Original Paper

The Protective Effect of Ligustilide in Osteoarthritis: An in Vitro and in Vivo Study

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Key Words

Ligustilide • Osteoarthritis • Chondrocytes • PI3K/NF-κB • IL-1β • Inflammation

Abstract

Background/Aims: Osteoarthritis is a degenerative joint disease characterized by cartilage degeneration and a chondrocyte inflammatory response that induces an inflammatory environment closely linked to extracellular matrix (ECM) degradation. Ligustilide (LIG) is a major component of the herb *Radix Angelicae Sinensis*, with demonstrated anti-inflammatory effects. To confirm whether LIG has an equally inhibitory effect on inflammation in human osteoarthritis chondrocytes, we performed *in vivo* and *in vitro* experiments to validate the above conjectures and determine the relevant mechanisms. **Methods:** Quantitative real-time PCR and western blotting were performed to evaluate the expression of MMP-3, MMP-13, ADAMTS-5, iNOS, and COX-2 at both gene and protein levels. An enzyme-linked immunosorbent assay was used to evaluate the levels of other inflammatory factors (PGE2, TNF-α, and IL-6). The PI3K/AKT and nuclear factor kappa B (NF-κB) signaling pathways were also analyzed by western blotting, whereas immunofluorescence was used to assess the expression of collagen II and aggrecan. The *in vitro* effect of LIG was evaluated by intraperitoneal injection into a mouse osteoarthritis model induced by destabilization of the medial meniscus. **Results:** LIG lowered the phosphorylation levels of p65, IκBα, and IKKα/β and suppressed the IL-1β-induced expression of MMP-3, ADAMTS-5, iNOS, and COX-2 and the inflammatory factors PGE2, TNF-α, and IL-6. LIG markedly decreased IL-1β-induced degradation of collagen II and aggrecan. *In vivo* results showed that LIG-treated mouse cartilage showed less damage than the control group; the Osteoarthritis Research Society International (OARSI) score was also lower. LIG further reduced the thickness of the subchondral bone plate and alleviated the synovitis.

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Conclusion: LIG may act as a promising therapeutic agent for osteoarthritis by attenuating IL-1 β -induced inflammation in chondrocytes and ECM degradation via suppression of NF- κ B activation by the PI3K/AKT pathway.

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Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by cartilage degeneration. Cartilage is an avascular, aneural, and alymphatic matrix synthesized by chondrocytes and the extracellular matrix (ECM), which mainly consists of type II collagen and aggrecan [1]. Cartilage degeneration includes ECM degeneration and cartilage tissue destruction. Type II collagen is cleaved by matrix metalloproteinases (MMPs), whereas aggrecan is cleaved by “a disintegrin and metalloproteinase with thrombospondin motifs” (ADAMTS) enzymes, most probably ADAMTS5 [2]. Many MMPs are increased in OA. Furthermore, MMP-13 not only degrades aggrecan, but also preferentially digests type II collagen, resulting in the destabilization of the collagen network. MMP-3 can also digest aggrecan [3-5].

There is mounting evidence that the cartilage destruction in OA is the result of cartilage inflammation at the molecular level [6, 7]. Pro-inflammatory cytokines such as interleukin-1 beta (IL-1 β) and tumor necrosis factor alpha (TNF- α) produced by activated synovocytes, mononuclear cells, or articular cartilage itself are strongly linked to the pathophysiology of OA [8, 9]. These factors serve to increase the catabolic activity of the chondrocyte, which results in the release of proteolytic enzymes, including ADAMTS and MMPs, which cause destruction of the ECM [10]. Among these cytokines, IL-1 β exerts its inflammatory effects by significantly increasing the secretion of inflammatory cytokines such as interleukin-6 (IL6), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2), and prostaglandin e2 (PGE2). PGE2 contributes to articular inflammation and destruction by enhancing the activation and production of MMPs and inhibiting the synthesis of anabolic macromolecules such as collagen and proteoglycan [8].

Studies aimed at unraveling the underlying molecular alterations induced by pro-inflammatory cytokines under chronic inflammatory conditions revealed that IL-1 β activates a ubiquitous central transcription factor known as NF- κ B, which is a key regulator of gene expression [11, 12]. In the inactive state, NF- κ B is present in the cytoplasm as a heterotrimer complex consisting of two subunits and an additional inhibitory subunit, I κ B α . Of the five different subunits, p65/p50 is one of the most prevalent combinations [13]. Activation of the NF- κ B pathway results in phosphorylation of p65 and I κ B α in the cytoplasm and ultimately the translocation of p65 from the cytoplasm to the nucleus [14]. The PI3K/AKT pathway, one of the most widely studied upstream signaling pathways of NF- κ B, is involved in both cellular and ECM alterations [15]. Currently, there are no effective target drugs for the treatment of OA, and inhibition of these inflammatory mediators would be reasonable therapeutic targets for OA.

Ligustilide (LIG) is the main bioactive component of Danggui, which is the root of *Angelica sinensis* and one of the most popular traditional Chinese medicines [16]. Recent studies showed that LIG has an anti-inflammatory effect because it reduces the production of LPS-induced pro-inflammatory mediators (e.g., pro-inflammatory cytokines and inflammatory enzymes) in microglia and macrophages and of LPS-induced chemokines in astrocytes [17-19]. LIG can inhibit the LPS-induced increase in inflammatory cytokines (TNF- α , IL-1, and IL-6) [20]. Additionally, LIG may help to protect PC12 cells against oxidative stress by activating hormetic pathways such as PI3K/AKT [21]. Moreover, the inhibitory effect of LIG on Prx-induced inflammation is probably associated with downregulation of TLR4/NF- κ B signaling activation in macrophages [22]. However, the anti-inflammatory effects of LIG in OA have not been reported. Therefore, we investigated the anti-inflammatory effects of LIG and the underlying mechanism in IL-1 β -stimulated human chondrocytes *in vitro* and the protective role of LIG in mouse OA models *in vivo*.

Materials and Methods

Chemicals and Reagents

The ligustilide (purity > 98.5%), recombinant human IL-1 β , collagenase type II, dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The ligustilide was dissolved in DMSO as a 50 mM stock solution and stored at 4 °C. Further dilution was done in cell culture medium. Cell-Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamoto, Japan). Primary antibodies against β -actin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary antibodies against COX-2, iNOS, MMP-3, MMP-13, ADAMTS-5 and collagen-II were purchased from Abcam (Cambridge, MA, USA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase conjugates were purchased from Bio\Rad Laboratories (Calif., USA). Fetal bovine serum (FBS), bovine serum albumin (BSA), Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium and 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were purchased from Gibco (Life Technologies Corp. Carlsbad, Calif., USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, Calif., USA). QuantiTect Reverse Transcription kit was purchased from Qiagen (Valencia, CA). ELISA kits of PGE2, TNF- α and IL-6 were purchased from R&D systems (Minneapolis, MN, USA). Griess reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China).

Primary human Chondrocyte Culture

Articular cartilage sample collection was according to the terms of the Medical Ethical Committee of the Second Affiliated Hospital, Wenzhou Medical University and following the guidelines of the Declaration of Helsinki and Tokyo. OA human cartilage tissues were obtained from four OA patients (aged 53–65 years, two men and two women) who underwent total knee replacement surgery at the Second Affiliated Hospital of Wenzhou Medical University. The OA patients met the American College of Rheumatology (ACR) classification criteria for the diagnosis of osteoarthritis [23]. Full ethical consent was obtained from all patients. Cartilage was separated from underlying bone and connective tissues, and the obtained cartilage tissues were cut into 1 × 1 × 1 mm³ pieces and washed three times with PBS. Afterwards, the joint cartilage pieces were digested with 0.25% trypsin-EDTA solution. After removing 0.25% trypsin-EDTA, they were digested in 0.2% collagenase type II for 5 h at 37 °C and then centrifuged at 1000 rpm for 5 min, and the supernatant was discarded. The inner cell mass was obtained and suspended in DMEM/F12 with 10% FBS and 1% antibiotic mixture (penicillin and streptomycin). Finally, cells were plated at a density of 1 × 10⁵ cells/ml in 6-well plates and incubated in a humidified atmosphere of 5% CO₂ at 37 °C. The media were changed every 2–3 days. Cells were passaged when at 80 to 90% confluence using 0.25% trypsin-EDTA solution. Only passages 1 to 3 were used in our study to avoid phenotype loss.

Cell viability

Cell viability was assessed using the Cell Counting Kit-8 (CCK8) assay. Human OA chondrocytes were cultured in 96-well plates at a density of 5 × 10³ cells per well for 24 h. In brief, human OA chondrocytes were pretreated with or without different concentrations (5, 25, 50, 100 and 250 μ M) of LiG for 24 h and 48 h. After that, 10 μ L CCK-8 was added to each well and incubated at 37 °C for 4 h. The optical density was read at a wavelength of 450 nm with a microplate reader (Leica Microsystems, Germany).

Griess Reaction and ELISAs

The nitrite levels in the culture medium were assessed by Griess reaction. The levels of PGE2, TNF- α and IL-6, MMP-3, MMP-13 and ADAMTS-5 in the culture medium were evaluated using commercial ELISA kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). All assays were performed in duplicate.

RNA isolation and qRT-PCR

Total RNA was isolated from human OA chondrocytes by TRIzol reagent according to the manufacturer's instructions. Its concentration was determined spectrophotometrically at 260 nm (Thermo Scientific NanoDrop 2000). The A260/A280 ratio was calculated to verify quality and purity. First-strand cDNA was synthesized using 1 μ g of total RNA and the QuantiTect Reverse Transcription kit. Quantitative real-time PCR (qRT-PCR) was performed using CFX96 Real-Time PCR System (Bio-Rad Laboratories, California, USA),

under the following conditions: 10 min 95 °C, followed by 40 cycles of 15 s 95 °C and 1 min 60 °C. The reaction was performed in a total volume of 10 µL, containing 4.5 µL diluted cDNA, 0.25 µL forward primer, 0.25 µL reverse primer and 5 µL SYBR Green Master Mix. The level of target mRNA was normalized to the level of GAPDH and compared with control. Data were analyzed using $2^{-\Delta\Delta CT}$ method. Each gene analysis was performed in triplicate. Primer's sequences of the targeted genes were listed in Table 1.

Western blotting

The proteins were extracted from chondrocytes using RIPA lysis buffer. Lysates were sonicated on ice and centrifuged at 12,000 rpm for 30 min at 4 °C. The protein concentration of the supernatant was determined using the BCA protein assay kit. 40 µg of total protein were resolved on 12% SDS-PAGE and transferred to PVDF membranes. Membranes were incubated with blocking buffer 5% non-fat in TBS containing 0.1% Tween-20 for 2 h at room temperature and then probed with the primary antibodies against COX-2, iNOS, ADAMTS-5, MMP-3, MMP-13, p65, p-p65, IκB-α, p-IκB-α, PI3K(P110), PI3K(P85), AKT, p-AKT and β-actin (dilution 1:1000) overnight at 4 °C. After washing three times with TBS containing 0.1% Tween-20 for 5 min, the membranes were incubated with HRP-conjugated secondary antibodies (dilution 1:3000) for 2 h. Finally membranes were detected by Enhanced Chemiluminescence (ECL) kit and quantified by the Quantity ONE (Bio-Rad, Hercules, CA, USA) software. β-actin was used as an internal control.

Immunofluorescence microscopy

Chondrocytes were seeded on 6-well plates on glass coverslips and incubated for 24 h. For collagen II staining, the cells were treated with 10 ng/ml IL-1β or being co-treated with 10 ng/ml IL-1β and 50 µM LIG for 24 h in medium after incubated with serum-starved medium overnights. For p65 staining, the duration of the IL-1β and LIG treatment was down to 2 h. After treatments, glass coverslips with chondrocyte monolayers were rinsed three times in PBS. Then cells were fixed with 4% paraformaldehyde for 15 min at room temperature and rinsed with PBS again. Cells and nuclear membranes were permeabilized with 0.1% Triton X-100 in for 5 min at room temperature. Later, cells were overlaid with 5% protease-free BSA for 1 h at room temperature, rinsed with PBS and incubated with primary antibody against collagen-II (1:200) and p65 (1:200) at 4 °C overnight. After washing with PBS, cells were incubated with fluorescein-conjugated goat anti-rabbit IgG antibody (1:500) for 1 h at room temperature. Finally, cells were washed three times with PBS and mounted in medium containing DAPI (Invitrogen). Slides were viewed with a confocal laser scanning microscope (Leica Microsystems, Germany). Fluorescence intensity was measured using Image J software 2.1 (Bethesda, MD, USA).

Mice OA models

Ten-week-old C57BL/6 male wild-type (WT) mice were purchased from Animal Center of Chinese Academy of Sciences, Shanghai, China. The protocol for animal care and use conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Animal Care and Use Committee of Wenzhou Medical University. The experimental mice were subjected to surgically induced OA by destabilization of the medial meniscus (DMM) as previously described [24]. In brief, after anaesthesia with peritoneal injection of 4% chloral hydrate, the cranial attachment of the medial meniscus to the tibial plateau (medial meniscotibial ligament) of the right knee was transected with a microsurgical knife. The lateral meniscotibial ligament was identified and protected during the surgery. A sham operation, consisting of an arthrotomy without the transection of medial meniscotibial ligament, was also performed in the right knee joint of mice in sham control group and sham LIG group.

Experimental animal design

The mice were randomly divided into three groups of 10 mice to establish a sham control group (sham), an osteoarthritis group (OA) and an osteoarthritis treated with LIG group (LIG). Mice

Table 1. Primer sequences used in qRT-PCR experiments

Gene	Forward primer	Reverse primer
COX-2	5'-GAGAGATGTATCCTCCACAGTCA-3'	5'-GACCAGGCACCAGACCAAG-3'
iNOS	5'-CCTTACGAGGCGAAGAAGGACAG-3'	5'-CAGTTTGAGAGAGGAGGCTCCG-3'
MMP-3	5'-CTGGCCTGCTGGCTCATGCTT-3'	5'-GCAGGGTCTTGGAGTGGTCA-3'
MMP-13	5'-CCAGAACTTCCCAACCAT-3'	5'-ACCTCCATAATGTCATACC-3'
ADAMTS-5	5'-GCAGAACATCGACCAACTTACTC-3'	5'-CCAGCAATGCCACCGAAC-3'
Collagen-II	5'-CTCAAGTCGCTGAACAACCA-3'	5'-GTCTCCGCTCTTCACTCTG-3'
GAPDH	5'-TCTCTCTGACTTCAACAGCGAC-3'	5'-CCCTGTTGCTGTAGCCAAATTC-3'

OA models were made by DMM surgery. LIG was dissolved in 2% Lutrol F68 poloxamer 188 (BASF Corporation). Mice in sham and sham-LIG group were made by sham operation. Mice in sham-LIG group and LIG group received a gavage of LIG (40 mg/kg) daily for 8 weeks after surgery while mice in OA group received a gavage of vehicle (2% Lutrol F68 poloxamer 188). Food and water were available. Mice were maintained under a constant temperature of $20 \pm 2^\circ\text{C}$, a relative humidity of $50\% \pm 10\%$, and a 12 h light/dark cycle. All animals were sacrificed at 8 weeks after surgery. Knee joint tissues were collected for further evaluation.

Histological analysis

Knee joint samples were fixed in 4% paraformaldehyde for 24 h at 4°C and decalcified in 10% EDTA solution at 4°C for two weeks. After that, the samples were dehydrated through an alcohol gradient, cleared, and embedded in paraffin blocks. Frontal serial sections ($5\ \mu\text{m}$ thick) across entire joints were obtained and 10 slides per joint at every $50\ \mu\text{m}$ were selected and stained with Safranin-O/Fast Green and Hematoxylin-Eosin staining to assess cartilage destruction. The stained sections were photographed digitally under a microscope. To determine the extent of cartilage degeneration, we used multiple separate scoring systems for articular cartilage destruction, synovitis and subchondral bone thickness. The destruction of articular cartilage was graded using the Osteoarthritis Research Society International (OARSI) scoring system for medial femoral condyle and medial tibial plateau [25]. Then we used a summed OARSI score (0–12) from medial femoral condyle and medial tibial plateau to evaluate the degree of articular cartilage destruction. The severity of synovitis was graded using a scoring system which was previously described [26]: Enlargement of the synovial lining cell layer on a scale of 0–3 (0 = 1–2 cells, 1 = 2–4 cells, 2 = 4–9 cells and 3 = 10 or more cells) and density of cells in the synovial stroma on a scale of 0–3 (0 = normal cellularity, 1 = slightly increased cellularity, 2 = moderately increased cellularity and 3 = greatly increased cellularity). We applied AxioVision software to measure the thickness of the medial subchondral bone plate according to Safranin-O-stained sections.

Statistical analysis

All experiments were performed independently at least three times. Data are expressed as mean $\pm$ standard deviation (SD). All analyses were performed using SPSS version 16.0 software. Statistical significance was assessed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

Results

Effects of LIG on chondrocyte viability

The cytotoxic effects of LIG on chondrocytes were determined at various concentrations (5, 25, 50, 100, and $250\ \mu\text{M}$) for 24 and 48 h using the Cell Counting Kit-8 assay. As shown in Fig. 1, treatment with 250 or $100\ \mu\text{M}$ LIG significantly reduced cell viability at 24 h ($P < 0.01$ vs. untreated cells) and 48 h ($P < 0.05$ vs. untreated cells), respectively, indicating that cell viability was not affected by LIG at concentrations up to $50\ \mu\text{M}$ before 48 h. Therefore, 5, 25, and $50\ \mu\text{M}$ LIG was used for subsequent experiments.

Fig. 1. Effects of Ligustilide on the cell viability of chondrocytes. Chemical structure of Ligustilide. (A). The cytotoxic effect of Ligustilide on chondrocytes was determined at various concentrations for 24 and 48 hours using a CCK8 assay. (B, C) The values presented are the means $\pm$ S.D. of three independent experiments. * $P < 0.05$, ** $P < 0.01$ vs. control group, $n = 5$.

Effects of *LIG* on inflammatory factor expression in *IL-1 β* -stimulated human chondrocytes

Human chondrocytes were pretreated with various concentrations of *LIG* (5, 25, and 50 μ M) for 2 h, followed by stimulation with or without *IL-1 β* (10 ng/ml) for 24 h. *iNOS* and *COX-2* levels were measured by both qRT-PCR and western blot analysis. As shown in Fig. 2, we found that *IL-1 β* markedly increased the mRNA expression of *iNOS* and *COX-2* compared with the control group. However, *LIG* dose-dependently inhibited the mRNA expression of *iNOS* and *COX-2* induced by *IL-1 β* . Consistent with the qRT-PCR results, the protein expression levels of *iNOS* and *COX-2* induced by *IL-1 β* were also significantly inhibited by *LIG* treatment in a dose-dependent manner. Moreover, *LIG* significantly inhibited *PGE2*, *TNF- α* , and *IL-6* generation at mRNA levels in a dose-dependent manner (Fig. 3). Taken together, these results suggest that *LIG* inhibits the production of these inflammatory cytokines.

Effects of *LIG* on ECM degradation in *IL-1 β* -treated human chondrocytes

The ECM mainly consists of aggrecan and type II collagen. As described above, type II collagen is cleaved by MMP (mainly MMP3 and MMP13), whereas aggrecan is cleaved by ADAMTS, most likely ADAMTS5. Western blot and qRT-PCR analysis showed that *IL-1 β* significantly upregulated the expression of MMP-3 and particularly MMP-13. However, *LIG* reversed this destructive effect in a dose-dependent manner. Similar results were obtained for ADAMTS-5 (Fig. 4).

Then, we investigated the effect of *LIG* on *IL-1 β* -induced collagen II degradation in human OA

Fig. 2. Ligustilide inhibit inflammatory effect in chondrocyte. Human OA chondrocytes were pretreated for 2 h with various concentrations of Ligustilide (5, 25, 50 μ M) and then stimulated or not stimulated with *IL-1 β* (10 ng/ml) for 24 h. The mRNA expression levels of *iNOS*(A) and *COX-2*(B) were assayed by qRT-PCR. The protein expressions of *iNOS* and *COX-2* in chondrocytes treated as above were visualized by western blot (C), quantified in (D, E). Significant differences among different groups are indicated as ##P<0.01, vs control group; **P<0.01, vs *IL-1 β* alone treatment group, n = 5.

Fig. 3. Ligustilide inhibit inflammatory effect in human OA chondrocytes. Human OA chondrocytes were pretreated for 2 h with various concentrations of ligustilide (5, 25, 50 μ M) and then stimulated or not stimulated with *IL-1 β* (10 ng/ml) for 24 h. The nitrite levels in the culture medium were assessed by Griess reaction(B). The levels of *PGE2*, *TNF- α* and *IL-6* were determined using ELISA (A,C,D). The data in the figures represent the averages $\pm$ S.D. Significant differences among different groups are indicated as ##P<0.01, vs control group; **P<0.01, vs *IL-1 β* alone treatment group, n = 5.

chondrocytes. Chondrocytes were pretreated with various concentrations of LIG (5, 25, and 50 μ M) for 2 h, followed by stimulation with or without IL-1 β (10 ng/ml) for 24 h. The mRNA expression of collagen II was determined by qRT-PCR, and the protein expression of collagen II was detected by immunofluorescence. As shown in Fig. 5B, chondrocytes showed a clear downregulation of the mRNA expression of collagen II after IL-1 β stimulation. Otherwise, LIG clearly inhibited the mRNA downregulation of collagen II. Moreover, the immunofluorescence results suggested that LIG signaling suppressed the protein degradation of collagen II, which is consistent with the results of qRT-PCR (Fig. 5A and C).

Effects of IL-1 β on the levels of phosphorylated IKK/ $\text{I}\kappa$ B α /p65

To further reveal the mechanism, the phosphorylation levels of p65, $\text{I}\kappa$ B α , and IKK α / β in IL-1 β -stimulated chondrocytes were assessed by western blot after 2 h. Subsequent analysis revealed that IL-1 β markedly induced the phosphorylation of IKK α / β , $\text{I}\kappa$ B α , and NF- κ B p65 (Fig. 6A and B).

Effects of LIG on IL-1 β -induced NF- κ B activation

To examine the effects of LIG on NF- κ B pathway activation, the phosphorylation levels of p65, $\text{I}\kappa$ B α , and IKK α / β in IL-1 β -stimulated chondrocytes were assessed by western blot at 2 h. IL-1 β markedly induced the phosphorylation of IKK α / β and $\text{I}\kappa$ B α , significantly so at 50 μ M (Fig. 7). Indirect immunofluorescence staining of p65 revealed the translocation of p65 from the cytoplasm to the nucleus in chondrocytes in response to NF- κ B activation by IL-1 β . The chondrocytes were divided into three groups: (1) control group; (2) IL-1 β -stimulated group; and (3) IL-1 β plus LIG treatment group. In the control group, most p65 protein was located in the cytoplasm. Upon IL-1 β stimulation, p65-positive proteins were stained intensively in the nucleus of chondrocytes, leaving the cytoplasm unstained, indicating nuclear translocation of the NF- κ B subunit. However, pretreatment with LIG inhibited the translocation of p65 subunits to the nucleus (Fig. 6C). These immunomorphological findings suggest an inhibitory effect of LIG on the IL-1 β -induced p65 nuclear dislocation in chondrocytes and are consistent with the inhibition of the NF- κ B pathway observed by western blot.

Fig. 4. Effect of Ligustilide on IL-1 β -induced ADAMTS-5, MMP-3 and MMP-13 expression in human OA chondrocytes. Human OA chondrocytes were pretreated for 2 h with various concentrations of Ligustilide (5, 25, 50 μ M) and then stimulated or not stimulated with IL-1 β (10 ng/ml) for 24 h. The mRNA expression levels of ADAMTS-5 (A), MMP-3 (B) and MMP-13 (C) were assayed by qRT-PCR. The protein expression levels of ADAMTS-5, MMP-3 and MMP-13 were determined by Western blot and quantification analysis (D-G). The data in the figures represent the averages $\pm$ S.D. Significant differences among different groups are indicated as ## P <0.01, vs control group; ** P <0.01, vs IL-1 β alone treatment group, n = 5.

Fig. 5. Ligustilide inhibit extracellular matrix reduction from IL-1 β induced in human OA chondrocytes. Human OA chondrocytes were pretreated for 2 h with ligustilide (5, 25, 50 μ M) and then stimulated with IL-1 β (10 ng/ml) for 24 h. The mRNA expression levels of collagen-II (B) were assayed by qRT-PCR. The representative collagen II (A) were detected by the immunofluorescence combined with DAPI staining for nuclei. The fluorescence intensity of Col II (C) were analysed by image J. The data in the figures represent the averages $\pm$ S.D. Significant differences among different groups are indicated as ###P<0.01, vs control group; **P<0.01, vs IL-1 β alone treatment group, n = 5.

Fig. 6. Effect of ligustilide on IL-1 β -induced NF- κ B activation in human OA chondrocytes. Chondrocytes were pretreated with ligustilide (5, 25, 50 μ M) for 2h, followed by stimulation with or without IL-1 β (10 ng/ml) for 1 h. The protein expression of I κ B, p-I κ B, p65 and p-p65 were determined by Western blot and quantification analysis(A,B). The nuclei translocation of p65 was detected by the immunofluorescence combined with DAPI staining for nuclei (original magnification $\times$ 400, scale bar: 10 μ m)(C). The data in the figures represent the averages $\pm$ S.D. Significant differences among different groups are indicated as ###P<0.01, vs control group; **P<0.01, vs IL-1 β alone treatment group, n = 5.

LIG ameliorates OA development in a mouse model involving destabilization of the medial meniscus

To investigate the protective effects of LIG on OA development *in vivo*, a surgically induced mouse model involving destabilization of the medial meniscus (DMM) was established, followed by intraperitoneal injection of 40 mg/kg LIG dissolved in 2% Lutrol F68 poloxamer 188 once daily for 8 weeks. Histological analysis of OA was performed using safranin O staining for cartilage and synovitis. Osteoarthritis Research Society International (OARSI) scores and synovitis scores were used for quantitative analysis. As revealed by the safranin O staining (Fig. 8A), the cartilage surface was smooth and showed positive red staining in the sham control group. The OA group showed cartilage erosion, apparent hypocellularity, and massive proteoglycan loss compared with the sham control group. However, the LIG

group exhibited less proteoglycan loss compared with the OA group. Consistent with the safranin O staining, the OARSI scores (Fig. 8B) of the OA group were markedly higher than those of the sham control. In contrast, the LIG group displayed lower OARSI scores than the OA group. In addition, synovial thickening and hypercellularity was present in the OA group, whereas LIG treatment alleviated the synovitis compared with the OA group (Fig. 8C). Together, these results indicate that LIG protects against OA development *in vivo* by inhibiting the loss of proteoglycan, cartilage calcification, and synovitis.

Discussion

Drugs currently used for OA, including non-steroidal anti-inflammatory drugs and steroids, act against the pain and joint inflammation but fail to prevent disease progression and often cause many adverse effects [27, 28], which suggests that these drugs are not ideal choices for the treatment of OA. Thus, there is an urgent need to identify safe and effective agents that can slow the progression of OA.

LIG, a quinone extracted from the roots of medicinal herbs of the *Plumbago* genus, has anti-inflammatory effects. Here, we found that LIG significantly inhibited the IL-1 β -induced inflammatory response in chondrocytes. Furthermore, several other *in vitro* and *in vivo* studies have confirmed that the inflammatory environment plays a negative role in the stability of the chondrocyte phenotype and homeostasis of the ECM, which aggravates cartilage destruction and promotes OA progression [29-31]. Thus, we further investigated whether LIG can reverse these inflammation-induced conditions and explored the potential molecular mechanisms involved.

The inflammatory mediator PGE2 plays a crucial role in the pathogenesis of OA[32]. Previous reports showed that inhibition of the production of inflammatory mediators such

Fig. 7. Effect of ligustilide on IL-1 β -induced PI3K/AKT phosphorylation in human OA Chondrocytes. Chondrocytes were pretreated with ligustilide (5, 25, 50 μ M) for 2h, followed by stimulation with or without IL-1 β (10 ng/ml) for 1 h. The protein expression levels of PI3K(P110), PI3K(P85), AKT and p-AKT were determined by Western blot and quantification analysis (A-C). The data in the figures represent the averages $\pm$ S.D. Significant differences among different groups are indicated as ^{##} P <0.01, vs control group; ^{**} P <0.01, vs IL-1 β alone treatment group, n = 5.

Fig. 8. Ligustilide ameliorates OA development in mouse DMM model *in vivo*. Representative S-O staining of cartilage and synovitis from different experimental groups at 8 weeks post-surgery(A) (original magnification $\times$ 100 or $\times$ 400, scale bar: 200 μ m or 50 μ m). Diagrams showed the OARSI scores of cartilage (B). Diagrams showed the scores of synovitis (C). The data in the figures represent the averages $\pm$ S.D. Significant differences among different groups are indicated as ^{##} P <0.01, vs sham group; ^{**} P <0.01, vs DMM group, n = 10.

as PGE2 could slow down OA progression [33]. PGE2, a principal mediator of inflammation, is converted from endogenous arachidonic acid induced by IL-1 β via COX-2 catalytic reactions and is associated with cartilage destruction due to its powerful activation for MMPs and other inflammatory mediators [34, 35]. All of these factors, as well as TNF- α and IL-6, are important inflammatory cytokines in OA development [36]. In this study, we found that overproduction of PGE2, TNF- α , and IL-6 after IL-1 β stimulation is inhibited by LIG at the gene level, whereas COX-2 and iNOS are inhibited at both the gene and protein levels. These results are consistent with those from Yang et al., whose research revealed that LIG inhibits LPS-induced inflammatory activation in RAW264.7 macrophages [37]. Therefore, we surmised that the anti-inflammatory effects of LIG may be associated with inhibition of TNF- α and IL-6 expression, as well as decreased production of PGE 2, via targeting of iNOS and COX-2.

As described above, the ECM consists of aggrecan and type II collagen, which can provide tensile strength and shock absorption under mechanical damage [38]. Inhibition of the degradation of collagen II and aggrecan may have positive effects on OA treatment. Type II collagen is cleaved by MMPs, whereas aggrecan is cleaved by ADAMTS[39-41]. In our study, in the presence of IL- β , LIG not only promoted the synthesis of type II collagen and aggrecan, but also inhibited the catabolism of ECM components by downregulating MMPs and ADAMTS-5, which helped to maintain ECM homeostasis.

To elucidate the mechanism by which LIG unites anti-inflammation and anti-catabolism in the ECM in chondrocytes, we further explored the cross-talk between the PI3K/AKT and NF- κ B signaling pathways. NF- κ B is a ubiquitously expressed transcription factor involved in the regulation of inflammatory mediators associated with OA[42, 43]. According to previous studies [44], IL-1 β stimulation triggers the phosphorylation of the catalytic subunits of IKK (IKK α and IKK β), as well as the binding protein of p65 (I κ B α), which consequently frees p65 and translocates it from the cytosol to the nucleus and leads to the production of catabolic enzymes, cytokines, and inflammatory mediators. Additionally, LIG inhibits inflammation in macrophages via NF- κ B inhibition. Our data reveal that LIG inhibits IL-1 β -induced inflammation and ECM degradation by suppressing NF- κ B signaling in chondrocytes.

Several upstream signaling molecules, such as STAT3, ERK1/2, TRAF6, IRAK1, and PI3K/AKT, have been reported to be involved in IL-1 β -induced inflammation in chondrocytes. The intracellular messengers PI3K/AKT are one of the best-studied upstream signaling pathways [15]. The pathway regulates a cascade of changes through a variety of target proteins, such as mechanistic target of rapamycin (mTOR), NF- κ B, glycogen synthase kinase 3 beta (GSK-3 β), and p53, all of which are involved in the OA process. Therefore, we further investigated the correlation between NF- κ B and PI3K/AKT signaling during LIG treatment with respect to OA. In the current study, IL-1 β stimulation markedly upregulated the protein levels of the catalytic subunit (PI3K-p110) and the regulatory subunit (PI3K-p85) of PI3K, which catalyze the production of PIP3 and leads to the phosphorylation of AKT and subsequent activation of NF- κ B. Our data revealed that LIG blocks IL-1 β -induced AKT activation via inhibition of PI3K-p110 and PI3K-p85, which further targets the downstream NF- κ B molecules. Thus, the results from previous studies along with ours indicate that the anti-inflammatory effects of LIG may be closely related to the inhibition of NF- κ B activation via the PI3K/AKT pathway.

DMM is a reliable and effective method to establish an animal OA model for *in vivo* analysis. Thus, in this study, mouse OA models induced by DMM were used to examine the protective effects of LIG *in vivo*. Treatment with LIG dramatically reduced the OARSI scores as well as the severity scores of synovitis in our mouse OA model.

In this study, we found for the first time that LIG significantly inhibits the IL-1 β -induced inflammatory response in chondrocytes, including the expression of PGE2, iNOS, COX-2, MMP-3, MMP-13, and ADAMTS-5, and decreases the degradation of aggrecan and collagen II in human OA chondrocytes via the PI3K/AKT pathway. Furthermore, our results showed that LIG treatment markedly suppresses the IL-1 β -induced NF- κ B activation. Moreover, *in vivo*, LIG treatment clearly prevented the development of OA in mouse OA models. Therefore, LIG may one day serve as an effective chemotherapeutic agent for the treatment of OA.

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Disclosure Statement

The authors declare no conflicts of interest.

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