe ERK activation in our sham rats receiving NPC31169. This suggests that there may be something unique about the RK model. Because the RK model is also associated with some

Figure 5. Infiltration of ED-1-positive and α -SMA-positive cells. Infiltration of ED-1-positive monocytes/macrophages (A, B) and α -SMA-positive cells (C, D) in glomeruli of RK rats (A, C) and RK+NPC31169 rats (B, D). Some of the ED-1-positive cells within the glomerulus are PCNA-positive (E, arrows). α -SMA- and PCNA double-positive cells were also observed in the interstitium (F, arrows). Original magnifications: $\times 400$ (A, B); $\times 200$ (C, D); $\times 600$ (E, F).

Figure 6. Changes in PCNA and apoptotic cells. PCNA-positive cells (A, B) and apoptotic cells (C, D) in RK rats (A, C) and RK+NPC31169 rats (B, D). Tubular proliferation is more pronounced in RK+NPC31169 (B) than RK rats (A). By contrast, more apoptotic cells are seen in RK rats (C, asterisks) compared to RK+NPC31169 rats (D, arrowheads). Original magnifications, $\times 200$.

Figure 7. Activation of ATF-2, ERK1/2, and JNK. **A:** p38 kinase assay (**top**) with densitometric analysis (**bottom**). RK rats showed enhanced p38 activation, as evidenced by an increase of p-ATF-2. Treatment with NPC31169 reduced p38 kinase activity. **B:** Western blot for ERK1/2 (**top**) and densitometric analysis for ratio between phosphorylated and total ERK1/2 expression (**bottom**). RK rats demonstrated ERK phosphorylation in the renal cortex, and this was significantly increased in the RK+NPC31169 group. In sham-operated rats, no activation of ERK in either control or NPC31169-treated rats. **C:** JNK/SAPK kinase assay (**top**) and densitometric analysis (**bottom**). Although RK rats with or without NPC31169 showed an apparently mild increase of JNK activity over sham groups, by densitometry no significant difference was noted among groups. N, NPC31169. Values are expressed as mean $\pm$ SD ($n = 4$). *, $P < 0.001$ compared to sham or sham+NPC31169 group; #, $P < 0.001$ compared to RK group.

ERK1/2 activation (Figure 7B), one might speculate that some ERK activation must be ongoing for chronic p38 blockade to potentiate this pathway.

The importance of ERK1/2 in renal disease is becoming evident with recent studies. ERK1/2 is known to have an important role in cell survival and proliferation whereas p38 and JNK are linked to induction of apoptosis.²¹ In a recent article ERK was found to be expressed in a model of progressive anti-Thy1 nephritis, and inhibition of ERK resulted in amelioration of the disease by suppressing the proliferation of mesangial cells.²⁴ Others have shown that activation of ERK precedes tubulointerstitial injury in unilateral ureteral obstruction nephropathy model, indicating that the ERK pathway may be involved in the proliferation of renal tubular epithelial cells that occurs in this model.²⁵ Taken together, it is conceivable that activation of ERK triggered a marked proliferation of a variety of cells including infiltrating and resident renal cells, resulting in marked exacerbation of the disease. Contrary to ERK, the p38 kinase is involved in induction of apoptosis. In bleomycin-induced lung fibrosis p38 inhibition reduced apoptosis in resident pulmonary cells, resulting in amelioration of pulmonary fibrosis.¹¹ In this respect, our findings seem consistent with the above basic concept for the MAPK family in that enhanced ERK activation and suppressed p38 were accompanied by more proliferative cells and less apoptotic cells in RK+NPC31169-treated rats.

Blockade of p38 may be most beneficial in severe and acute inflammatory diseases in which there are high levels of proinflammatory cytokines (such as interleukin-1 β and tumor necrosis factor- α). In contrast, this model is associated with low-grade platelet-derived growth factor-associated mesangial cell proliferation²⁶ and transforming growth factor- β -associated fibrosis,²⁷ and tumor necrosis factor- α expression was minimally expressed by enzyme-linked immunosorbent assay in both the RK and RK+NPC31169 groups (data not shown), suggesting more of an ERK-dominant as opposed to a p38-dominant disease. Because low-grade chronic inflammation is known to suppress normal cell growth, one might speculate that blocking p38-mediated inflammation may stimulate a proliferative response, which in this model is deleterious. We therefore speculate that treatment of this type of disease may require either a combination of an ERK and p38 inhibitor or an ERK inhibitor alone.

Another explanation for our finding might be that p38 inhibition hindered cells that have protective effects on disease progression. Interleukin-10 is a cytokine produced by various cells including T cells and macrophages, and is known to be protective in models of inflammation.²⁸ Administration of interleukin-10 diminishes mesangial proliferation and macrophage influx in experimental glomerulonephritis.^{29,30} However, p38 inhibition by SB203580 can suppress interleukin-10 production by T cells, in turn blocking its protective effects.³¹ Although a role of T cells in the RK model has not been proven, it is possible that p38 inhibition suppressed the function of infiltrating T cells that are protective in this model of renal progression.

In conclusion we have found that chronic p38 inhibition in the RK model was associated with worsening and not improvement of renal disease. We present evidence that this is because of a potentiation of the ERK MAPK resulting in a more severe proliferative response. These studies suggest that future therapies in models of slowly progressive renal disease may need to incorporate combinations of therapies that also block the ERK pathway. This study also emphasizes the potential for crosstalk of the various intracellular signaling pathways that are activated in diseased states.

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