INTRODUCTION

The knee is essential to movement, is more flexible, and more prone to injury. Osteoarthritis (OA) causes physical pain and even disability in patients and results in increased socio-economic costs. Identification of effective treatments for OA is necessary to improve public health worldwide [1].

P2X purinoceptor 7 (P2X7) is a purinergic receptor and trimeric adenosine triphosphate (ATP)-gated cation channel that is expressed in several types of eukaryotic cells, including immune and bone cells. Moderate activation occurs when the receptor binds to ATP. When the gated state of P2X7R is open, the receptor mediates Na+ and Ca2+ influx and K+ efflux, resulting in rapid depolarization [2]. When the activation time is prolonged, P2X7R can induce membrane pore formation, allowing molecules up to hundreds of Da to pass [3]. As a key inflammatory switch, the activation of P2X7R mediates several downstream reactions, such as inflammatory factor release, cell proliferation, cell death, and phenotypic changes [4]. K+ efflux is one of the factors for activation of the NLRP3 inflammasome [5]. Indeed, when cells undergo pyroptosis, cell swelling and rupture are accompanied by the uncontrolled release of many pro-inflammatory cytokines, such as interleukin (IL)-1β, leading to passive and rapid cell death [6]. IL-1β activated by caspase-1 cleavage not only induces apoptosis [7] but also stimulates the secretion of cartilage-degrading enzymes, such as matrix metalloproteinase (MMP) 3, MMP13, and a disintegrin and metalloproteinase with thrombospondin motifs-4 and -5 [8, 9], leading to the degradation of type II collagen and proteoglycan in the extracellular matrix and aggravating the occurrence and development of OA.

In addition, P2X7-mediated Ca2+ influx [10, 11] and increased AMP/ATP ratios can activate the energy receptor AMP-activated protein kinase (AMPK), inhibits mammalian target of rapamycin (mTOR), and promote autophagy [12]. Cells recover excess or damaged lipids, proteins, and organelles through autophagy; these proteins are then degraded and reused to maintain cell viability and relieve inflammation [9, 13]. Rapamycin (an autophagy inducer) promotes the degradation and clearance of damaged mitochondria, reduces IL-1β-induced reactive oxygen species (ROS) generation, and blocks the OA-like phenotype in chondrocytes [14]. mTOR overexpression inhibits chondrocyte autophagy and promotes apoptosis, leading to increased cartilage degeneration [15]. However, studies have...
shown that the activation state of P2X7 affects the level of autophagy in cells [16, 17]. In the early stage of activation, P2X7-mediated K\(^{+}\) efflux and Ca\(^{2+}\) influx activate mitochondrial ROS (mtROS), and Ca\(^{2+}\) activates Ca\(^{2+}\)/calmodulin-dependent protein kinase, which in turn activates the AMPK pathway, inhibits mTOR, and promotes mitophagy and lysosome biogenesis. During the later stages of activation, the lysosome proteinkine, which in turn activates the AMPK pathway, disrupted, cartilage degradation is intensified, and cells are destroyed, and it cannot be fused with autophagosomes to form autolysosomes for targeting and degrading harmful substances, such as inflammasome components, leading to apoptosis, pyroptosis [18], and ultimately cell death [19]. Based on the above research results, we speculate that modulation of the ion current by variations in the expression and activation of P2X7 can cause changes in the fate of cell autophagy and pyroptosis. When the chondrocyte (b) has been disrupted, cartilage degradation is intensified, which leads to the aggravation of OA.

Although our previous work initially explored the connection between P2X7 and OA [20, 21], the role of P2X7 in exercise prevention and treatment of OA is still unknown. P2X7 modulates IL-1β levels in the plasma after exercise, and the expression of P2X7 increases with the duration of exercise. After exercise, the levels of P2X7R and nuclear factor-kB decrease with the duration of exercise. In sedentary individuals, the levels of NLRP3 and caspase-1 increase after exercise; in contrast, NLRP3 levels decrease after exercise in individuals participating in normal or endurance exercise. Thus, exercise exerts pro-inflammatory effects in sedentary individuals and anti-inflammatory effects in individuals who exercise [22].

Therefore, in this study, we evaluated whether moderate-intensity exercise could promote autophagy and inhibit pyroptosis through the P2X7/AMPK/mTOR axis, thereby alleviating the occurrence and development of OA. Our findings are expected to help elucidate the role of P2X7 in OA and contribute to the development of reasonable and appropriate exercise regimens to prevent and treat OA.

RESULTS

Moderate-intensity exercise maintained low activation of P2X7, promoted autophagy, and inhibited pyroptosis

Our previous research work showed that moderate-intensity exercise reduced the occurrence and development of OA [23]. Disorders of the chondrocyte state balance, such as cell death, are the main factors aggravating OA. The animal tissues in this part, such as paraffin sections and joint cavity lavage fluid, came from previous experiments of our research group [23]. In this study, TUNEL assays (Fig. 1A, D) showed that moderate-intensity exercise significantly reduced cell death in the knee joint tissue of OA model rats compared with low- and high-intensity exercise. The unmerged images are shown in Fig. S3A. As a key switch for inflammation, P2X7 is closely related to cell death. IHC analysis (Fig. 1B, E) showed that P2X7 expression first decreased and then increased as the exercise intensity increased, with lower expression observed in the moderate-intensity exercise group. Moreover, the expression levels of pyroptosis-related indicators, such as NLRP3 and caspase-1, also decreased in the moderate-intensity exercise group. By contrast, autophagy-related indicators, such as LC3B and Beclin-1 were upregulated, and changes in the expression levels of AMPK/mTOR pathway indicators also confirmed that moderate-intensity exercise promoted autophagy. ELISAs (Fig. 1C) showed that IL-1β levels in IALF initially decreased and then increased as the exercise intensity increased. Based on the above results, we concluded that moderate-intensity exercise downregulated P2X7, promoted autophagy, and inhibited pyroptosis.

Moderate-intensity exercise inhibited pyroptosis and alleviated OA by moderately activating P2X7-mediated autophagy

In addition to the intervention of treadmill exercise in OA model rats, we also injected the P2X7 agonist BzATP (0.3 mg) or the autophagy inhibitor MHY1485 (0.3 mg) into the knee joint cavity of rats to explore whether the anti-inflammatory effects exerted by moderate-intensity exercise functioned via maintaining the appropriate activation of P2X7 and promoting autophagy. The results of micro-CT analysis (Fig. 2A, E) showed that bone destruction in rats in the OA group injected with MIA into the knee joint cavity was severe, and treadmill training with moderate intensity reduced osteophyte formation and joint space narrowing. In the group injected with BzATP or MHY1485, the anti-inflammatory effects of exercise disappeared, and even more serious OA symptoms appeared. The results of ELISA (Fig. 2B) showed that IL-1β levels in the IALF decreased after moderate-intensity exercise, and this trend was reversed after the additional injection of BzATP or MHY1485. The results of H&E and TB staining (Fig. 2C, D) showed that moderate-intensity exercise reduced cartilage loss and alleviated the occurrence and development of OA (Fig. 2F). BzATP upregulated P2X7, and MHY1485 inhibited autophagy; thus, the anti-inflammatory effects were blocked. Similar results were observed in TUNEL assays (Fig. 3A) and IHC analyses (Fig. 3B). The unmerged pictures are shown in Fig. S3B. Additional injection of BzATP significantly increased P2X7 expression, accompanied by the aggravation of pyroptosis and decreased autophagy, resulting in increased numbers of dead cells (Fig. 3C). Additionally, MHY1485 reduced the expression levels of autophagy-related indicators, leading to increased cell death and more obvious inflammation (Fig. 3D). Based on the above results, we inferred that moderate-intensity exercise inhibited pyroptosis and alleviated OA by maintaining the appropriate activation of P2X7 and promoting autophagy. Accordingly, overactivation of P2X7 or inhibition of autophagy blocked the anti-inflammatory effects of moderate-intensity exercise.

BzATP moderately activated P2X7, promoted autophagy, and inhibited pyroptosis, and excessive activation of P2X7 aggravated OA

In order to determine the appropriate concentration and treatment time for BzATP-induced P2X7 activation, we evaluated cell viability for various BzATP concentrations (0, 10, 20, and 40 μM) and treatment times (12 and 24 h). CCK-8 assays (Fig. 4A) showed that cell viability was significantly reduced by BzATP treatment for 6 h at a concentration of 20 μM. Moreover, when the BzATP concentration was 40 μM, the cell viability dropped to 70.1% that of the control group, and after treatment for 12 h, cell viability decreased significantly when the concentration of BzATP was 10 μM. Therefore, for subsequent cellular experiments, we treated cells with BzATP for 6 h.

LDH release experiments (Fig. 4B) and flow cytometry analysis (Fig. 4C, D) showed that when the concentration of BzATP was 10 μM, pyroptosis was not induced. However, as the concentration increased, cell death and pyroptosis became more obvious. Similarly, ELISA (Fig. 4E) showed that the IL-1β content in cell culture supernatants was not significantly different from the control group when the BzATP concentration was 10 μM. However, when the concentration of BzATP was increased, IL-1β levels in cell culture supernatants increased significantly.

Western blot analysis (Fig. 4F, G) and RT-qPCR (Fig. 4H) also confirmed the above results, demonstrating that P2X7 mRNA and protein levels were increased as the BzATP concentration increased. Indeed, 10 μM BzATP moderately activated P2X7 and increased the expression levels of autophagy-related targets, while decreasing the expression levels of pyroptosis-related
targets. At 20 and 40 μM, BzATP overactivated P2X7, inducing the synthesis of MMP13 and the degradation of collagen II, leading to the opposite phenomena and worsening the inflammatory response. Based on the above results, we inferred that BzATP moderately activated P2X7, promoted autophagy, inhibited pyroptosis, restored cell viability, reduced cell death, and alleviated OA. By contrast, excessive activation of P2X7 promoted pyroptosis, inhibited autophagy, and aggravated OA.

**Fig. 1** Moderate-intensity exercise downregulated P2X7, promoted autophagy, and inhibited pyroptosis. We set the following five groups: saline, MIA, MIA + low-intensity exercise, MIA + moderate-intensity exercise, MIA + high-intensity exercise. A total of 50 rats were randomly assigned, with 10 rats in each group. The animal tissues in this part, such as paraffin sections and joint cavity lavage fluid, came from previous experiments of our research group [23]. A TUNEL assays were used to evaluate cell death in tissue sections from each group. Red: dead cells; blue: nuclei stained by DAPI (scale bar: 50 μm). B IHC was used to evaluate P2X7, NLRP3, caspase-1, mTOR, AMPK, LC3B, and Beclin-1 expression in tissue sections from each group. Brown: stained cells; blue: hematoxylin-stained nuclei (scale bar: 500 μm). C ELISA were used to detect IL-1β content in the articular cavity lavage fluid from each group. D Statistical data for cell death in TUNEL assays. E Statistical data for stained cells in IHC analysis. The * in the histochemical images represents the positively stained cells. Data are presented as means ± standard deviations of at least three independent experiments. *p < 0.05, **p < 0.01.
promoted autophagy and thereby alleviated pyroptosis. In order to explore whether appropriate activation of P2X7 AMPK/mTOR signaling pathway to block pyroptosis. Moderate activation of P2X7 induced autophagy through the AMPK/mTOR signaling pathway, we treated cells with the mTOR activator MHY1485 (10 μM) and/or the AMPK inhibitor compound C (10 μM). CCK-8 assays (Fig. 6A) showed that application of MHY1485 alone did not cause significant changes in cell viability. However, when combined with BzATP (10 μM), cell viability decreased to 84.3% that of the control group. LDH release experiments (Fig. 6B) and flow cytometry analyses (Fig. 6C, D) showed that BzATP (10 μM) did not cause cell damage and pyroptosis. However, activation of mTOR resulted in increased LDH release and enhanced the ratio of inflammatory response. Cytofluorescence experiments (Fig. 6F, G) showed that LC3B aggregation was increased by treatment with BzATP (10 μM), but decreased after application of MHY1485, indicating that MHY1485 treatment blocked autophagy.

**Excessive activation of P2X7 aggragated the occurrence and development of OA by inducing pyroptosis**

In order to explore whether high-level activation of P2X7 induced pyroptosis to aggravate OA, we selected the NLRP3 inhibitor CY-09 for subsequent cell experiments. The results of CCK-8 assays (Fig. 5A) showed that CY-09 (10 μM) alone did not cause significant changes in cell viability, whereas BzATP (40 μM) significantly reduced cell viability. After treatment with CY-09, cell viability was restored. The results of LDH release assays (Fig. 5B) and flow cytometry (Fig. 5C, D) showed that cell damage and pyroptosis caused by BzATP were alleviated after application of the NLRP3 inhibitor. Additionally, ELISA (Fig. 5E) showed that CY-09 effectively reduced IL-1β levels in the cell culture supernatant, indicating that the inflammatory response was reduced. Cell fluorescence analyses (Fig. 5F) showed that the fluorescence intensity of caspase-1 and PI double staining decreased significantly after inhibition of NLRP3. Western blotting (Fig. 5G, H) and RT-qPCR analyses (Fig. 5I) confirmed the above results. Moreover, CY-09 reduced the expression levels of P2X7 and pyroptosis-related indicators, blocked the synthesis of MMP13, and inhibited the degradation of collagen II. Based on these results, we concluded that excessive activation of P2X7 aggragated the occurrence and development of OA by inducing pyroptosis. Inhibition of NLRP3 blocked pyroptosis and cell death, thereby alleviating OA.

**Moderate activation of P2X7 induced autophagy through the AMPK/mTOR signaling pathway to block pyroptosis**

In order to explore whether appropriate activation of P2X7 promoted autophagy and thereby alleviated pyroptosis through the AMPK/mTOR signaling pathway, we treated cells with the mTOR activator MHY1485 (10 μM) and/or the AMPK inhibitor compound C (10 μM). CCK-8 assays (Fig. 6A) showed that application of MHY1485 alone did not cause significant changes in cell viability. However, when combined with BzATP (10 μM), cell viability decreased to 84.3% that of the control group. LDH release experiments (Fig. 6B) and flow cytometry analyses (Fig. 6C, D) showed that BzATP (10 μM) did not cause cell damage and pyroptosis. However, activation of mTOR resulted in increased LDH release and enhanced the ratio of inflammatory effects of moderate-intensity exercise.
Fig. 3  Supplement to Fig. 2. A TUNEL assays were used to detect cell death in tissue sections from each group. Red: dead cells; blue: nuclei stained by DAPI (scale bar: 50 μm). B IHC was used to detect the expression levels of P2X7, NLRP3, caspase-1, mTOR, AMPK, LC3B, and Beclin-1 in tissue sections from each group. Brown: stained cells; blue: hematoxylin-stained nuclei (scale bar: 500 μm). C Statistical data for cell death in TUNEL assays. D Statistical data for stained cells in IHC analyses. The * in the histochemical images represents the positively stained cells. Data are presented as means ± standard deviations of at least three independent experiments. *p < 0.05, **p < 0.01.
mTOR or inhibition of AMPK blocked autophagy and promoted pyroptosis, leading to aggravation of OA.

**Promotion of autophagy alleviated pyroptosis induced by overactivation of P2X7**

Based on our above experiments, we knew that decreasing autophagy would enhance pyroptosis. Therefore, in order to explore whether increasing the level of autophagy could alleviate the pyroptosis induced by P2X7 overactivation, we used the mTOR inhibitor rapamycin (5 μM) and the AMPK activator A-769662 (5 μM) for subsequent cell experiments. CCK-8 assays (Fig. 7A) showed that rapamycin alone did not cause significant changes in cell viability, but significantly restored cell viability after cotreatment with BzATP (40 μM). Moreover, LDH release assays (Fig. 7B) revealed that rapamycin significantly reduced LDH release. ELISA and Western blotting were used to detect the levels of IL-1β and P2X7. The results showed that rapamycin decreased the expression of IL-1β and P2X7, indicating that rapamycin could alleviate pyroptosis. Finally, flow cytometry was used to detect the number and ratio of caspase-1/PI-stained cells, reflecting the severity of cell pyroptosis. The results showed that rapamycin significantly reduced the number of caspase-1/PI-stained cells, indicating that rapamycin could alleviate pyroptosis.

**Fig. 4** Activation of P2X7 with increasing concentrations of BzATP first promoted autophagy and then induced pyroptosis. A CCK-8 assays were used to evaluate cell viability in each group. Absorbance was measured at a wavelength of 450 nm. B LDH release assays were used to detect the degree of cell damage. C Flow cytometry was used to detect the number and ratio of caspase-1/PI-stained cells, reflecting the severity of cell pyroptosis. D Statistical data for stained cells. E ELISA was used to detect IL-1β levels in cell culture supernatants from each group. F, G Western blotting and H RT-qPCR were used to detect protein and mRNA expression levels of P2X7, NLRP3, caspase-1, mTOR, AMPK, LC3B, Beclin-1, MMP13, and collagen II. Data are presented as means ± standard deviations of at least three independent experiments. *p < 0.05, **p < 0.01.
and flow cytometry showed that cell damage, ROS production (Fig. 7F, G), and pyroptosis (Fig. 7C, D) caused by BzATP (40 μM) were partially blocked by mTOR inhibition. Additionally, LDH release was reduced, and the caspase-1/PI and ROS staining ratio decreased.ELISA (Fig. 7E) also showed that the promotion of autophagy significantly decreased IL-1β content in cell culture supernatants, indicating that pyroptosis was partially blocked. The results of TEM (Fig. 7H) also confirmed that increased autophagy promoted the morphological integrity of the cells; the cell membrane remained intact, and the number of autophagic lysosomes increased.

Similar results were obtained by western blotting (Fig. 7I, J) and RT-qPCR (Fig. 7L). Indeed, treatment with BzATP (40 μM) increased in the mRNA and protein expression levels of pyroptosis-related indicators. However, treatment with rapamycin significantly decreased the expression levels of these targets, whereas the mRNA and protein expression levels of autophagy-related indicators increased. Additionally, treatment with rapamycin blocked the synthesis of MMP13 and the degradation of collagen II. Co-IP experiments (Fig. 7K) confirmed that LC3B could bind to NLRP3 and that autolysosomes could target and degrade inflammasomes to inhibit pyroptosis. Similar results were
Fig. 6  Moderate activation of P2X7 reduced pyroptosis through AMPK/mTOR-induced autophagy. A CCK-8 assays were used to evaluate cell viability in each group. Absorbance was measured at a wavelength of 450 nm. B LDH release assays were used to detect the degree of cell damage. C Flow cytometry was used to detect the number and ratio of caspase-1/PI-stained cells, reflecting the severity of cell pyroptosis. D Statistical data for stained cells. E ELISA was used to detect IL-1β content in cell culture supernatants for each group. F Cell fluorescence experiments were used to detect the fluorescence intensity and puncta number of cell LC3B staining, reflecting the level of autophagy (scale bar: 10 μm). G Statistical data for LC3B puncta/cell. H, I Western blotting and J RT-qPCR were used to detect protein and mRNA expression levels of P2X7, NLRP3, caspase-1, mTOR, AMPK, LC3B, Beclin-1, MMP13, and collagen II. Data are presented as means ± standard deviations of at least three independent experiments. *p < 0.05, **p < 0.01.
observed after treatment with A-769662 (Fig. S5). Collectively, these findings suggested that the promotion of autophagy increased the targeted degradation of inflammasome components by autolysosomes, thereby alleviating the pyroptosis induced by high-level activation of P2X7 and delaying the occurrence and development of OA.

**DISCUSSION**

In this study, we found that under moderate-intensity exercise or stimulation with an appropriate concentration of BzATP, the activation state of P2X7 was low. Appropriate activation of P2X7 promoted autophagy through the AMPK/mTOR signaling pathway, inhibited pyroptosis, and then blocked the occurrence and development of OA.
Fig. 7 Increasing the level of autophagy effectively blocked pyroptosis induced by excessive activation of P2X7. A CCK-8 assays were used to detect cell viability for each group. Absorbance was measured at a wavelength of 450 nm. B LDH release assays were used to detect the degree of cell damage. C, F Flow cytometry was used to detect the number and ratio of caspase-1/P2X7 and ROS-stained cells, reflecting the severity of cell pyroptosis and ROS production. D, G Statistical data for stained cells. In the statistical analysis of ROS, we used the control group as a reference to normalize the absorbance value and expressed the results as the fold change. E ELISA was used to detect IL-1β content in cell culture supernatants for each group. H TEM analysis of cell morphology, including cell membrane rupture, nuclear membrane autophagy (red arrow), and autolysosome number (white arrow), reflecting the level of cell pyroptosis and autophagy (green arrow, autophagosome) (scale bar: 2 μm). K Co-IP experiments demonstrating the binding of LC3B and NLRP3. I, J Western blotting and L RT-qPCR were used to detect the protein and mRNA expression levels of P2X7, NLRP3, caspase-1, mTOR, AMPK, LC3B, Beclin-1, MMP13, and collagen II. Data are presented as means ± standard deviations of at least three independent experiments. *p < 0.05, **p < 0.01.

development of OA. By contrast, overactivation of P2X7, such as that occurring by stimulation with high-intensity exercise or a high concentration of BzATP, increased mTOR activation and decreased autophagy. These effects were not sufficient to counteract the massive activation of NLRP3 inflammasomes, resulting in pyroptosis, cell death, and cartilage degradation (Fig. 8).

As an important signaling pathway downstream of P2X7, AMPK signaling participates in the regulation of many physiological and disease functions [24], such as the promotion of autophagy by exercise [25]. During exercise, the interaction between the innate immune molecule Toll-like receptor 9 and Beclin-1 is strengthened to regulate AMPK activation in muscles [26]. AMPK interacts with sestrins to participate in exercise-induced autophagy and thereby maintains skeletal muscle glucose metabolism [27]. However, another study showed that autophagy plays important role in maintaining mitochondrial function during exercise, but has little effect on PRKAA1/AMPK activation, exercise-dependent glucose homeostasis, and energy supply to satisfy muscle contraction [28]. Despite these inconsistencies, the effects of AMPK activation-induced autophagy on alleviating inflammation are unquestionable. Post-exercise pretreatment (10 min rest for every 10 min running) promotes autophagy through AMPK/mTOR signaling to alleviate heart damage caused by exhaustive exercise [29], and downregulation of autophagy promotes accumulation of damaged mitochondria, increases ROS, and leads to tissue degeneration [30].

Our experimental results also showed that AMPK expression was higher in the moderate-intensity exercise group than in the high- and low-intensity exercise groups; mTOR expression showed the opposite trend. Thus, taken together with our findings of changes in the expression levels of autophagy- and pyroptosis-related indicators, we concluded that moderate-intensity exercise decreased P2X7 expression and activity, promoted autophagy, and inhibited pyroptosis. However, further studies are needed to clarify the involvement of P2X7 and the AMPK/mTOR signaling pathway in this mechanism.

As a key effector of inflammation [31], P2X7 mediates multiple signaling pathways to modulate autophagy, pyroptosis, metabolism, and nutrition [32]. Compared with other P2X receptors, P2X7 requires a higher concentration of ATP for activation [33] and has a higher affinity for the selective agonist BzATP, whose potency is dozens of times that of ATP [4]. Our cell experiments showed that as the concentration of BzATP increased, the expression of P2X7 increased. Notably, the P2X7-mediated ion current not only induces autophagy but also promotes pyroptosis. Moreover, during the early stages of activation, the P2X7-mediated cellular response is dominated by autophagy; in the later stages of activation, the main phenotype of the cell gradually changes from autophagy to pyroptosis. This finding is similar to the results of another study demonstrating that initial activation of P2X7 has protective effects on cells but as the activation time is increased, cell death is induced [19]. Ca2+ overload, caused by P2X7R-mediated Ca2+ influx under stimulation with high concentrations of ATP in macrophages, leads to mitochondrial dysfunction, which in turn causes cell pyroptosis. However, when P2X7R was stimulated with low ATP concentrations or was positively allosterically regulated by compound K, the accumulation of mROS and the activation of caspase-1 and -3 promoted apoptosis rather than pyroptosis as the mechanism of cell death [34].

The activation level of P2X7 is closely related to the direction of cell fate. Regulation of the AMPK/mTOR signaling pathway by P2X7 promotes autophagy, exerts antitumor effects [35], and protects against Mycobacterium tuberculosis infection in macrophages [36]. However, some studies have reported conflicting results. For example, P2X7 activates the phosphatidylinositol 3-kinase/AKT/mTOR pathway and other signaling pathways to promote the proliferation and metastasis of osteosarcoma and increase bone destruction [37]. Additionally, P2X7 expression is downregulated in an inflammatory environment, and its activation promotes the osteogenesis of periodontal ligament stem cells [38]. In our study, treatment with AMPK and mTOR activators or inhibitors showed that the AMPK/mTOR signaling pathway was a key signaling pathway through which P2X7 induced autophagy. Inhibition of AMPK or activation of mTOR blocked the autophagy-inducing effects of P2X7, even if P2X7 was only moderately activated, and insufficient autophagy would then lead to aggravation of pyroptosis. However, the link between P2X7-induced autophagy and pyroptosis remains unknown.

When cells undergo pyroptosis, autophagy usually plays protective roles. In the resting state of macrophages, autophagy is inhibited. However, in the presence of a low degree of infection, inhibition of autophagy is released, resulting in cell death; when autophagy cannot eliminate intracellular infection, caspase-1 is activated to initiate pyroptosis [39]. Autophagy often suppresses pyroptosis through the fusion of autophagosomes and lysosomes to form autolysosomes, which further degrade intracellular harmful substances, such as unfolded proteins and damaged mitochondria [40], as well as inflammasome components, such as NLRP3 [41] and ASC [42]. The interaction between LC3B and NLRP3 is also essential for autophagy-mediated degradation of NLRP3 and inhibition of pyroptosis [43]. In this study, we verified the interaction between LC3B and NLRP3 in chondrocytes through co-IP experiments and found that proper activation P2X7-induced autophagy inhibited pyroptosis by degrading NLRP3. In this study, we showed that AMPK activators or mTOR inhibitors effectively increased autophagy and significantly alleviated pyroptosis and inflammation caused by excessive activation of P2X7. Based on the above results, we concluded that regulation of the AMPK/mTOR signaling pathway by P2X7 played an important role in inducing autophagy and inhibiting pyroptosis.

In this study, we demonstrated the role of P2X7 in the prevention and treatment of OA by exercise for the first time. Moderate activation of P2X7 can effectively relieve inflammation, providing insights into novel approaches for OA treatment. However, further studies are still required. For example, cell models involving stress and stretching [44] may be more consistent with the in vivo environment under exercise conditions. Therefore, in subsequent studies, we will evaluate the effects of P2X7 expression on chondrocytes under different degrees of mechanical stress stimulation.
CONCLUSION
In summary, we explored the expression and activation of P2X7 under different intensities of exercise or stimulation and confirmed that the appropriate intensity of exercise or moderate stimulation led to activation of P2X7 to a certain level, exerting protective effects on cells. The AMPK/mTOR signaling pathway was found to play an indispensable role in P2X7-induced autophagy, and the formation of autolysosomes affected the degradation of inflammasome components and reduced cell damage. We also confirmed, for the first time, that moderate-intensity exercise promoted autophagy through the P2X7/AMPK/mTOR signaling axis, inhibited pyroptosis, and thereby alleviated OA. These findings regarding the role of P2X7 in exercise-based prevention and treatment of OA provide new perspectives for OA treatment.

MATERIALS AND METHODS
Antibodies and reagents
The antibodies used in this study were as follows: anti-P2X7 (Abcam, Cambridge, UK; cat. no. ab109054), anti-collagen II (Abcam; cat. no. ab34712), anti-MMP13 (Abcam; cat. no. ab39012), anti-AMPKα1 (Abcam; cat. no. ab32047), anti-mTOR (Abcam; cat. no. ab109268), anti-NLRP3 (Proteintech; cat. no. 19771-1-AP), anti-caspase-1 (Proteintech; cat. no. 22915-1-AP), anti-LC3B (Abcam; cat. no. ab192890), anti-Beclin-1 (Abcam; cat. no. ab62557), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Proteintech; cat. no. 10494-1-AP), and horseradish peroxidase (HRP)-labeled IgG (Beyotime; cat. no. A0208). The reagents used in the experiment were as follows: MIA (Sigma, St. Louis, MO, USA; cat. no. I2512), the P2X7 receptor agonist BzATP (Sigma; cat. no. B6396), the mTOR activator MHY1485 (Sigma; cat. no. SML0810), the mTOR inhibitor rapamycin (Sigma; cat. no. V900930), the NLRP3 inhibitor CY-09 (Sigma; cat. no. SML2465), the AMPK activator A-769662 (Sigma; cat. no. SML2578), and the AMPK inhibitor compound C (Sigma; cat. no. P5499). The concentrations, dosages, and preparation of the reagents were described previously [43, 45].

Animal models and extraction of rat tissue
Fifty Sprague-Dawley (SD) rats (male, 5 weeks old, 230–250 g) were purchased from HFK Bioscience (Beijing, China) and were randomly assigned into the following five groups (n = 10 each; Fig. S1): [1] control group (normal saline) [2], OA group (MIA) [3], exercise group (MIA + treadmill exercise) [4], agonist group (MIA + treadmill exercise + BzATP), and [5] inhibitor group (MIA + treadmill exercise + MHY1485 [an autophagy inhibitor]). We considered that male rats are more capable of running on a treadmill, plus the previous research results of our research group, so we chose male SD rats for animal experiments. Under the premise of ensuring sufficient data, we used as few animals as possible to conduct experiments. In the end, we used a total of 50 SD rats, which were randomly allocated, and 10 in each group were used for subsequent animal experiments. Animal-related experiments complied with the Animal Ethics Regulations of China Medical University (approval no. 2017PS237K) and were run in blinded fashion. During the...
experiments, we adhered to the 3R rules to ensure that rats were sacrificed comfortably.

MIA, BzATP, and MHY1485 were prepared as 50 μL aliquots and were injected into the knee joint cavity of rats after 3 days of adaptive feeding. The control group was injected with the same volume of normal saline, and the other four groups were injected with 0.5 mg MIA for each knee joint once. In our previous research [20, 23] and subsequent cell experiments, the dosage and treatment time of BzATP referred to our previous research results, and the dosage of other reagents was selected according to our previous research results, reagent instructions, and the results of preliminary experiments. After determining the appropriate dosage and processing time of the above reagents, they can be used in subsequent cell experiments.

Cell viability assays and lactate dehydrogenase (LDH) release tests

The cells were seeded in 96-well plates at a density of 5000 cells/well, and 200 μL culture medium was added to each well. Cells were then incubated for 24–48 h, and appropriate reagents were added. After the reaction was completed, 90 μL culture medium and 10 μL Cell Counting Kit (CCK)-8 (Dojindo, Japan) were added to each well. One hour before the detection time point, LDH release reagent provided by the kit (Beyotime; cat. no. C0042) was added to each well. Cells were incubated for an additional 2 h and then placed in a microplate reader (Synergy H1; BioTek, USA) to detect luminescence at a wavelength of 450 nm. The same approach was used for LDH release experiments. When the cell confluence reached 80–90%, appropriate drug treatments were applied. One hour before the detection time point, LDH release reagent provided by the kit (Beyotime; cat. no. C0042) was added to each well. Cells were then incubated for an additional 2 h, and the fold increase in LDH concentration was normalized to the control.

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blocked with 5% skim milk at room temperature for 2 h and incubated with primary antibodies (1:1000) overnight at 4 °C. Next, membranes were incubated with HRP-labeled IgG antibodies (1:2000) at room temperature for 2 h and scanned using a chemiluminescence detection system (GE Amersham Imager 600). Image J (NIH, USA) was used to analyze the gray values of the bands, and GAPDH was used as an internal control. Statistical analysis was performed after the values were normalized to GAPDH expression.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

First, 1 mL TRIzol (TaKaRa, Shiga, Japan; cat. no. 9109) was added to each well of a six-well plate containing treated cells. RNA was then isolated and reverse transcription-quantitative PCR system (Applied Biosystems, Foster City, CA, USA). Thermocycling conditions were as follows: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s and 60 °C for 35 s. The obtained Ct values were normalized using the 2^(-ΔΔCt) formula, with GAPDH as the housekeeping gene. The primer sequences are detailed in Supplementary Fig. S2.

Immunofluorescence and imaging analysis

Treated cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1–0.2% Triton X-100 for 10 min, and then blocked with 5–10% serum for 30 min. Cells were then incubated with primary antibodies (1:100) overnight at 4 °C, followed by incubation with secondary antibodies (1:200) for 1 h at room temperature in the dark. Finally, cells were incubated with DAPI to stain nuclei at room temperature for 5 min in the dark. A Zeiss LSM880 confocal microscope (Zeiss, Oberkochen, Germany) was used to image the cells, and the images were analyzed using Image J.

Co-immunoprecipitation (Co-IP) assay

RIPA was added to lyse the cells, and 600 μL protein sample (600 μg protein) was added to 1 μg ordinary IgG and 20 μL resuspended Protein G Agarose. Samples were shaken slowly at 4 °C for 30 min to 2 h and centrifuged at 1000 × g for 5 min. The supernatants were then used for subsequent immunoprecipitation. Briefly, 0.2–2 μg primary antibody was added, and samples were shaken slowly overnight at 4 °C. Next, 20 μL fully resuspended Protein G Agarose was added, and samples were shaken slowly at 4 °C for 1-3 h. Samples were centrifuged at 1000 × g for 5 min, supernatants were removed, pellets were washed with PBS, the supernatants were removed again, and 20–40 μL 1 × sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer was added. Samples were vortexed to resuspend the pellet and then centrifuged. Finally, samples were boiled at 100 °C for 10 min and subjected to SDS-PAGE.

Transmission electron microscopy (TEM)

Cultured cells were treated with 2.5% normal temperature glutaraldehyde fixative after removal of culture medium and fixed for 5 min at room temperature in the dark. A cell scraper was used to gently remove the cells, and the cell liquid was moved to a centrifuge tube and centrifuged at 1000 × g for 2 min. After discarding the fixative, 1% OsO4 was added, and the cell cluster was gently lifted and suspended in the fixative. Cells were fixed for 30 min at room temperature in the dark and then transferred to 4 °C until analysis. After dehydration, samples were embedded in resin. Ultrathin tissue sections (60 nm) were stained with uranyl acetate and lead citrate, and the cell morphology and subcellular structure were then observed using TEM (Hitachi 800; Hitachi, Tokyo, Japan).

Statistical analysis

We performed at least three independent experiments, and the average values of each experimental group were used in the statistical analysis. Data are expressed as means ± standard deviations. The between-group differences were determined using Student’s t-test or one-way analysis of variance with Tukey’s post hoc test in GraphPad Prism version 7.0c. Significance was set at p < 0.05.
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AUTHOR CONTRIBUTIONS

ZL: Conceptualization, methodology, and writing—original draft. ZL, HZ, JL, SP, and ZL: Formal analysis and resources. ZL, ZH, and LB: Writing—review and editing. ZL and LB: Funding acquisition and supervision.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ADDITIONAL INFORMATION

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