Delayed inhibition of p38 mitogen-activated protein kinase ameliorates renal fibrosis in obstructive nephropathy

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Abstract

Background. The p38 mitogen-activated protein kinase (MAPK) pathway is an important intracellular signalling pathway involved in the production of proinflammatory and profibrotic mediators. Previous reports indicated the role of p38 MAPK activation in renal fibrosis.

Methods. We administered a selective p38α MAPK inhibitor, FR167653, in a mouse model of unilateral ureteral obstruction (UUO) during the late stage (Days 7–14) after UUO, and the kidneys were examined at Day 14. p38 and phospho-p38 MAPK protein levels, the degree of renal fibrosis, the degree of myofibroblast accumulation and macrophage infiltration, and mRNA levels for TGF-β1 and α1(I) collagen in the kidneys were assessed.

Results. FR167653 treatment caused marked decreases in phospho-p38 MAPK levels along with decreased fibrosis at Day 14 after UUO. Although myofibroblast accumulation and α1(I) collagen mRNA level were decreased, no significant change was observed in the number of interstitial macrophages and TGF-β1 mRNA level with FR167653 treatment.

Conclusions. These results suggest that p38 MAPK blockade is an appealing therapeutic target, even after the emergence of established fibrosis.

Keywords: macrophage; obstructive nephropathy; p38 mitogen-activated protein kinase; renal fibrosis; unilateral ureteral obstruction

Introduction

The p38 mitogen-activated protein kinase (MAPK) pathway is an important intracellular signal transduction pathway involved in the production of proinflammatory and profibrotic mediators [1], and previous reports showed that pharmacological inhibition of p38 MAPK brings beneficial effects in various animal models involving inflammatory disorders [2,3]. These beneficial effects have also been reported recently in experimental models of renal fibrosis including obstructive nephropathy, showing that the blockade of p38 MAPK ameliorates renal fibrinogenesis [4,5]. In these reports, the blockade of p38 MAPK was started from the very beginning of the fibrinogenic insult, which shows that the blockade of p38 MAPK at the initial phase of inflammatory response results in reduced renal fibrosis as a consequence. However, the recent notion that showed the interrelation between inflammation and fibrosis indicated the need to consider fibrogenic response as a biological inflammatory reaction as a whole [6] and to study the process of renal fibrosis at each stage of inflammatory reaction, i.e. the initiation of tissue injury, followed by tissue remodelling and resolution of fibrosis. In this study, we examined the effect of inhibiting p38 MAPK at the late stage after unilateral ureteral obstruction (UUO) to further investigate the therapeutic potential of inhibiting p38 MAPK in renal fibrosis.

Subjects and methods

Experimental protocol

Experiments were performed on male C57BL/6 mice (10–12 weeks, 20–24 g). Renal fibrosis was induced in mice by a complete UUO as previously described (Day 0) [7]. Briefly, under sodium pentobarbital anaesthesia, the middle portion of the left ureter was ligated and cut between the two ligated points. To pharmacologically inhibit the p38 MAPK activity in the UUO kidney, a selective p38α MAPK inhibitor, FR167653 (Astellas Pharma Inc., Tokyo, Japan) [3,8,9], was injected subcutaneously once a day at a daily dose of 33 mg/kg body weight diluted in 0.5% methylcellulose. Two groups of mice were administered daily with FR167653 or vehicle during Day 7 (7 days after UUO) and Day 14. At Day 14 after surgery, the mice were sacrificed, and the obstructed kidneys were harvested and subjected to the studies. The experimental protocols were performed...
according to the regulations of the Kyoto Prefectural University of Medicine Animal Care Committee.

**Western blot analysis**

Western blot analysis was performed as previously described [4]. In brief, the obstructed kidneys were lysed in a lysis buffer containing 20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 100 mM NaF, 1 mM Na3VO4 and 1% proteinase inhibitor (Sigma-Aldrich Japan K.K., Tokyo, Japan). Lysates were centrifuged at 10 000 × g for 10 min, and supernatants (30 µg protein/lane) were separated by SDS–PAGE on a 10% acrylamide gel. Gels were electroblotted onto a nitrocellulose membrane (GE Healthcare UK Ltd, Little Chalfont, UK). The membranes were blocked with 2% nonfat dry milk and were incubated with either polyclonal rabbit anti-p38 MAPK (Cell Signaling Technology Inc., Danvers, MA, USA) or monoclonal rabbit anti-phospho-p38 MAPK (Cell Signaling Technology Inc.) overnight at 4°C. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology Inc., Danvers, MA, USA) or monoclonal rabbit anti-mouse F4/80 (Selotec, Oxford, UK) for 1 h at room temperature, followed by standard ABC immunostaining using an ABC-alkaline phosphatase kit (Vector, Burlingame, CA, USA) [7]. α-Smooth muscle actin (α-SMA) was stained by mouse monoclonal anti-human α-SMA (DAKO, Carpinteria, CA, USA) following the manufacturer’s protocol [10]. Macrophage infiltration was determined by enumerating F4/80-positive cells within the cortical interstitium in 10 randomly selected cortical fields under magnification (×400), and the numbers were averaged for each field [7]. The expression of α-SMA in the cortical interstitial area was assessed semiquantitatively by immunoreactivity for α-SMA as previously described [10].

**Histological study**

For histological examinations, the kidneys were fixed with 4% buffered paraformaldehyde for 6 h, embedded in paraffin and sectioned transversely with a thickness of 4 µm. A standard point-counting method was used to quantitate the collagen fractional volume in the renal cortical interstitium on Masson trichrome-stained sections (magnification, ×400), as previously described [7]. The index of interstitial collagen fractional volume was defined as the number of trichrome-positive points in every 1000 points evaluated. To detect infiltrating macrophages, sections were incubated with monoclonal rat anti-mouse F4/80 (Selotec, Oxford, UK) for 1 h at room temperature, followed by standard ABC immunostaining using an ABC-alkaline phosphatase kit (Vector, Burlingame, CA, USA) [7]. The amount of hydroxyproline in the renal cortex was measured as an index of collagen content as previously described [11]. Briefly, pieces of renal cortex for the hydroxyproline assay were weighed and snap-frozen in liquid nitrogen at sacrifice. After being hydrolyzed in 1 mL of 6 N hydrochloric acid and neutralized with sodium hydroxide, the concentrations of hydroxyproline of the samples were determined by high-performance liquid chromatography (HPLC) using HPLC system 800 series (JASCO Co., Tokyo, Japan).

**RT-PCR analysis**

Total RNA was isolated from the UUO kidneys using Sepasol (R)-RNA I Super (Nacalai Tesque, Inc., Kyoto, Japan) and real-time reverse transcription polymerase chain reaction (RT-PCR) for transforming growth factor (TGF)-β1, α1(I) collagen, and GAPDH mRNA was performed as previously described [9]. In brief, the sample RNA was reverse-transcribed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems). The forward and reverse primers for mouse TGF-β1 mRNA were 5′-CGTGGGCTTCTAGTGCTGACG-3′ and 5′-ACTGGCGAGCCTTAGTTGGG-3′, respectively, and those for mouse α1(I) collagen were 5′-TCTCGGCAACAAAGGAGACA-3′ and 5′-GGGCCTCTGGTTTCTC-3′, whereas those for GAPDH were 5′-GATGGGTGTAACACCTCAGA-3′ and 5′-TCATGAGCCCTTCCAAATT-3′.

**Collagen content**

The amount of hydroxyproline in the renal cortex was measured as an index of collagen content as previously described [11]. Briefly, pieces of renal cortex for the hydroxyproline assay were weighed and snap-frozen in liquid nitrogen at sacrifice. After being hydrolyzed in 1 mL of 6 N hydrochloric acid and neutralized with sodium hydroxide, the concentrations of hydroxyproline of the samples were determined by high-performance liquid chromatography (HPLC) using HPLC system 800 series (JASCO Co., Tokyo, Japan).

**Statistical analysis**

Data are presented as means ± SEM. Statistical analysis was performed by Mann–Whitney’s U test, and the significance was defined as P < 0.05.

**Results**

**Effect of FR167653 administration on phospho-p38 MAPK levels in the UUO kidneys**

By western blot analysis, there were marked increases in phosphorylated p38 MAPK (phospho-p38 MAPK) levels in the UUO kidneys at Day 14 after UUO compared with that in non-obstructed normal kidney. Administration of FR167653 significantly suppressed the phosphorylation of p38 MAPK at Day 14 after UUO (Figure 1).

**Effect of FR167653 administration on renal fibrosis**

Treatment with FR167653 resulted in a significant decrease in interstitial collagen index at Day 14 after UUO compared with that of control (97 ± 4 versus 44 ± 4/1000 points, P < 0.01, N = 9 in control and N = 10 in the FR167653 group) (Figures 2A,B, and Figure 3A). The collagen content in renal tissue assessed by HPLC was also significantly lower in mice treated with FR167653 compared to that in control (hydroxyproline; 16.1 ± 2.4 versus 8.3 ± 0.3 nmol/mg wet weight, P < 0.05, N = 7 in control and N = 5 in the FR167653 group) (3B).

**Effect of FR167653 administration on myofibroblast accumulation and interstitial macrophage infiltration**

The degree of interstitial myofibroblast accumulation assessed by immunostaining for α-SMA was significantly
Fig. 1. Effect of FR167653 administration on phospho-p38 MAPK levels in the UUO kidneys. Whole kidney lysates from obstructed kidneys at Day 14 after UUO with or without FR167653 treatment, and from non-obstructed normal kidney, were examined for p38 mitogen-activated protein kinase (MAPK) and phosphorylated p38 MAPK (phospho-p38 MAPK) levels by the western blot analysis. NOB, non-obstructed kidney.

Fig. 2. Histological assessment of interstitial fibrosis, myofibroblast accumulation and macrophage infiltration in the UUO kidneys. Masson trichrome staining of the renal cortex in control mice (A) and in FR167653-treated mice (B) at Day 14 after UUO. Immunohistochemical demonstration of myofibroblasts with an α-smooth muscle actin (α-SMA) antibody in control mice (C) and in FR167653-treated mice (D), and of macrophages with an anti-F4/80 antibody in control mice (E) and in FR167653-treated mice (F) at Day 14 after UUO. Original magnification, ×400.

lower in mice treated with FR167653 compared to that in control at Day 14 after UUO (α-SMA score; 2.16 ± 0.16 versus 1.58 ± 0.08, P < 0.05, N = 9 in control and N = 10 in the FR167653 group) (Figures 2C, D, and Figures 3C). No significant change was observed by treatment with FR167653 in the number of F4/80-positive interstitial macrophages at Day 14 after UUO (37.4 ± 1.3 versus 33.3 ± 1.7/×400 field, NS, N = 9 in control and N = 10 in the FR167653 group) (2E, F, and 3D).

Effect of FR167653 administration on TGF-β1 and α1(I) collagen mRNA expression

At Day 14 after UUO, treatment with FR167653 resulted in a significant decrease in mRNA expression for α1(I) collagen assessed by RT-PCR. However, no significant change was observed in mRNA expression for TGF-β1 by the treatment with FR167653 (the ratio to GAPDH mRNA; TGF-β1: 1.54 ± 0.09 versus 1.73 ± 0.04, NS; α1(I) collagen: 2.85 ± 0.13 versus 3.24 ± 0.16, NS).
2.47 ± 0.04 versus 1.68 ± 0.25, P < 0.05, N = 7 in control and N = 6 in the FR167653 group) (Figure 3E and F).

Discussion

Previous reports indicated that pharmacological inhibition of p38 MAPK in the early stage after the fibrotic insults exerts beneficial alleviative effect for renal fibrosis in experimental models, including UUO [4,5]. In this context, we have also performed another preliminary experiment, in which we inhibited p38 MAPK activity in the early stage after UUO by the daily administration of FR167653 during Day −2 (2 days before the implementation of UUO) and Day 5. Consistent with the previous reports, we have found in this experiment that treatment with FR167653 in the early stage after UUO resulted in decreased renal fibrosis assessed by Masson staining and hydroxyproline content in the kidney at Day 5 after UUO, along with decreased myofibroblast accumulation, decreased number of interstitial macrophage and decreased mRNA expressions for both TGF-β1 and α1(I) collagen in the UUO kidneys (unpublished data). A previous report also showed that, in a mouse model of adriamycin-induced nephropathy, the inhibition of p38 MAPK and TGF-β1/Smad signalling pathways, which was initiated at 2 weeks after fibrogenic insult, ameliorates subsequent renal fibrosis [12]. However, our present study showed, for the first time, that the single blockade of p38 MAPK after the emergence of established fibrosis is effective to reduce subsequent renal fibrosis also in the UUO model.

Although the precise role of p38 MAPK activation in renal fibrosis has not been fully elucidated, a previous report by Stambe et al. [4] showed that, at Day 7 after UUO, a reduction in myofibroblast accumulation as well as a significant reduction in renal fibrosis was observed by inhibiting p38α MAPK from the time of UUO surgery until being killed 7 days later in a rat UUO model. In their study, TGF-β1 mRNA and protein levels in the UUO kidney, and also the number of infiltrating interstitial leukocytes, were unaltered. They concluded that p38α MAPK plays an important role in renal fibrosis, acting downstream of TGF-β1. In considering the mechanisms of the ameliorated renal fibrosis by inhibiting p38 MAPK in our model, these results by Stambe et al. are similar to our results at the late stage after UUO with a delayed inhibition of p38 MAPK, in that both cases showed unaltered levels of TGF-β1 mRNA expression and interstitial macrophage number despite decreased fibrosis. In this regard, we also speculate that ameliorated renal fibrosis at a late stage after UUO with a delayed inhibition of p38 MAPK in our model is mainly due to the inhibition of downstream signalling of TGF-β1. This notion is supported by previous data that TGF-β1 induces myofibroblast transdifferentiation of tubular epithelial cells in UUO [13], and that TGF-β1-induced fibroblast transdifferentiation of mammary epithelial cell is dependent on p38 MAPK activation [14]. Furthermore, TGF-β1 has been shown in vitro to induce collagen synthesis via the p38 MAPK pathway in myoblasts [15]. Thus, p38 MAPK inhibition at the late stage after UUO may possibly inhibit TGF-β1-induced tubular epithelial cell transdifferentiation to myofibroblasts and may also inhibit TGF-β1-induced collagen production by myofibroblasts, both of which could have been contributed to the decreased renal interstitial fibrosis in our model.

Another previous report by Wada et al. showed decreased mRNA and protein levels for both TGF-β1 and monocyte chemoattractant protein (MCP)-1 and a decreased interstitial macrophage infiltration by the inhibition of p38 MAPK, along with a drastic reduction in interstitial fibrosis and glomerulosclerosis in a rat model of chronic allograft nephropathy [5]. They concluded that decreased renal fibrosis in their model is partly because of decreased MCP-1 and TGF-β1 expression in transplanted kidneys. However, the results of our present study that showed no significant changes either in the number of interstitial macrophages or in the level of TGF-β1 mRNA expression with the inhibition of p38 MAPK were contradictory to this notion. In this regard, Ma et al. showed that, using Mkk3−/− UUO mice that showed a decreased level of phospho-p38 MAPK in the UUO kidney, though upregulation of MCP-1 mRNA levels and macrophage infiltration seen on Day 3 in wild-type UUO mice was significantly reduced in Mkk3−/− mice, this difference was not evident by Day 7 [16]. They also found a decreased rate of apoptosis of tubular and interstitial cells in Mkk3−/− UUO mice compared with control and concluded that MKK3-p38 signalling plays discrete roles in renal cell apoptosis and early inflammatory response in the obstructed kidney. Our other preliminary experiment with the early-stage inhibition of p38 MAPK after ureteral ligation also showed a decrease of both the interstitial macrophage number and of the TGF-β1 mRNA expression level. Thus, these differences in the changes of the interstitial macrophage number and TGF-β1 mRNA expression level among experiments with p38 MAPK inhibition would be due to the different stages of the fibrotic process in which p38 MAPK inhibition was performed.

In summary, we have shown for the first time that delayed inhibition of p38 MAPK in obstructive nephropathy, as well as early-stage inhibition, ameliorates subsequent renal fibrosis. Further studies regarding the role of p38 MAPK signalling in the process of renal fibrosis will provide insights for the mechanisms of the alleviative effect of inhibiting p38 MAPK on renal fibrosis. Nevertheless, our present study suggests the inhibition of p38 MAPK as an appealing therapeutic target, even after the emergence of established fibrosis.

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Conflict of interest statement. None declared.

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