Original Paper

Licochalcone A Inhibits MMPs and ADAMTSs via the NF- κ B and Wnt/ β -Catenin Signaling Pathways in Rat Chondrocytes

Wei-Ping Chen^a Zhong-Nan Hu^b Li-Bin Jin^a Li-Dong Wu^a

^aDepartment of Orthopedics Surgery, The Second Hospital of Medical College, Zhejiang University, Hangzhou, ^bDepartment of Orthopedics Surgery, The Children's Hospital of Medical College, Zhejiang University, Hangzhou, China

Key Words

Osteoarthritis • Licochalcone A • Matrix metalloproteinase • NF- κ B • β -catenin

Abstract

Background: Osteoarthritis (OA) is a joint disease in which cartilage degradation is the central pathological change. In this study, we investigated the anti-osteoarthritic effects of licochalcone A (Lico A) in rat chondrocytes. **Methods:** Polymerase chain reaction and Western blotting were performed to evaluate the expression of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-5, ADAMTS-4, collagen II, matrix metalloproteinase (MMP)-13 and MMP-1 at both the gene and protein levels, respectively. In addition, the wnt/ β -catenin and nuclear factor kappa B (NF- κ B) signaling pathways were also analyzed by Western blotting. **Results:** Lico A downregulated ADAMTS-5, ADAMTS-4, MMP-13 and MMP-1 expression, and diminished the IL-1 β -induced inhibition of collagen II. In addition, the activation of β -catenin and phosphorylation of p65 and IKK α / β were suppressed by Lico A. **Conclusions:** Our results suggest that Lico A inhibits MMPs and ADAMTS partly via the NF- κ B and wnt/ β -catenin signaling pathways in rat chondrocytes. Thus, Lico A may have therapeutic effects in OA.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Osteoarthritis (OA) is a common joint disease that often causes pain and disability, particularly in older people [1-4]. Many factors such as mechanical, biochemical, and enzymatic factors are involved in its onset [5]. However, the mechanisms underlying this disease are still unclear. As a pro-inflammatory cytokine, interleukin-1 β (IL-1 β) leads to the apoptosis of chondrocytes and degradation of the extracellular matrix (ECM), which results in cartilage degeneration [6-8]. Previous studies have shown that the degeneration

of cartilage may be associated with matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS), and other catabolic enzymes [9]. MMP-1 and MMP-13 can cleave collagen II, whereas ADAMTS-4 and ADAMTS-5 can degrade the aggrecan in cartilage [10-13]. Nonsteroidal anti-inflammatory drugs (NSAIDs) are still the primary treatment for OA; however, they can not protect the cartilage from further damage [14, 15]. Thus, new effective agents that can protect joint structure and function are still needed.

Flavonoids are plant-derived compounds that can be purified from vegetables, fruits, and medical herbs [16]. Licochalcone A (Lico A) is a flavonoid isolated from licorice or *Glycyrrhiza inflata* [17]. It possesses a variety of pharmaceutical activities including anti-inflammatory, anti-oxidative, and anti-tumor properties [18-21]. Moreover, a recent study revealed that Lico A can suppress MMP expression [22]. Therefore, it may be a potential treatment for OA. However, little is known about the anti-osteoarthritic effects of Lico A in chondrocytes. In this study, we examined the anti-osteoarthritic effects of Lico A in IL-1 β -stimulated rat chondrocytes. The activation of wnt/ β -catenin and nuclear factor kappa B (NF- κ B) signaling pathways was also investigated to determine the probable regulatory mechanisms.

Materials and Methods

Reagents

Lico A, dimethylsulfoxide (DMSO), and IL-1 β were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), penicillin, streptomycin, and 0.25% trypsin, collagenase II were purchased from Gibco BRL (Grand Island, NY, USA).

Cell culture

With approval of the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China), articular cartilage was obtained from the knees and femoral heads of rats (Zhejiang Academy of Medical Sciences, Hangzhou, China). After digestion with 0.25% trypsin for 15 min, the cartilage was incubated with 1 mg/mL collagenase II at 37°C for 3 h. Then chondrocytes were separated from the cartilage and cultured in 25 cm² flasks in DMEM with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37°C with 5% CO₂. These cells were considered passage 0 (P0). Once they reached confluence, the chondrocytes were split in a 1:3 ratio. P3 cells were used for analysis.

Cell viability assay

Cells were cultured in a 96-well plate (5 $\times$ 10³ cells/well) with various concentrations of Lico A and IL-1 β (10 ng/mL) for 24 h. Then, 20 μ L per well of MTT (5 mg/mL) was added to each well for 4 h at 37°C. The culture medium was removed before adding 150 μ L/well DMSO. Finally, the absorbance at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was determined using the MTT assay as previously described [23]. All of the assays were performed in triplicate.

Cells treatment

Chondrocytes were serum-starved for 12 h before seeding into six-well plates at an initial density of 2 $\times$ 10⁵ cells/well. The cells were pretreated with Lico A (0, 1, 5, 10 μ M) for 1 h followed by IL-1 β (10 ng/mL) stimulation for 24 h to analyze the expression of MMPs, ADAMTS, and collagen II. Other chondrocytes were cultured in 60 mm dishes at an initial density of 5 $\times$ 10⁵ cells/well, and pretreated with Lico A (0, 1, 5, 10 μ M) for 2 h followed by IL-1 β (10 ng/mL) stimulation for 30 min to investigate activation of the wnt/ β -catenin and NF- κ B signaling pathways.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted and purified using TRIzol® Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA) and RNase-Free DNase Set (QIAGEN Translational Medicine Co., Ltd., Wilmington, DE). The Su-

perScript™ III First-Strand Synthesis SuperMix for qRT-PCR Kit (Invitrogen) was used to reverse transcribe total RNA to complementary DNA (cDNA) following the manufacturer's instructions. The amplification of MMPs, ADAMTS, and collagen II was performed using the Power SYBR® Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) under conditions of 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 60 s [24]. To normalize expression, 18S rRNA was amplified

as an internal control. The primers used are shown in Table 1. All of the experiments were performed according to the manufacturer's protocol in triplicate using three independent samples. Quantitative real-time PCR (qPCR) data were calculated using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

Cells were washed using ice-cold phosphate-buffered saline followed by addition of 40 μ L RIPA buffer containing 0.4 μ L protease inhibitors for 30 min to extract all of the proteins. Then the mixture was centrifuged and quantified using a BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China), and electrotransferred to nitrocellulose membranes. The membranes were incubated with Tris-buffered saline with tween (TBS-T) for 1 h and cut into sections based on protein molecular weights. Then the membranes were incubated with primary antibodies against ADAMTS-5, ADAMTS-4, collagen II, MMP-13, MMP-1, β -catenin, β -actin, (Santa Cruz Biotech, Inc., Dallas, TX, USA), I κ B alpha, p-IKK alpha/beta, IKK alpha/beta, p-65, and p-p65 (Abcam Inc., Cambridge, UK) for 12 h at 4°C. After washing with TBS-T, the membranes were incubated with secondary antibodies (Thermo Fisher, Waltham, MA, USA) at 25°C for 1 h. The membranes were washed again with TBS-T, and immunoreactive bands were detected by enhanced chemiluminescence (Thermo Fisher) and exposure to X-ray film (Kodak, Hangzhou, China) for densitometry analysis (BandScan 5.0 software). All of the assays were performed in triplicate.

Statistical analysis

All data are expressed as the mean $\pm$ SD. Data were analyzed using one-way ANOVA. Dunnett's test was used as the post-test for ANOVA. Differences were considered statistically significant when $P < 0.05$ (*) or $P < 0.01$ (**).

Results

Effects of Lico A on chondrocyte viability

MTT assays revealed that Lico A had no cytotoxicity at a concentration of 10 μ M (Fig.1); thus, this was the concentration used in subsequent experiments.

Table 1. Primers of targeted genes

Gene	Genbank Accession	Primer Sequences(5'to3')	Size(bp)
Rat MMP-1	NM_001134530.1	GCTTAGCCTTCCTTTGCTGTTGC	136
		GACGTCTTCACCCAAGTTGTAGTAG	
Rat MMP-13	NM_133530	CAACCTGTTCCTACCCACTTAT	85
		CTATGTCTGCCTTAGCTCCTGTC	
Rat Collagen II	L48440	GAGTGGGAAGAGCGGAGACTACTG	83
		GTCTCCATGTTGCAGAAGACTTTCA	
Rat Adams4	NM_023959	GCCAGCAACCGAGGTCCCAT	118
		CCACCACCAAGTGTCTCCACGAAT	
Rat Adams5	NM_198761.1	GACAAGAGTCTGGAGGTGAGCAA	113
		GCTGCATCGTAGTGCTCCTCAT	
Rat I κ B- α	NM_001105720.2	CAAGTACCCGGATACAGCAG	87
		ACACAGTCATCGTAGGGCAA	
Rat 18S rRNA	M11188	GAATTCACAGTAAGTGCAGGTCATA	105
		CGAGGGCCTCACTAAACCATC

Fig. 1. Effects of Lico A on cell viability. Cells were cultured in a 96-well plate (5×10^3 cells/well) at various concentrations (0, 1, 5, 10 μ M) of Lico A and IL-1 β (10 ng/mL) for 24 h followed by MTT assay analysis.

Fig. 2. Effects of Lico A on the gene expression of ADAMTS-5, ADAMTS-4, Collagen II, MMP-1 and MMP-13. Samples were pretreated with various concentrations of Lico A. The mRNA expressions of ADAMTS-5, ADAMTS-4, MMP-13, and MMP-1 decreased and collagen II levels increased in a dose-dependent manner after Lico A treatment. ** $P < 0.01$ compared to cells stimulated with IL-1 β in the absence of Lico A.

Fig. 3. Effects of Lico A on the protein expression of ADAMTS-5, ADAMTS-4, Collagen II, MMP-13, and MMP-1. Samples were pretreated with various concentrations of Lico A. Densitometry was performed to analyze the ratio of the density between the protein and β -actin. * $P < 0.05$ compared to samples stimulated with IL-1 β in the absence of Lico A.

Effects of Lico A on ADAMTS-5, ADAMTS-4, Collagen II, MMP-13 and MMP-1 expression in chondrocytes

qPCR was performed to measure the mRNA expression of ADAMTS-5, ADAMTS-4, collagen II, MMP-13, and MMP-1 in IL-1 β -stimulated chondrocytes. The expression of ADAMTS-5, ADAMTS-4, MMP-13 and MMP-1 was decreased and the expression of collagen II was increased by Lico A in a dose-dependent manner (Fig. 2). Western blot analysis revealed

Fig. 4. Effects of Lico A on the NF- κ B and Wnt/ β -catenin signaling pathways. Samples were pretreated with Lico A (0, 1, 5, 10 μ M) for 2 h followed by IL-1 β (10 ng/mL) for 30 min. Densitometry was performed to analyze the ratios of the density between phosphorylated p65 and p65, and between phosphorylated IKK α / β and IKK α / β . The expression of I κ B- α and β -catenin was compared to β -actin. The mRNA levels of I κ B- α were also investigated. *P<0.05 compared to samples stimulated with IL-1 β in the absence of Lico A.

that the protein expression of ADAMTS-5, ADAMTS-4, collagen II, MMP-13 and MMP-1 was similar to the mRNA expression (Fig. 3).

Effects of Lico A on NF- κ B and wnt/ β -catenin signaling pathways

We also investigated the effects of Lico A on both the NF- κ B and wnt/ β -catenin signaling pathways, and found that IL-1 β activated both pathways. However, treatment with Lico A reduced the activation of both pathways, and p-IKK α , β , p-p65, and β -catenin levels were downregulated. In addition, the gene and protein expression of I κ B α , an inhibitor of NF- κ B, significantly increased after Lico A treatment (Fig. 4).

Discussion

In this study, we investigated the effects of Lico A on IL-1 β -stimulated chondrocytes, and found that Lico A reduced expression of ADAMTS-5, ADAMTS-4, MMP-13 and MMP-1 in rat chondrocytes. In addition, the expression of collagen II was upregulated by Lico A.

Collagen II is an important component of the ECM, and the expression of collagen II is decreased in OA. Therefore, inhibiting collagen II degradation is a strategy for OA treatment. In this study, IL-1 β stimulation resulted in reduced collagen II expression. However, Lico A upregulated the expression of collagen II, which suggests that Lico A has protective effects in chondrocytes.

In OA, enzymes are secreted that degrade the ECM including ADAMTS-5, ADAMTS-4, MMP-13, and MMP-1. These enzymes can cleave collagen II and aggrecan in cartilage. Thus, downregulation of these enzymes could be beneficial to cartilage [25, 26]. In this study, stimulation of IL-1 β resulted in the increased expression of ADAMTS-5, ADAMTS-4, MMP-13, and MMP-1. Lico A significantly inhibited the upregulation of ADAMTS-5, ADAMTS-4, MMP-13, and MMP-1 in chondrocytes. In addition, Lico A diminished the IL-1 β -induced inhibition

of collagen II expression. A previous study reported that Lico A inhibits the expression of MMP-1 and MMP-3 in human lung cancer cells [22]. Our results demonstrate that Lico A has anti-catabolic properties in rat chondrocytes.

The importance of the NF- κ B pathway in the inflammatory process has been well established. Recently, Zhou *et al.* reported that by controlling the activation of NF- κ B signaling, IL-1 β can modulate ADAMTS expression [27]. Moreover, the critical role of NF- κ B in the regulation of MMP expression has also been recognized [28, 29]. For NF- κ B activation, inhibitors of κ B (I κ B) are ubiquitinated after phosphorylation by IKK, which releases NF- κ B dimers, after which p50 and p65 heterodimers are released [30-32]. In this study, we found that IL-1 β stimulation resulted in activation of the NF- κ B pathway, and that Lico A significantly suppressed the phosphorylation of IKK α / β and p65 and increased I κ B α expression. These results suggest that the NF- κ B pathway may be involved in the downregulation of MMPs and ADAMTS by Lico A in IL-1 β -stimulated chondrocytes.

The wnt/ β -catenin signaling pathway is related to cartilage homeostasis and joint remodeling [33]. In addition, a previous study showed that β -catenin was overexpressed in joint tissues from OA patients and animals [7]. In this study, the expression of β -catenin was significantly downregulated by Lico A in chondrocytes. Thus, we speculated that the anti-catabolic effects of Lico A may partly occur through inhibition of the wnt/ β -catenin signaling pathway.

Conclusion

This study demonstrated that Lico A can inhibit IL-1 β -induced ADAMTS-5, ADAMTS-4, MMP-13, and MMP-1 expression at both the gene and protein levels in rat chondrocytes. Moreover, the NF- κ B and wnt/ β -catenin signaling pathways may be involved in this process. Thus, Lico A may be a potential treatment for OA.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (81371996 and 81572173).

Disclosure Statement

The authors declare that there are no conflicts of interest.

References

- 1 Dubin A: Managing Osteoarthritis and Other Chronic Musculoskeletal Pain Disorders. *Med Clin North Am* 2016;100:143-150.
- 2 Kevorkian L, Young DA, Darrah C, Donell ST, Shepstone L, Porter S, Brockbank SMV, Edwards DR, Parker AE, Clark IM: Expression profiling of metalloproteinases and their inhibitors in cartilage. *Arthritis Rheum* 2004;50:131-141.
- 3 Silverwood V, Blagojevic-Bucknall M, Jinks C, Jordan JL, Protheroe J, Jordan KP: Current evidence on risk factors for knee osteoarthritis in older adults: a systematic review and meta-analysis. *Osteoarthritis Cartilage* 2015;23:507-515.
- 4 Felson DT: Epidemiology of hip and knee osteoarthritis. *Epidemiol Rev* 1988;10:1-28.
- 5 Priyanka V, Krishna D: ADAMTS-4 and ADAMTS-5: Key Enzymes in Osteoarthritis. *J Cell Biochem* 2011;112:3507-3514.

- 6 Pasternak B, Aspenberg P: Metalloproteinases and their inhibitors-diagnostic and therapeutic opportunities in orthopedics. *Acta Orthop* 2009;80:693-703.
- 7 Jin L, Xin-die Z, Kong-he Y, Teng-di F, Wei-ping C, Li-feng J, Bao JP, Wu LD, Xiong Y: Hinokitiol reduces matrix metalloproteinase expression by inhibiting Wnt/ β -Catenin signaling *in vitro* and *in vivo*. *Int Immunopharmacol* 2014;23: 85-91.
- 8 Chen WP, Xiong Y, Shi YX, Hu PF, Bao JP, Wu LD: Astaxanthin reduces matrix metalloproteinase expression in human chondrocytes. *Int Immunopharmacol* 2014;19:174-177.
- 9 Santangelo KS, Nuovo GJ, Bertone AL: *In vivo* reduction or blockade of interleukin-1 β in primary osteoarthritis influences expression of mediators implicated in pathogenesis. *Osteoarthritis Cartilage* 2012;20:1610-1618.
- 10 Bramono DS, Richmond JC, Weitzel PP, Kaplan DL, Altman GH: Matrix metalloproteinases and their clinical applications in orthopaedics. *Clin Orthop Relat Res* 2004;428:272-285.
- 11 Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, Rosenfeld SA, Caspers N, Wittwer AJ, Malfait A-M, Shieh H-S: Purification and cloning of aggrecanase-1: A member of the ADAMTS family of proteins. *Science* 1999;284:1664-1666.
- 12 Tortorella MD, Pratta M, Liu RQ, Austin J, Ross OH, Abbaszade I, Burn T, Arner E: Sites of aggrecan cleavage by recombinant human aggrecanase-1 (ADAMTS-4). *J Biol Chem* 2000; 275:18566-18573.
- 13 Gendron C, Kashiwagi M, Lim NH, Enghild JJ, Thøgersen IB, Hughes C, Caterson B, Nagase H: Proteolytic activities of human ADAMTS-5: Comparative studies with ADAMTS-4. *J Biol Chem* 2007;282:18294-18306.
- 14 Felson DT, Kim Y-J: The futility of current approaches to chondroprotection. *Arthritis Rheum* 2007;56:1378-1383.
- 15 Crofford LJ, Lipsky PE, Brooks P, Abramson SB, Simon LS, van de Putte LB: Basic biology and clinical application of specific cyclooxygenase-2 inhibitors. *Arthritis Rheum* 2000;43:4-13.
- 16 Dixon RA, Steele C L: Flavonoids and isoflavonoids-a gold mine for metabolic engineering. *Trends Plant Sci* 1999;4:394-400.
- 17 Hatano T, Kagawa H, Yasuhara T, Okuda T: Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chem Pharm Bull* 1988;36:2090-2097.
- 18 Kwon HS, Park JH, Kim DH, Kim YH, Park JH, Shin HK, Kim JK: Licochalcone a isolated from licorice suppresses lipopolysaccharide-stimulated inflammatory reactions in RAW264.7 cells and endotoxin shock in mice. *J Mol Med* 2008;86:1287-1295.
- 19 Bortolotto LF, Barbosa FR, Silva G, Bitencourt TA, Belebony RO, Baek SJ, Marins M, Fachin AL: Cytotoxicity of trans-chalcone and licochalcone A against breast cancer cells is due to apoptosis induction and cell cycle arrest. *Biomed Pharmacother* 2017;85:425-433.
- 20 Hongming Lv, Hua Ren, Lidong Wang, Wei Chen, Xinxin Ci: Lico A Enhances Nrf2-Mediated Defense Mechanisms against t-BHP-Induced Oxidative Stress and Cell Death via Akt and ERK Activation in RAW 264.7 Cells. *Oxid Med Cell Longev* 2015; 2015: 709845.
- 21 Egler J, Lang F: Licochalcone A Induced Suicidal Death of Human Erythrocytes. *Cell Physiol Biochem* 2015;37:2060-2070.
- 22 Huang HC, Tsai LL, Tsai JP, Hsieh SC, Yang SF, Hsueh JT, Hsieh YH: Licochalcone A inhibits the migration and invasion of human lung cancer cells via inactivation of the Akt signaling pathway with down regulation of MMP-1/-3 expression. *Tumour Biol* 2014;35:12139-12149.
- 23 Ding Q, Zhong H, Qi Y, Cheng Y, Li W, Yan S, Wang X: Anti-arthritis effects of crocin in interleukin-1 β -treated articular chondrocytes and cartilage in a rabbit osteoarthritic model. *Inflamm Res* 2013;62:17-25.
- 24 Wei-Ping C, Zhi-Gao Y, Peng-Fei H, Jia-Peng B, Li-Dong W: Acacetin inhibits expression of matrix metalloproteinases via a MAPK-dependent mechanism in fibroblast-like synoviocytes. *J Cell Mol Med* 2015;19:1910-1915.
- 25 Du G, Song Y, Wei L, Li L, Wang X, Xu Q, Zhan H, Cao Y, Zheng Y, Ding D: Osteole Inhibits Proliferation and Induces Catabolism in Rat Chondrocytes and Cartilage Tissue. *Cell Physiol Biochem* 2015;36:2480-2493.
- 26 Chen WP, Xiong Y, Hu PF, Bao JP, Wu LD: Baicalein Inhibits MMPs Expression via a MAPK-Dependent Mechanism in Chondrocytes. *Cell Physiol Biochem* 2015; 36: 325-333.
- 27 Sun Z1, Yin Z, Liu C, Liang H, Jiang M, Tian J: IL-1 β promotes ADAMTS enzyme-mediated aggrecan degradation through NF- κ B in human intervertebral disc. *J Orthop Surg Res* 2015; 10:159.

- 28 Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE: Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis Rheum* 2000;43:801-811.
- 29 Han ZN, Boyle DL, Manning AM, Firestein GS: AP-1 and NF-kappa B regulation in rheumatoid arthritis and murine collagen-induced arthritis. *Autoimmunity* 1998;28:197-208.
- 30 Tak PP, Firestein GS: NF-κB: a key role in inflammatory diseases, *J Clin Invest*. 2001;107:7-11.
- 31 Schottelius AJ, Mayo MW, Sartor RB, Baldwin AS, Jr: Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. *J Biol Chem* 1999;274:31868-31874.
- 32 Vincenti MP, Coon CI, Brinckerhoff CE: Nuclear factor kappaB/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1beta-stimulated synovial fibroblasts. *Arthritis Rheum* 1998;41:1987-1994.
- 33 Chen M, Zhu M, Awad H, Li TF, Sheu TJ, Boyce BF, Chen D, O'Keefe RJ: Inhibition of beta-catenin signaling causes defects in postnatal cartilage development. *J Cell Sci* 2008;121:1455-1465.