ASK1/p38 signaling in renal tubular epithelial cells promotes renal fibrosis in the mouse obstructed kidney

Frank Y. Ma, Greg H. Tesch, and David J. Nikolic-Paterson

Department of Nephrology and Monash University Department of Medicine, Monash Medical Centre, Clayton, Victoria, Australia

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Ma FY, Tesch GH, Nikolic-Paterson DJ. ASK1/p38 signaling in renal tubular epithelial cells promotes renal fibrosis in the mouse obstructed kidney. *Am J Physiol Renal Physiol* 307: F1263–F1273, 2014. First published October 8, 2014; doi:10.1152/ajprenal.00211.2014.—Stress-activated kinases p38 MAPK and JNK promote renal fibrosis; however, how the pathways by which these kinases are activated in kidney disease remain poorly defined. Apoptosis signal-regulating kinase 1 (ASK1/MAP3K5) is a member of the MAP3K family that can induce activation of p38 and JNK. The present study examined whether ASK1 induces p38/JNK activation and renal fibrosis in unilateral ureteric obstruction (UUO) using wild-type (WT) and Ask1−/− mice. Basal p38 and JNK activation in WT kidneys was increased three- to fivefold in day 7 UUO mice in association with renal fibrosis. In contrast, there was no increase in p38 activation in Ask1−/− UUO mice, whereas JNK activation was only partially increased. The progressive increase in kidney collagen (hydroxyproline) content seen on days 7 and 12 of UUO in WT mice was significantly reduced in Ask1−/− UUO mice in association with reduced α-smooth muscle actin-positive myofibroblast accumulation. However, cultured WT and Ask1−/− renal fibroblasts showed equivalent proliferation and matrix production, indicating that ASK1 acts indirectly on fibroblasts. Tubular epithelial cells are the main site of p38 activation in the obstructed kidney. Angiotensin II and H2O2, but not IL-1 or lipopolysaccharide, induced p38 activation and upregulation of transforming growth factor-β1, platelet-derived growth factor-B, and monocyte chemoattractant protein-1 production was suppressed in Ask1−/− tubular epithelial cells. In addition, macrophage accumulation was significantly inhibited in Ask1−/− UUO mice. In conclusion, ASK1 is an important upstream activator of p38 and JNK signaling in the obstructed kidney, and ASK1 is a potential therapeutic target in renal fibrosis.

apoptosis; apoptosis signal-regulating kinase 1; fibroblast; p38 mitogen-activated protein kinase; c-Jun NH2-terminal kinase

STRESS-ACTIVATED PROTEIN KINASES p38 MAPK and JNK are activated in response to a wide variety of stimuli, including ROS, hypoxia, proinflammatory cytokines (IL-1 and TNF-α), Toll-like receptor activation [lipopolysaccharide (LPS)], transforming growth factor (TGF)-β, aldosterone and ANG II (2, 25). Drug-based blocking studies (7, 10, 18, 24, 34, 37, 38, 48) have demonstrated that both p38 and JNK signaling play a functional role in renal inflammation, apoptosis, and fibrosis, and studies (6, 18, 39) of human kidney disease have identified that increased p38 and JNK activation is associated with renal injury, making these pathways attractive therapeutic targets. However, clinical trials of p38 inhibitors in rheumatoid arthritis have failed (2, 12), and a recent phase 2 trial to characterize the safety, pharmacokinetics, and biological activity of a JNK inhibitor in idiopathic pulmonary fibrosis (NCT01203943) was terminated as the benefit/risk profile did not support continuation. This raises questions as to whether p38 and JNK have both beneficial homeostatic functions as well as being important in disease pathogenesis, thus making therapeutic targeting of these kinases problematic. One approach to this issue is to identify the upstream kinases involved in the activation of p38 and JNK in the anticipation that activation of these kinases in disease pathogenesis could be targeted while sparing their homeostatic roles.

There are >20 members of the MAP3K (MAP3K) family, of which at least half have the potential to activated p38 via MAPK3 and MAPK6 (MKK3/6) and JNK via MAPK4 and MAPK7 (4). However, little is known of the upstream kinases involved in p38 and JNK activation in kidney disease. Apoptosis signal-regulating kinase 1 (ASK1/MAP3K5) is one member of the MAP3K family that is expressed in many tissues, of which kidney has the highest levels of expression (35, 45). ASK1 is held in an inactive state by binding to the redox-sensitive protein thioredoxin. Upon oxidation, thioredoxin dissociates from ASK1, enabling the kinase to undergo dimerization and activation via auto-phosphorylation of the activation loop (35). In contrast to the fetal lethality of p38α or JNK1/2 gene deletion (11), ASK1 gene-deficient (Ask1−/−) mice are born at the expected Mendelian frequency and have a normal phenotype (46).

Ask1−/− mice show protection from cardiac fibrosis induced by aldosterone/salt or ANG II and from bile duct ligation-induced peribiliary fibrosis (30–32). Activation of p38 and JNK signaling is prominent in tubular epithelial cells in different types of renal injury, including the unilateral ureteric obstruction (UUO) model of renal fibrosis (6, 24, 38, 39). ASK1 has been implicated in tubular p38 activation in ischemia-reperfusion injury (44), whereas a different MAP3K family member, TGF-β1-induced kinase 1 (TAK1), has been implicated in tubular cell p38 activation in response to proinflamatory stimuli (27). However, the role of ASK1 in renal fibrosis is unknown. The aim of the present study was to determine whether ASK1 plays a role in p38/JNK-dependent renal fibrosis. To this end, we examined wild-type (WT) and Ask1−/− mice in the UUO model of renal fibrosis.

MATERIALS AND METHODS

Antibodies. Rabbit antibodies used in this study were as follows: anti-JNK (10, 18, 24), anti-phosphorylated (p-JNK) (Thr183/Tyr185) (10, 18, 24, 27), anti-p-c-Jun (Ser63) (6, 10, 18, 24, 27), anti-p-MKK3/6 (Ser189/207) (26), anti-p-p38 (Thr180/Tyr182) (26, 27, 37–39), and anti-p-activating transcription factor 2 (ATF2; Thr198) (26) (Cell Signaling, San Diego, CA) as well as anti-ASK1 and anti-tubulin (Abcam, Cambridge, UK).
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UK). Other antibodies used were as follows: mouse anti-p38α (Upstate Biotechnology, Lake Placid, NY), mouse anti-α-smooth muscle actin (α-SMA; Sigma-Aldrich, Castle Hill, NSW, Australia) (24, 26, 27, 38), mouse anti-PCNA (DAKO) (21), goat anti-collagen type IV (Southern Biotechnology, Birmingham, AL) (26, 27), and F4/80 anti-macrophage (Soretex, Oxford, UK) (26, 27). Horseradish peroxidase-conjugated goat anti-mouse IgG and peroxidase-conjugated mouse anti-peroxidase complexes (PAP) were purchased from DAKO. Biotinylated rabbit anti-goat IgG was from Zymed-Invitrogen (Carlsbad, CA). The avidin-biotin complex 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).

Animals. A cloned Ask1 (Map3k5) “knockout first” mouse embryonic cell line (c57BL/6N) was obtained from the European Conditional Mouse Mutagenesis Program, which was used to establish an Ask1<−/−> mouse line by the Australian Phenomics Network (Monash University). WT control mice (c57BL/6j) were obtained from the Monash Animal Research Precinct. Mice were backcrossed onto the C57Bl/6j background. Consistent with a previous report (46), Ask1<−/−> mice had a normal phenotype. Groups of six male Ask1<−/−> and WT mice underwent UUO surgery to permanently obstruct the left ureter and were euthanized 7 or 12 days later, as previously described (26). Control groups consisted of six Ask1<−/−> and WT mice without experimentation. All animal experimentation was approved by the Monash Medical Centre Animal Ethics Committee and was performed in accordance with Australian National Health and Medical Research Council guidelines for animal experimentation.

Analysis of renal fibrosis and apoptosis. Kidney tissues were fixed in 4% buffered formalin and embedded in paraffin, and 4-μm sections were cut for immunostaining. Immunoperoxidase staining for α-SMA, collagen type IV, F4/80, and PCNA was performed using antigen retrieval [microwave oven heating in 0.1 M sodium citrate (pH 6.0) for 12 min] followed by a three-layer avidin-biotin complex peroxidase or PAP peroxidase method and developed with 3,3-diaminobenzidine to produce a brown colour, as previously described (21).

ApopTag Peroxidase In Situ Apoptosis Detection TUNEL Kit (Chemicon). Tubular and interstitial cells stained for TUNEL or PCNA were counted in high-power fields covering the entire cortex. The interstitial area of F4/80, α-SMA, and collagen type IV immunostaining was quantified in ×250 power fields covering >90% of the cortex by image analysis using Image-Pro Plus software (Media Cybernetics, Rockville, MD), and the results were expressed as the percentage of the cortical area stained (large blood vessels were excluded from the analysis for α-SMA and collagen type IV staining). All analyses were performed on blinded slides. The collagen content of normal and obstructed kidneys was measured using the Hydroxyproline Colorimetric Assay Kit (BioVision, Milpitas, CA).

Western blot analysis. Cultured tubular cells or frozen kidney samples were homogenized in 0.5 ml lysis buffer containing 1 mM EDTA, 5 mM NaF, 6 M urea, 0.5% Triton X-100, 1 mM NaVO4, 20 mM sodium pyrophosphate, 25 μg/ml leupeptin, 3 μg/ml aprotonin, 100 μg/ml PMSF, and 1% phosphatase inhibitor cocktail (Sigma) in 12 mM phosphate buffer (pH 7.2). Samples then were sonicated and incubated at 4°C for 60 min. After centrifugation, 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 being washed, blots were incubated with goat anti-rabbit Alexa fluor 680 or donkey anti-mouse IRDye 800 (Molecular Probes) for 1 h and detected using the Odyssey Infrared Image Detecting System (LI-COR). Densitometry analysis was performed by the Gel Pro analyzer program (Media Cybernetics).

PCR. Total RNA was extracted from cells or quarter kidney samples using RiboPure Reagents (Ambion) according to the manufacturer’s protocol. Reverse transcription was performed using SuperScript First-Strand Synthesis Kit with random primers (Life Sciences, Mulgrave, VIC, Australia). Real-time PCR using Taqman probes was performed on a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) with thermal cycling conditions of 37°C for 10 min and 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 have been published previously (1, 24, 27) or purchased [platelet-derived growth factor (PDGF)-B and collagen type I from Applied Biosystems] except for CD206, as follows: forward 5′-GACAGATGAG-CAAGCATTTCC-3′, reverse 5′-TGAACACCTGAGCTCTGTC-3′, and probe 5′-GTTGTGTTGATTGAGG-3′. Real-time SYBR Green PCR for ASK1 used (forward: 5′-AGACGGAGACTGTT-GAGGG-3′ and reverse: 5′-GCTCTGACATAGACGATCCCAT3′) with thermal cycling conditions of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative amount of mRNA was calculated using comparative threshold cycle method. All specific amplicons were normalized against 18S rRNA or β-actin controls (Applied Biosystems).

Primary cell cultures. Primary cultures of kidney fibroblasts were prepared from WT and Ask1<−/−> day 5 UUO kidneys. Dissected kidneys were incubated in collagen-coated wells in 10% FCS, DMEM, penicillin, and streptomycin. After 2 wk in the presence of a neutralizing antibody against mouse colony-stimulating factor receptor (20 μg/ml AF598), the remaining kidney cells were found to be fibroblasts, as previously characterized (15). After starvation in 0.5% FCS for 24 h, cells were incubated with and without recombinant human PDGF-AB (10 ng/ml, Peprotech, Rocky Hill, NJ), 0.05 or 0.1 mM H2O2, or 50–200 μM CoCl2 (Sigma) for 24 h in DMEM containing 0.1% BSA and 1% insulin-transferrin-selenium supplement (Invitrogen, Grand Island, NY). During the last 18 h of culture, [3H]thymidine (0.5 μCi/well, Amersham) was added to the medium. After incubation, cells were washed and lysed, and the incorporated label was quantified using a β-counter. Cells were also treated with 10 ng/ml TGF-β1 for 72 h to assess profibrotic gene expression.

Primary cultures of renal tubal epithelial cell culture were prepared from Ask1<−/−> and WT control mice as previously described (27). After starvation in 1% FCS for 18 h, cells then were stimulated for 15 min with 1 μM ANG II (Peprotech), 0.1 mM H2O2, 10 ng/ml IL-1α (R&D Systems, Minneapolis, MN), or 1 μg/ml LPS (Sigma-Aldrich). After 72 h, TGF-β1, PDGF-B, and monocyte chemotactant protein (MCP)-1 mRNA levels in cultured cells were measured by PCR, whereas TGF-β1 secretion into the culture media was determined by ELISA (Promega, Promega, Australia, NSW, Australia). To examine apoptosis, starved cells were stimulated for 24 h with 0.5 mM H2O2. Apoptosis in cultured cells was measured using the Cell Death Detection ELISA Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The results were normalized to the DNA content in cell lysates using a Quant-iT DNA Assay Kit (Molecular Probes) and expressed as the ratio of optical density to DNA content. All results are based on analyses of three independent experiments.

Statistics. Experimental data are presented as means ± SD. All animal data are based on groups of six mice. Results were analyzed by one-way ANOVA with post hoc analysis using Bonferroni’s multiple-comparison test. PCR results among multiple groups were analyzed using the Kruskal-Wallis test followed by Dunn’s multiple-comparison test. All analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA).

RESULTS

Role of ASK1 in p38 and JNK activation in normal and obstructed kidneys. The normal mouse kidney exhibited high levels of mRNA and protein for ASK1, and this was unchanged on day 7 of UUO (Fig. 1). Unfortunately, we were unable to detect ASK1 by immunohistochemistry to define the cellular distribution of ASK1 in the mouse kidney using...
currently available commercial antibodies. In addition, commercial antibodies against the phosphorylated active site of human ASK1 failed to detect mouse p-ASK1 by Western blot analysis or immunohistochemistry.

The normal mouse kidney exhibits a basal level of p38 activation, as shown by Western blot analysis for phosphorylation of Thr180/Tyr182 in the p38 kinase activation loop (Fig. 2A). Immunohistochemistry localized p-p38 to collecting ducts, interstitial cells, and some glomerular cells in the normal mouse kidney (Fig. 2F). Consistent with a previous study (38), a marked increase in p38 activation was evident in the WT UUO kidney. Western blot analysis identified a 5.1 ± 0.9-fold increase in p-p38 levels in WT UUO kidneys compared with control kidneys, and this was associated with increased phosphorylation of upstream MKK3/6 and the downstream p38 target ATF2 (Fig. 2, A, D, and E).

We established mice deficient in the Ask1 gene (Ask1−/− mice), which develop normally and have normal kidney structure and function (data not shown). We confirmed that Ask1−/− mice lack ASK1 mRNA and protein expression in the kidney (Fig. 1). In contrast to WT kidneys, basal p38 activation was reduced in Ask1−/− kidneys (Fig. 2). There was only a 1.6 ± 0.7-fold increase in p-p38 levels in Ask1−/− UUO kidneys compared with control kidneys, and this was associated with increased phosphorylation of upstream MKK3/6 and the downstream p38 target ATF2 (Fig. 2, A, D, and E).

Normal kidneys also exhibited basal activation of JNK, predominantly in collecting ducts (Fig. 3, A and C), although its downstream target c-Jun is not expressed in normal kidneys (24). Kidney JNK activation was increased 2.9 ± 0.8-fold in WT UUO mice, which was accompanied by JNK-dependent phosphorylation of c-Jun at Ser63 (Fig. 3). Ask1−/− mice showed reduced basal JNK activation in normal kidneys, and although they exhibited a similar fold increase in JNK activation on day 7 UUO (4.5 ± 1.8, P = 0.072 vs. the change in WT mice), this was still lower than the degree of JNK activation seen in WT UUO mice (Fig. 3).

Role of ASK1 in renal fibrosis in the obstructed kidney. The total collagen content of normal and obstructed kidneys was

Fig. 1. Expression of apoptosis signal-regulating kinase 1 (ASK1) in normal and unilateral ureteric obstruction (UUO) kidneys. A: Western blots showing ASK1 (≈155 kDa) expression in normal (control) and day 7 UUO kidneys of wild-type (WT) and Ask1 gene-deficient (Ask1−/−) mice. B: graph of Western blot analysis of ASK1 expression relative to tubulin control. C: graph of real-time RT-PCR analysis of ASK1 mRNA levels relative to β-actin control. Data are means ± SD from groups of six mice.

Fig. 2. Effect of ASK1 deletion on p38 signaling in normal and UUO kidneys. A: Western blots showing phosphorylation of p38 [phosphorylated (p-)p38; ≈ 38 kDa], MAPKK3 and MAPKK6 (p-MKK3/6; ≈ 40 kDa), and activating transcription factor 2 (p-ATF2; ≈ 70 kDa) in normal (control) and day 7 UUO kidneys from WT and Ask1−/− mice plus tubulin and p38α loading controls. B–E: quantification of Western blots for p-p38/p38α (B), p-p38/tubulin (C), p-MKK3/6/tubulin (D), and p-ATF2/tubulin (E). F–I: immunostaining of p-p38 in WT control (F), Ask1−/− control (G), WT UUO (H), and Ask1−/− UUO (I) groups. Original magnification: × 250. Scale bar = 40 μm. Data are means ± SD from groups of six mice.
determined using a hydroxyproline assay. WT mice showed a progressive increase in kidney hydroxyproline content on day 7 and 12 UUO, which was significantly reduced in Ask1−/− mice at both time points (Fig. 4). Further detailed analysis focused on the day 7 UUO timepoint. Marked renal fibrosis was also evident in WT day 7 UUO mice based on parameters of α-SMA-positive myofibroblast accumulation, increased deposition of collagen type IV, and upregulation of mRNA
levels for matrix molecules (collagen type I and IV) and profibrotic factors [α-SMA, TGF-β, PDGF-B, and plasminogen activator inhibitor (PAI)-1; Fig. 5]. All of these fibrotic parameters were significantly reduced by 25–40% in Ask1−/− UUO mice (Fig. 5).

Role of ASK1 in renal inflammation in the obstructed kidney. Inflammation is part of the response to UUO. A prominent interstitial infiltrate of F4/80-positive macrophages was evident in the WT UUO group, which was also quantified by increased CD68 mRNA levels (Fig. 6, A–E). The macrophage infiltrate was associated with upregulation of MCP-1. There was a significant increase in the expression of proinflammatory molecules characteristic of M1 type macrophages (nitric oxide synthase 2 and IL-12) as well as an increase in M2 type macrophage markers (arginase-1 and CD206) and an increase in the expression of more general proinflammatory molecules (TNF-α and IL-1α) in the WT UUO group (Fig. 6). The Ask1−/− UUO group showed a significant reduction in the macrophage infiltrate in association with reduced levels of MCP-1 mRNA (Fig. 6). White blood cell counts, including monocytes, were comparable in Ask1−/− and WT mice (data not shown). There was a partial reduction in the expression of both M1 and M2 type macrophage markers, which followed...
the pattern of an overall reduction in the macrophage infiltrate, but without an obvious change in the phenotype of macrophages in the Ask1−/− UUO group (Fig. 6). However, expression of TNF-α and IL-1β mRNA was not affected in the Ask1−/− UUO group (Fig. 6).

Role of ASK1 in cell apoptosis and proliferation in the obstructed kidney. A substantial increase in apoptosis of both tubular epithelial cells and interstitial cells was evident in the cortex in WT UUO kidneys (Fig. 7). Tubular damage in WT UUO mice was also evident on the basis of increased levels of kidney injury molecule (KIM)-1 mRNA and increased proliferation of both tubular and interstitial cells (Fig. 7, F–H). Ask1−/− UUO mice showed a significant reduction in the number of apoptotic cortical tubular and interstitial cells (Fig. 7, A–E). The number of PCNA-positive proliferating tubular and interstitial cells was also reduced in Ask1−/− UUO mice, although KIM-1 mRNA levels were unchanged compared with WT UUO mice (Fig. 7, F–H).

Role of ASK1 in cultured tubular epithelial cells and fibroblasts. Given that p38/JNK activation in the UUO kidney is most prominent in tubular cells, we investigated primary cultures of tubular epithelial cells isolated from WT and Ask1−/− kidneys. WT tubular cells showed clear ASK1 mRNA and protein expression (Fig. 8, A–C). Western blot analysis showed that H2O2, ANG II, IL-1β, and LPS all induced p38 activation in WT tubular cells (Fig. 8, D and G). Both H2O2- and ANG II-induced p38 activation were prevented in Ask1−/− tubular cells; however, neither IL-1β- nor LPS-induced p38 activation was affected (Fig. 8). Consistent with these findings, H2O2-induced apoptosis and ANG II- or H2O2-induced production of MCP-1, PDGF-B, and TGF-β1 were substantially reduced in Ask1−/− tubular cells (Fig. 9), whereas IL-1β- and

Fig. 6. Effect of ASK1 deletion on renal inflammation in UUO. A–C: immunostaining of F4/80-positive macrophages in WT normal (A), WT UUO (B), and Ask1−/− UUO (C) kidneys. Original magnification: ×400. Scale bar = 20 μm. D and E: kidney macrophages were quantified by image analysis of F4/80 immunostaining (D) and PCR analysis of whole kidney CD68 mRNA levels (E). F–L: PCR analysis of whole kidneys for mRNA levels of monocyte chemoattractant protein (MCP)-1 (F), nitric oxide synthase (NOS)2 (G), IL-12β (H), arginase-1 (I), CD206 (J), TNF-α (K), and IL-1α (L). Data are means ± SD from groups of six mice.
LPS-induced MCP-1, PDGF-B, and TGF-β production were unaltered in Ask1−/− tubular cells (Fig. 9 and data not shown).

We also examined whether ASK1 plays a direct role in the response of renal fibroblasts isolated from WT and Ask1−/− day 5 UUO kidneys. WT fibroblasts expressed ASK1 mRNA and protein, which were absent in Ask1−/− fibroblasts (Fig. 10, A–C). However, no differences were evident between WT and Ask1−/− fibroblasts in PDGF-induced cell proliferation or TGF-β1-induced upregulation of α-SMA, collagen type I, or PAI-1 mRNA levels (Fig. 10, D–G). Exposure of WT fibroblasts to hypoxic stress (200 μM CoCl2) induced p38 activation, which was reduced in Ask1−/− fibroblasts. However, the antiproliferative effects of oxidative or hypoxic stress on WT fibroblasts were unchanged in Ask1−/− fibroblasts (data not shown).

DISCUSSION

The present study demonstrated that ASK1 plays a functional role in the activation of p38 and JNK signaling and in the development of renal inflammation, apoptosis, and fibrosis in the mouse obstructed kidney.

Given the normal phenotype of Ask1−/− mice, we were surprised to find that ASK1 is an important inducer of basal p38 and JNK activation in the normal kidney. However, this is consistent with the relatively hypoxic environment of the kidney and the fact that ASK1 is activated in response to hypoxia (9, 20). The substantial reduction in basal p38 and JNK signaling in Ask1−/− mice suggests that this signaling is not essential for normal kidney function, although it may be involved in the response of tubules to hypertonic stress (50). Interestingly, the only other MAP3K enzyme to be studied in...
the kidney is TAK1 (MAP3K7), and acute deletion of TAK1 did not affect basal p38 or JNK activation in the contralateral kidney in the UUO model (27), indicating distinct functions of these MAP3K enzymes in the normal kidney.

In the obstructed kidney, p38 activation was entirely dependent on ASK1, whereas partial JNK activation occurred in the absence of ASK1. This provides an interesting comparison with a previous study (27) of TAK1 deletion in the UUO model, in which p38 activation was unaffected but JNK activation was substantially reduced, indicating dominant ASK1/p38 and TAK1/JNK signaling pathways in the UUO kidney. Indeed, genetic deletion of either ASK1 or TAK1 reduced myofibroblast accumulation and collagen deposition in the obstructed kidney, consistent with previous studies (24, 38).

Fig. 8. Effects of ASK1 deletion on p38 activation in cultured tubular epithelial cells. A–C: cultured tubular epithelial cells from WT mice showed clear ASK1 expression based on PCR analysis (A), Western blots (B), and a graph of Western blot analysis (C). D–F: Western blots showing phosphorylation of p38 in WT and Ask1−/− tubular cells in response to stimulation with 0.5 mM H2O2 or 1 µM ANG II (D), with data quantified in E and F. G–I: Western blot showing phosphorylation of p38 in WT and Ask1−/− tubular cells in response to stimulation with 10 ng/ml IL-1α or 1 µg/ml lipopolysaccharide (LPS) (G), with data quantified in H and I. All results are based on at least three independent experiments.

Fig. 9. Effects of ASK1 deletion on the response of cultured tubular epithelial cells. A: apoptosis was induced by stimulation with 0.5 mM H2O2 for 24 h in WT and Ask1−/− tubular cells. In other experiments, WT and Ask1−/− tubular cells were stimulated with 0.1 mM H2O2 (B), 1 µM ANG II (C–E), or 10 ng/ml IL-1α (G–I) for 48 h and then analyzed by PCR for PDGF-B (B, D, and H), MCP-1 (C and G), and TGF-β (E and I). WT and Ask1−/− tubular cells were stimulated with 1 µM ANG II (F) or 10 ng/ml IL-1α (J) for 72 h, and secreted TGF-β protein was then analyzed by ELISA. All results are based on at least three independent experiments.

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showing that blockade of either p38 or JNK is sufficient to suppress renal fibrosis in this model.

Blockade of ASK1-p38/JNK signaling may have reduced renal fibrosis in the obstructed kidney by exerting effects on various cell types, including fibroblasts, tubular epithelial cells, and macrophages. Myofibroblast accumulation was reduced in Ask1−/− UUO kidneys in association with reduced interstitial cell proliferation. We established that renal fibroblasts express ASK1 and that hypoxic stress-induced p38 activation in fibroblasts is partly dependent on ASK1. However, we did not identify a direct role for ASK1 in fibroblast proliferation based on studies of PDGF-induced proliferation of isolated renal fibroblasts from Ask1−/− and WT obstructed kidneys. In addition, we investigated whether ASK1 could be involved in the fibroblast profibrotic response to TGF-β1 stimulation; however, Ask1−/− and WT renal fibroblasts showed equivalent upregulation of α-SMA, collagen type I, and PAI-1 mRNA levels in response to TGF-β1 stimulation. Thus, ASK1 appears to affect fibroblasts in the obstructed kidney via indirect mechanisms.

ASK1 signaling may be an important mechanism by which the response of tubular cells to ureteric obstruction drives fibrosis. Tubules are the major site of p38 and JNK activation in the obstructed kidney, which was substantially diminished in Ask1−/− UUO mice. Consistent with this finding, ANG II and oxidative stress induced p38 activation in primary tubular cells, which was shown to be ASK1 dependent. Tubules are the main site of production of factors that induce myofibroblast recruitment and proliferation (PDGF), collagen production (TGF-β1), and inflammation (MCP-1) in the obstructed kidney (19, 22, 23, 26, 29, 36, 38, 43). The Ask1−/− UUO kidney showed reduced expression levels of TGF-β1, PDGF-B, and MCP-1, and studies of cultured tubular epithelial cells demonstrated that ANG II- and H2O2-induced TGF-β1, PDGF-B, and MCP-1 production were substantially inhibited in Ask1−/− tubular cells. This is consistent with the known roles of ANG II and oxidative stress in promoting renal fibrosis in the obstructed kidney (16, 40, 49, 51), that ANG II induces the production of intracellular ROS (33), and that ROS are pivotal in ASK1 activation (35). A previous study (31) has also shown protection of Ask1−/− mice from ANG II-induced cardiac fibrosis. In addition, an important role for ASK1-p38/JNK signaling in tubular cell activation can be inferred from a study (44) in which Ask1−/− mice were shown to be protected from renal ischemia-reperfusion injury with reduced MCP-1 production and macrophage infiltration. Tubules are the main site of the dramatic p38 and JNK activation that occurs upon reperfusion of the ischemic kidney, and blockade of p38 or JNK provides substantial protection against acute renal failure in ischemia-reperfusion injury with reduced macrophage infiltration (8, 18).

An interesting finding was that IL-1 and LPS induced p38 activation, and upregulation of MCP-1 and TGF-β1 in cultured tubular epithelial cells was independent of ASK1. This has several implications. First, it demonstrates that not all stimuli that induce p38 activation in tubular cells operate via ASK1. Indeed, we (27) have previously shown that IL-1, TNF-α, and LPS induced p38 activation and that a proinflammatory response operates via TAK1, providing another example of how ASK1 and TAK1 play complementary roles in regulating p38 signaling in tubular cells. Second, these findings indicate that ASK1 acts in a cell type-dependent fashion, since LPS-induced p38 signaling and the inflammatory response in spleen cells and dendritic cells are ASK1 dependent (28). Finally, these data indicate that neither IL-1 nor TLR4 ligands are important inducers of tubular p38 signaling in the UUO model. This is consistent with a study (3) in which Toll-like receptor 4-deficient and WT mice developed equivalent renal fibrosis in the UUO model, although mice lacking IL-1 receptor type I do show a mild reduction in renal fibrosis in this model (17).

ASK1 signaling may promote renal fibrosis via macrophage recruitment into the obstructed kidney. A number of studies have shown a role for macrophages in promoting fibrosis in the UUO model (14, 41), so the reduction in macrophage accumulation seen in the Ask1−/− UUO kidney may have contributed to the protection from renal fibrosis. The reduced macrophage accumulation in the Ask1−/− UUO kidney is presumably due to a reduction in tubular MCP-1 production, which would be
consistent with the reduction in MCP-1 production and macrophage accumulation seen in renal ischemia-reperfusion injury in Ask1−/− mice (44) and the role of ASK1-p38/JNK signaling in TNF-α-induced MCP-1 production in cultured synovial fibroblasts (47). However, despite of the effect on macrophage accumulation, the phenotype of the macrophage infiltrate was not substantially altered in terms of M1 versus M2 type markers in Ask1−/− mice.

Deletion of either ASK1 or TAK1 significantly reduced renal fibrosis in the obstructed kidney but had opposing effects upon cell apoptosis. ASK1 is best known for its proapoptotic role in oxygen radical-induced cell apoptosis (35, 42, 44), whereas TAK1 exerts an antiapoptotic action in most (but not all) cell types (5, 13). Given that both ASK1 and TAK1 deletion suppress proapoptotic p38 and JNK signaling, the most likely explanation for this difference is that TAK1 deletion also suppressed NF-κB signaling, which has a potent antiapoptotic action in most cell types (27).

There has been considerable commercial interest in developing inhibitors of p38 MAPK for the treatment of inflammatory and fibrotic disease. However, the failure of p38 inhibitors in trials of rheumatoid arthritis has necessitated a rethink about how to target p38 (12). The dramatic inhibition of p38 signaling and reduced renal fibrosis in Ask1−/− mice, plus the viability of Ask1−/− mice compared with the fetal lethality of mice lacking p38α (11, 46), indicate that the upstream kinase ASK1 may be an alternative therapeutic target for the treatment of p38-mediated disease.

In summary, this study has established a functional role for ASK1 in renal fibrosis and identified ASK1 as a major upstream kinase in the activation of p38 MAPK and, to a lesser degree, JNK in tubular cells in the obstructed kidney. A role for ASK1 in ANG II- and oxidative stress-induced tubular cell production of TGF-β1, PDGF-B, and MCP-1 was demonstrated. Finally, ASK1 and TAK1 appear to play complementary roles in the activation of p38 and JNK signaling in the normal and diseased kidney. In conclusion, this study has identified ASK1 as a potential therapeutic target in renal fibrosis.

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DISCLOSURES

D. J. Nikolic-Paterson is involved in studies with ASK1 inhibitor compounds with Gilead Sciences.

AUTHOR CONTRIBUTIONS

Author contributions: F.Y.M. performed experiments; F.Y.M. and D.J.N.-P. analyzed data; F.Y.M., G.H.T., and D.J.N.-P. interpreted results of experiments; F.Y.M. prepared figures; F.Y.M. drafted manuscript; F.Y.M., G.H.T., and D.J.N.-P. approved final version of manuscript; G.H.T. and D.J.N.-P. conception and design of research; G.H.T. and D.J.N.-P. edited and revised manuscript.

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