Original Paper

Inhibitory Effect of NH_4Cl Treatment on Renal Tgf β 1 Signaling Following Unilateral Ureteral Obstruction

Martina Feger Ioana Alesutan Tatsiana Castor Sobuj Mia Katharina Musculus
Jakob Voelkl Florian Lang

Department of Physiology, University of Tuebingen, Tuebingen, Germany

Key Words

Unilateral ureteral obstruction • NH_4Cl • Tgf β 1 • Sox9 • Renal fibrosis

Abstract

Background/Aims: Consequences of obstructive nephropathy include tissue fibrosis, a major pathophysiological mechanism contributing to development of end-stage renal disease. Transforming growth factor β 1 (Tgf β 1) is involved in the progression of renal fibrosis. According to recent observations, ammonium chloride (NH_4Cl) prevented phosphate-induced vascular remodeling, effects involving decrease of Tgf β 1 expression and inhibition of Tgf β 1-dependent signaling. The present study, thus, explored whether NH_4Cl influences renal Tgf β 1-induced pro-fibrotic signaling in obstructive nephropathy induced by unilateral ureteral obstruction (UUO).

Methods: UUO was induced for seven days in C57Bl6 mice with or without additional treatment with NH_4Cl (0.28 M in drinking water). Transcript levels were determined by RT-PCR as well as protein abundance by Western blotting, blood pH was determined utilizing a blood gas and chemistry analyser. **Results:** UUO increased renal mRNA expression of *Tgfb1*, Tgf β -activated kinase 1 (Tak1) protein abundance and Smad2 phosphorylation in the nuclear fraction of the obstructed kidney tissues, effects blunted in NH_4Cl treated mice as compared to control treated mice. The mRNA levels of the transcription factors nuclear factor of activated T cells 5 (*Nfat5*) and SRY (sex determining region Y)-box 9 (*Sox9*) as well as of tumor necrosis factor α (*Tnfa*), interleukin 6 (*Il6*), plasminogen activator inhibitor 1 (*Pai1*) and *Snai1* were up-regulated in the obstructed kidney tissues following UUO, effects again significantly ameliorated following NH_4Cl treatment. Furthermore, the increased protein and mRNA expression of α -smooth muscle actin (α -Sma), fibronectin and collagen type I in the obstructed kidney tissues following UUO were significantly attenuated following NH_4Cl treatment. **Conclusion:** NH_4Cl treatment ameliorates Tgf β 1-dependent pro-fibrotic signaling and renal tissue fibrosis markers following obstructive nephropathy.

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Prof. Dr. Florian Lang

Department of Physiology, University of Tuebingen,
Gmelinstr. 5, 72076 Tuebingen, (Germany)
Tel. +4970712972194, Fax +497071295618, E-Mail florian.lang@uni-tuebingen.de

Introduction

Renal fibrosis, particularly tubulointerstitial fibrosis, is a common pathophysiological pathway of several renal diseases leading to end-stage renal failure [1-3]. Renal fibrosis contributes to the pathophysiology of chronic kidney disease [1, 3]. Tubulointerstitial fibrosis is characterized by excessive accumulation of extracellular matrix (ECM) proteins, such as α -smooth muscle actin (α -Sma), collagen type I and fibronectin, leading to destruction of the functional tissue [1, 3, 4]. The increased production and deposition of ECM is mainly caused by myofibroblasts [5], which may be recruited from resident fibroblasts, bone marrow-derived cells or from epithelial to mesenchymal transition (EMT) [5, 6].

Mechanisms involved in the regulation of ECM protein expression leading finally to ECM deposition include transforming growth factor β 1 (Tgfβ1) [1, 3, 4]. Tgfβ1 signaling seems to be a major pathway inducing fibroblast activation and proliferation [7]. In renal fibrosis, Tgfβ1-induced EMT is mediated via Smad2/3 phosphorylation and subsequent nuclear translocation [3]. Activation of the Tgfβ1/Smad pathway up-regulates plasminogen activator inhibitor 1 (Pai1) which is closely linked to the development of EMT and ECM deposition [3]. In addition to activation of Smad-dependent signaling pathways, Tgfβ1 activates various Smad-independent pathways to induce its pro-fibrotic effects [7]. Tgfβ-activated kinase 1 (Tak1) has been identified as a major non-canonical signaling molecule in Tgfβ1-mediated fibrosis [7]. Moreover, Tak1 participates in the regulation of Tgfβ1-induced activation of Smads [7].

Tgfβ1, tumor necrosis factor α (Tnf α) and Pai1 are further implicated in excessive vascular remodeling, osteoinductive signaling and subsequent vascular calcification in *klotho* deficiency [8, 9]. According to recent observations, ammonium chloride (NH₄Cl) treatment prevented tissue calcification and reduced vascular senescence in *klotho*-hypomorphic mice [8]. In those mice, the excessive expression of *Tgfb1*, transcription factor nuclear factor of activated T cells 5 (*Nfat5*), *Nfat5*-downstream mediator SRY (sex determining region Y)-box 9 (*Sox9*) and *Pai1* was reversed following NH₄Cl treatment. NH₄Cl has previously been shown to interfere with Tgfβ1 [10]. Moreover, the expression of *Sox9*, a transcriptional regulator in the development of tissue fibrosis [11] was shown to be regulated by Tgfβ1 [12] and Tak1 [13].

The present study thus explored the effect of NH₄Cl treatment on renal Tgfβ1-dependent signaling and markers of fibrosis following obstructive nephropathy. To this end, mice were subjected to unilateral ureteral obstruction (UUO), a well established model to induce tubulointerstitial fibrosis [2, 14], with or without NH₄Cl treatment added to the drinking water [8].

Materials and Methods

Animals

All animal experiments were conducted according to the German law for the welfare of animals and were approved by local authorities. Experiments have been performed in male mice (C57Bl6) at the age of 8 weeks. Mice had free access to food (Sniff) and tap drinking water. Where indicated, NH₄Cl (0.28 M) was added to the drinking water 3 days before the surgical intervention and for the duration of the UUO treatment.

Unilateral ureteral obstruction

Renal fibrosis was induced by unilateral ureteral obstruction (UUO) [2]. Following surgical incision of the skin and abdominal wall, the left ureter was exposed and ligated twice with a non-resorbable 7-0 filament. Following ligation the surgical wound was closed by sutures. Mice were treated with metamizole for analgesia (200 mg/kg body weight) after the procedure and for the duration of the UUO experiment in drinking water. The mice were sacrificed 7 days after the ligation procedure and the obstructed as well as the non-obstructed kidney were rapidly removed and kidney tissues snap frozen in liquid nitrogen.

Blood pH analysis

For blood pH analysis, the retro-orbital plexus was punctured with a heparinized capillary. The blood samples were injected directly from the capillary into a blood gas analyzer (i15 blood gas and chemistry analyzer, Edan) to determine blood pH.

Quantitative RT-PCR

Total RNA was isolated from murine kidney tissues using Trifast Reagent (Pqclab) according to the manufacturer's instructions. Reverse transcription of 2 µg RNA was performed using oligo(dT)₁₂₋₁₈ primers (Invitrogen) and SuperScript III Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was performed with the iCycler iQTM Real-Time PCR Detection System (Bio-Rad Laboratories) and iQSybr Green Supermix (Bio-Rad Laboratories) according to the manufacturer's instructions. The following primers were used (5'→3' orientation):

a-Sma fw: CCCAGACATCAGGGAGTAATGG;
a-Sma rev: CTATCGGATACTTCAGCGTCA;
Col1a1f w: ACCCGAGGTATGCTTGATCTG;
Col1a1 rev: CATTGCACGTCATCGCACAC;
Fbn fw: GTGACACTTATGAGCGCCCTA;
Fbn rev: CCACTTGTCGCCAATCTTGTA;
Gapdh fw: AGGTCGGTGTGAACGGATTG;
Gapdh rev: TGTAGACCATGTAGTTGAGGTCA;
Il6 fw: TCTATACCACTTCACAAGTCGGA;
Il6 rev: GAATTGCCATTGCACAACCTCTT;
Nfat5 fw: GAGGGGTGTGGATTGGAATCT;
Nfat5 rev: CTGGTGCTCATGTTACTGAAGTT;
Pai1 fw: TTCAGCCCTTGCTTGCCTC;
Pai1 rev: AACTTTTACTCCGAAGTCGGT;
Snai1 fw: CACACGCTGCCTTGTGTCT;
Snai1 rev: GGTCAGCAAAAGCACGGTT;
Sox9 fw: AGTACCCGCATCTGCACAAC;
Sox9 rev: ACGAAGGGTCTCTTCTCGCT;
Tgfb1 fw: CTCCCGTGGCTTCTAGTGC;
Tgfb1 rev: GCCTTAGTTTGACAGGATCTG;
Tnfa fw: CTGAACTTCGGGGTGATCGG;
Tnfa rev: GGCTTGCTACTCGAATTTTGAGA.

The specificity of the PCR products was confirmed by analysis of the melting curves. All PCRs were performed in duplicate and relative mRNA fold changes were calculated by the 2^{-ΔΔCt} method using Gapdh as internal reference. Results are shown normalized to the mRNA expression in obstructed kidney tissues of control treated mice.

Extraction of nuclear and cytoplasmic proteins

Cytoplasmic and nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Protein concentration was determined by Bradford assay (Biorad Laboratories) and 20 µg of protein were boiled in Roti-Load1 Buffer (Carl Roth) at 100°C for 5 min. Smad2 phosphorylation in the nuclear and cytoplasmic fractions were further determined by Western blot analysis.

Western blot analysis

Murine kidney tissues were lysed with ice-cold lysis buffer (Thermo Fisher Scientific) supplemented with complete protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific). After centrifugation at 10000 rpm for 5 min, proteins were boiled in Roti-Load1 Buffer (Carl Roth) at 100°C for 5 min. Proteins were separated on SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated overnight at 4°C with the following primary antibodies: rabbit anti-α-smooth muscle actin, rabbit anti-collagen I (diluted 1:1000, Abcam), mouse anti-fibronectin (diluted 1:1000, BD Biosciences), rabbit anti-Tak1, rabbit anti-Smad2, rabbit anti-phosphorylated Smad2 (Ser^{465/467}, diluted 1:1000, Cell Signaling),

rabbit anti- α -tubulin and rabbit anti-Gapdh antibody (diluted 1:2000, Cell Signaling) and then with secondary goat anti-rabbit HRP-conjugated antibody (diluted 1:2000, Cell Signaling) or goat anti-mouse HRP-conjugated antibody (diluted 1:2000, Cell Signaling) for 1 hour at room temperature. For loading controls, the membranes were stripped with stripping buffer (Carl Roth) at 60°C for 10 min. Antibody binding was detected with the ECL detection reagent (Thermo Fisher Scientific). Bands were quantified with Quantity One Software (Bio-Rad Laboratories) and results are shown as the ratio of total protein to Gapdh. For cytoplasmic and nuclear phosphorylated Smad2, results are shown as the ratio of phosphorylated to total Smad2 to Hdac2 or α -tubulin protein for the nuclear fraction or the cytoplasmic fraction, respectively.

Statistics

Data are provided as means $\pm$ SEM, n represents the number of independent experiments. All data were tested for significance between control and NH_4Cl treated mice using unpaired Student t -test (normal distributed data) or Mann-Whitney test (non-normal distributed data) according to Shapiro-Wilk test. Only results with $p < 0.05$ were considered statistically significant.

Results

To explore whether NH_4Cl treatment influences renal pro-fibrotic signaling following obstructive nephropathy, UUO was performed in C57Bl6 mice with or without additional treatment with NH_4Cl . The mice were treated with 0.28 M NH_4Cl in the drinking water 3 days before the obstructive injury and for the duration of the UUO treatment. Seven days after the obstructive injury, the animals were sacrificed and the obstructed kidney tissues were compared between the treatment groups. NH_4Cl treatment significantly ($p < 0.01$) decreased blood pH from 7.37 ± 0.03 to 7.20 ± 0.04 ($n = 5$).

Fig. 1. Effect of NH_4Cl treatment on UUO-induced Tgf β 1 and Tgf β -activated kinase 1 (Tak1) expression in renal tissue. (A) Arithmetic means $\pm$ SEM ($n = 6$) of transforming growth factor β 1 (*Tgfβ1*) relative mRNA expression (arbitrary units, a.u.) in renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr; white bars) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water; black bars). (B) Representative original western blots and arithmetic means $\pm$ SEM ($n = 9$) showing Tgf β -activated kinase 1 (Tak1)/Gapdh protein ratio in renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr; white bars) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water; black bars). * ($p < 0.05$) statistically significant vs respective kidney tissues of control treated mice.

Fig. 2. Impact of NH_4Cl treatment on nuclear and cytoplasmic Smad2 phosphorylation in renal tissue following unilateral ureteral obstruction. (A) Representative original western blots showing phospho-Smad2 (Ser^{465/467}), Smad2, Hdac2 and α -tubulin protein abundance in nuclear and cytoplasmic fractions of renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water). (B, C) Arithmetic means $\pm$ SEM (n = 7) of nuclear (Nuc, B) and cytoplasmic (Cyt, C) phospho-Smad2 (Ser^{465/467})/Smad2 to Hdac2 or α -tubulin protein ratio in the nuclear fraction and cytoplasmic fraction respectively, in renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr; white bars) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water; black bars). * (p < 0.05) statistically significant vs respective kidney tissues of control treated mice.

Based on previous observations, Tgf β 1 signaling was investigated in renal obstructive injury. As shown in Fig. 1A, *Tgfb1* mRNA expression was higher in the obstructed kidney tissues than in non-obstructed control kidney tissues following UUO. The increased *Tgfb1* mRNA levels were significantly decreased in the obstructed kidney tissues from NH_4Cl treated mice as compared to control treated mice (Fig. 1A). The effects of NH_4Cl treatment on renal *Tgfb1* expression were paralleled by a significant reduction of the increased Tak1 protein levels in the obstructed kidney tissues (Fig. 1B). Moreover, the increased Smad2 phosphorylation at Ser^{465/467} in the nuclear fraction isolated from obstructed kidney tissues following UUO was significantly decreased in NH_4Cl treated mice as compared to control treated mice (Fig. 2A, B). No significant effects of NH_4Cl treatment on Smad2 phosphorylation in the cytoplasmic fraction isolated from the obstructed kidney tissues were observed (Fig. 2A, C). In non-obstructed control kidney tissues, NH_4Cl treatment did not significantly modify *Tgfb1* and Tak1 expression (Fig. 1) or Smad2 phosphorylation (Fig. 2).

To further define the effects of NH_4Cl on Tgf β 1-mediated pro-fibrotic signaling following UUO, the mRNA expression of the transcription factors *Nfat5* and *Sox9* was determined. Following UUO, *Nfat5* and *Sox9* mRNA levels were increased in the obstructed kidney tissues as compared to non-obstructed control kidney tissues, effects significantly suppressed following treatment with NH_4Cl (Fig. 3A, B). NH_4Cl treatment did not significantly modify *Nfat5* and *Sox9* mRNA levels in the non-obstructed control kidney tissues (Fig. 3A, B).

Fig. 3. Effect of NH_4Cl on renal *Nfat5* and *Sox9* mRNA expression following unilateral ureteral obstruction. Arithmetic means $\pm$ SEM (n = 6) of nuclear factor of activated T cells 5 (*Nfat5*; A) and SRY (sex determining region Y)-box 9 (*Sox9*; B) relative mRNA expression (arbitrary units; a.u.) in renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr; white bars) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water; black bars). ** (p < 0.01), *** (p < 0.001) statistically significant vs respective kidney tissues of control treated mice.

Fig. 4. NH_4Cl sensitive expression of Tgf β 1 target genes in renal tissue following unilateral ureteral obstruction. Arithmetic means $\pm$ SEM (n = 6) of tumor necrosis factor α (*Tnfα*; A), interleukin 6 (*Il6*; B), plasminogen activator inhibitor 1 (*Pai1*; C) and *Snai1* (D) relative mRNA expression (arbitrary units, a.u.) in renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr; white bars) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water; black bars). * (p < 0.05), ** (p < 0.01), *** (p < 0.001) statistically significant vs respective kidney tissues of control treated mice.

Further experiments addressed the transcript levels of Tgf β 1 target genes: *Tnfα*, the cytokine *Il6*, as well as of *Pai1* and *Snai1*. As shown in Fig. 4, the increased mRNA expression of *Tnfα*, *Il6*, *Pai1* and *Snai1* in obstructed kidney tissues following UUO were all significantly reduced in NH_4Cl treated mice as compared to control treated mice. NH_4Cl treatment did not

Fig. 5. NH_4Cl treatment ameliorates the expression of α -smooth muscle actin, fibronectin and collagen type 1 in renal tissue following unilateral ureteral obstruction. (A-C) Representative original western blots and arithmetic means $\pm$ SEM ($n = 9$) of α -smooth muscle actin (α -Sma)/Gapdh (A), fibronectin/Gapdh (B) and collagen type I/Gapdh (C) protein ratio in renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr; white bars) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water; black bars). (D-F) Arithmetic means $\pm$ SEM ($n = 6$) of α -smooth muscle actin (α -Sma; D), fibronectin (*Fbn*; E) and collagen type I (*Col1a1*; F) relative mRNA expression (arbitrary units, a.u.) in renal tissue from non-obstructed control kidney (CTR) and obstructed kidney (UUO) following 7 days of unilateral ureteral obstruction (UUO) in control treated mice (ctr; white bars) and in mice treated with NH_4Cl (0.28 M NH_4Cl added to the drinking water; black bars). * ($p < 0.05$), ** ($p < 0.01$) statistically significant vs respective kidney tissues of control treated mice.

significantly modify *Tnfa*, *Il6*, *Pai1* and *Snai1* mRNA expression in the non-obstructed control kidney tissues (Fig. 4).

In order to quantify the effect of NH_4Cl treatment on markers of the fibrotic response following UUO, the expression of α -Sma, fibronectin and collagen type I was determined. As a result, the increased α -Sma (Fig. 5A), fibronectin (Fig. 5B) and collagen type I (Fig. 5C) protein expression in obstructed kidney tissues following UUO were significantly reduced in NH_4Cl treated mice as compared to control treated mice. Accordingly, NH_4Cl treatment significantly blunted the increased renal mRNA expression of α -Sma (Fig. 5D), fibronectin (*Fbn*) (Fig. 5E) and collagen type I (*Col1a1*) (Fig. 5F) in obstructed kidney tissues following UUO. In non-obstructed control kidney tissues, NH_4Cl treatment did not significantly affect renal α -Sma, fibronectin and collagen type I protein expression and *Fbn* and *Col1a1* mRNA levels but significantly increased renal α -Sma mRNA expression (Fig. 5).

Discussion

The present study reveals a suppressive effect of NH_4Cl treatment on the signaling of renal interstitial fibrosis following UUO. NH_4Cl treatment significantly blunted the increase of renal

α -Sma, a mesenchymal marker mainly expressed by activated fibroblasts and myofibroblasts [5]. Consistent with reduced α -Sma abundance following obstructive injury, renal expression of ECM proteins such as fibronectin and collagen type 1 were mitigated in the mice treated with NH_4Cl .

In the development of renal fibrosis, Tgf β 1 is regarded as the most relevant pro-fibrotic cytokine [7]. Similar to previous findings [8], NH_4Cl treatment inhibited UUO-induced upregulation of *Tgfb1* mRNA expression in the obstructed kidney tissues. Dietary treatment with NH_4Cl leads to acidosis [15]. Moreover, NH_4^+ may further dissociate to H^+ and NH_3 , which permeates membranes and thus enters cells and cellular compartments [16]. NH_3 may bind H^+ and may thus be trapped as NH_4^+ within acidic cellular compartments [17]. The binding of H^+ alkalinizes those compartments [17]. By alkalinizing lysosomes, NH_4Cl has previously been shown to interfere with Tgf β 1 maturation [10].

The effects of NH_4Cl on *Tgfb1* expression were paralleled by inhibition of increased Tak1 protein levels and of increased nuclear phosphorylated Smad2, downstream signaling mediators of Tgf β 1 [3, 18]. Moreover, NH_4Cl treatment reduces the increased expression of the pro-fibrotic Tgf β 1 target genes: *Tnfa*, *Il6*, *Pai1* and *Snai1* following obstructive injury.

Beyond that, the cellular accumulation of NH_4^+ with Cl^- may lead to cell swelling [19] which suppresses expression of the cell volume sensitive transcription factor TONEBP (Tonicity-Responsive Enhancer Binding Protein) or Nfat5 [20]. Accordingly, NH_4Cl treatment significantly blunted UUO-induced renal *Nfat5* expression. Tgf β 1 up-regulates Nfat5 expression [21]. Nfat5 was in turn shown to up-regulate *Tnfa* and *Il6* in immune cells [22]. On the other hand, Nfat5 was shown to regulate the expression of several collagen subtypes [23] and chondrogenic differentiation dependent on Sox9 [23], a key transcription factor also in the development of fibrosis [11]. Previous studies show that Sox9 expression is up-regulated by Tgf β 1 [12] and Tak1 [13].

Tgf β is decisive in the triggering of fibrosis of the kidney [3]. Tgf β may be effective through serum- and glucocorticoid-inducible kinase 1 (Sgk1) and Akt [24]. Sgk1 expression is sharply up-regulated by cell shrinkage [24]. Tgf β significantly contributes to the stimulation of renal fibrosis following UUO [4]. Sgk1 is at least in part effective by phosphorylating glycogen synthase kinase 3 (Gsk-3) [14], which is accomplished by Sgk and Akt isoforms [25].

NH_4Cl similarly interferes with Tgf β 1 signaling in vascular tissue during hyperphosphatemia [8]. Moreover, renal dysfunction following 5/6 nephrectomy is ameliorated by NH_4Cl treatment [26]. The renoprotective effects of NH_4Cl drinking solution were independent of phosphate [27]. According to these observations, NH_4Cl may have beneficial properties in renal disease. On the other hand acidosis has been shown to aggravate and alkali treatment to delay progression of chronic kidney disease [28, 29].

Conclusion

Tgf β 1 signaling leading to renal tissue fibrosis may be counteracted by NH_4Cl treatment, an observation possibly opening novel therapeutic opportunities.

Abbreviations

Col1a1 (collagen type I); ECM (extracellular matrix); EMT (epithelial to mesenchymal transition); Fbn (fibronectin); Gapdh (glyceraldehyde 3-phosphate dehydrogenase); Gsk-3 (glycogen synthase kinase 3); Hdac2 (histone deacetylase 2); *Il6* (interleukin 6); Nfat5 (nuclear factor of activated T-cells); NH_4Cl (ammonium chloride); *Pai1* (plasminogen activator inhibitor 1); Sgk1 (serum- and glucocorticoid-inducible kinase 1); Smad2 (Sma and Mad (Mothers against decapentaplegic) 2); Sox9 (SRY (sex determining region Y)-box 9); Tak1 (Tgf β -activated kinase 1); Tgf β 1 (transforming growth factor β 1); *Tnfa* (tumor necrosis factor α); TONEBP (Tonicity-Responsive Enhancer Binding Protein); UUO (unilateral ureteral obstruction); α -Sma (α -smooth muscle actin).

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Disclosure Statement

All authors disclose that they have no potential conflict of interest.

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