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Original Article

Potential Effects of Indomethacin on Alleviating Osteoarthritis Progression *in Vitro*

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Abstract

Objective: To elucidate how indomethacin may slow the progression of osteoarthritis (OA). **Methods**: Chondrocytes were treated with IL-1β (10 ng/mL) for 12 hours to create an *in vitro* model of OA. Following this, 10 μM of indomethacin was added to the IL-1β-treated chondrocytes for an additional 4 hours to evaluate its effects on inflammation, anabolism, catabolism, apoptosis, and autophagy using ELISA, western blot, immunofluorescence and flow cytometry, respectively. **Results**: IL-1β significantly stimulated inflammatory responses, hampered anabolic processes, induced catabolic activity, accelerated apoptosis, and inhibited autophagy in chondrocytes, as well as activated the PI3K/AKT/mTOR signaling pathway. However, treatment with indomethacin reversed the effects of IL-1β stimulation on chondrocytes and simultaneously suppressed the activation of the PI3K/AKT/mTOR signaling pathway. **Conclusions**: Our findings indicate the mechanism of action of indomethacin in mitigating OA progression, indicating that it can inactivate the PI3K/AKT/mTOR signaling pathway, thereby regulating inflammation, metabolism, apoptosis, and autophagy in chondrocytes, which attenuates the development of OA.

Keywords: Apoptosis, Autophagy, Indomethacin, Inflammation, Osteoarthritis

Introduction

Osteoarthritis (OA) is a chronic joint disorder that is prevalent worldwide, particularly among the elderly population[1]. The primary symptom in patients with OA is joint pain, and the fundamental pathological features of the condition include synovial inflammation, cartilage degeneration, and chondrocyte apoptosis[2]. Recent studies have increasingly highlighted the critical role of chondrocyte autophagy in the progression of OA[3,4]. Consequently, there is an urgent need for further research into the molecular mechanisms underlying OA to identify potential therapeutic agents.

Traditional methods for managing osteoarthritis (OA) range from non-invasive techniques to surgical interventions, including joint replacement in severe cases. Currently, there is no definitive cure for OA[5]. The widespread use of nonsteroidal anti-inflammatory drugs (NSAIDs) aims to relieve symptoms[6]. Indomethacin, a member of the NSAID class, is known for its potent anti-inflammatory, analgesic, and antipyretic properties. Its anti-cancer effects have garnered significant attention; indomethacin has been shown to induce apoptosis and inhibit inflammation in colon cancer[7], overcome multidrug resistance in breast cancer[8], and significantly suppress

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Table 1. Primary antibodies for Western blotting and immunofluorescence tests.

Primary antibody	Catalog number	Dilution ratio	Source
Collagen II	28459-1-AP	1:800	Proteintech, Wuhan, China
Aggrecan	13880-1-AP	1:200	Proteintech, Wuhan, China
MMP13	18165-1-AP	1:100	Proteintech, Wuhan, China
MMP3	66338-1-Ig	1:200	Proteintech, Wuhan, China
IL-6	66146-1-Ig	1:500	Proteintech, Wuhan, China
TNF-α	#8184	1:500	Cell Signaling Technology, Boston, MA, USA
COX-2	#12282	1:200	Cell Signaling Technology, Boston, MA, USA
iNOS	#20609	1:500	Cell Signaling Technology, Boston, MA, USA
Bax	60267-1-Ig	1:1,000	Proteintech, Wuhan, China
Caspase 3	68773-1-Ig	1:1,000	Proteintech, Wuhan, China
Caspase 9	66169-1-Ig	1:1,000	Proteintech, Wuhan, China
Bcl-2	60178-1-Ig	1:500	Proteintech, Wuhan, China
LC3	14600-1-AP	1:1,000	Cell Signaling Technology, Boston, MA, USA
Beclin1	66665-1-Ig	1:1,000	Cell Signaling Technology, Boston, MA, USA
p-PI3K	#4228	1:1,000	Cell Signaling Technology, Boston, MA, USA
PI3K	#4249	1:1,000	Cell Signaling Technology, Boston, MA, USA
p-AKT	66444-1-Ig	1:2,000	Proteintech, Wuhan, China
AKT	60203-2-Ig	1:5,000	Proteintech, Wuhan, China
p-mTOR	67778-1-Ig	1:2,000	Proteintech, Wuhan, China
mTOR	66888-1-Ig	1:5,000	Proteintech, Wuhan, China
GAPDH	60004-1-Ig	1:50,000	Proteintech, Wuhan, China

tumor growth in non-small cell lung cancer patients[9]. Additionally, researchers are investigating indomethacin's therapeutic effects on OA, with promising results. For instance, Yassin et al. demonstrated the efficacy of a topical copper indomethacin gel in a rat OA model, confirming its strong anti-inflammatory and edema-reducing properties[10]. Koonrungsesomboon conducted a randomized controlled trial involving OA patients and demonstrated that applying an indomethacin solution is significantly more effective than two other formulation solutions[11]. Liu et al. reported that a relatively low dose of indomethacin can be beneficial for improving cartilage damage[12]. However, elucidating the specific mechanisms by which indomethacin acts in OA remains challenging, as the current understanding in this area is still limited.

The current study aims to elucidate the potential mechanisms by which indomethacin influences OA progression *in vitro*. It suggests that indomethacin can mitigate OA by regulating inflammation, metabolism, apoptosis, and autophagy through the modulation of the PI3K/Akt/mTOR signaling pathway.

Materials and Methods

Cells and Reagents

Indomethacin was provided by Merck (Darmstadt, Germany). Both human chondrocytes (cat. no. CP-H096) and specialized culture medium were obtained from Pricella Biotechnology (Wuhan, China). Human IL-6 and TNF-α ELISA kits were sourced from Beyotime (Shanghai, China), while human COX-2 and iNOS ELISA kits were provided by Abcam (Cambridge, UK). The primary antibodies for western blotting and immunofluorescence tests are listed in Table 1. Thermo Fisher Scientific (Rockford, MD, USA) supplied the BCA Kit and the Apoptosis Detection Kit. The ECL Kit was obtained from Tanon (Shanghai, China).

Cell culture and treatment

The obtained human chondrocytes were cultured in a specialized medium from Pricella Biotechnology, maintained at 37°C in a 5% CO_2. Chondrocytes were treated with 10 ng/mL of IL-1β for 12 hours to establish an OA cell model. Following this, indomethacin at a concentration of 10 μM was added to the IL-1β-treated chondrocytes for an additional 4 hours to assess its effects.

ELISA

The levels of IL-6, TNF-α, COX-2, and iNOS in IL-1β-induced chondrocytes were assessed using specialized commercial kits.

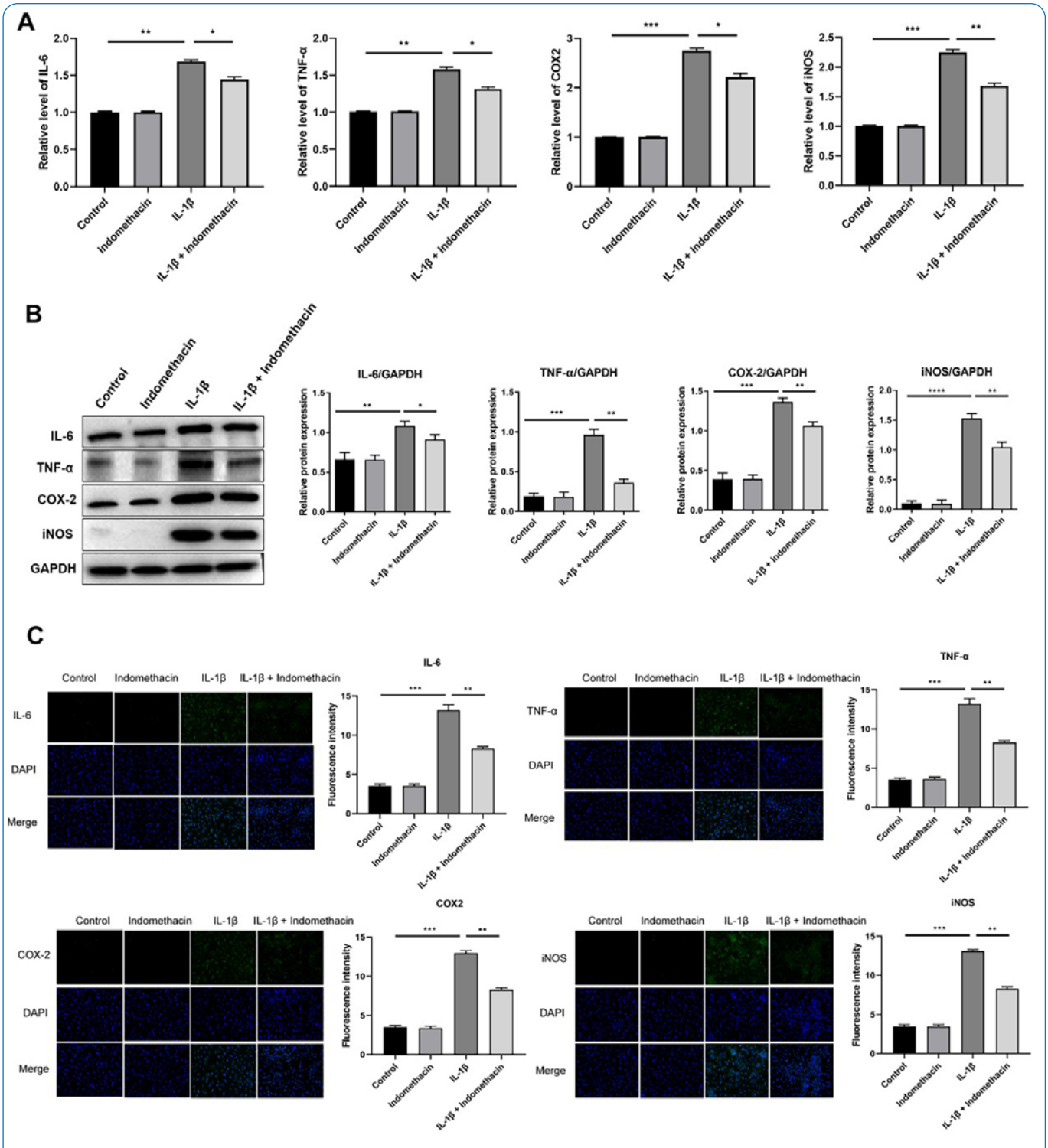

Figure 1. Indomethacin reduces IL-1β-induced inflammation in chondrocytes. (**A**) The relative levels of IL-6, TNF-α, COX-2, and iNOS were determined using specialized commercial kits. (**B**) The protein levels of these inflammatory markers were subsequently measured by Western blotting to quantify their expression. (**C**) Finally, immunofluorescence staining was conducted to assess the fluorescence intensity of IL-6, TNF-α, COX-2, and iNOS in the chondrocytes, providing a visual representation of the inflammatory changes induced by IL-1β treatment. Scale bar = 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Immunofluorescent tests

Chondrocytes were washed with PBS following various treatments and then fixed using 4% paraformaldehyde. Subsequently, they were permeabilized with 0.1% Triton X-100. The treated chondrocytes were incubated with goat serum for approximately 1 hour at room temperature to block non-specific binding. Afterward, the primary antibodies listed in Table 1 were applied and incubated with the chondrocytes for 12 hours. This was followed by a 1-hour incubation with the corresponding fluorescent secondary antibodies in the absence of light. The nuclei were stained with DAPI, and fluorescent images were captured using an EVOS FL Auto fluorescence microscope (Life Technologies, Delhi, India).

Apoptosis detection assay

Chondrocyte apoptosis was assessed using an apoptosis assay kit and analyzed via flow cytometry. In a light-protected environment, the re-suspended chondrocytes were stained with Annexin V-FITC and propidium iodide (PI). After approximately 30 minutes, the apoptotic chondrocytes were detected using a flow cytometer from Agilent Technologies (Santa Clara, CA, USA).

Western blot

The treated chondrocytes were lysed using RIPA lysis buffer to extract proteins. We then used a BCA kit to determine the protein concentrations. Subsequently, 15 μg of the protein samples were subjected to SDS-PAGE for separation and then transferred to PVDF membranes via electroblotting. After blocking with 5% nonfat milk, the membranes were incubated with the primary antibodies listed in Table 1, followed by the corresponding secondary antibodies. Finally, the protein bands were analyzed using an ECL kit and visualized with a Bio-Rad exposure device (Hercules, CA, USA).

Statistical analysis

Data analysis was performed using SPSS software (version 22.0). The results are expressed as the mean value ± standard deviation (SD). Differences among groups were assessed using one-way ANOVA, with a p-value of less than 0.05 considered statistically significant.

Results

Indomethacin reduces IL-1β-induced inflammation in chondrocytes

To evaluate the effects of indomethacin on inflammatory responses *in vitro*, levels of pro-inflammatory factors (IL-6, TNF-α, COX-2, and iNOS) in IL-1β-induced chondrocytes were measured. As shown in Figure 1A, treatment with IL-1β significantly increased the release of these pro-inflammatory markers ($P < 0.01$). In contrast, indomethacin treatment alleviated inflammatory responses, evidenced by a reduction in the expression of pro-inflammatory markers in chondrocytes following IL-1β stimulation ($P<0.05$). Additionally, protein levels of IL-6, TNF-α, COX-2, and iNOS were elevated after IL-1β treatment (Figure 1B, $P<0.01$) but significantly suppressed upon indomethacin administration ($P<0.05$). Similar trends were observed in the immunofluorescence results (Figure 1C, $P<0.01$).

Indomethacin induces anabolism and hampers catabolism in IL-1β-induced chondrocytes

The disruption of the balance between catabolism and anabolism in cells contributes to the degradation of the extracellular matrix (ECM) in cartilage, ultimately triggering the onset and progression of osteoarthritis (OA)[13]. In our study, we observed that protein levels of anabolic markers, specifically collagen II and aggrecan, were significantly reduced in chondrocytes treated with IL-1β (Figure 2A, $P<0.001$). However, these levels were partially restored following treatment with indomethacin ($P<0.01$). Immunofluorescence tests corroborated these findings, showing a notable decrease in the fluorescence intensity of collagen II and aggrecan in IL-1β-induced chondrocytes (Figure 2B, $P<0.001$), while indomethacin treatment significantly enhanced their fluorescence intensity ($P<0.05$). Conversely, the expression levels of catabolic markers, MMP13 and MMP3, exhibited diametrically opposite results, as illustrated in Figures 3A-B. IL-1β treatment led to the overexpression of MMP13 and MMP3 proteins in chondrocytes ($P<0.001$), whereas indomethacin administration dramatically inhibited the levels of these proteins ($P<0.01$).

Indomethacin suppresses IL-1β-induced chondrocyte apoptosis

The expression levels of apoptosis-related proteins were assessed using western blotting. As shown in Figure 4A, compared to the control group, the IL-1β group exhibited a significant increase in pro-apoptotic proteins, including Bax, caspase-3, and caspase-9 ($P<0.01$), while the anti-apoptotic protein Bcl-2 showed a notable decrease ($P<0.001$). In contrast, the combination of IL-1β and indomethacin resulted in a marked reduction of Bax, caspase-3, and caspase-9 levels ($P<0.05$) and a significant increase in Bcl-2 expression ($P<0.01$). These findings were further confirmed by flow cytometry analysis (Figure 4B, $P<0.05$).

Indomethacin restores impaired chondrocyte autophagy induced by IL-1β treatment

Figure 5 illustrates that a relatively high level of autophagy was observed in the indomethacin and control groups. In contrast, the IL-1β group demonstrated impaired autophagy, evidenced by a significant reduction in the levels of autophagy-related proteins, LC3II/I and Beclin 1 ($P<0.001$). Treatment with indomethacin significantly restored autophagy processes, as indicated by elevated protein levels of LC3II/I and Beclin 1 ($P<0.01$).

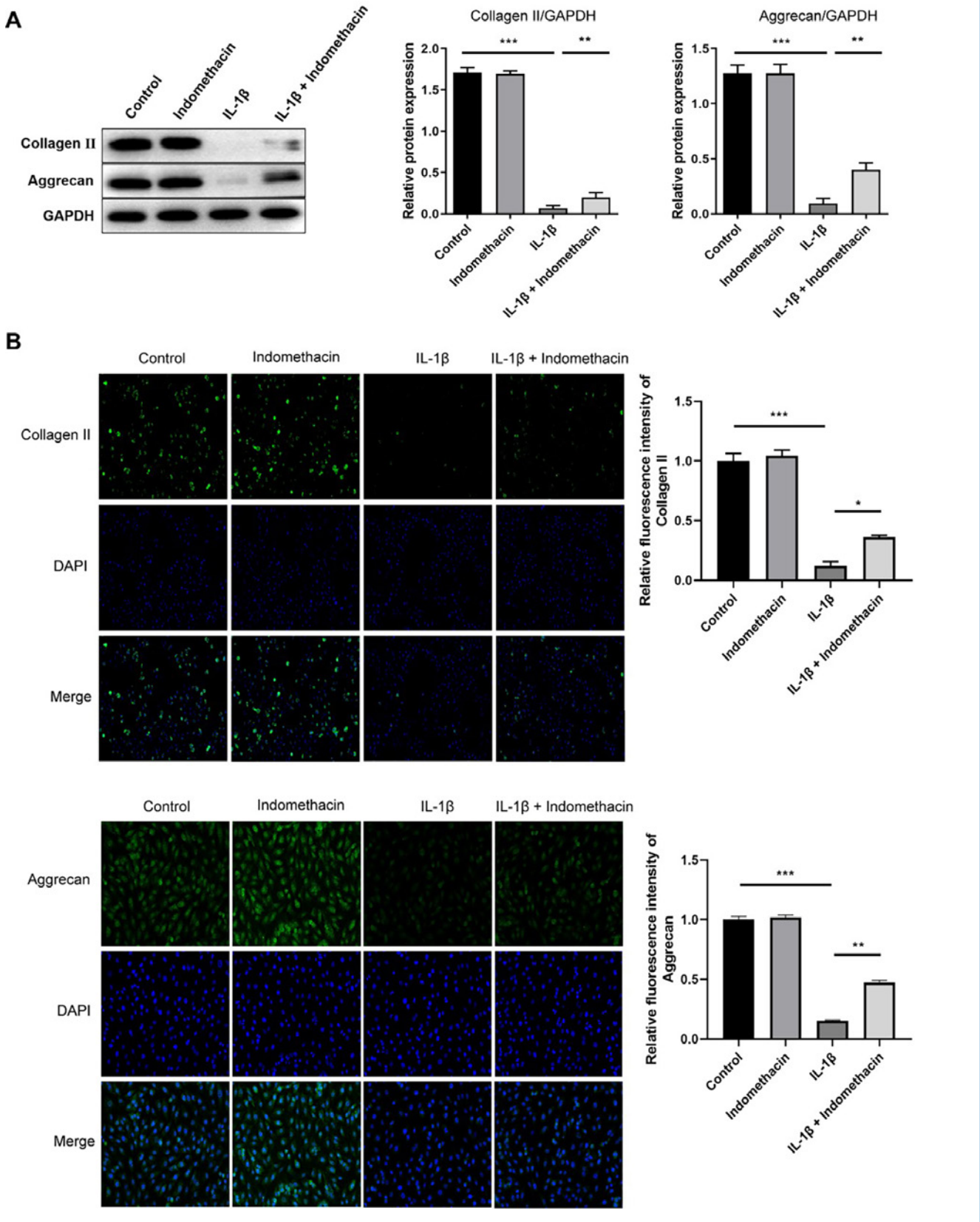

Figure 2. Indomethacin induces anabolism in IL-1β-induced chondrocytes. (**A**) The protein levels of anabolic markers (collagen II and aggrecan) in these chondrocytes were measured by Western blotting. (**B**) Immunofluorescence staining was performed to assess the fluorescence intensity of collagen II and aggrecan. Scale bar = 100 μm. *P < 0.05, **P < 0.01, ***P < 0.001.

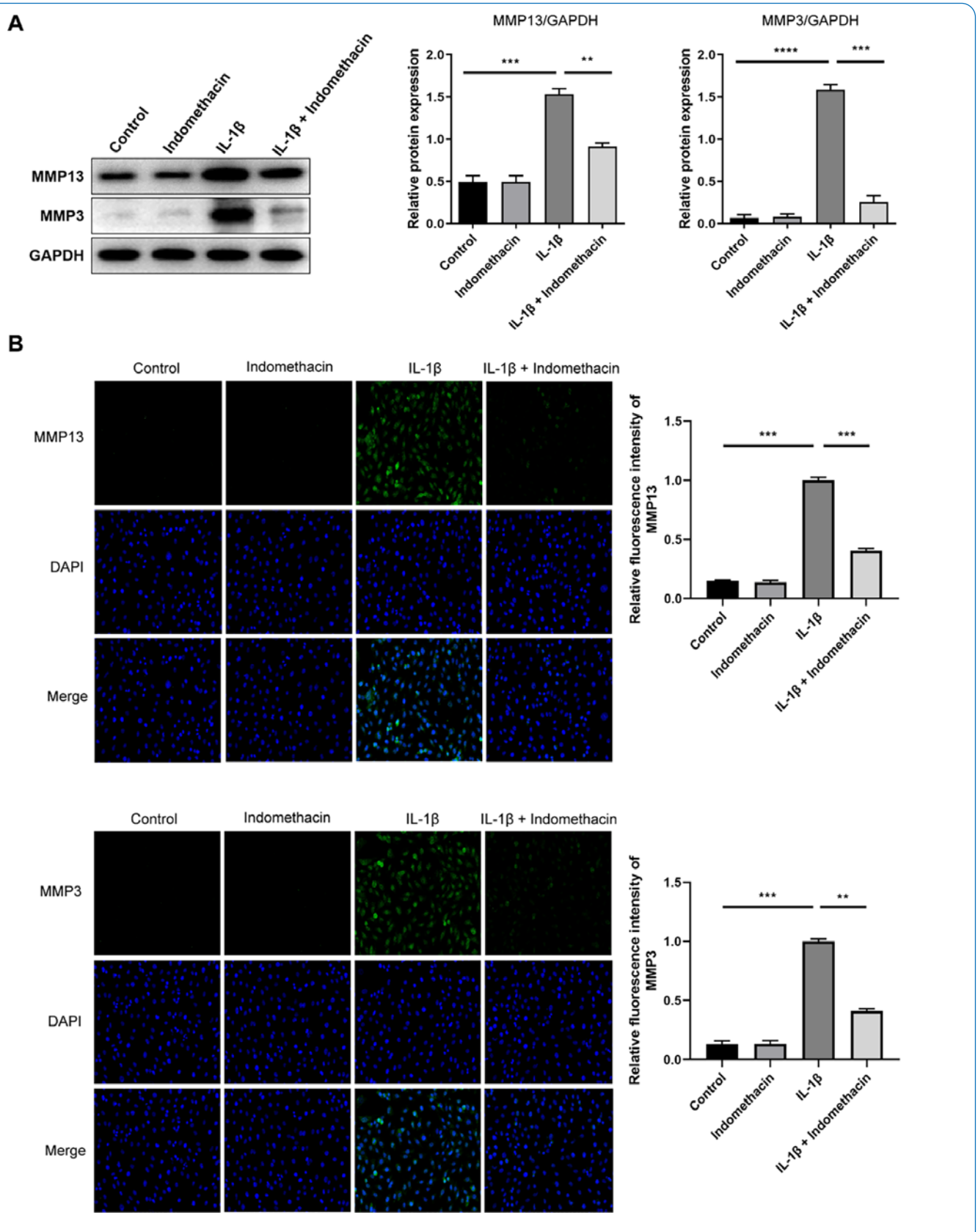

Figure 3. Indomethacin hampers catabolism in IL-1β-induced chondrocytes. (**A**) The protein levels of catabolic markers (MMP13 and MMP3) in these chondrocytes were measured by Western blotting. (**B**) Immunofluorescence staining was performed to assess the fluorescence intensity of MMP13 and MMP3. Scale bar = 100 μm. **P < 0.01, ***P < 0.001, ****P < 0.0001.

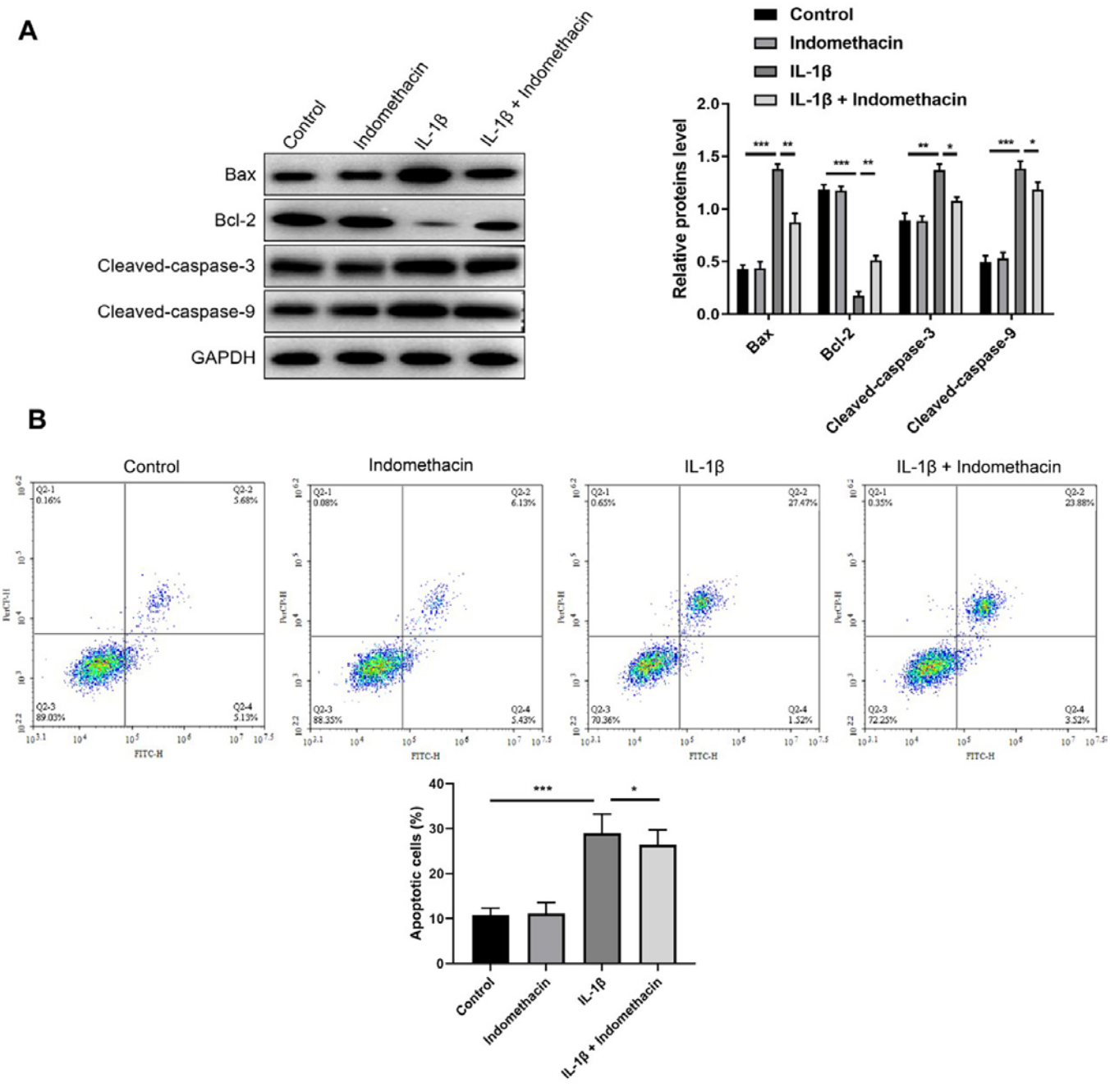

Figure 4. Indomethacin suppresses IL-1β-induced chondrocyte apoptosis. (**A**) The protein levels of Bax, Bcl-2, Cleaved-caspase3, and Cleaved-caspase9 in different groups were measured by Western blotting. (**B**) Following treatment with indomethacin, the apoptosis of IL-1β-induced chondrocytes was analyzed by flow cytometry. *P < 0.05, **P < 0.01, ***P < 0.001.

Indomethacin inactivates the PI3K/AKT/mTOR signaling pathway, leading to the inhibition of inflammation, regulation of anabolism and catabolism, and promotion of autophagy in IL-1β-induced chondrocytes

The PI3K/AKT/mTOR pathway has been implicated in the autophagic processes of articular chondrocytes[14]. We evaluated the interplay between indomethacin and the PI3K/AKT/mTOR pathway. As shown in Figure 6A, key proteins (p-PI3K, p-AKT, and p-mTOR) were significantly upregulated upon IL-1β stimulation, leading to the activation of the PI3K/AKT/mTOR pathway (P<0.001). In contrast, indomethacin administration exhibited a notable inhibitory effect on this

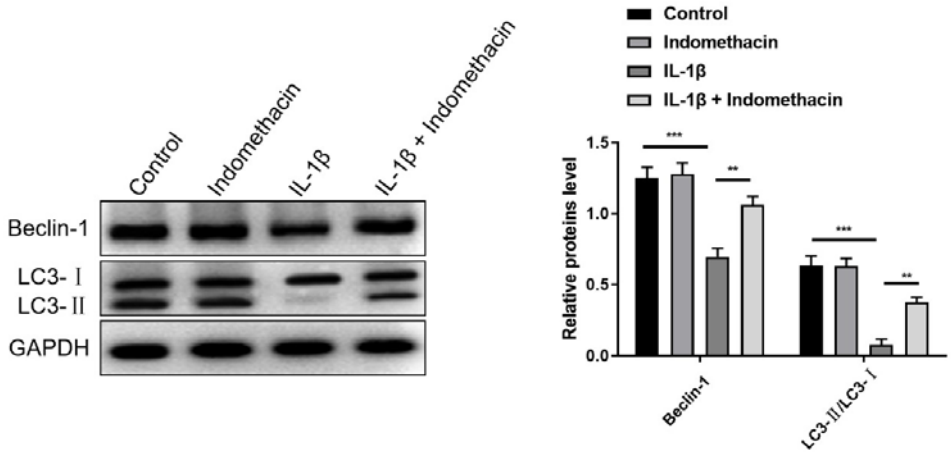

Figure 5. Indomethacin restores the impaired chondrocyte autophagy caused by IL-1β treatment. The protein levels of Beclin-1 and LC3II/I in different groups were measured by Western blotting. **P < 0.01, ***P < 0.001.

pathway's activation (P<0.05). Additionally, the introduction of 4 μL of IGF-1, an agonist of the PI3K/AKT/mTOR pathway, demonstrated that IGF-1 could partially negate the regulatory effects of indomethacin on inflammation, anabolism, catabolism, and autophagy in IL-1β-induced chondrocytes (Figure 6B-D, P<0.05).

Discussion

Global estimates indicate that at least 300 million individuals are affected by osteoarthritis (OA)[15]. Knee and hip OA significantly contribute to disability, accounting for 2.2% of all disability cases and greatly diminishing quality of life[16,17]. Additionally, the annual societal cost of OA is estimated to reach $303 billion[18]. Therefore, it is crucial to implement sustained efforts to reduce the prevalence of this chronic disease. In this study, we explored the potential mechanisms through which indomethacin may influence the progression of OA *in vitro*, suggesting its action on the PI3K/AKT/mTOR pathway to inhibit inflammation, prevent extracellular matrix (ECM) degradation, reduce apoptosis, and restore chondrocyte autophagy.

In recent years, there has been an increasing recognition of the role of chronic inflammatory reactions as a primary pathogenic factor in OA[19]. IL-1β is known to be associated with the development and progression of OA, as it triggers the release of inflammatory cytokines. Consequently, IL-1β is commonly utilized as a cytokine to establish *in vitro* cell models for OA research[20]. In this study, we established an IL-1β-induced OA cell model, revealing that IL-1β significantly elevated the concentrations of pro-inflammatory cytokines, including IL-6, TNF-α, COX-2, and iNOS in chondrocytes, which aligns with previous findings.

Additionally, articular cartilage is a crucial component of the joint, primarily composed of ECM and chondrocytes. The functional state of chondrocytes is vital for maintaining cartilage homeostasis, as these cells are responsible for synthesizing and repairing the cartilage matrix. The balance of chondrocyte metabolism is essential for maintaining healthy articular cartilage, and factors such as inflammation can disrupt this balance, leading to the onset of OA. The primary components of the extracellular matrix (ECM) are collagen II and aggrecan, which play critical roles in maintaining the physiological function of joint cartilage[21]. In contrast, MMP13 and MMP3 serve as key catabolic enzymes in chondrocytes, with the capability to degrade collagen II and aggrecan[22]. In this study, treatment with IL-1β significantly induced chondrocyte catabolism while inhibiting anabolic processes. Our data indicate that indomethacin administration not only suppressed the expression of pro-inflammatory proteins but also restored the balance between anabolic and catabolic activities in chondrocytes disrupted by IL-1β. These findings suggest that indomethacin possesses properties that can reduce inflammation and protect articular cartilage.

The viability of chondrocytes is critical for their normal physiological functions. Apoptosis is a recognized mechanism in various physiological and pathological processes, including chondrocyte death. The lack of a well-developed vascular network in cartilage makes it nearly impossible for chondrocytes to be replaced after apoptosis[23]. As the population of chondrocytes decreases, the cartilage ECM becomes increasingly vulnerable to damage, leading to a vicious cycle. The degradation of cartilage ECM accelerates OA progression and exacerbates its severity, ultimately contributing to debilitating symptoms such as joint pain, stiffness, and loss of mobility. Our results demonstrated that indomethacin administration inhibited the expression of pro-apoptotic proteins, including caspase-3, caspase-9, and Bax, while enhancing Bcl-2 expression. These findings suggest

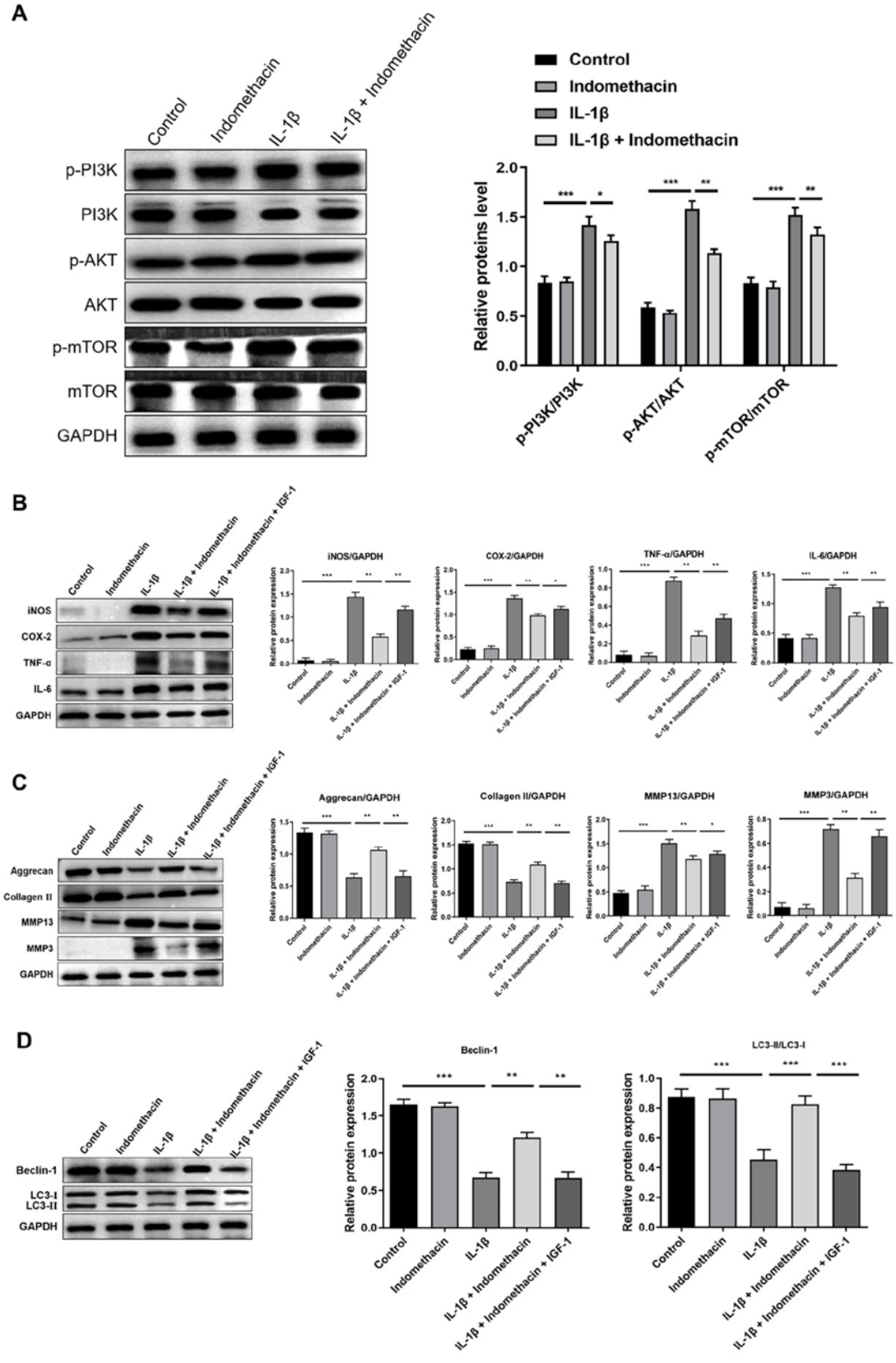

Figure 6. Indomethacin suppresses the PI3K/AKT/mTOR pathway in IL-1β-induced chondrocytes. (**A**) Following treatment with indomethacin, the protein levels of mTOR, p-mTOR, PI3K, p-PI3K, AKT, and p-AKT in IL-1β-induced chondrocytes were measured by Western blotting. After the addition of IGF-1, the protein levels of (**B**) pro-inflammatory markers (IL-6, TNF-α, COX-2, and iNOS), (**C**) anabolic markers (collagen II and aggrecan), and catabolic markers (MMP13 and MMP3), as well as (**D**) autophagy markers (Beclin-1 and LC3II/I) in IL-1β-induced chondrocytes were measured by Western blotting. *P < 0.05, **P < 0.01, ***P < 0.001.

that indomethacin may mitigate chondrocyte apoptosis, which is crucial for alleviating the ongoing deterioration of the cartilage ECM .

Autophagy is a crucial metabolic pathway that helps maintain homeostasis within the cell's internal environment[24]. It plays significant regulatory roles in both apoptosis and inflammation and influences the progression of inflammatory diseases[25]. Disruption of autophagy can lead to chondrocyte dysfunction or death, resulting in an imbalance of catabolic and anabolic processes, ECM degradation, and ultimately the development of osteoarthritis (OA)[26]. Indomethacin enhances the expression of autophagy-related proteins (LC3II/I and Beclin 1), indicating its potential to promote chondrocyte autophagy. The regulation of autophagy is closely linked to the PI3K/AKT/mTOR signaling pathway. mTOR, a key downstream component of this pathway, negatively regulates autophagic processes[27]. Inhibiting this signaling pathway may stimulate chondrocyte autophagy, thereby mitigating OA progression.

Our findings suggest the existence of reciprocal interactions between indomethacin and the PI3K/AKT/mTOR pathway. Western blotting results indicate that indomethacin administration significantly suppressed the expression levels of p-PI3K/PI3K, p-AKT/AKT, and p-mTOR/mTOR, confirming our hypothesis. Our *in vitro* experiments demonstrated that indomethacin can inactivate the PI3K/AKT/mTOR pathway in IL-1β-stimulated chondrocytes, regulating inflammatory responses as well as anabolic and catabolic processes.

Conclusion

In summary, our findings suggest a potential regulatory mechanism of indomethacin in alleviating OA. We observed that indomethacin may have the capacity to inactivate the PI3K/AKT/mTOR pathway, which could contribute to reducing inflammation, supporting the balance between catabolism and anabolism, inhibiting apoptosis, and promoting autophagy in chondrocytes. These insights may serve as a basis for further exploration of indomethacin's clinical application in the management of OA.

Authors' contributions

DS designed the study and drafted the manuscript. TW, NL and ZM were responsible for the collection and analysis of the experimental data. XZ, WW and BW revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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