Original Article

Paeonol inhibits IL-1β induced expression of iNOS, COX-2, and MMPs through NF-κB activation: an in vitro and in vivo study

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Abstract: Introduction: Paeonol (2'-hydroxy-4'-methoxyacetophenone) is a common traditional Chinese remedy that possesses diverse biological activities, including anti-inflammatory properties. Osteoarthritis (OA) is a degenerative joint disease with an inflammatory component that drives the degradation of cartilage extracellular matrix. In the present study, we investigated the anti-inflammatory properties of paeonol in chondrocytes. Methods: We assessed the effects of paeonol on MMPs, iNOS and COX-2 mRNA expression by quantitative real-time PCR and western blot in IL-1β-induced rabbit chondrocytes and evaluated the in vivo effects of paeonol in an experimental OA model in rabbits. The NO production was determined by Griess method, the PGE₂ production was detected by ELISA. Western blot and PCR were performed to investigate the protein level of inhibitor of IκB-α and the translocation of NF-κB. Results: We found that paeonol inhibited the production of PGE₂ and NO induced by IL-1β and significantly decreased IL-1β-stimulated gene expression and production of MMPs, iNOS and COX-2 in rabbit chondrocytes. Paeonol inhibited IL-1β-mediated activation of NF-κB by suppressing degradation of IκBα in the cytoplasm. In rabbits, morphological and histological analyses determined that paeonol decreased cartilage degradation. Down-regulation of MMP-1, MMP-3, MMP-13, iNOS and COX-2 expression and up-regulation of TIMP-1 expression were also detected in paeonol-treated cartilage compared with the control group, confirming these findings in an in vivo model. Conclusions: These findings suggest that paeonol may be a potential agent in the treatment of OA.

Keywords: Paeonol, osteoarthritis, matrix metalloproteinase, nitric oxide synthase, cyclooxygenase-2, nuclear factor kappaB

Introduction

Paeonol (2'-hydroxy-4'-methoxyacetophenone), the main active compound of the traditionally Chinese herb *Paeonia lactiflora Pallas*, is a major phenolic component of Moutan Cortex from the root bark of *Paeonia suffruticosa Andrews*. Paeonol has numerous biological activities, including anti-tumor, anti-oxidant, and anti-inflammatory functions [1, 2]. Moreover, a study on rat synoviocytes found that paeonol could reduce tumor necrosis factor-α (TNF-α) synthesis and interleukin (IL)-6 production induced by IL-1β [3]. Paeonol also down-regulated cyclooxygenase-2 (COX-2) in a dose-dependent manner in HT-29 cells [4] and matrix metalloproteinase (MMP)-2 and -9 activity in HT1080 human fibrosarcoma cells [5]. Himaya et al. reported that paeonol isolated from the sea horse suppressed lipopolysaccharide (LPS)-induced release of nitric oxide (NO) and prostaglandin E₂ (PGE₂) via nuclear factor-κB (NF-κB) signaling [6]. However, the role of paeonol in inflammatory responses in articular chondrocytes remains unclear.

Osteoarthritis (OA) is a slowly progressive degenerative disease characterized by gradual loss of articular cartilage. Chondrocytes are the unique cellular component of adult hyaline cartilage extracellular matrix (ECM) and are responsible for maintaining the synthesis and degradation of cartilage [7]. OA is considered an inflammatory disease, since many soluble mediators, such as cytokines or PGEs, can increase the production of MMPs by chondro-
cytes [8]. MMPs are a large group of extracellular endopeptidase proteinases involved in the degradation of ECM [9]. It’s worth mentioning, MMP-1, -3 and -13 account for important roles in degrading the components of the articular cartilage matrix, especially the collagens and aggrecan [10]. Activation of MMPs is regulated by the tissue inhibitors of metalloproteinases (TIMPs).

Pro-inflammatory cytokines, such as IL-1β, have been verified to up-regulate MMP expression in articular chondrocytes [11]. IL-1β is produced by activated synoviocytes and articular chondrocytes, where it can activate other mediators of inflammation, such as COX-2 and inducible nitric oxide synthase (iNOS). And that COX-2 and iNOS can lead to elevated production of PGE$_2$, and NO, respectively [12-14]. NO has been proved to up-regulate the production of MMPs and inflammatory cytokines, such as PGE$_2$, in OA [15], while PGE$_2$ itself exerts a catabolic effect in OA cartilage [16, 17]. Since these are all important mediators of inflammation in arthritis, the modulation of IL-1β activity in order to regulate MMP expression and PGE$_2$ and NO production would be an excellent target for drug development in the treatment of OA.

Increasing evidence indicates that NF-κB plays a prominent role in inflammation and cartilage degradation [18, 19]. The effects of IL-1β are bounded up with the activation of NF-κB in OA [20]. On behalf of the current pharmacological treatments of OA, non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors, however, do not reverse cartilage damage and have serious adverse effects with long-term use [21]. Therefore, it is of considerable clinical interest to identify novel naturally occurring pharmacotherapies for OA.

In the present study, we investigated the effects of paeonol on the mRNA and protein expression levels of MMPs, COX-2, and PGE$_2$ in IL-1β-induced rabbit chondrocytes and evaluated the in vivo effects of paeonol on cartilage in experimental OA induced by anterior cruciate ligament transaction (ACLT) in rabbits. The objective was to test the hypothesis that paeonol antagonizes the catabolic effects of IL-1β by suppressing NF-κB-activation and NF-κB-induced gene expression in articular chondrocytes.

Materials and methods

Reagents

Recombinant human (rh) IL-1β, paeonol, and 3-(4,5-dimethylthiazolyl-2)-2,5-diphe-nyltetrazoliumbromide (MTT) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), penicillin, fetal bovine serum (FBS), streptomycin, collagenase II, and 0.25% trypsin were purchased from Invitrogen (Burlington, ON, Canada). Paeonol was dissolved in dimethylsulfoxide (DMSO) with the concentration of 1.0 g/L and stored at room temperature.

Isolation and chondrocyte culture

This study was approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). Normal cartilage (from knee joint) was obtained from 5-week-old female China white rabbits from animal center in Zhejiang University. Chondrocytes were released from cartilage slices, which were digested with 0.2% collagenase II in DMEM at 37°C for 2 h. The isolated cells were cultured in tissue culture flasks in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO$_2$ atmosphere at 37°C. Confluent chondrocytes were passaged at a ratio of 1:3, and the second to the forth passaged chondrocytes were used.

MTT assays

Chondrocytes were cultured in 96-well plate (8000/well) and incubated with various concentrations (25-200 mg/L) of paeonol for 24 h. Then MTT (5 mg/ml) was added (20 ul/well) and cells were incubated with MTT for 4 h. The supernatants were aspirated and DMSO was added (150 ul/well). Absorbance at 570 nm was measured, using a microplate reader (Bio-Rad, Hercules, CA, USA). Repeat this step for three times to get average so as to reduce the error.

Treatments of cell

After serum starved overnight, cells were seeded in 6-well plates (1×10$^5$/well). Partial cells were pre-incubated with paeonol for 1 h followed by stimulation with IL-1β (10 ng/ml) for 24 h, and harvested for the analysis of MMPs, TIMP, COX-2 and iNOS expression. Conditioned media was collected for NO and PGE$_2$ measurement, cells were collected for real-time poly-
Table 1. Gene-specific primers used in the study

<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Genbank accession</th>
<th>Primer sequence (5'→3')</th>
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<td>MMP1</td>
<td>M25663</td>
<td>CAGGAGCCTTCCAAGGAGA</td>
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<td>CAGTTTGAGAGGAGGCTCCG</td>
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Figure 1. The effect of paeonol on cell viability. After treated with various concentrations of paeonol for 24 h, chondrocytes were analyzed by MTT assay. Paeonol treated cells have the same viability compared with cells incubated with culture medium only (100% viability); #P>0.05 by an unpaired t-test.

Measurement of NO and PGE₂

The nitrite levels in the culture medium were assessed by Griess reaction as previously described [22]. The PGE₂ levels were investigated using a commercially available enzyme-linked immuno sorbent assay (ELISA) kit according to the manufacturer's guidelines (R&D Systems, Minneapolis, MN, SA). All assays were performed in duplicate.

Western blot analysis

Cells were lysed with iced PBS, and the cytoplasmic and nuclear proteins were extracted from three samples using a total protein extraction kit, then quantified with a BCA quantification kit. The proteins were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluo-
ride membranes, and the blocked for 1 h with 5\% milk in Tris-buffered saline-Tween. After incubated with antibodies against IκB-α (anti-IκB-α antibody; Santa Cruz, USA), NF-κB p65 (anti-NF-κB p65; Santa Cruz, USA), and β-actin (Santa Cruz Biotechnology) overnight at 4°C, membranes were incubated with goat anti-mouse IgG-HRP and goat anti-rabbit secondary

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**Figure 2.** Effects of paeonol on nitrite and PGE\(_2\) production and iNOS and COX-2 mRNA expression in IL-1β-induced chondrocytes. Chondrocytes (1×10\(^5\)/well in a 6-well plate) were pre-treated with various concentrations of paeonol for 1 h prior to 10 ng/ml IL-1β for 24 h. A: Conditioned media were collected for nitrite and PGE\(_2\) measurement. B: mRNA expression of iNOS and COX-2 in chondrocytes was assessed by RT-PCR. Data are expressed as means ± standard deviation (SD); *P<0.01, **P<0.05 compared with cells stimulated with IL-1β. The experiment is representative of three experiments performed.

**Figure 3.** Expression of iNOS and COX-2 in cartilage in a rabbit model of osteoarthritis. Cartilage was collected for PCR analysis. The expression of iNOS and COX-2 decreased significantly after injection of paeonol (100 mg/L) into the cartilage of anterior cruciate ligament transection (ACLT) rabbits compared with expression in DMSO-treated articular cartilage. Rabbits receiving the sham-operation were regarded as the Normal group. Relative gene expression data were normalized to GAPDH. The experiment is representative of three experiments. Data are presented as means ± standard deviation (SD). *P<0.01 compared with the DMSO-treated group.
antibody at room temperature for 1 h, and signals were detected using an SuperSignal® West Dura Extended Duration Substrate (Pierce, USA) with exposure to X-ray film (Kodak, Hangzhou, China).

Animal experiments
Sixteen New Zealand white rabbits weighing about 2.0 kg were used (Animal Center of Zhejiang University). All experiments have the approval of the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). 12 rabbits which were divided into two groups randomly, were underwent ACLT in the bilateral knee joints to induce OA [23], four rabbits were used as controls, which involved all the experimental procedures but without cutting the anterior cruciate ligament. After surgery for 1 month, the paeonol group received intra-articular injection 0.3 ml of paeonol + DMSO (100 mg/L) in the both knee per week for 6 weeks. Under the same conditions, the OA group was only injected with 0.3 ml DMSO in both knees. The rabbits were sacrificed 1 week after the last injection.

Histological examination
Six samples were obtained from each group, then fixed in 4% paraformaldehyde [24], decalciﬁed, and embedded in paraffin, sliced at 3-μm sections, and stained with safranin O-fast green. The samples were graded according to Mankin score system [25]. Histological cartilage damage degree was evaluated blinded to two independent researchers.

Statistical analysis
Data were expressed as the mean ± standard deviation (SD). MTT assay data was analyzed using analysis of variance (ANOVA). One-way anova and LSD was selected in multiple comparison. The histological and gene expression data were analyzed by a paired t-test. P<0.05 were considered statistically significant.

Results

Effect of paeonol on cell viability
The chondrocyte toxicities of 25, 50, 100 and 200 mg/L paeonol were assessed using a MTT
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assay (Figure 1), which indicated that at these doses, paeonol was not toxic to chondrocytes. In subsequent experiments, we used 100 mg/L paeonol, as reported previously [26].

Effects of paeonol on IL-1β-induced NO and PGE₂ production and iNOS and COX-2 gene expression in chondrocytes and OA cartilage

Paeonol significantly inhibited IL-1β-induced NO and PGE₂ production in chondrocytes (Figure 2A), and as expected, treatment with paeonol suppressed IL-1β-induced expression of iNOS and COX-2 in chondrocytes (Figure 2B). Similar effects of paeonol on the expression of iNOS and COX-2 were observed in vivo (Figure 3).

Effects of paeonol on expression of MMP-1, MMP-3, MMP-13, and TIMP-1 in IL-1β-stimulated chondrocytes and OA cartilage

Significant increases in MMP-1, MMP-3, and MMP-13 gene expression and a decrease in TIMP-1 gene expression were observed following a 24-h treatment with IL-1β in chondrocytes. Treatment with paeonol (25, 50, 100, 200 mg/L) suppressed the expression of MMP-1, MMP-3, and MMP-13 and increased TIMP-1 gene expression in a dose-dependent manner (Figure 4). We identified similar changes in the expression of the MMPs and TIMP-1 in vivo (Figure 5).

Effects of paeonol on IκBα degradation and NF-κB translocation in chondrocytes and OA cartilage

Since NF-κB is important for the up-regulation of COX-2, iNOS and MMPs during inflammation, we investigated the activation of NF-κB using Western blot and RT-PCR. Degradation of IκBα, which is indicative of NF-κB activation, was noted in chondrocytes in response to IL-1β, and this was blocked by paeonol (Figure 6A). These results were consistent with RT-PCR results in vivo (Figure 6B).
Histological observations and gross morphological evaluation of OA cartilage

Varying degrees of cartilage damage were observed in the control and OA groups (Figure 7A). Histological analysis determined that dimethylsulfoxide (DMSO)-treated femoral condyles appeared to have more serious cartilage damage, showing obvious erosion and osteophyte formation, compared with the paeonol-treated group. Typical differences in safranin O-fast green staining of the cartilage are shown in Figure 7B, and Table 2 lists the Mankin score.

Discussion

Analgesics and NSAIDs are the only therapeutic treatment options for OA, although side effects...
and serious health risks limit their long-term use [27]. Thus, there is an urgent need to investigate safer and more-tolerated agents for the treatment of OA. In recent years, plant extracts have received increased attention because they are naturally occurring compounds. For inflammatory diseases, paeonol has been used as a traditional Chinese anti-inflammatory, although scientific reports on its role in OA remain limited.

The results presented here have led to several important findings in rabbit chondrocytes induced by IL-1β in vitro and in an experimental rabbit model of OA: (1) paeonol inhibited IL-1β-mediated mRNA expression of MMP-1, MMP-3 and MMP-13 in rabbit chondrocytes; (2) paeonol antagonized IL-1β-induced inhibition of TIMP-1 expression in rabbit chondrocytes; (3) paeonol inhibited the production of NO and PGE

<table>
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<th>Table 2. Histological score of articular cartilage</th>
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<td>Femoral condyle</td>
</tr>
<tr>
<td>Structural changes</td>
</tr>
<tr>
<td>Cellular changes</td>
</tr>
<tr>
<td>Safranin staining</td>
</tr>
<tr>
<td>Total score</td>
</tr>
</tbody>
</table>

Values represent the means ± SD; #P<0.05 normal group compared with the OA group, *P<0.05 normal group compared with the paeonol group.

OA is characterized by the degradation of collagenous and non-collagenous ECM components in articular cartilage. The increased expression and activity of MMPs is partly responsible for cartilage degradation [33]. MMP-1 and MMP-13, in particular, are interstitial collagens that degrade type II collagen in cartilage. MMP-3, also known as stromelysin-1, is known to activate both MMP-1 and MMP-13 by cleavage of their pro-peptide domains [34], which leads to increased collagen degradation. Thus, agents capable of suppressing MMP expression may be useful in arthritis therapy. Under physiological conditions, an imbalance exists between MMPs and TIMP, while in arthritis, this dynamic balance is often dysregulated. Here, we demonstrate that paeonol inhibits the IL-1β-mediated induction of TIMP-1 both in vivo and in vitro, which suggests a protective role of paeonol in arthritis.

IL-1β regulates activation of the transcription factor NF-kB, which further stimulates its expression, as well as the expression of iNOS and COX-2 [35]. COX-2 and iNOS, which subsequently produce PGE

NO is an inflammatory mediator synthesized by a family of NO synthases (NOSs), which include three isoforms: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). iNOS is expressed following stimulation by inflammatory mediators such as cytokines [37], and it promotes inflammation by enhancing the production of inflammatory cytokines and PGE

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model of OA, which confirms the anti-inflammatory effects of paeonol. Similar results were demonstrated in a LPS-induced acute lung injury model [2] and in LPS-treated RAW 264.7 cells [26].

PGE2, another inflammatory factor, is the most abundant prostanoid in the arthritic joint and one of the major catabolic mediators involved in cartilage resorption. The COX enzymes, COX-1 and COX-2, are PGE2 synthetases, and COX-2 is a target in the treatment of OA that can be induced by pro-inflammatory cytokines [41]. In our study, we found that induction of PGE2 production and COX-2 expression by IL-1β was blocked by paeonol. These findings are consistent with previous studies showing that paeonol prevented the production of PGE2 and COX-2 expression [26]. Moreover, our results show that paeonol reduced the production of NO and PGE2 in IL-1β stimulated chondrocytes via inhibition of the expression of iNOS and COX-2 in an OA rabbit model.

NF-κB activation is involved in many important physiological processes, such as inflammation. In the inactive state, NF-κB is present in the cytoplasm as a heterotrimeric complex consisting of two subunits and an additional inhibitory subunit, IkBα. The two subunits consist of five Rel-protein family members: p65, RelB, c-Rel, p52 and p50, which form homo- and heterodimers [42], and the most prevalent combination is p65/p50 [43]. NF-κB is activated by phosphorylation of IkBα, followed by dissociation of p65 from IkBα and translocation from the cytoplasm to the nucleus, where it triggers the transcription of specific target genes [44], such as MMP, iNOS and COX-2 [13, 45, 46]. IL-1β activates NF-κB via stimulation of IkBα phosphorylation. In the current study, paeonol inhibited IL-1β-induced phosphorylation and degradation of the IkBα protein, and subsequently reduced nuclear p65 in a concentration-dependent manner. Therefore, the anti-arthritic activity of paeonol may be associated with inhibition of NF-κB activation, and further studies are required to elucidate the molecular mechanism.

In conclusion, we have shown that paeonol inhibits suppression of IL-1β-induced NO and PGE2 production, iNOS and COX-2 mRNA expression, as well as MMP-1, 3, and 13 mRNA expression and TIMP-1 degradation. The mechanism by which paeonol attenuates IL-1β effects is independent of the DNA-binding activity of the transcription factor NF-κB. Thus, these data suggest that paeonol represents a promising therapy in the treatment of OA.

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Disclosure of conflict of interest

None.

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