Recombinant human irisin regulated collagen II, matrix metalloproteinase-13 and the Wnt/β-catenin and NF-kB signaling pathways in interleukin-1β-induced human SW1353 cells

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Received January 20, 2019; Accepted November 27, 2019

DOI: 10.3892/etm.2020.8562

Abstract. Osteoarthritis (OA) is a degenerative joint disease that seriously affects the quality of life of patients. Irisin has been reported to regulate bone metabolism via the cellular autocrine mechanism and play a protective role in rat OA. In the present study, a SW1353 chondrosarcoma cell line was treated with interleukin (IL)-1β and irisin. The present study evaluated cell viability, expression levels of collagen II (Col II) and matrix metalloproteinase-13 (MMP-13), and activity of the Wnt/β-catenin and NF-κB signaling pathways in treated SW1353 cells. The present results suggested that IL-1β could decrease Col II expression and increase MMP-13 expression at both the mRNA and protein levels, and also activate the Wnt/β-catenin and NF-κB signaling pathways in SW1353 cells. By contrast, irisin was identified to reverse the effects of IL-1β in IL-1β-induced SW1353 cells. The present results suggested that irisin treatment may have a cartilage-protective role in an IL-1β-induced SW1353 cell model.

Introduction

Osteoarthritis (OA), is the most prevalent joint disease characterized by loss of cartilage, subchondral bone sclerosis or cyst and osteophyte formation (1). The clinical manifestations of OA include joint stiffness, chronic pain and limited movement (2). Traditional treatment can only temporarily relieve clinical symptoms, and cannot effectively inhibit the pathological progress of OA (3). Therefore, it is important to understand the pathogenesis of OA and investigate novel safe and effective treatments. Degeneration of articular cartilage is one of the major pathological changes in OA (3). Many cytokines, growth factors and enzymes, such as interleukin (IL)-1β and collagenase, are involved in articular cartilage degeneration (4). IL-1β is secreted by synovial cells and OA inflammatory cells, and stimulates the production of proteolytic enzymes, such as matrix degrading enzymes and collagenase, causing synovial inflammation and bone resorption (4). Collagenase is upregulated in OA cartilage, and intra-articular injection of collagenase has successfully established an animal model of OA (4). In addition, matrix metalloproteinases (MMPs) have been shown to play an important role in the pathogenesis of OA (4). Inflammatory cytokines increase the secretion of MMP-13, and promote the degradation of collagen II (Col II) in chondrocytes, leading to the occurrence of OA (5).

Irisin, a myokine produced by skeletal muscle in response to physical exercise, promotes transdifferentiation of white adipose tissue into brown adipose tissue (6). Previous studies have suggested that irisin is also involved in the control of bone metabolism. Faienza et al (7) identified that irisin influences the treatment of pediatric patients with type 1 diabetes and promotes pediatric bone health. Previous studies have shown that irisin can directly enhance osteogenic differentiation of bone stromal cells and improve cortical quality (8). In addition, previous studies have suggested that irisin can activate the Wnt/β-catenin signaling pathway in MC3T3-E1 cells to promote osteoblast differentiation in OA mice (9,10). A previous study reported that irisin inhibits osteoclast differentiation by inhibiting the receptor of nuclear factor C1 of T cells activated by NF-κB ligand in RAW264.7 cells (9). Irisin can effectively enhance the osteogenesis process and reduce the occurrence of osteoporosis and fracture (9).

Many signaling pathways regulating joint formation and homeostasis are thought to be key factors in the pathogenesis of OA (10). The Wnt/β-catenin signaling pathway is considered to be one of the most important pathways associated with postnatal metabolism of articular cartilage matrix, differentiation and apoptosis of articular chondrocytes (10,11). β-catenin is a key factor in the Wnt/β-catenin signaling pathway and its expression level in the nucleus directly reflects the activation level of this signaling pathway (11). When the Wnt/β-catenin signaling pathway is activated, β-catenin can regulate the function of chondrocytes and change their physiological state, resulting in OA and other related diseases (10).
The SW1353 cell line was initiated in 1977, and was later considered to be a valuable in vitro system for investigating catabolic gene regulation with IL-1β, tumor necrosis factor-α and fibroblast growth factors (12,13). At present, previous studies have focused on the bone and subchondral bone in OA joints. To the best of our knowledge, there are no studies investigating whether irisin directly acts on cartilage and plays a protective role in the process of OA. Furthermore, to the best of our knowledge, there are no data showing the close interaction between irisin and the Wnt/β-catenin and NF-kB signaling pathways in SW1353 cells. The present results suggested that irisin inhibited the Wnt/β-catenin and NF-kB signaling pathways in SW1353 cells.

Materials and methods

Materials. Recombinant human full-length irisin protein (112 amino acids, FNDC5 sequence 32-143) was purchased from Phoenix Pharmaceuticals, Inc. Recombinant human IL-1β was purchased from Bio-Techne, and lithium chloride (LiCl; molecular weight, 42,39400) was purchased from Shanghai Mintchem Development Co., Ltd.

Cell culture. The chondrosarcoma cell line SW1353, originating from a 72-year-old woman, was purchased from Procell Life Science & Technology, Co., Ltd. Cells were cultured with DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO₂ incubator at 37°C.

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 kit (Gibco; Thermo Fisher Scientific, Inc.) was used to evaluate the cytotoxicity of IL-1β and irisin. The experiment was performed according to the manufacturer's instructions. Cells (100 µl/well; ~5,000/well) were incubated for 4 h in 96-well plates in a humidified incubator (at 37°C; 5% CO₂). Different concentrations of IL-1β (0, 10, 20 or 50 ng/ml) or irisin (0, 10, 20, 50 or 100 mM) were added for 12, 24, 36 or 48 h. Then, 10 µl of CCK-8 solution was added to each well of the plate using a repeating pipettor and incubated at 37°C for 4 h. The optical density was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Model 550; Bio-Rad Laboratories, Inc.).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). SW1353 cells (5 x 10⁵ in each dish) were seeded in a 6 cm dish and treated with 10 ng/ml IL-1β and/or 20 mM irisin at 37°C for 24 h, then RIPA lysis buffer (Cell Signaling Technology, Inc.) was used to extract protein. Protein concentration was determined using a bicinchoninic acid kit (Sigma-Aldrich; Merck KGaA). A total of 20 µg of protein was loaded into each well of a 10% SDS-PAGE gel. After gel electrophoresis, the protein bands were separated from the gel, transferred to PVDF membranes after blocking with 5% non-fat milk at room temperature for 1 h by transfer electrophoresis and then incubated at 37°C for 1 h with the following antibodies: MMP-13 (1:10,000; cat. no. DF6494; Affinity Biosciences), Co1II (1:10,000 dilution; cat. no. AF5456; Affinity Biosciences), phospho-NFκB (1:10,000; cat. no. AF2006; Affinity Biosciences), inhibitor of NF-kB (100 µM) α (1:10,000; cat. no. AF002; Affinity Biosciences), Wnt-1 (1:10,000; cat. no. DF514; Affinity Biosciences) and β-catenin (1:2,000; cat. no. ab1008; Abcam). β-actin (cat. no. ab5051, Abcam) was used as the endogenous control. The membranes were then incubated with a goat anti-rabbit secondary antibody (1:10,000; cat. no. ab97051; Abcam) at 37°C for 1 h. Protein bands were visualized with an ECL kit (Abcam). The data of study groups were quantitatively analyzed relative to the NC group using SPSS 19.0 (IBM Corp.).

Immunofluorescence analysis. SW1353 cells (6 x 10⁴) were cultured in a 24-well plate and treated with 10 ng/ml IL-1β and/or 20 mM irisin at 37°C for 24 h. After the culture solution was aspirated, cells were washed with PBS, and fixed with acetone at 4°C for 10 min, and blocked with 5% BSA in PBS at 37°C for 60 min. After the treatment with the primary antibody (rabbit LC3 antibody; 1:250; cat. no. DF674; Affinity Biosciences) and the secondary antibody (anti-rabbit IgG; 1:500; cat. no. AF313; Affinity Biosciences) at 37°C for 1 h, the DAPI staining solution was added dropwise at 37°C for 10 min, and the images were taken using a fluorescence microscope (magnification, 400x; Carl Zeiss AG).

Activation of the Wnt/β-catenin signaling pathway. LiCl is a specific activator of the Wnt/β-catenin signaling pathway. To investigate the effect of irisin on the activated Wnt/β-catenin signaling pathway in a non-inflammatory environment, the cells were pretreated with 10 mM LiCl at 37°C for 24 h and treated with 20 mM irisin at 37°C for a further 24 h. Phosphorylated-p65 (p-p65) primary antibody (1:500; cat. no. BC336) was purchased from Affinity Biosciences.

Statistical analysis. Data were analyzed using SPSS software version 12.0 (SPSS, Inc.) and are presented as the mean ± SD.
Table I. Primers used for reverse transcription-quantitative PCR.

| Gene       | Primer sequence (5'-3') | Length, bp |
|------------|-------------------------|------------|
| MMP-13     | F: ACCCAAACCTAAACATCC  | 155        |
|            | R: CGTTAAAAACAGCTCCGA  |            |
| Collagen II| F: TGGTCTGGGGTCTTTC    | 172        |
|            | R: CTTGATCAGCTGTTTTC   |            |
| β-catenin  | F: CAGTGGATTTTGTTT     | 170        |
|            | R: ATTGGAGACCTGTTGTT   |            |
| Wnt-1      | F: CACAAACGCTGCCC      | 142        |
|            | R: GCAGCTCGACGCCGTC    |            |
| β-actin    | F: CCAAGGCAACGGCGGAGAA | 187        |
|            | R: GCATGGGGGAGGGCATA   |            |

F, forward; R, reverse; MMP-13, matrix metalloproteinase-13.

Graphs were drawn using GraphPad Prism 7.00 (GraphPad Software, Inc.). One-way ANOVA with subsequent Tukey’s test was used for multiple comparisons. The experiment was repeated three times in each group. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of irisin and IL-1β on cell viability of SW1353 cells. Effects of irisin and IL-1β on the cytotoxicity of SW1353 cells were evaluated by CCK-8 assay. Irisin at concentrations of 0, 10, 20, 50 and 100 mM did not have a significant effect on cell viability, after 24 h (Fig. 1A) or 48 h (Fig. 1B) of incubation. The concentration of 20 mM was chosen for the following experiments as previously described.[12]. Furthermore, IL-1β at concentrations of 0, 5, 10, 20 and 50 ng/ml led to no difference in cell viability at 12 h (Fig. 1C) or 24 h (Fig. 1D). However, at 36 h (Fig. 1E) and 48 h (Fig. 1F) of incubation, IL-1β (10, 20 and 50 ng/ml) significantly decreased cell viability (P<0.05, P<0.001, P<0.001), and the effects of 50 ng/ml IL-1β were more significant (P<0.001). Therefore, 10 ng/ml IL-1β was used to stimulate cells.

Effect of irisin on IL-1β-induced expression of MMP-13 and Col II in SW1353 cells. The present study investigated the effect of irisin on IL-1β-induced MMP-13 and Col II expression levels using RT-qPCR, western blotting and immunofluorescence analysis. The present results indicated that the expression of Col II at the protein (Fig. 2A and C) and mRNA (Fig. 2D) levels was significantly decreased by IL-1β treatment, whereas irisin treatment reversed the IL-1β induced-decrease of Col II expression levels (P<0.05). Moreover, the expression of MMP-13 at the protein (Fig. 2B and C) and mRNA (Fig. 2E) levels in the SW1353 cells was upregulated by IL-1β treatment, and the effect of IL-1β was reversed by irisin treatment (P<0.05). Immunofluorescence analysis supported the present RT-qPCR results. The expression level of MMP-13 in SW1353 cells was downregulated after irisin treatment, whereas IL-1β treatment upregulated the expression level of MMP-13 in SW1353 cells compared with the control groups (Fig. 3A). However, the expression level of Col II in SW1353 cells was downregulated following IL-1β intervention, and subsequently upregulated by the irisin treatment compared with the control group (Fig. 3B).

Effect of irisin on IL-1β-induced activation of the Wnt/β-catenin signaling pathway in SW1353 cells. To study the anti-inflammatory mechanism of irisin, western blotting and RT-qPCR were used to investigated its effects on IL-1β-induced activation of the Wnt/β-catenin signaling pathway in SW1353 cells. The present results suggested that, compared with the negative control group, the protein and mRNA expression levels of β-catenin (Fig. 4A, C and D) and Wnt-1 (Fig. 4B, C and E) in SW1353 cells were upregulated by IL-1β treatment (P<0.05), indicating the activation of the Wnt/β-catenin signaling pathway. However, following irisin treatment, IL-1β-induced activation was suppressed (P<0.05). The present results suggested the IL-1β-induced activity of the Wnt/β-catenin signaling pathway was significantly decreased by irisin treatment.

Effect of irisin on the Wnt/β-catenin signaling pathway. LiCl significantly increased the mRNA expression levels of β-catenin (Fig. 5A) and Wnt-1 (Fig. 5B; P<0.05). Irisin treatment significantly decreased the expression levels of Wnt-1 and β-catenin that were induced by LiCl (P<0.05). SW1353 cells co-cultured with IL-1β and LiCl were also treated with irisin, and the present results indicated that treatment with irisin decreased the expression levels of β-catenin and Wnt-1 (Fig. 5C and D). The present results suggested that irisin may exerted its functions in SW1353 cells by inhibiting the Wnt/β-catenin signaling pathway (Fig. 5).

Effect of irisin on IL-1β-induced activation of NF-κB signaling in SW1353 cells. To investigate the effect of irisin on the NF-κB signaling pathway in IL-1β-induced SW1353 cells, the changes in IκBα and phosphorylated-p65 (p-p65) were detected by western blot analysis (Fig. 6C). Statistical analysis showed that the expression levels of p-p65 (Fig. 6A) and IκBα (Fig. 6B) were significantly increased by IL-1β, but were significantly decreased after irisin treatment compared with the IL-1β group. Therefore, the present results indicated that irisin could inhibit the level of cytoplasmic p-p65 and downregulate the activity of the NF-κB pathway.

Discussion

Different types of SW1353 cells have been applied in cell models of OA. Huang et al (14) utilized and treated SW1353 cells with IL-1β to imitate the microenvironment of OA for in vitro experiment. Feng et al (15) applied human SW1353 chondrocytes to evaluate the effect of salicin in OA. Lu et al (16) investigated the chondroprotective role of sesamol by downregulating MMP expression via retention of the NF-κB signaling pathway in activated SW1353 cells. In addition, Tetsunaga et al (17) analyzed the effect of runt-related transcription factor 2 on the mechanical stress-induced MMP-13 and A disintegrin and metalloproteinase with thrombospondin motifs 5 expression in SW1353 chondrocyte-like cells. Cheng et al (18) successfully established a cellular model
of OA by stimulating SW1353 cells with IL-1β. All these previous studies have shown that SW1353 cells are a feasible cell line with which to establish OA models.

The present study used RT-qPCR, western blotting and immunofluorescence analysis to investigate whether irisin can protect against OA induced by IL-1β in SW1353 cells. Previous studies have confirmed that IL-1β plays an important role in the development of OA and can stimulate chondrocytes to produce OA (19,20). The present study performed CCK-8 analysis to identify the optimum concentration of IL-1β for modeling OA. Cartilage degradation in OA is mediated by the MMP family (21). MMP-13, as a member of the MMP family, can degrade collagen and reduce cartilage composition (22). High levels of MMPs are considered to be one of the
Figure 3. Immunofluorescence assay of the expression levels of (A) MMP-13 and (B) Col II. SW1353 cells were treated with different concentrations of 20 mM irisin for 6 h, with or without IL-1β. The expression levels of MMP-13 and Col II were visualized using immunofluorescence. n=3/group. Scale bar, 10 µm. IL-1β, interleukin -1β; MMP-13, matrix metalloproteinase-13; Col-II, collagen II; NC, negative control.

Figure 4. Effect of irisin on the activation of the Wnt/β-catenin signaling pathway in IL-1β-induced SW1353 cells. SW1353 cells were treated with 20 mM irisin for 24 h, with or without IL-1β, and (A) β-catenin protein levels, (B) Wnt-1 protein levels and (C) β-catenin and Wnt-1 protein expression were evaluated by western blot analysis. n=5/group. mRNA expression levels of (D) β-catenin and (E) Wnt-1 were evaluated by reverse transcription-quantitative PCR. n=5/group. *P<0.05, **P<0.01 and ***P<0.001. IL-1β, interleukin-1β; NC, negative control.
Figure 5. Effect of irisin on SW1353 cells after the addition of a Wnt/β-catenin signaling pathway activator. SW1353 cells were treated with 10 mM LiCl for 24 h, with or without IL-1β. mRNA expression levels of (A) β-catenin and (B) Wnt-1 were evaluated by RT-qPCR. n=5/group. SW1353 cells were treated separately with 10 ng/ml IL-1β, 10 ng/ml IL-1β + 10 mM LiCl or 10 ng/ml IL-1β + 10 mM LiCl + 20 mM irisin for 24 h. **P<0.01. mRNA expression levels of (C) β-catenin and (D) Wnt-1 were evaluated by RT-qPCR. n=5/group. **P<0.01 vs. NC group; #P<0.05, ##P<0.01, ###P<0.001 vs. IL-1β+LiCl group. RT-qPCR, reverse transcription-quantitative PCR; IL-1β, interleukin-1β; NC, negative control; LiCl, lithium chloride.

Figure 6. Effect of irisin on IL-1β-induced activation of NF-κB signaling in SW1353 cells. SW1353 cells were treated with 20 mM irisin for 24 h, with or without IL-1β. Protein expression levels of (A) p-p65 and (B) IκBα were evaluated by (C) western blot analysis. n=5/group. **P<0.01 vs. the NC group/IL-1β group. IκBα, inhibitor of NF-κB α; p-p65, phosphorylated p65; NC, negative control; IL-1β, interleukin-1β.
main characteristics of OA (22,23), which can combine with low levels of chondrocyte-specific proteins, such as Sox9 and collagen II (24), leading to cartilage degeneration. Therefore, it is important to regulate the expression levels of MMPs to facilitate the prevention and treatment of OA. The present results indicated that five different concentrations of irisin did not affect the viability of SW1353 cells. However, treatment with 20 mM irisin significantly reduced the expression levels of MMP-13 in SW1353 cells. In addition, irisin treatment increased the expression level of Col-II. The present results suggested that irisin exerted its protective effects by decreasing the expression level of MMP-13 and increasing the expression level of Col-II in IL-1β-induced SW1353 cells.

Previous study has shown that exercise causes production of skeletal muscle and release of irisin into the blood, which has beneficial effects in increasing bone mass, lowering body mass index, increasing insulin sensitivity and releasing total body energy (25). Colaianni et al (25) found that, compared with a restricted exercise group, the expression of irisin in mice after 3 weeks of treadmill exercise was increased significantly at both the mRNA and protein levels. A recent study showed that irisin can directly act on bone stromal cells and enhance osteogenic differentiation (25). A clinical investigation into OA showed that irisin expression levels in circulating and synovial fluids, and CRP are elevated in patients with severe OA (26), suggesting that irisin is associated with OA. Cartilage degeneration is an important part of the pathogenesis of OA (26). To the best of our knowledge, the present study is the first to investigate the direct effects of irisin on IL-1β-induced SW1353 cells.

Studies have shown that several signaling pathways regulating joint formation and homeostasis are key factors in the pathogenesis of OA (26). The NF-κB and Wnt/β-catenin signaling pathways are two major pathways involved in OA development (26). Wnt protein is a classical activator of the Wnt pathway, and can regulate the expression level of Col-II in IL-1β-induced SW1353 cells. The present results indicated that irisin downregulated IL-1β-induced upregulation of Wnt-1 and β-catenin at both protein and mRNA expression levels in SW1353 cells. Therefore, the present results suggested that irisin could inhibit the Wnt/β-catenin signaling pathway. LiCl acts as a classical activator of the Wnt pathway, and can regulate GSK-3β (27). The primary role of GSK-3β is to phosphorylate free β-catenin (27). The present results suggested that irisin inhibited the Wnt pathway activated by LiCl, and this effect was also identified with irisin-treated cells co-treated with IL-1β and LiCl. Therefore, the present results suggested that irisin regulated the Wnt/β-catenin signaling pathway by inhibiting the phosphorylation of β-catenin.

The NF-κB pathway is another important pathway in the OA process, and could be a potential therapeutic target for OA (28). When the NF-κB pathway is activated by inflammatory mediators such as IL-1β, NF-κB-p65 in the cytoplasm, it is then translocated into the nucleus where it upregulates multiple inflammation-related genes, such as MMPs, cyclooxygenase-2 and prostaglandin E2 (28). exposed chondrocytes to a variety of inflammatory cytokines leads to the degradation of IkB and further translocation of p65 into the nucleus (29). In the present study, western blot analysis indicated that IL-1β induced a significant increase in IkBα and p-p65 expression levels in SW1353 cells; however, this effect was reversed by irisin treatment. The present results suggested that irisin may reduce p-p65 in the cytoplasm to inhibit the NF-κB pathway, activated by inflammatory factors. Previous studies have shown that there is some crosstalk between the Wnt/β-catenin and NF-κB pathways (28,29). Activation of the Wnt/β-catenin pathway can enhance β-transducing repeat-containing protein-mediated degradation of IkB, and IkB kinase can also inhibit the degradation of β-catenin protein (29). However, the exact target of irisin in mitigating the IL-1β-induced inflammatory response is still unclear, and future studies will need investigate this in depth.

In conclusion, the present results suggested that irisin suppressed the activation of the Wnt/β-catenin and NF-κB signaling pathways, and may facilitate the treatment of OA.

Acknowledgements

Not applicable.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81660373).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors’ contributions

XJL, YM and QHJ were responsible for the acquisition and interpretation of data. YL, QL and SW conducted the conception and design of the study, drafted the article. All authors read and approved final version of the manuscript.
Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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