Serelaxin Inhibits Inflammatory Response Induced by LPS in Cardiac Fibroblasts via Activating PPAR-γ and Suppressing NF-ϰB Signaling Pathway

xueping Wu[1]

[1]Shanghai University of Medicine and Health Sciences

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Abstract

BACKGROUND Various models of cardiovascular disease that have been used to prove the anti-fibrotic effects of serelaxin. However, whether anti-fibrotic effects of serelaxin are achieved by inhibiting inflammatory response have not been clarified. This research is intended to explore the role of serelaxin in lipopolysaccharide (LPS)-induced inflammation of cardiac fibroblasts (CFs) and elucidated the potential mechanisms. APPROACH Cardiac fibroblasts (CFs) were isolated from the hearts of 3 days neonatal rats. Effects of serelaxin on inhibiting inflammatory response after induction with LPS were examined. Cell proliferation was determined by cell-counting kit-8. The inflammatory cytokines secretion levels of IL-1β, IL-6 and TNF-α, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) respectively. The mRNA levels of α-SMA, collagen I/III, MMP-2, MMP-9, IL-1β, IL-6,TNF-α, IL-10, IκBα, p-IκBα, p65 subunit of nuclear factor-kappa B (NF-ϰB) and peroxisome proliferator-activated receptor-γ (PPAR-γ) were assessed by real-time quantitative PCR (RT-qPCR). The protein levels of α-SMA, collagen I/III, MMP-2, MMP-9, IκBα, p-IκBα, p65, p-p65 and PPAR-γ were examined by Western blotting. RESULTS Serelaxin inhibited LPS-induced IL-1β, IL-6 and TNF-α, α-SMA, collagen I/III, and elevated IL-10, MMP-2 and MMP-9 expression. And, LPS-induced activation of NF-ϰB pathways was suppressed by serelaxin treatment. Substantially, Further research demonstrated that serelaxin elevated the expression of PPAR-γ and PPAR-γ inhibitor GW9662 could reverse the inhibition of serelaxin on IL-1β, IL-6,TNF-α production. CONCLUSIONS These results suggested that serelaxin alleviates cardiac fibrosis by inhibiting inflammatory products of IL-1β, IL-6 and TNF-α, α-SMA, collagen I/III in LPS-induced CFs by stimulating PPAR-γ which subsequently abolished the expression of NF-ϰB signalling pathways.

Serelaxin Inhibits Inflammatory Response Induced by LPS in Cardiac Fibroblasts via Activating PPAR-γ and Suppressing NF-ϰB Signaling Pathway

Xueping WU, Yehui Lv, Zhihong LI, Zhifang YANG

Department of Anatomy, Histology and Embryology, Shanghai University of medicine & Health Sciences, 279 Zhouzhu Road, Pudong New District, Shanghai, China

BACKGROUND AND PURPOSE

Various models of cardiovascular disease that have been used to prove the anti-fibrotic effects of serelaxin. However, whether anti-fibrotic effects of serelaxin are achieved by inhibiting inflammatory response have not been clarified. This research is intended to explore the role of serelaxin in lipopolysaccharide (LPS)-induced inflammation of cardiac fibroblasts (CFs) and elucidated the potential mechanisms.

EXPERIMENTAL APPROACH

Cardiac fibroblasts (CFs) were isolated from the hearts of 3 days neonatal rats. Effects of serelaxin on inhibiting inflammatory response after induction with LPS were examined. Cell proliferation was determined

by cell-counting kit-8. The inflammatory cytokines secretion levels of IL-1β, IL-6 and TNF-α, and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) respectively. The mRNA levels of α-SMA, collagen I/III, MMP-2, MMP-9, IL-1β, IL-6,TNF-α, IL-10, IκBα, p-IκBα, p65 subunit of nuclear factor-kappa B (NF-κB) and peroxisome proliferator-activated receptor-γ (PPAR-γ) were assessed by real-time quantitative PCR (RT-qPCR). The protein levels of α-SMA, collagen I/III, MMP-2, MMP-9, IκBα, p-IκBα, p65, p-p65 and PPAR-γ were examined by Western blotting.

KEY RESULTS

Serelaxin inhibited LPS-induced IL-1β, IL-6 and TNF-α, α-SMA, collagen I/III, and elevated IL-10, MMP-2 and MMP-9 expression. And, LPS-induced activation of NF-κB pathways was suppressed by serelaxin treatment. Substantially, Further research demonstrated that serelaxin elevated the expression of PPAR-γ and PPAR-γ inhibitor GW9662 could reverse the inhibition of serelaxin on IL-1β, IL-6,TNF-α production.

CONCLUSIONS AND IMPLICATIONS

These results suggested that serelaxin alleviates cardiac fibrosis by inhibiting inflammatory products of IL-1β, IL-6 and TNF-α, α-SMA, collagen I/III in LPS-induced CFs by stimulating PPAR-γ which subsequently abolished the expression of NF-κB signalling pathways.

Key words: Serelaxin; PPAR-γ· NF-κB; LPS; Cardiac fibroblast; inflammation

INTRODUCTION

Cardiac fibroblasts (CFs) are abundant in adult mammalian heart and are involved not only play a pivotal role in maintaining structural integrity and function, but also in cardiac remodeling in connection with myocardial injury or pathologies [1]. More and more studies have shown that CFs secrets both pro- and anti-inflammatory cytokines are involved in cardiac inflammation [2]. As pro-inflammatory cytokine, IL-1β, IL-6 and TNF-α, which can regulate differentiation of CFs to myofibroblasts [3-4] and the secretion of various MMPs and of TIMP-1, as well as promoting extracellular matrix (ECM) deposition [5]. And yet, IL-10 can reduce the fibrosis by inhibiting the proliferation of rat CFs [6]. Accordingly, CFs may be regarded as an important cell and molecular target for inhibition of myocardial fibrosis, but the inflammatory mechanisms have bot been fully elucidated yet.

Many studies have shown that NF-κB signaling pathway is participated in the release of inflammatory factors in CFs via NF-κB activation. Recently, PPARs has been shown to have anti-inflammatory and anti-proliferative properties.[7]. Interestingly, PPAR-γ ligand has been shown to abate cardiac fibrosis in various in situ models of cardiac hypertrophy and failure [8]. More critically, PPAR-γ agonists can effectively inhibit the differentiation of human lung and dermal fibroblasts into myofibroblasts [9]. Activation of PPAR-γ can inhibit the secretion of inflammatory cytokines after LPS treatment [7]. To sum up, these researches put forward the possibility that anti-fibrotic effects of PPARs in the heart are mediated by inhibiting the proliferation and differentiation of CFs.

Up to the present, there is no effective measures to alleviate cardiac fibrosis. Over the years, great attention has been paid to the treatment of myocardial fibrosis. Serelaxin, a recombinant form of human relaxin-2, is considered to be a promising drug for the treatment of acute heart failure (AHF)[10]. Many studies have shown that

serelaxin can markedly decreased renal interstitial fibrosis, lung fibrosis [11] and cardiac fibrosis [12]. Furthermore, serelaxin has been reported to improve the inflammatory response in LPS-induced fibrosis[13]. Study showed that another signaling pathway activated by relaxin, involving peroxisome proliferator-activated receptor gamma (PPAR-γ)[14]. However, PPAR-γ also has anti-inflammatory and antifibrotic effects in several tissues [13]. Although the above-mentioned experimental and clinical data suggests various serelaxin effects on the heart, whether serelaxin alleviates cardiac fibrosis by inhibiting inflammatory effects and its detailed mechanisms have not been explored. This led us to examine whether PPAR-γ was involved in the cellular

effects of serelaxin. Thus, special attention was given to the study observing the anti-inflammatory effects of serelaxin on LPS-stimulated CFs and the potential anti-inflammatory mechanism.

MATERIALS AND METHODS

Chemicals and Reagents

Serelaxin was purchased from Peprotech (Rocky Hill, NJ, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (MD, USA). Lipopolysaccharides (LPSs, Escherichia coli 055:B5) was obtained from Sigma-Aldrich (USA). Rat IL-1β, TNF-α, and IL-6 ELISA kits were obtained from R&D (USA), and rat IL-10 ELISA kit was purchased from Invitrogen (USA). Rabbit polyclonal antibodies for vimentin, α-SMA, MMP-2, MMP-9, Collagen I and Collagen III were purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibodies for NF-ϰB p65 and phospho-NF-ϰB p65 (S276) were purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal antibodies for phospho-IϰB-α, and phosphorylated-IϰB-α,PPAR-γ were purchased from Santa Cruz Biotechnology (CA, USA). GW9662 was from Cayman Chemical (Ann Arbor, MI, USA); Rabbit polyclonal antibody for β-actin was from Trevigen (USA). Alexa Fluor 594 conjuncted goat anti-mouse IgG and Alexa Fluor 488 conjuncted goat anti-rabbit were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) 4',6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich (Saint Louis, MO). RNAiso Plus, PrimeScript RT MasterMix Perfect Real Time, and SYBR Premix Ex Taq II (Perfect Real Time) were purchased from Takara (Sangon Biotech, Shanghai).

Cell culture and treatment

The study was permitted by the Law of the People's Republic of China on the Protection of Wildlife, and the protocol was approved by the Institutional Animal Care Committee of Shanghai University of medicine & Health Sciences, China (Permit Number: JCLW01-08). Cardiac fibroblasts were isolated from the hearts of 3-day-old neonatal Sprague-Dawley rats and cultured as described previously. Briefly, the hearts were removed and washed with cold PBS. The atria and aorta were discarded. The ventricles were cut open and soaked with 75% ethanol for 30s to inactivate epicardial and endocardial endothelial cells. Then ventricles was finely minced into small pieces and digested with 0.125% trypsin and and 0.5 g/L collagenase II (Invitrogen, Carlsbad, CA) for 6 min in a 37 °C in six consecutive steps. Cells were centrifuged and resuspended in DMEM with 10 % FBS. The cells were seeded at a density of 1×10^5 cells/ml and incubated for 60 min. the Unattached cells were discarded by washing with PBS, and the attached cells were cultured in DMEM supplemented with 10 % FBS. CFs were identified by their morphology, positive staining for vimentin, and negative staining for α-smooth muscle actin (α-SMA) and von Willebrand factor (vWF). The purity of CFs in this study was more than 97 %. The cells were divided into control, LPS, serelaxin + LPS, serelaxin + LPS+GW9962 and LPS+ GW9962 groups. In LPS group, the cells were induced with LPS (1 μg/ml). In serelaxin and LPS groups, the cells were treated with LPS(1 μg/ml) and serelaxin (100 ng/ml). GW9662 with 100 nM in each groups.

Cell Proliferation Assay

The effect of serelaxin on cell proliferation assay was performed according to the manufacturer's instructions of cell counting kit-8 (CCK8). Firstly, cell suspension was inoculated in a 96-well plate at a density of 4 x 10^3 cells/well, three parallel wells for each group. After incubation for 24h, 10 μl of CCK-8 was added to the each well, and the cells continued to be incubate for 4 h. Absorbance values were determined at 450 nm with a microplate reader (Tecan, Männedorf, Switzerland).

Immunofluorescence Staining

CFs were cultured on sterile glass coverslips were fixed in 4% paraformaldehyde for 10 min, washed with PBS three times, and permeabilized with 0.3% Triton X-100 (Sigma-Aldrich, Saint Louis, MO) in PBS for 20 min. After blocking with 10% goat serum (Jackson ImmunoResearch, West Grove, PA) at room temperature for 30 min. Then, the cells were incubated with rabbit anti-vimentin (1:200), mouse anti-α-SMA (1:400) overnight in humidified incubator. The slides were then washed with PBS and incubated with Alexa Fluor 594 conjuncted

goat anti-mouse IgG (1:400) or Alexa Fluor 488 conjuncted goat anti-rabbit (1:200; Jackson, West Grove, PA) for 30 min at 37 °C. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma-Aldrich, Saint Louis, MO). Finally, the slides were mounted in fluoromount-G(Southern Biotech), and examined using a fluorescence microscope.

ELISA (enzyme linked immunosorbent assay)

Concentration of IL-1β, IL-6, TNF-α and IL-10 in CFs culture supernatants were determined using corresponding ELISA a kit in accordance with the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader. Results were compared with a standard curve constructed by titrating standards respectively. The level of IL-1β, IL-6, TNF-α and IL-10 in cell culture supernatants, expressed as pg/ml, were standardized based on each corresponding standard curve.Samples tested in triplicate and each experiment was repeated 3 times inindependently.

Real-Time Quantitative PCR (RT-qPCR).

mRNA levels of IL-1β, IL-6, TNF-α, IL-10, α-SMA, Collagen I,Collagen III, MMP-2, MMP-9, IkBα, p65 and PPAR-γ in CFs were evaluated by RT-qPCR. Total RNA was extracted from CFs using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the 1st strand cDNA was synthesized using a commercial kit (Takara, Otsu, Japan) according to the producer's instructions. Real-time PCR (RT-PCR) was completed on a 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The primers used in the present work were presented in Table 1. The mRNA levels of these targets were calculated using the 2^{-t} method and normalized against β-actin which was used as an internal reference gene. The results were expressed as fold changes to control.

Table 1: Primers sequences for quantitative real-time PCR (qRT-PCR)

Gene	Primer sequences 5/˜3/Forward
α-SMA Collagen I Collagen III MMP-2 MMP-9 IL-6 IL-1β TNF-α IL-10 P65 IkBα PARP-γ β-actin	CGATAGAACACGG

Western blot

For Western blot, the total protein were extracted from the cells by homogenization in RIPA lysis buffer supplemented with 1 mmol PMSF (Pierce, Rockford, IL). Upon centrifugation at 14,000g for 10 min at 4 °C, the supernatant was collected, and the total protein was quantified using the BCA protein assay kit (Beyotime, Shanghai) following the manufacturer instructions. The protein was separated by 10% SDS-polyacrylamide electrophoresis gels and electrotransferred onto a PVDF membrane (Millipore, Beijing). After blocking with 5% nonfat dry milk in TBST, the membrane was incubated with mouse anti-α-SMA, rabbit anti-MMP-2, rabbit anti-MMP-9, rabbit anti-collagen I, rabbit anti-collagen III (1:1000, Abcam), rabbit NF-ϰB p-P65 (1:500), NF-ϰB P65 (1:500),p-IϰBα (1:500), IϰBα (1:500), and PPAR-γ (1:500) (1:1000, Cell Signaling, Danvers, MA) and mouse anti-β-actin (1:2000, Abcam) overnight at 4 °C. Then, the membrane was washed with TBST and then incubated with goat anti-rabbit or goat anti-mouse (1:2000, santa cruz, CA). The proteins were visualized using enhanced chemiluminescence (Bio-Rad, Hercules, CA).

Statistical analysis

The results are expressed as means ± SD of three independent experiments. Statistical evaluation of the data was performed by o by using Student's unpaired t-test and one-way ANOVA with Scheffe's post-hoc multiple-comparison analysis. $P < 0.05$ was considered statistical significance.

Results

Characterization of cardiac fibroblasts.

CFs were cultured for 10 d. Phase contrast images and immunocytochemistry confirmed that CFs from all

strains exhibit similar mesenchymal morphology (Figure 1A) and immunostaining with an antibody against Vimentin (green). Almost all cells expressed the fibroblast marker Vimentin (Fig.1B) negative for α-SMA, vWF or cTnT(not showed) .

Serelaxin attenuated the CFs proliferation induced by LPS

The CCK-8 assay was used to measure proliferation of the CFs. As shown in Figure 2, The proliferation of CFs was induced by the LPS stimulation. Compared with the control group, the OD values were significantly higher in the LPS group ($P < 0.05$). However, compared with the LPS group, CCK-8 assay showed that the OD values in the serelaxin treated group were decreased markedly. Further analysis showed that GW9662 could reverse the inhibitory effect of serelaxin and increase the proliferation of CFs.

Serelaxin blocked the CFs activation induced by LPS

Studies have shown that the differentiation of CFs into myofibroblasts plays an important role in myocardial fibrosis. CFs were stained for the expression of α-smooth muscle actin (α-SMA), a marker associated with activated fibroblasts. Accordingly, we detected expression of α-SMA. At 72 h after treatment with LPS, most of the cells expressed α-SMA obviously. After treatment with serelaxin, the expression of α-SMA in cells was significantly reduced(Fig. 3). In addition, immunofluorescence staining also revealed an increased cell size of CFs stimulated with LPS compared with control, which was significantly reduced by serelaxin. qRT-PCR showed that mRNA expression level of α-SMA was increased by LPS induced, on the contrary serelaxin inhibits α-SMA expression (Fig 4A). Western blot results showed that the expression level of α-SMA was increased in LPS-treated cells, and significantly reduced after treatment with serelaxin(Fig. 4B and C). Moreover, the PPAR-γ inhibitor, GW9662, abated the inhibitive effect of serelaxin on α-SMA production.

Serelaxin inhibits LPS-induced inflammatory cytokines secretion

To assess the anti-inflammatory effects of serelaxin on LPS-induced inflammation, the inhibitory effect of serelaxin on the products of inflammatory cytokines treated with LPS was tested. Compared with the control group, the results illustrated that secretion of IL-1β, IL-6 and TNF-α increased significantly, IL-10 decreased marked with LPS treated. Nonetheless, treatment of serelaxin suppressed LPS-induced IL-1β, IL-6 and TNF-α secretion markedly (Fig. 5). The results of mRNA showed that compared with control group, the mRNA expression of IL-1β, IL-6 and TNF-α increased significantly, IL-10 mRNA decreased in LPS stimulation. However, treatment of serelaxin inhibited LPS-induced IL-1ß, IL-6 and TNF-α mRNA expression significantly, and IL-10 in LPS-induced cells increased significantly in serelaxin (Fig. 5). Additionally, GW9662, a PPAR-γ inhibitor, lessened the inhibitory effect of serelaxin on IL-1ß, IL-6 and TNF-α production.

Serelaxin inhibits LPS-induce expression of ECM-related protein in CFs

We investigated the expression of collagen I and collagen III, Compared with control, LPS induced significantly increased the mRNA and protein levels of collagen I and collagen III, and was significantly reduced the expression of collagen I and collagen III in CFs following treatment with serelaxin(Fig. 6A and B). Then, we analyzed the effects of serelaxin on the expression of MMP-2 and MMP-9 using qRT-PCR and western blotting. The results of qRT-PCR showed that serelaxin strongly upregulated LPS-induced the mRNA expression levels of MMP-2 and MMP-9 in CFs (Fig. 6C and D). Besides, the production of MMP-2 and MMP-9 remarkable decrease in LPS-induced. In addition, GW9662, a PPAR-γ inhibitor, reduced the inhibitory effect of serelaxin on collagen I and collagen III production.

Ινηιβιτιον οφ ΝΦ-κΒ Εξπρεσσιον Ις Ινολεδ ιν τηε Προτεςτιε Εφφεςτς οφ σερελαξιν. To test the mechanism of serelaxin anti-inflammatory effects, we detected the expression of IκBα and P65 NF-κB. In Figures 7A and B, results showed that LPS induced significant increase of IκBα mRNA and protein expression in CFs. Compared with the control group, the phosphorylation levels of NF-κB P65 increased markedly with LPS treatment(Figures 7C and D). However, the increase was strongly inhibited by serelaxin treatment. However, GW9662, a PPAR-γ inhibitor, reduced the inhibitory effect of serelaxin on P65 NF-κB and IκBα production.

Serelaxin exhibits its anti-inflammatory effects through activating PPAR-

Then, we studied the mechanism of serelaxin inhibition of P65 NF-κB expression. In order to elucidate the anti-inflammatory mechanism of serelaxin on LPS-induced inflammation, the effects of serelaxin on PPAR-γ expression were tested. Compared with the control group, expression of PPAR-γ reduced with LPS treatment. However, treatment of serelaxin upregulated the expression of PPAR-γ (Fig. 7E and F). Furthermore, the increasing of serelaxin on PPAR-γ expression were prevented by PPAR-γ inhibitor GW9662.

ΠΠΑΡγ ινηιβιτορ ρεερσεδ τηε αντι-ινφλαμματορψ εφφεςτς οφ σερελαξιν

In order to prove the anti-inflammatory mechanism of serelaxin, PPARγ was inhibited by GW9662. As shown in Fig.7, GW9662 significantly inhibited the expression of PPARγ. However, inhibitory effect of serealxin on IL-1β, IL-6 and TNF-α was reversed by GW9662(Fig. 5). These results demonstrated that serelaxin exerts an anti-inflammatory effect by activating PPARγ pathway.

Discussion

Serelaxin has been documented to have broad clinical application prospects Because it has a wide range of pharmacological effects, such as anti-inflammatory, anti fibrosis, anti heart failure and so on. In this paper, our results showed that LPS induced inflammatory cytokine IL-1β, IL-6 and TNF-α were increased significan in CFs, while serelaxin inhibited the expression of these inflammatory cytokines. In addition, the expression of MMP-2, MMP-9 and IL-10 were increased, collagen I and III were decreased and fibrosis was alleviated. The results showed that the antifibrosis effect of serelaixn was to reduce the release of inflammatory factors by activation of the PPAR-γ which following abolished the activation of NF-κB signalling pathways.

Increasing evidences show that CFs may be proinflammatory and can produce a wide range of proinflammatory cytokines and chemokines in response to stimulation with hypoxia, mechanical stress, or endotoxin[15]. The role of these cytokines or chemokines in cardiovascular system involves inspiring cell proliferation, division and differentiation. There are many reports that infammation plays a crucial role in the progress of cardiac disease [2]. Hence, controlling infammation may be helpful for treatment and prevent cardiac fibrosis [8]. And inflammatory response is mainly regulated by cytokine network[16]. It is common knowledge that a variety of harmful stimuli induce the release of inflammatory factors IL-1β, IL-6 and TNF-α and participate in the occurrence and development of acute inflammation. LPS is a potent stimulant that increases IL-1β, IL-6 and TNF-α expression by activating host immune and inflammatory cells. Expression of IL-1β was increased significant in the heart tissue of acute myocardial infarction. The experimental mouse model showed that IL-1β is very important for the recruitment of leukocytes, especially neutrophils and monocytes after myocardial infarction.[18]. Furthermore, it has been reported that blocking IL-1 signal can attenuate heart failure in mice and men after myocardial infarction[19]. TNF-α is known as the main cytokine because it plays an important role in initiating acute inflammatory response[20]. Emerging evidence demonstrates that TNF-α positively correlate with the progression of immune activation and cardiac remodeling[21]. IL-6 is an inflammatory cytokine with pleiotropic effects in diverse cells and organs, has been implicated in cardiovascular pathologies [22]. Previous research have shown that serelaxin could suppress IL-1β, IL-6 and TNF-α in renal ischaemia/reperfusion (I/R) injury[23]. In this paper, indeed, the results showed that IL-1β, IL-6 and TNF-α expression were significantly increased in LPS treated cells, indicating that CFs may be the source of proinflammatory cytokines in the development of cardiac disease. Besides, our results showed that serelaxin markedly inhibited LPS-induced productions of IL-1β, TNF-α and IL-6 in CFs, confirming our results in vitro are able to exert an anti-inflammatory effect on CFs stimulated by LPS. These results suggest that serelaxin can inhibit myocardial inflammation may help to avoid inflammation induced excessive proliferation of CFs and subsequent cardiac fibrosis and cardiac remodeling.

Studies have shown that the production of IL-1β, IL-6 and TNF-α is regulated by NF-κB[24]. CFs are the key cell mediators of injury and inflammation that cause cardiac fibrosis[25]. It is generally believed that MI leads to cytokine/chemokine gene expression in resident cardic cells by NF-κB-mediated signaling pathway [17]. NF- κB pathway has been considered as a typical pro-inflammatory signaling pathway, because of its

role in increasing the expression of inflammatory cytokines including IL-1β, IL-6 and TNF-α [24]. In this study, expression of IL-1β, IL-6 and TNF-α were signifcantly elevated by LPS-induced but down-regulated by serelaxin, suggesting the anti-infammatory capability of serelaxin. In addition, the results also showed that serelaxin treatment can significantly promote the expression of anti-inflammatory cytokines IL-10, IL-10 is an anti-inflammatory cytokine that can suppress the expression of pro-inflammatory cytokines[26]. After treatment with serelaxin, it significantly inhibited the expression of collagen I/III, and increased the expression of MMP-2/9. These results indicate that serelaxin has the ability to reduce the inflammation and anti-fibrosis of CFs induced by LPS.

It is crucial that serelaxin inhibited the expression of NF-ϰB and its signal target gene TNF-α. It is very likely that the anti-inflammatory activity of serelaxin is mainly through the regulation of key components of NF-ϰB.

Inflammatory mediators such as LPS triggers the activation of a heterodimer composed of RelA/p65 and p50 subunits, which is usually defined as the classical NF-ϰB pathway[27]. Due to the presence of NF-ϰB (IϰBs), the NFϰB p65/p50 heterodimer is usually isolated in the cytoplasm, and the most distinctive member of IϰBs is IϰBα. Under the stimulation of LPS, IϰBα was phosphorylation and degradation lead to the release of p65/P50 heterodimer, which translocates to the nucleus and interacts with NF-ϰB common site. As a result, the the target gene transcriptional activity of NF-ϰB is activated. Our study further explores the potential anti-inflammatory mechanism of serelaxin, we tested the activity of NF-ϰB. The results showed that the expression of IϰBα and p65/p50 were increased in LPS group and decreased with the treatment of serelaxin. Thus, serelaxin may reduce inflammation by blocking the activation of NF-ϰB signaling pathway.

PPARγ is a key transcription factor appertain to nuclear receptor family that plays an important role in regulating a great quantity of biological processes including inflammation[28]. The linkage between serelaxin and PPARγ has been investigated in a model of hepatic fibrosis[29] and it has been reported that PPARγ played a key impact in anti-inflammatory activities [28]. It has been proved that PPAR-γ agonists could down regulate the inflammatory response induced by LPS [30]. Therefore, we further examined the effects of serelaxin on PPAR-γ expression. In this paper, the result showed that serelaxin significantly increases the expression of PPAR-γ. We also discovered that the inhibitory effect of IL-1β, IL-6 and TNF-α by serelaxin can be reversed by the PPAR-γ specific antagonist GW9662. Our results indicated that anti-inflammatory ability through serelaxin in CFs was rely on the PPAR-γ pathway. In the present study, the results show that the expression of IϰBα was managed by PPARγ, and yet use of GW9662 abated the expression of IϰBα and enhanced the expression of p-IϰBα. In addition, the expression of p-p65, a subunit of NF-ϰB, was analogue to the level of p-IϰBα. Besides, the activation of PPAR-γ has been shown to attenuate LPS induced inflammation is related to the inhibition of NF-ϰB [31]. Consequently, the results show that serelaxin debilitates LPS-induced infammatory response in CFs through triggering PPAR-γ which afterwards abrogated the activation of NF-ϰB. Actually, more inhibitory experiments are required in our future research to demonstrate whether serelaxin treatment could suppress the nuclear translocation of NF-p65 by immunofuorescence staining and the activation of NF-ϰB using a luciferase reporter gene assay. Furthermore, a NF-ϰB agonist can apply to demonstrate whether it can inverse the anti-inflammatory effects of serelaxin.

In conclusion, the results showed that serelaixn could reduce the inflammatory factor IL-1β, IL-6 and TNF-α in CFs induced by LPS. Serelaxin play anti-infammatory role in CFs by stimulating PPAR-γ means and suppressed NF-ϰB pathway. The anti-inflammatory mechanism of serelaxin needs more in vivo experiments to confirm. The current research shows that serelaxin has great potential in the treatment of myocardial fibrosis.

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Authors' contributions

WXP has substantial contributions to design the experiments, analysis and interpretation of data and mainly drafting the manuscript. YZF has contributed to conception, acquisition of data, analysis and interpretation of data and has been involved in revising the manuscript critically. LYH and LZH have contributed to analysis and interpretation of data and have been involved in revising the manuscript critically. All authors have read and approved the final manuscript.

Conflict of interest

The authors have no conflicts of interest.

Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Material, and further inquiries can be directed to the corresponding authors.

Ethics approval and consent to participate

The study was permitted by the Law of the People's Republic of China on the Protection of Wildlife, and the protocol was approved by the Institutional Animal Care Committee of Shanghai University of medicine & Health Sciences, China (Permit Number: JCLW01-08).

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Legend

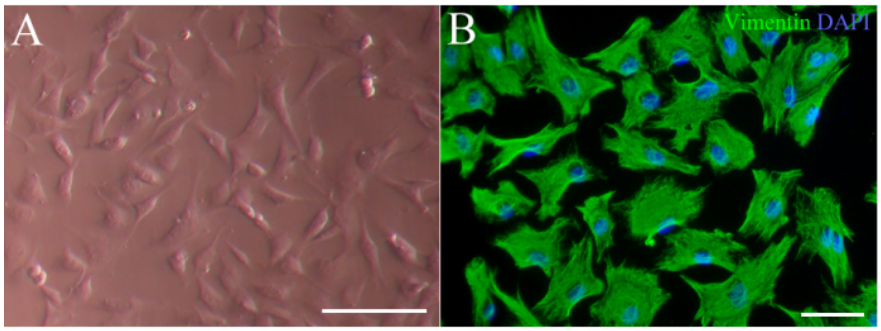

Fig. 1. Shape and Immunocytochemical staining of cardiac fibroblasts. (A) Cells cultured for 10d Phase contrast image of the cells. Bar = 200 μm.(B) Stained with Vimentin antibody (green).The nuclei, visualized with DAPI (blue). Bar = 100 μm.

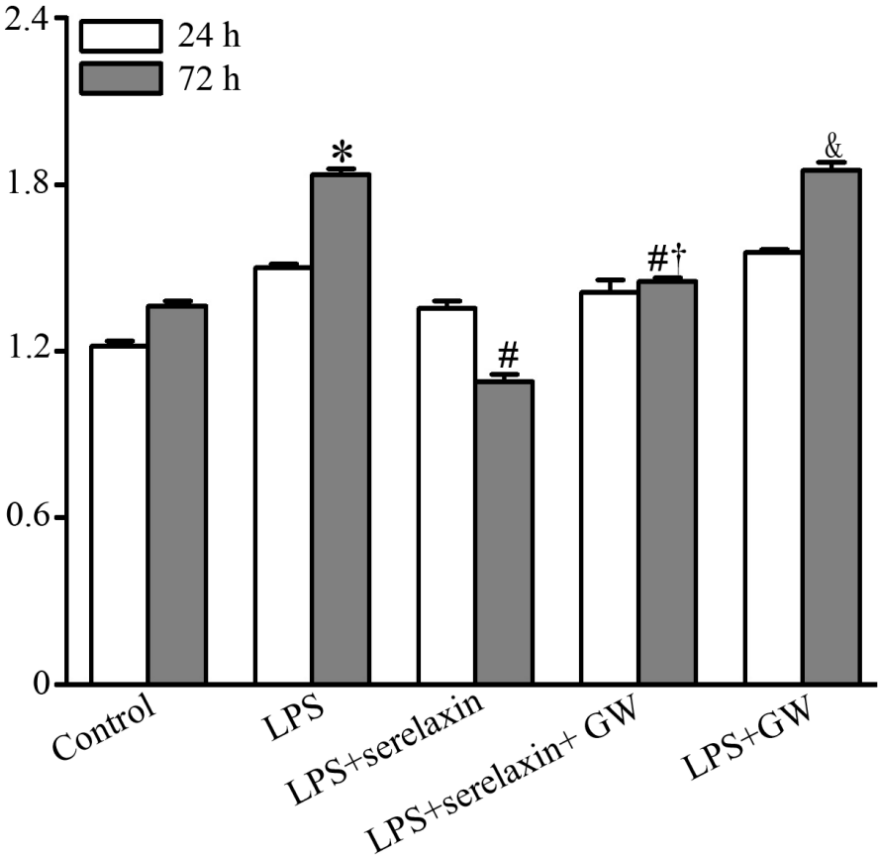

Fig. 2. Effects of serelaxin on CFs proliferation. The effect of serelaxinon CFs proliferation was detected by CCK-8 assay after treatment for 24 and 48 h. The optical density was normalized to a relative value of 100% for untreated cells. Data were represented as means ± standard deviation (SD) from three independent experiments. *p < 0.05 versus control, #p < 0.05 versus LPS group.

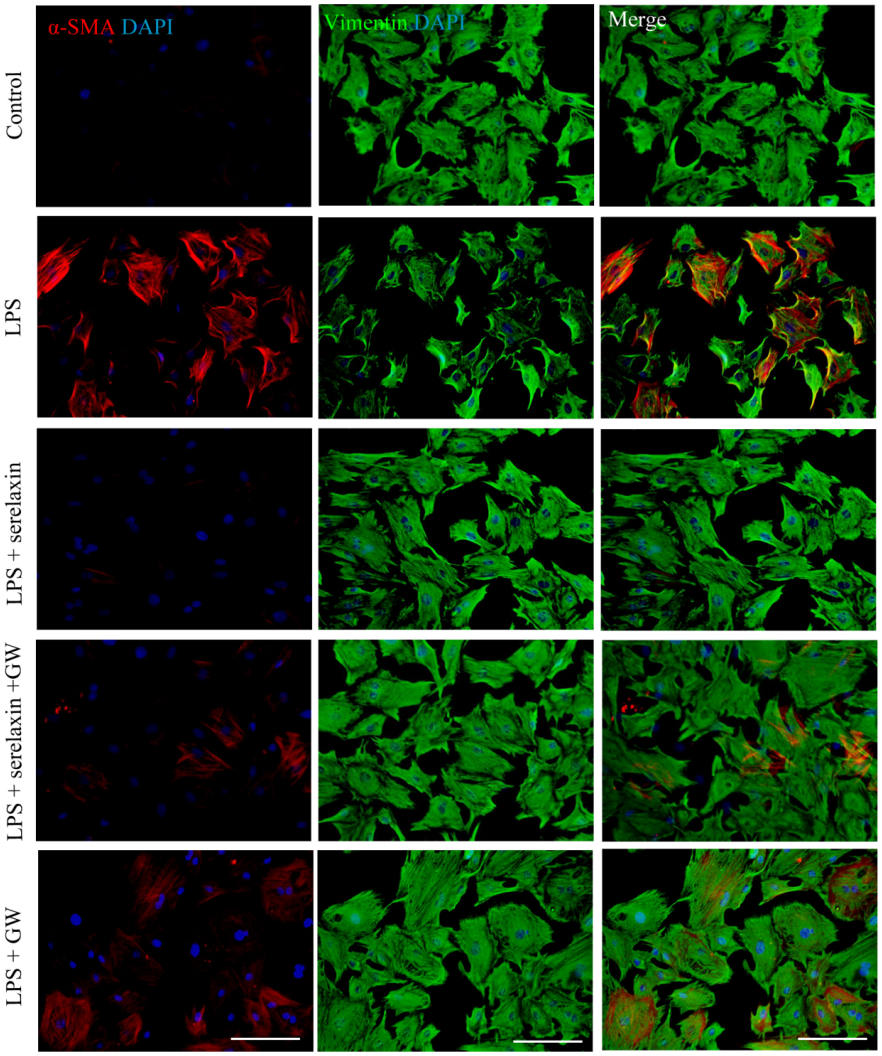

Fig. 3. Changes of α-SMA expression after treatment with serelaxin. Immunostaining of α-SMA(red)and vimentin(green). Bar = 100 μm.

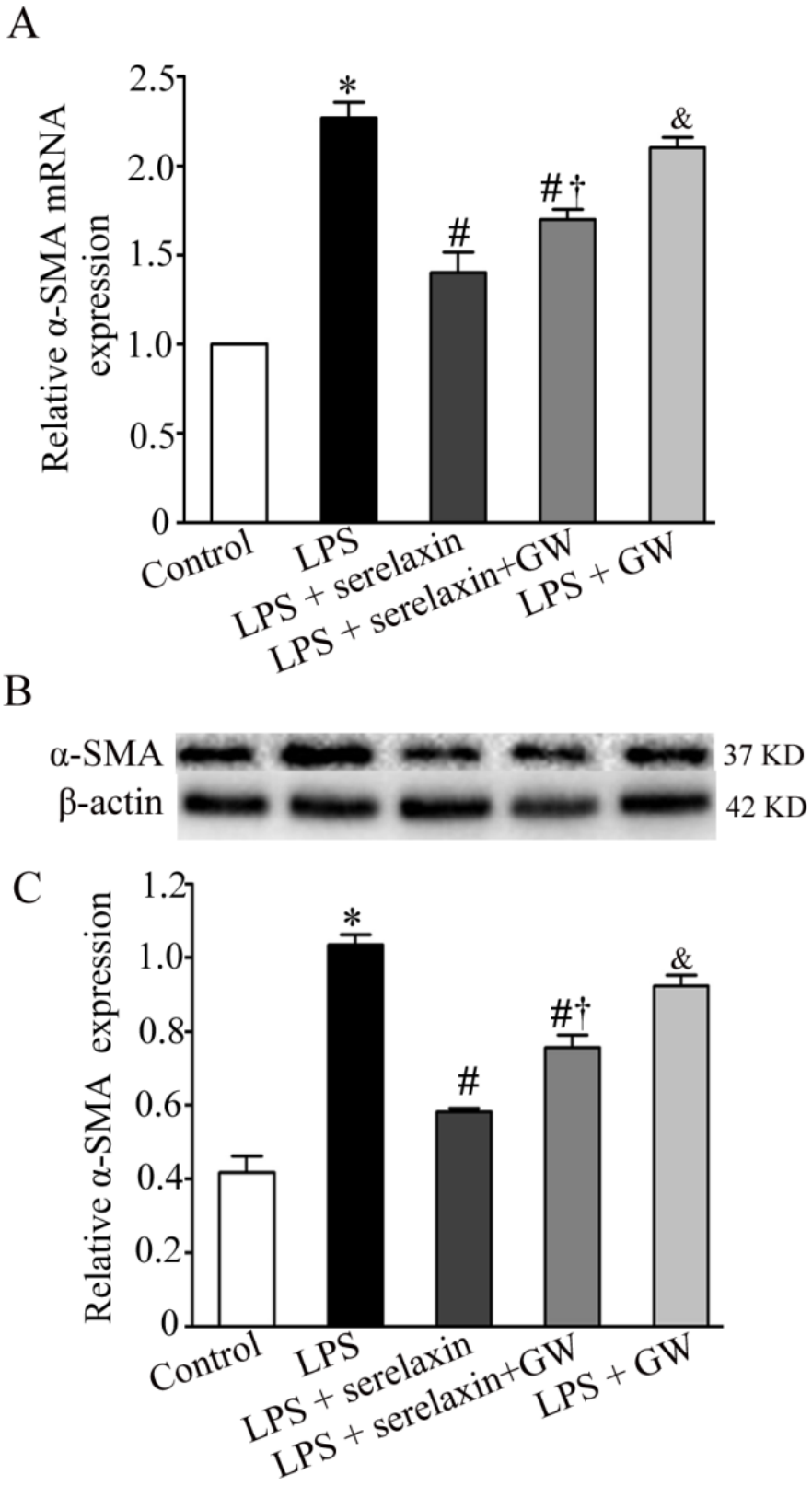

Fig. 4. Changes of α-SMA expression after treatment with serelaxin. (A) Immunostaining of α-SMA. Bar = 100 μm. (A) Expression of α-SMA mRNA assessed with RT-PCR. (B) Expression of α-SMA assessed with Western blot. (C) The statistical result of α-SMA expression. Serelaxin inhibits α-SMA expression in TGF-β1-induced cells. *p < 0.05 versus control group,#p < 0.05 versus LPS group. +p < 0.05 versus LPS+serelaxin group.$^{\&}$p < 0.05 versus LPS+serelaxin+GW group.

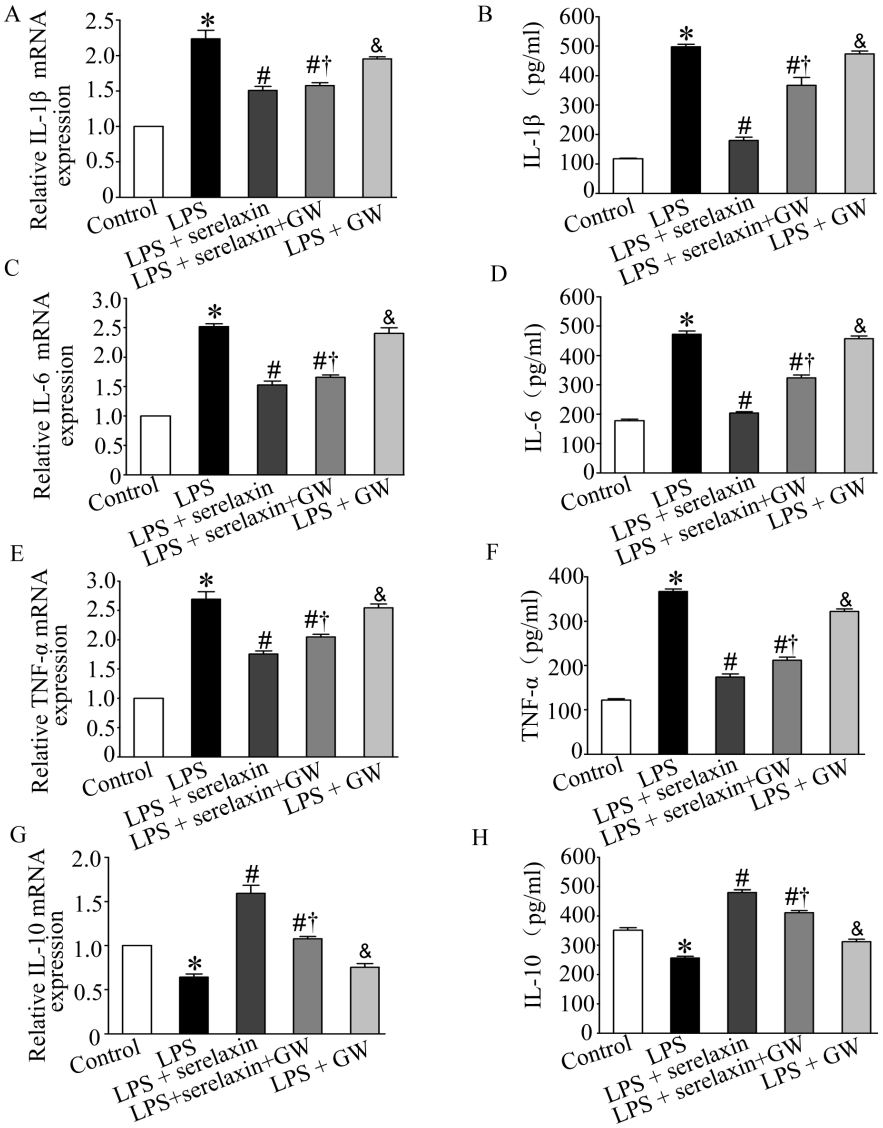

Fig 5 Effects of serelaxin on LPS-induced inflammatory cytokines, (A, B, E-H) The gene level of IL-1β (A), IL-6 (C), TNF-α (E), and IL-10 (G) were determined with qRT-PCR. Culture supernatants were collected to evaluate the protein level of IL-1β (B), IL-6 (D), TNF-α (F), and IL-10 (H) by ELISA. Histograms represented means ± SD of relative quantification from three independent experiments. *p < 0.05 versus control group,#p < 0.05 versus LPS group. +p < 0.05 versus LPS+serelaxin group.$^{\&}$p < 0.05 versus LPS+serelaxin+GW group.

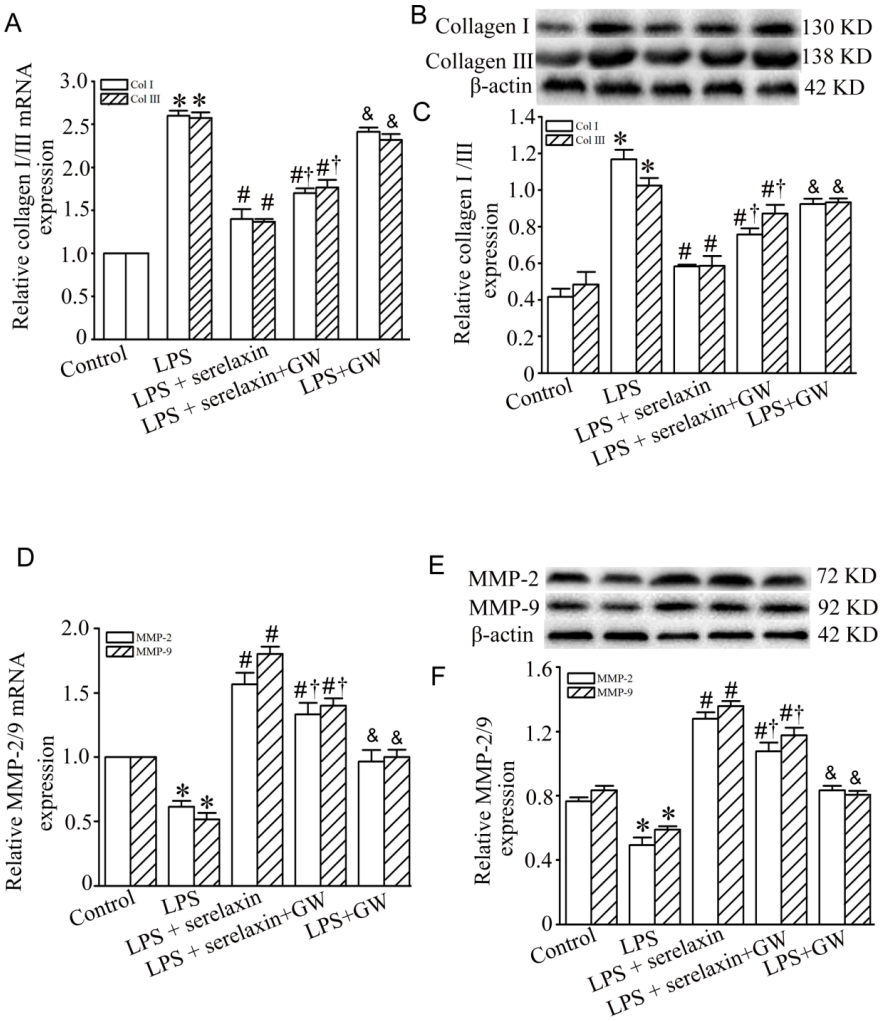

Fig. 6 Expression of Collagen I, Collagen III, MMP-2 and MMP-9. (A) Expression of Collagen I/ III mRNA analyzed with RT-PCR. The statistical result of Collagen I, Collagen III mRNA expression. (B) Expression of Collagen I/ III detected with Western blot. (C) The statistical result of expression of Collagen I, Collagen III proteins. (D) Expression of MMP-2 and MMP-9 mRNA analyzed with RT-PCR. The statistical result of MMP-2 and MMP-9 mRNA expression. (E) Expression of MMP-2 and MMP-9 proteins. (F) The statistical result of expression of MMP-2 and MMP-9 proteins. Histograms represented means ± SD of relative quantification from three independent experiments. *$p < 0.05$ versus control group,#$p < 0.05$ versus LPS group. +$p < 0.05$ versus LPS+serelaxin group.$^{\&}p < 0.05$ versus LPS+serelaxin+GW group.

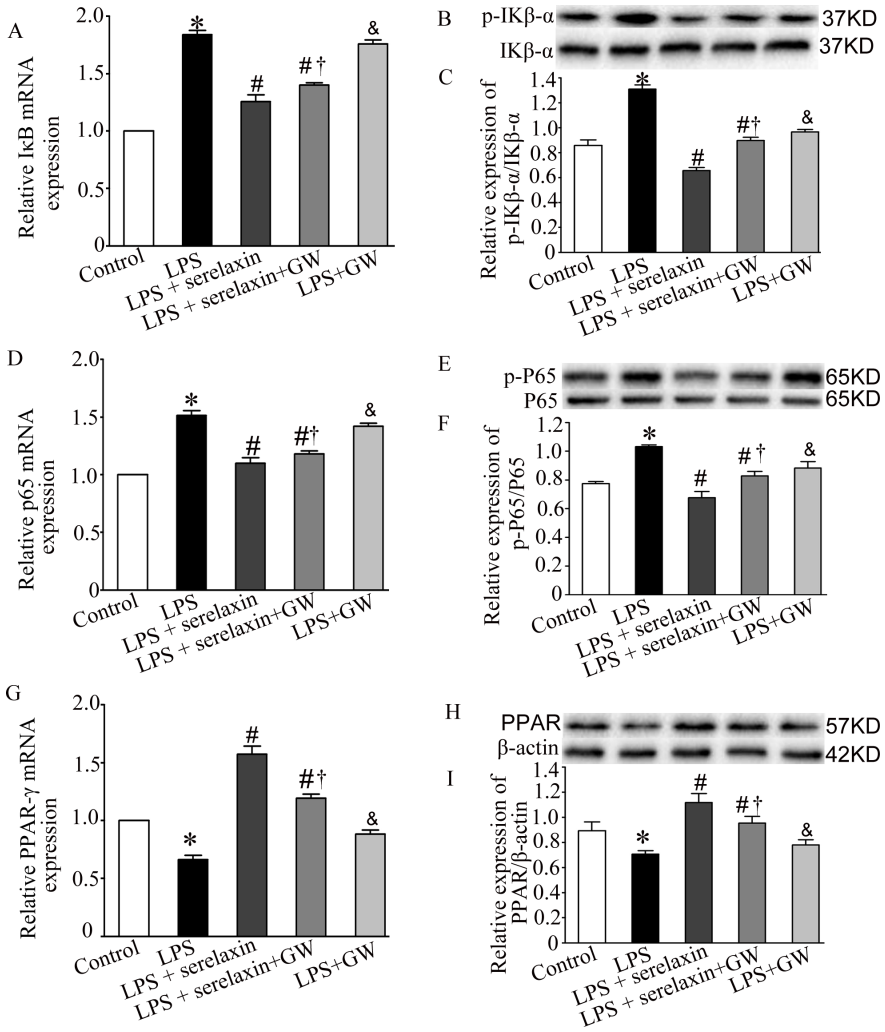

Fig. 7. Effects of serelaxin on LPS-induced activation of NF-ϰB and PPAR-γ pathways. (A) Expression of IϰB mRNA analyzed with RT-PCR. The statistical result of IϰB mRNA expression. (B) Expression of p-IϰB detected with Western blot. (C) The statistical result of expression of p-IϰB proteins. (D) Expression of P65 mRNA analyzed with RT-PCR. The statistical result of P65 mRNA expression. (E) Expression of p65 detected with Western blot. (F) The statistical result of expression of p65 proteins. (G) Expression of P65 mRNA analyzed with RT-PCR. The statistical result of P65 mRNA expression. (H) Expression of p65 detected with Western blot. (I) The statistical result of expression of p65 proteins. Histograms represented means ± SD of relative quantification from three independent experiments. *p < 0.05 versus control group,#p < 0.05 versus LPS group. +p < 0.05 versus LPS+serelaxin group.&p < 0.05 versus LPS+serelaxin+GW group.