

Original Article

# PPAR $\gamma$ agonists inhibit TGF- $\beta$ -PKA signaling in glomerulosclerosis

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**Aim:** To study the probable mechanisms of the anti-glomerulosclerosis effects induced by peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) agonists in rat intraglomerular mesangial cells (MCs).

**Methods:** Cells were transfected with the pTAL-PPRE-tk-Luc<sup>+</sup> plasmid and then treated with different concentrations of PPAR $\gamma$  agonist, either troglitazone or telmisartan, for the indicated times. Promega luciferase assays were subsequently used for the detection of PPAR $\gamma$  activation. Protein expression levels were assessed by Western blot, and PepTag<sup>®</sup> assays were used for the non-radioactive detection of protein kinase A (PKA) activity. The deposition of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and p-cyclic AMP responsive element binding protein (pCREB) were analyzed by confocal laser scanning.

**Results:** Both troglitazone and telmisartan remarkably inhibit the PKA activation and pCREB expression that is stimulated by TGF- $\beta$ . The PPAR $\gamma$  agonists also inhibited  $\alpha$ -SMA and collagen IV protein expression by blocking PKA activation.

**Conclusion:** PPAR $\gamma$  ligands effectively suppress the activation of MCs and the accumulation of collagen IV stimulated by TGF- $\beta$  *in vitro*. The renal protection provided by PPAR $\gamma$  agonists is partly mediated via their blockade of TGF- $\beta$ /PKA signaling.

**Keywords:** PKA signal pathway; glomerulosclerosis; PPAR $\gamma$ ; rat intraglomerular mesangial cells; troglitazone; telmisartan

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## Introduction

Glomerulosclerosis, characterized by as a phenotype transition of mesangial cells and an increase in extracellular matrix formation<sup>[1,2]</sup>, is a final common pathway leading to the loss of renal function in a variety of underlying kidney diseases. Multiple injuries to the glomerulus stimulate the glomerular intrinsic cells to proliferate, secrete pro-inflammatory factors, undergo cell death or necrosis, and lay down extracellular matrix<sup>[3]</sup>.

TGF- $\beta$ , known as a pivotal driver of glomerulosclerosis and tubulointerstitial fibrosis, is a multifunctional cytokine that regulates cell proliferation, differentiation, and production of extracellular matrix proteins in a variety of cells including mesangial cells<sup>[4]</sup>. Many different cellular responses are elicited by TGF- $\beta$ , and these are often cell-type specific<sup>[5]</sup>. The classic cellular response pathway involves Smad-mediated

changes in target gene transcription, which have become well understood in the past few years. Other signaling pathways, including mitogen activated protein (MAP) kinases, PI3 kinases, and Rho-like GTPases, can modulate Smad-dependent or independent cellular responses<sup>[6,7]</sup>. In particular, it has been proven that TGF- $\beta$  also mediates protein kinase A (PKA) signal transduction *in vivo*<sup>[8]</sup>. After TGF- $\beta$  combines with its receptor, the receptor phosphorylates PKA directly, with the formation of complexes between Smads and the regulatory subunits of PKA. Inhibition of the PKA signal pathway could block the matrix protein synthesis stimulated by TGF- $\beta$ <sup>[9]</sup>.

Multiple drugs with different pharmacological profiles are employed in the experiments on glomerulosclerosis, because it is one of the major pathological features of primary glomerular diseases. The more recently introduced drugs include the peroxisome proliferator-activated receptor (PPAR) $\gamma$  agonists (thiazolidinediones or glitazones).

PPAR $\gamma$ , a nuclear receptor that regulates specific gene transcription<sup>[10]</sup> is involved in the regulation of lipid and glucose metabolism<sup>[11]</sup>, and inflammatory responses<sup>[12]</sup>. Through PPAR $\gamma$  activation, PPAR $\gamma$  agonists exert some beneficial effects on different renal diseases, such as anti-

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inflammatory effects<sup>[13]</sup>, anti-fibrotic effects<sup>[14]</sup>, and vascular protective effects<sup>[15]</sup>, although the exact mechanism is not well understood<sup>[16]</sup>. Recently, it has been reported that the promoter activity and phosphorylation levels of cyclic AMP responsive element binding protein (CREB) could be inhibited by the activation of PPAR $\gamma$ , although it is still unclear what the exact regulation mechanism is and whether additional regulation exists at the level of CREB Ser133 phosphorylation. Because CREB phosphorylation is mainly modulated through PKA activation, similar modulation effects are also performed by the PPAR $\gamma$  agonist, raising the possibility that PPAR $\gamma$  activation might mediate the PKA signal pathway. These observations allow the speculation that a probable direct modulation on PKA signaling by PPAR $\gamma$  exists in the complex cellular signal networks. Therefore, the interaction between PPAR $\gamma$  and PKA signaling was examined. The probable mechanisms of anti-glomerulosclerosis effects mediated by PPAR $\gamma$  agonist in mesangial cells (MCs) were also investigated *in vivo*. On the other hand, telmisartan, a subclass of angiotensin receptor blockers (ARBs), has been reported to associate with PPAR $\gamma$  as a partial agonist<sup>[17]</sup>. Therefore, we chose both troglitazone and telmisartan as PPAR $\gamma$  ligands for these experiments.

## Materials and methods

### Reagents

Recombinant TGF- $\beta_1$  was purchased from R&D Systems (Minneapolis, MN). Telmisartan was obtained from Boehringer Ingelheim (Ingelheim, Germany). Troglitazone, a selective ligand for PPAR $\gamma$ , was kindly provided by Dr Ming HAN (Sankyo, Tokyo, Japan). Telmisartan and troglitazone were dissolved in DMSO, with a final concentration of 0.05% in the culture medium. Polyclonal anti-PPAR $\gamma$  (sc-7196) and anti-collagen IV (sc-9301) were purchased from Santa Cruz Biotechnology (California, USA). Monoclonal antibodies against CRE binding protein (CREB, #9197) and phospho-CREB (#9198), which recognizes phosphorylated Ser133, were obtained from Cell Signaling Technology (Boston, USA). Monoclonal anti- $\alpha$ -smooth muscle actin ( $\alpha$ -SMA, ab32575) was obtained from Abcam (Cambridge, UK), and horseradish peroxidase (HRP)-conjugated anti-mouse, anti-rabbit immunoglobulin were obtained from Dako (Glostrup, Denmark). The ECL detection system was obtained from Pierce Biotechnology (Rockford, USA). GW9662, a specific PPAR $\gamma$  antagonist, was from Cayman chemical (Michigan, USA). H89, a selective inhibitor of PKA, was from Alexis Biochemicals (San Diego, USA).

### Cell culture

Rat intraglomerular mesangial cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO, USA), at 37 °C in 95% air /5% CO<sub>2</sub>. Throughout this study, cells from passages 5 to 15 were used. All treatments were done in a serum-free medium in confluent cells that had been cultured in medium without FBS for 24 h before stimulation.

### Transient transfection and luciferase assay

To assess the PPAR $\gamma$  activation, MCs at 80% confluence were transiently transfected with 2.0  $\mu$ g of luciferase reporter plasmids containing either three consensus PPAR $\gamma$  response elements (pTAL-3xPPRE-TK-Luc) or the corresponding empty vector (pTAL-Luc) (a kind gift from Dr Qiu-jun LÜ) along with a  $\beta$ -galactosidase vector (Promega) for 4 h using Lipofectamine (Invitrogen Life Technologies, USA). The PPREx3-Luc reporter plasmid was designed to contain PPRE sites (5'-GTC-GACAGGGGACCAGGACAAAGGTCACGTTCCGGGAGGTCAC-3', three copies) attached to a luciferase vector (pTAL-Luc) as described<sup>[18]</sup>. After a 16-h recovery period in serum-free medium, cells were treated with troglitazone or telmisartan. The transfections were done in triplicate and repeated at least three times to ensure the reproducibility of the results. Firefly luminescence and  $\beta$ -galactosidase values were measured in cell lysates according to the instructions of the manufacturer from Promega. PPRE activities were expressed as the ratio of relative light units to the  $\beta$ -galactosidase values. In all cases, the luciferase activity of the empty vectors was negligible. Results (mean $\pm$ SD) were expressed as the fold increase in relative luciferase units, corrected to  $\beta$ -galactosidase, and compared with unstimulated cultures.

### Western blotting

After being intervened with troglitazone or telmisartan, washed cells (PBS, 4 °C) were harvested under non-denaturing conditions (4 °C/30 min) with lysis buffer (50 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1% TritonX-100, 0.1% SDS, 1% NP-40, 0.5% deoxysodium cholate, 1 mmol/L EGTA, 0.5 mmol/L benzamidine, 1.5 mmol/L sodium fluoride, 30  $\mu$ mol/L sodium vanadate, 10  $\mu$ mol/L sodium pyrophosphate, 2 mmol/L PMSF, 10  $\mu$ g/mL aprotinin, 10  $\mu$ g/mL leupeptin). Proteins were separated by electrophoresis (10% sodium dodecyl sulfate-polyacrylamide gel) and electroblotted to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH). The membranes were blocked with 5% nonfat dry milk in TBST (25 mmol/L Tris, 140 mmol/L NaCl, 3 mmol/L KCl, 0.05% Tween-20, pH 8.0, 37 °C, 1 h), and then were incubated with anti-PPAR $\gamma$  (1:200 dilution), anti-CREB (1:200 dilution), anti-pCREB (1:200 dilution), anti- $\alpha$ -SMA (1:200 dilution), and anti-collagen IV (1:100 dilution) antibodies at 4 °C overnight with continuous shaking in TBST containing 5% nonfat dry milk. Membranes were then washed with TBST (10 min $\times$ 3 times) and were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution) at room temperature for 1 h. Appropriate bands were identified with ECL Chemiluminescence Reagent and were followed by exposure to X-ray film (X-OMAT, Kodak, USA). Subsequently, immune complexes were removed from the membrane (5% NaOH, 37 °C, 5 min). Then, the immune complexes were re-blocked with 5% non-fat milk (37 °C, 1 h). Protein was assessed by re-blotting with anti-actin antibody (1:200 dilution) and a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1000 dilution). The bands were

photographed using Chemilmager 5500 (Alpha Innotech, San Leandro, CA).

#### *In vitro* kinase assay for PKA activity

After treatment with TGF- $\beta_1$ , troglitazone, telmisartan, or H89 for the indicated time periods, the cells were washed with ice-cold PBS and were harvested on ice. Then, cells were suspended in 0.5 mL of cold PKA extraction buffer (25 mmol/L Tris-HCl, pH 7.4, 0.5 mmol/L EDTA, 0.5 mmol/L EGTA, 10 mmol/L  $\beta$ -mercaptoethanol, 1  $\mu$ g/mL leupeptin, 1  $\mu$ g/mL aprotinin) and homogenized using a cold homogenizer. The supernatant was mixed with other compositions after centrifuge (5 min, 4 °C, 14000 r/min). Subsequently, all reaction components were added on ice in a final volume of 25  $\mu$ L of the following mixture: PKA Reaction 5 $\times$ Buffer 5  $\mu$ L, A1 Peptide (0.4  $\mu$ g/ $\mu$ L) 5  $\mu$ L, PKA Activator 5 $\times$ Solution 5  $\mu$ L, Peptide Protection Solution 1  $\mu$ L, cAMP-Dependent Protein Kinase (2  $\mu$ g/mL in PKA dilution buffer) 5  $\mu$ L. The mixture was incubated for 30 min at room temperature. Then, the reaction was stopped by heating at 95 °C for 10 min, and the samples were loaded onto the agarose gel (0.8% agarose in 50 mmol/L Tris-HCl, pH 8.0) for electrophoresis. The phosphorylated peptide migrates towards the negative electrode (cathode) while the non-phosphorylated one migrates toward the positive electrode (anode). The negative control lacks PKA enzyme and contains only buffer, while only the positive control contains the PKA catalytic subunit (final concentration 16 U/mL) supplied with the kit. The intensity of the bands was quantified as above.

#### Confocal microscopy

Extracellular matrix accumulation and the p-CREB translocation into the nucleus were determined using confocal microscopy of monolayers stained with antibodies to collagen IV and p-CREB. The cells were plated in a chamber-slide for 24 h in regular medium and 24 h in serum-free medium. Accompanied with TGF- $\beta_1$  stimulation, treatments with troglitazone, telmisartan, or H89 were applied for 24 h. This was followed

by thorough washes with PBS. Then, the cells were fixed with 4% fresh paraformaldehyde at 4 °C for 30 min and were permeabilized in PBS containing 0.2% TritonX-100. To block the nonspecific reaction, the cells were incubated with 5% BSA in PBS for 60 min. Then, the cells were incubated with the specific primary antibodies against collagen IV at a dilution of 1:50 at 4 °C overnight. After washes, cells were incubated with FITC conjugated donkey anti-goat IgG (1:50) for 1 h in the dark at room temperature. Cells were double stained with PI (propidium iodide) to visualize the nuclei. Slides were washed three times with PBS and glass coverslips were applied after the addition of one drop of mounting media. Confocal microscopy was performed using a Zeiss confocal laser scanning microscope (Carl Zeiss, Inc, Thornwood, NY). Basal and apical membrane locations were determined visually in the Z-plane using light field microscopy. Two to three photomicrographs per monolayer at the basal and apical membranes were then scanned with an omnichrome laser filtered at 480 nm to detect FITC and 530 nm to detect PI.

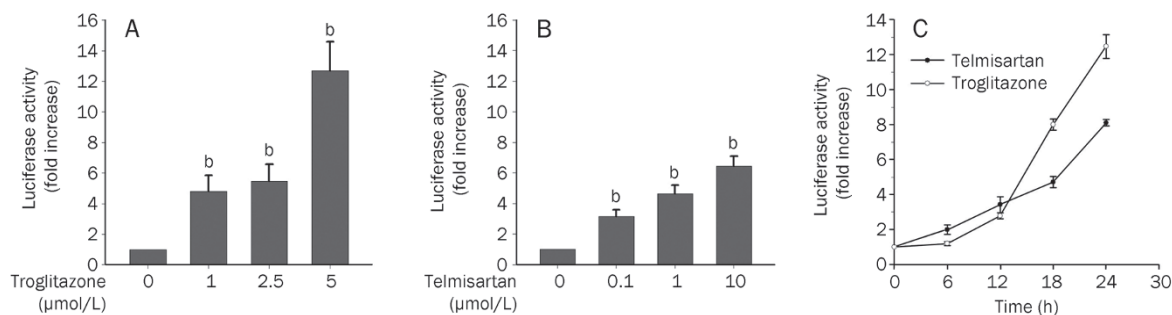
#### Statistical analyses

Data were expressed as mean $\pm$ SD. Difference of means was compared by one-way ANOVA and Student-Newman-Keuls *post hoc* test for the comparison of multiple means using SPSS 13.0 software. Statistical significance was defined as  $P < 0.05$ .

## Results

### PPAR $\gamma$ ligands enhanced activity of PPAR $\gamma$ response elements (PPRE) in MCs

As shown in Figure 1, treatment with different concentrations of PPAR $\gamma$  agonist caused an effective activation of promoter plasmid. The differences of luciferase levels between the treatment group and the control group were statistically significant. Both troglitazone and telmisartan enhanced luciferase expression in a dose-dependent manner. Figure 1C shows the time course of the effects of 5  $\mu$ mol/L troglitazone or 10  $\mu$ mol/L telmisartan on PPRE activities. The PPAR $\gamma$  activation was detected as early as 12 h after the start of incubation, and

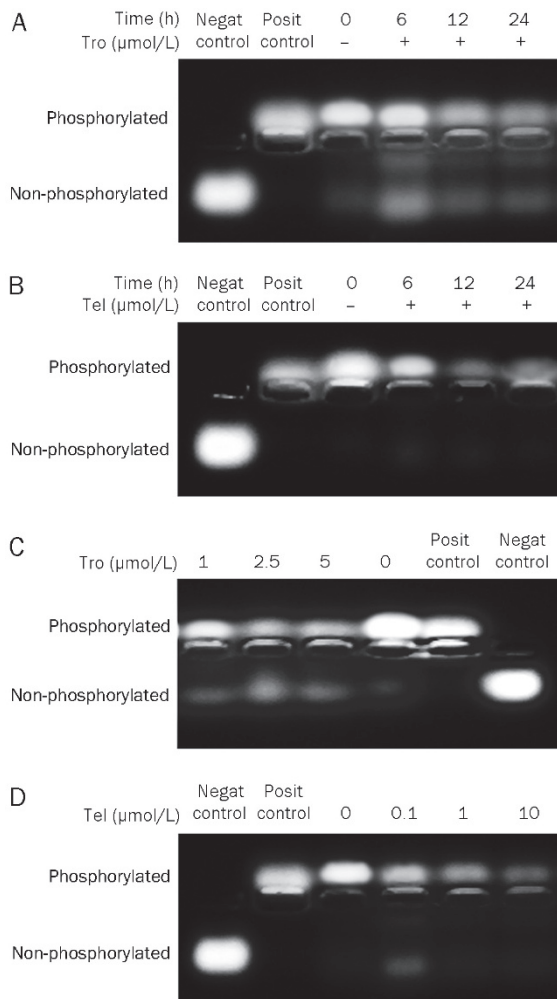


**Figure 1.** Both troglitazone and telmisartan activated PPAR $\gamma$  response elements (PPRE) effectively. MCs were transfected with 2.0  $\mu$ g tk-PPREx3-Luc and 0.2  $\mu$ g  $\beta$ -galactosidase vectors. After transfection, cells were treated with 1, 2.5, 5  $\mu$ mol/L troglitazone (A) or 0.1, 1, and 10  $\mu$ mol/L telmisartan (B) for 24 h. Meanwhile, transfected cells were treated with 5  $\mu$ mol/L troglitazone or 10  $\mu$ mol/L telmisartan for the indicated periods of time (C). Luciferase and  $\beta$ -galactosidase assays were performed at least three times, and the results were expressed as percentage of control considering the values in untreated samples as 100%. Luciferase activity represents data that have been normalized with  $\beta$ -galactosidase activity. The data represent the mean $\pm$ SD of three independent experiments. <sup>b</sup> $P < 0.05$  vs the un-stimulated controls.

enhanced remarkably in 24 h after treatments. Both troglitazone and telmisartan enhanced luciferase expression in a time-dependent manner.

### The suppression on PKA signaling induced by PPAR $\gamma$ agonists in normal MCs

To elucidate whether the influence on PKA pathway by PPAR $\gamma$  agonist existed in MCs, we measured the expression of major proteins of the PKA signal pathway. The inhibition of PKA activity was accelerated between 12 and 24 h after troglitazone or telmisartan exposure and then performed moderately. Meanwhile, both troglitazone and telmisartan inhibited PKA activation in variety of concentrations (Figure 2). Because



**Figure 2.** PPAR $\gamma$  agonists inhibited PKA signaling in normal MCs. (A) Time course 1. MCs were treated with troglitazone (Tro, 5  $\mu\text{mol/L}$ ) for the indicated time periods, and PKA activity was measured by PepTag $^{\text{®}}$  Non-Radioactive Protein Kinase Assays (promega) as described. Purified PKA catalytic subunit was used as a positive control (negative control contained only buffer), while all other samples contain cell lysates from treated and untreated cells. (B) Time course 2. Telmisartan (Tel, 10  $\mu\text{mol/L}$ ). PKA activity was estimated as in (A). (C) Dose response 1. MCs were treated with troglitazone (1, 2.5, and 5  $\mu\text{mol/L}$ ) for 24 h. (D) Dose response 2. Telmisartan (0, 0.1, 1, and 10  $\mu\text{mol/L}$ , 24 h).

the CREB phosphorylation depends on the PKA activation, we extended our observations by measuring the pCREB expression levels in response to the PPAR $\gamma$  agonist. Accordingly, significant pCREB signals at Ser-133 were detected within 24 h of stimulation by troglitazone or telmisartan (Figure 3A). The pCREB expression was suppressed markedly after exposure to either troglitazone or telmisartan. The inhibition of pCREB stimulated by PPAR $\gamma$  agonist could be abolished by a specific PPAR $\gamma$  antagonist-GW9662 (Figure 3B). However, PPAR $\gamma$  agonist had little effect on the total CREB expression (Figure 3). These findings indicated that PPAR $\gamma$  activation could directly influence the activation of PKA/CREB signaling in normal mesangial cells.

### Inhibition of TGF- $\beta_1$ -PKA signaling by PPAR $\gamma$ agonists

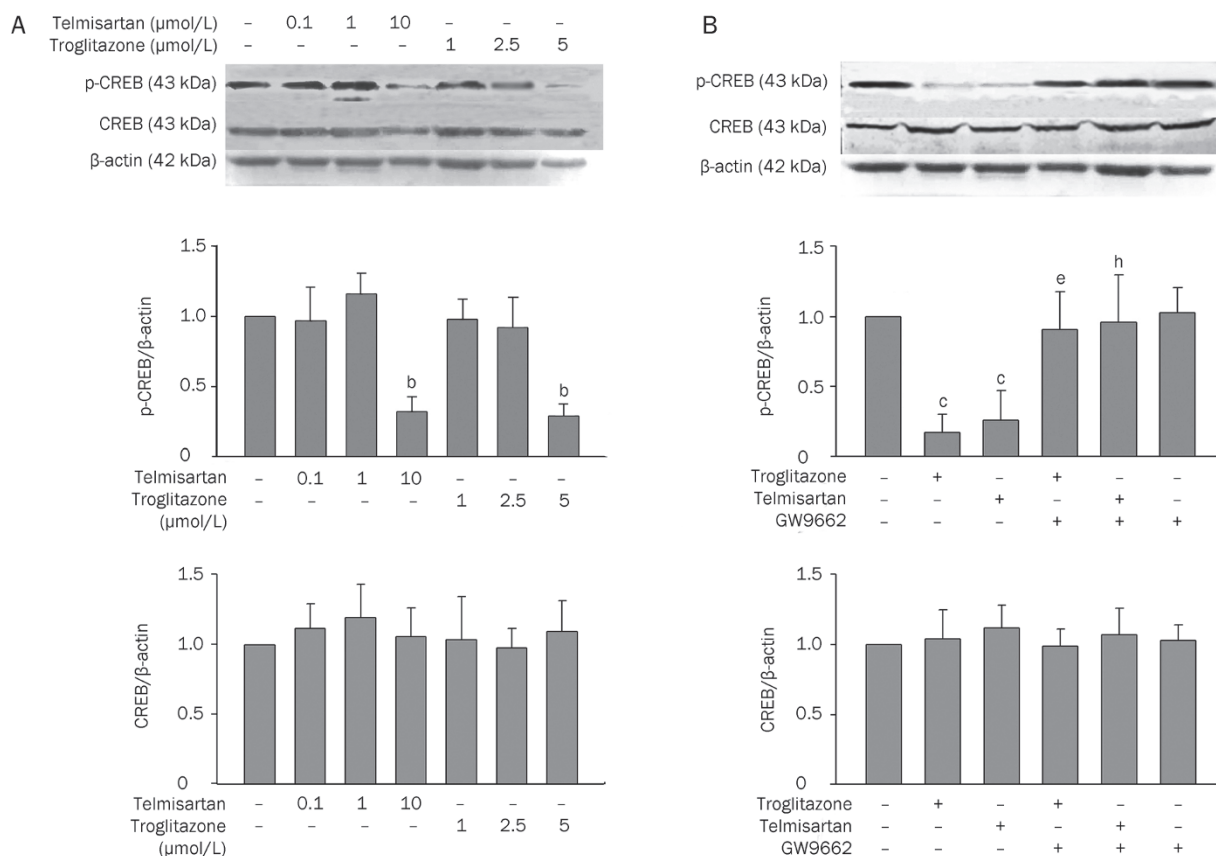
Because CREB cooperates with Smads to mediate TGF- $\beta_1$ -induced glomerulosclerosis process, we tested whether the PKA signal pathway, under the influence of TGF- $\beta_1$ , was suppressed by PPAR $\gamma$  agonist. In harmony with the former results, PPAR $\gamma$  agonist remarkably suppressed PKA activation induced by TGF- $\beta_1$  (Figure 4A). pCREB expression was also suppressed after PPAR $\gamma$  agonist treatments (Figure 4B), which was recovered by GW9662 (Figure 4B). We also examined the inhibition effects on PKA signaling performed by H89, a selective inhibitor of PKA. The mimic effects were also obtained after pre-incubation with 10  $\mu\text{mol/L}$  H89 (Figure 4C, 4D). Similar results were also obtained by confocal microscopy assays. It is demonstrated that a visible expression of pCREB in TGF- $\beta_1$ -treated MCs (Figure 4F) and noticeable suppression in PPAR $\gamma$  agonist-treated (Figure 4G) and H89-treated MCs compared with control (Figure 4E).

### PPAR $\gamma$ agonists suppressed TGF- $\beta_1$ -induced MCs phenotype alteration

The induction of  $\alpha$ -SMA is a hallmark for MCs phenotype alteration in glomerulosclerosis process. As shown in Figure 5, both troglitazone and telmisartan dramatically suppressed the  $\alpha$ -SMA expression mediated by TGF- $\beta_1$ . The suppression effects performed by PPAR $\gamma$  agonists were abolished by GW9662 pre-incubation.

### PPAR $\gamma$ agonists inhibit TGF- $\beta_1$ -mediated collagen IV expression

ECM accumulation is a dynamic process that results from the delicate balance between matrix synthesis and degradation. The collagen VI distribution, which was characterized as the important component of ECM<sup>[19]</sup>, was shown by confocal microscopy. As shown in Figure 6, TGF- $\beta_1$  markedly induced collagen IV expression in the extracellular matrix. Either telmisartan or troglitazone significantly abolished collagen IV over-production stimulated by TGF- $\beta_1$  (Figure 6A). In harmony with immunoblotting, similar results were obtained when the MCs were stained with a specific antibody against collagen IV (Figure 6B–6F). The collagen IV over-expression induced by TGF- $\beta_1$  was notably reduced after both PPAR $\gamma$  agonists and H89 treatments.



**Figure 3.** Both troglitazone and telmisartan inhibited the phosphorylation levels of cyclic AMP responsive element binding protein (CREB). Cells lysates were subjected to Western analysis with the pCREB and CREB antibody.  $\beta$ -actin was used as a loading control. (A) MCs were treated with troglitazone (0–5  $\mu$ mol/L) or telmisartan (0–10  $\mu$ mol/L) for 24 h. The expression levels of pCREB were represented as mean $\pm$ SD. <sup>b</sup> $P$ <0.05 vs control (untreated cell),  $n=3$ . (B) MCs were treated with either troglitazone (5  $\mu$ mol/L) or telmisartan (10  $\mu$ mol/L) in the presence or absence of GW9662 (20  $\mu$ mol/L).  $n=3$ . The data was represented as mean $\pm$ SD. <sup>c</sup> $P$ <0.01 vs control (untreated cell). <sup>e</sup> $P$ <0.05 vs troglitazone group. <sup>h</sup> $P$ <0.05 vs telmisartan group.

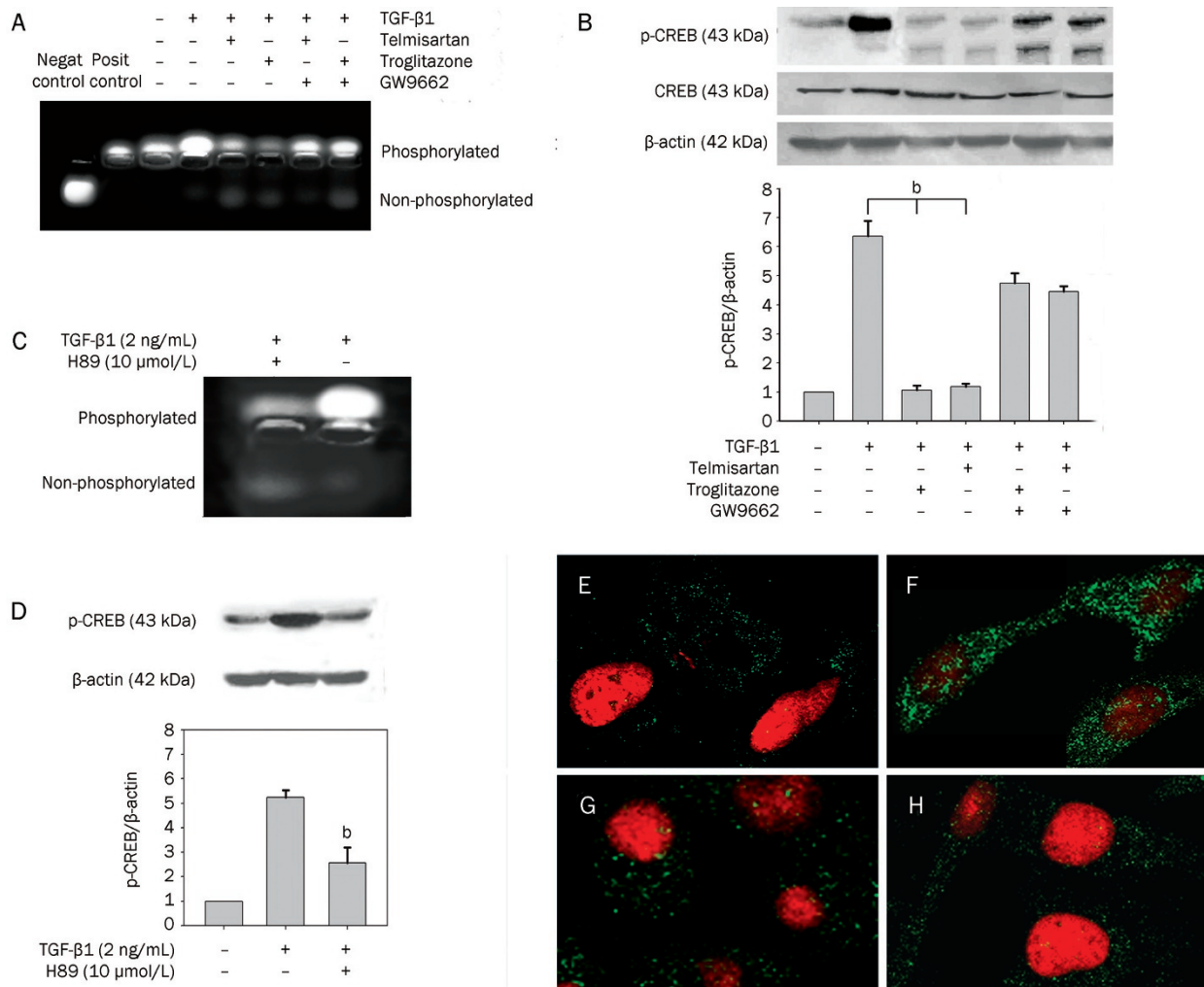
## Discussion

PPAR $\gamma$  is a ligand-activated transcription factor belonging to the nuclear hormone receptor superfamily. Like other nuclear receptors, PPAR $\gamma$  has a modular structure consisting of an agonist-dependent activation domain (AF-2), a DNA binding domain, and an agonist-independent activation domain (AF-1)<sup>[20]</sup>. Many of its biological effects are mediated by receptor regulation of target gene transcription in a ligand-dependent manner. It has been recognized increasingly that PPAR $\gamma$  agonists have protective effects on the progression of glomerulosclerosis<sup>[21, 22]</sup>, possibly by a mechanism that is independent of insulin/glucose effects and associated with the regulation of glomerular cell proliferation, hypertrophy, and decreased PAI-1 and TGF- $\beta$  expression<sup>[21]</sup>. Through a PPAR $\gamma$ -dependent manner, PPAR $\gamma$  agonists exert some beneficial effects on the kidney, although the molecular mechanism is not well understood.

In this paper, we demonstrated that both troglitazone and telmisartan suppressed PKA signal transduction in both dose-dependent and time-dependent manners in normal MCs. Pre-incubation with GW9662 abolished the suppression effects induced by both troglitazone and telmisartan, suggesting that

the suppression effects were caused by PPAR $\gamma$  activation. It suggests that PPAR $\gamma$  agonists indeed influenced PKA signaling cascades in MCs by PPAR $\gamma$  dependent mechanisms.

The inactive holoenzyme PKA consists of two regulatory (R) and two catalytic subunits. Unlike many protein kinases whose activity is regulated by the transient addition of a phosphate to the activation loop, PKA is assembled as a fully active enzyme that is kept in its inactive state by inhibitory proteins<sup>[23]</sup>. There are two classes of inhibitors: the heat-stable protein kinase inhibitors (PKIs) and the regulatory subunit. PKI binds with high affinity to the free catalytic subunit, but, unlike the R subunit, it includes a nuclear export signal (NES) that mediates active nuclear export of the catalytic subunit. The NES at the C terminus of the PKI provides a mechanism for rapidly exporting the catalytic subunit from the nucleus<sup>[24]</sup>. Protein-protein interactions are important for many signaling processes. The research focused on PPAR $\gamma$  localizations disclosed the protein-protein interactions between PPAR $\gamma$  and MEKs in its nuclear translocation<sup>[25]</sup>. Indeed, several nuclear receptors were reported to interact with regulatory proteins via the AF2 domain of PPAR $\gamma$ , including glucocorticoid receptors, estrogen receptors, and NF- $\kappa$ B interactions with



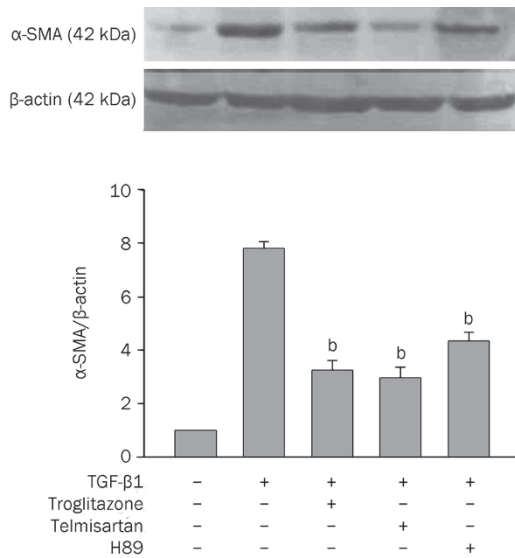
**Figure 4.** PPAR $\gamma$  agonists inhibited TGF- $\beta$ 1 /PKA signaling. (A and B) MCs were treated with troglitazone or telmisartan after exposure to TGF- $\beta$ 1, in the presence or absence of GW9662. PKA activation was measured in MC lysates as described (A). The expression levels of pCREB and total CREB were measured by Western blot (B). <sup>b</sup> $P < 0.05$ ,  $n = 3$ . (C and D) MCs were pre-incubated with or without 10  $\mu$ mol/L H89, then exposed to TGF- $\beta$ 1. PKA activation was measured (C). The expression levels of pCREB were measured by Western analysis (D). <sup>b</sup> $P < 0.05$  vs TGF- $\beta$ 1-treated cells.  $n = 3$ . (E–H) Assess nuclear distribution of pCREB by confocal microscopy in TGF- $\beta$ 1-treated cells without or with PPAR $\gamma$  ligands. (E) normal group; (F) TGF- $\beta$ 1-treated group (2 ng/mL); (G) troglitazone (5  $\mu$ mol/L) with TGF- $\beta$ 1; (H) telmisartan (10  $\mu$ mol/L) with TGF- $\beta$ 1.

PPAR $\gamma$ <sup>[26]</sup>. How did PPAR $\gamma$  affect the PKA activities? One speculation is, through nuclear export processes of catalytic subunits, an interaction may exist in the protein complexes of the catalytic subunit and PKI caused by PPAR $\gamma$ . Whether the protein-protein interaction indeed exists between PPAR $\gamma$  and the catalytic subunit, and how it works, are questions that require further research.

As a multi-functional factor, TGF- $\beta$  is a pivotal driver of glomerulosclerosis and tubulointerstitial fibrosis. The classical TGF- $\beta$ /Smads pathway in the fibrotic process has been well illustrated<sup>[27]</sup>. In recent years, some signaling molecules other than Smads were found to be involved in TGF- $\beta$  signaling. For example, p38 MAPK was previously recognized to play a critical role in the apoptosis of AML-12 cells induced by TGF- $\beta$ <sup>[28]</sup>. Additionally, interactions between the TGF- $\beta$ /Smads pathway components with the PKA/CREB signal pathway have been

widely reported<sup>[8, 29, 30]</sup>. The phosphorylation of Smad3 and Smad4 by TGF- $\beta$  have a direct protein-protein interaction with the regulatory subunit of PKA, leading to the release of the PKA catalytic subunit from the regulator subunit. When the PKA catalytic subunit translocates into the nucleus to phosphorylate its cellular substrate (CREB), the pCREB binds to conserved CREs in the promoters of many cAMP-responsive genes and regulates related gene transcription.

TGF- $\beta$ /PKA signaling plays an important role in many aspects of physiologic and pathologic processes. For example, TGF- $\beta$  induced apoptosis and Epithelial-to-Mesenchymal Transition (EMT) in AML-12 murine hepatocytes was associated with PKA activation<sup>[31]</sup>. Singh *et al* reported that laminin and fibronectin (Fn) expression induced by TGF- $\beta$ 1 could be down-regulated by PKA signaling blockage; however, the inhibition of PKC did not have significant effects on TGF- $\beta$ 1



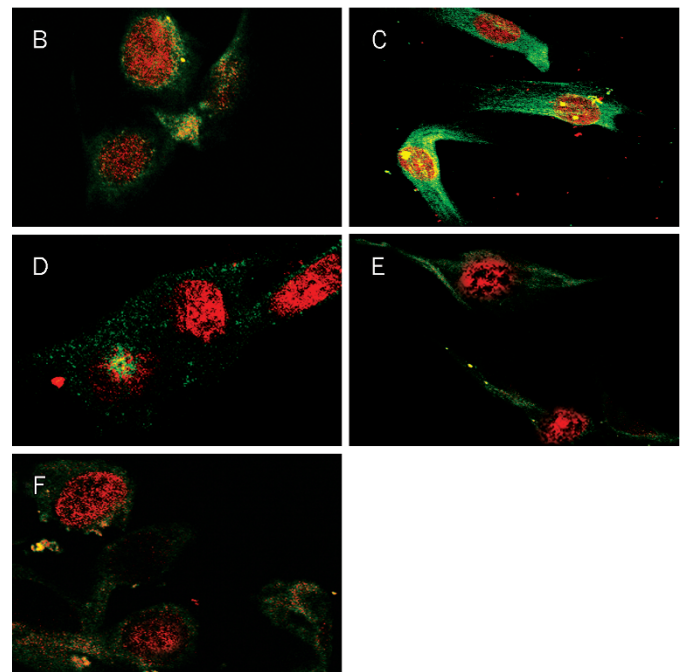
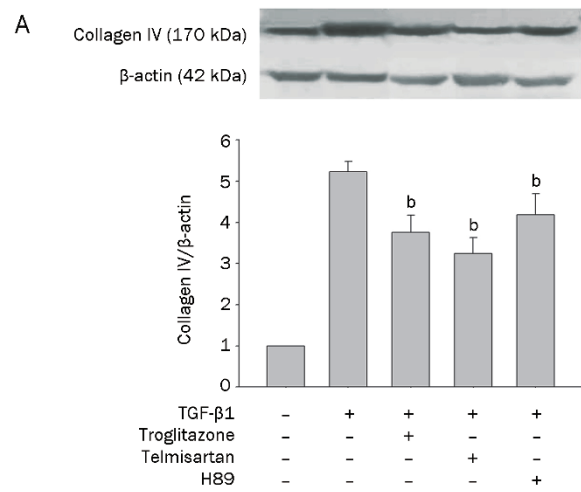
**Figure 5.** PPAR $\gamma$  ligands inhibit the activation of MCs mediated by TGF- $\beta$ 1. The expression of  $\alpha$ SMA mediated by TGF- $\beta$ 1 was measured by Western blot. MCs were treated with troglitazone (5  $\mu$ mol/L), telmisartan (10  $\mu$ mol/L) or H89 (20  $\mu$ mol/L) after exposure to TGF- $\beta$ 1. <sup>b</sup> $P$ <0.05 vs TGF- $\beta$ 1 treated group.  $n$ =3.

mediated laminin synthesis<sup>[32]</sup>. With these observations, it was also reported that the secretion of Fn was downregulated by PPAR $\gamma$  activation in lung carcinoma cells, which was mediated by antagonizing the transactivating activity of CREB binding to one CRE site in the Fn promoter<sup>[33]</sup>. These results indicate a potential involvement of PKA signaling in TGF- $\beta$  mediated pro-glomerulosclerosis process.

In our study, we proved that PPAR $\gamma$  agonists remarkably suppressed the PKA signaling stimulated by TGF- $\beta$ 1. The suppression effects were dependent on PPAR $\gamma$  activation. These results suggest that the suppression of PKA signaling cascades induced by PPAR $\gamma$  agonists not only in normal MCs, but also in TGF- $\beta$  stimulated cells. Because of the tight interaction between TGF- $\beta$  and the PKA/CREB pathway, which is important for the glomerulosclerosis process, these observations provided possibilities that PPAR $\gamma$  agonists might inhibit the TGF- $\beta$  mediated glomerulosclerosis process through the suppression of the PKA pathway.

Furthermore, the inhibition of TGF- $\beta$ /PKA signaling enacted by the PPAR $\gamma$  agonists efficiently blocked the  $\alpha$ SMA expression and collagen IV accumulation. The results demonstrate that TGF- $\beta$ 1 itself could stimulate MCs to differentiate into myofibroblasts; and that treatment with PPAR $\gamma$  agonists, accompanied by TGF- $\beta$ 1-stimulated differentiation, could inhibit the differentiation of MCs. Moreover, the inhibition effects of both telmisartan and troglitazone were mediated by PPAR $\gamma$  activation.

In summary, PPAR $\gamma$  activation in MCs leads to beneficial effects for TGF- $\beta$ -associated glomerulosclerosis. These reno-protective effects combine to suppress the activation of MCs and ECM accumulation. The relevant regulation mechanisms



**Figure 6.** PPAR $\gamma$  agonists inhibited the collagen IV accumulation. (A) The expression levels of collagen IV were measured by Western blot. MCs were treated with troglitazone (5  $\mu$ mol/L), telmisartan (10  $\mu$ mol/L) or H89 (20  $\mu$ mol/L) after exposure to TGF- $\beta$ 1. <sup>b</sup> $P$ <0.05 vs TGF- $\beta$ 1 treated group,  $n$ =3. (B-F) The localization of collagen IV in MCs was shown by confocal laser scanning microscopy. (B) Normal group; (C) TGF- $\beta$ 1-treated group (2 ng/mL); (D) Troglitazone (5  $\mu$ mol/L) with TGF- $\beta$ 1; (E) Telmisartan (10  $\mu$ mol/L) with TGF- $\beta$ 1; (F) H89 (20  $\mu$ mol/L) with TGF- $\beta$ 1.

act through the suppression of PKA signaling, which is tightly associated with TGF- $\beta$ . Our study thus suggests a novel inhibition of PKA signaling that is mediated by PPAR $\gamma$  activation, and explores the role of the reno-protective mechanisms induced by PPAR $\gamma$  agonists. This pathway will provide an additional therapeutic approach for the treatment of TGF- $\beta$ -mediated glomerulosclerosis.

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## Author contribution

Ying YAO designed research; Rong ZOU performed research; Gang XU, Xiao-cheng LIU, Min HAN, Jing-jing JIANG, Qian HUANG, and Yong HE contributed new analytical tools and reagents; Rong ZOU and Ying YAO analyzed data; Rong ZOU wrote the paper.

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