Rosiglitazone alleviates lipopolysaccharide-induced inflammation in RAW264.7 cells via inhibition of NF-κB and in a PPARγ-dependent manner

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Abstract. Rosiglitazone is a synthetic peroxisome proliferator-activated receptor (PPAR)γ agonist widely used for the treatment of type 2 diabetes. Recent studies have demonstrated that rosiglitazone displays anti-inflammatory effects. The present study aimed to investigate whether rosiglitazone alleviates decreases in RAW264.7 cell viability resulting from lipopolysaccharide (LPS)-induced inflammation, as well as exploring the underlying mechanism. A macrophage inflammatory injury model was established by treating RAW264.7 cells with 100 ng/ml LPS. Cells were divided into LPS and rosiglitazone groups with different concentrations. Cell viability was assessed by performing an MTT assay. The expression of inflammatory cytokines was detected by conducting enzyme-linked immunosorbent assays and reverse transcription-quantitative PCR. Nitric oxidesecretion was assessed using the Griess reagent system. The expression levels of key nuclear factor-κB pathway-associated proteins were detected via western blotting. Rosiglitazone alleviated LPS-induced decrease in RAW264.7 cell viability and inhibited inflammatory cytokine expression in a concentration-dependent manner. Rosiglitazone significantly inhibited LPS-induced upregulation of p65 phosphorylation levels and downregulated IκBα expression levels. However, rosiglitazone-mediated inhibitory effects were reversed by PPARγ knockdown. The results of the present study demonstrated that rosiglitazone significantly inhibited LPS-induced inflammatory responses in RAW264.7 macrophage cells, which was dependent on PPARγ activation and NF-κB suppression.

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Introduction

Inflammation is a complex pathological response caused by harmful stimuli to the internal and external environment (1). *In vitro* inflammatory models are typically established by lipopolysaccharide (LPS) or interferon-γ induction in macrophages. Macrophages, which are central cells that produce inflammatory mediators and modulate inflammatory responses *in vivo,* can be immunomodulated by the secretion of various cytokines or lysosome release (2,3).

The RAW264.7 cell line is derived from mouse mononuclear macrophage leukemia cells (4). RAW264.7 cells are widely used to evaluate the immunomodulatory effects of mononuclear macrophages on nitric oxide (NO) secretion and associated inflammatory signaling pathways (5). LPS stimulates cells to secrete a variety of inflammatory mediators, including nitric oxide (NO), tumor necrosis factor (TNF)-α and interleukin (IL)-1β, via binding to the corresponding receptors on the cell membrane to regulate immune response (6).

NO is the major mediator of oxidative stress, which exacerbates inflammatory responses. Therefore, NO levels are closely associated with the pathogenesis of numerous inflammatory diseases (7). Inducible NO synthase (iNOS) is a vital indicator of the inflammatory response (8). At present, the regulation of NO synthesis and iNOS expression is considered to be a novel therapeutic strategy for inflammatory diseases. Proinflammatory cytokines, including TNF-α, IL-1β and IL-6, can interact with the anti-inflammatory cytokine IL-10 to participate in inflammation regulation (9).

LPS is a major component of the cell wall of Gram-negative bacteria; identification and signal transduction of LPS is an essential step in the self-defense response of the body (10). Previous studies have reported that LPS can promote the development of acute kidney injury by inducing the production of proinflammatory cytokines, including TNF-α, IL-6 and IL-1β (11,12). LPS induction in tyrosine hydroxylase immunoreactive cells selectively inhibit cell viability and increases the culture medium contents of IL-1β, TNF-α and NO (13). Toll-like receptor (TLR)-4 mediates LPS-induced inflammatory responsesin human coronary artery endothelial cells (13). Moreover, LPS can induce inflammatory effects by regulating the nuclear factor (NF)-κB signaling pathway in

A549 cells (14). The primary downstream signaling pathways involved in LPS-induced inflammatory responses include the NF-κB, MAPK and JAK-STAT signaling pathways. Activation of the aforementioned signaling pathways further regulates a variety of inflammatory mediators (15).

Previous studies have reported that LPS-induced production of proinflammatory cytokines is associated with the NF-κB signaling pathway (16-19). It is considered to be the central step in LPS-induced macrophage inflammation that exerts a crucial role in promoting iNOS and proinflammatory cytokine expression (20). LPS activates TLR-4 and binds to heat shock protein 60 via activating the NF-κB signaling pathway (21). LPS also induces the production of proinflammatory cytokines by macrophages, thus leading to myocardial hypertrophy and ischemia (22).

LPS-induced inflammation is also associated with peroxisome proliferator-activated receptors (PPARs). PPARγ belongs to the nuclear hormone receptor superfamily and is a ligand-activated transcription factor. PPARγ regulates cell proliferation, differentiation, carbohydrate lipid metabolism and inflammatory responses. PPARs can be divided into three types: PPARα, PPARβ and PPARγ, among which PPARγ is primarily distributed in adipose tissue and the immune system, suggesting its role in fat metabolism and body immunity (23).

Recent studies have demonstrated that PPARγ activation downregulates the expression of NOS, matrix metalloproteinases and adhesion molecules in the mononuclear phagocyte cell line, thereby inhibiting the inflammatory response (24-26). PPARγ agonists are capable of inhibiting the production of proinflammatory cytokines in mononuclear macrophages (23). Pretreatment with a PPARγ ligand can significantly decrease the expression of proinflammatory cytokines in tissues, and alleviate tissue damage at local and distant sites of inflammation (27). PPARγ agonist ligands are split into two major classes, natural ligands and synthetic ligands. Natural ligands are primarily 15-deoxy prostaglandin J2 (15d-PGJ2) and linoleic acid oxidation products, whereas synthetic ligands are primarily thiazolidinedione (TZDs), including pioglitazone, troglitazone and rosiglitazone. Rosiglitazone is the most commonly used drug with the highest bioavailability, strongest drug effect and fewest side effects (28). Previous studies have demonstrated the anti-inflammatory effects of rosiglitazone in diverse models (29). Rosiglitazone upregulatesheme oxygenase-1 expression via the reactive oxygen species-dependent nuclear factor, erythroid 2 like 2-antioxidant response elements axis (30).

Moreover, rosiglitazone could also impair colonic inflammation in mice with experimental colitis (31). However, the mechanism underlying the anti-inflammatory effects of rosiglitazone is not completely understood.

The present study aimed to explore the role of the PPARγ agonist rosiglitazone in the regulation of LPS-induced inflammatory responses and decreases in viability in RAW264.7 cells, as well as its potential underlying mechanisms.

Materials and methods

Cell culture. The RAW264.7 cell line is a mouse mononuclear macrophage leukemia cell line that was obtained from the American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37˚C. Culture medium was replaced every 2 days.

MTT assay. RAW264.7 cells at the logarithmic growth phase were digested with PBS supplemented with 0.25% EDTA and prepared for cell suspension. After the cell density was adjusted to $2x10^5$/ml, 100 μl cell suspension was added to each well of a 96-well plate. RAW264.7 cells were treated with 100 ng/ml LPS (Sigma-Aldrich; Merck KGaA; L4391), or 1, 2, 5, 10 or 20 μM rosiglitazone (Sigma-Aldrich; Merck KGaA; cat. no. R2408) for 48 or 72 h at 37˚C. Each group consisted of three replicates. Subsequently, cells were incubated with 200 μl 0.5% MTT solution (0.5 mg/ml) for 4 h. The purple formazan was dissolved with DMSO solution. Absorbance was measured at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Enzyme-linked immunosorbent assay (ELISA). RAW264.7 cells in the logarithmic growth phase were seeded ($1x10^4$/ml; 100 μl/well) into 96-well plates. Following culture for 24 h, cells were pretreated with rosiglitazone at different concentration for 1 h and then treated with LPS for 24 h. Subsequently, the culture medium was collected and cytokine levels were detected using an IL-6 (cat. no. M6000B) and TNFα (cat. no. MTA00B) ELISA kit (R&D Systems, Inc.) according to the manufacturer's protocol.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cells using TRIzol® (Thermo Fisher Scientific, Inc.). The RNA concentration of each sample was detected using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Takara Bio, Inc.) at 42˚C for 30 min. Subsequently, qPCR was performed for 40 cycles using Hieff™ qPCR SYBR®-Green Master Mix (with ROX; Yeasen Technology, Inc.). The sequences of the primers used for qPCR are presented in Table I. The $2^{-\Delta\Delta Cq}$ method was used for quantitative analysis (32).

Western blotting. Total protein was extracted from cells using RIPA solution (cat. no. 89900; Thermo Fisher Scientific, Inc.). The BCA method was used to determine the protein concentration. A total of 20 μg protein was separated via 10% SDS-PAGE gel and transferred to PVDF membranes. Following blocking with 5% skimmed milk at room temperature for 1 h, the membranes were incubated overnight at 4˚C with primary antibodies (1:1,000 dilution). Subsequently, the membranes were incubated with a HRP-conjugated anti-mouse (1:5,000; cat. no. #7076; Cell Signaling Technology, Inc.) or HRP-conjugated anti-rabbit IgG (1:5,000; cat. no. #7074; Cell Signaling Technology, Inc.) at room temperature for 1 h. p65 (cat. no. 66535-1-Ig), IkB-α (cat. no. 10268-1-AP), PPAR (cat. no. 16643-1-AP) and β-actin (cat. no. 60008-1-Ig) primary antibodies were purchased from ProteinTech Group, Inc. Thephosphorylated (p)-p65 (cat. no. 3033) primary antibody was purchased from Cell Signaling Technology, Inc. Protein bands were visualized using an ECL system

Table I. Sequences of primers used for quantitative reverse transcription PCR.

Gene	Sequence, 5'-3'
GAPDH	F: CGGAGTCAACGGATTTGGTCGTAT
	R: AGCCTTCTCCATGGTGGTGAAGAC
IL-1β	F: GAAAGACGGCACACCCACCCT
	R: GCTCTGCTTGTGAGGTGCTGATGTA
TNF-α	F: TTCTGTCTACTGAACTTCGGGGTGAT CGGTCC
	R: GTATGAGATAGCAAATCGGCTGACGG TGTGGG
IL-10	F: ATAACTGCACCCACTTCCCA
	R: GGGCATCACTTCTACCAGGT
iNOS	F: CCTTGTTCAGCTACGCCTTC
	R: CTGAGGGCTCTGTTGAGGTC

F, forward; R, reverse; iNOS, inducible nitric oxide synthase; IL, interleukin; TNF, tumor necrosis factor.

with an ImageQuant LAS 500 imager (GE Healthcare). The protein bands were quantified by ImageQuant TL version 8.0 (GE Healthcare).

Cell transfection. Small interfering RNA (si)-PPARγ-1, si-PPARγ-2 and si-negative control were purchased from Shanghai GenePharma Co., Ltd. Briefly, 0.8 μg si RNA or 3 μl Viromer blue transfection reagent (Lipocalyx GmbH) were diluted in 350 μl buffer blue, mixed and stored at room temperature for 15 min. Subsequently, cells were seeded at $1x10^5$ cells/well in a six-well plate and then were incubated with the reagent mixture for 48 h. Culture medium was replaced every 2 days. The siRNA sequences were as follows: si-PPARγ-1: 5'-CCGGGCTCCACACTATGAAGACATTCTCGAGAATGT CTTCATAGTGTGGAGCTTTTT-3'; si-PPARγ-2: 5'-CCG GGCCTCCCTGATGAATAAAGATCTCGAGATCTTTAT TCAGGGAGGCTTTTT-3'.

Determination of NO secretion. NO secretion levels were determined using the Griess reagent system kit (Beyotime Institute of Biotechnology). Cells were seeded ($1x10^4$/ml) into 96-well plates and incubated for 24 h. Following different treatments for 24 h, 50 μl cell supernatant was collected and plated into 96-well plates at room temperature. Subsequently, 50 μl Griess I and Griess II reagent were added in order at room temperature. Absorbance was measured at a wavelength of 540 nm with microplate reader.

Dual-luciferase reporter gene assay. A total of $2x10^4$ cells were co-transfected with 1 μg mass luciferase-coupled reporter gene for NF-κB and 1 μg *Renilla* luciferase reporter (Beyotime Institute of Biotechnology) using Viromer blue transfection reagent (High-Tech Gründerfonds). At 48 h post-transfection, cells were pre-treated with rosiglitazone for 1 h and then treated with LPS for 5 h at 37˚C. Following washes with cold PBS, cells were lysed with luciferase lysis buffer (Beyotime Institute of Biotechnology) and luciferase activities were measured using the Dual Luciferase Assay System and a Victor luminometer (Promega Corporation) according to the manufacturer's instructions. Relative NF-κB luciferase activity was normalized to *Renilla* activity.

Statistical analysis. Statistical analyses were performed using GraphPad software (version 7; GraphPad Software, Inc.). Data are presented as the mean ± SD. Comparisons between groups were analyzed using Kruskal-Wallis test and Dunn's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed three times.

Results

Rosiglitazone reverses LPS-induced decrease in cell viability. Rosiglitazone is an insulin sensitizer and is commonly used for the treatment of type 2 diabetes (33). In order to explore the effect of different concentrations of rosiglitazone on LPS-induced decrease in cell viability, RAW264.7 cells were treated with 1, 2, 5, 10 or 20 μM rosiglitazone to detect the cell cytotoxicity of rosiglitazone. Following treatment for 48 h, cell viability was measured using the MTT assay. Compared with the control group, 1-20 μM rosiglitazone showed no obvious cytotoxic effect on RAW264.7 cells (Fig. 1A). Therefore, 1, 5, 10 and 20 μM rosiglitazone were selected as the extremely low-, low-, middle- and high-dose rosiglitazone groups, respectively. Subsequently, RAW264.7 cells were treated with 100 ng/ml LPS for 48 h. LPS treatment decreased RAW264.7 cell viability compared with the control group. However, middle- and high-dose rosiglitazone treatment for 48 h reversed LPS-induced decrease in cell viability (Fig. 1B); similar results were observed following treatment for 72 h (Fig. 1C).

Effect of rosiglitazone on LPS-induced proinflammatory and anti-inflammatory cytokine expression. In order to explore the effect of rosiglitazone on LPS-induced alterations to the expression of proinflammatory and anti-inflammatory cytokines, mRNA expression levels of IL-1β, TNF-α and IL-10 were detected via RT-qPCR. The results demonstrated that treatment with 100 ng/ml LPS for 48 h remarkably upregulated IL-1β, TNF-α and IL-10 mRNA expression levels. Compared with the LPS group, rosiglitazone treatment downregulated IL-1β, IL-10 and TNF-α mRNA expression levels in a dose-dependent manner (Fig. 2A-C). In order to further verify the aforementioned results, IL-6 and TNF-α contents in the culture medium of different groups were assessed. The ELISA results demonstrated that IL-6 and TNF-α contents in the culture medium of the LPS group were remarkably elevated. However, IL-6 and TNF-α contents were downregulated in the middle- and high-dose groups in a dose-dependent manner (Fig. 2D and E). NO and iNOS mRNA expression levels in RAW264.7 cells, following exposure to LPS and different concentrations of rosiglitazone, were also detected. The results demonstrated that different concentrations of rosiglitazone treatment decreased NO secretion in a dose-dependent manner (Fig. 2F). Similar results were obtained for the detection of iNOS mRNA expression levels via RT-qPCR (Fig. 2G).

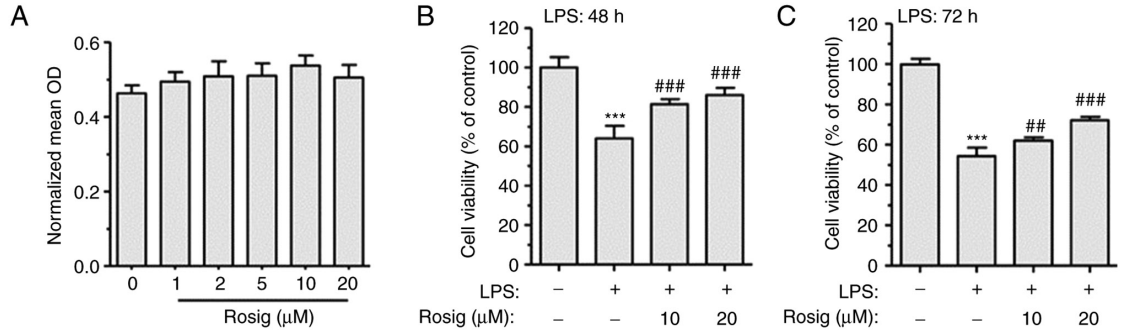

Figure 1. Effect of rosiglitazone on RAW264.7 cell viability with or without LPS stimuli. (A) Effect of different concentrations of rosiglitazone on RAW264.7 cells. (B and C) Effect of LPS treatment for 48 or 72 h and different concentrations of rosiglitazone on RAW264.7 cell viability. ***P<0.001 vs. PBS; ##P<0.01 and ###P<0.001 vs. LPS. LPS, lipopolysaccharide; rosig, rosiglitazone.

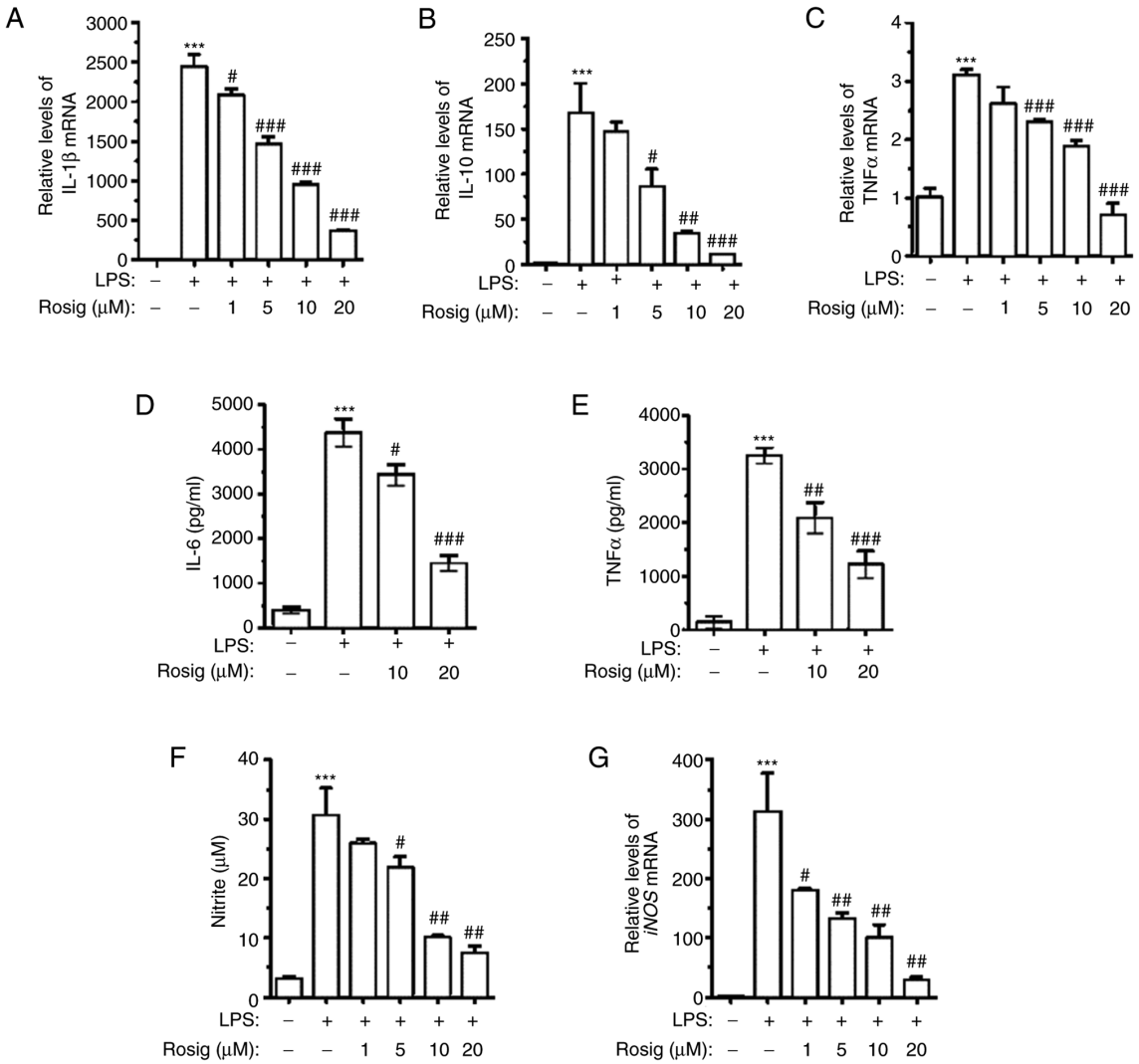

Figure 2. Effect of different concentrations of rosiglitazone on LPS-induced expression. (A-C) Effect of different concentrations of rosiglitazone on LPS-induced mRNA expression levels of cytokines in RAW264.7 cells. Effect of rosiglitazone on the expression of inflammatory mediators (D) TNF-α and (E) IL-6 in LPS-treated RAW264.7 cells. Effect of different concentrations of rosiglitazone on (F) NO secretion and (G) iNOS mRNA expression in LPS-treated RAW264.7 cells. ***P<0.001 vs. PBS; #P<0.05, ##P<0.01 and ###P<0.001 vs. LPS. LPS, lipopolysaccharide; TNF, tumor necrosis factor; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL, interleukin; rosig, rosiglitazone.

Rosiglitazone inhibits the NF-κB signaling pathway in LPS-treated RAW264.7 cells. It has been reported that the NF-κB signaling pathway is greatly involved in inflammatory responses (34). Therefore, whether rosiglitazone could regulate RAW264.7 cell inflammation via the NF-κB signaling pathway was investigated. The activity of the NF-κB-driven luciferase reporter gene was markedly elevated after LPS induction for 48 h. However, middle- and high-dose rosiglitazone treatment

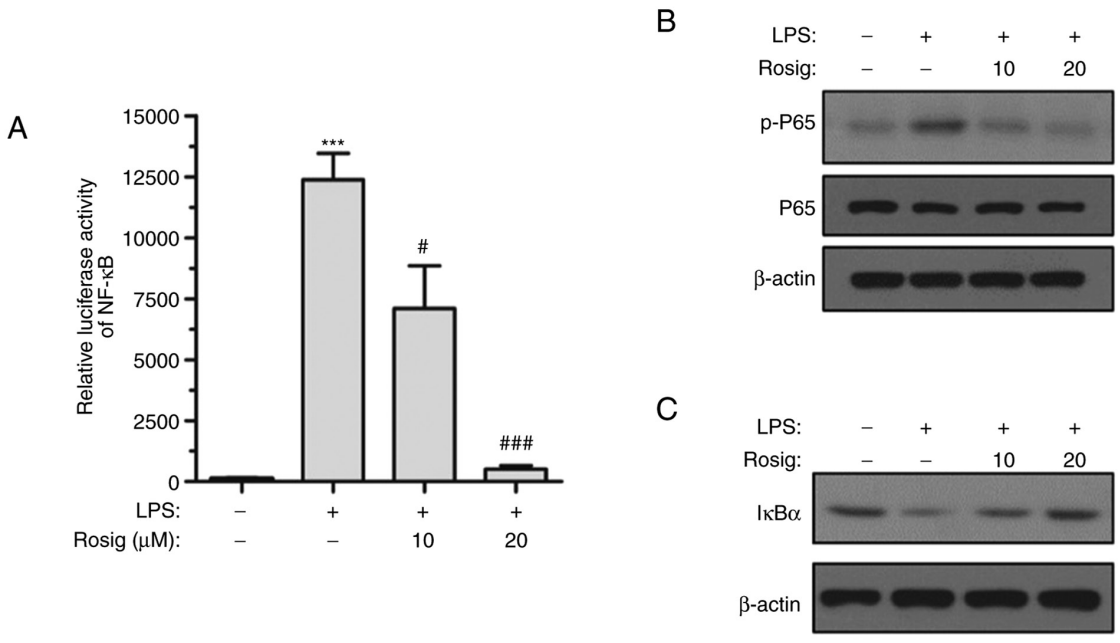

Figure 3. Effects of rosiglitazone on NF-κB activity induced by LPS in RAW264.7 cells (A) Effect of rosiglitazone on NF-κB activity assessed by luciferase reporter gene. (B) Effect of rosiglitazone on the level of p-p65 level induced by LPS in RAW264.7. (C) RAW264.7 cells were pre-treated with rosiglitazone, and then subjected to the LPS treatment; the expression level of IκBα was measured by western blotting. ***P<0.001 vs. PBS; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 vs. LPS. LPS, lipopolysaccharide; p-, phosphorylated; NF-κB, nuclear factor-κB; rosig, rosiglitazone.

inhibited the activity of the NF-κB-driven luciferase reporter gene (Fig. 3A). Moreover, the phosphorylation level of p65 was gradually decreased and IκBα expression was increased with increasing concentrations of rosiglitazone (Fig. 3B and C), indicating that the NF-κB signaling pathway was inhibited by rosiglitazone in a concentration-dependent manner.

PPARγ knockdown impairs the anti-inflammatory effect of rosiglitazone on RAW264.7 cells. In order to verify whether the effect of rosiglitazone on inflammation regulation was mediated via PPARγ, si-PPARγ-RAW264.7 cell lines were constructed. The transfection efficacy of si-PPARγ was verified via western blotting (Fig. 4A). The results indicated that PPARγ knockdown upregulated IL-1β and TNF-α mRNA expression levels (Fig. 4B and C). Similarly, PPARγ knockdown reversed rosiglitazone-induced decrease in p65 phosphorylation levels and increased IκBα expression (Fig. 4D-F).

Discussion

As innate immune cells, macrophages trigger inflammatory and immune responses for self-defense. LPS is a potent inducer of monocyte and macrophage immune responses. When activated by LPS, macrophages release a variety of proinflammatory cytokines and anti-inflammatory cytokines (35). Excessive release of cytokines may lead to extensive tissue damage and pathological alterations (36). Macrophages produce a number of inflammatory mediators, including IL-1β, IL-6, TNF-α and NO (37). LPS induction stimulates the secretion of proinflammatory mediators by macrophages, eventually leading to cell injury and even cell death (38). Therefore, the present study used LPS as an *in vitro* model of inflammation.

PPARγ is a type of ligand-dependent transcription factor that regulates the proliferation, invasion, differentiation and apoptosis of various cells at the transcriptional level. PPARγ serves a crucial role in various inflammatory injury processes (39-40). Rosiglitazone is a synthetic PPARγ agonist and is widely used for the treatment of type 2 diabetes (41). Previous studies have demonstrated that rosiglitazone serves a neuroprotective role via anti-inflammatory and antioxidant mechanisms after brain trauma (41-43). In the present study, 1-20 μM rosiglitazone showed no obvious cytotoxic effect on RAW264.7 cells. However, rosiglitazone reversed the inhibitory effect of LPS on cell viability, potentially via inhibiting cytokine expression. Moreover, rosiglitazone inhibited LPS-induced proinflammatory cytokine and enzyme expression, including IL-1β, TNF-α, IL-6 and iNOS in RAW264.7 cells. Interestingly, LPS also elevated the expression of IL-10, an anti-inflammatory cytokine, potentially to overcome the proinflammatory cytokines, which is a phenomenon derived from cell self-protective mechanisms (44). Rosiglitazone not only inhibited proinflammatory cytokines, but also repressed anti-inflammatory cytokines, suggesting that it might serve a vital role in balancing the process of inflammation.

To confirm whether the anti-inflammatory effect of rosiglitazone was mediated via PPARγ, si-PPARγ-RAW264.7 cells were constructed. The results indicated that PPARγ knockdown attenuated the inhibitory effect of rosiglitazone on proinflammatory cytokines. Therefore, the aforementioned results suggested that rosiglitazone regulated inflammation via PPARγ activation.

NF-κB is an important transcription factor that regulates the expression of immune and inflammatory response factors (45). Previous studies have demonstrated that the PPARγ/NF-κB signaling pathway is involved in the dynamic balance of the inflammatory response (46-48). Besides, PPARγ agonists, including rosiglitazone, were reported to inhibit the activity of the NF-κB signaling pathway in osteoclastogenesis. The

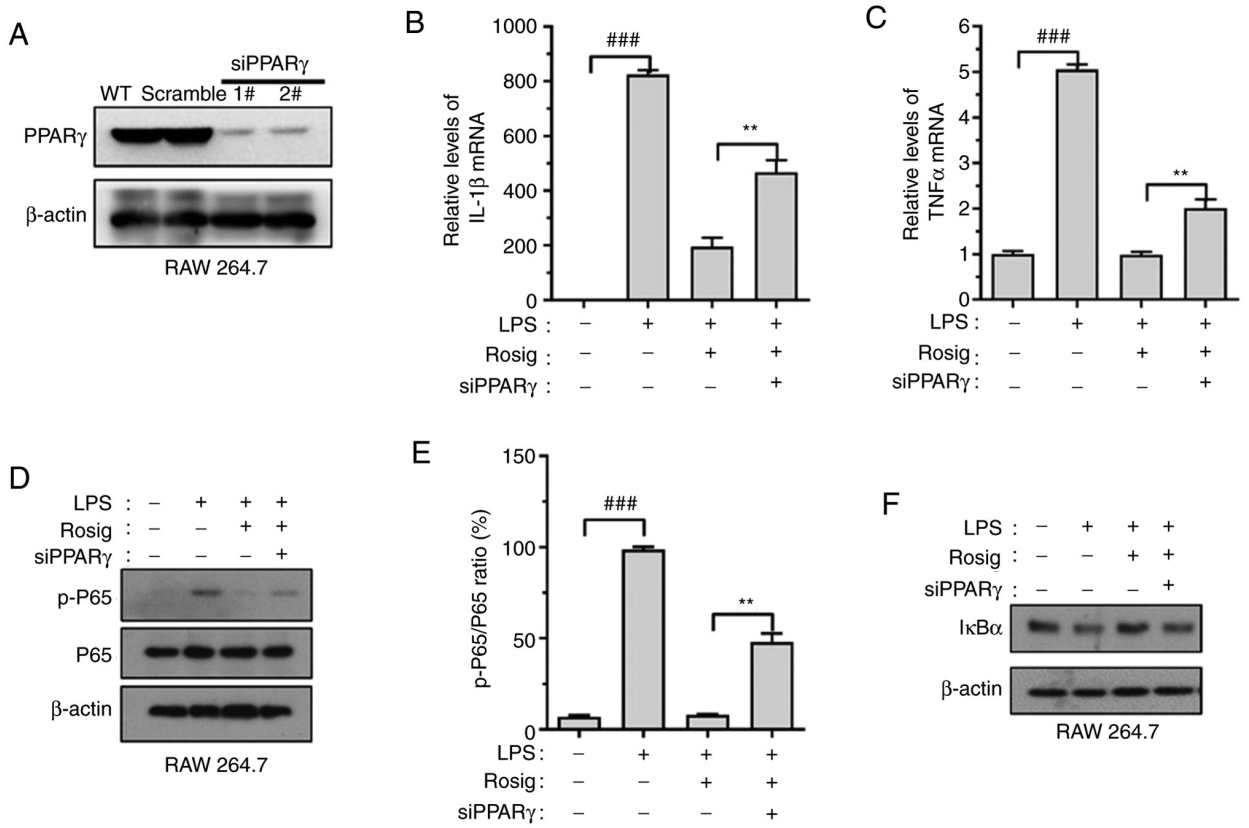

Figure 4. Rosiglitazone exerts anti-inflammatory effect via PPARγ (A) PPARγ was knocked down by siRNA transfection, and the expression of PPARγ was assessed by western blotting. Scramble siRNA was used as a negative control. (B and C) PPARγ was knocked down by siRNA and then subjected to the pre-treatment with rosiglitazoneand LPS induction, then the mRNA levels of IL-1β and TNF-α were measured by reverse transcription-quantitative PCR. (D and F) Effect of rosiglitazone on the levels of p-p65 and IκBα when PPARγ was knocked down. (E) The semi-quantification of p-P65/P65 ratio was measured by Quantity One software for panel. (D) $^{###}$P<0.001 vs. PBS control. **P<0.01 vs. LPS combined with rosiglitazone treatment. si, small interfering RNA; PPARγ, peroxisome proliferator-activated receptor γ; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; p-, phosphorylated; rosig, rosiglitazone.

aforementioned studies suggested that PPARγ could partly regulate the level of the NF-κB signaling pathway. NF-κB signaling pathway activation may be the control point for the expression of abundant inflammatory response genes (49). In the present study, rosiglitazone inhibited NF-κBp65 phosphorylation and increased IKBα expression, reversing LPS-induced activation of NF-κB. PPARγ knockdown impaired the effect of rosiglitazone on NF-κB activation. Therefore, the results suggested that the PPARγ/NF-κB signaling pathway might serve as a crucial target for controlling inflammatory responses.

NF-κB is a transcription factor family that regulates a number of genes that are involved in several physiological and pathological processes. In the canonical pathway, NF-κB dimers and molecules of IκB family form a stable complex which prevent dimers translocating to the nucleus. When stimulated by extracellular stimuli, IκB kinase (IKK) is phosphorylated causing the dimers to translocate to the nucleus and activate downstream gene expression (50). Due to the limitation of funding, p-IKKβ as well as the translocation of cytosolic p65 to the nucleus, and other signaling such as MAPK substances were not detected. The effect of IL-1β, TNF-α, IL-6 on NF-κB transcriptional activity were not studied.

In conclusion, the present study demonstrated that rosiglitazone significantly inhibited the LPS-induced inflammatory response in RAW264.7 cells and improved cell viability. Rosiglitazone inhibited the expression level of proinflammatory cytokines, potentially via activating PPARγ and inhibiting NF-κB. The results of the present study provided an experimental basis for the new application of old drugs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JPZ and XNY contributed to the study design. YQH, LGC and JJL contributed to the interpretation of data. FZ performed the experiments and YS was responsible for statistical analysis. All authors read and approved the final manuscript. JPS and XNY confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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