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Oridonin inhibits LPS-induced inflammation in human gingival fibroblasts by activating PPARγ

Tianliang Yu, Weili Xie, Yu Sun*

First Clinical Hospital of Harbin Medical University, Harbin 150001, China

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ABSTRACT

Oridonin, the major terpene isolated from *Rabdosia rubescens*, has been used as dietary supplement. Recently, it has been known to exhibit anti-inflammatory effect. This study we employed an *in vitro* model of LPS-stimulated human gingival fibroblasts to investigate the anti-inflammatory effects and mechanism of oridonin. Oridonin (10–30 μg/mL) was administrated 1 h before LPS treatment. The results showed that oridonin significantly inhibited inflammatory mediators PGE_2, NO, IL-6, and IL-8 production. Immunoblotting experiments revealed that oridonin reduced the expression of phosphorylation levels of NF-κB p65 and IκBα. Furthermore, the expression of PPARγ was up-regulated by the treatment of oridonin. Further studies showed that PPARγ inhibitor GW9662 could reverse the inhibition of oridonin on PGE_2, NO, IL-6, and IL-8 production. In conclusion, oridonin inhibited LPS-induced microglia activation through activating PPARγ.

1. Introduction

Periodontal disease is a chronic inflammatory disease of microbial origin that often causes tooth loss in adult humans [1]. *Porphyromonas gingivalis* is the major bacteria that lead to periodontal disease, such as periodontitis [2]. LPS from *Porphyromonas gingivalis* could induce the inflammatory response in gingival fibroblasts [3]. LPS could induce the activation of NF-κB, which subsequently leads to the release of inflammatory mediators [4]. These inflammatory mediators, including PGE_2, NO, IL-6, and IL-8, result in bone resorption and inhibit bone formation [5]. Previous studies showed that increased inflammatory mediators were observed in patients of periodontitis [6]. Increased PGE_2 production could lead to the tissue destruction and bone resorption [7]. IL-6 has been known as a bone resorbing factor. A large body of evidences suggested that IL-6 plays a critical role in pathological conditions exhibiting bone loss [8]. IL-8 is a potent polymorphonuclear neutrophils chemoattractant that could induce the recruitment of neutrophils to inflammatory gingival sites and amply the inflammatory response [9]. Usually, inflammation is beneficial to automatic defense response. However, excessive inflammation is also harmful and attacks the body's own tissues. During the development of periodontitis, increased inflammatory mediators and inflammatory response were observed [10]. A large body of studies showed that inhibition of these inflammatory mediators had protective effects against periodontitis [5,11]. These inflammatory mediators were regulated by the NF-κB signaling pathway [12]. A previous study showed that inhibition of NF-κB activation could inhibit the development of periodontitis [13]. Therefore, inhibition of LPS-induced inflammation and NF-κB activation may have protective effects against periodontitis. Furthermore, there is increasing evidence that many natural herbal medicines have protective effects against periodontitis through inhibiting inflammatory response [14].

The peroxisome proliferator-activated receptor γ (PPARγ), an important transcription factor belongs to the nuclear receptor family, plays a critical role in the regulation of lipid and glucose metabolism [15]. Also, it has been known to have anti-inflammatory activity [16]. Activation of PPARγ had protective effects against LPS-induced lung injury [17]. PPARγ ligands could inhibit LPS-induced inflammatory response in rat synovial fibroblasts [18]. Furthermore, PPARγ agonists could inhibit LPS-induced neuronal death in mixed cortical neurons [19]. Also, PPARγ agonist could attenuate inflammatory periodontal bone loss in mice [20]. PPARγ can be activated by natural fatty acids and some natural herbal compounds [21]. Studies showed many herbal compounds exhibited their anti-inflammatory effects through activating PPARγ [22]. Previous studies showed that PPARγ agonists could inhibit LPS-induced inflammatory cytokine production through blocking NF-κB signaling pathway [23].

Herbal medicines have been used for the treatment of periodontal disease for a long time [24]. *Rabdosia rubescens*, also called 'Dong-ling-cao' in traditional Chinese medicine, often used to treat stomach ache,

* Corresponding author.
E-mail address: sunyu20060212@126.com (Y. Sun).

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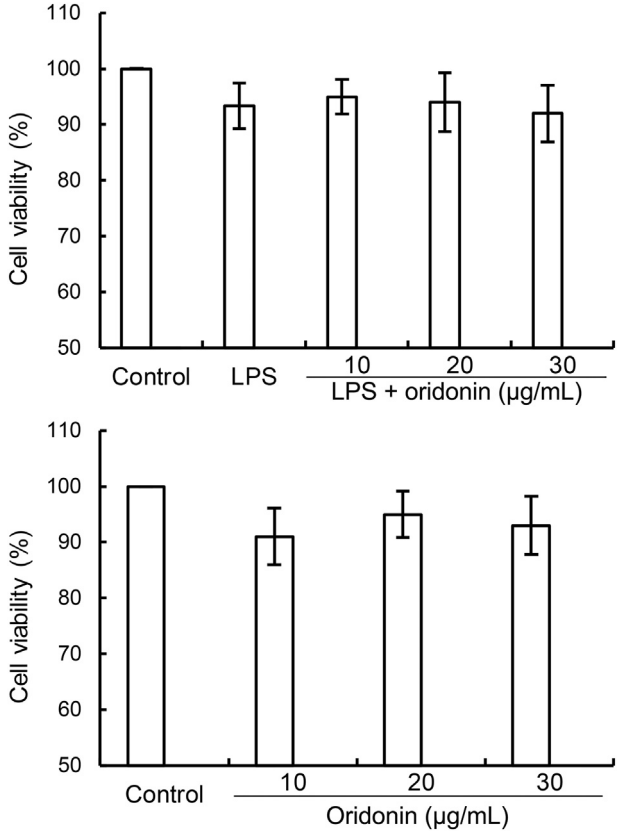

Fig. 1. Effects of oridonin on the cell viability of human gingival fibroblasts. The cell viability was determined by MTT assay. The values presented are the means ± SEM of three independent experiments.

sore throat, cough, periodontitis and tumors [25,26]. *Rabdosia rubescens* contains a variety of active components, including diterpenoids, flavonoids, and phenolic acids. Among these compounds, the diterpenoid compound oridonin has been known to exhibit anti-inflammatory effects. A previous study showed that the content of oridonin could reach 7.02 mg/g using the shaking extraction method [27]. Oridonin, the major terpene isolated from *Rabdosia rubescens,* has been reported to have anti-oxidative, anti-tumor, and anti-inflammatory activities [28]. A previous study showed that oridonin induced growth inhibition and apoptosis in human gastric carcinoma cells by enhancement of p53 expression and function [29]. Oridonin has been reported to induce mitochondria-dependent apoptosis in esophageal cancer cells [30]. Oridonin has been reported to protect against LPS/D-galactosamine-induced liver injury in mice [31]. A previous study demonstrated that oridonin inhibited LPS-induced endometritis in mice through attenuating TLR4/NF-κB signaling pathway [32]. Oridonin also had protective effects against LPS-induced lung injury in mice [33]. Meanwhile, oridonin was found to inhibit LPS-induced inflammatory cytokines production in LPS-stimulated microglia [34]. In LPS-activated RAW264.7 macrophages, oridonin also suppressed inflammatory cytokines production [33]. Oridonin inhibits LPS-induced IL-6 production in hepatic stellate cells [35]. Oridonin was found to attenuate vascular inflammation through inhibiting MAPK and NF-κB activation [36]. In addition, oridonin was found to activating PPARγ to exhibit anticancer effects [37]. However, whether oridonin could inhibit inflammatory response in LPS-stimulated human gingival fibroblasts have not been reported. The anti-inflammatory mechanism of oridonin on LPS-stimulated human gingival fibroblasts has not been clarified. The present study, we detected the anti-inflammatory effects and mechanism of oridonin on LPS-stimulated human gingival fibroblasts.

2. Materials and methods

2.1. Materials

Oridonin, DMSO, GW9662, and MTT were purchased from Sigma (St. Louis, MO, USA). LPS from *Porphyromonas gingivalis* was obtained from InvivoGen (San Diego, CA, USA). PGE_2, IL-8, and IL-6 ELISA kits were purchased from R&D systems (CA, USA). NF-κB p65, IκBα, NF-κB p-p65, p-IκBα, and β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). PPARγ antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated (HRP) secondary antibodies were purchased from Tianjin Sungene Biotech Co., Ltd. (Tianjin, China). The Griess reagent was

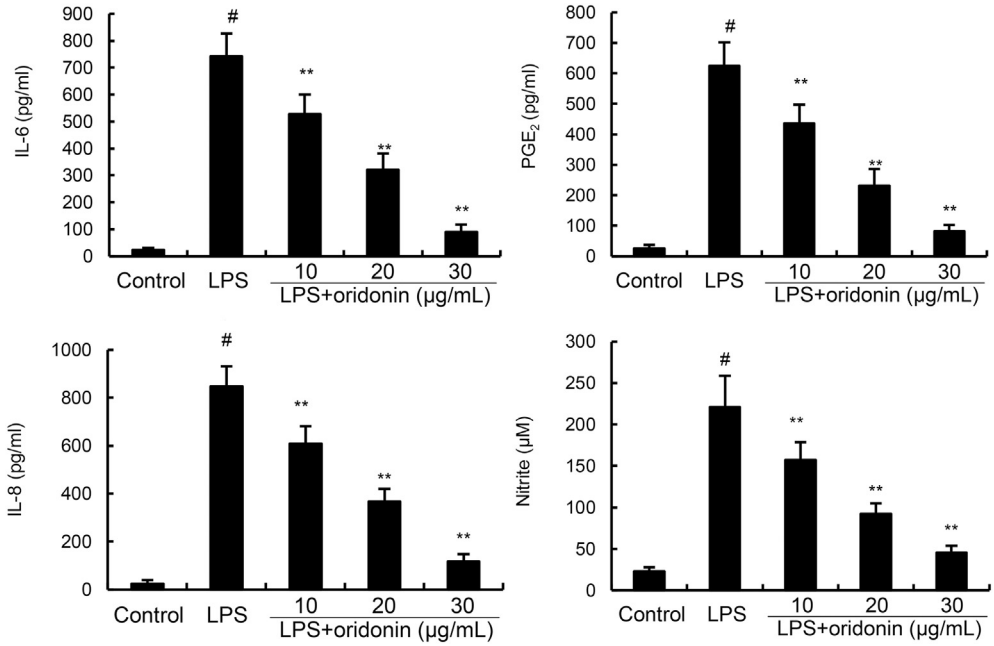

Fig. 2. Oridonin inhibits LPS-induced IL-6, IL-8, PGE_2 and NO production in human gingival fibroblasts. The data presented are the means ± SEM of three independent experiments. $^{\#}p < 0.05$ *vs.* control group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* LPS group.

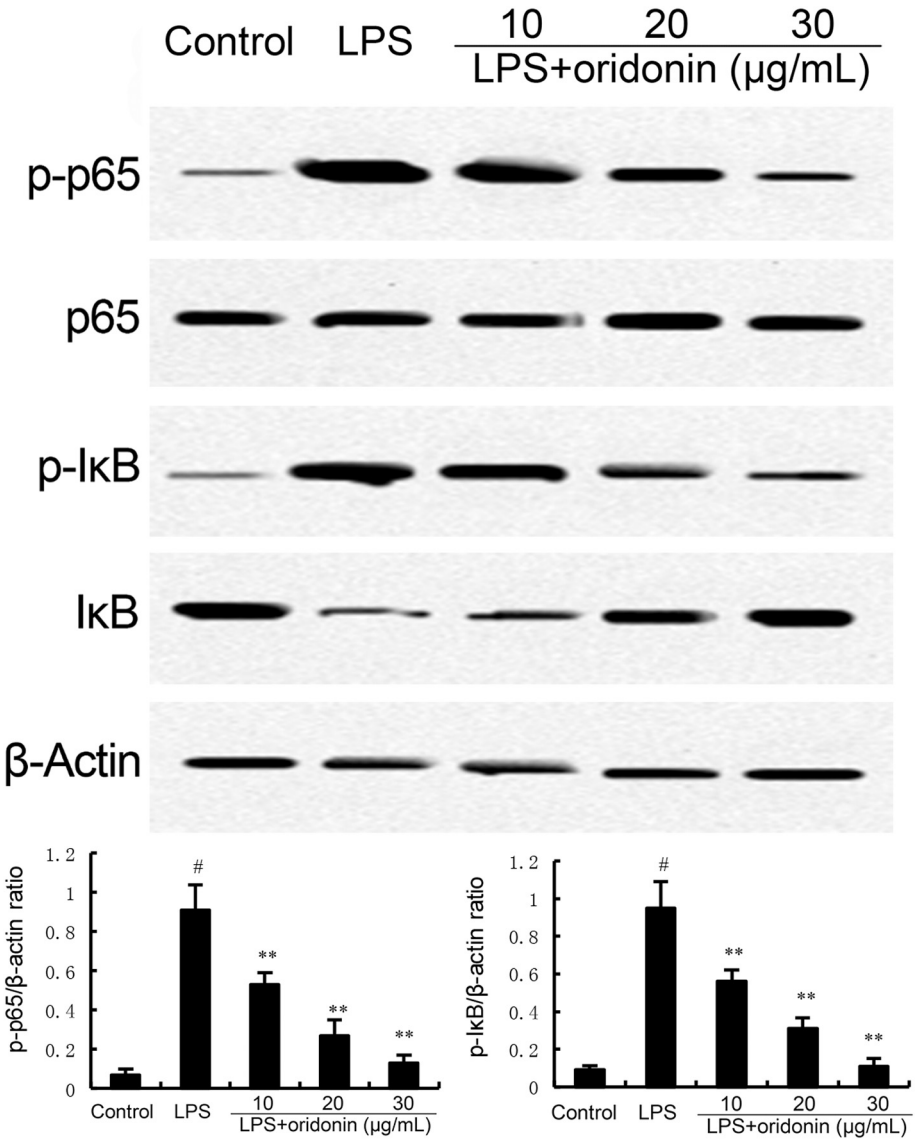

Fig. 3. Effects of oridonin on LPS-induced NF-κB activation. The values presented are the means ± SEM of three independent experiments. $^{\#}p < 0.05$ *vs.* control group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* LPS group.

purchased from Beyotime (Shanghai, China). All other chemicals were of reagent grade.

2.2. Cell culture

The samples of human normal gingival tissues were collected form 6 patients who were clinically free of periodontal disease. Gingival specimens were taken from the non-inflamed periodontal tissues of 6 patients who represented systemically healthy, non-smoking donors. Informed consent was obtained from all donors. Human gingival fibroblasts were isolated and cultured as described previously [38]. The experiment was in accordance with the Declaration of Helsinki and Tokyo and got the approval of the ethical committee of Harbin Medical University (approval number: 201742).

2.3. Cell viability assay

MTT assay was carried to detect the cytotoxicity of oridonin on human gingival fibroblasts [39]. Human gingival fibroblasts were cultured in 96-well plates for 12 h. Then, different concentrations of oridonin were added 1 h before LPS treatment. 24 h later, MTT (20 μL, 5 mg/ml) was added and incubated for 4 h. Finally, the 150 μL MTT was added to each well. Optical density was detected at 570 nm using a Bio-Rad Microplate Reader.

2.4. ELISA assay

24 h after LPS treatment, the supernatants were collected. The concentration of PGE_2 in the culture medium was measured by ELISA (R&D systems, USA) according to the manufacturer's instructions. The concentrations of IL-8 and IL-6 in the culture medium were tested by ELISA kits (Biolegend, USA) according to the manufacturer's instructions. For PPARγ inhibition assay, the cells were pretreated with oridonin (30 μg/mL) for 1 h, or 10 μM GW9662 for 30 min before oridonin incubation, and stimulated with LPS.

2.5. Nitrite determination

24 h after LPS treatment, the supernatants were collected. The level of NO in the culture medium was assessed through measuring nitrite level through Griess reagent (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, 100 μL Griess reagent was

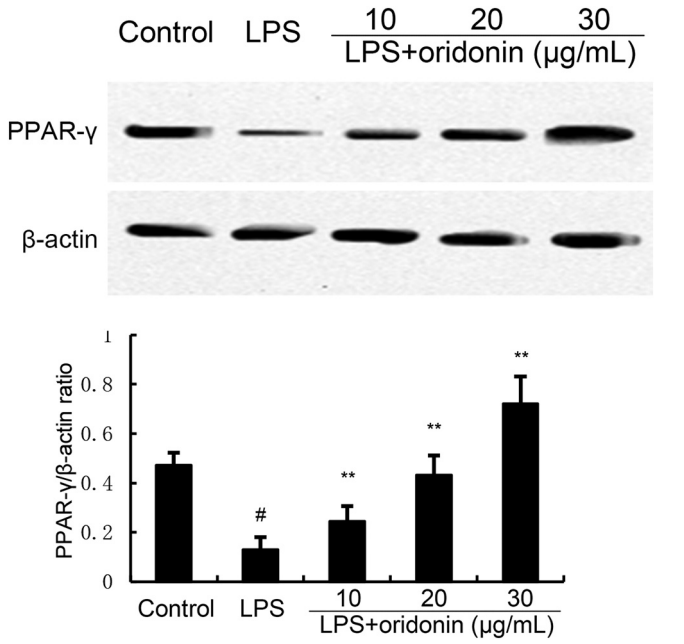

Fig. 4. Effects of oridonin on PPARγ expression. The values presented are the means ± SEM of three independent experiments. $^{\#}p < 0.05$ *vs.* control group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* LPS group.

added to 100 μL supernatant. The absorbance was detected at 520 nm.

2.6. Western blot analysis

1 h after LPS treatment, the cells were washed three times with phosphate buffer saline (PBS), collected, and lysed using M-PER Mammalian Protein Extraction Reagent (Thermo, USA) according to the manufacturer's instructions. Then, the proteins (30 μg) were loaded in each lane, separated on 12% SDS-PAGE, and electroblotted onto PVDF membranes. After blocking with 5% skimmed milk, the membranes were washed with PBST three times and each time contained 10 min. Then, the membranes were probed with primary antibodies (1:1000 dilutions in TBST): NF-κB p65, IκBα, NF-κB p-p65, p-IκBα, and β-actin at 4 °C for 24 h. The membranes were washed three times using TBST and incubated with secondary antibodies (1:2000 dilutions in TBST) at room temperature for 2 h. After wash three times with TBST, the bands were visualized using chemiluminescence reagents. The bands were quantified using Image J software (NIH, Bethesda, MD, USA).

2.7. Statistical analysis

The results of this study were expressed as means ± SEM and analyzed using SPSS 15.0 software (Chicago, USA). The differences between groups were performed using one-way analysis of variance (one-way ANOVA) followed by Tukey's multiple comparison test.

3. Results

3.1. Effects of oridonin on cell viability

To detect the cytotoxicity of oridonin, human gingival fibroblasts were treated with different concentrations of oridonin in the presence or absence of LPS. As shown in Fig. 1, oridonin at the concentration of 10, 20, and 30 μg/mL did not affect the cell viability of human gingival fibroblasts (Fig. 1). Meanwhile, treatment of LPS did not affect the viability of human gingival fibroblasts. In the presence of LPS, oridonin (10, 20, 30 μg/mL) did not affect the cell viability of human gingival fibroblasts (Fig. 1).

3.2. Oridonin inhibits LPS-induced IL-6 and IL-8 production

Cytokines IL-6 and IL-8 play critical roles in the development of periodontitis. Human gingival fibroblasts were pretreated with oridonin (10, 20, 30 μg/mL) 1 h before LPS treatment. 24 h after LPS treatment, the supernatants were collected and we evaluated the effects of oridonin on LPS-induced IL-6 and IL-8 production. As shown in Fig. 2, compared with the vehicle group, LPS caused a significant increase in IL-6 and IL-8 production. However, compared with the LPS group, LPS-induced IL-6 and IL-8 production were significantly suppressed by pretreatment of oridonin (Fig. 2).

3.3. Oridonin inhibits LPS-induced PGE_2 and NO production

Human gingival fibroblasts were pretreated with oridonin (10, 20, 30 μg/mL) 1 h before LPS treatment. 24 h after LPS treatment, the supernatants were collected and we assessed the effects of oridonin on LPS-induced PGE_2 and NO production. As shown in Fig. 2, compared with the vehicle group, LPS caused a significant increase in PGE_2 and NO production. However, LPS-induced PGE_2 and NO production were significantly suppressed by pretreatment of oridonin (Fig. 2).

3.4. Oridonin inhibits LPS-induced NF-κB activation

To explore the mechanism of oridonin on the inhibition of inflammatory mediator production, NF-κB activation was detected. Human gingival fibroblasts were pretreated with oridonin (10, 20, 30 μg/mL) 1 h before LPS treatment. 1 h after LPS treatment, the cells were collected and NF-κB activation was measured by western blot analysis. As shown in Fig. 3, compared with the vehicle group, LPS caused a significant increase in phosphorylation levels of NF-κB p65 and IκBα. However, LPS-induced NF-κB activation was significantly suppressed by pretreatment of oridonin (Fig. 3).

3.5. Effects of oridonin on PPARγ expression

To identify the upstream signaling by which oridonin mediated the inhibition of NF-κB activation, PPARγ expression was measured. Human gingival fibroblasts were pretreated with oridonin (10, 20, 30 μg/mL) 1 h before LPS treatment. 1 h after LPS treatment, the cells were collected and the expression of PPARγ was measured by western blot analysis. As shown in Fig. 4, compared with the vehicle group, LPS caused a significant decrease in PPARγ expression. However, the decreased expression of PPARγ was significantly increased by pretreatment of oridonin (Fig. 4).

3.6. PPARγ inhibitor reversed the anti-inflammatory effects of oridonin

To confirm the anti-inflammatory mechanism of oridonin, PPARγ was inhibited by GW9662. Human gingival fibroblasts were pretreated with oridonin (30 μg/mL) for 1 h, or 10 μM GW9662 for 30 min before oridonin incubation, and stimulated with LPS for 24 h. As shown in Fig. 5, the expression of PPARγ was inhibited by GW9662. And the inhibition of oridonin on inflammatory mediators IL-6, IL-8, PGE_2, and NO production were prevented by GW9662 (Fig. 5). These results suggested oridonin exhibited its anti-inflammatory effects through activating PPARγ.

4. Discussion

In the development of periodontitis, human gingival fibroblasts can be activated by bacteria and released the expression of inflammatory mediators, such as IL-6, IL-8, NO, and PGE_2. These inflammatory mediators could induce the injury and destruction of periodontal tissues [40]. The present study, we aimed to investigate the anti-inflammatory effects and mechanism of oridonin on LPS-stimulated human gingival

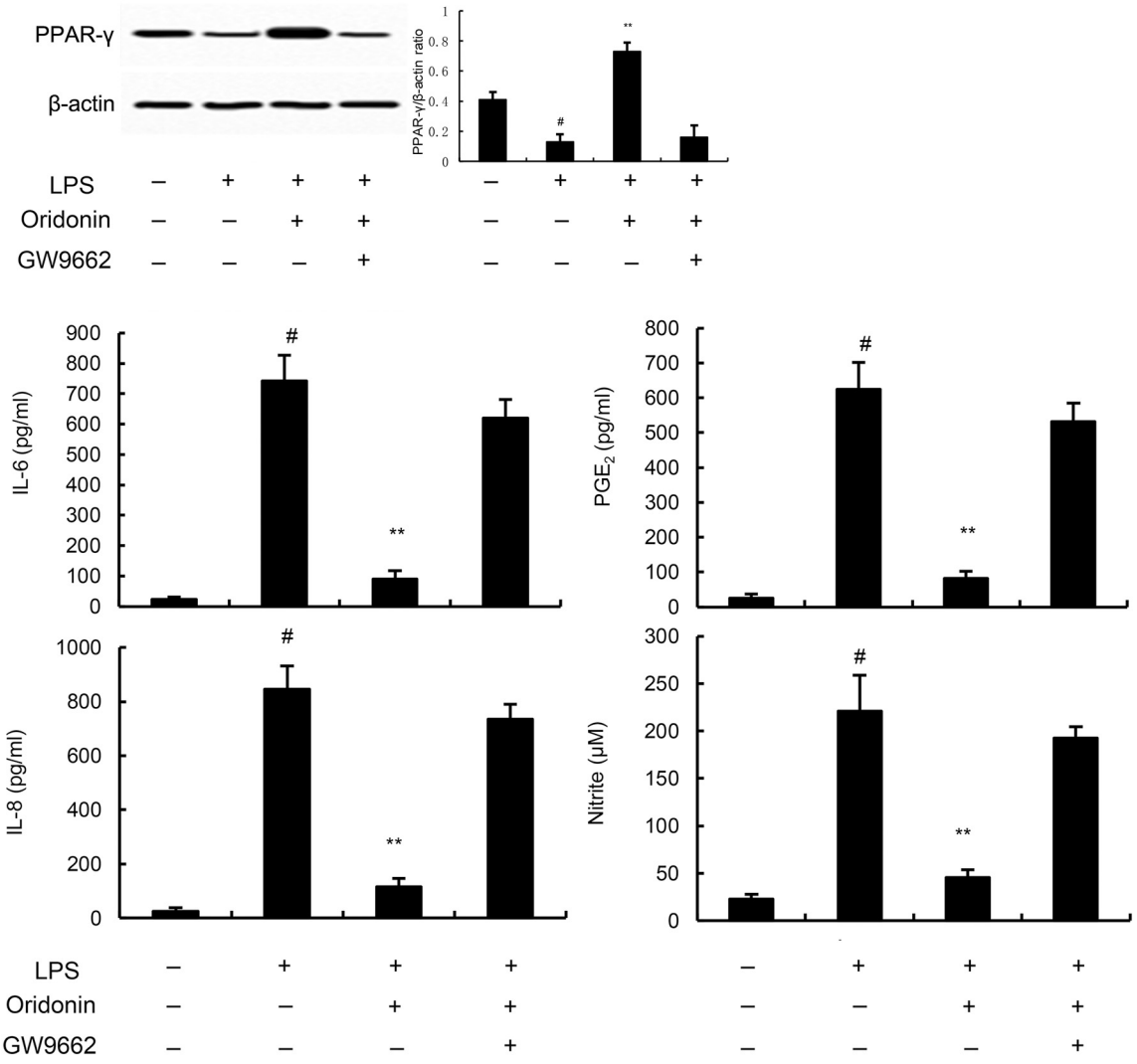

Fig. 5. Anti-inflammatory effects of oridonin were PPARγ dependent. The values presented are the means ± SEM of three independent experiments. $^{\#}p < 0.05$ *vs.* control group; $^{*}p < 0.05$, $^{**}p < 0.01$ *vs.* LPS group.

fibroblasts. In the present study, our results demonstrated that oridonin significantly attenuated LPS-induced inflammatory mediator production in human gingival fibroblasts. Oridonin may be used as an anti-inflammatory agent for the treatment of periodontitis.

Periodontitis, a multifactorial microbial disease with a destructive inflammatory course, is characterized by destruction of periodontal support tissues. Human gingival fibroblasts are the most abundant structural cell in periodontal tissue that has been known to play important roles in the pathogenesis of periodontal disease [41]. Studies showed that gingival fibroblasts were critical in sustaining inflammation in periodontal disease [41]. Therefore, we chose gingival fibroblasts in this study. Previous studies showed that many bacteria could induce the development of periodontitis [42]. Among these bacteria, *Porphyromonas gingivalis* is the major bacterium that leads to periodontitis [43]. *Porphyromonas gingivalis* was abundant in patients with periodontitis and studies demonstrated that this bacterium was associated with patient clinic-pathological characteristics [44]. LPS from *Porphyromonas gingivalis* has been known to have the ability to induce inflammation and promote tissue destruction [45]. It plays a critical role in the initiation and progression of chronic periodontitis [46]. Studies demonstrated that the elimination of pathogens and their component LPS were essential for the successful treatment of periodontitis [47]. LPS could stimulate the cells to release the production of inflammatory mediators, such as IL-6, NO, and PGE_2 [48]. The inflammatory mediators play a critical role in the pathogenesis of periodontitis [7]. Increased inflammatory cytokines could result in the destruction of periodontal supporting tissues [49]. Inflammation is a hallmark of many diseases and the continuance of this process can result in tissue injury. IL-6 is a major inflammatory cytokine of the host response to tissue injury, infection and bone resorption [50]. NO plays a critical role in the pathogenesis of periodontal disease [51]. It stimulates cyclooxygenase and metalloproteinases which results in periodontal tissue damage [51]. The production of PGE_2 could induce collagen synthesis and regulate bone density, which subsequently lead to bone resorption [7]. Studies showed that the inhibition of inflammation was a beneficial treatment for periodontitis [52]. Oridonin, the major terpene isolated from *Rabdosia rubescens*, has been reported to have anti-inflammatory activity [34]. Meanwhile, a large body of recent studies showed that natural herbal medicines and compounds isolated from these herbal medicines could attenuate the inflammatory response in LPS-stimulated human gingival fibroblasts [53]. Therefore, we assessed the effects of oridonin on inflammatory mediator production. In order to find the most safe and effective concentration of oridonin, MTT assay was used in this study. Our results showed that in the presence or absence of LPS, oridonin at the doses of 10, 20, and 30 μg/mL did not affect the cell viability of human gingival fibroblasts. Therefore, we chose the doses of 10, 20, and 30 μg/mL in this study. As shown in Fig. 2, LPS caused a significant increase in the production of

inflammatory mediators IL-6, IL-8, PGE_2, and NO. The increases were concentration-dependent inhibited by oridonin. These results indicated that oridonin could inhibit LPS-induced inflammation in human gingival fibroblasts.

Previous studies showed that LPS could trigger inflammatory response through NF-κB signaling pathway which subsequently leads to the production of inflammatory cytokines [48]. These inflammatory mediators, such as IL-6, IL-8, PGE_2, and NO were regulated by NF-κB, an important transcription factor [54,55]. Normally, NF-κB is existed as a latent form in the cytoplasm and bound to its inhibitor IκB proteins. Once stimulated by LPS, p65 is released from the IκB and translocated into the nucleus to regulate the inflammatory gene transcription [56]. NF-κB has been known as a target for many inflammatory diseases [57]. A previous study demonstrated that inhibition of NF-κB could protect mice against LPS-induced acute lung injury and kidney injury [58]. Previous studies indicated that the inhibition of NF-κB activation could protect rats against periodontitis [59]. Moreover, NF-κB is known as a target for the treatment of periodontitis [13]. To investigate the anti-inflammatory mechanism of oridonin, NF-κB activation was measured in this study. It has been demonstrated that NF-κB is activated in LPS-stimulated human gingival fibroblasts and our results were consistent with it. As shown in Fig. 3, LPS-induced NF-κB activation was significantly attenuated by the treatment of oridonin. This was consistent with a previous study showing that oridonin inhibited LPS-induced NF-κB activation in RAW264.7 cells. These results suggested that oridonin inhibited LPS-induced inflammatory response by inhibiting NF-κB activation in human gingival fibroblasts.

PPARγ is an important transcription factor belongs to the nuclear receptor family. Increasing evidence suggests that PPARγ is involved in the regulation of the inflammatory response [60]. Activation of PPARγ by its agonists could inhibit NF-κB signaling pathway and inflammatory cytokine production [61]. Recent studies demonstrated that many herbal compounds had the ability to activate PPARγ [62]. Many herbal compounds inhibited LPS-induced inflammatory response through activating PPARγ [22,63]. A previous study showed that the activation of PPARγ could reduce the development of periodontitis in rats [20]. Additionally, the activation of PPARγ could prevent inflammatory periodontal bone loss [64]. And recent studies demonstrated that asiatic acid and protocatechuic acid could inhibit LPS-induced inflammatory response in human gingival fibroblasts through activating PPARγ [65,66]. In this study, our results showed that the expression of PPARγ was decreased in LPS-treated group. And this was consistent with previous studies which showed LPS could inhibit the expression of PPARγ [67,68]. Meanwhile, treatment of oridonin significantly increased the expression of PPARγ. Treatment with GW9662 inhibited the expression of PPARγ. Furthermore, LPS-induced inflammatory mediator production can be reversed by the treatment of PPARγ specific inhibitor GW9662. These results indicated oridonin exhibited an anti-inflammatory mechanism by activating PPARγ. Oridonin has been reported to exhibit anti-tumor activity [69]. A previous study showed oridonin targeted AE (AML1-ETO) oncoprotein [70]. And silencing AE represented the increase of expression of cell cycle-related mRNAs such as: BCL2, GATA1, TCF12, Sp1, and ERK2 (MAPK1), which activates PPARs in AML cell line Kasumi-1 [71]. In the present study, our results showed oridonin could increase the expression of PPARγ. PPARγ belongs to the nuclear hormone receptor superfamily that can be activated by various xenobiotics and natural fatty acids. Also, it can be activated by many natural compounds. Whether oridonin activates PPARγ through targeting AE needs to be clarified in further study.

In conclusion, our results demonstrated that oridonin significantly inhibited LPS-induced inflammatory mediators PGE_2, NO, IL-6, and IL-8 production in human gingival fibroblasts. We demonstrated a novel mechanism involved in the anti-inflammatory activity of oridonin in LPS-stimulated human gingival fibroblasts. The mechanism is through activating PPARγ, which subsequently leads to the inhibition of NF-κB activation and inflammatory cytokine production. Oridonin may be used as an anti-inflammatory agent for the treatment of periodontitis.

Conflict of interest statement

All authors declare that they have no conflict of interest.

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