Original Paper

Morin Exhibits Anti-Inflammatory Effects on IL-1 β -Stimulated Human Osteoarthritis Chondrocytes by Activating the Nrf2 Signaling Pathway

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Key Words

Morin • Chondrocytes • IL-1 β • NF- κ B • Nrf2

Abstract

Background/Aims: Osteoarthritis (OA) is a multifactorial disease that is associated with inflammation in joints. The purpose of the present study was to investigate the anti-inflammatory activity and mechanism of morin on human osteoarthritis chondrocytes stimulated by IL-1 β . **Methods:** The levels of NO and PGE2 were measured by the Griess method and ELISA. The levels of MMP1, MMP3, and MMP13 were also measured by ELISA. **Results:** The results revealed that IL-1 β significantly increased the production of NO, PGE2, MMP1, MMP3, and MMP13. Additionally, the increases were significantly attenuated by treatment with morin. Furthermore, IL-1 β -induced NF- κ B activation was suppressed by morin. In addition, the expression of Nrf2 and HO-1 were increased by morin and knockdown of Nrf2 could prevent the anti-inflammatory effects of morin. **Conclusion:** In conclusion, this study suggested that morin attenuated IL-1 β -induced inflammation by activating the Nrf2 signaling pathway.

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Introduction

Osteoarthritis (OA) is a common form of arthritis that often leads to pain and disability in older people [1]. OA, also called degenerative joint disease, is a disease of the whole joint in which all articular structures are affected [2]. It is a chronic articular disease that affects millions of people in the world that is characterized by a loss of articular cartilage leading to narrowing of the joint space, increased joint friction, persistent pain, and functional impairment [3]. Until now, pharmacologic and nonpharmacologic modalities were commonly used to manage OA [4]. Among the drugs used for the treatment of OA, traditional NSAIDs and COX-2 selective inhibitors are commonly used [5]. Previous studies have shown that inhibition of COX-2 could prevent cartilage damage and protect against OA

[6]. The pathogenesis and mechanism of OA have not been clarified yet. In addition, a large body of previous studies have indicated that inflammation was involved in the pathogenesis of OA [7, 8]. Increased inflammatory cytokines such as TNF- α and IL-1 β were observed in patients of OA [9]. These inflammatory cytokines led to the degeneration of cartilage [10]. Previous studies have shown that these cytokines could lead to the production of matrix metalloproteinases (MMP), which play an important role in cartilage destruction [11]. Therefore, inhibition of these inflammatory cytokines could attenuate the injury to articular cartilage.

Herbal medicines have long been used in the treatment of OA [12]. Accumulated evidences suggested that herbal medicines could attenuate inflammatory response and pain in the joints [13]. Morin, a natural flavonoid that isolated from the Chinese herbs of the *Moraceae* family, has been known to possess anti-inflammatory effects [14]. Morin has been reported to attenuate inflammatory mediator production in LPS-stimulated bovine mammary epithelial cells [15]. Also, morin has been reported to protect mice from LPS-induced acute lung injury through inhibiting inflammatory mediator production [16]. A previous study showed that morin inhibited LPS-induced inflammatory mediator production in BV2 microglial cells [17]. Morin also has protective effects against cisplatin-induced kidney injury in mice [18]. In addition, morin has been reported to inhibit IL-1 β -induced inflammatory mediator production in human chondrocytes [19]. However, the anti-inflammatory mechanism of morin on OA remains unclear. The purpose of this present study was to investigate the anti-inflammatory activity and mechanism of morin on human osteoarthritis chondrocytes stimulated by IL-1 β .

Materials and Methods

Chemicals and reagents

Morin (purity>98%) and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human IL-1 β was purchased from R&D systems (Minneapolis, MN, USA). Nrf2 and HO-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). I κ B α , p-I κ B α , NF- κ B p-p65, and NF- κ B p65 were obtained from Cell Signaling Technology (Beverly, MA, USA). NO detection kit was purchased from Beyotime (China). All the ELISA kits were purchased from R&D systems (Minneapolis, MN, USA).

Cell culture

Samples of human articular cartilage were collected from 8 OA patients (age: 54 $\pm$ 8) according to the guidelines of the Declaration of Helsinki and Tokyo. The human chondrocytes were isolated and cultured as described previously [20]. In brief, cartilage was digested with 0.15% type II collagenase at 37°C for 6 h. After centrifugation at 800 g for 8 min, the pellets were collected and resuspended. The cells were cultured with DMEM containing 10% FBS at 37°C with 5% CO₂. The medium was changed every 2 days and passage 2 to 4 were used in the present study [21].

Cell treatment

The cells were pretreated with morin (2.5, 5, 10 μ M) 1 h before IL-1 β treatment. 20 mg morin was dissolved in 200 μ L DMSO and then further dissolved in PBS to the concentration of 2.5, 5, 10 μ M. 24 h after IL-1 β treatment, the supernatants were collected and inflammatory mediator production was detected. 1 h after IL-1 β treatment, the protein was extracted and the NF- κ B and Nrf2 signaling pathways were measured.

MTT assay

The effects of morin on the viability of chondrocytes were measured by MTT assay. In brief, chondrocytes were incubated with different concentrations of morin or morin and IL-1 β for 24 h. Then, MTT was added to each well for 3 h. After that, 150 μ L DMSO was added to each well. Finally, absorption in each well was read at 570 μ m on a microplate reader (TECAN, Austria).

Inflammatory mediator assay

The supernatants were collected 24 h after IL-1 β treatment. The level of NO was measured by Griess reagent (Beyotime) according to the manufacturer's instructions. The level of PGE2 was measured by ELISA (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

MMP assay

24 h after IL-1 β treatment, the supernatants were collected and the production of MMP1, MMP3, and MMP13 in the supernatants was measured by ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Western blot analysis

Western blot analysis was performed to detect the changes in NF- κ B and Nrf2 signaling pathways. Total proteins from chondrocytes were obtained using the M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). The proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes. After blocking, membranes were incubated with primary antibodies at 4°C overnight to detect the target protein, and then the membranes were incubated with secondary antibodies to develop the blots.

Nrf2 siRNA transfection

For Nrf2 siRNA transfection, human chondrocytes were transfected with Nrf2 siRNA and or Nrf2 negative control siRNA using Lipofectamine 2000 according to the manufacturer's instructions. 48 h later, the cells were incubated with morin and IL-1 β .

Statistical analysis

All the data were presented as the mean $\pm$ S.E.M and the data were analyzed using SPSS 17.0 software. Differences among groups were analyzed by one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ were considered to indicate statistical significance.

Results

Effects of morin on chondrocyte viability

The cytotoxicity of morin on chondrocytes was detected at a concentration range from 0 to 15 μ M, using the MTT assay. As shown in Fig. 1A, morin at the concentration of 0-10 μ M did not affect the viability of chondrocytes. Morin at the concentration of 15 μ M decreased the viability of chondrocytes. As shown in Fig. 1B, IL-1 β decreased the viability of chondrocytes and morin did not affect the viability decreased by IL-1 β .

Morin inhibits IL-1 β -induced NO and PGE2 production

In order to assess the anti-inflammatory effects of morin, the levels of NO and PGE2 were measured. The results showed that the levels of NO and PGE2 in IL-1 β treated group were higher than the control group. However, the increases in the production of NO and PGE2 were significantly attenuated by morin (Fig. 2). Treatment of morin (10 μ M) alone did affect the production of inflammatory mediators NO and PGE2.

Fig. 1. Effects of morin and IL-1 β on the cell viability of chondrocytes. The cell viability was determined by MTT assay. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.01$ vs. control group; ^{**} $p < 0.01$ vs. IL-1 β group.

Fig. 2. Morin inhibits IL-1 β -induced NO and PGE₂ production. The data presented are the means $\pm$ SEM of three independent experiments. [#] p <0.01 vs. control group; ^{**} p <0.01 vs. IL-1 β group.

Fig. 3. Morin inhibits IL-1 β -induced MMP1, MMP3, and MMP13 production. The data presented are the means $\pm$ SEM of three independent experiments. [#] p <0.01 vs. control group; ^{**} p <0.01 vs. IL-1 β group.

Morin attenuates MMP1, MMP3, and MMP13 production

Furthermore, we detected the effects of morin on MMP1, MMP3, and MMP13 production by ELISA. The results showed that the levels of MMP1, MMP3, and MMP13 in IL-1 β treated group were higher than the control group. However, the increases in the production of MMP1, MMP3, and MMP13 were significantly attenuated by morin (Fig. 3).

Effects of morin on NF- κ B activation

NF- κ B is involved in the regulation of inflammatory mediator production. Next, we detected the effects of morin on NF- κ B activation. The results showed that the levels of phosphorylation of NF- κ B p65 and I κ B α in IL-1 β treated group were higher than the control group. However, the increases in the expression of NF- κ B p65 and I κ B α were significantly attenuated by morin (Fig. 4).

Effects of morin on Nrf2 and HO-1 expression

To further clarify the anti-inflammatory mechanism of morin, Nrf2 signaling pathway was measured. The results showed that the levels of Nrf2 and HO-1 in IL-1 β treated group were higher than the control group. However, the increases were amplified by morin in a concentration-dependent manner (Fig. 5).

Fig. 4. Morin inhibits IL-1 β -induced NF- κ B activation. The data presented are the means $\pm$ SEM of three independent experiments. [#]p<0.01 vs. control group; ^{**}p<0.01 vs. IL-1 β group.

Fig. 5. Effects of morin on Nrf2 and HO-1 expression. The data presented are the means $\pm$ SEM of three independent experiments. [#]p<0.01 vs. control group; ^{**}p<0.01 vs. IL-1 β group.

The anti-inflammatory effects of morin were Nrf2 dependent

Next, we detected the effects of morin on inflammatory mediator production when Nrf2 was knocked down. As shown in Fig. 6, Nrf2 siRNA reduced the expression of Nrf2. Additionally, the inhibitory effects of morin on NF- κ B activation, as well as NO and PGE2 production, were reduced when Nrf2 was knocked down. The results indicated morin exhibited its anti-inflammatory effects through the Nrf2 signaling pathway.

Fig. 6. The effects of Nrf2 siRNA on Nrf2 expression and the anti-inflammatory effects of morin are Nrf2 dependent. The values presented are the means $\pm$ SEM of three independent experiments. [#] $p < 0.01$ vs. control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. IL-1 β group.

Discussion

Recent studies have demonstrated the anti-inflammatory effects of morin on lung inflammation, mastitis, and OA [16, 22]. However, the anti-inflammatory mechanism of morin on OA remains unclear. In this study, we detected the anti-inflammatory effects and mechanism of morin on OA. We reported the anti-inflammatory role of morin on OA through suppressing inflammatory mediator production. Previous studies showed that some COX-2 selective inhibitors could also inhibit inflammatory cytokine-induced inflammatory response and cartilage damage [6, 23]. These results showed that VA694, Naproxen and Naproxen could inhibit IL-1 β -induced inflammatory response by attenuating NF- κ B activation [24]. The results are also consistent with our study, which showed that morin inhibited IL-1 β -induced inflammation via suppression of NF- κ B activation. However, morin is a compound isolated from natural herbal medicines and these compounds are useful in the development of new drugs. These results indicated that inhibition of NF- κ B activation could protect OA and NF- κ B could be used as a target for the treatment of OA.

Inflammatory cytokines, such as TNF- α and IL-1 β , have been known to be involved in the development of OA [25]. These inflammatory cytokines could induce other inflammatory mediators and amplify the inflammatory response. *In vitro*, studies have shown that stimulation of chondrocytes with IL-1 β could induce the activation of NF- κ B [26]. The activation of NF- κ B could lead to the transcription of its target genes, such as NO, PGE₂, and MMPs [27]. Increased inflammatory mediator levels were observed in synovial fluid from patients with OA [28]. These inflammatory mediators could lead to the injury of joint [29]. In this study, our results showed that these inflammatory mediators were significantly

inhibited by morin.

Nrf2 is an important transcription factor that plays critical roles in the regulation of oxidative response [30]. Recently, Nrf2 has been known to be involved in the regulation of cartilage integrity and has protective effects against OA [31]. A previous study demonstrated that the Nrf2/HO-1 signaling pathway played a critical role in the regulation of the inflammatory response of type 2 diabetes-associated osteoarthritis [32]. Meanwhile, upregulation of HO-1 could reduce the severity of osteoarthritis [33]. Additionally, previous studies showed that activating Nrf2 could attenuate the activation of NF- κ B [34]. Furthermore, a large body of studies showed that many natural herbal compounds could inhibit the inflammatory responses of OA by activating the Nrf2 signaling pathway [35, 36]. In the present study, our results showed that the expression of Nrf2 and HO-1 were significantly up-regulated by morin. This indicated that morin could activate the Nrf2 signaling pathway. In the subsequent experiments, our results showed that the anti-inflammatory effects of morin were prevented when Nrf2 was knocked down. These results suggested that morin exhibited its anti-inflammatory response in chondrocytes through activation of the Nrf2 signaling pathway. Previous studies demonstrated that morin inhibit IL-1 β -induced inflammatory mediator production in human chondrocytes by inhibiting NF- κ B activation [19]. However, the precise molecular mechanism remains unclear. The present study, we demonstrated that morin inhibited IL-1 β -induced inflammation in human chondrocytes by activating the Nrf2 signaling pathway. Further studies need to investigate the effects of morin on the treatment of OA in animal model and human patients.

Conclusion

In summary, the present study suggests that morin inhibits the IL-1 β -induced inflammatory mediators by activating the Nrf2 signaling pathway, which subsequently attenuates IL-1 β -induced NF- κ B activation. Morin may be used as an anti-inflammatory agent in the treatment of OA. In further research, we will carry out *in vivo* experiments to investigate the protective effects of morin on OA.

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Disclosure Statement

All authors declare that they have no competing interests.

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