

I,25-(OH)₂D₃ ameliorates renal interstitial fibrosis in UUO rats through the AMPK α /mTOR pathway

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Abstract

Objective: To investigate the effects of I,25(OH)₂D₃ on renal fibrosis associated with the AMP-activated protein kinase (AMPK) α /mechanistic target of rapamycin (mTOR) signalling pathway in a rat model of unilateral ureteral obstruction (UUO).

Methods: A total of 54 male Sprague Dawley rats were randomly divided into three groups: sham-operation group, UUO group, and UUO plus calcitriol (3 ng/100 g) group. Renal tissue was excised for histological examination by immunohistochemistry and Western blot, and for gene expression analysis using real-time polymerase chain reaction.

Results: I,25(OH)₂D₃ enhanced AMPK α levels, inhibited mTOR levels and slowed the development of interstitial fibrosis in kidney tissue. Compared with the UUO plus calcitriol group, UUO rats demonstrated more severe renal damage characterized by marked tubular atrophy, interstitial fibrosis and significant induction of fibrogenic transforming growth factor- β 1 and increased extra-cellular matrix proteins (α -smooth muscle actin and collagen type III), and decreased E-cadherin.

Conclusion: Treatment with I,25(OH)₂D₃ altered the AMPK α /mTOR signalling pathway to suppress excessive fibroblast activation observed in UUO rats. This may serve as a novel mechanism to ameliorate renal dysfunction and fibrotic lesions.

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Introduction

Chronic kidney disease (CKD), as a worldwide public health issue, is gaining increasing attention. Clinical studies have shown that the circulating level of active Vitamin D, 1,25(OH)₂D₃ (calcitriol), is substantially reduced in patients with chronic renal insufficiency, and supplementation with active Vitamin D results in significant amelioration of renal dysfunction and fibrotic lesions.¹ Furthermore, calcitriol has been indicated to exert a renoprotective effect in various kidney diseases. In addition to its actions on calcium and phosphorus metabolism, and on bone formation and mineralization, vitamin D exerts other biological activities, including modulation of the renin–angiotensin system via renin suppression, decreasing urinary albumin excretion, and anti-inflammatory and anti-fibrosis effects.¹

Renal fibrosis is a typical and common pathway leading to end-stage organ diseases, including acute kidney injury and CKD.² Renal inflammation is an initial response to kidney injury and is a key process leading to CKD. The whole process of CKD is manifested by persistent inflammatory injury, excessive extracellular matrix (ECM) accumulation, and decreased parenchymal cells, and finally leads to the destruction of renal parenchyma, progressive loss of kidney function, and end-stage renal disease.³ Transforming growth factor (TGF)- β 1, a multifunctional cytokine, is an essential mediator in the pathogenesis of fibrosis. In the setting of renal obstruction, ischaemia hypoxia stimulates the renal production of TGF- β 1 and sets in motion a

signalling cascade that promotes fibrogenesis. Stimulation of TGF- β 1 has been shown to aggravate renal fibroblast activation and ECM synthesis.⁴ Meanwhile, epithelial-mesenchymal transition, which involves loss of the epithelial marker E-cadherin, expression of α -smooth muscle actin (SMA) and actin reorganization, is essential in the process of ECM.⁵

Calcitriol has been reported to exert anti-fibrosis effects through various mechanisms to protect intestinal and renal function and structure.^{6–8} AMP-activated protein kinase (AMPK) α is a member of a serine (Ser)/threonine (Thr) kinase family expressed in various organs (heart, lung, liver, kidney), and regulates the activities of a number of enzymes through phosphorylation. In addition to regulating energy homeostasis and metabolism, AMPK α is also a pivotal energy sensor that alleviates or delays the process of fibrogenesis.⁹ Increasing evidence has revealed that AMPK α protects against fibrosis in the heart,¹⁰ liver,¹¹ lung¹² and kidney.¹³ Mechanistic target of rapamycin (mTOR) is one of the downstream targets of AMPK α that senses the cellular environment and is activated by hormones, nutrients, and various stress conditions.^{14,15} AMPK α and mTOR play critical and opposing roles in cellular metabolism, energy homeostasis and cell growth.^{16,17} Furthermore, mTOR has been highlighted as an important regulator of renal diseases, and mTOR phosphorylation was shown to be significantly increased in the kidneys of rats with unilateral ureteral obstruction (UUO).¹⁸ Activation of mTOR in the kidney promotes upregulation of proinflammatory and profibrotic factors,

which leads to tubulointerstitial fibrosis and atrophy.¹⁹

Active vitamin D has been reported to activate chondrocyte autophagy to reduce osteoarthritis via mediating the AMPK/mTOR signalling pathway.²⁰ In addition, vitamin D3 has been shown to potentiate the growth inhibitory effects of metformin in DU145 human prostate cancer cells via the AMPK/mTOR signalling pathway.²¹ The aim of the present study was to explore whether calcitriol may delay renal fibrosis through activation of AMPK α and inhibition of mTOR in a well-established rat model of UO that demonstrates renal interstitial fibrosis (RIF)-like histology and pathology, characterized by tubular dilatation, epithelial cell sloughing and inflammatory cell infiltration.²² To the best of the authors knowledge, this is the first study to evaluate the effect of calcitriol on RIF through regulation of the AMPK/mTOR pathway.

Materials and methods

Animal model and study design

A total of 54 healthy male Sprague Dawley rats that weighed approximately 190–210 g (aged 6–8 weeks), were provided by the Laboratory Animal Centre of Shanxi Medical University, Taiyuan, China. All rats were housed under controlled environmental conditions ($22 \pm 3^\circ\text{C}$, 50–55% humidity and a 12-h light/ 12-h dark cycle). All experimental protocols were approved by the Ethics Committee of Shanxi Medical University (approval No. 20150001) and performed in accordance with the National Institute of Health (NIH) guidelines (NIH Publication, No. 80-23, revised 1978). All efforts were made to minimize the number of animals used and their suffering.

The animals were randomly divided into three groups ($n=18$ per group):

sham-operated group, UO group, and UO plus calcitriol group. Rats were housed in a standard facility with free access to food and water for 7 days prior to the experiment, then rats were subjected to the UO operation. Briefly, UO was performed under anaesthesia using 100 mg/kg body weight ketamine plus 5 mg/kg body weight xylazine, by intraperitoneal injection. A midline incision was made and the left ureter was exposed and tied off at two points. The sham groups received the same procedure except for ureteral ligation. Following the operation, the UO plus calcitriol group received calcitriol soft capsules (Rocaltrol; Roche, Shanghai, China) by daily gavage at the dose of 3 ng calcitriol/100 g body weight. As a control, the other two groups received a daily gavage of 3 ml peanut oil. Groups of rats ($n=6$ from each study group) were randomly selected on day 1, 3 and 7 following the UO operation, and kidney tissues were removed under anaesthesia (100 mg/kg body weight ketamine plus 5 mg/kg body weight xylazine, by intraperitoneal injection) for various analyses. Rats were then immediately euthanized under anaesthesia.

Histological analysis

Renal tissues were excised, fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (4- μm thick) were deparaffinized, rehydrated, and stained with haematoxylin and eosin (H&E). Tissue fibrosis levels were assessed with Masson staining using the ponceau – brilliant green staining method, in which collagen fibres are shown as green. All samples were mounted in resin following staining. A total of 20 random high-power fields (magnification, $\times 200$) of renal parenchyma (excluding the glomerulus) were selected using an Olympus microscope and Olympus Cell F software (Olympus, Tokyo, Japan). The relative value of tissue

fibrosis was defined as relative renal interstitial area as a percentage of the vision field area. Subsequent semi-quantitative analyses were performed using Image Pro Plus software, version 6.0 (Viscon Medical Electronics Company, Shanghai, China).

Real-time PCR

To analyse relative *TGF-β1* gene expression in kidney tissue, *TGF-β1* mRNA was assessed using reverse-transcription followed by real-time polymerase chain reaction (PCR). Total RNA was extracted from 25 mg renal tissue using a TRIZOL RNA extraction kit (Bao Bioengineering; Dalian, China) according to the manufacturer's protocol, and cDNA was then reverse transcribed from 1 μg total RNA using a cDNA Reverse Transcription kit (Bao Bioengineering). The *TGF-β1* cDNA was then amplified by real-time PCR using SYBR Green PCR Master Mix (Bao Bioengineering) and the following primer pairs: *TGF-β1*, upstream 5'-GGACTAC TACGCCAAGAAG-3', and downstream 5'-TCAAAAGACAGCCACTAGG-3'.

β -actin was amplified as an endogenous control for normalization using the following primers: upstream 5'-TGAAGTACC CCATTGAACACGG-3', and downstream 5'-TGCTCGAAGTCTAGGGCAACATA-3'. PCR was performed using an Applied Biosystems 7900 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), and the following cycling conditions: initial denaturation at 95°C for 30 s, and then 35 cycles of denaturation at 95°C for 5 s and annealing and elongation at 65°C for 35 s. Relative levels of mRNA were calculated using the $\Delta\Delta$ CT method of analysis. Each assay was performed in triplicate.

Immunohistochemical analysis

Immunohistochemistry was performed on paraffin-embedded tissues. Briefly, tissue

sections were mounted onto glass slides, and deparaffinized and rehydrated using standard techniques. 3% H₂O₂ was used to inactivate endogenous peroxides. After microwave radiation antigen retrieval, non-specific binding was blocked with bovine serum albumin (BSA). Primary antibodies (rabbit polyclonal anti- α -SMA, rabbit monoclonal anti-collagen III, and anti-E-cadherin), at working dilutions of 1: 200 (all Abcam; Cambridge, UK) were added to the slides and incubated for 2 h at 37°C. Slides were then washed with phosphate buffered saline (PBS) before incubating with biotinylated goat anti-rabbit IgG secondary antibody (Beyotime Biotechnology; Jiangsu, China) for 40 min at 37°C. Slides were washed again with PBS and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Boster Biological Technology; Pleasanton, CA, USA) for 20 min at 37°C, and the immunoreactivity was visualized with 3,3-diaminobenzidine (Zhongshan Golden Bridge Biotechnology; Beijing, China). PBS replaced the primary antibody as the negative control. Positive immunostaining was evaluated using Image Pro Plus software, version 6.0. The mean densities of α -SMA, collagen III, and E-cadherin were calculated as integrated optical density (IOD) of the positive signal per field divided by the total area of the measured region.

Western blot analysis

Renal tissue was lysed in RIPA buffer (25 mM tris-HCl [pH 8.0], 1% Nonidet-P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS], and 125 mM NaCl) containing 1% PMSF for 30 min at 4°C. Total protein extract samples (about 50 μg) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE Gel Preparation kit [Beyotime Biotechnology]) and transferred onto a polyvinylidene fluoride (PVDF) membrane.

The membrane was blocked with 2% BSA for 2 h at room temperature, then incubated overnight at 4°C with primary antibodies (rabbit monoclonal anti-p-AMPK α [Thr172] and anti-AMPK α , mouse monoclonal anti-p-mTOR [Ser 2448] and anti-mTOR, rabbit monoclonal anti-TGF β 1, and β -actin [control]; dilution 1: 200; all Abcam). After washing three times (15 min each) with Tris-HCl-Tween buffer (pH 7.4), the membrane was incubated with HRP-conjugated goat anti-mouse (or anti-rabbit) IgG secondary antibodies (dilution 1: 5000; Beyotime Biotechnology) for 1 h at room temperature. Protein signal was visualized with an enhanced chemiluminescence detection kit (Boster Biological Technology) according to the manufacturer's instructions. Relative band intensities normalised to β -actin were determined by densitometry using NIH Image J software, version 1.8.0.

Statistical analyses

Data are presented as mean \pm SD. Between-group differences were analysed using one-way analysis of variance followed by Least Significant Difference test. All statistical analyses were carried out using GraphPad Prism software, version 7 (GraphPad Software, La Jolla, CA, USA) with statistical significance set at $P < 0.05$.

Results

Calcitriol ameliorates the histopathology changes and collagen deposition in RIF

To investigate the therapeutic potential of calcitriol in CKD, 3 ng calcitriol/100 g of body weight was administered via daily gavage for 7 days in UUO rats. On day 1, 3 and 7 following the UUO operation and after the calcitriol gavage, rat renal tissue was obtained for H&E and Masson staining to analyse the effect of calcitriol on renal histopathology changes.

Analysis of H&E-stained sections (Figure 1a) revealed that the sham group demonstrated no anomalous renal pathological changes in morphology during the 7-day period, such as glomerular

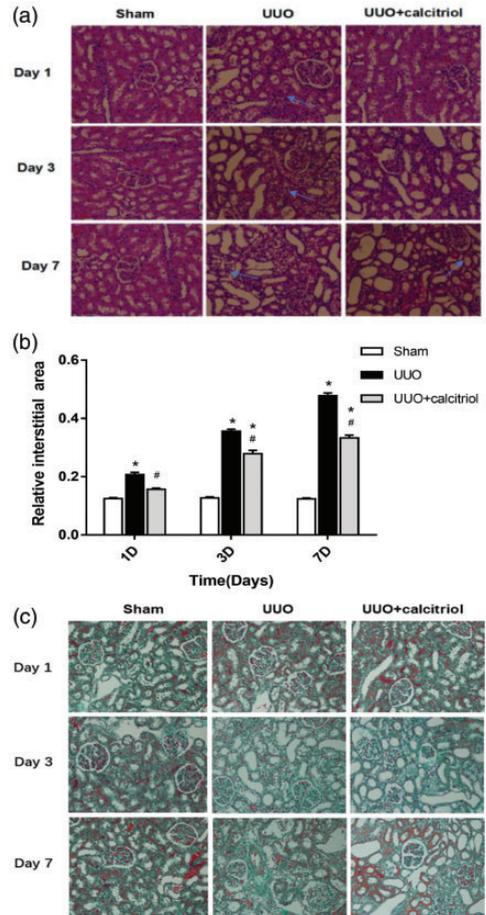


Figure 1. Calcitriol ameliorates histopathological changes and attenuates renal fibrosis in unilateral ureteral obstruction (UUO) rats. (a) Representative photomicrographs of haematoxylin and eosin-stained kidney sections; (b) bar graph showing semiquantitative analysis of renal interstitial collagen content based on Masson staining; and (c) representative photomicrographs of Masson-stained kidney sections. Data presented as mean \pm SD; * $P < 0.05$ versus sham group, # $P < 0.05$ versus UUO group ($n = 6$ rats per group; original magnification, $\times 200$).

hypertrophy, mesangial cell proliferation, tubular dilatation or atrophy, tubular cast formation, degeneration and sloughing of tubular epithelial cells, or interstitial widening. In the UUO group, day 1 presented with infiltration of a small number of inflammatory cells, negligible tubular dilatation and basically normal glomeruli. On day 3, H&E staining demonstrated slight tubular dilatation, and increased inflammatory cell infiltration in the interstitium along with mild interstitial widening. Renal histology worsened over time and presented as denatured epithelial cells, and disordered interstitial structure accompanied by wider interstitium. In the UUO plus calcitriol group, the above changes were dramatically attenuated compared with those in the UUO group on all test days.

Masson-stained tissue sections were analysed, and the renal-interstitium/visual-field ratio (relative interstitial area) was measured to assess the extent of renal interstitial fibre. The areas covered in green represented the level of fibrosis. On days 1, 3 and 7, relative areas in the calcitriol treatment group were significantly decreased compared with the UUO group (0.207 ± 0.032 versus 0.157 ± 0.004 , 0.356 ± 0.007 versus 0.279 ± 0.012 , and 0.478 ± 0.009 versus 0.333 ± 0.011 , respectively; all $P < 0.05$; Figure 1b). When relative interstitial area was ranked in the order of largest to smallest area, the UUO group was ranked highest, followed by UUO plus calcitriol, followed by the sham group ($P < 0.05$; Figure 1b). Representative photomicrographs of Masson-stained tissue fibrosis are shown in Figure 1c.

Calcitriol inhibits TGF- β 1 and fibrogenic protein levels to ameliorate fibrosis

Western blot analysis showed that TGF- β 1 protein levels were significantly increased in the UUO versus sham group ($P < 0.05$), and the increase was significantly

attenuated by treatment with calcitriol ($P < 0.05$) on all test days (Figure 2Aa and b). RT-PCR results showed that TGF- β 1 mRNA levels were also significantly increased in the UUO group compared with the sham group, and the increase was significantly suppressed by calcitriol ($P < 0.05$; Figure 2Ac). Thus, TGF- β 1 was shown to be up-regulated in the obstructive kidney at both the protein and mRNA level, while calcitriol down-regulated its expression.

Immunohistochemical analyses showed that α -SMA and collagen III increased over time in response to obstructive injury, whereas E-cadherin decreased over time (all $P < 0.05$ versus sham group; Figure 2B, C and D). In the calcitriol-treated UUO group, changes in fibrogenic protein were revealed to corresponded with the inhibition of TGF- β 1, such as attenuation of α -SMA and collagen III, and augmentation of E-cadherin (all $P < 0.05$ versus UUO group; Figure 2B, C and D).

The results suggest that calcitriol may be an important protective factor in rat renal interstitial cells following fibroblast activation, and this protective function may be partly attributable to inhibition of the profibrotic factor TGF- β 1. Thus, it was considered that calcitriol may suppress the involvement of RIF in a direct or indirect manner.

Calcitriol attenuates RIF in UUO rats via the AMPK α /mTOR signalling pathway

Western blot analysis was performed to quantify the changes in phosphorylated and total AMPK α and mTOR levels in UUO rats and UUO rats treated with calcitriol. Levels of p-AMPK α were inhibited and p-mTOR levels were increased in UUO rats ($P < 0.05$ versus sham group). Calcitriol treatment significantly enhanced the p-AMPK α levels and inhibited the p-mTOR levels ($P < 0.05$ versus UUO

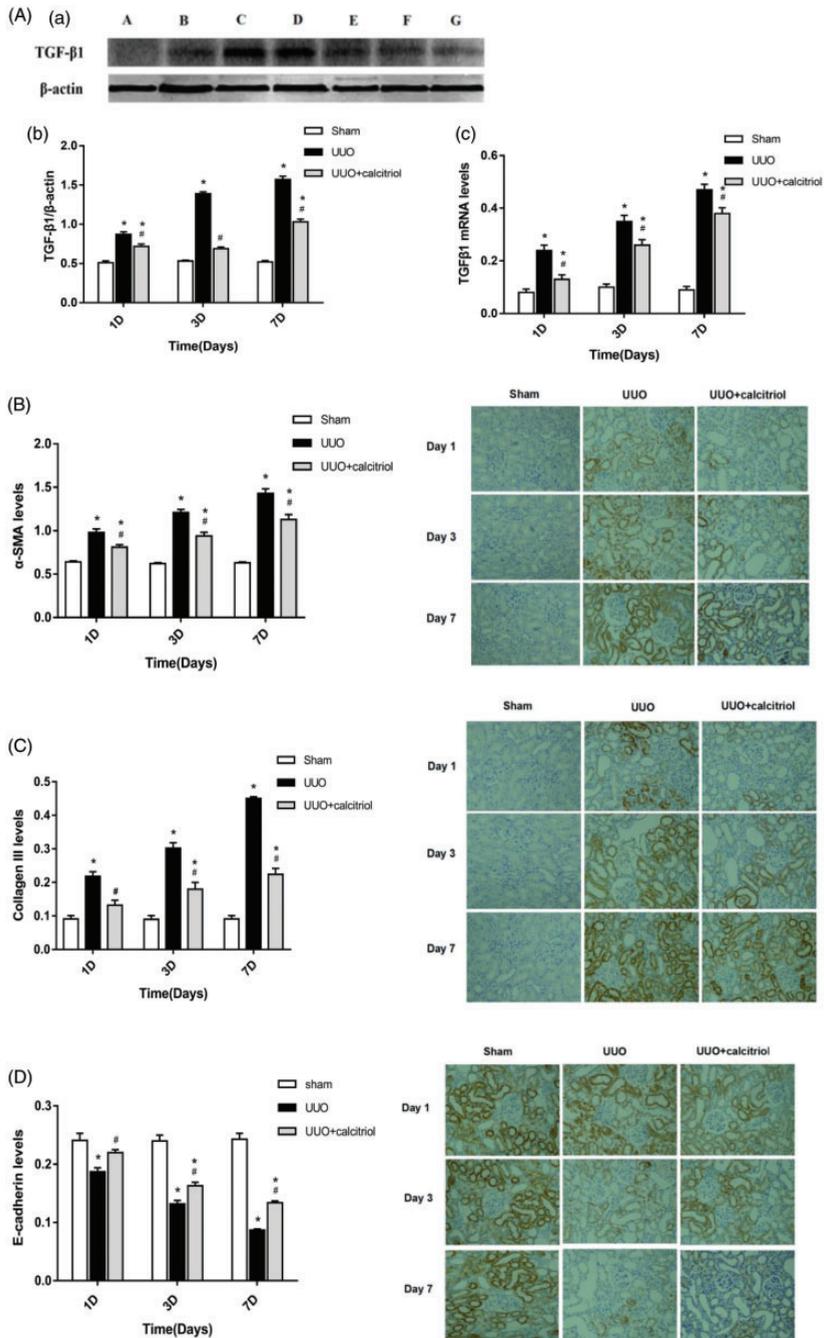


Figure 2. Calcitriol diminishes expression of the fibrosis index in unilateral ureteral obstruction (UO) rats. (Aa) Representative Western blot of transforming growth factor (TGF)- β 1 in different study groups at different time-points (A, sham group; B–D, UO group at days 1, 3 and 7; E–G, UO plus calcitriol group at days 1, 3 and 7), (Ab) bar graph showing TGF- β 1 protein levels normalised to β -actin, and (Ac) bar graph showing TGF- β 1 mRNA levels; (B, C and D) bar graphs of integrated optical density and representative photomicrographs of kidney sections immunostained for α -smooth muscle actin (SMA), collagen III, E-cadherin (brown) and counter-stained with haematoxylin. Data presented as mean \pm SD; * $P < 0.05$ versus sham group, # $P < 0.05$ versus UO group ($n = 6$ rats per group; original magnification, $\times 200$).

group; Figure 3). Interestingly, in the UUO group, p-AMPK α levels showed a mild numerical peak on day 3, but t-AMPK α did not show the same trend (data not shown).

Discussion

Chronic kidney disease (CKD) has emerged as a world-wide public health issue,²³ with an estimated prevalence rate of 8–16% worldwide.²⁴ To date, there is no effective therapy for this devastating disorder. Thus, it is essential to understand the mechanisms behind renal fibrosis to facilitate the development of therapies to prevent or reverse

this process and slow down the progression of CKD. Considerable evidence indicates that AMPK activators may attenuate renal fibrosis by activating AMPK. Thus, the present study aimed to establish a therapeutic relationship between Vitamin D and RIF via the AMPK α /mTOR signalling pathway.

AMP-activated protein kinase has been proven to protect the kidneys from injury-induced fibrosis by counteracting TGF- β /Smad3 and mTOR signalling.^{25,26} Qiu et al.²⁷ discovered that the knockdown of AMPK α enhances renal epithelial trans-differentiation. Likewise, AMPK α deficiency was shown to enhance

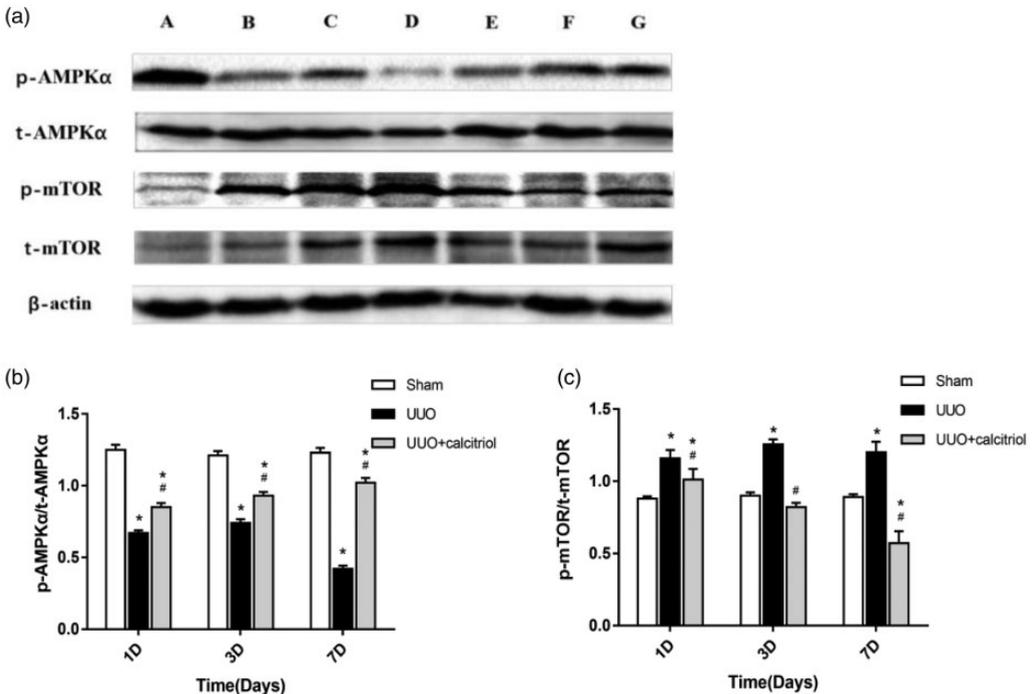


Figure 3. Altered activities of AMP-activated protein kinase (AMPK) α and mechanistic target of rapamycin (mTOR) in response to calcitriol in renal tissues of unilateral ureteral obstruction (UUO) rats. (a) Representative Western blots showing protein levels of phosphorylated (p)-AMPK α , p-mTOR, total (t)-AMPK α , and t-mTOR in the kidney at days 1, 3, and 7 of calcitriol treatment (A, sham group; B–D, UUO group at days 1, 3 and 7; and E–G, UUO plus calcitriol group at days 1, 3 and 7); (b) bar graph showing levels of p-AMPK α /t-AMPK α normalised to β -actin; and (c) bar graph showing levels of p-mTOR/t-mTOR normalised to β -actin. Data presented as mean \pm SD; * P < 0.05 versus sham group, # P < 0.05 versus UUO group (n = 6 rats per group).

epithelial-mesenchymal transition and inflammatory infiltration in a mouse unilateral ureteral obstruction (UUO) model, and correspondingly exacerbate renal fibrosis.²⁷ In contrast, activated AMPK α may reverse renal epithelial-mesenchymal transition and reduce the level of fibrosis.²⁸ Furthermore, with an insufficiency of calcitriol, AMPK activity has been shown to decrease.²⁹ These findings reveal that knockout or inactivation of AMPK α promotes fibrogenesis, while activation of AMPK α might be a possible treatment for fibrosis. Consistent with the above studies, the present results show that *AMPK* expression is inhibited in UUO rats, and the level of AMPK recovers under calcitriol treatment, suggesting that calcitriol may serve as an AMPK agonist to delay renal interstitial fibrosis.

In terms of AMPK/mTOR, previously published studies have indicated an association between the expression of *AMPK* and *mTOR*.^{30,31} mTOR is a protein kinase regulated by the AMPK signalling molecule. AMPK phosphorylates TSC complex subunit 2 and Raptor to inhibit the mTOR complex1 (mTORC1) pathway, which results in the inhibition of mTOR.¹⁵ mTOR regulates the expression of TGF- β 1 and mTOR activation is critically involved in epithelial-mesenchymal transition, an important process in kidney fibrosis.³² Levels of p-mTOR have been shown to be significantly increased in the UUO kidney, and treatment with an mTOR inhibitor ameliorated kidney injury through inhibition of TGF- β 1 and proinflammatory cytokines.¹⁸ As the present results showed, levels of phosphorylated AMPK were decreased in UUO rats, and the inhibition of AMPK further induced the activation of mTOR, subsequent to the increase of TGF- β and fibrotic proteins. Vitamin D supplementation can stimulate the expression of *AMPK α* but inhibit the mTOR pathway.^{33,34} Similarly, calcitriol was found to significantly attenuate p-mTOR levels in

the present study while significantly up-regulating levels of p-AMPK α , which also concurs with a previous report.³⁵ Interestingly, in the UUO group, p-AMPK α levels showed a mild peak on day 3, but t-AMPK α did not show the same trend (data not shown). AMPK α is activated to curtail energy consumption while cellular energy is depleted. Furthermore, TGF- β -activated kinase (Tak1), the key factor in the renal fibrotic response, has been shown to activate AMPK α to promote renal fibrosis. It may be that the vigorous biochemical reaction following UUO depleted cellular energy, and Tak1-induced RIF plays the dominant role in the early period of RIF, and thus the earlier level of AMPK α was higher than the later period.³⁶ In summary, calcitriol was shown to regulate AMPK/mTOR signalling by inducing p-AMPK α activity and attenuating mTOR phosphorylation. Such an effect of calcitriol on signalling molecules might have contributed to its involvement in ameliorating UUO-induced RTF.

There are some limitations to the results of the present study. Vitamin D is being shown to play an increasingly important role in diverse organ and tissue fibrosis associated with many complicated signalling pathways. However, the present study only explored the AMPK α /mTOR pathway. Therefore, the underlying interaction between Vitamin D and RIF requires further exploration. Furthermore, although Vitamin D may be effective in renal fibrosis, the associated dysfunction of serum calcium homeostasis may prevent its clinical application. Therefore, the drug safety of Vitamin D in humans should be examined in future studies. In addition, a mild peak in AMPK α was observed at day 3. Contrary to the present results, Wang et al.³⁶ observed that AMPK α 1 contributes to the development of renal fibrosis. Given the fact that AMPK α induces various signalling pathways to work in different periods and

to exert diverse effects on different tissue,³⁰ the interaction between AMPK α -induced signalling pathways requires further research.

In conclusion, the present results demonstrate that 1,25(OH)₂D₃ may have prophylactic effects on renal interstitial fibrosis associated with the AMPK α /mTOR pathway. The study yields a novel insight into the signalling mechanism between 1,25(OH)₂D₃ and RIF. Despite these results, further research is required to explore the underlying mechanisms and potential limitations of treatment.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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