Original Article
Gallic acid inhibits LPS induced hypertrophic scar inflammation via toll-like receptor 4/nuclear factor-κB/peroxisome proliferator-activated receptor γ signaling

Pengju Fan[1], Shuyue Chen[1], Zhen Li[2], Xinghua Yang[1], Shaorong Lei[1], Wuyuan Tan[1]

[1]Department of Plastic and Esthetic Surgery, Xiangya Hospital of Central South University, Changsha, P. R. China;
[2]Department of Anaesthesia, The Maternity and Child Health Hospital of Hunan Province, Changsha, P. R. China

Abstract: Prolonged and enhanced inflammation is a common pathogenic feature of hypertrophic scars (HTS). Gallic acid (GA) is a naturally occurring plant phenol with lots of pharmacological activities, including antimicrobial, anti-inflammatory, anticancer, antioxidant, and anti-fibrosis effects. The objective of this study was to evaluate the effects of GA in LPS induced inflammatory response. Results suggest that there is significant production of TNF-α, IL-6, IL-1β, and IL-8 in HSFs treated with LPS. However, treatment with GA significantly decreased levels of TNF-α, IL-6, IL-1β, and IL-8 in HSFs. Results also show that LPS reduced both PPARγ mRNA and protein expression in HSFs, but GA can upregulate expression of PPARγ and downregulate expression of TLR-4. Results indicate that LPS negatively regulates expression of PPARγ in HSFs but GA can antagonize these effects of LPS. Furthermore, LPS-induced inhibition of PPARγ was not observed in HSFs treated with a TLR-4 siRNA. Additionally, overexpression TLR-4 can induce the inhibition of PPARγ, but GA can increase PPARγ expression in HSFs that have been engineered to overexpress TLR-4. This study further proved that GA can significantly inhibit LPS-induced NF-κB expression. Results indicate that GA has a positive effect on HTS by attenuating LPS induced inflammatory response via TLR-4/NF-κB/PPARγ signaling. Results also suggest a novel potential role for GA, which can be used as an effective drug for treatment of hypertrophic scars, keloids, and so forth.

Keywords: Gallic acid, hypertrophic scar fibroblasts, peroxisome proliferator-activated receptor γ, toll-like receptor 4, nuclear factor-κb, inflammatory

Introduction

Hypertrophic scars (HTS) are a fibro-proliferative disorder which result in excess deposition of collagen and other extracellular matrix (ECM) molecules. They damage the deep dermis by thermal injuries or other forms of trauma, normally causing severe cosmetic and functional impairment [1]. Current therapeutic approaches include superficial compression therapy, radiation therapy, cytotoxic drugs, and surgical procedures. However, none of these approaches provide effective results. These deficiencies are largely because of a poor understanding of the molecular mechanisms of HTS formation [2].

Prolonged and enhanced inflammation is a common pathogenic feature of HTS. Corticosteroid anti-inflammatory drugs are not suitable for long-term and high dosage use. Superficial compression is not suitable for scars in visceral organs or on large surface area. Therefore, bioactive compounds with anti-inflammation activities would be more effective in the management of HTS [3]. Gallic acid (GA) is a naturally occurring plant phenol with lots of biological and pharmacological activities, including antimicrobial, antioxidant, anticancer, anti-fibrosis, and anti-inflammatory effects. A previous study proved that GA could inhibit collagen gel contraction in hypertrophic scar fibroblasts (HSFs) [4]. Recent studies have demonstrated that GA can suppress RhoA/Rho-kinase pathways and attenuate TGF-β1-stimulated collagen gel contraction in hypertrophic scar fibroblasts [5].

Recently, peroxisome proliferator-activated receptor γ (PPARγ) has been confirmed as a po-

tential target for treatment of inflammatory diseases, such as ulcerative colitis, atherosclerosis, and rheumatoid arthritis. Furthermore, naturally occurring or synthetic ligands of PPARγ have been shown to inhibit myofibroblast transdifferentiation, proliferation [6], and collagen synthesis in hepatic and pancreatic stellate cells. Toll-like receptors (TLRs) are essential to the innate immune system [7]. Activation of TLR signaling can activating protein 1 (AP-1) activity and induce nuclear factor-κB (NF-κB) expression. They release some pro-inflammatory molecules, such as chemokines, nitric oxide, cytokines, and cell surface adhesion molecules. These molecules not only drive the immune response but also interfere with expression of key regulatory factors involved in regulating physiological inflammation. TLR-4 has been reported to contribute to barrier dysfunction and microvascular inflammation in thermal injuries [8]. Previous research has demonstrated that TLR-4 is involved in the mechanisms of hyperplastic scarring via TGF-β [9].

Based on these studies, the present research was aimed at determining whether GA may have a positive effect on HTS by attenuating LPS induced inflammatory response via TLR-4/NF-κB/PPARγ signaling. Results suggest a novel potential role for GA, which can be used as an effective drug for treatment of hypertrophic scars, keloids, and so forth.

Materials and methods

Reagents

Lipopolysaccharide (LPS) was purchased from Sigma (St. Louis, MO). SB20380, U0126, SP600125, and curcumin were purchased from Calbiochem, (San Diego, CA). Gallic acid (GA), Ammonium pyrrolidinedithiocarbamate (PDTC), and Bay 11-7085 were purchased from Sigma (St Louis, MO). I-κB kinase c (IKKc) NEMO binding domain (NBD) inhibitory peptide set was purchased from Imgenex (San Diego, CA).

Cell culture and LPS treatment

Hypertrophic scar fibroblasts (HSFs) were isolated and cultured *in vitro*, as previously described [9]. Prior written informed consent was obtained from patients and the study received approval from Xiangya Hospital's Institutional Review Board. The study was conducted according to the Helsinki Declaration of 1975 (revised 1983). HSFs were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 1% vitamins, 2 mM L-glutamine, and 1% penicillin/streptomycin (all Gibco, NY, USA) at 37°C in an atmosphere of 5% CO_2. For all experiments, HSFs were studied between passages third to tenth. HSFs were treated with LPS (0.1 µM) with or without GA (100 µM) for 24 hours.

TLR-4 knockdown

TLR-4 small interfering (si) RNAs and scrambled siRNAs (negative control) were purchased from Santa Cruz Biotechnology. The siRNAs were transfected into the HSFs based on Santa Cruz Biotechnology siRNA transfection protocol. Briefly, after culturing the fibroblasts in antibiotic-free DMEM medium at 37°C in an atmosphere of 5% CO_2 for 24 hours, the siRNA duplex solution diluted with siRNA transfection medium (Santa Cruz Biotechnology) was added to the fibroblasts. After transfection for 24 hours, the medium was replaced with normal DMEM medium and the fibroblasts were treated with drugs.

TLR-4 overexpression

The constructs pcDNA3-YFP-TLR4 and pcDNA3-YFP were obtained from Addgene (Cambridge, MA). HSFs were plated in a 6-well plate or 24-well plate one day before transfection and were grown to 80% confluence. Cells were transfected with Lipofectamine 2000 (Invitrogen) and harvested for total RNA or cell-lysate after 24 hours or 48 hours. Overexpression of genes was determined by real time RT-PCR or Western blot analysis. After transfection for 24 hours, the medium was replaced with normal DMEM medium and the fibroblasts were treated with drugs.

RNA isolation and quantitative reverse transcription-polymerase chain reaction analysis

Total RNA was isolated using TRIzol Reagent, reverse-transcribed into cDNA using the TaKaRa RT reagent kit, and quantified using the ABI 7500 fast RT-PCR System. Primers and probes detecting PPARγ, TLR-4, tumor necrosis factor-α (TNF-α), CD36, IL-6, IL-1β, IL-8, and GAPDH were purchased from Applied Bio-
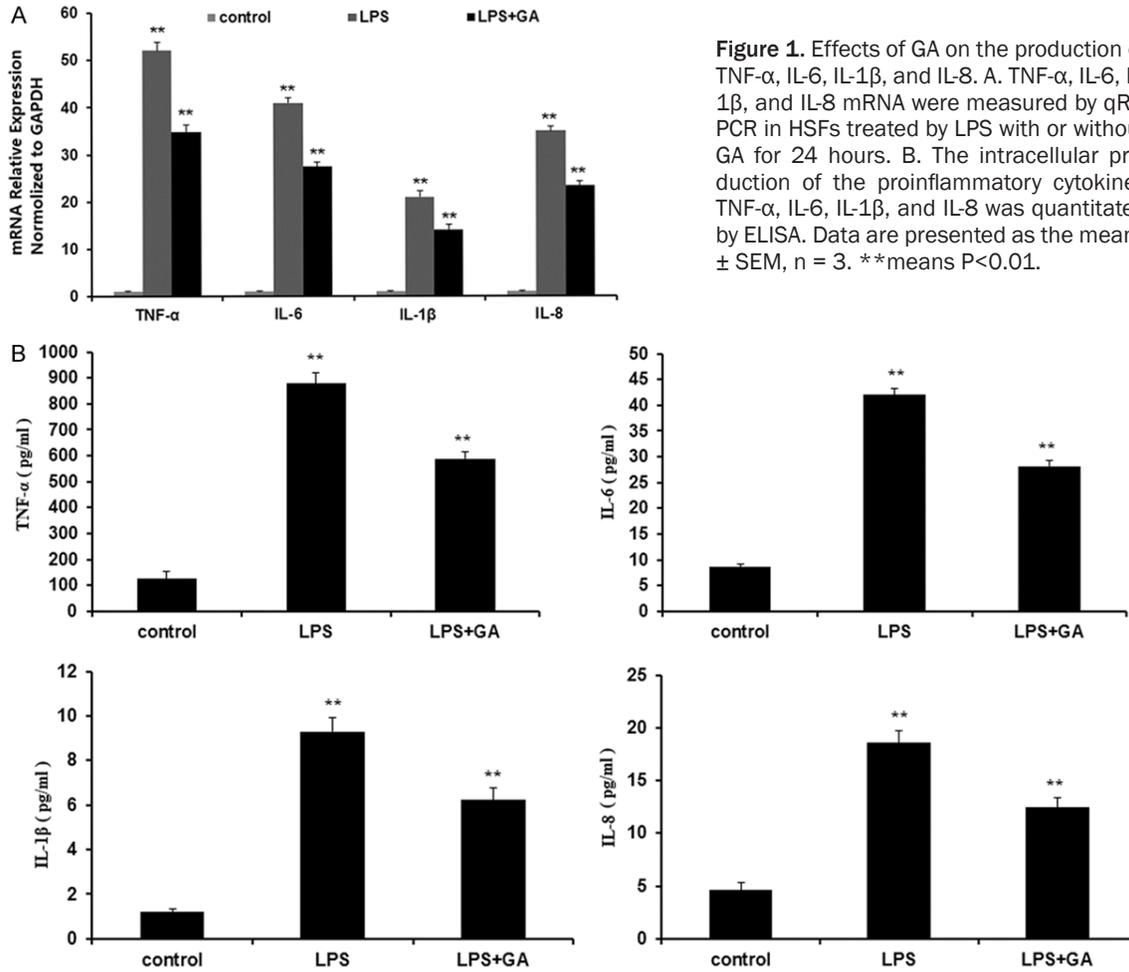
Figure 1. Effects of GA on the production of TNF-α, IL-6, IL-1β, and IL-8. A. TNF-α, IL-6, IL-1β, and IL-8 mRNA were measured by qRT-PCR in HSFs treated by LPS with or without GA for 24 hours. B. The intracellular production of the proinflammatory cytokines TNF-α, IL-6, IL-1β, and IL-8 was quantitated by ELISA. Data are presented as the means ± SEM, n = 3. **means P<0.01.

systems. Cycle threshold (Ct) values were obtained graphically for the target genes and GAPDH. $\Delta Ct = Ct_{target\ genes} - Ct_{endogenous\ reference\ gene}$. $\Delta\Delta Ct = \Delta Ct_{treated\ samples} - \Delta Ct_{control\ samples}$. The relative fold change in gene expression was calculated as $2^{-\Delta\Delta Ct}$.

Western blot

Cells were lysed with ice-cold lysis buffer (5 mM EDTA, 50 mM HEPES, 1% TritonX-100, 100 mM NaCl, pH 4) in the presence of a protein inhibitor cocktail (Roche, Germany). Protein concentrations were measured with the BCA protein assay kit (Pierce, Rockford, IL). Protein samples (30 μg) were loaded on 10% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membranes in a semi-dry system (Bio-Rad, Hercules, CA). Membranes were incubated with specific antibodies against PPARγ, TLR-4, and β-actin. β-actin was used as a loading control. Signals were revealed with

chemiluminescence and visualized by exposure to X-ray film.

Cytokine measurements

Cytokine production was determined using human TNF-α, IL-6, IL-1β, and IL-8 ELISA kits (Boster, Wu Han, China), according to manufacturer instructions.

NF-κB DNA binding enzyme-linked immunosorbent assay

Human skin fibroblasts were pretreated for 1 hour with or without PDTC, NBD, or Bay 11-7085. HSFs were then incubated with or without LPS for an additional 24 hours. Nuclear extracts were isolated with the Nuclear Extraction Kit (Panomics, Fremont, CA) and stored at -80°C in aliquots until use. Protein concentrations were measured with the BCA protein assay kit (Pierce, Rockford, IL). Binding
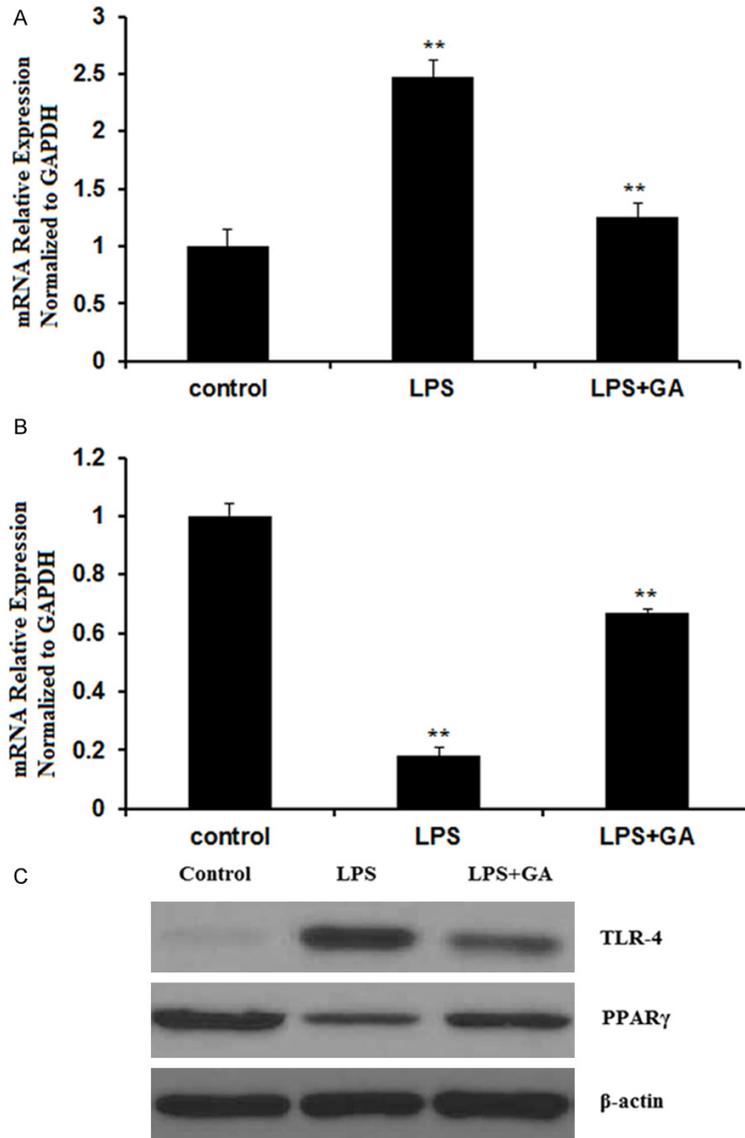
A

B

C

Figure 2. GA upregulated the expression of PPARγ by inhibited LPS stimulated TLR-4 activation. A. PPARγ mRNA was measured by qRT-PCR in HSFs treated by LPS with or without GA for 24 hours. B. TLR-4 mRNA was measured by qRT-PCR in HSFs treated by LPS with or without GA for 24 hours. C. PPARγ and TLR-4 protein was measured by Western blotting in HSFs treated by LPS with or without GA for 24 hours, and the loading control was β-actin. In all experiments, the mRNA abundance was normalized to GAPDH and is presented as the mean ± SD, n = 3. **means P<0.01.

of the NF-κB p65 (RelA) subunit to an NF-κB consensus binding site was detected in nuclear extracts (10 µg) using the NF-κB Transcription Factor ELISA Kit (Panomics), according to manufacturer instructions.

Statistical analysis

Data were analyzed using the SPSS (version 13.0) statistical package. Descriptive data and measurement data were analyzed by both t-test and analysis of variance. Data are presented as the mean ± SD. A *p*-value of < 0.05 indicates statistical significance.

Results

Effects of GA on the production of TNF-α, IL-6, IL-1β, and IL-8

As shown in **Figure 1**, there was significant production of TNF-α, IL-6, IL-1β, and IL-8 in HSFs treated with LPS. Compared with the LPS group, treatment with GA significantly decreased levels of TNF-α, IL-6, IL-1β, and IL-8 in HSFs.

GA upregulated expression of PPARγ by inhibited LPS stimulated TLR-4 activation

First, the consequence of TLR-4 signaling activation on PPARγ expression in HSFs was evaluated using agonists for TLR-4. As shown in **Figure 2**, LPS reduced both PPARγ mRNA and protein expression in HSFs. To further verify the specific effects of GA on TLR4/PPARγ pathways, HSFs were incubated in the presence or absence of GA for 24 hours. Results show that GA upregulated expression of PPARγ and downregulated expression of TLR-4. Data indicates that LPS negatively regulated expression of PPARγ in HSFs but GA antagonized the effects of LPS.

GA regulation of PPARγ is TLR-4 dependent

Activation of the TLR-4 pathway by LPS has been studied thoroughly. Even though this study used highly purified LPS, results still confirmed that the observed effects of LPS on PPARγ expression were regulated specifically through TLR-4 and were not the result of a contaminant that might activate another pathway. It was also found that co-treatment of HSFs with LPS antagonist polymyxin can block downregulation of PPARγ (**Figure 3A**). Furthermore,
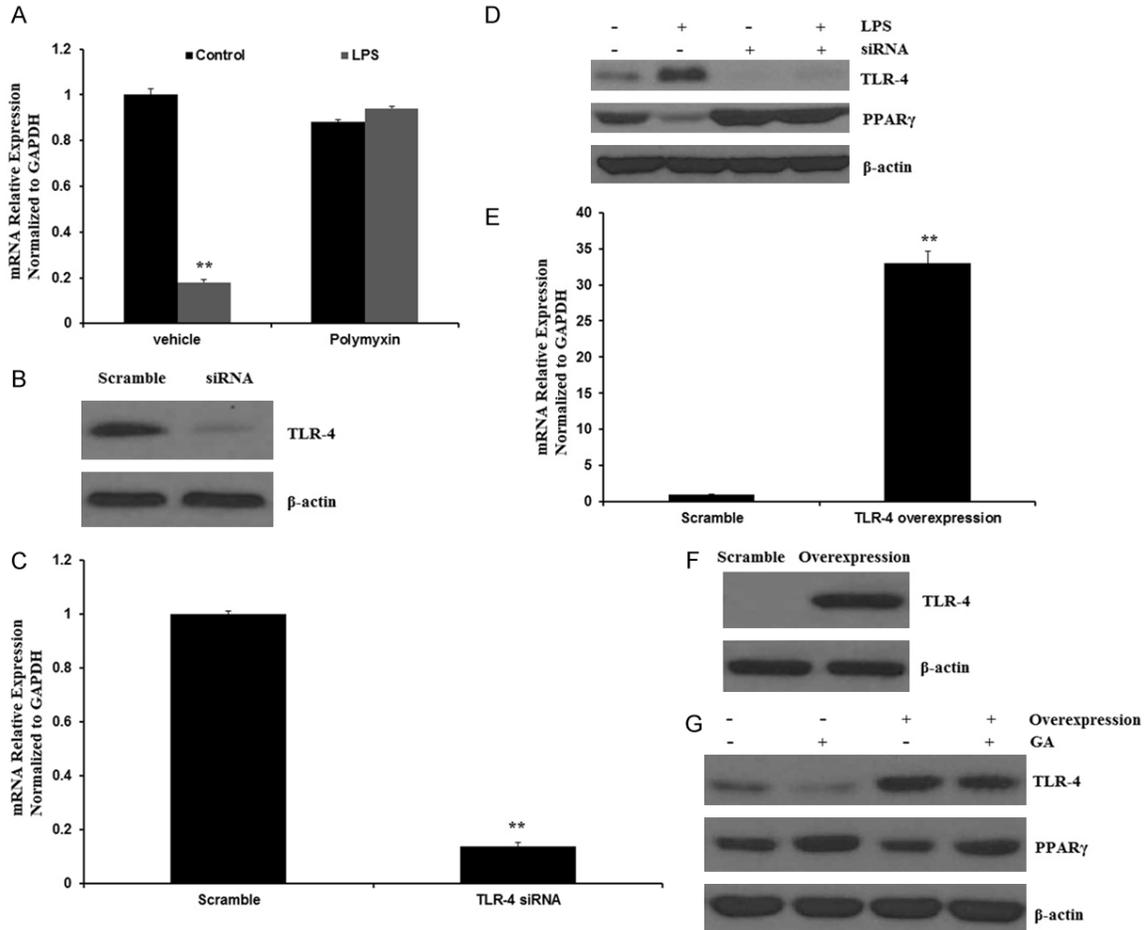
Figure 3. Regulation of PPARγ is TLR-4 dependent. A. qRT-PCR was used to measure the PPARγ mRNA in HSFs treated with vehicle and the LPS antagonist polymyxin (50 μg/ml) ± LPS. B. The effects of a TLR-4 siRNA in HSFs were confirmed by qRT-PCR. C. The effects of a TLR-4 siRNA in HSFs were confirmed by Western blotting and the loading control was β-actin. D. HSFs after TLR-4 siRNA treatment were stimulated with vehicle or LPS. Western blot was used to measure the PPARγ protein. E. The effects of TLR-4 overexpression in HSFs were confirmed by qRT-PCR. F. The effects of TLR-4 overexpression in HSFs were confirmed by Western blotting and the loading control was β-actin. G. HSFs engineered to overexpress TLR-4 were exposed to vehicle or GA and the expression of PPARγ protein were determined by Western blot. In all experiments, mRNA was normalized to GAPDH and expressed as the fold change relative to the control. Each bar represents the mean ± SD, n = 3. **means $P < 0.01$.

LPS-induced inhibition of PPARγ was not observed in HSFs treated with a TLR-4 siRNA (**Figure 3B-D**). Additionally, overexpression TLR-4 induced the inhibition of PPARγ, but GA increased PPARγ expression in HSFs that were engineered to overexpress TLR-4 (**Figure 3E-G**). Present data demonstrates that the effects of GA/LPS on PPAR γ expression are specifically regulated by TLR-4.

GA regulates PPARγ expression by TLR-4 needs the nuclear factor-κB (NF-κB) signaling

This study examined the molecular mechanisms of how TLR-4 activation by LPS contributes to the inhibition of PPARγ mRNA synthe-sis. Previous studies have proven that TLR-4 signaling is related to the activation of proinflammatory transcription factor AP-1 through MEK1/2-, p38 MAPK-, and JNK-dependent phosphorylation, but IKK-dependent processes are related to NF-κB activation. Therefore, this study used inhibitors of MEK1/2 (U0126), p38 MAPK (SB203580), JNK (SP600125), and AP-1 (curcumin) to determine which pathways are required for LPS-mediated regulation of PPARγ. It was found that treatment of HSFs with these inhibitors for 6 hours had no effect on PPARγ regulation by LPS (**Figure 4A**), indicating that the TLR-4-dependent activation of AP-1 is not involved in the downregulation of PPARγ expression by LPS.
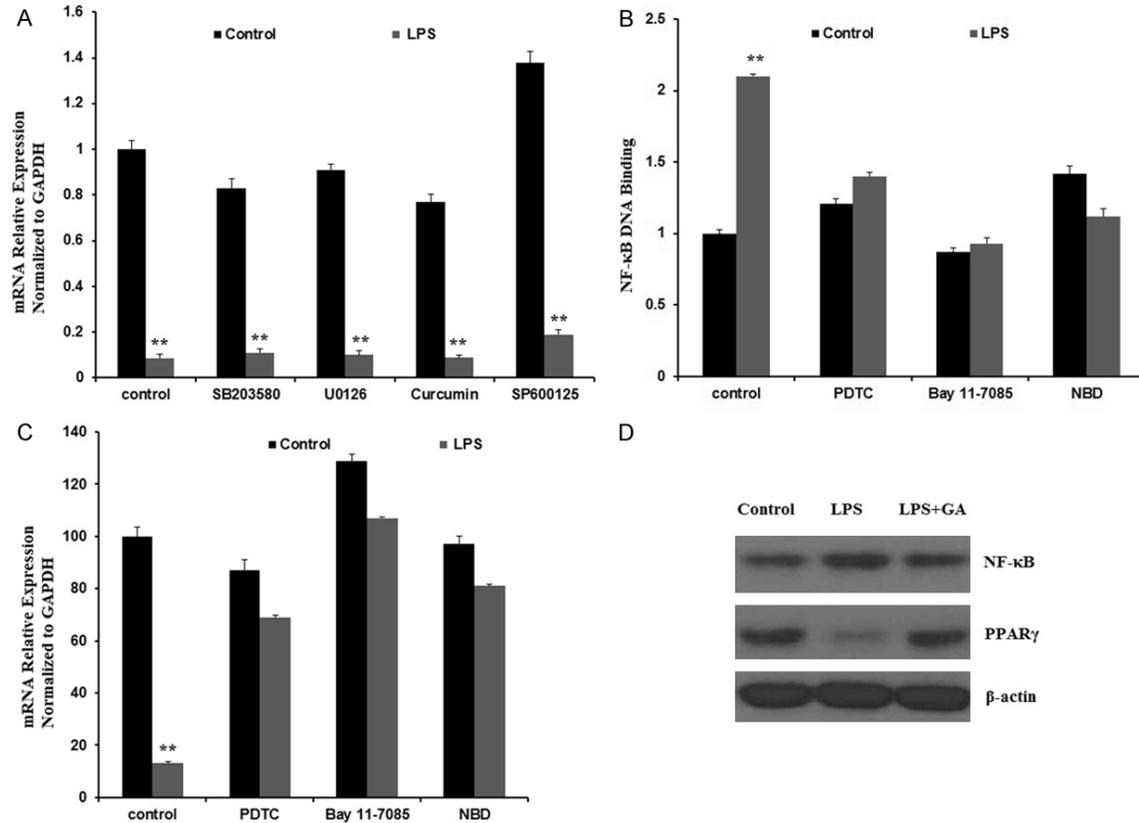
Figure 4. GA regulates PPARγ expression by TLR-4 needs the nuclear factor-κB (NF-κB) signaling. A. HSFs were pre-treated for 1 hour with inhibitors of the MEK/JNK/AP-1 signaling axis, including p38 (SB203580, 20 μM), MEK1/2 (U0126, 10 μM), AP-1 (curcumin, 20 μM), and JNK (SP600125, 10 μM), and were subsequently exposed for ± LPS. qRT-PCR was used to measure the PPARγ mRNA from each sample. B. HSFs were pretreated for 1 hour with up-stream inhibitors of NF-κB, including IKK (Bay 11-7085, 5 μM), IκB (NBD, 100 μM), and NF-κB (PDTC, 100 μM), and were then exposed for ± LPS. NF-κB activity was determined by the measurement of the NF-κB DNA binding activity. C. The PPARγ mRNA was determined by qRT-PCR in HSFs pretreated for 1 hour with upstream inhibitors of NF-κB. D. Expression of TLR-4, NF-κB P65, and PPARγ proteins was measured by Western blotting in HSFs treated by LPS with or without GA for 24 hours and the loading control was β-actin. All data are expressed as a fold change relative to the control. Each bar represents the mean ± SD, n = 3. **means $P<0.01$.

Moreover, this study evaluated the roles of NF-κB pathways in LPS-regulated PPARγ expression. This study detected the effects of different inhibitors that target the IKK complex (PDTC), NEMO (NBD, NEMO binding domain), and IκB (Bay 11-7085), and upstream components required for activation of NF-κB. Those inhibitors efficacy were shown through the inhibition of LPS-induced NF-κB DNA binding activity (**Figure 4B**). Each of these NF-κB inhibitors effectively blocked the LPS-mediated downregulation of PPARγ (**Figure 4C**). It was further proven that GA can significantly inhibit LPS-induced NF-κB expression (**Figure 4D**). Present results indicate that GA upregulation of PPARγ expression mediates TLR4/NF-κB signaling in HSFs.

Discussion

HTS is a common and serious problem after cutaneous injuries, such as burns, traumas, abrasions, and deep donor site excisions [10]. Although mechanisms underlying its pathogenesis are not well understood, the roles of mechanical stress and inflammation have been validated in its occurrence, de-development, and progress [11, 12]. Compounds targeting either mechanical stress or inflammation may serve as effective anti-fibrotic agents. However, lots of anti-inflammatory compounds, like corticosteroids, are not suitable as anti-fibrotic drugs because of their severe side effects. Thus, compounds with biology inhibitory effects on either mechanical stress or inflammation

may be potential candidates for the development of new anti-fibrotic drugs. The present study demonstrated that GA decreased expression of pro-inflammatory cytokines TNF-α, IL-6, IL-1β, and IL-8 in HSFs. More importantly, this study documented that TLR-4/NF-κB/PPARγ pathways are specifically involved in the anti-inflammatory effects of GA in HSFs.

TLRs are important pattern-recognition receptors involved in host defense against a variety of pathogenic microorganisms. Activation of TLRs can accelerate the secretion of chemokines, cytokines, antimicrobial peptides, and the increase of adhesion molecules levels involved in innate and adaptive immune responses [13]. The present molecular mechanism studies focused on TLR-4, a well-known role in the development of inflammatory conditions, such as sepsis, atherosclerosis, and ulcerative colitis [14-18]. The critical roles of TLR-4 pathways in the pathogenesis of HTS were explored in a previous work. Dermal fibroblasts were found to express TLR-4 and fibroblasts from HTS enhanced TLR-4 expression, compared with site matched normal fibroblasts. This study found significant increases in mRNA and protein levels of TLR-4, MyD88, transforming growth factor-beta1 (TGF-β1), and procollagen I when normal and hyperplastic scar fibroblasts (HSFB) were treated with LPS, suggesting that TLR-4 plays an important role in the pathogenesis of HTS [9].

PPARs are a family of ligand-activated nuclear transcription factors that have emerged as important factors in organ fibrosis. The present study shows that PPARs regulate important cellular functions, including cell proliferation, differentiation, and inflammation. The anti-inflammatory effects of PPARs are important because pro-inflammatory responses play important roles in the pathogenesis of HTS. Additionally, PPAR signals have been demonstrated to be related to skin development and barrier formation [19]. Recent studies have demonstrated that activation of PPARγ inhibits transforming growth factor-1 induction of connective tissue growth factor and extracellular matrix in HSFs [20].

Gallic acid (GA), a polyhydroxy phenolic compound, is found in various natural products, such as tea leaves, gallnuts, grapes, green tea, strawberries, lemons, pineapples, and in red and white wines. Increasing evidence has shown that GA has anti-inflammatory and pro-apoptotic activities. Based on a previous work and these reports, the present study aimed to extend these observations by elucidating the effects of GA on HSFs inflammation after LPS stimulation, as well as the underlying mechanism relationship. It was found that GA could decrease the basal levels of pro-inflammatory molecules, including TNF-α, IL-6, IL-1β, and IL-8. The inactivation of TLR-4 by GA upregulates PPARγ through the inactivation of NF-κB. These findings suggest that the anti-inflammatory actions of GA by TLR-4/NF-κB/PPARγ signaling are critical for HSFs. Other studies have found that LPS can reduce expression of PPARγ in macrophages and lung cells [21, 22]. Downregulation of PPARγ by TLR-4 may also be relevant to other diseases, such as obesity-related insulin resistance. Free fatty acids can activate TLR-4 on macrophages to induce an inflammatory response [23] and the macrophage PPARγ is required to maintain glucose homeostasis and insulin sensitivity. Similarly, TZD, the PPARγ agonist, mediates insulin-sensitizing effects through anti-inflammatory effects on macrophages [24, 25].

In summary, the present study demonstrates that GA may suppress the secretion of inflammatory cytokines in HSFs by TLR-4/NF-κB/PPARγ signaling pathways. However, whether GA can be used clinically to treat HTS or if it acts upon other signaling pathways to affect HTS remains to be elucidated. Therefore, further detailed studies are required to evaluate the therapeutic use of GA.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Pengju Fan, Department of Plastic and Esthetic surgery, Xiangya Hospital of Central South University, Changsha 410008, P. R. China. E-mail: pfan1207@hotmail.com

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