Osthole Regulates TGF-β1 and MMP-2/9 Expressions via Activation of PPARα/γ in Cultured Mouse Cardiac Fibroblasts Stimulated with Angiotensin II

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ABSTRACT - PURPOSE. Our previous studies have demonstrated that osthole, an active constituent isolated from the fruit of *Cnidium monnieri* (L.) Cusson, can prevent isoprenaline-induced myocardial fibrosis in mice, but the underlying mechanism is still unclear. **METHODS.** The mouse cardiac fibroblasts (CFs) stimulated with angiotensin II (Ang II) were cultured and treated with different concentrations of osthole. The mRNA expressions of peroxisome proliferator-activated receptor (PPAR) α/γ, transforming growth factor β1 (TGF-β1), and matrix metalloproteinase (MMP)-2/9 were detected by reverse transcription polymerase chain reaction method, and the protein expressions of nuclear factor-κB (NF-κB) and TGF-β1 were detected by Western blot method, respectively. **RESULTS.** Following treatment of cells with osthole at 2.5, 5, 10 and 20 μg/mL, the NF-κB and TGF-β1 expressions were dose-dependently decreased, while the expressions of PPARα/γ and MMP-2/9 were dose-dependently increased. After the cells were preincubated with PPARα antagonist (MK886) or/and PPARγ antagonist (GW9662), the inhibitions of osthole on the NF-κB and TGF-β1 expressions were decreased or completely halted and the increment of osthole on the MMP-2/9 expressions were also decreased or completely cancelled. **CONCLUSION.** Osthole could inhibit the NF-κB and TGF-β1 expressions by activation of PPARα/γ, and subsequently enhance the MMP-2/9 expressions in cultured CFs, and these effects of osthole may play the beneficial roles in the prevention and treatment of myocardial fibrosis.

INTRODUCTION

Myocardial fibrosis is a common pathological feature seen in many patients with heart diseases, and is hypothesized to be the final common pathway that ultimately results in irreversible organ failure (1). The renin-angiotensin system is a major regulator of systemic blood pressure (2). However, it is widely accepted that activation of the renin-angiotensin system and generation of angiotensin II (Ang II) play an important role in the pathogenesis of cardiac remodeling due to the induction and stimulation of transforming growth factor-β1 (TGF-β1) (3). TGF-β1 has been shown to be a powerful fibrogenic cytokine (4), and may rapidly cause the deposition of the extracellular matrix (ECM) via the enhancement of ECM synthesis and reduction of ECM degradation (5). The biological effects of TGF-β are mediated via a heteromeric serine/threonine receptor complex signalling through the Smad signaling pathway. In general, the predominant characteristic of fibrosis, ECM deposition, is from the activities of fibroblasts and myofibroblasts. Cardiac fibroblasts (CFs) may be activated in response to hypertrophic stimuli, including Ang II (6-9). In order to keep the balance between synthesis and degradation of ECM, CFs can also synthesize and secrete the ECM-regulatory proteins, namely, matrix metalloproteinases (MMPs), which are the predominant proteases responsible for degradation of ECM.

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Osthole, a coumarin derivative, is extracted from the fruit of *Cnidium monnieri* (L.) Cusson has been used as a traditional medicine in China for the treatment of skin diseases and gynecopathy (10). Recently, Liang et al. have reported that osthole could activate peroxisome proliferator-activated receptor (PPAR) α/γ in cultured cells, and have suggested that osthole might be a dual PPARα/γ agonist (11). In our previous studies, we have found that osthole could prevent isoprenaline-induced myocardial fibrosis by reduction of TGF-β1 and increment of PPARα/γ expressions in myocardial tissues (12), but the underlying mechanism is still unclear. Several documents have demonstrated that PPARα/γ agonists possess the anti-inflammatory and anti-proliferative properties (13, 14). In the present study, we wanted to further illustrate whether osthole decreases TGF-β1 and increases MMP-2/9 expressions via the PPARα/γ pathway in the cultured CFs stimulated with Ang II.

MATERIALS AND METHODS

Materials

Osthole was provided by Dr. Jia Zhou of Green Fount Natural Product Co., Ltd. (Xi'an, China). The purity of the drug was ≥98 % as determined by high performance liquid chromatography. Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Taq DNA polymerase and reverse transcriptase were bought from Sangon Gene Company (Shanghai, China) and Fermentas (Vilnius, Lithuania), respectively. The primers used for amplification by reverse transcription polymerase chain reaction (RT-PCR) were synthesized by Sangon Gene Company (Shanghai, China). The first antibodies to nuclear factor (NF)-κB-p65 and TGF-β1 were purchased from CST Company (Boston, USA) and Abcam Company (Hong Kong, China), respectively. MK886 (purity > 99 %) and GW9662 (purity > 98 %) were obtained from Cayman Chemical Company. All other reagents used in this study were of analytical grade.

Isolation and Culture of CFs

Kunming mice (male, 20±2 g in weight) were obtained from Animal Breeding Center of Soochow University (Suzhou, China). All experiments with animals were approved by the Ethics Committee of Soochow University and conducted according to the regulations for the use and care of experimental animals at Soochow University.

The mouse CFs were isolated according to the method described previously with minor modification (15). In brief, mouse hearts were dissected, minced, and digested with collagenase, the debris and myocytes were removed by unit gravity sedimentation. The obtained fibroblasts were then suspended in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum. After a 60-min period of attachment to uncoated culture plates, the cells that were weakly attached or unattached were rinsed free and discarded. The attached cells were cultured in an incubator with 5 % CO_2 at 37 °C. The culture medium was changed every other day. The cultured cells contained >95 % CFs as indicated by positive vimentin expression. When CFs reached 80 % of confluence, they were trypsinized by 0.25 % trypsin, and the amounts of cells were then adjusted to 5.0×10^5/mL in each culture flask. Cells between third and fifth passage were utilized for subsequent experiments.

Measurements of PPARα/γ, NF-κB, TGF-β1, and MMP-2/9 Expressions in Osthole-treated CFs Stimulated with Ang II

The CFs were divided into 6 groups: control group, model (Ang II) group, and Ang II with osthole 20, 10, 5, and 2.5 μg/mL groups. After pretreatment with osthole for 2 h, Ang II (a final concentration was 10^{-6} mol/L) was added and incubated with the cells for 24 h. The mRNA expressions of PPARα/γ, TGF-β1, and MMP-2/9 in cultured CFs were detected by RT-PCR method, and the protein expressions of NF-κB-p65 and TGF-β1 were detected by Western blot method, respectively.

Effects of PPARα/γ Inhibitors on NF-κB, TGF-β1, and MMP-2/9 Expressions in Osthole-treated CFs Stimulated with Ang II

The CFs were divided into 8 groups: control group, model (Ang II) group, Ang II with osthole 20 μg/mL group, Ang II with PPARα antagonist（MK886, 4 μmol/L）plus osthole 20 μg/mL group, Ang II with PPARα antagonist（MK886, 4 μmol/L）group, Ang II with PPARγ antagonist（GW9662, 20 μmol/L）plus osthole 20 μg/mL group, Ang II with PPARγ antagonist（GW9662, 20 μmol/L）group, Ang II with PPARα/γ antagonists （MK886 4

μmol/L and GW9662 20 μmol/L） plus osthole 20 μg/mL group. The cultured CFs were pretreated with PPARα/γ antagonists for 2 h, and osthole was then added and incubated with the cells for an additional 2 h, finally, the cells were stimulated with 10^{-6} mol/L Ang II for 24 h. The mRNA expressions of TGF-β1 and MMP-2/9 as well as protein expressions of NF-κB-p65 and TGF-β1 in cultured CFs were detected by RT-PCR and Western blot methods, respectively.

RT-PCR Assay

RT-PCR was used to measure the mRNA expressions of PPARα/γ, TGF-β1, MMP-2/9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in CFs. The harvested CFs were washed twice with ice-cold PBS and then placed in Trizol reagent, and total RNA was extracted following the manufacturer instructions. The final RNA pellet was resolved by 0.1% diethyl pyrocarbonate-treated water. The concentration and purity of the RNA were spectrophotometrically determined by the absorbance ratio of 260:280 nm. Total RNA (2 μg) was used as the RT reaction following the manufacturer instruction. After RT, a total of 22 μL of a PCR master mix, including all PCR components and primers (Table 1), were added to tubes containing 3 μL of cDNA. These tubes were then placed in a DNA thermal cycler. The PCR conditions were as follows: 32 cycles of denaturation at 94 °C for 30 s, annealing (Table 1) for 45 s, and extension at 72 °C for 45 s after an initial step of 94 °C for 5 min. A final extension was performed at 72 °C for 10 min. The PCR products were separated on a 1.5 % agarose gel, stained with ethidium bromide (0.5 g/L), and quantitated by densitometry using the Image Master VDS system and associated software (Pharmacia, USA). Data were expressed as ratio of the signals of interest band to those of GAPDH band. The latter acted as the internal control in the experiments.

Table 1. Sequences of the Primers used in RT-PCR Analysis

Gene	Primer Sequence (Sence/Anti-Sence)	Length (bp)	Annealing Temperature (°C)
PPARα	5′-CCTGGAAAGTCCCTTATCT-3′ 5′-GCCCTTACAGCCTTCACAT-3′	319	56
PPARγ	5′-CTCACAATGCCATCAGGTTT-3′ 5′-CTCTTGCACGGCTTTCTACGG-3′	359	51
TGF-β1	5′-CGGAAGCGCATCGAAGCCATCC-3′ 5′-GCAAGCGCAGCTCTGCACGG-3′	350	60
MMP-2	5’-AGATCTTCTTCTTCAAGGACCGGTT-3’ 5’-GGCTGGTCAGTGGC TTGGGGTA-3’	224	63
MMP-9	5’-CGACAGCACCTCCCACTATG-3’ 5’-CCCAACTTATCCA GACTCCT-3’	480	56
GAPDH	5′-GTATGACGTGGAGTCTACTG-3′ 5′-TACTCCTTGGAGGCCATGTA-3′	728	56

Western Blot Assay

Western blot assay was used to measure the protein expressions of NF-κB-p65, TGF-β1, and GAPDH in CFs. The harvested CFs were washed twice with ice-cold PBS and lysed in a buffer containing Tris-HCl (pH 7.4) 10 mmol/L, NaCl 150 mmol/L, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % SDS, edetic acid 5 mmol/L, phenylmethysulfonyl

fluoride (PMSF) 1 mmol/L, aprotinin 0.28 kU/L, leupeptin 50 mg/L, benzamidine 1 mmol/L, and pepstatin A 7 mg/L. The protein concentration was determined by a bicinchoninic acid (BCA) kit. 50 μg of protein from each sample was loaded onto 10 % SDS-polyacrylamide gel and subjected to electrophoresis, and then transferred to nitrocellulose membranes. The membrane was blocked with 5 % BSA and the primary antibody of rabbit anti-mouse NF-κB-p65 (1:100 dilution) or mouse anti-mouse TGF-β1 (1:75 dilution) or GAPDH was added overnight at 4 °C in Tris-buffered saline containing 0.1 % Tween 20 containing 5 % skimmed milk. The membrane was then washed and incubated with fluorescent secondary antibody for 1 h. The ratio of the protein interested was subjected to GAPDH and was densitometrically analyzed by Odyssey infrared imaging system.

Statistical Analysis

Data are indicated as mean ± SD. For statistical analysis, one-way ANOVA was performed with a post hoc LSD test for comparisons among groups at $p<0.05$.

RESULTS

Effects of Osthole on PPAR α/γ, TGF-β1, and MMP-2/9 mRNA Expressions in Mouse CFs Stimulated with Ang II

The results showed that after stimulation with Ang II 10^{-6} mol/L, the mRNA expressions of PPARα/γ and MMP-2/9 in CFs were significantly decreased （$P<0.01$）, while the TGF-β1 mRNA expression was markedly increased （$P<0.01$）as compared with the control group. The addition of 2.5-20 μg/mL osthole could prevent the cells from undergoing downregulation of PPARα/γ and MMP-2/9 mRNA expressions and upregulation of TGF-β1 mRNA expression in CFs stimulated with Ang II （$P<0.05$ or $P<0.01$）(Figs. 1 and 2), and these effects showed a good dose-effect relationship in the osthole-treated groups.

Osthole Decreased NF-κB and TGF-β1 Protein Expressions in CFs Stimulated with Ang II

Compared with the control group, the protein expressions of NF-κB and TGF-β1 in the Ang II-stimulated group were high（$P<0.01$）. After the addition of 2.5-20 μg/mL osthole, the protein expressions of NF-κB and TGF-β1 were dose-dependently decreased （$P<0.05$ or $P<0.01$）(Fig. 3), indicating that osthole could inhibit the upregulation of NF-κB and TGF-β1 proteins in the CFs stimulated with Ang II.

Effects of PPARα/γ Antagonists on Osthole-regulated mRNA Expressions of TGF-β1 and MMP-2/9 in CFs Stimulated with Ang II

After pretreatment of CFs with PPARα antagonist (MK886) or PPARγ antagonist (GW9662) for 2 h, the osthole-reduced TGF-β1 mRNA expression and -enhanced MMP-2/9 mRNA expression were attenuated（$P<0.05$ or $P<0.01$）(Fig. 4). Likewise, after pretreatment of CFs with MK886 plus GW9662 for 2 h, the effects of osthole on the TGF-β1 and MMP-2/9 mRNA expressions were almost canceled（$P<0.01$）. The effects of MK886 or GW9662 per se on the three gene expressions were not found.

Effects of PPARα/γ Antagonists on Osthole-reduced Protein Expressions of NF-κB and TGF-β1 in CFs Stimulated with Ang II

After pretreatment of CFs with PPARα antagonist MK886 or PPARγ antagonist GW9662 for 2 h, the osthole-reduced protein expressions of TGF-β1 and NF-κB were attenuated（$P<0.05$ or $P<0.01$）(Fig. 5). After the cells were pretreated with MK886 plus GW9662 for 2 h, the inhibition of TGF-β1 and NF-κB protein expressions by osthole were almost canceled （$P<0.01$）. The effects of MK886 or GW9662 per se on TGF-β1 and NF-κB expressions were not found.

DISCUSSION

Our results show that after stimulation with Ang II 10^{-6} mol/L, the PPARα/γ and MMP-2/9 expressions in cultured CFs are decreased, while the TGF-β1 and NF-κB expressions are increased. These were in accordance with previous reports (16-18). Importantly, we found that osthole could enhance the mRNA expressions of PPARα/γ and MMP-2/9 and reduce the mRNA expression of TGF-β1 and the protein expressions of TGF-β1 and NF-κB. The results were also consistent with our previous animal experimental results (12). Hence, we assumed that osthole might regulate the TGF-β1 and MMP-2/9 expressions through the PPARα/γ pathways in CFs. The finding also led us to the

same conclusion as Liang et al. that osthole might be a dual PPARα/γ agonist (11).

PPARs are ligand-activated nuclear transcription factors that have many biological effects on cardiovascular system, such as anti-inflammation and antiproliferation (13, 14). There are three subtypes, namely, PPARα, PPARβ/δ, and PPARγ. Intriguingly, it has been reported that PPARα and PPARγ ligands might attenuate myocardial fibrosis in various models of myocardial hypertrophy and infarction (19-22). Ogata et al. further demonstrated that the activation of PPARα/γ could inhibit the fibrotic gene expressions through interference with the inflammatory transcription factor NF-κB (21). Under normal conditions, NF-κB forms a cytoplasmic complex with its inhibitory κB proteins (IκB). On cell stimulation with inflammatory factors, the IκB is phosphorylated and degraded, the free NF-κB subsequently translocate to the nucleus where it initiates the gene transcription of proinflammatory and profibrogenic mediators (23), such as TGF-β1. In the present study, we examined the effects of osthole on NF-κB protein expression. The results indicated that the NF-κB protein expression in the osthole-treated groups was significantly decreased. So, we thought that inhibitory effect of osthole on TGF-β1 expression was from the reduction of NF-κB through the activation of PPARα/γ.

Collagen content of ECM depends not only on its production, but also on its degradation, which relies on enzymes such as MMP-2/9. It has been reported that TGF-β may block TNF-α-stimulated the upregulation of MMP-2 expression in lung fibroblasts (24), suggesting that the reduction of MMP-2 might be in response to TGF-β itself (25). But MMP-2 can activate TGF-β (26, 27), which in turn can promote the fibroblast-to-myofibroblast transition. It is well known that MMP-9 has a NF-κB binding site in the promoter regions (28, 29), suggesting that MMP-9 expression is associated with NF-κB. In this study, we found that Ang II could increase the expressions of NF-κB and TGF-β1 and decrease the expressions of MMP-2/9 in cultured CFs, while osthole pretreatment might reverse the effects of Ang II. Our present results, together with data in the literature, suggested that increments of MMP-2/9 expressions in the osthole-treated CFs might be related to the inhibition of NF-κB and TGF-β1 via the activation of PPARα/γ.

In order to further verify whether osthole regulated the NF-κB, TGF-β1, and MMP-2/9 expressions in CFs via PPARα/γ pathways, a specific PPARα inhibitor MK886 and a specific PPARγ inhibitor GW9662 were used (30, 31). The results showed that after pretreatment with MK886 and/or GW9662, the inhibitions of osthole on the NF-κB and TGF-β1 expressions were decreased or completely halted, and the increment of osthole on the MMP-2/9 expressions were also decreased or completely cancelled, suggesting that the increase in PPARα/γ mRNA expressions by osthole might be one of its mechanisms of regulating the NF-κB, TGF-β1, and MMP-2/9 expressions in cultured CFs. The present results further demonstrated that PPARα/γ-mediated mechanisms involved in the effects of osthole. But the effects of NF-κB and TGF-β1 on MMP-2/9 expressions seem to overlap to a certain extent, hence, further research is needed for clarification.

CONCLUSION

Osthole could inhibit the NF-κB and TGF-β1 expressions by activating the PPARα/γ, and subsequently enhance the MMP-2/9 expressions in cultured CFs. These effects of osthole may play the beneficial roles in the prevention and treatment of myocardial fibrosis.

ACKNOWLEDGEMENTS

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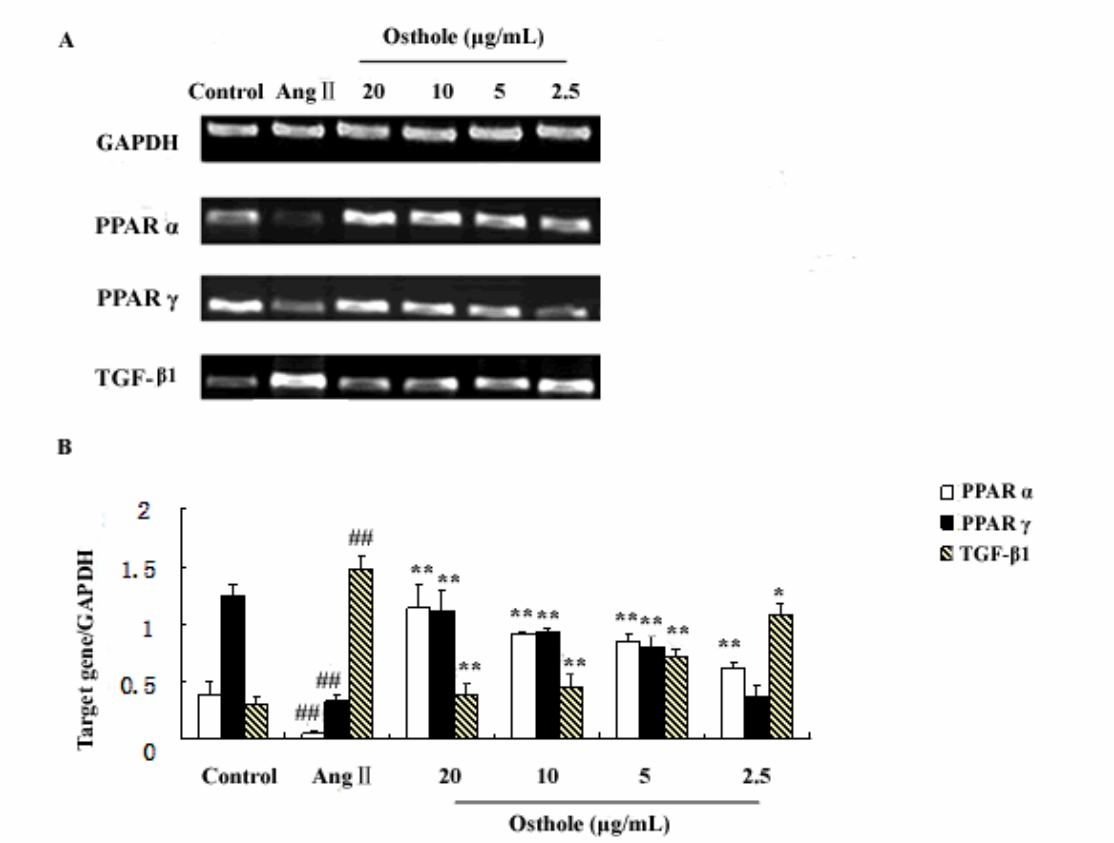

Figure 1. Effects of osthole on PPARα/γ and TGF-β1 mRNA expressions in the cultured mouse CFs stimulated with Ang II. $\bar{x} \pm$ SD, n=4. **A** is the electrophoresis gel photo of polymerase chain reaction products. **B** is the ratio of PPARα/γ or TGF-β1/GAPDH. Compared with the control group: $^{\#\#}P$<0.01; Compared with the Ang II group; $^{**}P$<0.01, $^{*}P$<0.05.

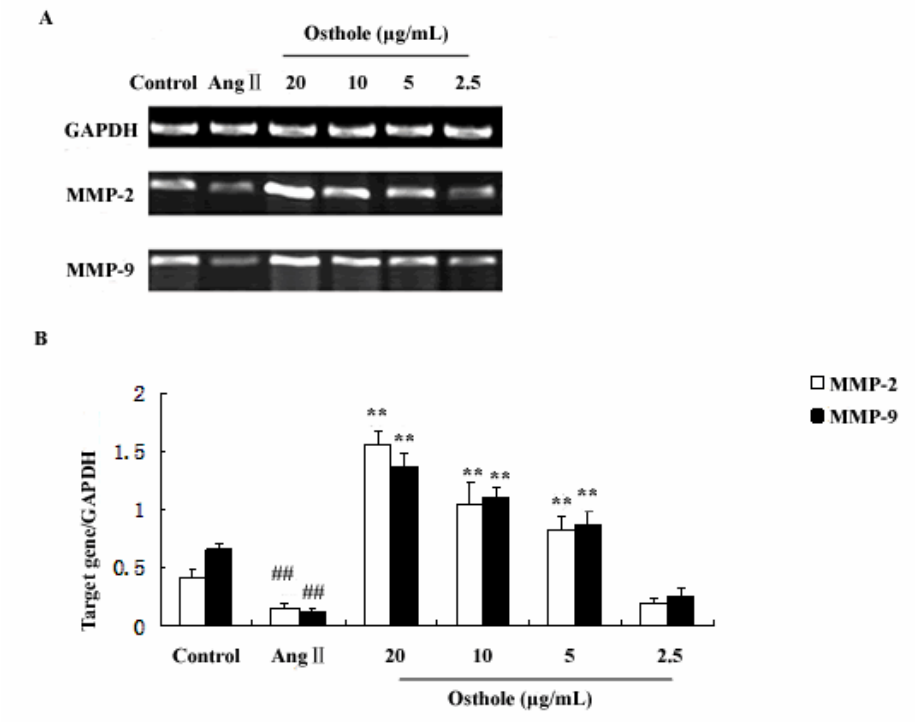

Figure 2. Effects of osthole on MMP-2/9 mRNA expressions in the cultured mouse CFs stimulated with Ang II. $\bar{x} \pm$ SD, n=4. **A** is the electrophoresis gel photo of polymerase chain reaction products. **B** is the ratio of MMP-2/9/GAPDH. Compared with the control group: $^{\#\#}P$<0.01; Compared with the Ang II group: $^{**}P$<0.01.

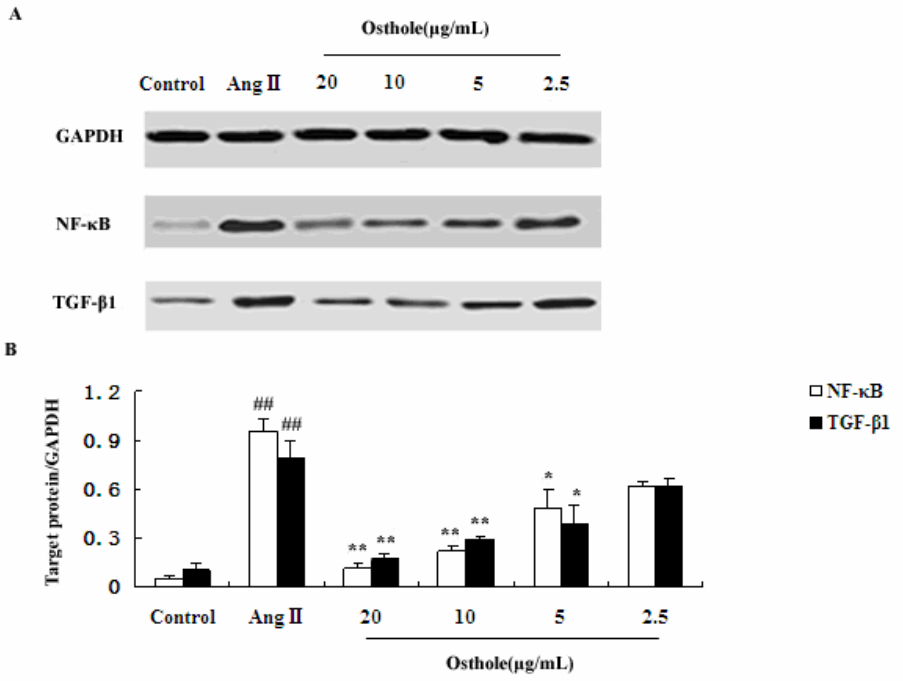

Figure 3. Effects of osthole on NF-κB and TGF-β1 protein expressions in the cultured mouse CFs stimulated with Ang II. $\bar{x} \pm$ SD, n=4. **A** is the photo of Western blot analysis. **B** is the ratio of NF-κB or TGF-β1/GAPDH. Compared with the control group: $^{\#\#}P$<0.01; Compared with the Ang II group: $^{**}P$<0.01, $^{*}P$<0.05.

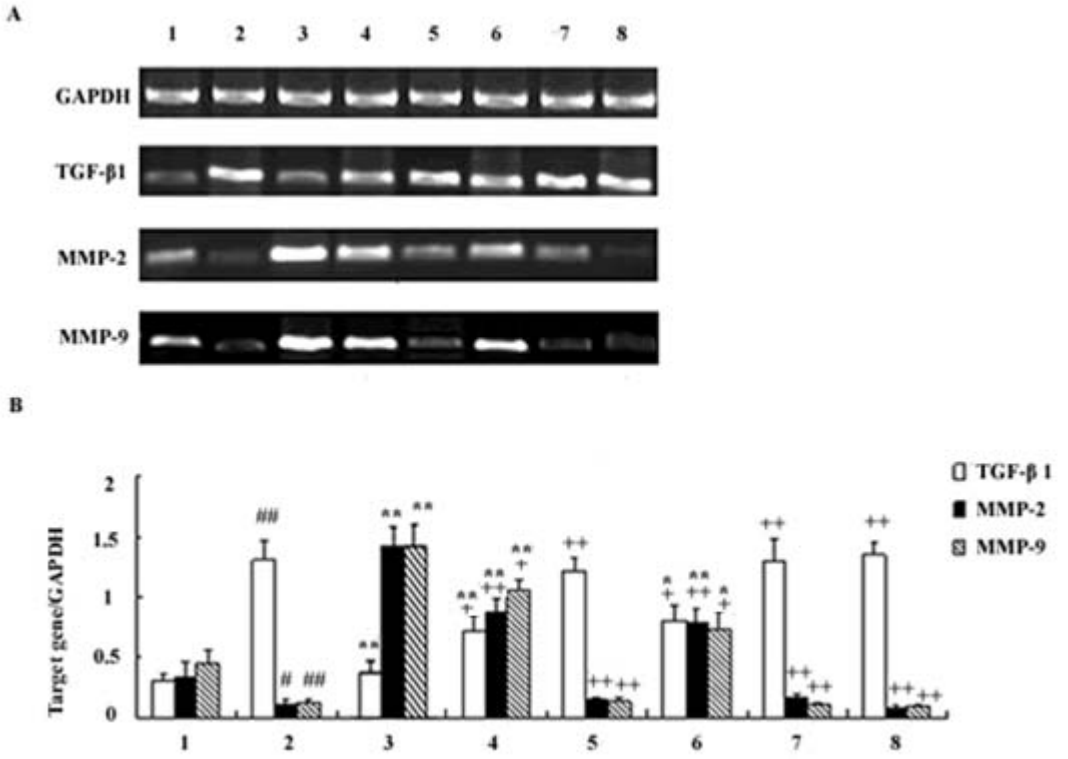

Figure 4. Effects of osthole on TGF-β1 and MMP-2/9 mRNA expressions in the cultured mouse CFs stimulated with Ang II after the cells were preincubated with PPARα/γ antagonists. $\bar{x} \pm$ SD, n=4. **A** is the electrophoresis gel photo of polymerase chain reaction products. **B** is the ratio of TGF-β1 or MMP-2/9/GAPDH. Compared with the control group: $^{\#\#}P<0.01$, $^{\#}P<0.05$; Compared with the Ang II group: $^{**}P<0.01$, $^{*}P<0.05$; Compared with the osthole plus Ang II group: $^{++}P<0.01$, $^{+}P<0.05$.

1: Control group; 2: Ang II group; 3: Osthole + Ang II group; 4: PPARα antagonist (MK886) 4 μmol/L plus osthole plus Ang II group; 5: PPARα antagonist (MK886) 4 μmol/L plus Ang II group ; 6: PPARγ antagonist (GW9662) 20 μmol/L plus osthole plus Ang II group ; 7: PPARγ antagonist (GW9662) 20 μmol/L plus Ang II group; 8: PPARα antagonist (MK886) 4 μmol/L plus PPARγ antagonist (GW9662) 20 μmol/L plus osthole plus Ang II group.

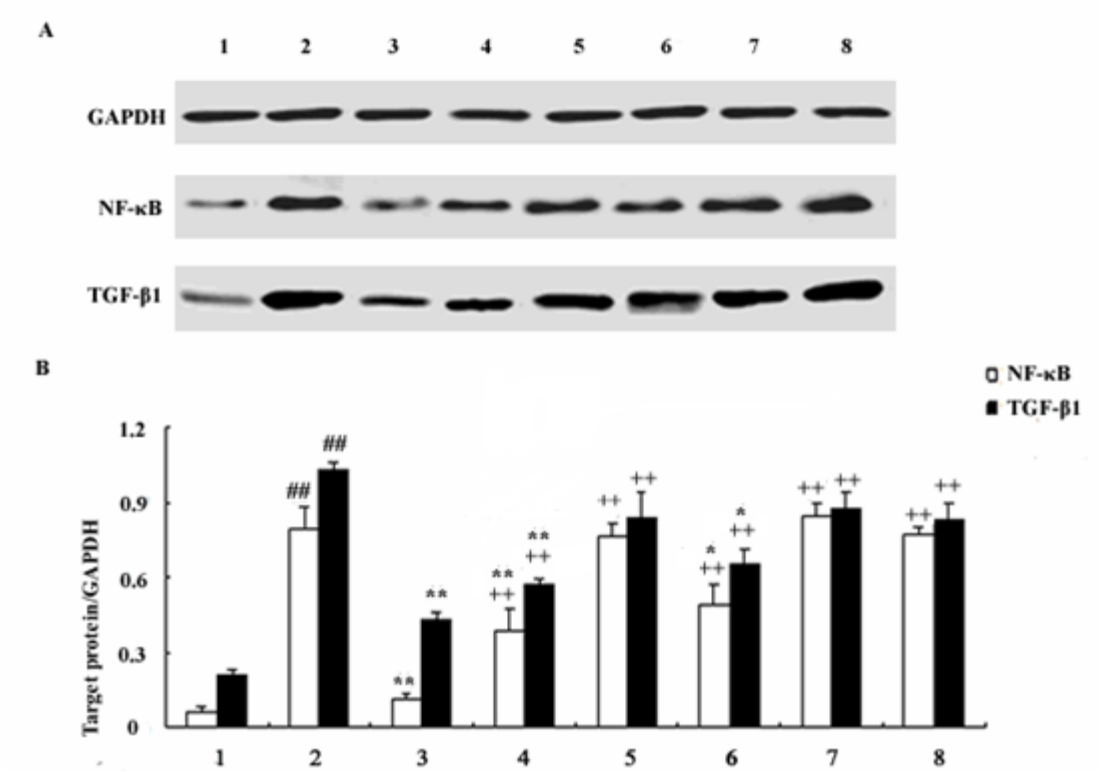

Figure 5. Effects of osthole on NF-κB and TGF-β1 protein expressions in the cultured mouse CFs stimulated with Ang II after the cells were preincubated with PPARα/γ antagonists. $\overline{x} \pm$ SD, n=4. **A** is the photo of Western blot analysis. **B** is the ratio of NF-κB or TGF-β1/GAPDH. Compared with the control group: $^{\#\#}P$<0.01; Compared with the Ang II group: $^{**}P$<0.01, $^{*}P$<0.05; Compared with the osthole plus Ang II group: ^{++}P<0.01.

1: Control group; 2: Ang II group; 3: Osthole + Ang II group; 4: PPARα antagonist (MK886) 4 μmol/L plus osthole plus Ang II group; 5: PPARα antagonist (MK886) 4 μmol/L plus Ang II group ; 6: PPARγ antagonist (GW9662) 20 μmol/L plus osthole plus Ang II group ; 7: PPARγ antagonist (GW9662) 20 μmol/L plus Ang II group; 8: PPARα antagonist (MK886) 4 μmol/L plus PPARγ antagonist (GW9662) 20 μmol/L plus osthole plus Ang II group.