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Original Article

mTORC2 inhibition by JR-AB2-011 improves IL-1β-induced inflammation, catabolic response, and apoptosis in human chondrocytes through IκB-α/NF-κB p65

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Abstract

Osteoarthritis (OA) is a very common chronic joint condition marked by inflammation and cartilage loss. mTOR is a well-known mediator of inflammation, cell survival, and aging; however, its role in OA has not been determined. To explore the role of mTORC2 in OA-and associated pathological changes, we examined the contribution of mTORC2-mediated Akt, rictor and IκB-α/NF-κB p65 pathway in interleukin (IL)-1β-treated human chondrocytes. We focused on the protein expression of proinflammatory cytokines and catabolic and apoptotic factors, including TNF-α, IL-6, iNOS, MMP13, Bax, and caspase3, which may occur through this signalling pathway in IL-1β-treated chondrocytes. Chondrocytes were cultured and treated with either 2 ng/mL IL-1β alone or in combination with increasing concentrations of JR-AB2-011 (50, 100, or 250 μM), a selective mTORC2 inhibitor. The protein levels of phosphorylated (p)-Akt, Akt, rictor, p-NF-κB p65, NF-κB p65, IκB-α, p-IκB-α, iNOS, MMP13, Bax, and caspase3 were evaluated by Western blotting. In IL-1β-stimulated chondrocytes, mTORC2 activity was increased with increased phosphorylation of Akt and expression of rictor. IL-1β increased the expression of p-IκBα, p-NF-κB p65, NF-κB p65, IL-6, TNF-α, iNOS, Bax, and caspase3 proteins and decreased the expression of IκB-α. All of these IL-1β-induced alterations were prevented by JR-AB2-011. The main novel finding in the present study is that selective mTORC2 inhibition by JR-AB2-011 prevents the inflammatory, catabolic, and apoptotic responses induced by IL-1β via modulation of IκB-α/NF-κB activity. Therefore, we demonstrated a previously unknown function of mTORC2 inhibition that seems to be a potential therapeutic target for OA.

Keywords: mTORC2, IL-1β, Chondrocytes, Inflammation, Catabolic response, Apoptosis

1. Introduction

Osteoarthritis (OA) is a most common chronic, heterogeneous, degenerative joint disease characterised by alterations to the morphology, structure, and function of chondrocytes that can affect the many tissues of the joint [1, 2]. The pathogenic mechanism behind OA is currently unknown, although current theories imply that mechanical, inflammatory, and metabolic components are involved in the disease's complicated etiology, leading to the failure and destruction of the joint [3, 4].

One of the key hallmarks of OA is low-grade chronic inflammation. Overproduction of reactive oxygen species, matrix-degrading enzymes, and inflammatory mediators promotes the gradual deterioration of cartilage, synovium, and subchondral bone [5]. It appears that local proinflammatory mediator concentrations and inflammation have a direct role in the development of OA joint pain [6]. Inflammatory substances like IL-1β can cause cartilage degeneration, matrix breakdown, chondrocyte apoptosis, oxidative stress, cell senescence, and defective autophagy in chondrocytes, which starts and accelerates OA [7]. The development of OA is often accompanied by an increase in cell death, a decrease in chondrocyte proliferation, quantity, and viability, extracellular matrix (ECM) disintegration, deterioration in metabolic status, and chondrocyte inflammation [8]. Therefore, completely understanding the regulatory mechanisms linked to cell apoptosis, inflammation, and signal transduction pathways in chondrocytes and preserving the homeostasis of chondrocytes is an important goal when seeking more efficient therapeutic approaches for OA [9].

It has been suggested that the NF-kB pathways play a crucial role in the pathogenesis of OA [10]. Following IL 1β stimulation, NF-kB p65 is translocated from the

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cytoplasm to the nucleus, where it activates the expression of numerous inflammatory or catabolic genes, such as inducible NO synthase (iNOS), TNF α, and MMPs which degrade articular cartilage and cause the onset and progression of OA [11]. These results suggest that NF-B, along with its cofactors, regulators, and downstream effectors, may represent prospective therapeutic targets for the treatment of OA.

Complex signalling networks are known to mediate these cellular events. In particular, the mTOR signalling pathway is essential for maintaining joint health and is correlated with OA pathogenesis [12]. Concurrently, it is worth noting that different roles for mTORC1 and mTORC2 have been identified in skeletal development. It is known that rictor-mediated mTORC2 signalling plays a role in skeletal growth and bone formation with its important effects on both chondrocytes and osteoblasts by affecting chondrocyte hypertrophy and bone growth under physiological and pathological conditions. Although the exact mechanism by which mTORC2 regulates cartilage width and metabolism is yet unknown, it appears to involve modifications to the overall chondrocyte proliferation, survival, or anabolic response.

To the best of our knowledge, no prior studies have investigated the contribution of mTORC2 in the pathogenesis of OA with respect to inflammation, catabolic and apoptotic response, most likely through IκB-α/NF-κB activation, and subsequently, expression of IL-6 and TNF-α, MMP13 and iNOS, caspase3, and Bax protein, which is well known to play a role in the above-mentioned processes. Therefore, in this study, we investigated whether mTORC2 contributes to inflammation, catabolic responses, and apoptosis via the above signalling pathways induced by IL-1β in human chondrocyte cell culture.

2. Materials and methods

2.1. Reagent

The recombinant human IL-1β protein was obtained from Millipore (California, USA). JR-AB2-011 was purchased from Aobious Inc. (Gloucester, MA, USA). Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were obtained from Gibco (New York, NY, USA). Fetal bovine serum (FBS) and trypsin-EDTA were purchased from Biological Industries (Israel). Primary antibodies for rictor (SC-271081), p-Akt Ser473 (SC-293125), Akt (SC-5298), IκB-α (SC-1643,), p-IκB-α Ser32 (SC-7977), NF-κB p65 (SC-8008), p-NF-κB p65 (SC-33020), TNF-α (SC-33639), IL-6 (SC-32296), MMP13 (MA5-14247), iNOS (SC-7271), Bax (SC-23959), caspase3 (SC-56053), and β-actin (SC-47778) were purchased from Santa Cruz Biotechnology (Texas, USA) and Invitrogen (Massachusetts, USA). Additionally, secondary antibodies (goat anti-mouse IgG-horseradish peroxidase (HRP) (ab6808) and goat anti-rabbit IgG-HRP (ab6721)) were purchased from Abcam (Cambridge, United Kingdom).

2.2. Experimental groups and drug administration

The human chondrocyte cell line (HC, 402-05A) was purchased from Sigma Aldrich (St. Louis, MO, USA). The cell culture was performed in DMEM/F12 containing 10% FBS, 1% penicillin-streptomycin, and L-glutamine at 37 °C in 5% CO2 incubator. When cell confluence reached 80%, the subculture was carried out. In 6-well plates, experimental cells were cultured at a density of 2 x 10^4 cells per well and grown to 80%-90% confluence [13, 14]. Through the stimulation of cultured chondrocytes with 2 ng/mL of recombinant IL-1β for 24 hours, an in vitro osteoarthritis model has been established [13, 15]. IL-1β was used to activate the cells for 24 hours in either the absence or presence of JR-AB2-011 (50, 100, or 250 μM), a selective mTORC2 inhibitor. JR-AB2-011 (50, 100, or 250 μM) was administered to chondrocytes simultaneously with IL-1β. Cells were divided into five groups: control; IL-1β (2 ng/mL); IL-1β (2 ng/mL) + 50 μM JR-AB2-011; IL-1β (2 ng/mL) + 100 μM JR-AB2-011; and IL-1β (2 ng/mL) + 250 μM JR-AB2-011. All cells were incubated at 37 °C in a 95% air, 5% CO_2 environment.

2.2. Immunoblot assay

Cells were quickly rinsed with PBS and lysed in situ for 10 min on ice with radioimmunoprecipitation assay (RIPA) lysis buffer, including 1 % protease and phosphatase inhibitors. Whole-cell lysates were obtained after centrifugation at 14,000 rpm for 15 min. The protein content was then assessed using the Bradford assay. 100 μg of total protein was loaded into 10% SDS-PAGE and transferred to nitrocellulose membranes. Afterward, the membranes were blocked with TBST supplemented with 5 % non-fat dry milk solution for 1 h at room temperature. The membranes were subsequently examined using certain primary antibodies [Anti-rictor (1:1000), anti-AKT (1:1000), anti-p-AKT Ser473 (1:1000), anti-p-IκB-α Ser32 (1:500), anti-IκB-α (1:500), anti-p-NF-κB p65 (1:500), anti-NF-κB p65 (1:1000), anti-TNF-α (1:1000), anti-IL-6 (1:1000), anti-MMP13 (1:5000), anti-iNOS (1:1000), anti-BAX (1:1000), anti-caspase3 (1:1000), and anti-β- actin (1:5000)] in TBST, including 5% BSA overnight at 4 °C. Membranes were rinsed with TBST and then incubated with appropriate secondary antibodies (1:1000) for 2 h at room temperature. Finally, the membranes were visualized by the detection system using the western blotting detection reagent. Image J software (1.54d National Institutes of Health) was used to analyse the band intensity.

2.3. Statistical analysis

GraphPad Prism 5.0 was used for all statistical analysis (GraphPad Software Inc., California, USA). Data were presented as mean ± SEM and analysed by one-way ANOVA followed by the Newman–Keuls multiple comparison test and an unpaired *t*-test when appropriate. $p < 0.05$ was used to determine statistical significance.

3. Results

3.1. JR-AB2-011, the selective mTOR2 inhibitor, prevented IL-1β-induced increase in activation of mTORC2

To investigate the effect of JR-AB2-011 on IL-1β-induced mTOR2 activation and associated signalling molecules in cultured chondrocytes, we examined the protein expressions of rictor, Akt, and p-Akt. We found that phosphorylation of Akt (ser473), the principal downstream target of mTORC2, was increased, whereas protein expression of Akt was unchanged in response to IL-1β chondrocytes in the different experimental groups ($p < 0.05$) (Fig. 1). JR-AB2-011 reversed this change in IL-1β-treated chondrocytes. IL-1β-induced rictor expression, which is the main component of mTORC2, was also suppressed by

JR-AB2-011 ($p < 0.05$) (Fig. 1). In IL-1β-treated chondrocytes, Akt phosphorylation and rictor expression were upregulated, which is consistent with enhanced mTORC2 activity.

3.2. JR-AB2-011 prevented IL-1β-induced NF-κB activation in human chondrocytes

To determine the contribution of NF-κB activation to IL-1β induced inflammatory, catabolic, and apoptotic processes in the chondrocytes, we examined IκB-α/NF-κB protein expression and phosphorylation. The results revealed an increase in the phosphorylation of NF-κB p65 by IL-1β stimulation, which was inhibited by pretreatment with JR-AB2-011 ($p<0.05$) (Fig. 2). Additionally, IL-1β caused an increase in IκB-α phosphorylation and a decrease in IκB-α expression in chondrocytes ($p<0.05$) (Fig. 2). The degradation and phosphorylation of IκB-α in chondrocytes treated with JR-AB2-011 were decreased ($p<0.05$) (Fig. 2). These data suggested that JR-AB2-011 blocked IL-1β-induced NF-κB p65 activation by inhibiting the phosphorylation of NF-κB p65 and the degradation of IκB-α in human chondrocytes.

3.3. Effect of JR-AB2-011 on the expression of inflammatory mediators in human chondrocytes stimulated with IL-1β

The effects of JR-AB2-011 on TNF- and IL-6 protein expression were examined in order to confirm whether mTORC2 mediates the IL-1β-induced inflammatory response in human chondrocytes via proinflammatory cytokines. The data showed an increase in the protein expression of TNF-α and IL-6 in the IL-1β-treated group compared to the control and JR-AB2-011 treated groups; the increase in protein expression of TNF-α and IL-6 were markedly inhibited in human chondrocytes ($p < 0.05$) (Fig. 3).

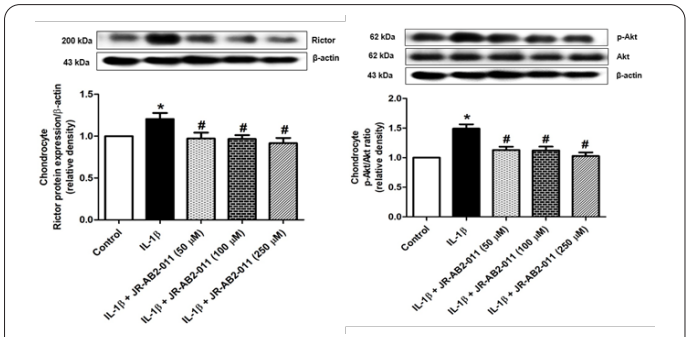

Fig. 1. JR-AB2-011 inhibits the increased protein expression and/or phosphorylation of rictor and Akt in IL-1β-treated chondrocytes. The chondrocytes were treated with IL-1β (2 ng/ml) with or without JR-AB2-011 (50, 100, and 250 μM) for 24 h. The density of bands was analyzed using Image J 1.54d software. The values are presented as the means ± SEM. * $p < 0.05$ versus control group; # $p < 0.05$ versus IL-1β alone group.

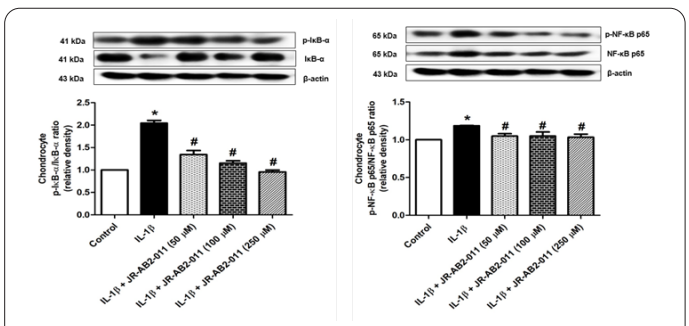

Fig. 2. JR-AB2-011 inhibits the protein expression of IκB-α, p-IκB-α, NF-κB p65, and p- NF-κB p65 in IL-1β-treated chondrocytes. The chondrocytes were treated with IL-1β (2 ng/ml) with or without JR-AB2-011 (50, 100, and 250 μM) for 24 h. The density of bands was analyzed using Image J 1.54d software. The values are presented as the means ± SEM. * $p < 0.05$ versus control group; # $p < 0.05$ versus IL-1β alone group.

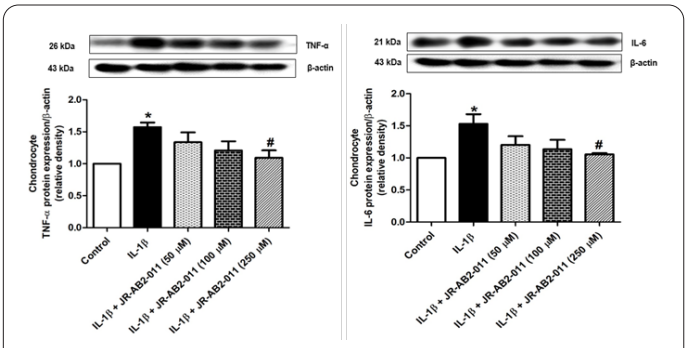

Fig. 3. Effects of JR-AB2-011 on inflammatory response induced by IL-1β. The chondrocytes were treated with IL-1β (2 ng/ml) with or without JR-AB2-011 (50, 100, and 250 μM) for 24 h. Western blotting was used to determine the expression levels of TNF-α and IL-6. The density of bands was analyzed using Image J 1.54d software. The values are presented as the means ± SEM. * $p < 0.05$ versus control group; # $p < 0.05$ versus IL-1β alone group.

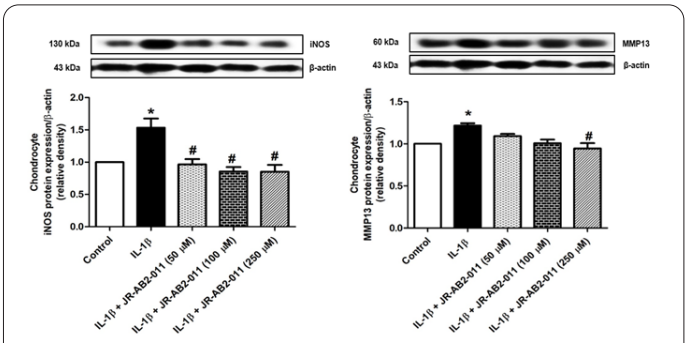

Fig. 4. Effects of JR-AB2-011 on catabolic response induced by IL-1β The chondrocytes were treated with IL-1β (2 ng/ml) with or without JR-AB2-011 (50, 100, and 250 μM) for 24 h. Western blotting was used to determine the expression levels of MMP13 and iNOS. The density of bands was analyzed using Image J 1.54d software. The values are presented as the means ± SEM. * $p < 0.05$ versus control group; # $p < 0.05$ versus IL-1β alone group.

3.4. Effect of JR-AB2-011 on the expression of catabolic factors in human chondrocytes stimulated with IL-1β

The effect of JR-AB2-011 on IL-1β-induced MMP13 and iNOS production was evaluated using Western blot assay. MMP13 and iNOS protein levels were significantly elevated in IL-1β-treated cells compared to the control cells ($p<0.05$). However, treatment with JR-AB2-011 markedly inhibited IL-1β-induced MMP13 and iNOS expression ($p<0.05$) (Fig. 4). Our results demonstrated that JR-AB2-011 alleviates IL-1β-induced catabolic response, thereby promoting MMP13 and iNOS formation.

3.5. Effect of JR-AB2-011 on the expression of apoptotic markers in human chondrocytes stimulated with IL-1β

Finally, we evaluated the contribution of mTORC2 to

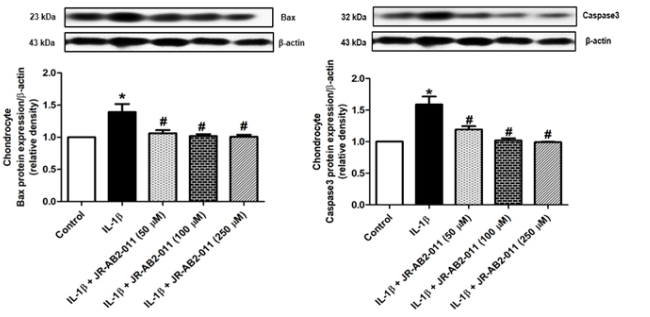

Fig. 5. Effects of JR-AB2-011 on apoptosis induced by IL-1β. The chondrocytes were treated with IL-1β (2 ng/ml) with or without JR-AB2-011 (50, 100, and 250 μM) for 24 h. Western blotting was used to determine the expression levels of Bax and cleaved caspase3. The density of bands was analyzed using Image J 1.54d software. The values are presented as the means ± SEM. * $p < 0.05$ versus control group; # $p < 0.05$ versus IL-1β alone group.

apoptotic markers in human chondrocytes. To determine IL-1β-induced cell death, we measured protein expression of main proapoptotic factors cleaved caspase3 and Bax. Western blot analysis indicated that IL-1β stimulation increased cleaved caspase3 and Bax protein levels. The addition of JR-AB2-011 decreased cleaved caspase3 and Bax, which indicates that JR-AB2-011 attenuated IL-1β-induced apoptotic response in chondrocyte ($p<0.05$) (Fig. 5).

4. Discussion

This study demonstrates for the first time that mTORC2 plays a crucial role in IL-1β-stimulated inflammation, catabolic responses, and apoptosis mediated by IκB-α/NF-κB p65 activity in chondrocytes which is known as the in vitro OA model. This conclusion is based on our demonstration that inhibition of mTORC2 by JR-AB2-011, the first selective mTORC2 inhibitor discovered, prevented the inflammatory and catabolic response and apoptosis, which is linked to decreased activity of IκB-α/NF-κB p65, resulted in reduced TNF-α, IL-6, iNOS, MMP13, Bax and caspase3 protein expression as well as attenuated rictor expression and Akt Ser473 phosphorylation in human chondrocytes induced by IL-1β. Therefore, our study highlights a previously unknown role of mTORC2 in the IL-1β-induced responses of chondrocytes, an in vitro model of OA.

Until now, both in vitro and in vivo studies related to OA have focused on explaining the contribution of mTOR through the activity of mTORC1. In particular, mTORC1 has been shown to contribute to the changes in cartilage homeostasis, chondrocyte proliferation, apoptosis, inflammation, and autophagy through the PI3K/Akt signalling pathway, which causes OA development [16-18]. Therefore, mTOR seems to be an important link to events that mediate the pathogenesis of OA. Compared to mTORC1, the regulation and specific roles of mTORC2 activity at the tissue level in whole organisms are less well understood. However, no study to date examined the function of mTORC2 in chondrocyte homeostatic, apoptotic, or inflammatory processes. In light of all these pieces of information, two important events were tried to be emphasized in this study: IL-1β administration increases mTORC2 activity in chondrocytes, which is indicated by increased Akt Ser473 phosphorylation and rictor expression, and in chondrocytes, increased mTORC2 activity mediates inflammatory, catabolic, and apoptotic responses via IκB-α/NF-κB p65.

The roles of mTORC2 in inflammation, catabolic response, and apoptosis are still not understood. To investigate how mTORC2 signalling is activated in these cases, known as the essential markers of OA, we focused on the contribution of this mTOR complex in an in vitro OA model generated by IL-1β stimulation of the chondrocytes. IL-1β is well known for its involvement in OA pathogenesis, and it may cause in significant changes in the cartilage, including matrix breakdown, inflammation, chondrocyte phenotypic abnormalities, and chondrocyte apoptosis [19, 20]. Previous research by us and others has shown that 2 ng/ml IL-1β causes a striking inflammatory response in chondrocytes [13, 15]. To gain further insight into the potential role of mTORC2 in chondrocyte inflammation, chondrocytes were incubated with IL-1β and the selective inhibitor of mTORC2, JR-AB2-011. As expected, mTORC2's scaffold protein rictor expression and phosphorylation of the effector protein Akt accepted as the indicators of activity of mTORC2 were increased by IL-1β treatment which was prevented by JR-AB2-01. Similarly, recent studies have shown that IL-1β increased mTORC2 activity in different tissues and cells like macrophages and T cells [21]. Rictor is a key component of mTORC2 interacting closely with mTOR and contributes to mTORC2 assembly, which is necessary for mTORC2 activation [22]. Also, in literature, rictor-deficient mice have been shown to have a remarkably decreased mTORC2 activity [23]. Although there are few studies on this topic, IL-1β has been demonstrated to be a crucial mTORC2 activator, especially in complicated inflammatory processes [24]. Based on the above evidence, our results support these similar reports implicating the critical role of mTORC2 signalling in proinflammatory responses that occur in IL-1β-treated chondrocytes during the progression of OA, and targeting mTORC2 may be a new and valuable strategy in chondrocyte homeostasis.

There is growing evidence for the hypothesis that inflammatory responses accompany OA-related pathophysiological changes, and therefore antiinflammatory treatments could be a useful intervention for OA. It is well-accepted that IL-1β can mimic OA by inducing an inflammatory response in chondrocytes [25, 26]. Also, multiple signals are known to be involved in OA progression, particularly NF-κB signalling [11, 27, 28]. NF-κB is a transcription factor that plays a well-established and important role in inflammation. The IL-1β activated NF-κB pathway is linked to numerous inflammatory pathologies. The NF-κB molecules could cause the articular joint to disintegrate, accelerating the development of OA [11, 29, 30]. Lin et al. [31] found that pharmacological inhibition of NF-κB could reduce IL-1β-induced inflammatory response in chondrocytes. Therefore, besides the inhibitory effect of JR-AB2-011 on inflammation; this study investigated the possible relationships between mTORC2 activity and NF-κB signalling activation were investigated. The results of this study showed that JR-AB2-011 reduced the expression and/or phosphorylation of IκB-α, NF-κB p65 in chondrocytes under IL-1β stimulation. These results illustrate that JR-AB2-011 exhibits antiinflammatory activities in chondrocytes by preventing the activation of NF-κB signalling. Also, it is known that IL-1β promotes the NF-κB p65 signalling pathway in chondrocytes and subsequently

induces an increase in the expression of proinflammatory mediators, eventually contributing to the progression of OA as critical factors [32]. This increased expression leads to proinflammatory phenotypic changes in OA, including the activation of iNOS and COX-2, TNF-α, and IL-6 expression. Supporting this view were our findings that IL-1β increased TNF-α and IL-6 protein expression in chondrocytes, and this cytokine-mediated inflammatory response was prevented with JR-AB2-011 treatment. As is known, there are several signalling pathways capable of activating NF-κB. One of them is the regulation of NF-κB by Akt/IKKα signalling pathway activation, a striking finding obtained for the first time in the study of Choi et al. on skin aging. It has also been shown that mTORC2 may play an important but inconsistent role in regulating inflammation and NF-kB signalling in various cell types [33-36]. Further studies are needed to fully understand the mechanisms underlying the modulation of inflammation by mTORC2 and NF-κB and to identify potential therapeutic targets for the treatment of inflammatory diseases.

Evidence is accumulating that IL-1β activates the NF-κB p65 signalling pathway in chondrocytes and subsequently induces the upregulation of the expression of procatabolic mediators as well as proinflammatory cytokines, eventually contributing to the degradation of the cartilage ECM [37]. There is growing support demonstrating the key role of NF-κB in inducing, in turn, cytokine expression and cytokine-induced secretion of MMPs, since its inhibitors could significantly block the induction of MMP1 and 13 [38, 39]. Changes in ECM content and structure indicate the progression of OA [40]. Degradation and aberrant homeostasis of the ECM are regarded as therapeutic targets in the regeneration, and reconstruction of articular cartilage [41, 42]. IL-1 stimulates the upregulation of matrix-degrading enzymes such as ADAMTS-5, MMP1, 3, and 13 in chondrocytes [43]. Since IL-1β induces ECM degradation, drugs that prevent its destruction can be employed as alternative OA treatments. As a prominent, MMP13 has been proven as a target in OA. A key member of the MMP family, MMP-13, primarily degrades ECM by cleaving type II collagen [44]. In the present study, JR-AB2-011 suppressed the IL-1β-mediated expression of MMP13 and iNOS, indicating that JR-AB2-011 might have chondroprotective properties against IL-1β-stimulated OA deterioration. Overall, our findings showed that the mechanism of JR-AB2-011's preventative effect on ECM degradation could be explained by suppressing IκB-α/NF-κB p65 activity through inhibition of mTORC2.

In order to slow and manage the progression of OA, chondrocyte proliferation and apoptosis inhibition have been key strategies [45, 46]. Chondrocyte apoptosis is one of the relevant causes of damage and loss of articular cartilage and is an essential mechanism for the formation and progression of OA [47]. At the same time, inflammatory cytokines tend to trigger apoptosis of the chondrocytes [48, 49]. To this end, in this study, we evaluated the contribution of mTORC2 to the apoptotic markers in IL-1β-induced chondrocytes with the effects of JR-AB2-011. Our results indicates that JR-AB2-011 reduces the protein expression of the apoptosis-related markers Bax and caspase3 in chondrocytes, suggest that JR-AB2-011 inhibits chondrocyte apoptosis to lessen cartilage damage and delay OA progression. Despite the paucity of research on the interplay between mTORC2 and apoptosis, a recent study using JR-AB2-011 to examine mTORC2's potential role in brain injury in epileptic rats showed that JR-AB2-011 treatment minimized brain damage and neuronal apoptosis. According to this study, mTORC2 inhibition may have anti-neuronal damage properties in epileptic brain injury by suppressing apoptotic markers [50].

5. Conclusion

In conclusion, chondrocyte cells are frequently used as an in vitro model for osteoarthritis in studies investigating the mechanisms underlying the pathophysiology of osteoarthritis, including inflammation, catabolic responses, and apoptosis, and to identify potential new therapeutic options for the treatment of OA. The data presented in this study for the first time demonstrate that mTORC2 plays a role in the inflammation, catabolic response, and apoptosis caused by IL-1β in chondrocytes. In addition, we identified mTORC2's stimulation of IκB-α/NF-κB p65 as the mechanism causing increased TNF-α, IL-6, iNOS, MMP13, Bax, and caspase3 expression, resulting in apoptosis, catabolic response, and inflammation in human chondrocytes. This is the first observation that highlights the significance of mTORC2 inhibition, which may represent a strategic goal for managing OA-related processes.

Conflict of Interests

The authors declare no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Informed Consent

The authors declare that no patients were used in this study.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Meryem Temiz-Resitoglu: concepts, design, experimental studies, data analysis, writing, original draft preparation. Seyhan Sahan-Firat: concepts, design, experimental studies, supervision, reviewing and editing, Zainab Sabrie: experimental studies, reviewing and editing, Rukiye Nalan Tiftik: experimental studies, reviewing and editing, Taskın Kalkan, experimental studies, reviewing and editing, Ayca Aktas-Sukuroglu: experimental studies, reviewing and editing, Kafait U. Malik: reviewing and editing. All the authors approved the final version.

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