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# Rosiglitazone inhibits angiotensin II-induced CTGF expression in vascular smooth muscle cells—Role of PPAR-γ in vascular fibrosis

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ABSTRACT

Angiotensin (Ang) II plays a pivotal role in vascular fibrosis, which leads to serious complications in hypertension and diabetes. Connective tissue growth factor (CTGF) is a potent profibrotic factor implicated in the Ang II-induced pathologic fibrosis process. PPAR-γ activators thiazolidinediones have been recently reported to have beneficial vascular effects. However, their effects and related molecular mechanisms on extracellular matrix (ECM) turnover in vascular smooth muscle cells (VSMCs) are unknown. The present study evaluated the regulation of Ang II-induced CTGF, ECM production and cell growth by rosiglitazone in VSMCs. In aorta of Ang II-infused rats, CTGF expression was markedly increased, and type III collagen and fibronectin overexpression was observed. Cotreatment with rosiglitazone diminished these changes, whereas increased nuclear PPAR-γ expression in VSMCs. In growth-arrested VSMCs, rosiglitazone attenuated the proliferation and apoptosis, increased PPAR-γ production and activation, and reduced CTGF and ECM production in response to Ang II in a dose-dependent fashion. These inhibitory effects were attenuated by the pretreatment of cells with PPAR-γ antagonist GW9662 or bisphenol A diglycidyl ether (BADGE). Furthermore, rosiglitazone inhibited Ang II-induced Smad2 production and phosphorylation but had no effect on transforming growth factor-$\beta_1$ (TGF-$\beta_1$) expression. These results suggest that in Ang II-stimulated VSMCs, rosiglitazone caused an antiproliferative, antiapototic effect and reduces ECM production through mechanisms that include reducing CTGF expression, and a crosstalk between PPAR-γ and Smad may be involved in the inhibitory effects of rosiglitazone. This novel finding suggests a role of PPAR-γ activators in preventing Ang II-induced vascular fibrosis.

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## 1. Introduction

Vascular fibrosis, characterized by excessive deposition of extracellular matrix (ECM) (e.g., collagen and fibronectin), is a major complication of hypertension and diabetic mellitus [1]. Increasing evidence shows that angiotensin (Ang) II plays a critical role in the process of vascular fibrosis. It has been shown that infusion of Ang II is able to induce vascular fibrosis in experimental animals [2,3] and blockage of Ang II inhibits vascular fibrosis in diabetic, nephrectomy rats and NO-deficient mice [4–6]. After binding to its receptors, Ang II activates multiple downstream intracellular signaling pathways, induces proliferation, apoptosis and migration of smooth muscle cells, leads to vascular inflammation and increases deposition of ECM proteins which constitute the hallmark of vascular fibrosis [7].

Connective tissue growth factor (CTGF) is a potent profibrotic factor implicated in pathologic fibrosis processes, including skin disorder, tumor development, lung fibrosis, and renal disease [8]. In cardiovascular system, CTGF is over-expressed in the atherosclerotic lesions [9], myocardium of infracted rats and patients with cardiac ischemia [10,11], and arteries of hypertensive animals [12]. In vascular smooth muscle cells (VSMCs), it is involved in cell proliferation, migration and apoptosis [13,14]. Moreover, Ang II increases the production of CTGF and ECM, indicating that CTGF is an intracellular mediator of Ang II-induced vascular fibrosis [15].

Thiazolidinediones (TZDs), such as rosiglitazone, are high-affinity ligands for peroxisome proliferator-activated receptor-γ (PPAR-γ), a transcription factor of the nuclease hormone receptor superfamily [16]. They are mainly used as insulin-sensitizing drugs in type 2 diabetes mellitus. Increasing evidence shows that TZDs not only improve insulin resistance in patients with type 2 diabetes but also exert a broad spectrum of pleiotropic vascular effects in vitro and in animal models [17]. Being activated by TZDs, PPAR-γ heterodimerizes with retinoic X receptor and recognizes PPAR-response element in the promoters of target genes to regulate their expression [18]. Expression of PPAR-γ, initially thought to be restricted to adipose tissue, now has been documented in multiple vascular cell types (inclusive of endothelial cells [19], smooth muscle cells [20,21], and monocytes/macrophages [22]) and regulates gene expression of key proteins involved in vascular inflammation, proliferation and apoptosis.

TZDs possess potential actions in cardiovascular fibrosis. In vitro, TZDs attenuate cardiovascular fibrosis by preventing ECM production and cell growth, inhibiting the inflammatory response and apoptosis in fibroblast [23]. In vivo, it has been shown that TZDs potentially prevented cardiovascular fibrosis in myocardium infarcted rats [24], Ang II-infused and deoxycorticosterone acetate (DOCA)-salt rats [25,26]. Despite these findings, the underlying mechanisms for the regulatory effects of TZDs on the vascular fibrosis process are largely unknown. In light of the observations that VSMCs are the main target of Ang II-induced vascular damage and CTGF acts as an important mediator in both Ang II- and endothelin-1 (ET-1)-induced vascular fibrosis [27,28], we sought to examine whether rosiglitazone, a widely used insulin sensitizer, could suppress the Ang II-induced expression of CTGF, and related ECM production and cell growth in VSMCs.

## 2. Materials and methods

### 2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and TRIzol reagent were purchased from Gibco Brl (Carlsbad, CA). Ang II, 15-deoxy-delta$^{12,14}$-prostaglandin J2 (15-d-PGJ2), GW9662 and bisphenol A diglycidyl ether (BADGE) were from Sigma (St. Louis, MO, USA). Rosiglitazone and pioglitazone were from Alexis (Lausen, Switzerland). Rabbit polyclonal antibodies against CTGF and phosphorylated Smad2 were from ABCAM (Cambridge, UK). Rabbit anti-PPAR-γ antibody was from UPSTATE Inc. (Chicago, IL, USA). Rabbit anti-Smad2, anti-type III collagen and anti-fibronectin antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### 2.2. Animal experiments

The animal protocol conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Male Sprague-Dawley rats (weight 200–220 g) were anesthetized with methoxyflurane and then osmotic minipumps (model 2001, Durect corporation, Cupertino, CA) were inserted subcutaneously to deliver Ang II (150 ng/(kg min)) or 0.9% saline. One group was treated with rosiglitazone (5 mg/(kg day) in drinking water) for 8 days, starting the day before Ang II infusion. Systolic blood pressure (SBP) was measured by the tail-cuff method. At the indicated time points, the animals were sacrificed by injecting excess amount of pentobarbital. One portion of aorta was dissected and cleaned of fat, then frozen in liquid nitrogen for RNA extraction. Another portion of aorta was fixed in 4% formaldehyde solution and embedded in paraffin for immunohistochemical analysis.

### 2.3. Cell culture

VSMCs were obtained from thoracic aorta of normal Sprague-Dawley rats by the collagenase method as described previously [29] and cultured in DMEM containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml). VSMCs between passages 3 and 7, confirmed positive (99%) for smooth muscle α-actin immunostaining, were used in the experiments. For subsequent experiments, cells at 80% confluence in culture wells were growth-arrested by serum-starvation for 48 h.

### 2.4. Cell viability assay

Cells were grown in 96-well plates at the density of $1 \times 10^4$ per well. After indicated treatments, 3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) at 5 mg/ml was added to each well for 4 h. Subsequently, the culture medium was removed. Then, 150 μl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at 490 nm by using a microplate spectrophotometer (POLARstar, OPTIMA, Germany).

### 2.5. Apoptosis assay

Cell apoptosis was detected with flow cytometry using the AnxA5 FITC apoptosis detection kit (Merck KGaA, Darmstadt, Germany). Briefly, cells were washed with PBS, detached with 0.25% trypsin, resuspended in medium ($1 \times 10^6$ cells/ml) and incubated with the FITC-conjugated annexin V antibody and propidium iodide for 30 min. Annexin V binding was analyzed by FACScan (Becton Dickinson GmbH, Heidelberg, Germany), collecting the fluorescence of 10,000 cells according to the manufacturer's instructions. Cell apoptosis was expressed as percentages of annexin V-FITC-positive cells compared to the total cells.

### 2.6. Real-time reverse-transcriptase polymerase chain reaction

Total RNA was isolated with TRIzol reagent. Real-time quantitative reverse-transcriptase polymerase chain reaction with SYBR (real-time RT-PCR) was performed with Super-Script™ III Platinum® Two-Step qRT-PCR kit (Invitrogen, Carlsbad, CA) on an ABI PRISM 7000 sequence detection PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primers for rat PPAR-γ, $AT_1$, CTGF, fibronectin (FN), type III collagen (Col III) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with Beacon designer v 4.0 (Premier Biosoft, USA) (see Table 1 for the sequences). GAPDH was used as an internal control. Results were expressed as the ratio between each gene and GAPDH relative quantities.

To validate our real-time PCR protocol, gene-specific standard curves for each gene and GAPDH were generated from serial 10-time dilutions of the cDNA. Slopes of each gene were similar to GAPDH. A melting-curve analysis was also performed to check the absence of primer dimers.

### 2.7. Immunohistochemistry

For in vivo study, paraffin-embedded sections of rat thoracic aorta were examined by using immunohistochemistry. Primary antibodies, PPAR-γ (1:300), CTGF (1:400), FN (1:250), Col III (1:200), were added and incubated overnight at 4 °C, respectively. Biotinylated and affinity purified goat anti-rabbit IgG (Zymed, USA) was used as a secondary antibody and incubated for 1 h at 37 °C. A streptavidin–enzyme conjugate was sequentially added for 20 min and incubated with substrate 3′,3′-diaminobenzidine (DAB), followed by haematoxylin nuclear counterstaining. Negative controls without the primary antibody were incubated to check for nonspecific staining. Quantitative analyses were using Qwin 550 quantitative image analysis system (Leica, German) by measuring the gray scale. For in vitro study, cells were fixed with 4% paraformaldehyde, permeablized with 0.3% Triton X-100, and incubated with primary antibody at 4 °C overnight, and then subjected to a three-step staining procedure by using the streptavidin–biotin complex method. Visualization was performed by horseradish peroxidase-based colorimetric reaction with DAB.

### 2.8. Western blot analysis

Cells were lysed with 200 μl of ice-cold lysis buffer (50 mM HEPES, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, pH 4) in the presence of protein inhibitor cocktail (Roche, Germany) and phosphatase inhibitors (50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 nM microcystin). Protein concentrations were determined with the BCA protein assay kit (Pierce, Rockford, IL). Protein samples (20 μg) were resolved on 10% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane in semi-dry system (Bio-Rad, Hercules, CA). The membranes were incubated with specific antibodies against PPAR-γ (1:500), CTGF (1:1000), TGF-$\beta_1$ (1:200), Smad2 (1:200), phosphorylated Smad2 (1:200) and β-actin (1:500). In experiments of cell-associated proteins, β-actin was used as loading control. Signals were revealed with chemiluminescence and visualized by exposure to X-ray films. Optical densities of the bands were scanned and quantified with the Gel Doc 2000 (Bio-Rad). Data were normalized against those of the corresponding β-actin. Results were expressed as fold increase over control.

### 2.9. DNA-binding assay

Nuclear proteins were extracted by using a Pierce NE-PER kit (Pierce). PPAR-γ DNA-binding activity was detected by an ELISA-based method using the PPAR-γ transcription assay kit (Cayman Chemical, USA). Briefly, 10 μg nuclear protein was added to the 96-well plate pre-coated with PPAR specific double-strand DNA containing the sequence for peroxisome proliferators-response element (PPRE), and then incubated over night at 4 °C. Bound PPAR-γ was detected by the specific

**Table 1 – Primer sequence used for real-time RT-PCR**

| Gene | Primer sequence | Accession number | Expected size (bp) |
|---|---|---|---|
| PPAR-γ | 5′-TGGAG-CCTAA-GTTTG-AGTTTG-3′, 5′-ATCTT-CTGGA-GCACC-TTGG-3′ | NM_013124 | 226 |
| CTGF/$CCN_2$ | 5′-AAGAA-GACTC-AGCCA-GACC-3′, 5′-AGAGG-AGGAG-CACCA-AGG-3′ | NM_022266 | 260 |
| Col III | 5′-AGATG-CTGGT-GCTGA-GAAG-3′, 5′-TGGAA-AGAAG-TCTGA-GGAAG-G-3′ | NM_032085 | 134 |
| FN | 5′-GTGAA-GAACG-AGGAG-GATGT-G-3′, 5′-GTGAT-GGCGG-ATGAT-GTAGC-3′ | NM_019143 | 267 |
| $AT_1$ | 5′-CTCAG-CCACC-TAACT-TCC-3′, 5′-TTGTG-TTCCA-GAGTA-GCC-3′ | NM_030985 | 243 |
| GAPDH | 5′-GCCTT-CTCCA-TGGTG-GTGAA-3′, 5′-GGTCG-GTGTG-AACGG-ATTTG-3′ | NM_017008 | 309 |

PPAR-γ antibody. A horseradish peroxidase-conjugated secondary antibody was then added for colorimetric reading at 450 nm.

### 2.10. Statistical analysis

Statistical significance between groups was assessed by using one-way ANOVA, followed by post hoc Duncan multiple comparisons with the SPSS 10.0 program (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. Rosiglitazone attenuated CTGF and ECM expression in Ang II-infused rats

As assessed by real-time RT-PCR, in the rats infused with Ang II for 3 days, CTGF mRNA expression in the aorta was induced and remaining elevated at day 7 (Fig. 1A), and this change was significantly inhibited by rosiglitazone treatment. To define the cellular localization and protein expression of CTGF after rosiglitazone treatment, we examined expression of CTGF by immunohistochemistry. As shown in (Fig. 1B and C), robust CTGF expression observed in the aortic VSMCs of Ang II-treated animals was significantly decreased by rosiglitazone treatment ($P < 0.05$ versus control).

We then evaluated whether rosiglitazone diminished ECM production in parallel with CTGF downregulation. Infusion of Ang II for 7 days resulted in ECM deposition and increased expression of Col III and FN within the thoracic aorta of Ang II-infused rats (Fig. 1D and E). Significant blunting of these Ang II-mediated effects was observed in rosiglitazone-treated group.

### 3.2. Vascular PPAR-γ expression in Ang II-infused rats

After rosiglitazone treatment, the increase of PPAR-γ mRNA expression was observed (Fig. 2B). Immunohistochemistry showed that PPAR-γ protein expression was also elevated by rosiglitazone treatment, and was mainly located in nuclei of VSMCs, indicating that PPAR-γ activation may be involved in the suppression of Ang II-induced CTGF and ECM expression by rosiglitazone in vivo (Fig. 2A and B). Ang II also induced PPAR-γ protein production ($P < 0.05$ versus control) and elicited a trend to an induction of PPAR-γ mRNA expression, although it did not achieve statistical significance ($P > 0.05$ versus control).

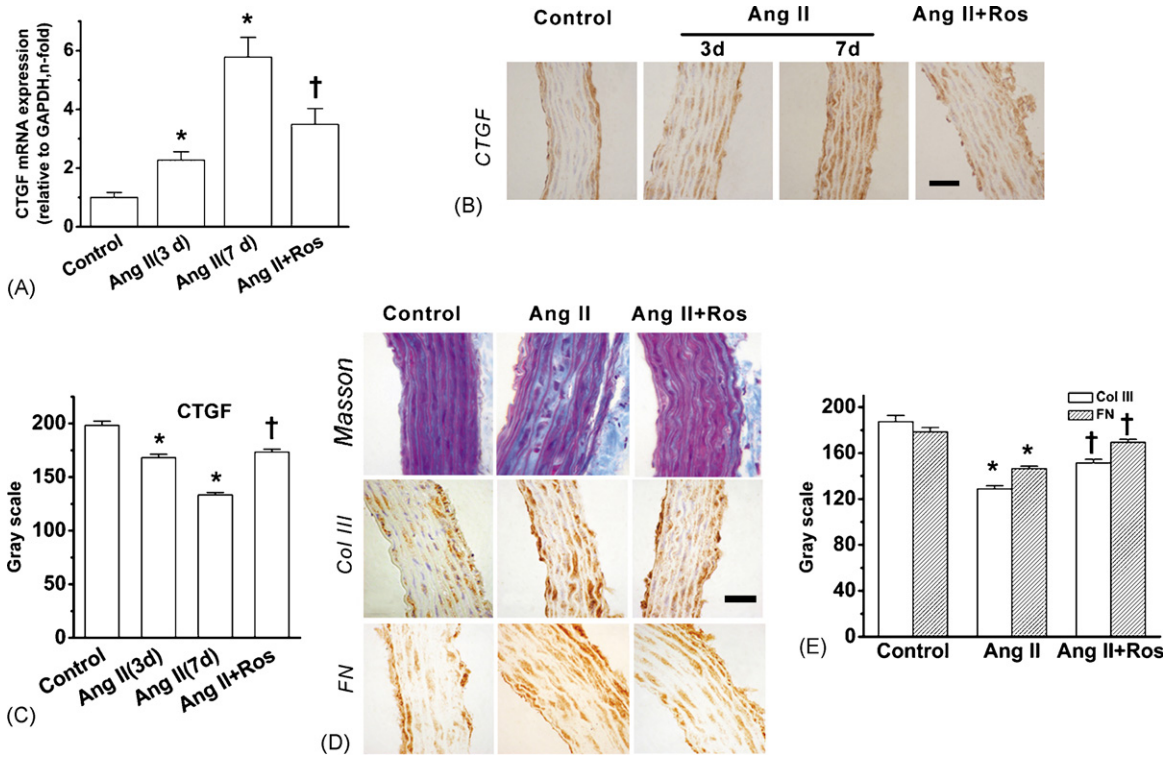


**Fig. 1 – In vivo induction of CTGF in response to Ang II and altered expression in response to rosiglitazone. (A) CTGF expression as measured by real-time RT-PCR in the thoracic aorta of Ang II-infused rats (150 ng/(kg min)), 3 and 7 days with or without rosiglitazone. Values are expressed as fold induction compared with control group. (B and C) Immunohistochemical staining and quantification of CTGF protein in each group. Figures show a representative of six animals studied in each group. Scale bar: 50 μm. Quantification results are presented as gray scale levels and mean ± S.E.M. data of 24 measurements in six slides. *P < 0.05 vs. control; †P < 0.05 vs. Ang II. (D and E) Masson staining, immunohistochemical staining and quantification of FN and Col III protein in each group. Figures show a representative of six animals studied in each group. Scale bar: 50 μm. Quantification results are presented as gray scale levels and mean ± S.E.M. data of 24 measurements in six slides. *P < 0.05 vs. control; †P < 0.05 vs. Ang II.**

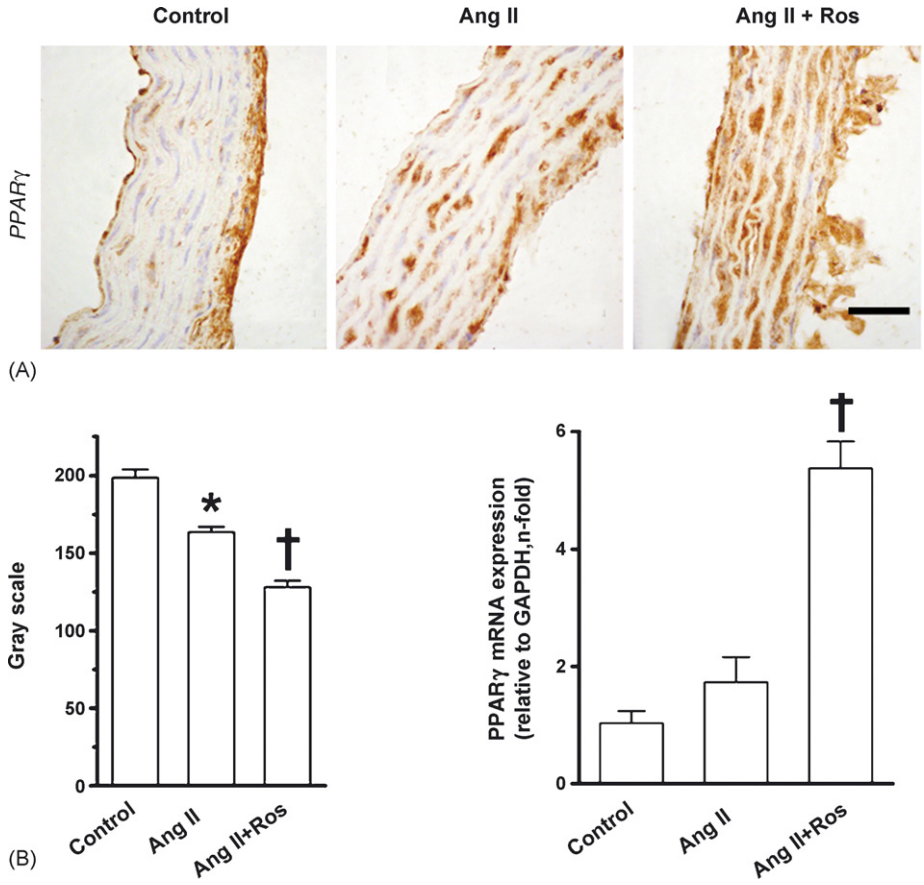


**Fig. 2 – Rosiglitazone upregulated vascular PPAR-γ expression. (A) Immunohistochemical staining of PPAR-γ in the thoracic aorta of rosiglitazone-treated and -untreated rats after Ang II-infusion. Figures show a representative of six animals studied in each group. Scale bar: 50 μm. (B) Quantification of PPAR-γ protein and mRNA contents in aorta after Ang II-infusion. Results were expressed as fold changes compared with controls. Ros indicates rosiglitazone. *n* = 6 per group. Results are presented as mean ± S.E.M. *P < 0.05 vs. control; †P < 0.05 vs. Ang II.**

### 3.3. *SBP in Ang II-infused rats*

The blood pressure of the rats was evaluated at baseline, 3 and 7 days after initiation of the Ang II-infusion. At third day, there was no difference in the SBP observed among each group (Fig. 3). At seventh day, Ang II-infusion induced a significant increase in SBP (165 ± 9 mmHg, $P < 0.05$ versus control), which was attenuated by rosiglitazone ($P < 0.05$ versus Ang II).

### 3.4. *Effects of rosiglitazone on proliferation and apoptosis of VSMCs in response to Ang II*

As shown in (Fig. 4A), Ang II (0.1 μM) induced a significant increase in the cell viability ($P < 0.05$ versus control). Pretreatment of the cells with rosiglitazone (1, 5 and 10 μM) or PPAR-γ nature ligand 15-d-PGJ2 (5 μM) markedly attenuated Ang II-induced proliferation in VSMCs. Moreover, this effect was substantially inhibited by the PPAR-γ antagonists GW9662 (3 μM) or BADGE (1 μM) ($P < 0.05$ versus Ang II + Ros). We then analyzed the effect of rosiglitazone on cell apoptosis. As shown in (Fig. 4B), Ang II (0.1 μM) significantly induced apoptosis in VSMCs. Pretreatment of cells with rosiglitazone (1, 5 and 10 μM) or 15-d-PGJ2 (5 μM) abolished the Ang II-induced apoptosis, and

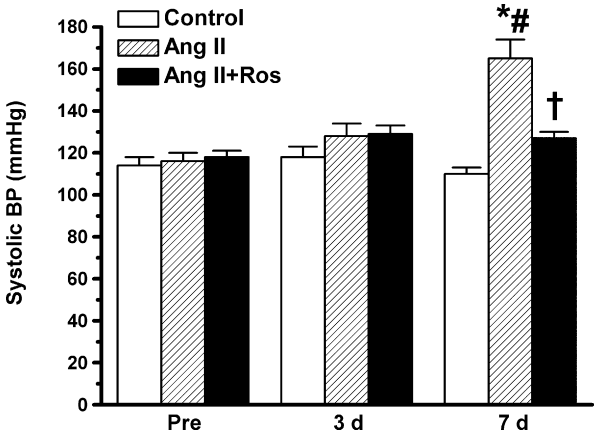


**Fig. 3 – SBP in Ang II-infused rats ± rosiglitazone. Blood pressure of Ang II-infused rats was measured before (Pre) and after Ang II-infusion for 3 and 7 days. *P < 0.05 vs. control; †P < 0.05 vs. Ang II; #P < 0.05 vs. Pre in the same group.**

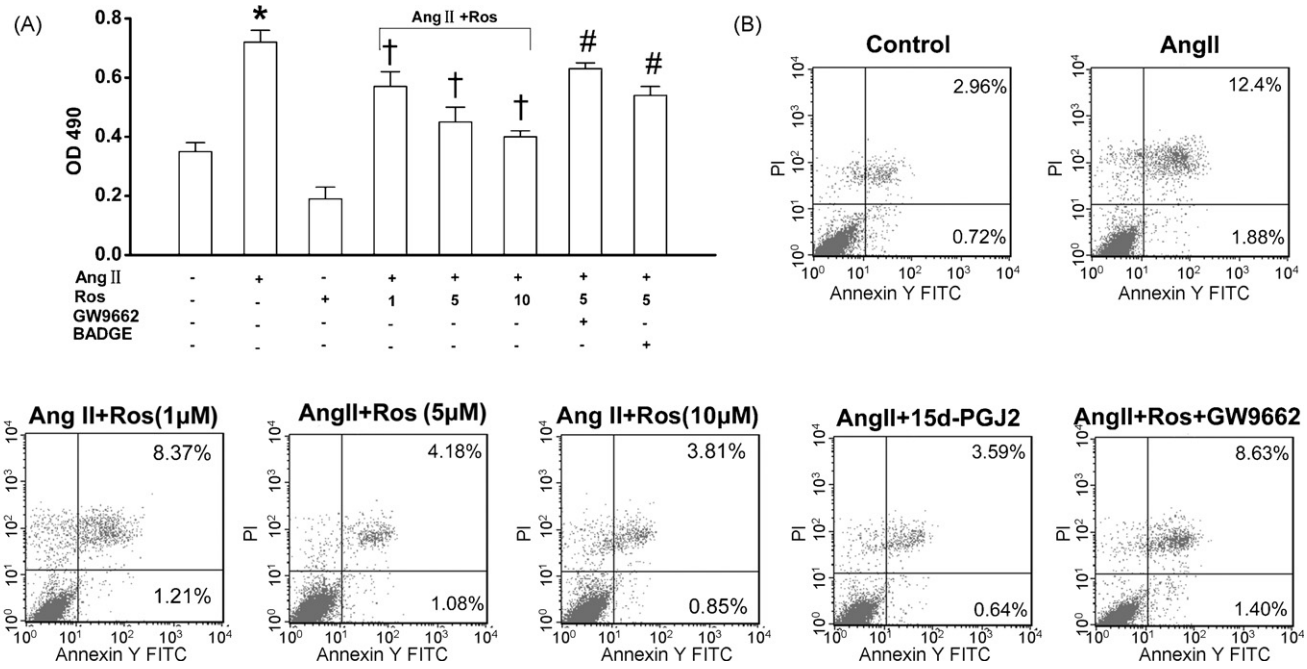


**Fig. 4 – Effects of rosiglitazone on proliferation and apoptosis in Ang II-stimulated VSMCs. (A) Rosiglitazone inhibited Ang II-induced cell proliferation in a PPAR-γ dependent fashion. Growth-arrested cells were switched to fresh medium containing 0.5% of serum when pretreated with or without PPAR-γ antagonist GW9662 (3 μM) or BADGE (1 μM) for 30 min prior to the addition of rosiglitazone (1, 5 and 10 μM) or PPAR-γ nature ligand 15-d-PGJ2 (5 μM). Ang II (0.1 μM) was then added for 24 h and MTT assays were carried out. Values represent the mean ± S.E.M. ($n = 12$) minus optical density of the vehicle control. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. Ang II; $^{\#}P < 0.05$ vs. Ang II + Ros. (B) PPAR-γ was involved in the anti-apoptotic effect of rosiglitazone in Ang II-stimulated VSMCs. Growth-arrested cells were switched to fresh medium containing 0.5% of serum when pretreated with or without PPAR-γ antagonist GW9662 (3 μM) prior to the addition of rosiglitazone (1, 5 and 10 μM) or 15-d-PGJ2 (5 μM). Ang II (0.1 μM) was then added for 24 h and annexin V-FITC based apoptosis assay was analyzed by FACScan.**

PPAR-γ antagonist GW9662 attenuated this effect, suggesting a PPAR-γ-dependent mechanism.

### 3.5. *Rosiglitazone inhibited Ang II-induced CTGF, Col III and FN expression in VSMCs in vitro*

To demonstrate whether a direct cellular mechanism was involved in the regulatory effect of rosiglitazone on Ang II-induced CTGF and ECM expression in VSMCs, we performed experiments in cultured VSMCs. As illustrated in (Fig. 5A), Ang II (0.1 μM) treatment for 24 h markedly increased the mRNA expression of CTGF, Col III and FN in VSMCs ($P < 0.05$ versus control). Rosiglitazone (1, 5 and 10 μM) dose-dependently abolished this effect ($P < 0.05$ versus Ang II). Similar results were observed when using 15-d-PGJ2 (data not shown). Notably, rosiglitazone had little effect on the basal expression level of Col III and FN in VSMCs.

Next, we examined the effect of rosiglitazone on CTGF protein production and secretion in Ang II-stimulated VSMCs. As shown in (Fig. 5), we found that CTGF protein was weakly detected in the untreated VSMCs (Fig. 5C) or in the conditioned medium (Fig. 5B) by Western blotting, whereas Ang II significantly induced CTGF protein production in VSMCs and the release into the extracellular medium (soluble fraction) after 24 h of treatment. Clearly, these effects of Ang II were inhibited by rosiglitazone (1, 5 and 10 μM) in a dose-dependent fashion. Immunocytochemistry showed that growth-arrested VSMCs presented a slight CTGF staining, and stimulation for 24 h with Ang II clearly increased cytoplasmic staining (Fig. 5D). This effect was also inhibited by rosiglitazone (5 μM).

### 3.6. *Suppression of CTGF expression is mediated by PPAR-γ*

To further demonstrate that PPAR-γ activation was involved in the regulation of Ang II-induced CTGF expression, first, we studied the expression and activation of PPAR-γ in response to Ang II and rosiglitazone treatment. As shown in (Fig. 6A–C), Ang II treatment for 24 h upregulated PPAR-γ protein expression whereas significantly decreased its activity when compared with control group. Pretreatment of cells with rosiglitazone significantly increased PPAR-γ expression and activation in Ang II-stimulated VSMCs ($P < 0.05$ versus Ang II).

If PPAR-γ activation is obligatory in the suppression of Ang II-induced CTGF expression by rosiglitazone, we would expect that other PPAR-γ activators would have a similar effect and that PPAR-γ antagonists would negate this action. To test this

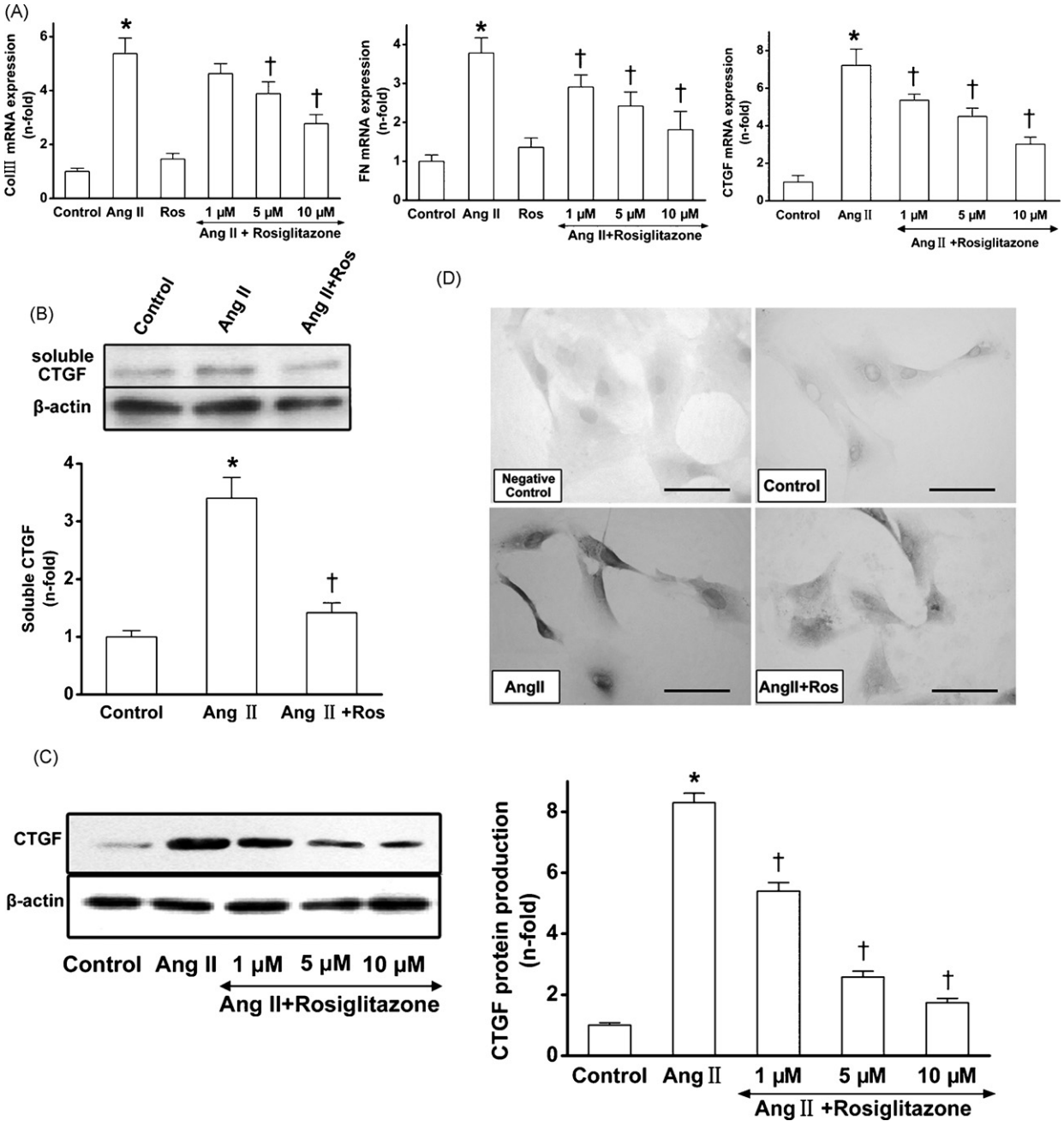


**Fig. 5 – Rosiglitazone inhibited Col III, FN and CTGF expression in Ang II-stimulated VSMCs. Cells were pretreated with increasing concentrations of rosiglitazone (1, 5 and 10 μM) for 1 h and subsequently stimulated with Ang II (0.1 μM) for 24 h. (A) Real-time RT-PCR revealed that rosiglitazone inhibited Ang II-induced CTGF, FN and CTGF mRNA expression in VSMCs. Results are expressed as fold increase over control and mean ± S.E.M. data of three independent experiments are shown. GAPDH was served as an internal control (*P < 0.05 vs. control; †P < 0.05 vs. Ang II). Western blots documented that rosiglitazone significantly inhibited Ang II-induced CTGF protein secretion (B) and production (C) in VSMCs. All results are representative of three independent experiments. β-Actin in cell fragment was served as an internal control. All values are showed as mean ± S.E.M. of three experiments (*P < 0.05 vs. control; †P < 0.05 vs. Ang II). Immunocytochemical staining (D) documented that rosiglitazone significantly inhibited CTGF cytoplasmic staining in VSMCs. Scale bar: 100 μm.**

hypothesis, VSMCs were pretreated with 15-d-PGJ2 (5 μM) or pioglitazone (50 μM), and subsequently stimulated with Ang II (0.1 μM) for 24 h. Western blot and real-time RT-PCR showed that either 15-d-PGJ2 or pioglitazone suppressed Ang II-induced CTGF expression (Fig. 6D and E). In contrast, pretreatment of cells with PPAR-γ antagonists GW9662 or BADGE partly diminished the effect of rosiglitazone. These results indicate that the effect of rosiglitazone on Ang II-induced CTGF expression is mediated at least in part by PPAR-γ activation.

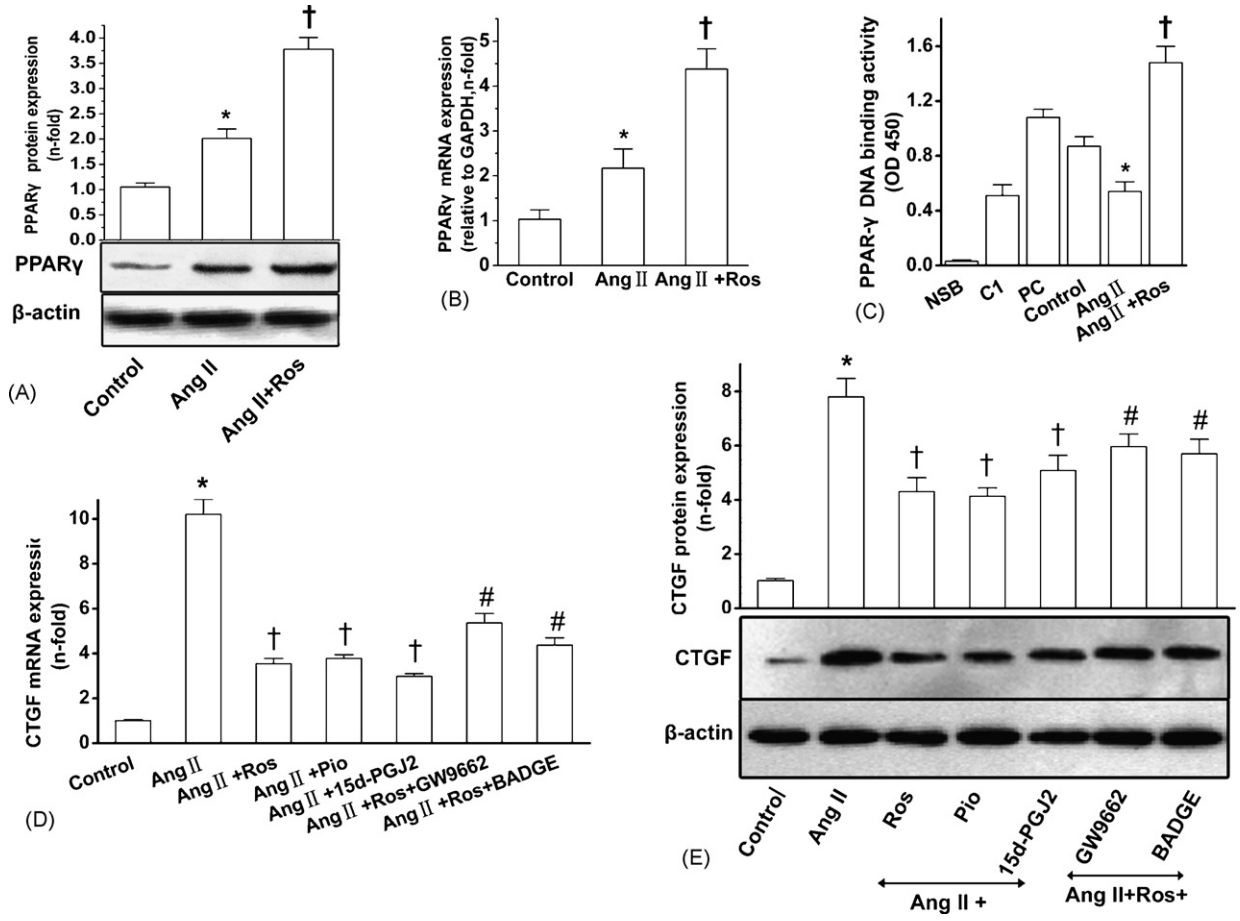


**Fig. 6 – Suppression of CTGF expression is mediated by PPAR-γ. Cells were pretreated with or without GW9662 (3 μM) or BADGE (1 μM) for 30 min prior to the addition of rosiglitazone (5 μM), 15-d-PGJ2 (5 μM) or pioglitazone (50 μM) for 1 h, and subsequently stimulated with Ang II (0.1 μM) for 24 h. (A) A representative immunoblots for PPAR-γ and β-actin from three separate experiments are shown. PPAR-γ protein expression is shown as fold increase over control. Results are presented as mean ± S.E.M. β-Actin was used as an internal control. (B) Real-time RT-PCR results are expressed as fold increase over control (mean ± S.E.M.). Data from three independent experiments are shown. GAPDH was served as an internal control (*P < 0.05 vs. control; †P < 0.05 vs. Ang II). (C) PPAR-γ activation was analyzed by DNA-binding assay using PPAR-γ transcription assay kit, where NSB indicates non-specific binding, C1 for competitor, and PC for positive control. Results are mean ± S.E.M. of three independent experiments, expressed as OD 450 (*P < 0.05 vs. control; †P < 0.05 vs. Ang II). (D) Real-time RT-PCR revealed that PPAR-γ antagonists attenuated CTGF mRNA expression in response to Ang II in VSMCs. Results are expressed as fold increase over control and mean ± S.E.M. data of three independent experiments are shown. GAPDH served as an internal control (*P < 0.05 vs. control; †P < 0.05 vs. Ang II, #P < 0.05 vs. Ang II + Ros). (E) Western blot indicated that PPAR-γ antagonists also attenuated Ang II-induced CTGF protein production. A representative Western blotting (upper panel) and mean ± S.E.M. data (bottom panel) of three independent experiments are shown. Results are expressed as fold increase over control. β-Actin served as an internal control (*P < 0.05 vs. control; †P < 0.05 vs. Ang II; #P < 0.05 vs. Ang II + Ros).**

### 3.7. *Effect of rosigltiazone on type I Ang II receptor ($AT_1$) expression*

Previous study indicated that PPAR-γ activation transcriptional suppressed $AT_1$ gene expression in vascular smooth muscle cells [30]. We then investigated whether this mechanism was involved in the regulation of CTGF expression by rosiglitazone. As demonstrated in (Fig. 7), Ang II (0.1 μM) treatment for 24 h downregulated $AT_1$ mRNA expression ($P < 0.05$ versus control), whereas compared with Ang II group, cotreatment of cells with rosiglitazone showed little effect on $AT_1$ mRNA expression.

### 3.8. *Rosiglitazone downregulated Ang II-induced CTGF expression in a TGF-β independent manner*

It has been shown that TGF-β is a major regulator of CTGF expression [31], and that PPAR-γ activators regulate TGF-β expression in many types of cells [32,33]. Therefore, we examined TGF-$\beta_1$ expression in response to Ang II and its

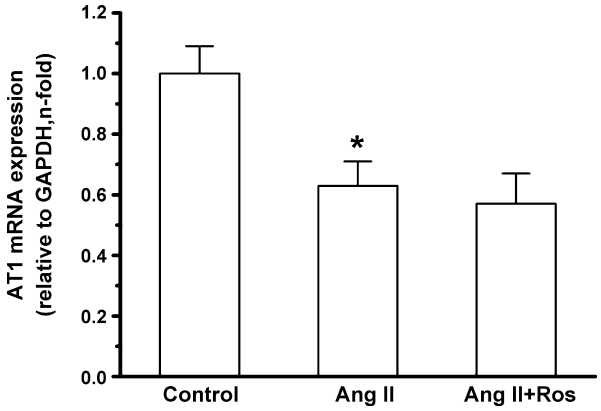


**Fig. 7 – Real-time RT-PCR revealed that rosiglitazone had little effect on $AT_1$ mRNA expression in Ang II-stimulated VSMCs. Results are expressed as fold increase over control and mean ± S.E.M. data of three independent experiments are shown. GAPDH served as an internal control (*$P < 0.05$ vs. control; †$P < 0.05$ vs. Ang II).**

modulation by rosiglitazone. As illustrated in (Fig. 8A), Ang II increased TGF-$\beta_1$ expression. However, rosiglitazone has little effect on Ang II-induced TGF-$\beta_1$ production.

### 3.9. *Rosiglitazone decreased Ang II-induced Smad2 expression and phosphorylation*

Rosiglitazone (5 μM) markedly diminished Ang II-induced Smad2 expression and phosphorylation ($P < 0.05$ versus Ang II) as shown in (Fig. 8B and C). This data suggested that rosiglitazone might regulate CTGF expression by interfering with Smad pathway.

## 4. Discussion

Hypertension and diabetic mellitus are risk factors for the development of coronary diseases and atherosclerosis [34]. Fibrosis is one of the vascular changes caused by hypertension and diabetic mellitus [35,36]. In the present study, we show that PPAR-γ ligand rosiglitazone is capable of attenuating Ang II-induced ECM and CTGF production in VSMCs both in vitro and in vivo. More importantly, we document that these effects are mediated partly by PPAR-γ activation likely through interference with the Ang II/Smad pathway. These findings extend the understanding of the important role of PPAR-γ in vascular fibrosis and provide novel evidence for the beneficial vascular effect of rosiglitazone.

Previous studies have shown that Ang II stimulated ECM synthesis in VSMCs [37,38]. The increase of ECM expression in response to Ang II in VSMCs was confirmed in the present study both in vivo and in vitro. More importantly, we, for the first time, demonstrated that the PPAR-γ activator rosiglitazone, both in vivo and in vitro, decreased both collagen and none-collagen expression in Ang II-challenged VSMCs. Notably, rosiglitazone did not affect the basal level of ECM production in these cells. Our study was in accordance with the previous study which showed that PPAR-γ activation decreased FN production in response to TGF-$\beta_1$ in VMSCs [39]. In contrast, it has been described that, rosiglitazone did not affect vascular collagen deposition in aorta of Ang II-infused rat [26]. This discrepancy might be due to the differences in the dosage of Ang II and animals used. In addition, Sirius Red staining was used to detect ECM content in the previous study, whereas immunohistochemistry and real-time RT-PCR used in the present study offered additional sensitivity to elicit the early change in ECM production in aorta.

Vascular wall size depends on a relative balance between proliferation and apoptosis. It is well known that Ang II is a potential trigger of proliferation and apoptosis [40,41]. Consistent with previous studies [21], we found that rosiglitazone has an anti-proliferative effect, which was PPAR-γ dependent. Some previous studies showed that PPAR-γ activators have a pro-apoptotic effect on VSMCs [42,43]. In contrast, we found in VSMCs, pretreatment of cells with rosiglitazone inhibited Ang II-induced apoptosis in a PPAR-γ dependent fashion. We speculated that the effects of rosiglitazone on VSMCs apoptosis may represent a both context- and dose-specific action, which is closely related to the type of pro-apoptotic stimuli as well as the dose of TZDs used. In the above-mentioned study [42], the effect of rosiglitazone on cell apoptosis took place at 30 μmol/l, whereas the anti-apoptotic effect on Ang II-induced apoptosis was found at a concentrations up to 10 μM. Recent reports have also demonstrated an anti-apoptotic effect of rosiglitazone in Ang II-stimulated kidney mesengial cells [44]. Therefore, one can hypothesizes that PPAR-γ activation with rosiglitazone either inhibits Ang II-induced proliferation or has an anti-apoptotic effect in VSMCs, thereby regulating vascular remodeling in aorta.

In VSMCs, as the major targets for Ang II in vascular remodeling, CTGF regulates cell proliferation and apoptosis, migration and fibrosis [13–15,45]. To evaluate the effect of rosiglitazone on Ang II-induced CTGF expression in vivo, we used the model of systemic infusion of Ang II. In this model, CTGF and ECM overexpression were not the result of blood pressure elevation but rather primarily of Ang II [15]. In the arteries of control animals, we observed a weak CTGF mRNA expression and staining of protein. Systemic infusion of Ang II into rats, which only slightly increased SBP (3 days), caused a marked aortic CTGF induction, and prolonged for at least 7 days, when ECM overexpression was observed. This finding further documented the notion that CTGF upregulation in this model was more likely due to the direct cellular effect of Ang II. Rosiglitazone treatment for 7 days significantly downregulated CTGF production, whereas upregulated the expression of PPAR-γ. These findings suggest that rosiglitazone downregulates CTGF expression and a PPAR-γ dependent pathway may be involved in this effect.

To clearly demonstrate that rosiglitazone downregulates Ang II-induced CTGF expression in VSMCs in a PPARγ-dependent manner and independent of depressurization- or hemodynamic-induced changes, since rosiglitazone has blood pressure-lowing effect, we performed experiments in cultured VSMCs. In these cells, Ang II increased CTGF expression, but decreased DNA-binding activity of PPAR-γ to PPRE. Pretreatment of cells with rosiglitazone significantly upregulated PPAR-γ expression and the DNA-binding activity, meanwhile, abrogated CTGF production and secretion. Furthermore, by

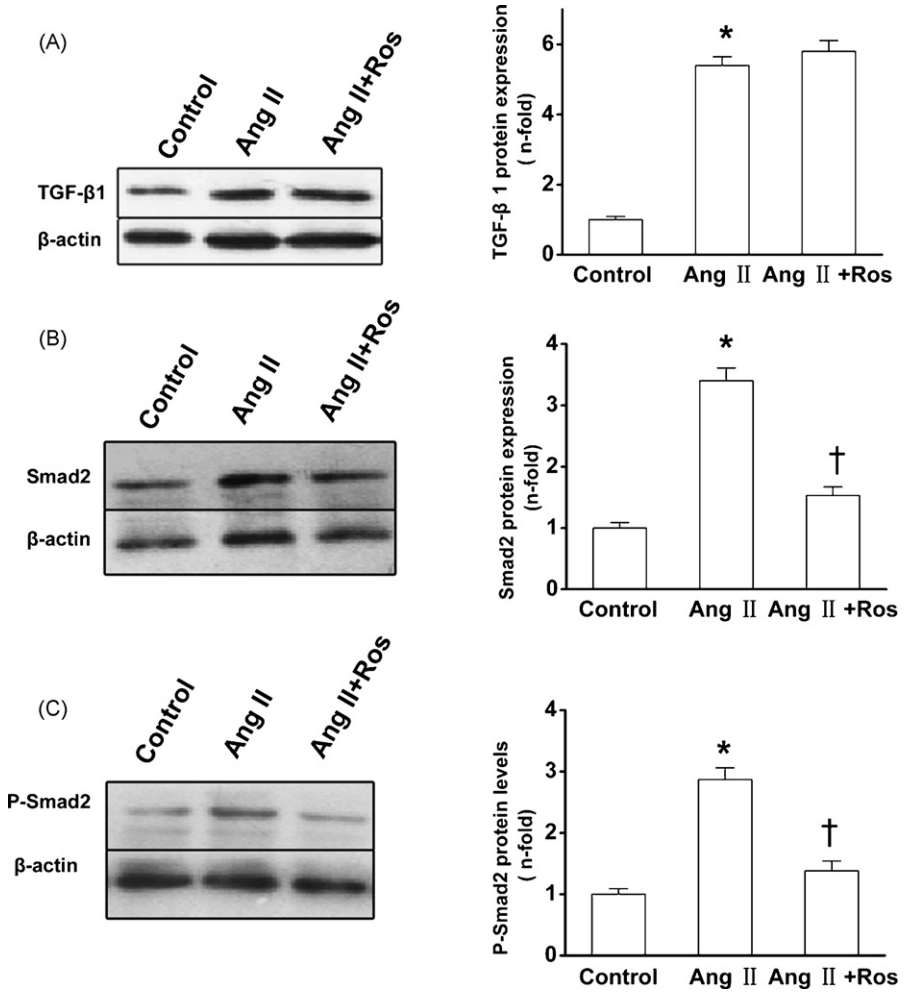


**Fig. 8 – Rosiglitazone inhibited Ang II-induced Smad2 expression and phosphorylation without affecting TGF-$\beta_1$ expression. (A and B) Rosiglitazone inhibited Ang II-induced Smad2 expression without affecting TGF-$\beta_1$ expression. Cells were pretreated with 5 μM rosiglitazone for 1 h, and subsequently stimulated with 0.1 μM Ang II for 24 h. A representative Western blot of Smad2, TGF-$\beta_1$ (upper panel) and mean ± S.E.M. data of three experiments (bottom panel) are shown. Results are expressed as fold increase over control. β-Actin was served as an internal control. (C) Rosiglitazone inhibited Ang II-induced Smad2 phosphorylation. Cells were treated with 0.1 μM Ang II for 20 min, and then were pretreated for 30 min with 5 μM of rosilitazone before Ang II treatment. A representative Western blot of phosphorylated-Smad2 (upper panel) and mean ± S.E.M. data of three experiments (bottom panel) are shown. $^{*}P < 0.05$ vs. control; $^{\dagger}P < 0.05$ vs. Ang II.**

using the PPAR-γ antagonists GW9662 and BADGE, we demonstrate that the effect of rosiglitazone on Ang II-induced CTGF expression is mediated partly by PPAR-γ. Our findings clearly indicate that direct cellular effects are involved in the regulatory effects of rosiglitazone on Ang II-induced CTGF expression. Furthermore, our study illustrate a potential role of PPAR-γ in the process of Ang II-induced vascular-fibrosis, because Ang II-induced ECM production and CTGF expression was accompanied by the inhibition of PPAR-γ activation, and when PPAR-γ activation was preserved by PPAR-γ ligands, CTGF and ECM production was diminished. Fu et al. [46] demonstrated that PPAR-γ activation suppressed TGF-β induced CTGF expression in cultured VSMCs. Our study extends these results to the Ang II-induced fibrosis process both in vitro and in vivo, because rosiglitazone prevented Ang II-induced CTGF not only in growth arrested VSMCs but also in rat aorta. Moreover, judging by the effects of PPAR-γ antagonists in this study, GW9662 and BADGE only partially reversed the effect of rosiglitazone on Ang II-induced CTGF expression. It has been shown that in cardiac fibroblast, PPAR-γ pioglitazone inhibited Ang II-induced ECM production through activation of NF-κB and AP-1 [47]. So we hypothesized that this discrepancy might because of that other PPAR-γ independent signal pathways, such as NF-κB and AP-1, were involved in the regulation effect of rosiglitazone on Ang II-induced CTGF and ECM production.

Ang II acts through its binding to specific $AT_1$ receptor that regulated CTGF expression. Previous studies demonstrated that PPAR-γ activation may transcriptionally regulate $AT_1$ expression [30]. We showed that rosiglitazone cotreatment had little effect on $AT_1$ receptor mRNA expression in Ang II-challenged VSMCs, which indicate that this mechanism may

not be involved in the inhibitory effect of rosiglitazone on Ang II-induced CTGF expression. Our finding was in accordance with those of Takeda et al. [48] and Sugawara et al. [49], who demonstrated that 24 h treatment with rosiglitazone had little effect on $AT_1$ expression in VSMCs when compared with untreated cells.

On binding to the $AT_1$ receptor, Ang II activates multiple signaling pathways, such as TGF-β that participate in regulating CTGF and ECM production [15,50,51], and more importantly, CTGF prompter has a TGF-β binding element [46], so we tested whether the deduction of CTGF by rosiglitazone was due to the downregulation of TGF-$\beta_1$ expression. Our data showed that Ang II-induced TGF-$\beta_1$ expression was not affected by rosiglitazone, indicating that rosiglitazone attenuated Ang II-induced CTGF expression by a TGF-β-independent mechanism. Some recent studies also support this term. It is documented that Ang II may induce CTGF production by a TGF-β-independent mechanism [28]. Moreover, in human kidney fibroblasts, pioglitazone inhibits cell growth and reduces matrix production by a TGF-β independent way [52]. These data indicate that PPAR-γ may affect post-TGF-β signals in regulating CTGF expression.

Smad signals are TGF-β downstream pathways in regulating CTGF expression. There are several Smad proteins in which Smad2, Smad3 and Smad4 are the mediators of TGF-β pathways [53,54]. In VSMCs, it is interesting that, independent of TGF-β signaling, Ang II activates Smad2 and Smad4, and regulates CTGF and ECM [28]. Moreover, PPAR-γ agonists and PPAR-γ receptor itself may interact with the TGF-β signaling pathway by inhibiting Smad3 [46]. We hypothesized that PPAR-γ activator may exert its effect on CTGF expression by crosstalk with Smad signal pathway. Our results indicated that PPAR-γ activator rosiglitazone inhibited Ang II-induced Smad2 expression and phosphorylation, suggesting the possible mechanism of PPAR-γ in Ang II-induced vascular fibrosis. Previous studies have shown that in glomerular mesangial cells, PPAR-γ activator pioglitazone has no effect on TGF-β-induced activation of Smad2 [55]. But in VSMCs, pioglitazone induced TGF-β production and caused phospho-Smad2 nuclear recruitment [33]. The difference between our results and previous studies may be caused by different signal transduction mechanisms between the Ang II/Smad and TGF-β/Smad pathways, or different phenotypes of cells were used in the study. For instance, it was documented that Ang II was unable to cause TGF-β activation in the VSMCs which was derived from Wistar rats [56].

In summary, the present study demonstrates that the PPAR-γ activator rosigltaizone inhibits Ang II-induced CTGF production in VSMCs, and in parallel, attenuates ECM production. The cross-talk between PPAR-γ and Smad signal pathway may be involved in the process. These observations point to a potential mechanism of PPAR-γ activator in preventing vascular fibrosis.

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