Paeonol prevents IL-1β-induced inflammatory response and degradation of type II collagen in human primary chondrocytes

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ABSTRACT
Osteoarthritis (OA) is a common joint disease for which a safe and reliable treatment has yet to be developed. Here, we demonstrated the potential benefit of treatment with paeonol, a derivative of Paeonia suffruticosa, in the treatment and prevention of OA. Chondrogenic cell line ATDC5 cells were cultured with IL-1β and the effects of paeonol were assessed through qRT-PCR, western blot analysis, MTT, ELISA, and NF-κB luciferase reporter gene assay. Our findings demonstrate a novel ability of paenol to inhibit numerous factors of OA, including expressions of IL-6, TNF-α, NOX2, PTGS2, NUCB2/nesfatin-1, ICAM-1, VCAM-1, MMP-3/13, degradation of type II collagen, and NF-κB activation through the rescue of iKBα. Additionally, we assessed the effects of paeonol on cell viability to confirm its safety. These findings implicate a valuable potential role of paeonol in the treatment and prevention of OA.

Introduction
Although osteoarthritis (OA) is a common debilitating disease worldwide, the pathophysiology of OA remains poorly understood. Overexpression of cytokines such as IL-1β plays a key role in the pathogenesis of OA by upregulating expression of degradative enzymes and proinflammatory cytokines, among other things [1]. One of the major hallmarks of OA is excessive loss of extracellular matrix (ECM) in the articular tissues, including type II collagen, which provides cartilage tissue with structural rigidity, thereby allowing joints to withstand heavy mechanical loading. It is degraded by matrix metalloproteinases (MMPs), which are upregulated in OA chondrocytes [2,3]. MMP-3 and MMP-13 are shown to induce degradation of type II collagen [4]. Additionally, MMP-3 induces expression of various pro-MMPs, including pro-MMP-13 [5]. MMP-13 is reported to specifically cleave type II collagen, making it a valuable target for therapies against cartilage degradation [6].

Another major factor driving the development and progression of OA is oxidative stress. The NADPH oxidase isoform NOX2 has been shown to induce the generation of ROS upon incubation with IL-1β. While the role of NOX2 in OA has not yet been fully elucidated, a recent study using human chondrogenic cell line ATDC5 cells showed that NOX2 may be involved in the loss of articular ECM by the activation of hyaluronidase through intra- and extra-cellular acidification [7]. Prostaglandin-endoperoxide synthase 2 (PTGS2) is an isoenzyme produced during inflammation that has been shown to regulate prostaglandin biosynthesis and pain response in OA [8]. Inhibition of PTGS2 has been considered as a potential method for managing pain and inflammation in OA [9]. The adipokine nucleobindin 2 (NUCB2/nesfatin-1) is a calcium-binding protein that regulates calcium homoeostasis and inflammation by inducing IL-6 and IL-8 when administered in combination with IL-1 in ATDC5 chondrocytes [10]. As a recent discovery in chondrocytes, there exists very little information on the function of NUCB2/nesfatin-1 in OA.

Numerous investigations have revealed the participation of proinflammatory cytokines in the pathological development of OA by driving expression of MMPs and degradative enzymes [11–14]. Two vascular adhesion molecules, ICAM1 and VCAM1, are important for promoting the inflammation in OA synovial tissue by regulating the recruitment of leukocytes through the vascular wall and into inflamed synovial tissue, where they interact with other cell types [15]. Increased expression of ICAM1 and VCAM1 have been shown to be correlated with the development of knee OA [16,17]. Additionally, ICAM1 and VCAM1 have been associated with the senescence-associated secretory phenotype (SASP), which causes chondrocytes to lose their normal functionality and instead promote inflammation and expression of cytokines, chemokines, and MMPs, thus perpetuating the inflammatory response and driving degradation of ECM [18,19]. One of the most important factors in OA is the NF-κB signalling...
pathway. Activation of NF-κB governs the expression of numerous genes in joint destruction, inflammation and homeostasis. NF-κB activation is triggered by cytokines and chemokines, activation of other signalling pathways, and byproducts of cartilage degradation. [20,21]. Phosphorylation of IκBα induced by IL-1β triggers translocation of the dimers of NF-κB, including p65 protein, to the nucleus where activation of NF-κB transcription occurs [22,23].

Paeonol is the active compound found in *Paeonia lactiflora* Pallas, *Cynanchum paniculatum*, and *Paeonia suffruticos* Andr and was recently shown to have anti-inflammatory effects in a rheumatoid arthritis model using human fibroblast-like synoviocytes stimulated with IL-1β [24]. Here, we examined the roles of treatment with paeonol on IL-1β-induced expression of cytokines, chemokines, adhesion molecules, ECM degradation and activation of IκBα/NF-κB in chondrogenic cell line ATDC5 cells. Our findings demonstrate that paeonol has a significant protective role against these factors of OA, thereby suggesting the potential value of paeonol in OA treatment.

**Materials and methods**

**Cell cultures and treatments**

ATDC5 cells were cultured in DMEM with 10% FBS and 1% P/S and allowed to differentiate into mature chondrocytes [25]. Paeonol was obtained from the National Institute for Food and Drug Control (Beijing, China). Cells were stimulated with IL-1β (10 ng/mL) with or without paeonol (50, 100 μM) for 24 h. Paeonol was purchased from the National Institute for Food and Drug Control (Beijing, China) and was recently shown to have anti-inflammatory effects in a rheumatoid arthritis model using human fibroblast-like synoviocytes stimulated with IL-1β [24]. Here, we examined the roles of treatment with paeonol on IL-1β-induced expression of cytokines, chemokines, adhesion molecules, ECM degradation and activation of IκBα/NF-κB in chondrogenic cell line ATDC5 cells. Our findings demonstrate that paeonol has a significant protective role against these factors of OA, thereby suggesting the potential value of paeonol in OA treatment.

**Quantitative real time PCR**

RNA from ATDC5 cells was extracted using Qiazol (Qiagen, USA). The isolated RNA (2 μg) was reverse-transcribed into cDNA. Expression of target genes was determined using real-time PCR with an SYBR Green PCR Master Mix. The 2^{-ΔΔCt} approach was applied to calculate the level of detected genes, using GAPDH as an internal control [26].

**Western blot analysis**

Proteins in ATDC5 cells were isolated from using RIPA buffer with protease inhibitors. Nuclear protein from ATDC5 cells was extracted using a Nuclear/Cytosol Fractionation Kit (Biovision Incorporated, USA). Total protein or nuclear protein was extracted using a Nuclear/Cytosol Fractionation Kit (Biovision Incorporated, USA). Total protein or nuclear protein (20 μg) was then separated on 8–12% Bis-Tris Precast Gels (Bio-Rad, USA) by electrophoresis. Proteins were then transferred onto nitrocellulose membranes. After blocking with 5% milk, membranes were probed with the following primary antibodies: type II collagen (1:2000, CAT# 14695–1-AP, Proteintech, Chicago, USA), p-IκBα (1:1000, Cat# sc-8404, Santa Cruz Biotechnology, Santa Cruz, USA), IκBα (1:1000, Cat#9242, Cell Signaling Technology, Danvers, USA), lamin B1 antibody (1:3000, CAT#sc-377000, Santa Cruz Biotechnology, Santa Cruz, USA), β-actin antibody (1:5000, CAT# sc-10731, Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4°C. and appropriate secondary antibodies. Bands were then detected using enhanced chemiluminescence [27].

**Measurement of cell viability**

Viability of ATDC5 cells was measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Briefly, MTT (5 mg/mL) was put into each well of cell culture. After incubation at 37°C for 4 h, the product formazan was dissolved in DMSO and OD values recorded at 570 nm were used to index the viability percentage [28].

**ELISA assay**

ATDC5 cells were cultured with IL-1β (10 ng/mL) with or without paeonol (50, 100 μM) for 24 h. Secretions of TNF-α, IL-6 and intracellular levels of ICAM-1, VCAM-1, MMP-3 and MMP-13 were examined using ELISA kits purchased from R&D Systems (Minneapolis, USA).

**NF-κB luciferase reporter gene assay**

The activity of NF-κB was examined using an NF-κB luciferase reporter gene assay. Briefly, NF-κB promoter-luciferase and β-galactosidase plasmids (Clontech, USA) were transfected into ATDC5 cells [29]. 24 h later, stimulated with IL-1β (10 ng/mL) with or without paeonol (50, 100 μM) for 24 h. Cells were then lysed and centrifuged at 15,000 × g. Supernatants were then harvested and used to determine luciferase and β-galactosidase activity (Thermo Fisher Scientific, USA) on a luminometer. Luciferase activity of NF-κB in ATDC5 cells was normalized to β-galactosidase activity.

**Statistical analysis**

The results are demonstrated as means ± SEM. Comparisons between different groups were made using analysis of variance (ANOVA) method. P values < .05 was considered of statistical significance.

**Results**

**Determination of cell viability**

The molecular structure of paeonol is shown in Figure 1. To measure the effects of paeonol on cell viability, human chondrocytic cell line ATDC5 cells were stimulated with 0, 0.1, 1, 10, 50, 100 and 500 μM paeonol for 48 h. As shown in Figure 2, the dose of 100 μM has a slight effect on cell viability, but only the dose of 500 μM paeonol elicited a significant decline in cell viability, thereby indicating that lower concentrations of paeonol are safe. Therefore, we chose the highest concentrations considered to be safe, 50 and 100 μM, for the following experiments.

**Paeonol reduces expression of NOX2, PTGS2 and NUCB/nesfatin-1**

Next, we examined the effects of paeonol on factors of oxidative stress in ATDC5 chondrocytes. As shown in Figure 2(A), NOX2 is barely expressed by ATDC5 cells at baseline, but IL-1β increased expression of NOX2 by roughly 11-fold. However,
IL-1β-induced upregulation of NOX2 was prevented by paeonol (50, 100 μM), with 100 μM paeonol reducing NOX2 expression to approximately 3-fold. Similarly, treatment with IL-1β increased expression of PTGS2 by roughly 10-fold, which was reduced by paeonol. Notably, the dose of 100 μM paeonol returned PTGS2 expression to almost baseline levels.

**Paeonol inhibits expression of proinflammatory cytokines**

Proinflammatory cytokines play a vital role in the pathogenesis of OA. Thus, we investigated the effects of paeonol on IL-1β-induced IL-6 and TNF-α in ATDC5 chondrocytes. Briefly, cells were exposed to insult from IL-1β for 48 h with or without 50 and 100 μM paeonol. As shown in Figure 3(A), exposure to IL-1β increased TNF-α and IL-6 at the gene level by about 4.5- and 5.7-fold, respectively. However, paeonol reduced expression of these cytokines, with 100 μM paeonol decreasing TNF-α and IL-6 to only 1.6 and 2.1-fold, respectively. We further confirmed these effects of paeonol using ELISA analysis. As shown in Figure 3(B), treatment with paeonol significantly reduced IL-1β-induced TNF-α and IL-6, reducing the expression of both cytokines to roughly 2-fold basal levels. These findings implicate a strong anti-inflammatory property in chondrocytes treated with paeonol, suggesting that paeonol may be of use in the treatment of OA.

**Paeonol attenuates expression of ICAM-1 and VCAM-1**

Expressions of the adhesion molecules ICAM1 and VCAM1 have been shown to be elevated in OA and high levels of these molecules are associated with greater risk of knee or hip replacement [17,30]. To assess the effect of treatment with paeonol on IL-1β-induced expression of cellular adhesion molecules, ATDC5 chondrocytes were exposed to insult from IL-1β with or without 50 and 100 μM paeonol for 24 h. As shown in Figure 4, IL-1β induced an about 3-fold increase in expression of both ICAM1 and VCAM1, which was attenuated by paeonol. The dose of 100 μM significantly lowered the mRNA levels of the two cytokines to less than 2-fold baseline. Concordantly, western blot analysis revealed that IL-1β led to a more than 3-fold increase in protein expression, which was lowered to roughly 1.5-fold baseline by the higher dose of paeonol.

**Paeonol reduces expression of MMP-3/13 and reduction of type II collagen**

Expression of MMPs degrades type II collagen. To investigate the effect of paeonol on the expression of MMPs, ATDC5 cells were cultured with IL-1β (10 ng/mL) with or without paeonol (50, 100 μM) for 24 h. The results show that IL-1β gave rise to approximately 10- and 12-fold increases in MMP-3 and MMP-13, respectively, at the gene level, which were reduced to 3- and 4-fold basal levels by paeonol. Expression of both ICAM1 and VCAM1, which was attenuated by paeonol. The dose of 100 μM significantly lowered the mRNA levels of the two cytokines to less than 2-fold baseline. Concordantly, western blot analysis revealed that IL-1β led to a more than 3-fold increase in protein expression, which was lowered to roughly 1.5-fold baseline by the higher dose of paeonol.
Importantly, an insult from IL-1β obviously induced reduction of type II collagen, which was rescued to near basal levels by paeonol (Figure 6). These findings suggest a new biological function of paeonol in preventing loss of type II collagen by suppressing MMP-3 and MMP-13.

**Inhibits phosphorylation of IκBα and activation of NF-κB**

Activation of the NF-κB pathway is strongly correlated with the inflammatory response in OA. To elucidate the effects of paeonol on this pathway, we assessed the phosphorylation of IκBα and activity of NF-κB. As demonstrated in Figure 7, an insult from IL-1β (10 ng/mL) increased phosphorylation of IκBα by more than 3.5-fold, which was decreased by paeonol to about 1.75-fold basal levels. Finally, we assessed the influence of paeonol on nuclear levels of p65 and subsequent luciferase activity of NF-κB. As shown in Figure 8, there was a remarkable increase in nuclear p65 and luciferase activity of NF-κB by IL-1β, which was reduced by paeonol.

**Discussion**

OA is a disease characterized by excessive degradation of cartilage, chronic inflammation, infiltration of synovial tissue by immune cells, and eventual irreversible joint destruction. In the pathophysiology of OA, proinflammatory cytokines and cellular signalling pathways contribute greatly to disease progression and to sustaining a mild chronic inflammatory state [31]. Therefore, inhibition of the release of proinflammatory cytokines and blockage of cellular signalling pathways is regarded as an attractive option for the management and treatment of OA. Paeonol is a commonly used compound in traditional Chinese medicine (TCM) and has been demonstrated to have antimicrobial, anticancer, antioxidant, analgesic, antipyretic and anti-inflammatory activities [24,32,33]. However, little is known regarding the potential of this natural therapeutic agent until recently. Here, we used human chondrogenic cell line ATDC5 cells cultured with IL-1β to explore the potential effects of paeonol treatment in OA. We confirmed the safety of treatment with paeonol by performing a cell viability assay, which showed that doses under
500 μM had little to no effect on the cell viability of ATDC5 cells (Figure 1). Additionally, our findings demonstrate that paeonol possesses anti-oxidative stress and anti-inflammatory effects by inhibiting expression of NOX2, PTGS2 and NUCB2/nesfatin-1 in ATDC5 chondrocytes, which have been suggested as a potential treatment targets for OA [34–36]. This beneficial effect suggests a novel role of paeonol against the expression of chemokines and adipokines by OA chondrocytes.

Expression of cytokines, especially IL-1β, IL-6 and TNF-α, are often considered to be the main factors driving the pathogenesis of OA. Hence, blockade or inhibition of these is considered a valuable approach for the management of OA. In the present study, we used insult from IL-1β to simulate the condition of OA in ATDC5 chondrocytes as previously described [37]. Our findings demonstrate a remarkable ability of paeonol to inhibit TNF-α and IL-6 induced by IL-1β. Another factor driving the pathology of OA is the recruitment of monocytes to the synovium by ICAM-1 and VCAM-1, resulting in chronic low-grade inflammation [38]. Our findings show that paeonol significantly reduced ICAM-1 and VCAM-1 in ATDC5 cells. Thus, treatment with paeonol may be a safe therapeutic option for reducing the expression of these molecules in a myriad of diseases. To our knowledge, this study is the first to demonstrate the beneficial role of paeonol in inhibiting ICAM-1 and VCAM-1 in an OA chondrogenic cell model.

Perhaps the most prominent feature of OA, inhibition of impairment of the articular cartilage matrix is a primary treatment target. Here, we demonstrated the ability of paeonol to suppress MMP-3 and MMP-13 and further confirmed the ability of paeonol to prevent reduction of type II collagen, a primary component of articular cartilage, in chondrogenic ATDC5 cells. These findings agree with the results of a recent

Figure 5. Paeonol suppresses the expression of matrix metalloproteinases in chondrogenic cell line ATDC5 cells. ATDC5 cells were incubated with 10 ng/mL IL-1β with or without paeonol (50, 100 μM) for 24 h. (A) mRNA levels of MMP-3 and MMP-13; (B) Elisa results of MMP-3 and MMP-13 (*, #, $, P < .01 vs. previous control group).

Figure 6. Paeonol ameliorates reduction of type II collagen in chondrogenic cell line ATDC5 cells. ATDC5 cells were incubated with 10 ng/mL IL-1β with or without paeonol (50, 100 μM) for 24 h. Protein level of type II collagen was measured (*, #, $, P < .01 vs. previous control group).

Figure 7. Paeonol inhibits the phosphorylation and degradation of IkBα. ATDC5 cells were incubated with 10 ng/mL IL-1β with or without paeonol (50, 100 μM) for 24 h. Phosphorylated and total IkBα was measured (*, #, $, P < .01 vs. previous control group).
study demonstrating the ability of paeonol to inhibit expression of MMPs and loss of type II collagen in rabbit chondrocytes. Also in accordance with the findings of the same study demonstrating the ability of paeonol to inhibit expression of MMPs and loss of type II collagen in rabbit chondrocytes. Thus, blockade of NF-κB signalling is often cited as a valuable therapeutic target. Importantly, we found that treatment with paeonol could inhibit activation of NF-κB by significantly inhibiting IkBα and nuclear translocation of p65 protein induced by IL-1β.

In summary, the results of this study are among the first to demonstrate the potential of paeonol as an effective therapeutic agent against the pathogenesis of OA by virtue of its ability to downregulate expression of proinflammatory cytokines, chemokines, cellular adhesion molecules, degradative enzymes and to inhibit the IkBα/NF-κB signalling pathway.

Disclosure statement
No potential conflict of interest was reported by the authors.

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