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Cilostazol prevents the degradation of collagen Type II in human chondrocytes

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ABSTRACT

The alteration of extracellular matrix (ECM) in cartilage during the pathological development of Osteoarthritis (OA) changes the biomechanical environment of chondrocytes, which further drives the progression of the disease in the presence of inflammation. Healthy cartilage matrix mainly contains collagen type II, which is degraded by matrix metalloproteinase13 (MMP13), an important molecules responsible for joint damage in OA. Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone) is a medication approved by the US Food and Drug Administration and used in the alleviation of the symptom of intermittent claudication in individuals with peripheral vascular disease. In this study, we reported that cilostazol is able to suppress the degradation of type II collagen in human chondrocytes induced by IL-1β. Mechanistically, cilostazol treatment leads to inhibiting the expression of IRF-1, thereby prevents the induction of MMP-13. Signal transducers and activator of transcription 1 (STAT1) has been reported to play an essential role in regulating the activation of IRF-1. Our results indicated that cilostazol suppresses the activation of STAT1 by mitigating the phosphorylation of STAT1 at Ser727 and tyrosine phosphorylation of STAT1 at position 701 (Tyr701).

1. Introduction

Osteoarthritis (OA), the most common joint disorder in aging people, is a degenerative disease that affects various tissues surrounding joints such as articular cartilage, subchondral bone, synovial membrane, and ligaments [1]. Inflammation is one of these factors affecting the development and progression of OA. Although the role of inflammation in OA has been unclear for a long time, more and more attracts have been made in recent years. The alteration of extracellular matrix (ECM) in cartilage during the pathological development of OA changes the biomechanical environment of chondrocytes, which further drives the progression of the disease in the presence of inflammation [2]. Healthy cartilage matrix mainly contains collagen type II, which is degraded by matrix metalloproteinase13 (MMP13), which is an important molecule responsible for joint damage in OA [3]. MMPs are induced by proinflammatory cytokines such as TNF-α and IL-1β mediated by interferon response factor-1 (IRF-1), which is a transcriptional factor induced by interferons, proinflammatory cytokines, and other stimuli [4]. Inhibition of type II collagen breakdown has been considered as a potential pharmacological strategy for OA treatment.

Cilostazol (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone) is a medication approved by the US Food and Drug Administration and used in the alleviation of the symptom of intermittent claudication in individuals with peripheral vascular disease [5]. Cilostazol and several of its metabolites are phosphodiesterase (PDE) inhibitors, which selectively inhibits PDE3, suppressing cAMP degradation with a resultant increase in cAMP in platelets and blood vessels, leading to inhibition of platelet aggregation and vasodilation [6]. It has been reported that cilostazol activates phosphorylation of cAMP response element-binding protein (CREB) in various cell types and tissues [7]. Anti-inflammation property has been found for cilostazol in various tissues [8]. The findings of anti-inflammatory properties imply that cilostazol might have a potential therapeutic effect in OA. In this study, we reported that cilostazol is able to suppress the degradation of type II collagen in human chondrocytes. Mechanistically, cilostazol treatment inhibits the expression of IRF-1, thereby

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prevents the induction of MMP-13 as well as the degradation of type II collagen.

2. Materials and methods

2.1. Human cartilage samples

Human subject researches were guided by the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. In addition, this study was approved by the ethics committee of Shantou University. Written informed consent was obtained from each participant. Normal specimens of knee joint cartilage were from normal femoral heads of patients undergoing prosthetic replacement due to fracture of the femoral neck or distal femoral tumor. Isolated chondrocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin–streptomycin) in a humid incubator with 5% CO_2 at 37 °C. Normal human chondrocytes were pretreated with or without cilostazol at the concentrations of 5, 10, 20 μM for 24 h, followed by incubated with IL-1β (10 ng/ml, R&D Systems) for another 24 h.

2.2. Real time polymerase chain reaction (PCR) analysis

Total RNA was isolated using Qiazol (Qiagen, USA) from cells according to the manufacturer's instruction. RNA concentration and quality were determined by absorbance at 260 and 280 nm. First-strand cDNA was synthesized using reverse transcriptional reaction with iScript reverse transcription kit (Bio-Rad). Real time PCR was carried out by a StepOnePlus Realtime PCR System using TaqMan gene expression assays (Applied Biosystems) in a 20-μl reaction volume. The comparison has been conducted by using the ΔΔCT method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primer sequences used for real time PCR were: IRF-1 (human): forward, 5′-CTGGAGAGGGTCTCGCTGT-3′; reverse, 5′-TTCTGGGGTCACTGGTCTGT-3′; MMP-13 (human): 5′-GCT TAG AGG TGA CTG GCA AC-3′, 5′-CCG GTG TAG GTG TAG ATA GGA AC-3′; GAPDH (human): 5′-GAA GGC CAT GCC AGT GAG CTT CC-3′, 5′-CCA TCA ACG ACC CCT TCA TTG ACC-3′.

2.3. Western blot analysis

Cells were harvested and resuspended with cell lysis buffer (Cell signaling, USA).

Protein concentration was determined by the bicinchoninic acid assay (BCA assay).

The protein lysates were subject to SDS–polyacrylamide gel electrophoresis using 10% gels and transferred to a high-quality polyvinylidene difluoride (PVDF) membrane [9]. Membranes were blocked with 5% non-fat milk in Tris buffered saline for 1 h then incubated with primary antibodies at 4 °C overnight. Horseradish peroxidase-conjugated secondary antibody was then applied and the blots were developed by using enhanced chemiluminescence (Roche, Germany) according to the manufacturer's protocol. Images were scanned and band intensities were analyzed with the ImageJ software (National Institute of Health, USA).

2.4. Immunocytochemistry

After indicated treatment, the cells were fixed by incubating them in 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature (RT), followed by incubated in 0.1% Triton X-100 in PBS for 15 min at RT. Then cells were blocked in 10% goat serum in PBS for 1 h at RT. Then cells were sequentially incubated with the primary antibody/antibodies at 4 °C overnight and the Alexa-594-conjugated secondary antibodies for 1 h at RT [10]. Fluorescence signals were observed using a deconvolution fluorescence microscope system (BZ-8000, Keyence, Japan).

2.5. Statistical analysis

Data in this study are shown as mean ± SEM from at least three independent experiments. Statistical comparison between different groups was performed using one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test using SPSS software (version 11; SPSS, USA). Differences were considered significant at $P < 0.05$.

3. Results

Cilostazol is a quinolinone-derivative medication, the molecular structure of which is shown in Fig. 1A. One of the objectives of this study was to investigate the contributory roles of cilostazol in inhibiting degradation of collagen type II. To address this, we examined the effects of cilostazol on IL-1β-induced degradation of collagen type II. Data in Fig. 1B showed that cilostazol prevented IL-1β-mediated decrease of collagen type II in a dose dependent manner.

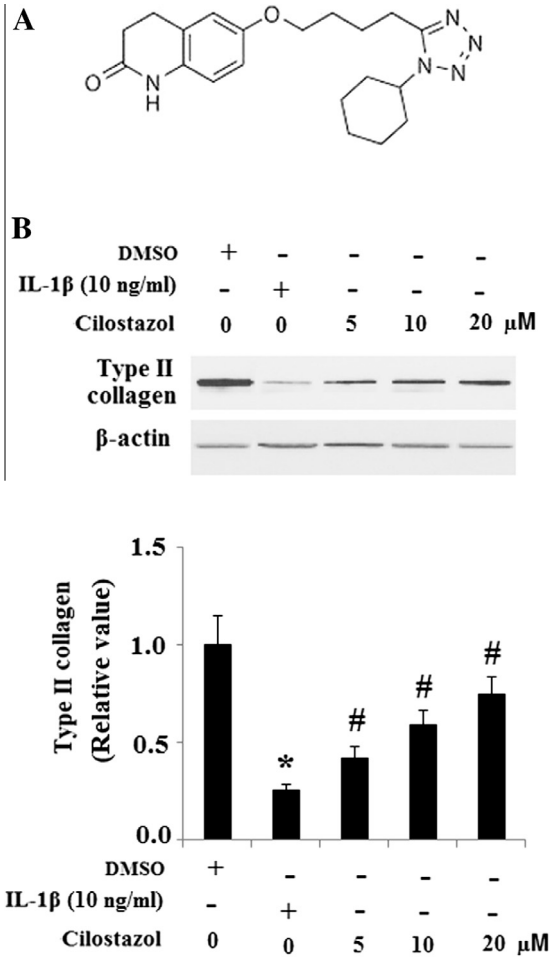

Fig. 1. Cilostazol ameliorates the degradation of collagen type II induced by IL-1β in human chondrocytes. (A) Molecular structure of cilostazol; (B) Normal human chondrocytes were pretreated with various doses of cilostazol or the solvent, DMSO, for 24 h and then stimulated with IL-1β for 24 h. The levels of collagen type II in total cell lysates were measured by Western blot analysis ($P < 0.01$ vs control group; $^{\#}P < 0.01$ vs IL-1β treated group).

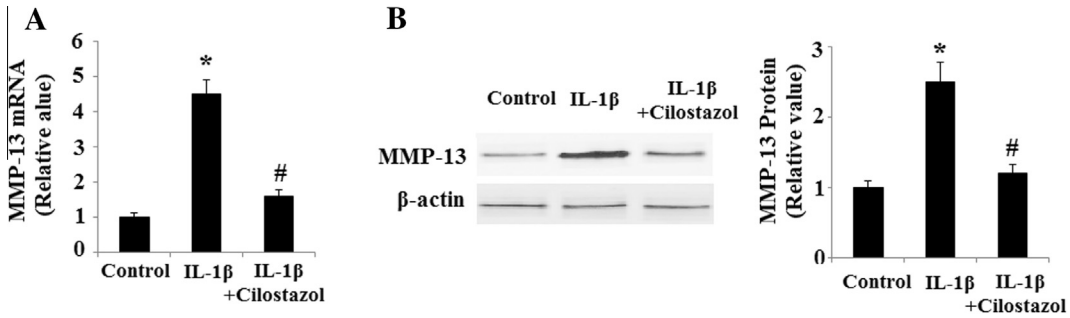

Fig. 2. Cilostazol ameliorates the expression of MMP-13 induced IL-1β. Normal human chondrocytes were pretreated with cilostazol (10 μM) or the solvent, DMSO, for 24 h and then stimulated with IL-1β (10 ng/ml) for another 24 h and (A) the expression of MMP-13 at the mRNA level was determined by real time PCR ($P < 0.01$ vs control group; $^{\#}P < 0.01$ vs IL-1β treated group). (B) The expression of MMP-13 at the protein level was determined by Western blot analysis ($P < 0.01$ vs control group; $^{\#}P < 0.01$ vs IL-1β treated group).

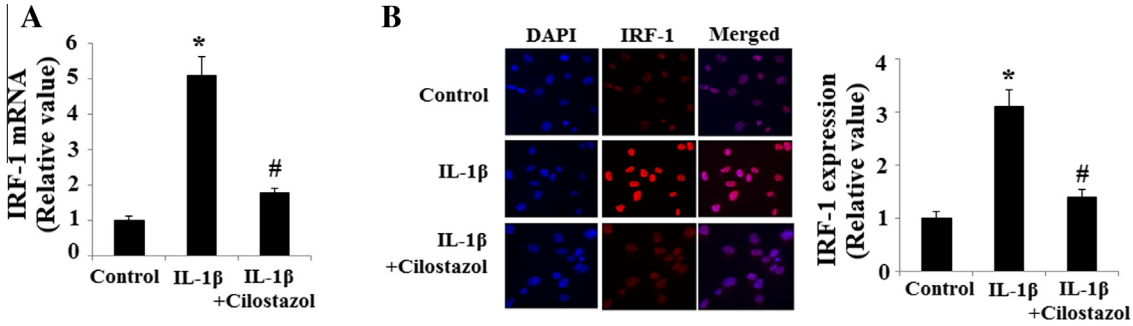

Fig. 3. Cilostazol mitigates the upregulation of interferon response factor-1 (IRF-1) induced by IL-1β. Normal human chondrocytes were pretreated with cilostazol (10 μM) or the solvent, DMSO, for another 24 h and then stimulated with IL-1β (10 ng/ml) for 24 h and (A) the expression of IRF-1 at the mRNA level was determined by real time PCR ($P < 0.01$ vs control group; $^{\#}P < 0.01$ vs IL-1β treated group). (B) The expression of IRF-1 at the protein level was determined by immunofluorescence.

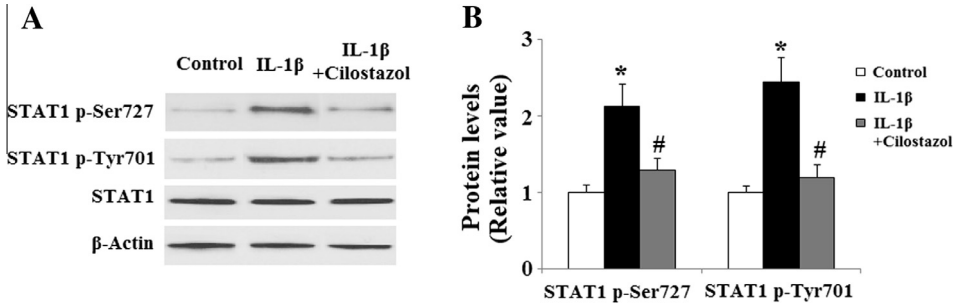

Fig. 4. Cilostazol prevents the activation of STAT1. Normal human chondrocytes were pretreated with cilostazol (10 ng/ml) or the solvent, DMSO, for 24 h and then stimulated with IL-1β (10 ng/ml) for another 24 h. Immunoblot and quantification analyses revealed that pretreatment with cilostazol mitigated the increased phosphorylation of STAT1 at Ser727 and Tyr701; (A) representative immunoblot bands; (B) quantification analyses ($P < 0.01$ vs control group; $^{\#}P < 0.01$ vs IL-1β treated group).

MMP-13 is directly responsible for damaging cartilage matrix. Importantly, collagen type II is preferentially cleaved by MMP-13. We then determined the effects of cilostazol on IL-1β-induced increase in MMP-13 expression. Our results indicated that IL-1β-induced expression of MMP-13 was suppressed by cilostazol (10 μM) at both the mRNA levels (Fig. 2A) and the protein levels (Fig. 2B). IRF-1 is responsible for the transcriptional regulation of MMP-13. Therefore, we checked the effect of cilostazol on the change in IRF-1 expression upon IL-1β treatment. As shown in Fig. 3A, real time PCR results showed that pretreatment with cilostazol (10 μM) suppressed the increase in IRF-1 induced by IL-1β in a dose-dependent manner at mRNA levels. The immunofluorescence study verified this finding at the protein levels.

Signal transducers and activator of transcription 1 (STAT1) has been reported to play an essential role in regulating the activation of IRF-1 [11]. In addition, it has been reported that cAMP could completely inhibit STAT1 activation [12]. Thus, we speculate that STAT1 activation participates in the regulatory roles of cilostazol in IRF-1 expression. Serine phosphorylation of STAT1 at amino acid 727 (Ser727) plays a critical role in the interaction of STAT1 with transcriptional co-activators. Our results indicated that cilostazol mitigated the phosphorylation of STAT1 at Ser727 (Fig. 4A). Moreover, tyrosine phosphorylation of STAT1 at position 701 (Tyr701) is an indispensable signature of STAT1 activation. Our results also indicated that cilostazol ameliorated tyrosine phosphorylation of STAT1 at amino acid 701 (Tyr701) induced

by IL-1β. These findings suggest that cilostazol might prevent the expression of IRF-1 and MMP-13 through inhibiting the activation of STAT1.

4. Discussion

The progressive degeneration of articular cartilage is a critical event in the pathogenesis of Osteoarthritis (OA). There is no therapy currently available, which can completely prevent the initiation or progression of the disease partly due to poor understanding of the mechanisms of the disease pathology. Chondrocyte is the only one cell type in articular cartilage. The remainder in cartilage is occupied by an extensive extracellular matrix. The structural backbone of this matrix is the collagen fibril, which is composed mainly of type II collagen [13]. Increasing evidence shows a measurable increase in type II collagen denaturation in early OA with a net loss of this molecule accompanying this damage [14]. The degradative changes involving collagen accompany the loss of proteoglycan, all of this usually starting at the articular surface [15]. MMPs play an essential role in the degradation. It has been shown that MMP-13 is probably the collagenase which plays the greatest part in the pathology of OA degrading the "resident" collagen fibrils more remote from the cell in the territorial and interterritorial matrix [16]. The initial cleavage by collagenases causes denaturation and loss of collagen type II and phenotypic change of chondrocytes. The activation of chondrocytes by these matrix degradation products involves the expression of IL-1β and TNF-α, which are directly involved in the cleavage of collagen and appear to have a key role in cartilage degeneration in OA. Studies have reported that IL-1β is actively generated by chondrocytes in a majority of patients. Inhibition of cytokines such as IL-1β is able to arrest the resultant degradation of extracellular matrix. Notably, a recent study reported that concurrent administration of cilostazol and methotrexate suppressed production of cytokines, including TNF-α, IL-1β, IL-6 and CCL2 in an additive manner, with prevention of joint damage in rheumatoid arthritis (RA) via the interactive action of adenosine A2A receptors and cAMP–protein kinase [17].

MMP-13 is selectively modulated by IRF-1. IRF-1 expression is up-regulated in many chronic inflammatory diseases [18]. A recent study reported the elevated expression of IRF-1 in OA chondrocytes [19]. However, little information with regarding to the role of IRF-1 has been reported in chondrocytes. Our finding that cilostazol could suppress IL-1β-induced activation of IRF-1 might suggest its significance in IL-1β-mediated damage of cartilage. The expression of IRF-1 is regulated by activation of STAT1 through phosphorylation at Ser727. Consistent with our finding, it has been recently shown that MMP-13 induction by IL-1β is mediated by STAT1 in chondrocytes [20]. Our results indicated that treatment with cilostazol is able to suppress the induction of IRF-1 by inhibiting the activation of STAT1. In addition to IRF-1, there are the other two main transcriptional factors are involved in inflammatory pathways of IL-1β, c-Jun and NF-κB [21]. Importantly, cilostazol has displayed its anti-inflammatory property and this property is ascribed to cAMP-dependent protein kinase activation coupled suppression of NF-κB gene transcription [22]. Another report showed that cilostazol significantly suppressed LPS-stimulated increase of TLR4 expression by blocking PU.1 transcriptional activity in RA macrophages. Cilostazol also suppressed LPS-induced increase of cytokine production (TNF-α, IL-1β) by inhibiting IkBα degradation and NF-κB p65 nuclear translocation. [23].

Taken together, the present results demonstrated that cilostazol suppressed IL-1β-induced degradation of collagen type II and damage of cartilage. Mechanistically, cilostazol mitigated MMP-13 expression by suppressing STAT1/IRF-1 activity. Future study will provide further information to confirm the therapeutic potentials of cilostazol in patients with OA.

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