

Original Paper

Role and Association of Inflammatory and Apoptotic Caspases in Renal Tubulointerstitial Fibrosis

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Key Words

Caspase-1 • Caspase-7 • Renal Tubulointerstitial Fibrosis • TGF- β 1

Abstract

Background/Aims: Caspases, an evolutionary conserved family of aspartate-specific cystein proteases, play pivotal roles in apoptotic and inflammatory signaling. Thus far, 14 mammalian caspases are identified and categorized into 3 distinct sub-types: inflammatory caspases, apoptotic initiator and apoptotic executioner. Caspase-1 is an inflammatory caspase, while caspase-7 belongs to apoptotic executioner. The roles and association of these two distinct types of caspases in renal tubulointerstitial fibrosis (TIF) have not been well recognized. **Methods:** Caspase-1 inhibitor Z-YVAD-FMK and caspase-7 siRNA were used in tubular epithelial cell line NRK-52E (TECs) to test their effects on transforming growth factor-beta1 (TGF- β 1) stimulation. *In vivo*, Unilateral ureteral obstruction (UUO) animal model was employed in wild-type (WT) and caspase-1 knock out (KO) (caspase-1^{-/-}) mice. **Results:** In current study, we found that caspase-7 was obviously activated in cultured TECs stimulated by TGF- β 1 and in UUO model of WT mice. While in UUO model of caspase-1 KO mice, the increased caspase-7 activation was suppressed significantly along with reduced trans-differentiation and minimized extracellular matrix (ECM) accumulation, as demonstrated by western blot, Masson trichrome staining and immunohistochemistry. In addition, pharmacological inhibition of caspase-1 dampened caspase-7 activation and TECs' transdifferentiation induced by TGF- β 1 exposure, which was consistent with *in vivo* study. Notably, caspase-7 gene knock down by specific siRNA abrogated TGF- β 1 driven TECs' trans-differentiation and reduced ECM accumulation. **Conclusions:** Our study associated inflammatory and apoptotic caspases in TIF for the first time and we further confirmed that caspase-1 activation is an upstream event of apoptotic caspase-7 induction in TIF triggered by UUO and in TECs mediated by TGF- β 1 induced transdifferentiation.

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Introduction

Chronic kidney disease (CKD) is a public health problem worldwide [1]. CKD ultimately progresses to renal failure and the need for dialysis or renal transplantation [2]. Many factors are involved in the onset and progression of CKD. Renal tubulointerstitial fibrosis, characterized by ECM deposition, interstitial myofibroblast proliferation, and the infiltration of inflammatory mononuclear cells, is thought to play an important role in the pathogenesis of CKD [3]. Therefore, preventing renal tubulointerstitial fibrosis remains a major target for clinicians. Although much progress has been made, our understanding of the cellular and molecular mechanisms of interstitial fibrosis still remains dismal.

Caspases are cysteinyl aspartate-specific proteases that play critical roles in apoptosis and inflammation [4, 5]. Currently, 14 caspases have been identified from mammalian cells [6]. They are synthesized as zymogens with an N-terminal prodomain of variable length preceding the catalytic domain. On the basis of their biological functions, caspases can be classified into 3 groups: inflammatory caspases, such as caspase-1, -4, -5, -11 and -12, initiator caspases, including caspase-2, -8, -9 and -10, and executioner caspases like caspase-3, -6, -7 and -14 [7, 8]. Caspase-1 is the prototypical member of the inflammatory caspases and responsible for the maturation of inflammatory cytokines like pro-IL-1 β and pro-IL-18 [9, 10]. It is activated within a cytoplasmic multiprotein complex named the inflammasome, which consists of a receptor like NLRP3, an adaptor ASC and caspase-1 [11]. Once activated, caspase-1 can also induce an apoptotic caspase cascade, which is required for executing proteolytic activity of the executioner caspases [12]. Many DAMPs released during renal injury are capable of activating the NLRP3 inflammasome, including reactive oxygen species (ROS), extracellular ATP, uric acid, nucleic acids and extracellular matrix components such as hyaluronan and biglycan [13-15]. The role of the inflammasome in acute renal failure is further demonstrated by the finding that caspase-1-deficient mice are more resistant to cisplatin-induced apoptosis and acute tubular necrosis [16]. Recently, NLRP3 inflammasome has been proven to promote renal inflammation and contribute to the pathogenesis of CKD [17]. Moreover, NLRP3 gene deletion can protect against renal fibrosis in mouse with 5/6 nephrectomy [18]. Thus far, there is still limited information on the role of caspases in renal tubulointerstitial fibrosis. Herein, it needs to be further explored as to how caspase-1 is involved in renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction (UUO).

In vitro experiments revealed that caspase-1 directly processed caspase-3 and caspase-7 [19, 20]. Nevertheless, the biological role of this cascade activation is unknown. Active caspase-3 and caspase-7 cleave a large set of substrates and ultimately result in apoptosis or necrosis of the cells [21]. Studies showed that C57BL/6 mice deficient for both caspase-3 and -7 died shortly after birth, while mice lacking either caspase-3 or -7 had a normal life span and displayed a limited apoptotic phenotype in this genetic background [22, 23]. The executioner caspase-6 and -7 but not caspase-3 were identified as transcriptional targets of p53 in cisplatin injured renal cells [24]. Taken into consideration of the complexity of renal tubulointerstitial fibrosis, the roles of executioner caspases in this process remain to be explored. It is necessary to determine the downstream executioner caspase in the process of TIF. The present study provides first evidence that caspase-1 and caspase-7 signaling participates in renal tubulointerstitial fibrosis and caspase-1 serves as the upstream activator of caspase-7.

Materials and Methods

Animals

Wild type C57/BL6 and caspase-1 knockout (KO) mice were from Jackson Laboratories (Bar Harbor, Maine, USA). All experiments were performed according to the guidelines for use and care of laboratory animals of National Institutes of Health (NIH), and approved by Animal Care and Use Committee (ACUC) of

Tongji Medical College. Wild-type (WT) and caspase-1 KO male mice (8 weeks of age) were anesthetized. After that, unilateral ureteral obstruction (UUO) or sham surgery was performed. The left ureter was ligated completely near the renal pelvis using a 6-0 silk tie. In sham-operated mice, the left ureter was exposed but not ligated. After 1, 3 or 7 days of UUO or sham operation, the mice were sacrificed and renal tissues were harvested.

Cell Culture and Treatment

Renal tubular epithelial cell line NRK-52E (TECs) (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle medium (DMEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA) and 100 U/ml penicillin-streptomycin in 37°C incubator with 5% CO₂. After the cells reached 70% confluence, the medium was changed into serum-free medium. After starvation for 12 h, recombinant human TGF-β1 (PeproTech, London, UK) was added into the medium at a final concentration of 5 ng/ml for indicated time [25, 26].

For caspase-1 inhibition, 10 μM caspase-1 inhibitor Z-YVAD-FMK (Biovision, Mountain View, CA, USA) was added into the medium 1 h before TGF-β1 treatment.

Transfection

Caspase-7 siRNA was purchased from RiboBio (Ribobio Co., Guangzhou, China). Briefly, cells were seeded in 6-well plates and were grown up to 60% confluence before transfection. Transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then TGF-β1 (5 ng/ml) was added as stated above and proteins were extracted 48 h after transfection.

Western Blot Analysis

In brief, proteins from mouse renal tissues or cultured TECs were extracted with RIPA Lysis Buffer (Beyotime, Jiangsu, China). Lysates were denatured and subjected to SDS-PAGE, transferred to PVDF membranes. After blocked for 2 h in 5% BSA in TBST (10 mM Tris-HCL and 0.05% Tween 20), the membranes were incubated with primary antibodies overnight at 4°C and then secondary antibodies for 2 h at room temperature. Band signals were visualized using ECL (Pierce, Rockford, IL, USA) for chemiluminescence detection. The luminescent signals were detected with ChemiDocXRS (Bio-Rad, Hercules, CA, USA) and analyzed by ImageLab software 3.0 (Bio-Rad, Hercules, CA, USA). The primary antibodies used were caspase-7 (Cell Signaling, Danvers, MA), α-SMA (Sigma, MO, USA), collagen I (Santa Cruz, CA, USA), collagen III (Santa Cruz, CA, USA), β-actin (Santa Cruz, CA, USA) and GAPDH (Santa Cruz, CA, USA).

Caspase-1 Activity Assay

Caspase-1 activity was examined on cytosolic extracts of kidneys or whole cell lysates by a commercially available colorimetric assay (Biovision, Mountain View, CA, USA) according to the manufacturer's instructions [27]. The activity was measured after 1 h at 37°C using a microplate reader (Tecan, Sunrise™, Männedorf, Switzerland) at 405 nm. The data was calculated as fold changes compared to control group.

Immunohistochemistry and Masson Trichrome Staining

Sections from paraffin-embedded tissues were prepared at 4 μm thickness and incubated at 4°C with α-SMA (Sigma, MO, USA) or fibronectin (Santa Cruz, CA, USA) overnight. Sections were then incubated with secondary antibody (Santa Cruz, CA, USA) for 1 h at room temperature. After staining, the sections were randomly chosen to calculate positively stained area using Image Pro Plus 6.0 software (Media Cybernetics, MD, USA). Six mice were analyzed for each group. For Masson trichrome staining, the 4 μm slides were stained by routine procedures [28]. The blue staining area represented the collagen.

Statistical Analysis

All the values were presented as mean ± SD. Significant differences among groups were evaluated by one-way ANOVA using SPSS 13.0. *P* < 0.05 was considered to be significant.

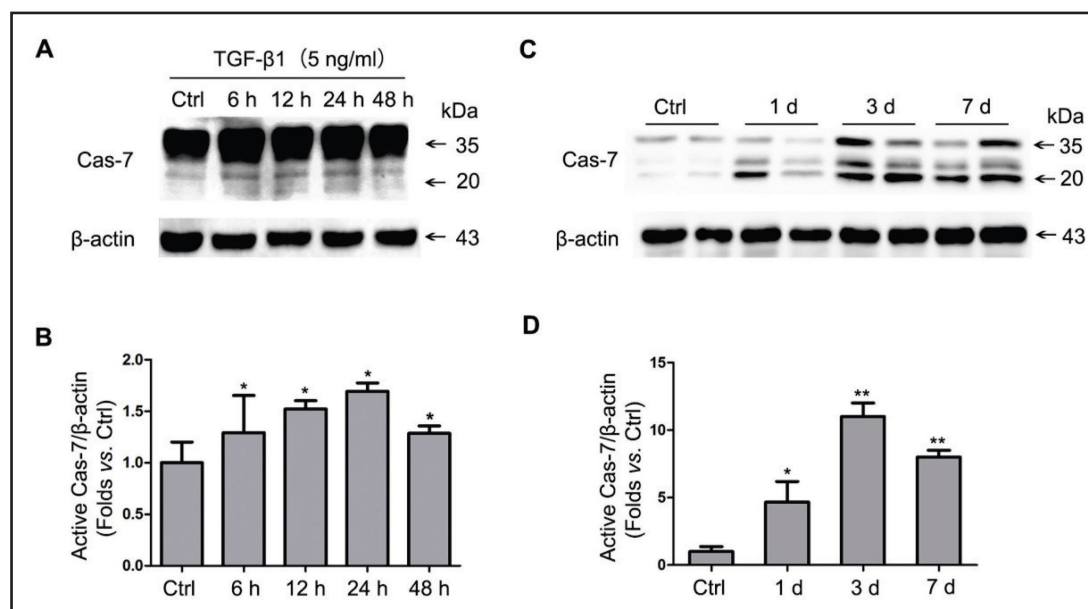


Fig. 1. Caspase-7 is activated in TGF- β 1 stimulated TECs and UUO mice. NRK-52E cells (TECs) were incubated with TGF- β 1 (5 ng/ml) for different time span as indicated. A. Representative western blot images showing the expression and activation of caspase-7 in whole cell lysates. B. Summarized data presenting the relative active caspase-7 levels (n=6). For *in vivo* study, wild-type mice were subjected to unilateral ureteral obstruction (UUO) for 1, 3 or 7 days and kidney extracts were analyzed by western blot. Control was the sham-operated mice group. C. Representative western blot images of caspase-7 expression and activation in mice. D. Summarized data of active caspase-7 level (n=6). * $P < 0.05$, ** $P < 0.01$ vs. Ctrl. Cas-7: caspase-7; Ctrl: control; 1d: UUO of 1 day; 3d: UUO of 3 days; 7d: UUO of 7 days.

Results

Caspase-7 is activated in TGF- β 1 stimulated TECs and UUO mice

To determine the role of caspase-7 in fibrotic kidney, we first analyzed whether TGF- β 1 could directly activate caspase-7 in cultured TECs. As shown in Figure 1A and 1B, active caspase-7 (20 kDa) protein level was elevated in TGF- β 1 treated TECs within 48 h, reaching the peak at 24 h ($P < 0.05$). Next we examined the expression and activation of caspase-7 in WT mouse kidney samples after 1, 3 and 7 days of UUO. By western blot analysis, we observed that the full-length (35 kDa) and cleaved forms (20 kDa) of caspase-7 were gradually increased after 1, 3 and 7 days of UUO (Figure 1C and 1D). The caspase-7 activation was significantly increased in 3 and 7 days kidney samples ($P < 0.01$). In order to uncover the functional changes of kidney caspase-7 in UUO mouse model, we chose to conduct the model for 7 days in all experiments hereafter.

Loss of caspase-1 suppresses caspase-7 activation and TEC's transdifferentiation in UUO mice

To explore whether caspase-1 is involved in renal interstitial fibrosis and participates in caspase-7 activation, we first confirmed the caspase-1 activity in WT and KO mice (Figure 2A). As expected, ligation of the kidney dramatically increased activity of caspase-1 in WT mice ($P < 0.01$) which was lacking in caspase-1 knock-out UUO kidneys ($P < 0.05$). Then we assessed caspase-7 activation in caspase-1 KO and WT mouse kidneys by western blot analysis. It was found that caspase-7 was notably activated in WT-UUO mice as compared to sham-operated (control) mice ($P < 0.05$) (Figure 2B and 2C). However, caspase-1 deficient mice that underwent UUO showed remarkable suppression of caspase-7 activation ($P <$

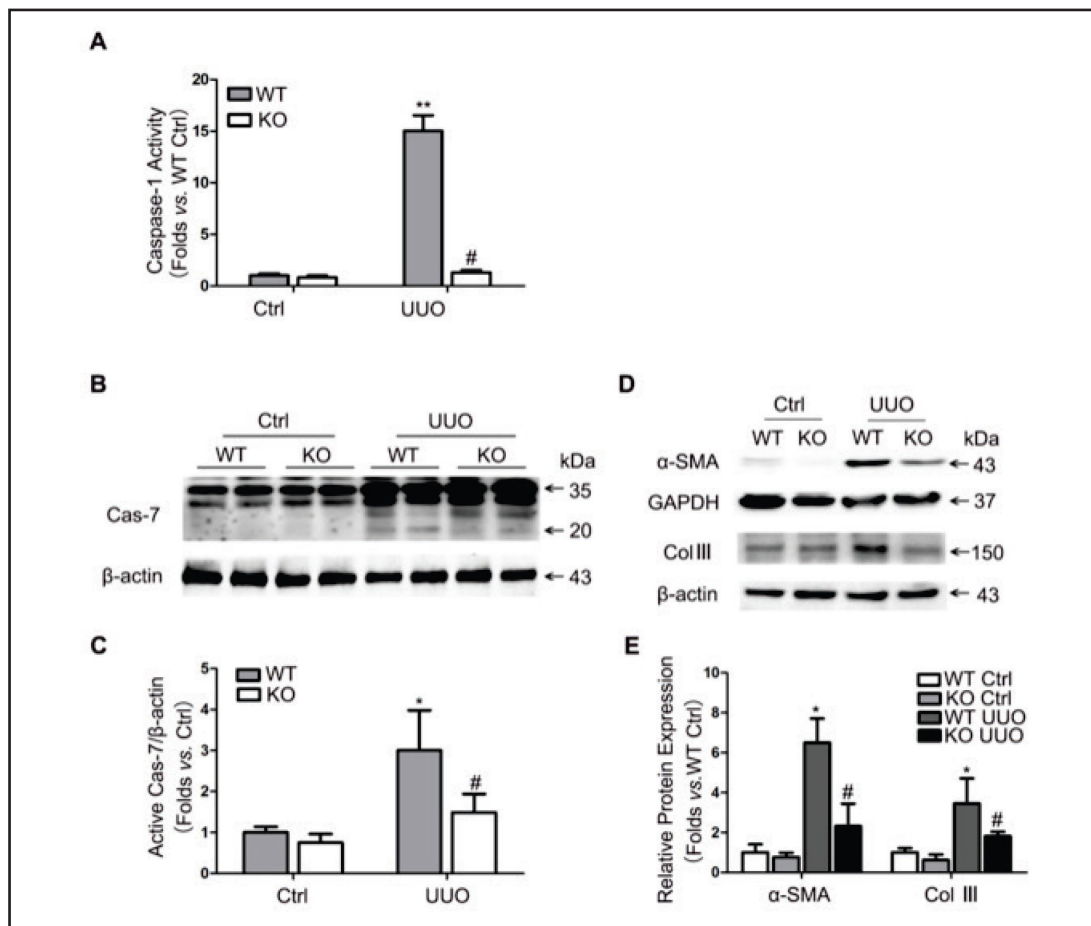


Fig. 2. Caspase-1 deficiency suppresses caspase-7 activation and TEC's transdifferentiation in UUO mice. Wild-type (WT) mice and caspase-1 KO mice were conducted UUO or sham operation for 7 days. Kidney samples of the above 4 groups were harvested and examined. **A.** Caspase-1 activity detected by enzyme activity assay (n=6). ** $P < 0.01$ vs. WT Ctrl, # $P < 0.05$ vs. WT UUO. **B** and **C.** Representative Western blot images (**B**) and summarized data (**C**) of the active caspase-7 levels (n=6). * $P < 0.05$ vs. WT Ctrl, # $P < 0.05$ vs. WT UUO. **D** and **E.** Representative Western blot images (**D**) and summarized data (**E**) displaying that caspase-1 deficiency suppresses TEC's transdifferentiation as implicated by the decreased production of α-SMA and collagen III in KO UUO mice (n=5). * $P < 0.05$ vs. WT Ctrl, # $P < 0.05$ vs. WT UUO. WT: wild-type; KO: knock-out; Ctrl: control; Cas-7: caspase-7; Col III: collagen III.

0.05). Similarly, α-SMA (the myofibroblasts marker), and collagen III expression in kidneys of caspase-1 KO UUO group demonstrated a dramatic decrease compared with that in WT UUO mouse kidneys ($P < 0.05$) (Figure 2D and 2E). Next, we evaluated the ECM deposition by Masson's trichrome staining and immunohistochemistry. It was observed that UUO increased collagen deposition in wild type mouse kidneys ($P < 0.05$), but this increase was largely attenuated in caspase-1 KO mice ($P < 0.05$) (Figure 3A and 3B). Consistently, the positive staining regions of α-SMA ($P < 0.05$) (Figure 3C and 3D) and fibronectin ($P < 0.05$) (Figure 3E and 3F) were also markedly elevated in WT-UUO mice but not in caspase-1 KO mice.

Inhibition of caspase-1 largely abrogates TGF-β1 induced caspase-7 activation and TECs' transdifferentiation in vitro

In an effort to test whether attenuation of caspase-1 would have effects on TGF-β1 induced caspase-7 activation and α-SMA accumulation, we conducted an experiment to

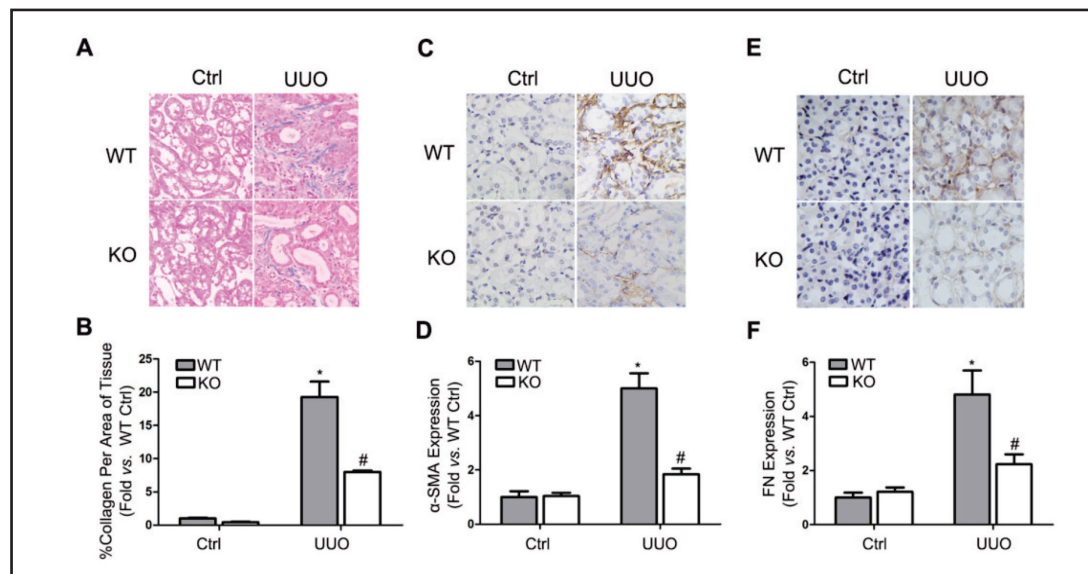


Fig. 3. Caspase-1 deficiency alleviates ECM accumulation in UUO mice. Sections from mice at 7 days after UUO or sham-operation were stained and analyzed. A and B. Masson trichrome staining (A) and the quantification of the positive staining (B) in tubulointerstitial region of 4 groups of mice (n=6). Magnification: ×400. * $P < 0.05$ vs. WT Ctrl, # $P < 0.05$ vs. WT UUO. C to F. Immunohistochemistry staining (C and E) and quantification of the positive staining (D and F) of α-SMA and fibronectin (FN) levels in tubulointerstitial region of 4 groups of mice, respectively (n=6). Magnification: ×400. * $P < 0.05$ vs. WT Ctrl, # $P < 0.05$ vs. WT UUO. WT: wild-type; KO: knock-out; Ctrl: control; Col III: collagen III; FN: fibronectin.

pharmacologically inhibit caspase-1 by Z-YVAD. Firstly, the effectiveness of caspase-1 inhibitor Z-YVAD was confirmed ($P < 0.05$) (Figure 4A). The results indicated that inhibition of caspase-1 significantly suppressed caspase-7 activation induced by TGF-β1 in TECs ($P < 0.05$) (Figure 4B and 4C). In addition, inhibition of caspase-1 could also markedly decrease α-SMA abundance driven by TGF-β1 ($P < 0.05$) (Figure 4D and 4E).

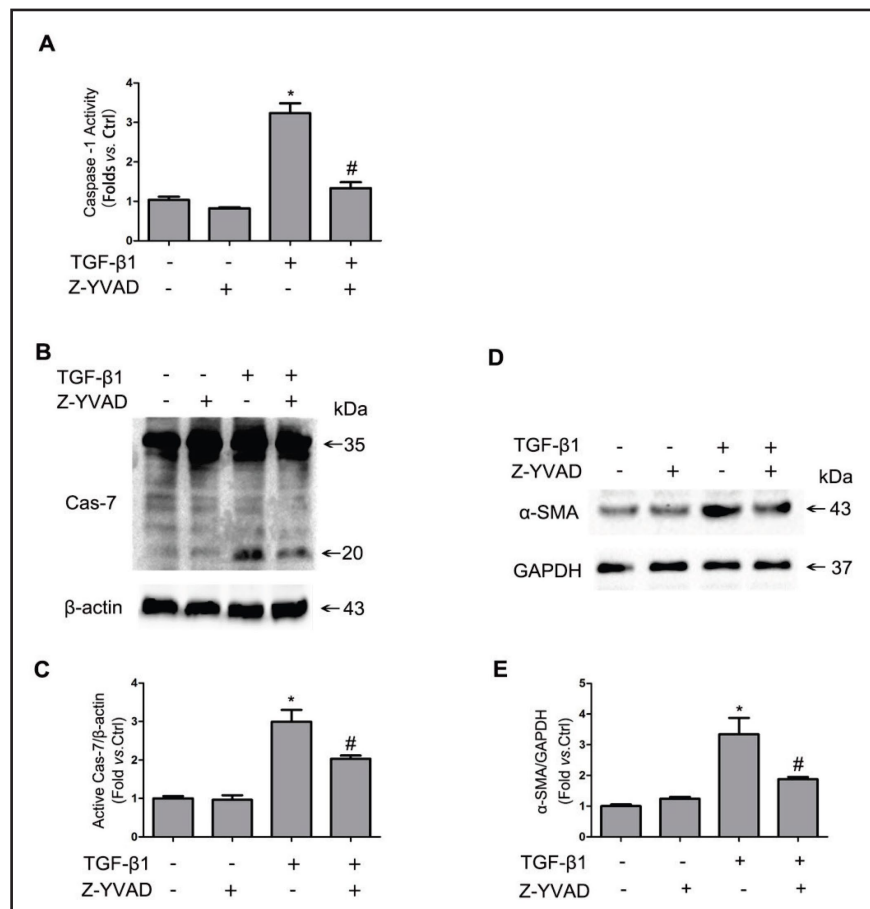
Genetic deletion of caspase-7 attenuates TGF-β1 stimulated TEC's transdifferentiation and ECM aggregation in vitro

To further explore the possibility that caspase-7 contributed to TGF-β1 induced interstitial fibrosis in the kidney, we investigated the effect of caspase-7 deficiency on renal interstitial fibrosis in TGF-β1 treated TECs by using specific caspase-7 siRNA. TGF-β1 stimulation, as expected, increased the α-SMA expression ($P < 0.05$) (Figure 5A and 5B). However, knocking down the caspase-7 gene in the cells largely reversed the aforementioned effect ($P < 0.05$). Likewise, the levels of collagen I and collagen III induced by TGF-β1 stimulation were reversed by caspase-7 siRNA transfection ($P < 0.05$) (Figure 5C and 5D).

Discussion

In the present study, we showed for the first time that executioner caspase-7 and inflammatory caspase-1 were involved in renal tubulointerstitial fibrosis in TGF-β1 treated TECs *in vitro* and mice induced by UUO *in vivo*. Our results demonstrated that caspase-7 was markedly activated in TGF-β1 treated TECs and UUO mouse model, as manifested by increased active caspase-7 (20 kDa) expression. The results implied that caspase-7 might be involved in renal interstitial fibrosis. Loss of caspase-1 inhibited caspase-7 activation and attenuated renal fibrosis after 7 days of UUO mice or in cultured TECs stimulated by TGF-β1.

Fig. 4. Inhibition of caspase-1 largely abrogates TGF- β 1 induced caspase-7 activation and TECs' transdifferentiation *in vitro*. NRK-52E cells (TECs) were incubated with TGF- β 1 (5 ng/ml) and Z-YVAD (a caspase-1 inhibitor; 10 μ M) for 48 hours. A. Caspase-1 activity (n=6). * P < 0.05 vs. Ctrl, # P < 0.05 vs. TGF- β 1. B. Representative Western blot images displaying the expression and activation of caspase-7 in whole cell lysates. C. Summarized data presents the relative active caspase-7 levels (n=5). * P < 0.05 vs. Ctrl, # P < 0.05 vs. TGF- β 1. D and E. Representative Western blot images (D) and summarized data (E) presenting caspase-1 inhibitor Z-YVAD reduces the abundance of α -SMA induced by TGF- β 1 (n=6). * P < 0.05 vs. Ctrl, # P < 0.05 vs. TGF- β 1. Ctrl: control; Cas-7: caspase-7.



Ctrl, # P < 0.05 vs. TGF- β 1. D and E. Representative Western blot images (D) and summarized data (E) presenting caspase-1 inhibitor Z-YVAD reduces the abundance of α -SMA induced by TGF- β 1 (n=6). * P < 0.05 vs. Ctrl, # P < 0.05 vs. TGF- β 1. Ctrl: control; Cas-7: caspase-7.

Moreover, knocking down of caspase-7 gene *in vitro* also demonstrated an anti-fibrotic effect. Taken together, these results suggest caspase-1 and caspase-7 signaling is one of the major mechanisms responsible for renal tubulointerstitial fibrosis, and executioner caspase-7 is the downstream effector of inflammatory caspase-1 during development of UUO induced kidney fibrosis.

Progressive tubulointerstitial fibrosis is the final common pathway for all kidney diseases leading to chronic renal failure [29]. Renal interstitial fibrosis typically resulted from chronic inflammation through production of related molecules, such as angiogenic factors, growth factors, fibrogenic cytokines and proteinase [30]. Renal inflammation is a universal response to infectious and noninfectious triggers. Non-microbial inflammation is an important component of many acute and chronic renal diseases [31-33]. NLRP3 inflammasome is an important contributor of inflammation via cleavage of pro-IL-1 β and -18 [34]. Growing evidences show that NLRP3 inflammasome plays a role in renal diseases, such as acute kidney injury (AKI), diabetic nephropathy and CKD [35-37]. As such, caspase-1, a typical inflammatory caspase, is activated within inflammasome. After activation, caspase-1 can process the effector caspases, like caspase-3 and caspase-7. These proteolytic caspase activation cascades presumably constitute an intracellular mechanism for signaling. Although long assumed to be functionally redundant with caspase-3, caspase-7 is revealed to play distinct roles in apoptosis and inflammation [38, 39]. Caspase-7 $^{-/-}$ MEFs are more resistant

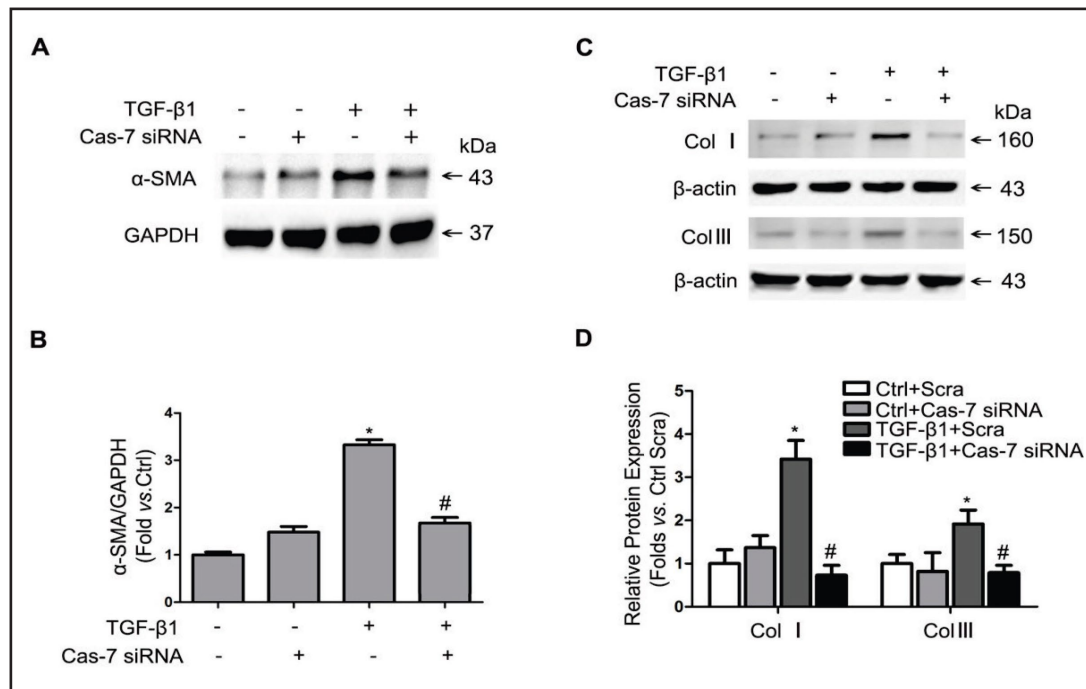


Fig. 5. Genetic deletion of caspase-7 attenuates TGF-β1 stimulated TEC's transdifferentiation and ECM aggregation *in vitro*. NRK-52E cells (TECs) were transfected with caspase-7 siRNA or scrambled siRNA. After 24 hours, TGF-β1 (5 ng/ml) was added and cells continued to be cultured for 48 hours. A and B. Representative Western blot images (A) and summarized data (B) showing caspase-7 siRNA transfection alleviates TGF-β1 stimulated α-SMA elevation (n= 6). *P < 0.05 vs. Ctrl, #P < 0.05 vs. TGF-β1. D and E. Representative Western blot images (D) and summarized data (E) showing that caspase-7 siRNA transfection reverses collagen I and collagen III accumulation induced by TGF-β1 exposure (n=5). *P < 0.05 vs. Ctrl Scra, #P < 0.05 vs. TGF-β1+ Scra. Ctrl: control; Col III: collagen III; Scra: scrambled siRNA; Cas-7 siRNA: caspase-7 siRNA.

to FasL- and UV-induced apoptosis than caspase 3^{-/-} MEFs, yet double knockout MEFs are even more resistant [22]. In macrophages stimulated with lipopolysaccharides (LPS) and ATP or infected with the Gram-negative pathogens *Salmonellatyphymurium* and *Legionella pneumophila*, caspase-7 activation requires caspase-1 complexes named inflammasome, but not the caspase-8 and -9 protein complexes involved in apoptosis [19, 38]. In addition, targeted peptide-centric proteomics identified caspase-7 as a caspase-1 substrate [40]. However, a direct link between these caspases remains to be formally established. So far, there is no evidence whether this kind of caspase cascades are involved in the pathogenesis of renal interstitial fibrosis. Therefore, we firstly examined caspase-7 expression and activation in TGF-β1 treated TECs. Caspase-7 activation in TGF-β1 treated TECs was indeed observed, with the peak at stimulation for 24 h. *In vivo* study of UUO mouse model showed enhanced caspase-7 activation in 1, 3 and 7 days of UUO. The results are in agreement with our initial hypothesis that caspase-7 contributes to renal fibrosis.

As it has long been recognized, α-SMA is an indicative marker for myofibroblasts activation. Although its precise origin is diverse and still controversial [41], myofibroblast remains the main effector cells during renal fibrosis. In an attempt to further clarify the role and associated cascades of caspase-7 in renal fibrosis, we performed UUO on caspase-1^{-/-} mice, because the NLRP3 inflammasome is involved in CKD and caspase-1 is a critical component and mediator of the function of NLRP3 inflammasome. Our current data demonstrated that caspase-1 abortion suppressed caspase-7 activation in UUO mouse

kidney. UUO mice of caspase-1 deficiency showed lower kidney α -SMA expression and less ECM deposition than WT mice. There were also studies indicating that TGF- β 1 induced epithelial-mesenchymal transition (EMT) in cultured tubular epithelial cells (TECs) [42]. In our experiment, the expression of α -SMA was markedly elevated by TGF- β 1 stimulation on TECs. However, this elevation was reduced by pretreatment with caspase-1 specific inhibitor. Previous work reported that the NLRP3 inflammasome promoted renal inflammation and contributed to CKD [37]. Very recently, another study found NLRP3 deletion protected against renal fibrosis in mouse with 5/6 nephrectomy model [18]. Our results are in accordance with previous studies. Together, these data suggests caspase-1 is the upstream inducer of caspase-7, and this signaling cascade plays a major role in UUO induced kidney fibrosis. In addition, direct inhibition of caspase-7 by specific siRNA in TGF- β 1 treated TECs decreased myofibroblasts activation and ECM deposition. Collectively, our study demonstrates that caspase-1 activation is an upstream event of caspase-7 cleavage and activation in renal tubulointerstitial fibrosis. Inhibition of caspase-1 and caspase-7 signaling can protect kidney from fibrotic injury.

Conclusion

Inflammatory caspase-1 and apoptotic caspase-7 together participate in TIF. Caspase-1 activation is an upstream event of caspase-7 cleavage and activation in TIF. Inhibition of caspase-1-caspase-7 pathway can alleviate renal tubulointerstitial fibrosis injuries. So the cascade signaling is a promising target for treatment of renal fibrosis in clinical practice in the future. Yet, the downstream event of caspase-1 and caspase-7 signaling in TIF still needs to be explored further.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No. 30900682, No.81570657, No. 31200872, No. 81470964, No. 81570671, No. 81400720, and No. 81522010).

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