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Original Research

Triptolide Inhibits Renal Fibrosis Through Promotion of PGC1α/PCK1-mediated Renal Gluconeogenesis

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Abstract

Background: Recent studies have identified impaired renal gluconeogenesis as a hallmark of chronic kidney disease. Triptolide is a natural compound widely used in China for the treatment of renal diseases. This study investigated whether triptolide mitigates renal fibrosis by promoting renal gluconeogenesis. **Methods**: Renal fibrosis was induced *in vivo* by unilateral ureteral obstruction (UUO) surgery in mice. Transforming growth factor-β (TGF-β)-stimulated human kidney-2 (HK-2) cells were used as an *in vitro* model to investigate renal fibrosis. Metabolomics, western blotting, immunohistochemistry (IHC), and metabolic assays were performed to investigate the underlying mechanisms. **Results**: Triptolide reduced the expression of several fibrotic markers in the kidneys of UUO mice. Metabolomic analysis revealed enhanced renal gluconeogenesis following treatment with triptolide, which was confirmed by analyzing gluconeogenic enzyme expression and lactate concentration in UUO kidneys. The pro-gluconeogenic effect of triptolide was further confirmed in TGF-β-stimulated HK2 cells. Inhibition of phosphoenolpyruvate carboxykinase 1 (PCK1) reversed the anti-fibrotic and pro-gluconeogenic effects of triptolide in TGF-β-stimulated HK2 cells. We further demonstrated that peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC1α) expression was downregulated in TGF-β-stimulated HK2 cells and UUO kidneys, and that triptolide reversed this downregulation. Moreover, the PGC1α inhibitor reversed the effect of triptolide on PCK1 expression and glucose metabolism. Finally, IHC analysis revealed that triptolide inhibited histone lactylation in UUO kidneys, which was associated with a decreased production of inflammatory factors and reduced macrophage infiltration. **Conclusions**: Triptolide may inhibit renal fibrosis by increasing the PGC1α/PCK1 axis, thereby promoting renal gluconeogenesis. This cascade may reduce histone lactylation and renal inflammation, providing a mechanistic pathway for its anti-fibrotic effect.

Keywords: triptolide; gluconeogenesis; fibrosis; lactic acid; kidney

1. Introduction

Chronic kidney disease (CKD) is a significant public health concern, affecting more than 10% of the global population [1]. Renal fibrosis is a common pathological pathway and key manifestation of CKD progression [2]. It is characterized by the excessive deposition of extracellular matrix (ECM) in the kidney, such as fibronectin and collagen-I in renal interstitial regions [3]. Furthermore, the upregulation of epithelial-mesenchymal transition (EMT) markers and activation of the transforming growth factor-beta (TGF-β) signaling pathway are well-recognized hallmarks of renal tubulointerstitial fibrosis [4].

Impaired renal gluconeogenesis has recently been identified as a key feature of acute and chronic kidney diseases [5–7]. Clinically, this impairment is associated with poor renal prognosis and increased mortality in patients with acute or chronic kidney dysfunction [5,7]. Overexpression of tubular phosphoenolpyruvate carboxykinase 1 (PCK1), an important enzyme involved in renal gluconeogenesis, inhibits disease progression in an animal model of CKD [8]. These findings suggest that improving renal gluconeogenesis is a novel therapeutic strategy to halt CKD progression.

Given the critical role of gluconeogenesis in various disease states, the regulatory mechanisms governing

this process have been the focus of extensive research [9,10]. Peroxisome proliferator-activated receptor-gamma co-activator 1 alpha (PGC1α) is widely recognized for its role in mitochondrial quality control [11]. Studies have demonstrated that PGC1α can upregulate the expression of key gluconeogenic genes, including PCK1 and fructose-bisphosphatase 1 (FBP1), through both Forkhead Box Protein O1 (FOXO1)-dependent and -independent pathways [6,12].

Triptolide, a diterpenoid trioxide isolated from the medicinal plant *Tripterygium wilfordii* Hook F [13,14], has been demonstrated to confer renoprotective effects through various mechanisms, including anti-fibrotic, anti-inflammatory, and immunosuppressive actions [15]. Clinically, triptolide-containing formulations have been used to treat human renal diseases such as nephritis, minimal change disease, and membranous nephropathy [16,17]. Preclinical studies in animal models further substantiate its advantages. Triptolide inhibits renal fibroblast activation, attenuates renal tubular EMT, and reduces collagen deposition in the renal interstitium [16]. However, the complete spectrum of mechanisms underlying triptolide-mediated renoprotection remains unclear. Notably, recent studies have reported that triptolide improves glucose metabolism in intrahepatic cholangiocarcinoma and rheumatoid arthritis models [18,19], prompting the question of whether it exerts similar effects on glucose metabolism in CKD, a prospect that remains unexplored.

In this study, we aimed to evaluate the hypothesis that triptolide inhibits renal fibrosis by improving renal gluconeogenesis.

2. Materials and Methods

2.1 Animal Studies

Wild-type male C57BL/6 mice (body weight 20–25 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd., and housed in a specific pathogen-free-grade animal facility in the Shanghai University of Traditional Chinese Medicine under the local regulations. Shanghai University of Traditional Chinese Medicine has approved the animal experiments (PZSHUTCM18111601).

Mice were anesthetized with pentobarbital sodium (P3761; Sigma-Aldrich Co., St. Louis, MO, USA) at a dose of 100 mg/kg via intraperitoneal (i.p.) injection before surgery. The UUO surgery was performed by ligating the left ureter twice using 4-0 nylon sutures. Mice were randomly divided into four groups: (1) Sham/vehicle (n = 6), (2) Sham/triptolide (n = 7), (3) UUO/vehicle (n = 7), and (4) UUO/triptolide (n = 7). Triptolide (Topscience, T2179, Shanghai, China) was dissolved in dimethylsulfoxide (DMSO) and diluted with normal saline to prepare a working solution containing 1% DMSO. Sham or UUO mice were treated with vehicle or 0.25 mg/kg triptolide daily by i.p. injection for 10 days starting from day 0. The triptolide dosage (0.25 mg/kg/day) was selected based on a previous study that demonstrated its protective effect against renal disease in mice [20]. Mice were sacrificed on day 10. Mice were euthanized by cervical dislocation under anesthesia with 100 mg/kg pentobarbital sodium at the experimental endpoint for kidney collection.

2.2 Cell Culture

Renal proximal tubular epithelial (HK2) cells were obtained from the Cell Bank of Shanghai Institute of Biological Sciences (Chinese Academy of Science, Cat. BFN60700259). The cell line used in this study was identified by short tandem repeat profiling and tested negative for mycoplasma. HK2 cells were seeded in 6-well plates to 40%–50% confluence and starved overnight with DMEM/F12 medium (GNM12400, GENOM, China) containing 0.5% fetal bovine serum. The following day, the medium was replaced with fresh medium containing 0.5% fetal bovine serum (04-001-1ACS, Biological Industries, Israel), and the cells were exposed to 2.5 ng/mL TGF-β (Peprotech, Rocky Hill, NJ, USA) for 48 h in the presence of different concentrations of triptolide. The triptolide concentration range (10–100 nM) was determined based on a previous study reporting a protective effect of triptolide on renal cells within this range [21]. In specific experiments, cells were treated with either 1 mM PCK1 inhibitor 3-mercaptopicolinic acid (3MPA, T12929, TargetMol, Shanghai, China) or 20 µM PGC1α inhibitor SR-18292 (T4353, TargetMol, Shanghai, China).

2.3 Masson's Trichrome

Mouse kidneys were sliced, fixed, and embedded in paraffin, and cut into 4 µm-thick sections. Paraffin-embedded kidney sections were stained with hematoxylin, followed by ponceau red liquid dye acid complex, and then incubated in phosphomolybdic acid solution. Finally, the tissues were stained with aniline blue liquid and acetic acid. Images were acquired using a microscope (Nikon 80i, Tokyo, Japan).

2.4 Western Blotting Analysis

Cell or kidney proteins were extracted using lysis buffer (P0013; Beyotime Biotech, Nantong, Jiangsu, China). The BCA Protein Assay Kit (P0012S, Beyotime Biotech, Nantong, Jiangsu, China) was used to quantify the protein concentration. Protein samples were dissolved in 5× SDS-PAGE loading buffer (P0015L; Beyotime Biotech, Nantong, Jiangsu, China) and subjected to SDS-PAGE. After electrophoresis, the proteins were electro-transferred to a polyvinylidene difluoride (PVDF) membrane (ISEQ00010; Merck Millipore, Darmstadt, Germany). Unspecific binding on the PVDF membrane was blocked by incubating with the blocking buffer (5% non-fat milk, 20 mM Tris-HCl, 150 mM NaCl, pH = 8.0, 0.01% Tween 20) for 1 h at room temperature, followed by incubation with primary anti-

bodies, including anti-fibronectin (1:1000, ab23750, Abcam), anti-collagen-I (1:500, sc-293182, Santa Cruz), anti-α-smooth muscle actin (α-SMA) (1:1000, ET1607-53, HUABIO), anti-β-actin (1:50,000, 66009-1-Ig, proteintech), anti-N-cadherin (1:1000, sc-59887, Santa Cruz), anti-vimentin (1:1000, R1308-6, HUABIO), anti-Snail (1:1000, A11794, ABclonal), anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:5000, 60004-1-lg, Proteintech), PCK1 (A2036, Abclonal, Port Talbot, UK, 1:1000), FBP1 (1:1000, 14405406, Raybiotech), PGC1α (1:1000, 144-62296-100, Raybiotech), and FOXO1 (1:1000, 144-60502-200, Raybiotech) overnight at 4 °C. Binding of the primary antibody was detected using an enhanced chemiluminescence method (SuperSignal™ West Femto, 34094, Thermo Fisher Scientific) with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, 1:1000, A0208, Beyotime or goat anti-mouse IgG, 1:1000, A0216, Beyotime).

2.5 Measurement of Gluconeogenic Metabolites

The renal cortex was collected for protein extraction. Protein samples were isolated using 10$\times$ normal saline via mechanical disruption. Lactate content was measured using a lactate test kit (A019-2-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) by measuring the optical density (OD) at 530 nm. Renal glucose concentration was determined by measuring OD at 505 nm using a glucose assay kit (F006-1-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China). All measurements were performed according to the manufacturer's instructions and standardized by protein concentration.

The supernatant was collected from HK2 cells and centrifuged to remove cell debris. Glucose and lactate concentrations were determined using commercial kits, as described previously.

2.6 Immunohistochemistry (IHC)

Paraffin-embedded tissue sections were dewaxed and immersed in a retrieval vessel containing citrate-based antigen retrieval buffer (pH 6.0, RC03; Shanghai Huilan Biotech) for high-pressure antigen retrieval. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. The sections were incubated overnight at 4 °C with primary antibodies: Histone H4 (Lys12) (PTM-1411RM, PTM bio, Hangzhou, Zhejiang, China, 1:100), F4/80 (HA721745, HUABIO, Hangzhou, Zhejiang, China, 1:100), or interleukin-1 beta (IL-1β; A1112, ABclonal, Wuhan, Hubei, China, 1:100). Subsequently, the sections were incubated with a secondary antibody (sheep anti-rabbit HRP, RCA054/RC0080RM, Shanghai Huilan Biotech) at 37 °C for 30 min, followed by 3,3′-diaminobenzidine (DAB) color development (RCD002; Shanghai Huilan Biotech) for nuclear staining. Kidney sections were counterstained with hematoxylin, dehydrated sequentially, mounted with neutral gum, and examined under a microscope. Images were acquired using a scanner system (KF-PRO-005; KFBIO Technology Co., Ltd., Ningbo, Zhejiang, China).

2.7 Non-targeted Metabolomics

Non-targeted metabolomics analysis was performed by Beijing Biomarker Technologies Co., Ltd. (Beijing, China) on six mouse kidney samples using LC-QTOF. Metabolites were extracted and analyzed, and the data were processed using Progenesis QI for peak alignment and identification against METLIN, HMDB, LIPID MAPS, and in-house databases. The quality control sample reproducibility was high (correlation $>$0.8). PCA and OPLS-DA were used to assess group separation and identify differential metabolites (FC $\geq$1, VIP $\geq$1, $p < 0.05$). Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis was performed to identify perturbed pathways. All analyses were performed using the BMKCloud platform.

2.8 Statistical Analysis

The results are presented as mean $\pm$ standard deviation (SD). Differences among multiple groups were analyzed using one-way analysis of variance (ANOVA), and comparisons between two groups were performed using the unpaired Student's *t*-test using GraphPad Prism (Version 8, GraphPad Software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Triptolide Inhibits Tubulointerstitial Fibrosis in Obstructive Mouse Kidneys

The effect of triptolide on renal tubulointerstitial fibrosis was assessed using a UUO mouse model. Masson staining revealed extensive interstitial collagen deposition in the kidneys of UUO mice at 10 days postoperation. Notably, triptolide therapy significantly reduced collagen deposition (Fig. 1A,B). Western blotting analysis further demonstrated that ECM markers, including collagen-I and fibronectin, were upregulated in UUO kidneys, whereas triptolide treatment significantly decreased their levels (Fig. 1C,D). Importantly, triptolide treatment did not significantly affect the expression of these ECM markers in Sham-operated kidneys (Fig. 1C,D).

Furthermore, we evaluated the effect of triptolide on EMT in the fibrotic kidneys. The expression levels of three key EMT markers, vimentin, α-smooth muscle actin (α-SMA), and Snail, were elevated in UUO kidneys, and triptolide treatment significantly reversed this upregulation (Fig. 1E,F).

Collectively, these findings demonstrate that triptolide exhibits anti-fibrotic effects in mice with obstructive kidney injury.

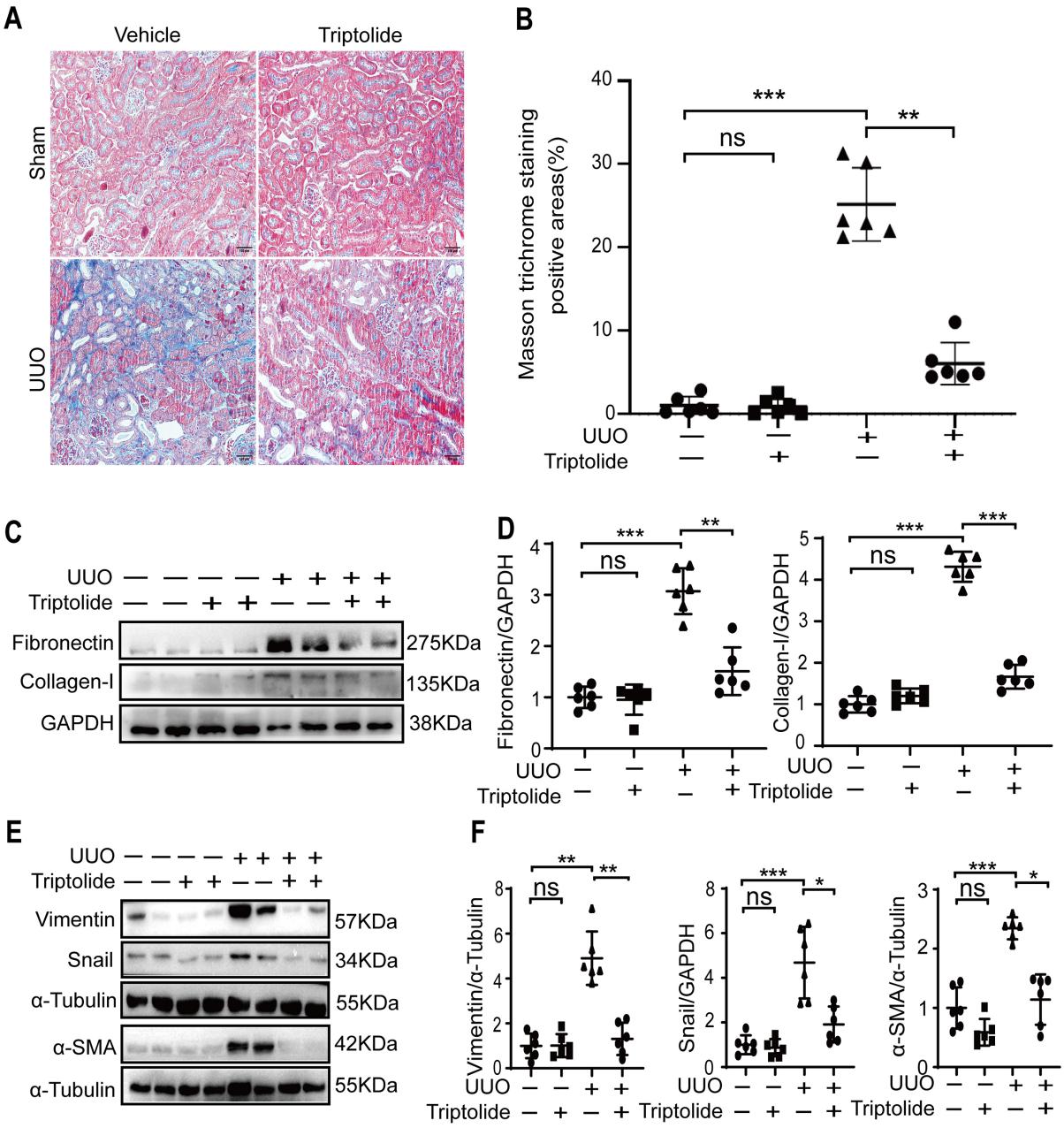

Fig. 1. Triptolide inhibits tubulointerstitial fibrosis in obstructive mouse kidneys and renal cells. Sham (-) or UUO (+) operation was performed on wild-type C57BL/6 mice, followed by 10 days of treatment with DMSO or triptolide. (A,B) Renal fibrosis was assessed using Masson's trichrome staining and quantified. Scale bar = 100 μm. (C,D) Expression levels of fibronectin and collagen-I were analyzed using Western blotting and quantified. Data are presented as mean ± SD. (E,F) Expression levels of vimentin, α-SMA, and Snail were analyzed using Western blotting and quantified. Representative results of at least three independent experiments are presented. ns indicates not significant. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Differences among multiple groups were analyzed using one-way ANOVA, and comparisons between two groups were performed using an unpaired Student's t-test. UUO, unilateral ureteral obstruction; α-SMA, α-smooth muscle actin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

3.2 Renal Gluconeogenesis is Promoted by Triptolide in Fibrotic Kidneys

To explore the mechanism underlying the renoprotective effects of triptolide in the fibrotic kidneys, we performed untargeted metabolomics analysis. A total of 260 differentially expressed metabolites (comprising 85 upregulated and 175 downregulated) were identified between the kidneys of UUO mice treated with vehicle and those treated

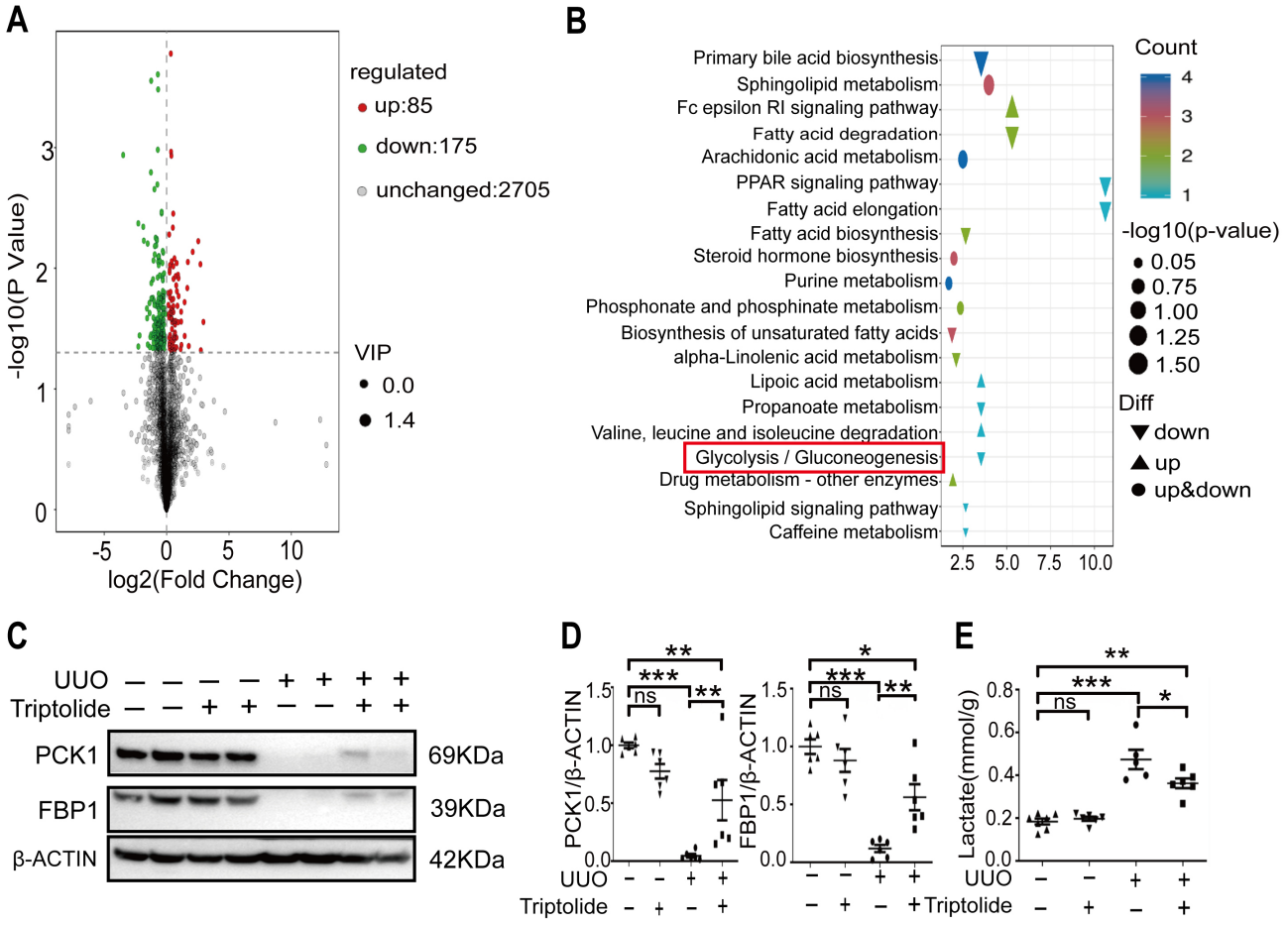

Fig. 2. Renal gluconeogenesis is promoted by triptolide in fibrotic kidneys. (A) Volcano plots of differentially expressed metabolites in mouse kidneys between UUO/DMSO and UUO/triptolide groups based on untargeted metabolomics analysis. (B) KEGG pathway enrichment analysis of triptolide-targeted metabolites by untargeted metabolomics. (C,D) Expression levels of PCK1 and FBP1 in Sham (-) or UUO (+) kidneys were analyzed using Western blotting and quantified. (E) Lactate levels in whole kidney samples were determined. Data from at least three independent experiments are presented as one sample. Data are presented as mean ± SD. ns represents not significant. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Differences among multiple groups were assessed using one-way ANOVA, and comparison between two groups was performed using an unpaired Student's *t*-test. UUO, unilateral ureteral obstruction; PCK1, phosphoenolpyruvate carboxykinase 1; FBP1, fructose-1,6-bisphosphatase 1.

with triptolide (Fig. 2A). KEGG enrichment analysis revealed that glucose metabolism, including gluconeogenesis and glycolysis, was among the most significantly dysregulated pathways affected by triptolide (Fig. 2B).

Subsequently, we validated the effects of triptolide on renal gluconeogenesis in fibrotic kidneys. The expression levels of PCK1 and FBP1, two rate-limiting enzymes involved in renal gluconeogenesis, were significantly downregulated in UUO kidneys compared to those in Sham-operated kidneys (Fig. 2C,D). Notably, triptolide treatment significantly increased the expression of these two enzymes in UUO kidneys; however, it failed to fully restore PCK1 and FBP1 expression in UUO kidneys to the levels observed in Sham-operated kidneys (Fig. 2C,D). Importantly, triptolide exhibited no significant effect on the expression of these two enzymes in Sham-operated kidneys (Fig. 2C,D).

Furthermore, renal lactate concentration was higher in UUO kidneys than in Sham kidneys (Fig. 2E), and triptolide treatment reduced lactate levels in UUO kidneys (Fig. 2E). However, triptolide did not completely reverse the UUO-induced increase in renal lactate to the baseline levels of Sham-operated kidneys (Fig. 2E). No significant difference in renal glucose concentration was observed between Sham and UUO kidneys (data not presented).

Collectively, these findings demonstrate that triptolide promotes renal gluconeogenesis in the fibrotic kidneys.

3.3 Triptolide Promotes Gluconeogenesis in TGF-β-stimulated HK2 Cells

The anti-fibrotic effect of triptolide was validated *in vitro* using TGF-β-stimulated HK2 cells. Triptolide inhibited the expression of fibronectin and collagen-I in TGF-β-

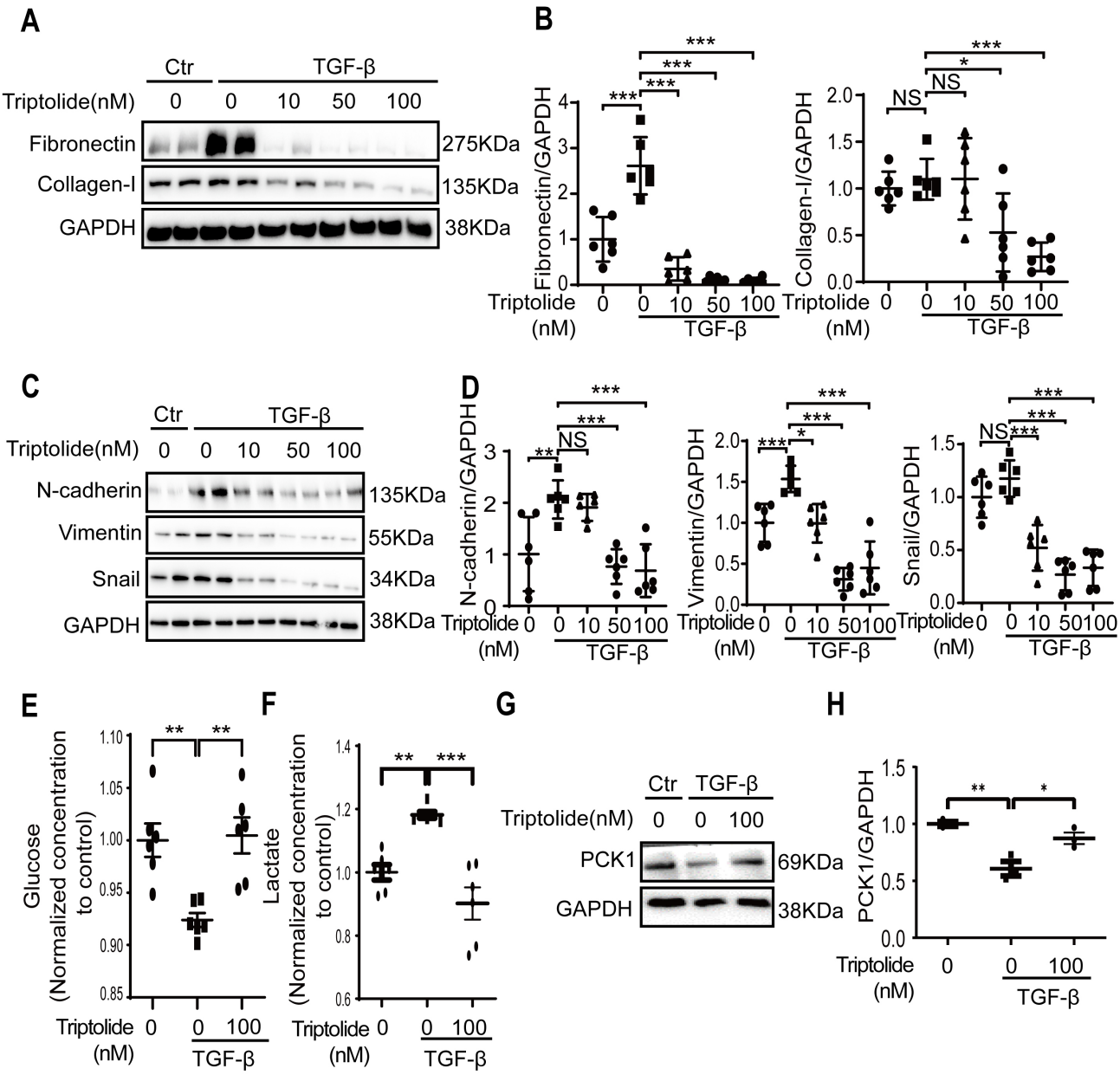

Fig. 3. Triptolide prevents fibrotic changes and promotes glucose metabolism in TGF-β-stimulated HK2 cells. HK2 cells were starved overnight, stimulated with 2.5 ng/mL TGF-β, and treated with different concentrations of triptolide for 48 h. Cell lysates were then extracted. (A,B) Expression levels of fibronectin and collagen-I were examined using Western blotting and quantified. (C,D) Expression levels of N-cadherin, vimentin, and Snail were assessed using Western blotting and quantified. (E,F) Glucose and lactate concentrations in the supernatant were measured. (G,H) PCK1 expression was analyzed using Western blotting and quantified. Data from at least three independent experiments are presented as one sample. Data are presented as mean ± SD. NS represents not significant. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Differences among multiple groups were analyzed using one-way ANOVA, and comparison between two groups was performed using an unpaired Student's *t*-test. TGF-β, Transforming growth factor beta; PCK1, phosphoenolpyruvate carboxykinase 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

stimulated HK2 cells in a dose-dependent manner at concentrations ranging from 10 to 100 nM. The maximum inhibitory effect on both proteins was observed at 100 nM (Fig. 3A,B). Similarly, triptolide decreased the expression of EMT markers (N-cadherin, vimentin, and Snail) in TGF-β-stimulated HK2 cells in a dose-dependent manner (10–100 nM), with the most significant inhibition observed at 100 nM (Fig. 3C,D).

We further observed that TGF-β stimulation decreased glucose levels and increased lactate levels in HK2 cells, whereas treatment with 100 nM triptolide reversed these metabolic changes (Fig. 3E,F). These changes in glu-

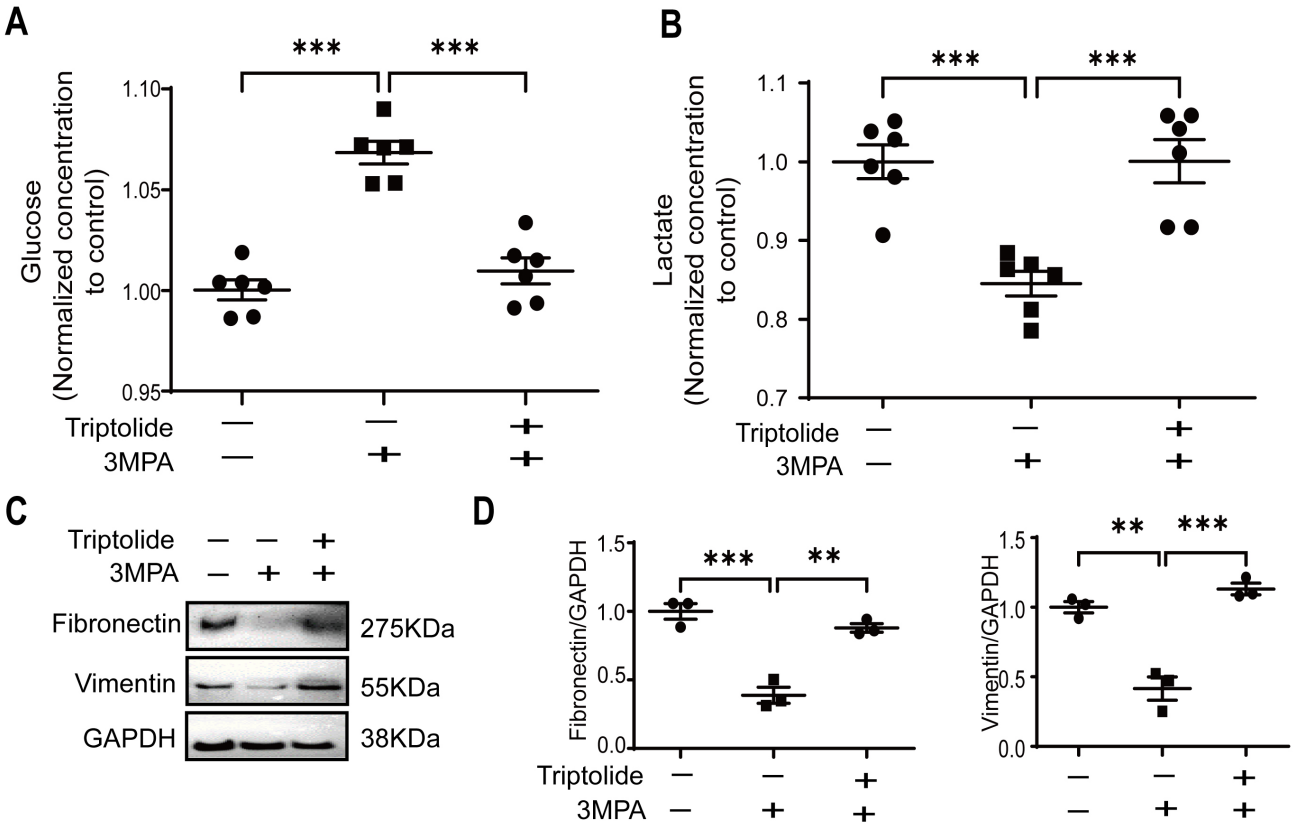

Fig. 4. Triptolide inhibits renal fibrosis by promoting gluconeogenesis. (A,B) Glucose and lactate levels in HK2 cells treated with (+) or without (-) 1 mM PCK1 inhibitor 3MPA were measured. (C,D) Western blotting was performed to evaluate the expression of fibronectin and vimentin in HK2 cells treated with (+) or without (-) the PCK1 inhibitor 3MPA and triptolide. The results were quantified. Data are presented as mean ± SD. $^{**}p < 0.01$. $^{***}p < 0.001$. Differences among multiple groups were analyzed using one-way ANOVA, and comparison between two groups was performed using an unpaired Student's *t*-test. 3MPA, 3-Mercaptopicolinic acid; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

coneogenesis were associated with increased PCK1 expression in TGF-β-stimulated HK2 cells following triptolide therapy (Fig. 3G,H).

3.4 Triptolide Inhibits Renal Fibrosis by Promoting Gluconeogenesis

To determine whether the anti-fibrotic effects of triptolide depend on gluconeogenesis, we treated TGF-β-stimulated HK2 cells with triptolide, followed by 3MPA, a selective PCK1 inhibitor. 3MPA reversed the triptolide-induced increase in glucose production and decrease in lactate levels (Fig. 4A,B). Furthermore, 3MPA abrogated the triptolide-mediated decrease in the expression of fibrotic markers (fibronectin and vimentin) (Fig. 4C,D). These results suggest that triptolide inhibits renal fibrosis by promoting PCK1-dependent gluconeogenesis.

3.5 Triptolide Promotes Glucose Metabolism Through PGC1α in Renal Fibrosis

We subsequently investigated the expression levels of PGC1α and FOXO1, two key regulators of gluconeogenesis, in fibrotic kidneys. UUO surgery significantly downregulated the expression of PGC1α and FOXO1 (Fig. 5A,B). Triptolide treatment slightly increased FOXO1 expression and significantly upregulated PGC1α expression in UUO kidneys (Fig. 5A,B). We further investigated the effect of triptolide on PGC1α expression *in vitro*. TGF-β-induced downregulation of PGC1α in HK2 cells was reversed by triptolide (Fig. 5C,D). *In vitro* experiments further confirmed that SR-18292 (a PGC1α inhibitor) reversed triptolide-induced increase in glucose concentration and decrease in lactate concentration in TGF-β-stimulated HK2 cells (Fig. 5E,F). Furthermore, SR-18292 suppressed the triptolide-mediated upregulation of PCK1 (Fig. 5G,H) and reversed the suppressive effect of triptolide on fibronectin and vimentin expression (Fig. 5I,J).

Collectively, these findings suggest that PGC1α mediates the pro-gluconeogenic effect of triptolide in fibrotic kidneys.

3.6 Triptolide Inhibits Inflammation in UUO Kidneys Through Histone Lactylation

A recent study has demonstrated that lactate, previously considered only a metabolic waste product, pro-

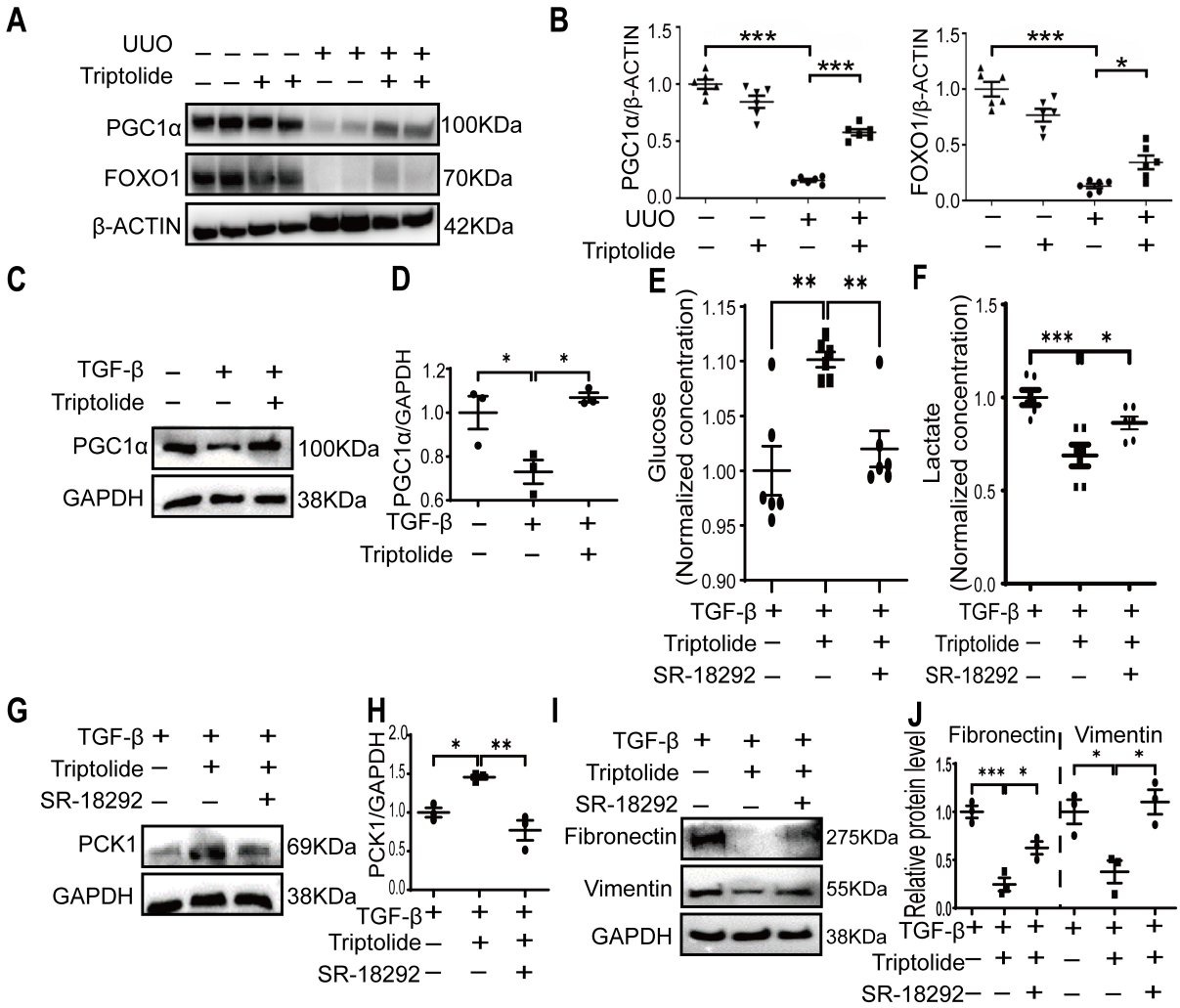

Fig. 5. Triptolide promotes glucose metabolism through PGC1α in renal fibrosis. (A,B) Expression levels of PGC1α and FOXO1 in Sham (-) or UUO (+) kidneys were analyzed using Western blotting and quantified. (C,D) HK2 cells were stimulated with TGF-β and treated with (+) or without (-) 100 nM of triptolide for 48 h. PGC1α expression was analyzed using Western blotting and quantified. (E,F) HK2 cells were starved overnight, stimulated with TGF-β, and treated with (+) or without (-) 20 μM of PGC1α inhibitor SR-18292 and 100 nM of triptolide for 48 h. Glucose and lactate concentrations in the supernatant were measured. (G,H) Western blotting analysis and quantification of PCK1 expression in HK2 cells treated with (+) or without (-) the PGC1α inhibitor SR-18292 and triptolide. (I,J) Western blotting was performed to analyze the expression of fibronectin and vimentin in HK2 cells treated with (+) or without (-) PGC1α inhibitor SR-18292 and triptolide. The results were subsequently quantified. Data from at least three independent experiments are represented as one sample. Data are presented as mean ± SD. *$p < 0.05$. **$p < 0.01$. ***$p < 0.001$. Differences among multiple groups were analyzed using one-way ANOVA, and comparison between two groups was performed using an unpaired Student's *t*-test. UUO, unilateral ureteral obstruction; TGF-β, transforming growth factor beta; FOXO1, Forkhead box protein O1; PGC-1α, peroxisome proliferator-activated receptor-gamma co-activator 1 alpha; PCK1, phosphoenolpyruvate carboxykinase 1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

motes renal fibrosis by inducing H4K12 histone lactylation-mediated inflammatory responses in tubular cells [22]. Therefore, we investigated whether triptolide inhibits renal fibrosis by suppressing histone lactylation-mediated inflammation, a mechanism that lies further downstream of gluconeogenesis.

IHC staining revealed elevated H4K12 lactylation in the renal tubular cells of fibrotic kidneys (Fig. 6A,B). This elevation was associated with increased macrophage infiltration, as indicated by F4/80 staining (Fig. 6C,D), and upregulated the expression of inflammatory factors such as IL-1β (Fig. 6E,F). Triptolide treatment reduced H4K12

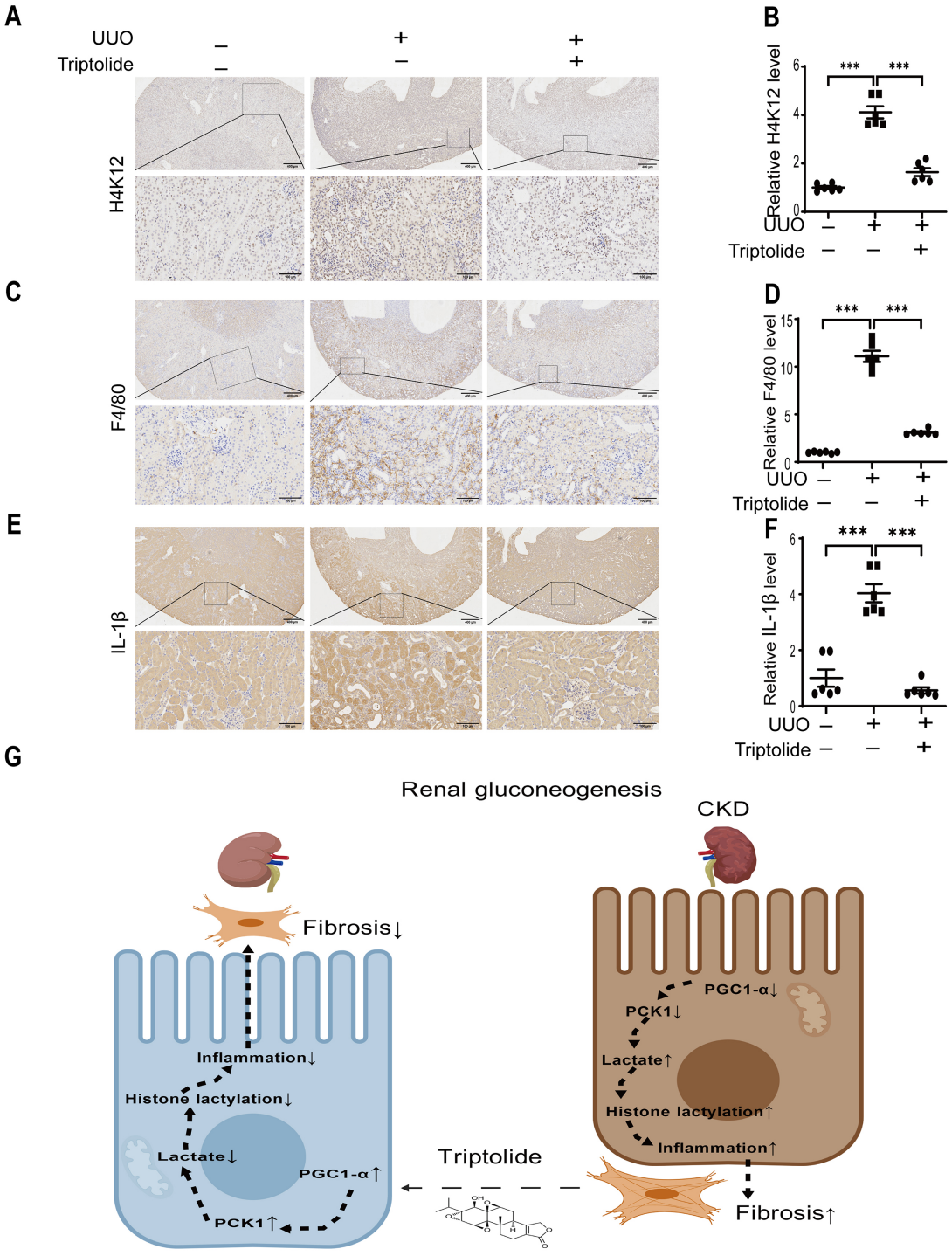

Fig. 6. Triptolide inhibits histone lactylation and inflammatory responses in UUO kidneys. (A,B) ICH analysis and quantification of H4K12 in sham (-) or UUO (+) mouse kidneys with (+) or without (-) triptolide treatment. Scale bar = 400 μm or 100 μm. (C,D) ICH analysis and quantification of F4/80 in the kidneys of triptolide-treated UUO mice. Scale bar = 400 μm or 100 μm. (E,F) ICH analysis and quantification of IL-1β in the kidneys of triptolide-treated UUO mice. Scale bar = 400 μm or 100 μm. (G) Schematic summary of the molecular mechanism underlying triptolide-induced recovery of renal gluconeogenesis in CKD. "↑" stands for up-regulation/activation; "↓" stands for down-regulation/inactivation. The schematic diagram was generated with BioGDP.com. Data from at least three independent experiments are represented as one sample. Data are presented as mean ± SD. ***$p < 0.001$. Differences among multiple groups were analyzed using one-way ANOVA, and comparison between two groups was performed using an unpaired Student's *t*-test. UUO, unilateral ureteral obstruction; H4K12, histone H4 Lys12; IL-1β, interleukin-1 beta.

lactylation levels and attenuated these inflammatory responses, as indicated by decreased F4/80 and IL-1β staining (Fig. 6A–F).

These findings imply that the anti-fibrotic effect of triptolide involves the suppression of histone lactylation, a pathway that contributes to inflammation in fibrotic kidneys.

4. Discussion

Formulations containing triptolide are widely used in China to treat patients with CKD [23]. In this study, we elucidated a novel mechanism underlying the renoprotective effect of triptolide, which inhibits renal fibrosis by promoting renal gluconeogenesis. First, we confirmed the anti-fibrotic effect of triptolide in UUO mice. Metabolomics analysis revealed that glucose metabolism is a downstream pathway mediating the anti-fibrotic effects of triptolide in UUO kidneys. This finding was further corroborated by analyses of gluconeogenic enzyme expression and metabolite levels. Furthermore, triptolide enhanced the expression of gluconeogenic enzymes and improved glucose metabolism in a cellular model of renal fibrosis. Moreover, *in vitro* studies demonstrated that the inhibition of gluconeogenesis by a PCK1 inhibitor reversed the anti-fibrotic effect of triptolide.

This study has several significant advancements in the current understanding of the mechanism of action of triptolide and the pathogenesis of renal fibrosis. First, it reveals a previously unrecognized role of triptolide in regulating renal glucose metabolism, particularly promoting gluconeogenesis, which differs fundamentally from its previously reported metabolic effects in other diseases [18,19]. Previous studies have demonstrated that triptolide improves glucose metabolism by inhibiting glycolysis, as observed in intrahepatic cholangiocarcinoma via the Akt/mammalian target of rapamycin pathway signaling pathway to suppress cancer cell proliferation [18], and rheumatoid arthritis by reducing glycolytic enzyme expression and lactate concentrations to alleviate joint inflammation [19]. Notably, these previously documented beneficial effects of triptolide on glucose metabolism are all associated with its inhibition of glycolysis. This study is the first to demonstrate that triptolide improves glucose metabolism by promoting gluconeogenesis.

Second, we demonstrated that triptolide upregulated PGC1α expression in fibrotic kidneys, and a PGC1α inhibitor reversed the effects of triptolide on glucose production and lactate clearance in renal cells. These findings indicate that triptolide promotes renal gluconeogenesis in fibrotic kidneys by activating the PGC1α signaling pathway. Notably, the therapeutic benefits of triptolide in other diseases have also been linked to its activation of the PGC1α pathway [24,25]. For instance, triptolide mitigated cognitive dysfunction in a rat model by upregulating PGC1α [25], and it exerts protective effects in an *in vitro* model of coronary heart disease by activating the PGC1α signaling pathway [24]. These findings suggest that triptolide may be developed as an agent to activate the PGC1α signaling pathway, a key therapeutic target for various kidney diseases [11]. As a transcriptional co-activator, PGC1α enhances gluconeogenesis not only through FOXO1 but also by interacting with hepatocyte nuclear factor 4α (HNF4α) [6]. Although not directly investigated in this study, the PGC1α/HNF4α axis is a well-recognized regulator of hepatic and renal gluconeogenesis, and its potential role in mediating the effects of triptolide requires further investigation.

Third, we identified a novel link between metabolic regulation (gluconeogenesis promotion) and epigenetic/inflammatory modulation (histone lactylation inhibition) in the anti-fibrotic mechanism of triptolide. Recent study has demonstrated that lactate (a byproduct of impaired gluconeogenesis) promotes renal fibrosis via H4K12 histone lactylation-mediated inflammatory responses [22]. Notably, histone lactylation, including at the H4K12 site, can exhibit context-dependent roles in inflammation. While our data and a recent study support a pro-inflammatory role of H4K12 lactylation in renal tubular cells [22], emerging evidence also suggests that lactate and lactylation may promote macrophage polarization toward an M2 phenotype [26]. In this study, we observed a positive correlation between IL-1β and H4K12 lactylation, consistent with a pro-inflammatory role in our model. Moreover, triptolide therapy reduced both H4K12 lactylation and macrophage infiltration, as indicated by F4/80 staining. Whether triptolide further modulates the M1/M2 balance through lactylation-dependent mechanisms remains an open question. Future studies using specific M1 (for instance, iNOS and CD86) and M2 (such as Arg-1 and CD206) markers will help elucidate how triptolide-induced metabolic reprogramming influences the immune microenvironment in renal fibrosis. Our study builds on these findings by demonstrating that triptolide-induced gluconeogenesis (which reduces lactate accumulation) correlates with decreased H4K12 lactylation in UUO kidneys, attenuated macrophage infiltration, and reduced production of inflammatory mediators. These findings connect three previously isolated processes: gluconeogenesis, histone lactylation, and renal inflammation, providing a more comprehensive, multi-layered understanding of how triptolide regulates renal fibrosis. Unlike previous studies that focused on either metabolic or inflammatory/epigenetic mechanisms independently, our study integrates these domains, providing a comprehensive perspective on the mechanism of action of triptolide that may guide the development of multi-targeted therapeutic strategies for CKD.

Furthermore, our findings have implications for translating preclinical research into clinical practice. Clinically approved agents targeting renal gluconeogenesis are

scarce, and triptolide-containing formulations are already used in China to treat renal diseases such as nephritis and membranous nephropathy [16,17]. By identifying the PGC1α/PCK1-gluconeogenesis-histone lactylation axis as a key mechanism of triptolide-induced renoprotection, we provide critical preclinical evidence supporting the use of triptolide to target metabolic defects in CKD. This not only strengthens the clinical relevance of triptolide but also opens avenues for optimizing its therapeutic potential. For instance, it may be combined with other agents that modulate the PGC1α/PCK1 axis or histone lactylation to improve anti-fibrotic efficacy.

This study has several limitations. First, although we observed changes in the protein expression of PCK1 and FBP1, and alterations in the levels of downstream metabolites (such as glucose and lactate) that suggested changes in enzymatic activity, we were unable to directly measure the enzymatic activities of PCK1 and FBP1. Similarly, while treatment with inhibitors (3-MPA and SR-18292) produced the expected metabolic and molecular phenotypic changes, we did not provide direct evidence for the inhibition of PCK1 enzymatic activity or PGC1α transcriptional co-activator function (for instance, via enzyme activity assays, PGC1α reporter gene assays or measurement of PCK1 mRNA levels). Second, the precise molecular mechanism by which triptolide upregulates PGC1α expression remains to be investigated. Third, although we observed that activation of the PGC1α/PCK1 axis was correlated with reduced H4K12 histone lactylation, the causal relationship remains to be established. Future "rescue" experiments, such as supplementation with exogenous lactate, could elucidate the direct regulatory link.

5. Conclusions

In conclusion, our findings suggest that triptolide may attenuate renal tubulointerstitial fibrosis by modulating the PGC1α/PCK1 axis, thereby promoting renal gluconeogenesis (Fig. 6G).

Disclosure

The paper is listed as, "Triptolide ameliorates renal tubulointerstitial fibrosis through EZH2" as a preprint on bioRxiv at: https://www.biorxiv.org/content/10.1101/2023.01.29.526092v1.

Availability of Data and Materials

All relevant data were included in the study. The datasets generated during this study are available from the corresponding author upon reasonable request.

Author Contributions

LL, DC, LY, and MW funded the project. LL, GW, MW and DC conceived this project. MW coordinated the study and wrote the paper. YW, YJ and GW conducted the in vitro experiments. MW, YW, LY, and DC performed the animal experiments. YW, LL and LY performed and analyzed the Western blotting. All authors reviewed the results and approved the final version of the manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments involving animals were performed in accordance with the National Institutes of Health guidelines and approved by the Animal Experimentation Ethics Committee of Shanghai University of Traditional Chinese Medicine (PZSHUTCM18111601).

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Conflict of Interest

The authors declare no conflict of interest.

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