

Curcumin Inhibits Transforming Growth Factor- β 1-Induced EMT via PPAR γ Pathway, Not Smad Pathway in Renal Tubular Epithelial Cells

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Abstract

Tubulointerstitial fibrosis (TIF) is the final common pathway in the end-stage renal disease. Epithelial-to-mesenchymal transition (EMT) is considered a major contributor to the TIF by increasing the number of myofibroblasts. Curcumin, a polyphenolic compound derived from rhizomes of Curcuma, has been shown to possess potent anti-fibrotic properties but the mechanism remains elusive. We found that curcumin inhibited the EMT as assessed by reduced expression of α -SMA and PAI-1, and increased E-cadherin in TGF- β 1 treated proximal tubular epithelial cell HK-2 cells. Both of the conventional TGF- β 1/Smad pathway and non-Smad pathway were investigated. Curcumin reduced TGF- β receptor type I (T β R-I) and TGF- β receptor type II (T β R-II), but had no effect on phosphorylation of Smad2 and Smad3. On the other hand, in non-Smad pathway curcumin reduced TGF- β 1-induced ERK phosphorylation and PPAR γ phosphorylation, and promoted nuclear translocation of PPAR γ . Further, the effect of curcumin on α -SMA, PAI-1, E-cadherin, T β R I and T β R II were reversed by ERK inhibitor U0126 or PPAR γ inhibitor BADGE, or PPAR γ shRNA. Blocking PPAR γ signaling pathway by inhibitor BADGE or shRNA had no effect on the phosphorylation of ERK whereas the suppression of ERK signaling pathway inhibited the phosphorylation of PPAR γ . We conclude that curcumin counteracted TGF- β 1-induced EMT in renal tubular epithelial cells via ERK-dependent and then PPAR γ -dependent pathway.

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Introduction

Renal fibrosis, characterized by accumulation of fibroblasts and excessive matrix proteins along with loss of functioning nephrons, is a major pathological feature of progressive kidney disease. Tubulointerstitial fibrosis is considered the final common pathway of renal fibrosis. Recent studies have demonstrated that a critical step in the pathogenesis of tubulointerstitial fibrosis is Epithelial-mesenchymal transition (EMT) [1], a process whereby fully differentiated epithelial cells undergo transition to a mesenchymal phenotype. EMT causes a substantial increase in the number of myofibroblasts, one of main effector cells that contributes to the development of progressive renal fibrosis [2].

TGF- β is known as a major inducer of EMT. TGF- β 1 induced EMT via Smad-dependent and Smad-independent pathways [3]. Through the Smad mediated pathway, TGF- β signals are transduced by transmembrane serine/threonine kinase type II and type I receptors (T β R II and T β R I) and intracellular mediators Smads [4]. In the non-Smad signaling pathway, TGF- β receptors interact with the MAPK pathway [5]. There are also

reports that Peroxisome proliferator-activated receptor- γ (PPAR- γ) activation exerts antiproliferative and antifibrotic effects via the modulation of TGF- β 1-mediated pathways [6].

PPAR- γ is a member of the nuclear receptor family of transcription factors. Ligands for PPAR- γ include a variety of natural and synthetic compounds. Synthetic ligands are often used as insulin sensitizing agents for treatment of type 2 diabetes [7]. Studies have demonstrated that PPAR γ agonists exert protective effects in the models of renal diseases [8,9]. PPAR γ agonists rosiglitazone significantly attenuated glomerulosclerosis, tubulointerstitial expansion and collagen IV deposition in the apolipoprotein E knockout mouse [10]. Troglitazone, another PPAR γ agonists, also attenuated renal interstitial fibrosis and inflammation in the unilateral ureteral obstruction's animal (UUO) [11], a classic renal fibrosis model.

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural polyphenolic compound derived from the root of curcuma longa that has been widely used in India for medical, culinary and other purposes. A large body of evidence from *in vitro* and *in vivo* studies of both animals and human have

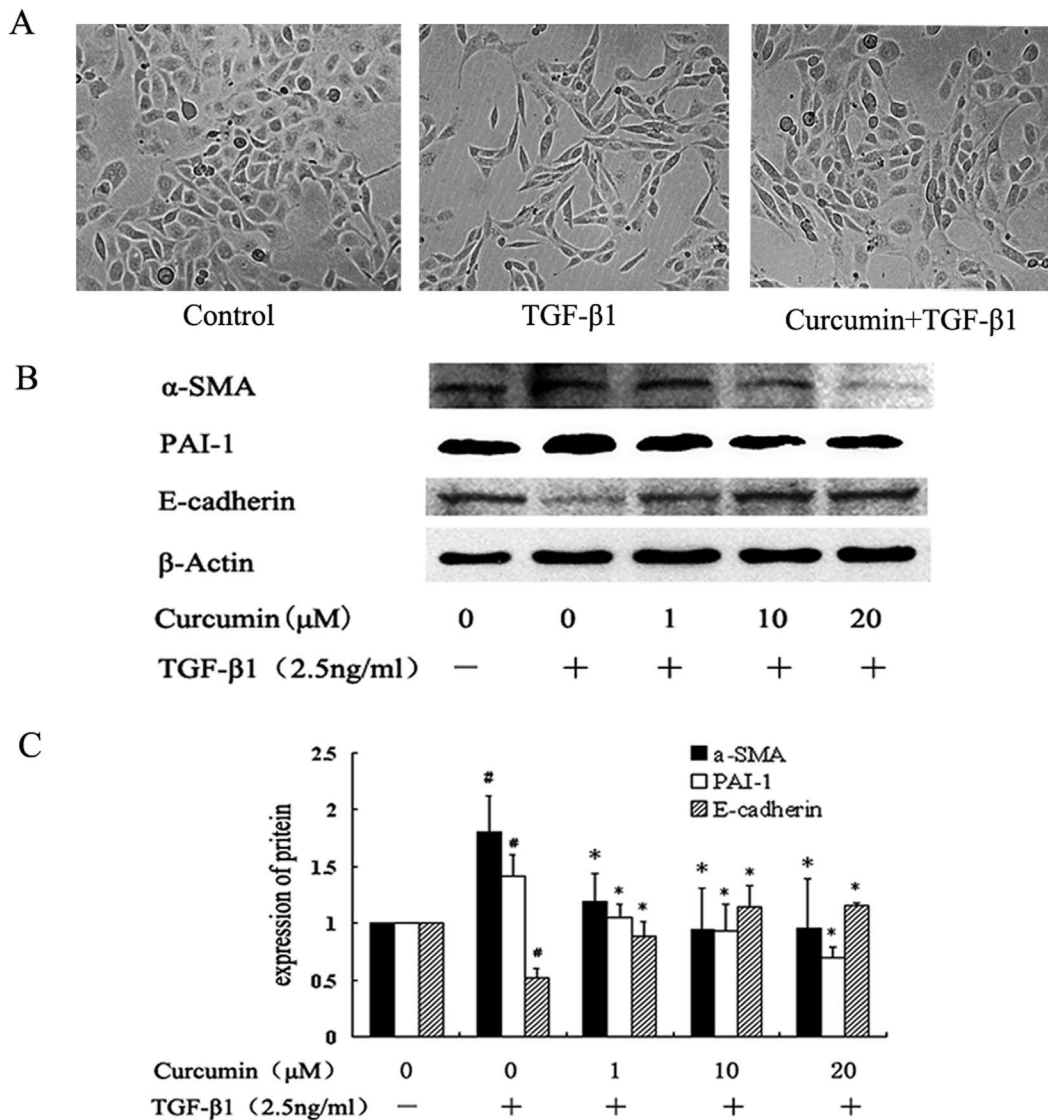


Figure 1. Effects of curcumin on morphology and the expression of α -SMA and PAI-1 and E-cadherin in TGF- β 1-induced HK-2 cells. HK-2 cells were incubated with curcumin at the indicated dose for 12 hours followed by treatment with TGF- β 1 for 36 hours. (A) Effects of 10 μ M of curcumin on morphology of HK-2 cells (B) Western blotting analyses for α -SMA, E-cadherin and PAI-1 expression in HK-2 cells. (C) Graphical presentation of the relative expression of α -SMA, E-cadherin and PAI-1. The values were represented as the density of α -SMA, E-cadherin or PAI-1 vs β -actin (%). The mean \pm SD (n=3) of values were obtained from densitometric analysis of all individual experiments. *p<0.05 vs TGF- β 1 alone. doi:10.1371/journal.pone.0058848.g001

indicated that curcumin exhibits a variety of biological effects such as anti-oxidant, anti-inflammatory, anti-tumor and wound healing properties [12]. In particular, some recent studies have shown that curcumin has anti-fibrotic effect in liver, lung and cystic fibrosis [13,14,15]. In UUO rat kidney fibrosis model curcumin has been reported to inhibit the renal interstitial inflammation and fibrosis by inhibiting of the NF- κ B-dependent pathway [16]. In immortalized rat kidney interstitial fibroblasts (NRK/49F), curcumin attenuated TGF- β -induced fibrosis through down-regulation of T β R II [17].

Curcumin has been reported to activate PPAR- γ as well [18], but it is unclear the effect is depend on binding to the receptor of PPAR- γ [18] or is the result of indirect effects [19]. Thus, we hypothesized that curcumin may inhibit renal fibrosis as assessed by EMT through PPAR γ pathways in the TGF- β signaling. On the other hand, researches on the anti-renal fibrosis effect of

curcumin have been concentrated on the mediating role of Smad pathway and little is known about curcumin's effects through the non-Smad pathway such as MAPK, and whether there is any cross talk between MAPK and PPAR γ is still elusive. Here, we reported the effect of curcumin on TGF- β 1-induced EMT in renal tubular epithelial cells, and its underlying mechanisms related to non-Smad ERK1/2 and PPAR γ pathways.

Materials and Methods

Cell culture and treatment

Human proximal tubular epithelial cells (HK-2 cells) were cultured in RPMI 1640 containing 2000 mg/L NaHCO₃, supplemented with 10% FBS, 100 units/ml penicillin and 100 ug/ml streptomycin in an atmosphere of 5% carbon dioxide and 95% air at 37°C. Normal Rat Kidney(NRK-52E) proximal

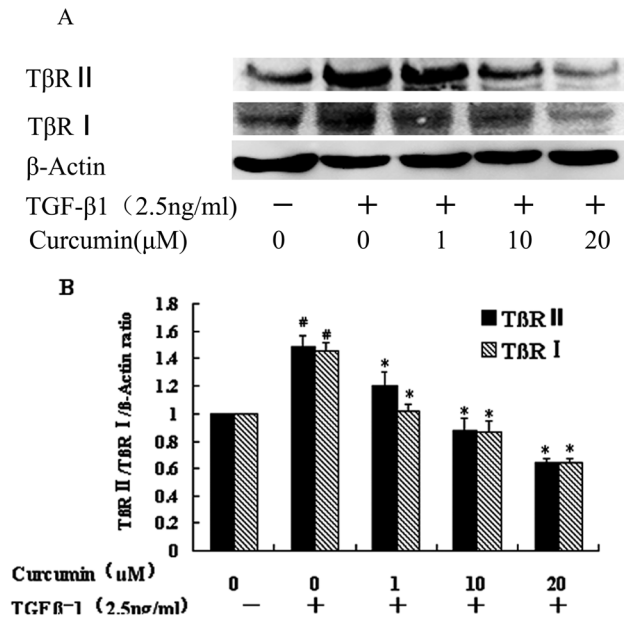


Figure 2. Effects of curcumin on TGF- β 1-induced T β R I and T β R II expression. HK-2 cells were incubated with curcumin at the indicated dose for 12 hours followed by treatment with TGF- β 1 for 36 hours. (A) Western blotting analyses for T β R I and T β R II expression in HK-2 cells. (B) Graphical presentation of the relative expression of T β R I and T β R II vs β -actin (%). The values were represented as the density of T β R I or T β R II vs β -actin (%). The mean \pm SD (n=3) of values were obtained from densitometric analysis of all individual experiments. #P<0.05 vs. control alone, *P<0.05 vs TGF- β 1 alone.
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cells were cultured in DMEM containing 2000 mg/L NaHCO₃, supplemented with 10% FBS, 100 units/ml penicillin and 100 ug/ml streptomycin in an atmosphere of 5% carbon dioxide and 95% air at 37°C.

At about 90% confluent, the cells were trypsinized by treatment with trypsin-EDTA and seeded in 6-well plates, grown to confluence, and rested in medium without FBS overnight.

Reagents

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] was purchased from Sigma (St. Louis, MO, USA). Stock solutions of curcumin were prepared in dimethyl sulfoxide and used at a final concentration \leq 0.1% (v/v). Recombinant Human transforming growth factor beta 1 (TGF- β 1) were purchased from R&D Systems (USA). Antibodies to phospho-smad2, smad2, phospho-smad3, smad3, phospho-ERK1/2, ERK 1/2, PPAR γ and TGF- β receptor II were purchased from Cell Signaling Technology (USA). Antibodies to p-PPAR γ were purchased from Bioworld (USA). TGF- β receptor I was purchased from Santa Cruz Biotechnology (CA, USA). Antibodies to PAI-1, E-cadherin and α -SMA were purchased from Epitomics (USA). BCA Protein Assay Kit was purchased from Shanghai Biocolor BioScience & Technology Company (China).

Western blot analysis

At the time of harvest, cells were washed twice with PBS (135 mM, 2.7 mM KCL, 1.5 mM KH₂PO₄, 8 mM K₂HPO₄), homogenized in RIPA lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PSMF). The supernatants were collected after centrifugation at 10,000 \times g at 4°C for 15 min. Protein concentration was de-

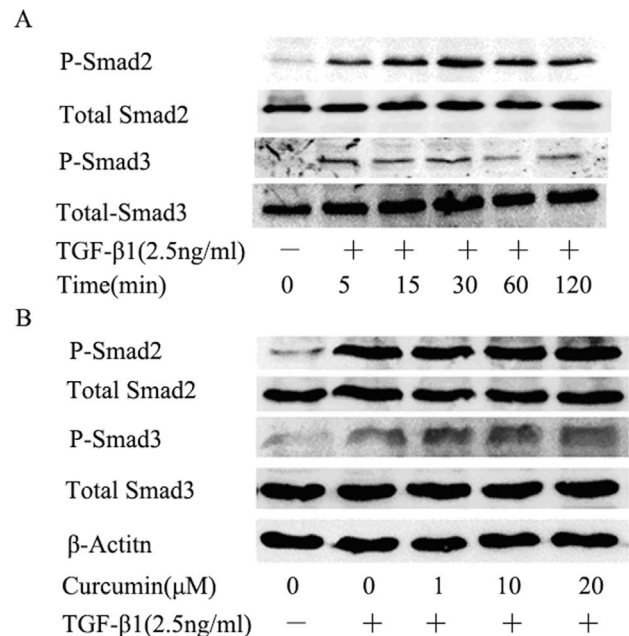


Figure 3. Effects of curcumin on TGF- β 1-induced Smad2 and Smad3 phosphorylation in HK-2 cells. (A) HK-2 cells were stimulates with TGF- β 1 (2.5 ng/ml) for the indicated times, and the phosphorylation of Smad2 and Smad3 were analyzed by western blotting. (B) Western blotting analyses for p-Smad2 and p-Smad3 expression in HK-2 cells. HK-2 cells were incubated with curcumin at the indicated doses for 12 hours followed by treatment with TGF- β 1 for 30 minutes.
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termined using a BCA Protein Assay Kit. Equal amounts of protein were boiled at 95°C for 5 min after the addition of 5 \times Laemmli buffer. Protein samples were separated by 10% SDS gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. After blocking with 5% dry milk in Tris-buffered saline with 0.1% Tween (TBST) at room temperature for 2 h, the membranes were incubated with primary antibodies at 4°C overnight. After being washed in TBST three times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. The signals were visualized using the enhanced chemiluminescent (ECL) substrate.

Stable shRNA mediated repression of PPAR γ in HK-2 cells

Human PPAR γ expression in HK-2 cells was silenced by shRNA interference. The lentiviral mediated shRNA against PPAR γ (NM_138712.3) system was purchased from Shanghai Sunbio Medical Biotechnology. An adopted non-silencing control shRNA sequence (TTCTCCGAACGTGTCACGT) that was not complementary to any human gene was used as a control shRNA. Lentiviruses were prepared in HEK293T cells followed by infecting HK-2 cells. Cells were selected using 0.5 μ g/ml puromycin and subjected to western blot to test the expression level of PPAR γ in the infected HK-2 cells.

Immunofluorescent staining and uorescent microscopy

HK-2 cells grown on cover slips were washed with cold PBS three times and fixed in 5% paraformaldehyde for 25 minutes. The cells were then extensively washed three times each for 10 minutes with PBS and permeabilized with 0.1% TritonX-100 for 5 minutes. After blocking in 5% bovine serum albumin (BSA)

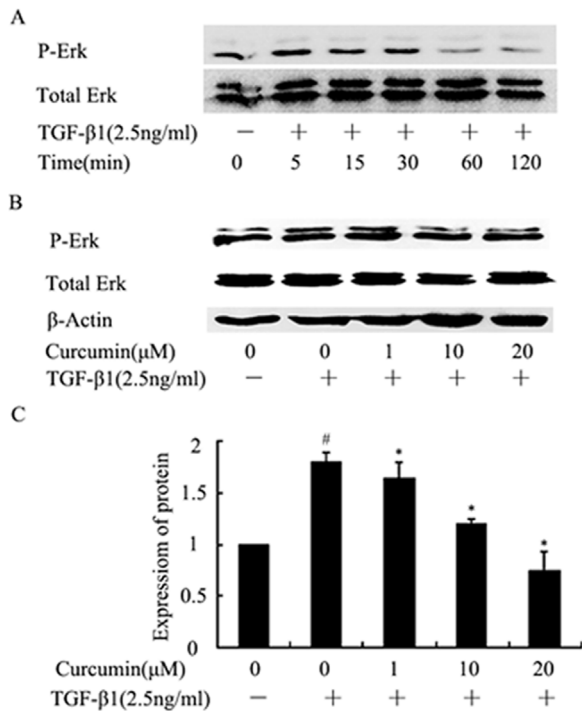


Figure 4. Effects of curcumin on TGF- β 1-induced ERK phosphorylation in HK-2 cells. (A) HK-2 cells were stimulated with TGF- β 1 (2.5 ng/ml) for the indicated times, and the phosphorylation of ERK was analyzed by western blotting. (B) Western blotting analyses for p-ERK expression in HK-2 cells. HK-2 cells were incubated with curcumin at the indicated doses for 12 hours followed by treatment with TGF- β 1 for 30 minutes. (C) Graphical presentation of the relative expression of p-ERK. The values were represented as the density of p-ERK vs total ERK (%). The mean \pm SD (n = 3) of values were obtained from densitometric analysis of all individual experiments. [#]P<0.05 vs. control alone, ^{*}P<0.05 vs TGF- β 1 alone. doi:10.1371/journal.pone.0058848.g004

in PBS buffer for 1 hour at room temperature, cells were incubated with the anti-PPAR γ antibody at 4°C overnight with gentle rocking. Cells were next washed three times in PBS and stained with Cy-2-conjugated secondary antibody at room temperature for 2 hours. Stained cells were mounted with antifade mounting medium on slides and viewed with a fluorescence microscope.

Statistical Analysis

The data are shown as mean \pm SD. Statistical significance was assessed using a Student's paired t test when there were only two groups involved. P<0.05 was considered statistically significant. All data analyses were performed using the SPSS software.

Results

1. Curcumin inhibited the expression of α -SMA and PAI-1 and increased E-cadherin in TGF- β 1 treated HK-2 cells

As shown in **Fig. 1**, the amount of α -SMA and PAI-1 proteins were very low while the epithelial cell marker, E-cadherin was prominent in normal HK-2 cells (**Fig. 1 b**). TGF- β 1 induced a significant increase in α -SMA and PAI-1 and decrease in E-cadherin (**Fig. 1 b & c**). Curcumin down-regulated α -SMA and PAI-1 and up-regulated E-cadherin (**Fig. 1 b & c**).

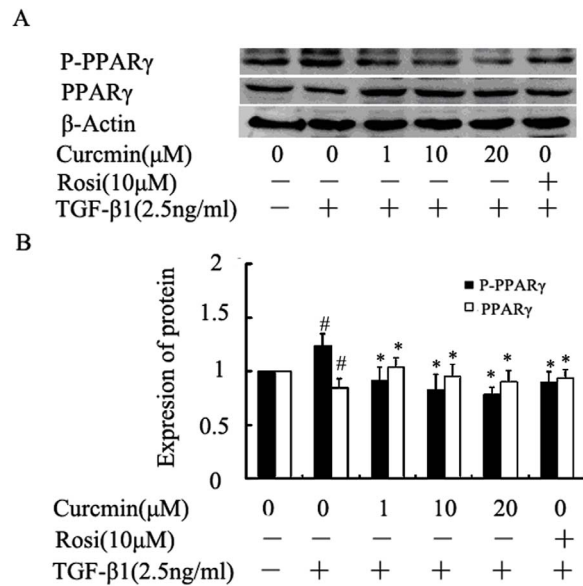


Figure 5. Curcumin reduced TGF- β 1-induced PPAR γ phosphorylation and reversed the reduction of PPAR γ . HK-2 cells were incubated with curcumin at the indicated dose for 12 hours or rosiglitazone for 1 hour followed by treatment with TGF- β 1 for 36 hours. (A) Western blotting analyses for PPAR γ and p-PPAR γ expression in HK-2 cells. (B) Graphical presentation of the relative expression of PPAR γ and p-PPAR γ . The values were represented as the density of PPAR γ and p-PPAR γ vs β -actin (%). The mean \pm SD (n = 3) of values were obtained from densitometric analysis of all individual experiments. [#]P<0.05 vs control alone, ^{*}P<0.05 vs TGF- β 1 alone. doi:10.1371/journal.pone.0058848.g005

2. Curcumin attenuated the TGF- β 1-induced expression of TGF- β receptor type I (T β R I) and TGF- β receptor type II (T β R II) in HK-2 cells

Binding to the TGF- β receptor type II (T β R II) is the first step of TGF- β signaling, and the T β R II in turn phosphorylates TGF- β receptor type I (T β R I) [20]. To determine whether the anti EMT effect of curcumin is targeting TGF- β 1-related signaling, we

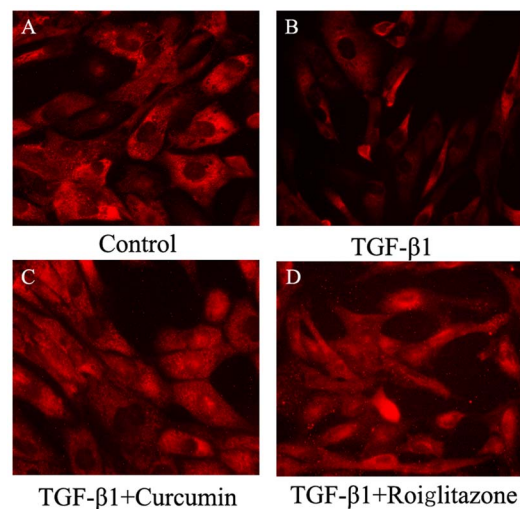


Figure 6. Effects of curcumin on nuclear translocation of PPAR γ . HK-2 cells were pretreated with curcumin (10 μ M) for 12 hours (C) or rosiglitazone (10 μ M) for 1 hour (D) following by incubated with TGF- β 1 for 36 hours. Red for PPAR γ . doi:10.1371/journal.pone.0058848.g006

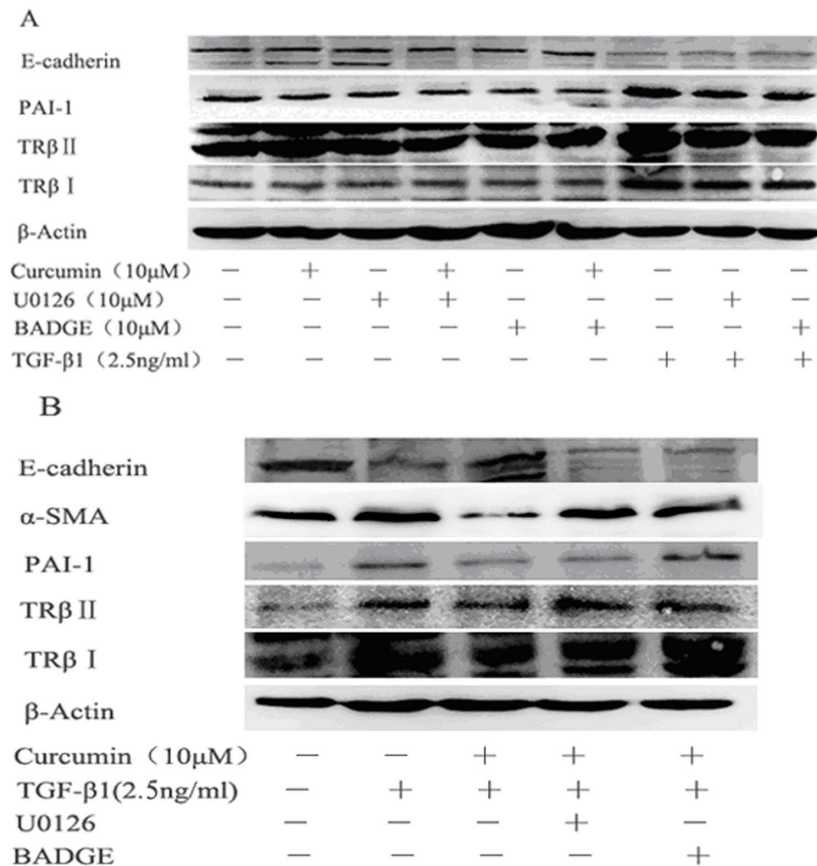


Figure 7. ERK1/2 inhibitor U0126 and PPAR γ inhibitor BADGE blocked the effects of curcumin on TGF- β 1-induced EMT in HK-2 cells. (A) Effects of U0126, BADGE on the expression of α -SMA, PAI-1, E-cadherin, T β R I and T β R II with or without TGF- β 1. HK-2 cells were pretreated with curcumin (10 μ M) for 12 hours followed by treatment with U0126 (10 μ M) or BADGE (10 μ M) for 36 hours or HK-2 cells were pretreated with U0126 (10 μ M) or BADGE (10 μ M) for 1 hour before addition of TGF- β 1 (2.5 ng/ml) for 36 hours. (B) Effects of curcumin on the expression of α -SMA, PAI-1, E-cadherin, T β R I and T β R II with TGF- β 1 and U0126 or BADGE. HK-2 cells were pretreated with curcumin (10 μ M) for 12 hours at indicated dose followed by treatment with U0126 (10 μ M) or BADGE (10 μ M) for 1 hour before addition of TGF- β 1 (2.5 ng/ml) for 36 hours. doi:10.1371/journal.pone.0058848.g007

studied the effects of curcumin on T β R II and T β R I. As shown in **Fig. 2**, curcumin decreased both T β R I and T β R II expression in HK-2 cells.

3. Effects of curcumin on TGF- β 1-induced Smad2 and Smad3 phosphorylation in HK-2 cells

We then studied the effects of curcumin on Smad2 and Smad3, two important mediators of the TGF- β /Smads signaling, which has been known to play a major role in TGF- β 1-induced EMT [21].

Smad2 and Smad3 were phosphorylated at 5 min and the phosphorylations reached the peak levels at 30 min (**Fig. 3a**). Pretreatment with curcumin for 12 hours and following by treatment with TGF- β 1 for 30 minutes has no effect on phosphorylation of Smad2 and Smad3 (**Fig. 3b**). These data indicated that the inhibition of TGF- β 1-induced EMT by curcumin is not through TGF- β /Smads signaling pathway.

4. Curcumin reduced TGF- β 1-induced ERK phosphorylation in HK-2 cells

Our studies showed that curcumin had little effect on TGF- β /Smads signaling. Since the activation of T β R I and T β R II can lead to the activation of other EMT associated signaling pathways, such as ERK [22], we examined the influence of curcumin on

ERK signaling. ERK was phosphorylated at 5 min and reached the peak levels at 30 min post TGF- β 1 treatment (**Fig. 4a**). Pretreatment with curcumin for 12 hours and following by treatment with TGF- β 1 for 30 minutes decreased phosphorylation of ERK (**Fig. 4b**). These data indicated that curcumin may inhibit TGF- β 1-induced EMT through ERK-related signaling pathway.

5. Curcumin reduced TGF- β 1-induced PPAR γ phosphorylation and promoted nuclear translocation of PPAR γ

Total PPAR γ expression was reduced while its phosphorylation was increased after being treated with TGF- β 1 (**Fig. 5**). Pretreatment with curcumin for 12 hours increased PPAR γ expression level and inhibited the phosphorylation of PPAR γ . As expected, PPAR γ agonist rosiglitazone increased PPAR γ expression. We also found that rosiglitazone inhibited the phosphorylation of PPAR γ . These results indicated that curcumin is a PPAR γ agonist.

PPAR γ is a nuclear transcription factor, which requires nuclear translocation. As shown in **Fig. 6**, TGF- β 1 decreased the expression of PPAR γ , and curcumin increased the expression of PPAR γ and promoted nuclear translocation of PPAR γ in HK-2 cells. Rosiglitazone also promoted nuclear translocation of PPAR γ .

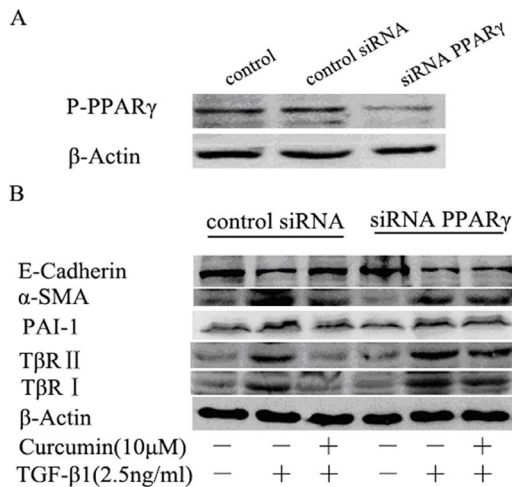


Figure 8. Effect of curcumin on TGF- β 1-induced EMT in HK-2 cells is dependent on PPAR γ . (A) Knockdown of PPAR γ in HK-2 cells. HK-2 cells were infected with PPAR γ -specific shRNA or scrambled shRNA for 3 hours and then changed the medium. The expression of PPAR γ was examined by western blotting. (B) After infected with PPAR γ -specific shRNA or scrambled shRNA for 3 hours, HK-2 cells were treated with curcumin (10 μ M) for 12 hours at indicated dose followed by treatment with TGF- β 1 (2.5 ng/ml) for 36 hours. Expression of α -SMA, PAI-1, E-cadherin, T β R II and T β R I were detected by western blotting.

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6. ERK pharmacological inhibitor and PPAR γ pharmacological inhibitor blocked the inhibitory effect of curcumin on TGF- β 1-induced EMT of HK-2 cells

To elucidate whether the inhibition of ERK and PPAR γ signaling are necessary for the effect of curcumin on TGF- β 1-induced EMT of HK-2 cells, we pretreated HK-2 cells with U0126, a pharmacological inhibitor of ERK, or BADGE, a pharmacological inhibitor of PPAR γ . In order to exclude the influence of curcumin or inhibitors on the protein expression, we have designed a control experiment with or without TGF- β 1. As shown in **Fig. 7a**, in the absence of TGF- β 1, curcumin, U0126, BADGE and curcumin combined with U0126, or BADGE did not have any effect on the protein levels of α -SMA, E-cadherin, PAI-1, T β RI and T β RII, and there was no significant difference compared with the control group. Similarly, in the presence of TGF- β 1, U0126 or BADGE did not have any effect on establishment of model, and there was no significant difference compared with TGF- β 1 treated group. Based on the above results, we studied the effects of curcumin with U0126 or BADGE on α -SMA, E-cadherin, PAI-1, T β R I and T β R II. The U0126 or BADGE treatment was shown to reverse the increases in α -SMA, PAI-1, T β R I and T β R II and the decreases in E-cadherin caused by curcumin (**Fig. 7b**).

7. Effect of curcumin on TGF- β 1-induced EMT in HK-2 cells is dependent on PPAR γ

Furthermore, we knocked down the expression of PPAR γ at cellular level using shRNA interference via lentiviral infection of HK-2 cells. Results from western blotting showed that the PPAR γ protein in knocked-down cells was significantly lower compared to that in normal cells and scrambled shRNA infected cells (**Fig. 8a**). With the significantly decreased PPAR γ expression by shRNA the effects of curcumin on α -SMA, PAI-1, E-cadherin, T β R I and T β R II were inhibited (**Fig. 8b**).

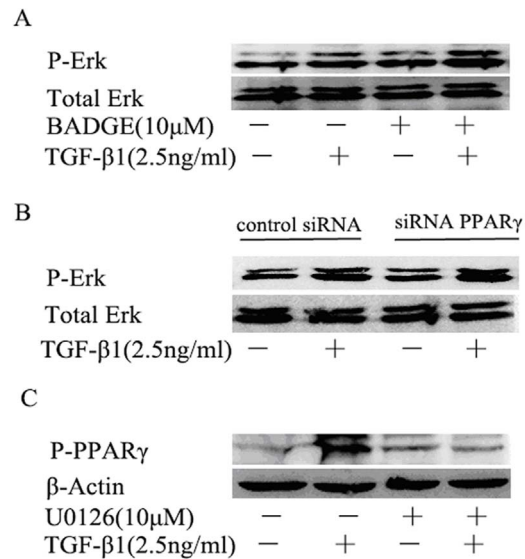


Figure 9. Crosstalk between ERK and PPAR γ signaling. (A) Effect of blocking PPAR γ signaling by pharmacological inhibitor on phosphorylation of ERK. HK-2 cells were pretreated with BADGE (10 μ M) 1 hour before addition of TGF- β 1 (2.5 ng/ml) for 30 minutes and the expression of p-ERK were analyzed by western blotting. (B) Effect of knockdown of PPAR γ at cellular level on phosphorylation of ERK. HK-2 cells infected with PPAR γ -specific shRNA or scrambled shRNA were treated TGF- β 1 (2.5 ng/ml) for 30 minutes and the expression of p-ERK were analyzed by western blotting. (C) Effect of blocking ERK signaling on phosphorylation of PPAR γ . HK-2 cells were pretreated with U0126 (10 μ M) for 1 hour before addition of TGF- β 1 (2.5 ng/ml) for 36 hours and the expression of PPAR γ were analyzed by western blotting.

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8. ERK phosphorylates PPAR γ in HK-2 cells

Blocking PPAR γ signaling pathway by inhibitor BADGE or shRNA (**Fig. 9a & 9b**) had no effect on the phosphorylation of

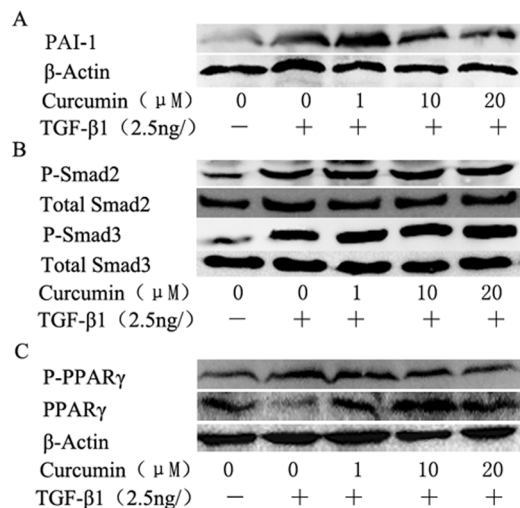


Figure 10. Effects of curcumin on TGF- β 1-induced EMT in NRK-52E cells. NRK-52E cells were incubated with curcumin at the indicated dose for 12 hours followed by treatment with TGF- β 1 for 36 hours (PAI-1, p-PPAR γ and PPAR γ) or 30 min (P-Smad2 and P-Smad3). (A) Western blotting analysis for PAI-1 expression. (B) Western blotting analysis for p-Smad2 and p-Smad3 expression. (C) Western blotting analysis for PPAR γ and p-PPAR γ expression.

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ERK whereas the suppression of ERK signaling pathway inhibited the phosphorylation of PPAR γ (Fig. 9c). These data suggest that ERK signaling pathway is the upstream of PPAR γ signaling pathway in the regulation of EMT in HK-2 cells.

9. Effect of curcumin on TGF- β 1-induced EMT in NRK-52E cells

Curcumin is also able to block TGF- β 1-induced EMT in NRK-52E cells, another renal tubular epithelial cell line (Fig. 10a). As shown in Fig. 10b & 10c, curcumin had no effect on p-Smad2 and p-Smad3, whereas reduced TGF- β 1-induced PPAR γ phosphorylation and increased the expression of total PPAR γ , which is similar to the effects of curcumin on HK-2 cells.

Discussion

In this study, we investigated the effect of curcumin on TGF- β -induced EMT in HK-2 cells and the underlying mechanism. Increased expression of α -SMA and PAI-1 and decreased expression of E-cadherin are the hallmarks of renal tubular epithelial cells undergoing EMT [1,23,24]. Suppression of E-cadherin is considered the earliest changes in TGF- β 1-induced EMT [25]. α -SMA is an important marker of fibroblast, contributing to the morphology of the transferred cells and to their ability to migrate and invade. PAI-1, a potent inhibitor of uPA/tPA (urokinase/tissue-type plasminogen activators), plays a major role in extracellular matrix (ECM) accumulation and degradation [26], which is a principal feature in fibrosis. In this study, we observed that with pretreatment of curcumin, the increase of α -SMA and PAI-1 induced by TGF- β 1 was attenuated, and the loss of E-cadherin was reversed, confirming the previous reports [17,27]. These results indicated that curcumin inhibits the process of EMT in renal tubular cell lines.

The mechanisms underlying the effect of curcumin on EMT of HK-2 cells were further investigated. Since binding of TGF- β 1 to T β R I and T β R II is the first step of TGF- β signaling [28], we first examined the effect of curcumin on the levels of T β R I and T β R II. Our studies showed that curcumin could inhibit the expression of both T β R I and T β R II, but Gaedeke et al found that curcumin selectively inhibited T β R II in renal fibroblasts cells. It suggested that the signaling might be different in tubular and fibroblasts cells.

TGF- β /Smads signaling is considered the most important pathway in EMT and most of the TGF- β -induced EMT appears to be dependent on this signaling. The phosphorylation of Smad2 and Smad3 are essential steps in the signaling cascade. Curcumin was previously demonstrated to suppress the phosphorylation of Smad2 but not that of Smad3 in HK-2 cells [27]. Li et al reported that curcumin inhibited the phosphorylations of both Smad2 and Smad3 in UUO rat model [29]. However, in our study, curcumin failed to affect the phosphorylations of both Smad2 and Smad3 and the expression of total Smad2 and Smad3 in HK-2 cells and NRK-52E cells. The different findings of the phosphorylations of Smad2 and Smad3 by curcumin treatment may be cell type specific, suggesting that the phosphorylation status and effect of Smad2 and Smad3 in EMT are complex and involve many factors.

Besides TGF- β /Smads pathway, TGF- β receptors activate some non-Smad signaling pathways as well, including ERK pathway et al. Studies have demonstrated that ERK is phosphorylated during TGF- β 1-induced EMT [30], ERK activity

is required for disassembly of adherens junctions and induction of cell motility, and blockade of ERK inhibited key morphological features of EMT in the mammary gland epithelial cells [31], hence the inhibition of phosphorylation of ERK is a potential target for suppressing renal fibrosis. In our study in HK-2 cells, ERK phosphorylation was detected at 5 min after treating with TGF- β 1, whereas curcumin attenuated the TGF- β 1-induced increase of p-ERK. We pretreated HK-2 cells with U0126, a pharmacological inhibitor of ERK, the inhibitory effect of curcumin on EMT was reduced, indicating that the inhibitory effect of curcumin on EMT is related to its inhibition on ERK signaling.

PPAR γ is present and active in multiple renal cell type including the cultured glomerular mesangial cells, podocytes, proximal epithelial cells and epithelial cells of collecting ducts [32,33]. Studies have shown that PPAR γ agonists could ameliorate renal fibrotic lesions in diabetic nephropathy and nondiabetic chronic kidney diseases [34,35,36]. In this study, we demonstrated, for the first time, that curcumin induces the expression of PPAR γ , inhibits the phosphorylation of PPAR γ and promotes the nuclear translocation of PPAR γ in renal tubular epithelial cells. Curcumin has been reported to activate PPAR γ , but whether it is a ligand is still a debate [19]. The mechanisms by which curcumin induces PPAR γ expression remain poorly understood. Two possibilities may be involved: firstly, curcumin binds to its own receptor and the complex stimulates the PPAR γ signaling pathway resulting in the up-regulation of PPAR γ [19]; secondly, curcumin is a ligand of PPAR γ , and binds directly to PPAR γ , leading to the activation of PPAR γ [18]. Activation of PPAR γ by curcumin is important for the inhibition of EMT by curcumin. Down-regulation of PPAR γ by its pharmacology inhibitor BADGE or shRNA significantly weakened the inhibition of EMT by curcumin in HK-2 cells, indicating that PPAR γ signaling is actively involved in EMT process and curcumin treatment. With both ERK pathway and PPAR γ pathway implicated in the mechanism of the inhibition of EMT by curcumin, what is the cross talk between the two pathways in EMT in HK-2 cells? In the inflammatory animal model and cyst formation cell model, studies reported the activating effect of PPAR γ by curcumin is attributed to its ability to reduce the intracellular protein p-ERK [37,38,39]. But the crosstalk between ERK and PPAR γ is still controversial, and it is cell type-dependent. Two possible mechanisms are that either p-ERK phosphorylated and inactivated PPAR γ or PPAR γ activated ERK [40,41,42]. In HK-2 cell, it is very interesting to note in our study that the inhibition of PPAR γ by shRNA or BADGE has no effect on the activation of ERK, whereas suppression of ERK using U0126 blocks the phosphorylation of PPAR γ . These findings suggest that there is one way communication between ERK and PPAR γ , in which ERK affects PPAR γ but not in the reverse order in HK-2 cells.

In conclusion, our studies, for the first time, provided evidence for a protective role of curcumin in counteracting TGF- β 1-induced EMT in renal tubular epithelial cells via ERK-dependent and then PPAR γ -dependent pathway.

Author Contributions

Modified the draft: XMZ HC. Conceived and designed the experiments: XMZ WP. Performed the experiments: RL YMW. Analyzed the data: RL YJL. Contributed reagents/materials/analysis tools: WCF HW QJC. Wrote the paper: RL.

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