MKK3-p38 signaling promotes apoptosis and the early inflammatory response in the obstructed mouse kidney

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THE p38 MITOGEN-ACTIVATED protein kinase (MAPK) is one of the three major MAPK signaling pathways which is triggered by a wide range of extracellular ligands and stresses such as proinflammatory cytokines (IL-1, TNF-α), growth factors (TGF-β1, PDGF), reactive oxygen species, stretch, osmotic stress, and UV irradiation (12). There are four isoforms of the p38 kinase, of which p38α, β, γ, and δ are expressed in the kidney. The p38 kinase is activated by phosphorylation of a conserved Thr-Gly-Tyr motif in the activation loop. The activated p38 is then able to phosphorylate a wide variety of targets in the cytoplasm and nucleus, resulting in cellular responses such as apoptosis, inflammation, or fibrosis (12). The activation loop of the p38 kinase is phosphorylated through the action of the upstream kinases MAPKKK3 (MKK3) and MAPKKK6 (MKK6), although other mechanisms of p38 activation have been described in response to specific stimuli (9). Components of the p38 signaling pathway are present in many cell types. However, activation of this pathway can lead to different outcomes depending on the nature of the activating signal and the cell type involved. For example, p38 signaling can either enhance or suppress apoptosis of cultured tubular epithelial cells depending on the nature of the stimulus (5, 7).

Studies in human biopsies have identified a correlation between increased p38 phosphorylation and the degree of renal dysfunction and pathological changes in human glomerulonephritis (24). Studies on the functional role of p38 kinase signaling in experimental kidney disease have been restricted to the use of p38 inhibitor drugs given that genetic deletion of the p38α isoform is fetally lethal (26). Administration of the p38α/β inhibitor FR167653 has been shown to suppress proteinuria and histological damage in anti-glomerular basement membrane (GBM) glomerulonephritis and puromycin nephrosis in the rat (11, 28). Blockade of p38α with NPC31145 was shown to suppress acute rat anti-GBM disease, while treatment with another p38α inhibitor, NPC31169, suppressed renal fibrosis in the obstructed rat kidney (22, 23). However, not all studies have shown beneficial effects of p38 blockade in experimental models of renal injury (3, 20).

In most tissues, including the kidney, very little is known of the contribution of MKK3 or MKK6 to activation of the p38 MAPK pathway, and thus to induction of renal injury. In vitro, cultured mouse mesangial cells require MKK3 in TGF-β1 activation of p38α and p38δ and collagen production (30). However, the contribution of MKK3-p38 signaling in the development of kidney disease is unknown. To address this important question, we used mice deficient for Mkk3 to investigate the contribution of MKK3-p38 signaling in the development of renal inflammation, apoptosis, and fibrosis in a mouse model of unilateral ureteric obstruction (UUO).

MATERIALS AND METHODS

Reagents. Antibodies used in this study were rabbit anti-MKK3, rabbit anti-phospho-MKK3/6 (this antibody recognizes a phosphorylated activation loop conserved between MKK3 and MKK6), rabbit anti-phospho-p38 (Thr180/Tyr182), and rabbit anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA); mouse anti-p38α and rabbit anti-MKK6 (Upstate Biotechnology, Lake Placid, NY); goat anti-collagen IV (Southern Biotechnology Associates, Birmingham, AL); mouse anti-α-smooth muscle actin (α-SMA; Sigma-Aldrich, St. Louis, MO); goat anti-MKK3 (Cell Signaling Technology, Beverly, MA); and mouse anti-p65 (Cell Signaling Technology, Beverly, MA). The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Castle Hill, NSW, Australia), rabbit anti-tubulin (Abcam, Cambridge, UK), rabbit anti-TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA), and F4/80 (Serotec, Oxford, UK). Horseradish peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated mouse anti-peroxidase complexes (PAP) were purchased from Dako (Glostrup, Denmark). Biotinylated goat anti-rabbit IgG was from Zymed-Invitrogen (Carlsbad, CA). The avidin-biotin complex (ABC) kit was from Vector Labs (Burlingame, CA). Goat anti-rabbit Alexa Fluor 680 and donkey anti-mouse IRDye 800 were from Molecular Probes (Eugene, OR).

Mouse model of obstructed kidney. Wild-type C57BL/6J mice were obtained from Monash Animal Services (Clayton, Australia). Mkk3−/− mice, which have been described previously (31), were backcrossed for eight generations onto the C57BL/6J background and then bred in-house at Monash Medical Centre, Clayton. Mice were maintained on a normal diet under conventional animal house conditions.

Female mice (18–22 g) were anesthetized, a midline incision was made, and the left ureter was ligated. Groups of mice were killed on day 3 (n = 6) or day 7 (n = 8) after UUO. Bromodeoxyuridine (BrdU; 50 mg/kg) was given by intraperitoneal injection 3 h before mice were killed. All animal experimentation was approved by the Monash Medical Centre Animal Ethics Committee.

Western blotting. A quarter kidney was snap-frozen and stored at −80°C until use. Frozen sections were homogenized in 0.5 ml of lysis buffer (pH 7.2) containing 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 135 mM NaCl, 2.7 mM KCl, 1 mM EDTA, 5 mM NaF, 6 μM urea, 0.5% Triton X-100, 1 mM Na3VO4, 20 mM sodium pyrophosphate, 25 μg/ml leupeptin, 3 μg/ml aprotinin, 100 μM phenylmethylsulfonyl fluoride, and 1% phosphatase inhibitor cocktail (Sigma-Aldrich). Samples then were sonication for 5 s, left at room temperature for 1 h, centrifuged, and the supernatant was collected and stored frozen until use. Samples were separated using SDS-PAGE and then transferred to nitrocellulose membranes. Blots were blocked using Odyssey Blocking Buffer (LI-COR, Lincoln, NE) and incubated with primary antibodies in Odyssey Blocking Buffer with 0.05% Tween 20 overnight at 4°C. After washing, the blots were then incubated with goat anti-rabbit Alexa Fluor 680 or donkey anti-mouse IRDye 800 for 1 h and detected using the Odyssey Infrared Image Detecting System (LI-COR). Densitometric analysis was performed by the Gel Pro analyzer program (Media Cybernetics).

Histochemistry and immunostaining. Kidney tissues were fixed in 4% buffered formalin for 4 h, embedded in paraffin, and 2-μm sections were cut for periodic acid-Schiff (PAS) staining or 4-μm sections cut for immunostaining (cleaved caspase-3, α-SMA, TGF-β1, and F4/80). Alternatively, tissue was fixed in 2% paraformaldehyde-hyde-peroxide-periodate (PLP) for 3.5 h, snap-frozen and 4-μm cryostat sections were prepared for collagen IV immunostaining. Immunohistochemical staining was performed as described previously (23). Briefly, paraffin-embedded sections were dewaxed (or frozen sections were hydrated) and microwave oven heated in 0.1 M sodium citrate buffer for 12 min (for cleaved caspase 3, TGF-β1 and F4/80). After the serum block, sections were incubated with primary antibodies in PBS with 3% BSA overnight at 4°C. Sections were washed, and the primary antibodies were detected using either the ABC (cleaved caspase-3, F4/80) or the PAP peroxidase method (m-SMA, TGF-β1 and collagen IV) and developed with 3,3-diaminobenzidine to produce a brown color.

Quantification of histochemistry and immunostaining. All analyses were performed on blinded slides. Interstitial volume was assessed on PAS-stained sections at ×250 magnification by counting the number of intersecting points that fall between tubules on a defined 100-point grid. Glomeruli and large vessels were excluded. A total of 20 cortical fields were counted per animal, and the results are expressed as a percentage of the total number of grid points counted. Tubular damage was also scored on PAS-stained sections. For each case, all of the tubular cross sections in 20 fields of the renal cortex (×250) were assessed for tubular dilatation and/or atrophy, and the number of damaged tubules is expressed as a percentage of the total number.

Tubular and interstitial cells stained for cleaved caspase-3 were counted in high-power fields (×400) covering the entire cortex. The interstitial area of F4/80, α-SMA, and collagen IV immunostaining was quantified in ×250-power fields covering >90% of the cortex by image analysis using Image-Pro Plus software (Media Cybernetics), and the results are expressed as the percentage of the cortical area stained (large blood vessels were excluded from the analysis).

Real-time RT-PCR. Total RNA was extracted from quarter kidney samples using RiboPure Reagents (Ambion) according to the manufacturer’s protocol. Reverse transcription was performed using a Superscript First-Strand Synthesis Kit with random primers (Invitrogen). Real-time PCR was performed on the Rotor-Gene 3000 System (Corbett Research, Sydney, NSW, Australia) with thermal cycling conditions of 37°C for 10 min, 95°C for 5 min, followed by 50 cycles of 95°C for 15 s, 60°C for 20 s, and 68°C for 20 s. The primer pairs and probes used were TGF-β1 (forward: GGACACACAGTACAAAA; reverse: GACCCAGTAGAGAAGA; probe: ACAACGCAACCACC); TNF-α (forward: GGCTGCCCACTACGCT; reverse: TTTCTCTGGATGAGAAGAATAC; probe: TACCCACACCGTCAG); collagen IV (forward: GGCCTTACAGCATGCACT; reverse: GGATAGCGATCCAGTGA; probe: CAGTGCCCTAACGGT); and MCP-1 (forward: GACCCGTAATCTGAAAGCTAA; reverse: CACACTGTTACTCCTACAGA; probe: ACAACCACCTCAAGC). The relative amount of mRNA

![Image](http://ajprenal.physiology.org/ by 10.221.33.6 on October 21, 2017 http://ajprenal.physiology.org/)
was calculated using the comparative Ct (ΔCt) method. All specific amplicons were normalized against GAPDH mRNA or 18S ribosome RNA (Applied Biosystems, Scoresby, Victoria, Australia), whose expression was determined in the same reaction as an internal control.

In vitro studies of tubular cell apoptosis. Proximal tubular epithelial cells were prepared from diced wild-type (WT) or Mkk3−/− kidney tissue by digestion with 5 mg/ml collagenase class II in Hank's buffered salt solution for 30 min at 37°C. Tissue fragments then were isolated by sieving, checked for purity by microscopy, and cultured in K1 medium (DMEM/F12 supplemented with 5% FCS, ITS, 25 mM HEPES, 100 μg/ml penicillin, 100 μg/ml streptomycin, 1 mg/ml proctaglandin, 5 × 10−11 M triiodothyronine, 5 × 10−8 M hydrocortisone, and 25 μg/ml mouse epidermal growth factor) at 37°C with 5% CO2. Culture medium was changed every 3 days.

In the apoptosis studies, cells were seeded into six-well plates and then starved in serum-free media for 24 h. Cells were treated with varying concentrations of H2O2 for 18 h in serum-free media, and then cell apoptosis was measured in cell lysates using a Cell Death Detection ELISA Kit according to the manufacturer's instructions (Roche, Mannheim, Germany). Apoptosis was normalized on the basis of DNA content of the cell lysates using a Quant-iT DNA Assay Kit (Molecular Probes) and expressed as the ratio of optical density to DNA content. In each experiment, two or three samples per condition were analyzed. Results are based on analyses of all five experiments performed.

Statistical analysis. Experimental data are presented as means ± SD. Immunohistochemical results were analyzed by one-way ANOVA with post-hoc analysis using Bonferroni’s multiple comparison test. PCR results among multiple groups were analyzed by the Kruskal-Wallis test followed by Dunn’s multiple comparison test. All analysis was performed with GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA).

RESULTS

p38 phosphorylation in normal and obstructed kidney. Western blotting identified phosphorylated p38 (p-p38) in lysates of WT kidney, and this was significantly increased in the WT UUO kidney (Fig. 1, A and B). In WT mice, MKK3 is readily detected in the kidney and its expression is unaltered in the UUO kidney. In contrast, expression of M KK6 is markedly increased in WT UUO compared with normal kidney (Fig. 1A). Low levels of p-MKK3/6 were detected in the normal kidney, and this was dramatically elevated in the UUO kidney (Fig. 1, A and B). Unfortunately, we were unable to localize M KK3 or M KK6 to specific cell types within the kidney since the antibodies were not suitable for immunohistochemistry.

p38 phosphorylation in normal and obstructed Mkk3−/− kidney. Mkk3−/− mice show no distinct phenotype, and the kidney exhibits normal morphology (34). Western blotting showed a reduced level of p-p38 in the Mkk3−/− kidney compared with WT mice despite an apparent compensatory upregulation of M KK6 expression (Fig. 1, A and B). Similarly, p38 phosphorylation was significantly reduced in the Mkk3−/− UUO vs. WT UUO kidney. However, a marked increase in p38 phosphorylation was still evident in the Mkk3−/− kidney in response to UUO (Fig. 1).

Fig. 2. Macrophage infiltration and MCP-1 mRNA expression in the obstructed kidney. A–D: immunostaining of F4/80+ macrophages on days 3 and 7 of UUO in WT and Mkk3−/− mice. Note the partial reduction in F4/80+ macrophages on day 3 in Mkk3−/− mice. E: area of immunostaining for interstitial F4/80+ macrophages on days 3 and 7 of UUO. Real-time RT-PCR analysis of whole kidney RNA samples shows the ratio of MCP-1 (F) and TNF-α (G) relative to control. Values are means ± SD; n = 6–8/group by ANOVA with Bonferroni’s multiple comparison test (E) or Kruskal-Wallis test followed with Dunn’s multiple comparison test (F and G).
M KK3-p38 signaling promotes early renal inflammation in the obstructed kidney. Inflammatory changes can be seen on days 3 and 7 in the obstructed kidney in terms of upregulation of MCP-1 and TNF-α mRNA and infiltration of F4/80+ macrophages (Fig. 2). A significant reduction in interstitial macrophage accumulation was evident on day 3 of UUO in Mkk3−/− mice, and this was associated with suppression of MCP-1 mRNA levels (Fig. 2). This suppression of renal inflammation was no longer evident at day 7 of UUO. In contrast to the reduction in MCP-1 mRNA at day 3, no change in TNF-α mRNA levels were evident on days 3 or 7 in Mkk3−/− UUO mice (Fig. 2).

M KK3-p38 signaling promotes renal cell apoptosis in the obstructed kidney. Apoptosis of tubular epithelial cells and, to a lesser extent, interstitial cells, is a feature of the UUO model (10). Apoptotic tubular and interstitial cells were identified in the renal cortex on day 7 of UUO by immunostaining for cleaved caspase-3 (Fig. 3). Despite marked tubular dilatation in the Mkk3−/− UUO kidney, there was an ~50% reduction in the number of apoptotic tubular and interstitial cells (Fig. 3). However, this reduction in apoptosis did not affect the percentage of tubules exhibiting dilatation and/or atrophy in the obstructed kidney (54.2 ± 5.5 vs. 48.1 ± 8.2% in WT and Mkk3−/− UUO mice, respectively, P = not significant). In addition, the marked proliferative response evident in tubular and interstitial cells in the WT UUO kidney was not altered in the Mkk3−/− UUO kidney (data not shown).

In vitro studies were performed to investigate whether the reduction in tubular cell apoptosis seen in the obstructed Mkk3−/− kidney may be due to resistance of Mkk3−/− cells to proapoptotic stimuli. Tubular injury in the UUO model is associated with oxidative stress, which is known to induce

Fig. 3. Apoptosis on day 7 in the obstructed kidney. Apoptotic cells were identified by immunostaining for cleaved caspase-3 (arrows). Low- and high-power views are shown for cleaved caspase-3 staining in WT normal (A and B), WT UUO (C and D), and Mkk3−/− UUO (E and F) kidney. G and H: graphs quantifying the number of cleaved caspase-3-stained tubular cells and interstitial cells, respectively. Values are means ± SD; n = 8/group by ANOVA with Bonferroni’s multiple comparison test.
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Apoptosis in cultured Mkk3−/− tubular epithelial cells. Primary cultures of WT and Mkk3−/− tubular epithelial cells were treated with varying concentrations of H2O2 for 18 h, and cell apoptosis was then measured using a Cell Death Detection ELISA Kit and normalized on the basis of DNA content (expressed as arbitrary units [a.u.] for the ratio of optical density to DNA content). WT cells, solid line; Mkk3−/− cells, dashed line. Values are means ± SD combined from 5 individual experiments. *P < 0.05 for WT vs. Mkk3−/− by unpaired t-test.

DISCUSSION

This study has identified a discrete role for MKK3-p38 signaling in renal cell apoptosis and in the early inflammatory response in the obstructed kidney, whereas MKK3-p38 signaling was found to be redundant in the fibrotic response.

There was a marked increase in MKK6 levels in the absence of MKK3. This compensatory upregulation of MKK6 expression in the Mkk3−/− kidney is consistent with a previous in vitro study showing that inhibition of p38α results in upregulation of MKK6 expression (2). Similar to WT mice, Mkk3−/− mice showed a marked increase in p-p38 MAPK in the obstructed kidney. However, the actual levels of p38 phosphorylation in normal and obstructed kidney were significantly reduced in Mkk3−/− mice, and this could not be entirely compensated for by the increase in MKK6 expression. The question arises of whether this reduction in p38 MAPK signaling in the Mkk3−/− kidney has functional significance? Based on the lack of effect of Mkk3 gene deletion on the upregulation of TNF-α mRNA and the development of renal fibrosis in the obstructed kidney, it would appear that, for these responses at least, this reduction in p38 MAPK signaling has no functional significance.

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A number of in vitro studies have shown that p38 signaling plays an important role in the induction of tubular epithelial cell apoptosis (4, 7). Our studies show that apoptosis of tubular epithelial cells and interstitial cells in the obstructed kidney is dependent on MKK3-p38 signaling, and this could not be compensated for by MKK6-p38 signaling or other potential MKK3/6-independent mechanisms of p38 activation, such as MKK4-dependent p38 phosphorylation or TAB1-associated MKK3/6-independent mechanisms of p38 activation, dependent on MKK3-p38 signaling, and this could not be compensated for by MKK6-p38 signaling or other potential MKK3/6-independent mechanisms of p38 activation.

Many in vitro studies have shown that p38 signaling plays an important role in the induction of tubular epithelial cell apoptosis (4, 7). Our studies show that apoptosis of tubular epithelial cells and interstitial cells in the obstructed kidney is dependent on MKK3-p38 signaling, and this could not be compensated for by MKK6-p38 signaling or other potential MKK3/6-independent mechanisms of p38 activation, such as MKK4-dependent p38 phosphorylation or TAB1-associated MKK3/6-independent mechanisms of p38 activation, dependent on MKK3-p38 signaling, and this could not be compensated for by MKK6-p38 signaling or other potential MKK3/6-independent mechanisms of p38 activation.

Figs. 6 A–D: immunostaining for TGF-β1 in tissue sections. Weak expression of TGF-β1 protein is seen in tubules in normal kidney in WT (A) and Mkk3−/− mice (B). C: induction of UUO causes a substantial increase in tubular TGF-β1 immunostaining, particularly in damaged tubules, although glomeruli remain largely negative. D: a similar pattern of increased tubular TGF-β1 immunostaining is seen in UUO Mkk3−/− mice. Real-time RT-PCR was used to analyze TGF-β1 (E) and collagen IV (F) mRNA expression on days 3 and 7 of UUO in WT compared with Mkk3−/− kidney. Open bars, WT normal; filled bars, WT UUO; hatched bars, Mkk3−/− UUO. Values are means ± SD; n = 8/group. Expression in WT UUO and Mkk3−/− UUO groups at days 3 and 7 is significantly increased compared with WT normal (P < 0.01) by the Kruskal-Wallis test followed with Dunn’s multiple comparison test.

Fig. 6. Transforming growth factor-β1 (TGF-β1) and collagen IV expression in the obstructed kidney. A–D: immunostaining for TGF-β1 in tissue sections. Weak expression of TGF-β1 protein is seen in tubules in normal kidney in WT (A) and Mkk3−/− mice (B). C: induction of UUO causes a substantial increase in tubular TGF-β1 immunostaining, particularly in damaged tubules, although glomeruli remain largely negative. D: a similar pattern of increased tubular TGF-β1 immunostaining is seen in UUO Mkk3−/− mice. Real-time RT-PCR was used to analyze TGF-β1 (E) and collagen IV (F) mRNA expression on days 3 and 7 of UUO in WT compared with Mkk3−/− kidney. Open bars, WT normal; filled bars, WT UUO; hatched bars, Mkk3−/− UUO. Values are means ± SD; n = 8/group. Expression in WT UUO and Mkk3−/− UUO groups at days 3 and 7 is significantly increased compared with WT normal (P < 0.01) by the Kruskal-Wallis test followed with Dunn’s multiple comparison test.
significant, reduction in the area of α-SMA staining was evident in Mkk3−/− UUO mice. The apparent discrepancy between unaltered renal fibrosis and reduced α-SMA myofibroblast accumulation in the Mkk3−/− UUO group may be explained by studies showing that the TGF-β1-induced transduction of α-SMA− fibroblasts to α-SMA+ myofibroblasts operates, in part, through a p38 MAPK-dependent mechanism (14, 15).

The current study has shown that specific targeting of Mkk3-p38 signaling can suppress tubular and interstitial cell apoptosis and reduce the early inflammatory response in the obstructed kidney. The inhibition of these responses, despite a significant increase in MKK6-p38 signaling in the obstructed kidney, suggests that this inhibition specifically relates to obstructed kidney. The inhibition of these responses, despite a


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