Original Research

Activation of the cannabinoid receptor 1 by ACEA suppresses senescence in human primary chondrocytes through sirt1 activation

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Impact statement

Senescence of chondrocytes and cartilage degeneration induced by the proinflammatory cytokine interleukin-1β (IL-1β) are associated with the pathogenesis of osteoarthritis (OA). Here we found that: (a) the CB1 agonist ACEA abolished IL-1β-induced senescence and cell arrest in chondrocytes; (b) the CB1 agonist ACEA also abolished IL-1β-induced expression of caveolin-1, PAI-1, and p21; (c) ACEA regulated the expression of sirt1; (d) the inhibitory effects of ACEA on senescence were mediated by sirt1.

Abstract

Senescence of chondrocytes and cartilage degeneration induced by the proinflammatory cytokine interleukin-1β is associated with the pathogenesis of osteoarthritis. The cannabinoid receptor 1 has been involved in the pathological development of various diseases. Here, we evaluated whether activation of cannabinoid receptor 1 using its selective agonist arachidonyl-2-chloroethylamide had an influence on cellular senescence induced by interleukin-1βin human chondrocytes. Our findings demonstrate that agonist arachidonyl-2-chloroethylamidedecreased senescence-associated β-galactosidase activity and cell cycle arrest in the G0/G1 phase induced by interleukin-1β. Importantly, our results display interleukin-1βtreatment significantly increased the expressions of senescence genes (caveolin-1, PAI-1 and p21), which were prevented by agonist arachidonyl-2-chloroethylamide treatment. However, it was noticed that these functions of agonist arachidonyl-2-chloroethylamide were abolished by the cannabinoid receptor 1 selective antagonist AM251, suggesting the involvement of cannabinoid receptor 1. Also, our results indicate that agonist arachidonyl-2-chloroethylamide enhanced the expression of sirt1. These findings suggest that activation of cannabinoid receptor 1 by agonist arachidonyl-2-chloroethylamide might have a protective effect against pro-inflammatory cytokines such as interleukin-1β-induced chondrocytes senescence in osteoarthritis patients.

Keywords: Osteoarthritis, cannabinoid receptor 1, senescence, Sirt1

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Introduction

Osteoarthritis (OA), the most prevalent disabling disease of articular cartilage, affects millions of people worldwide.[1] Chondrocytes play an essential role in the production of extracellular matrix (ECM) and the maintenance of proper articular cartilage.[2] Dysfunction of chondrocytes in OA patients has been reported to be the result of aging and exposure to stresses such as oxidative stress and inflammation. Chondrocytes do not have normal proliferative capacity in the articular cartilage of adults.[3] Recent studies have shown that OA chondrocytes have a senescent-like phenotype. Several senescent genes are present in senescent chondrocytes.[4] However, the exact biological mechanisms of senescence in chondrocytes remain rather enigmatic. Chronic secretions of pro-inflammatory cytokines such as interleukin-1β (IL-1β) play a key role in articular degradation and OA chondrocyte phenotypes.[5,6] In addition, IL-1β treatment resulted in extrinsic stress-induced senescence of articular chondrocytes.[7]

"Inflammaging," referring to a low-grade and chronic inflammatory state presented in the process of aging, has been associated with OA. "Chondrosenescence" has been used to characterize age-dependent dysfunction of chondrocytes in OA.[8,9] Cartilage that is injured during the progression of OA needs to be re-established due to its poor self-healing capacity. However, aging of human mesenchymal stem cells (hMSCs) results in the loss of the cell proliferation and differentiation capacity of hMSCs and disrupts

MSC-derived chondrocyte hypertrophic differentiation, which limits the use of these cells in approaches focused on cartilage tissue regeneration.[10] Notably, it has been reported that selective removal of senescent chondrocytes blocked the progression of post-traumatic OA.[11] Senescent chondrocytes have been considered as a potential target for OA treatment.

The endocannabinoid system (ECS) has emerged in a wide range of physiological and pathological processes.[12] Importantly, recent studies have implicated that the ECS has become a possible therapeutic target for OA.[13] The cannabinoid receptor 1 (CB1) is one of the most important endocannabinoid system receptors.[14] CB1 is expressed in human chondrocytes.[15] N-arachidonyl ethanolamide (anandamide, AEA) is the main endogenous ligand (endocannabinoid) of CB1.[16,17] AEA is identified in the synovial fluid from OA patients, but not from normal volunteers, implicating a potential role in pathophysiological processes.[18] Cannabinoids exert chondroprotective effects and are useful for OA treatment.[19] Moreover, biologically stable synthetic cannabinoids prevented IL-1-induced cartilage matrix breakdown in bovine cartilage dependent on CB receptors.[15] Interestingly, it has been reported that the selective CB1 receptor agonist arachidonyl-2-chloroethylamide (ACEA) decreased the mechanosensitivity of afferent nerve fibers in rat knee joints.[18] In this study, we investigated whether treatment with the CB1 receptor agonist ACEA could ameliorate inflammatory stress-induced senescent features of OA chondrocytes.

Materials and methods

Chondrocyte isolation, culture, and treatments

This study was approved by the Medical Ethics Committee of Jinan Military General Hospital (JMGME20140719). Written informed consent was collected from all of participants. Healthy articular cartilage samples were collected from 15 patients (8 males, 7 females, age: 45.2 ± 8.2) undergoing hip replacement surgery due to femoral neck or distal femoral tumor.[20,21] Cartilage pieces were finely minced, followed by sequential digestion in 0.25% trypsin (R&D Systems, USA) for 3 h at 37°C and 3 mg/ml collagenase type I overnight at 37°C. Released cells were filtered through a nylon mesh filter to isolate single cells. Isolated chondrocytes were maintained in Dulbecco's Modified Eagles Medium/Ham's F-12 (DMEM/Ham's 12) (Thermo Fisher Scientific, USA) containing 20 mM HEPES (Thermo Fisher Scientific, USA) and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 2 mM glutamine (Thermo Fisher Scientific, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml) (Thermo Fisher Scientific, USA) ($pH = 7.4$). Arachidonyl-2-chloroethylamide (ACEA) (#A9719, Sigma-Aldrich, USA) and the selective CB1 antagonist AM251 (ab120088, Abcam, USA) were dissolved in dimethylsulfoxide (DMSO). Chondrocytes were cultured with 10 ng/ml IL-1β (R&D Systems, USA) with or without 1 μM ACEA or 10 μM AM251 for 24 h.

Western blot analysis

After the indicated treatment, human chondrocytes were lysed with cell lysis buffer (Cell Signaling Technologies, USA) containing cocktail inhibitors (Thermo Fisher Scientific, USA). The concentration of total intracellular proteins was measured using a BCA method (Thermo Fisher Scientific, USA); 20 μg of total proteins were separated on a 10% SDS-PAGE and transferred to a PVDF membrane. After blocking in TBST with 5% non-fat milk at room temperature (RT) for 1 h, the membranes were sequentially probed with primary antibodies at RT for 2 h and horse radish peroxidase (HRP)-linked anti-rabbit IgG antibody (##7074, Cell Signaling Technologies, USA). Proteins were visualized by enhanced chemiluminescence detection (Pierce, USA). The following antibodies were used: Rabbit monoclonal antibody (mAb) against p21 (1:1000, #2947, Cell Signaling Technologies, USA); Rabbit mAb against PAI-1(1:2000, #11907, Cell Signaling Technologies, USA); Rabbit mAb against Caveolin-1 (1:1000, #3267, Cell Signaling Technologies, USA); Rabbit mAb against β-actin (1:5000, #4970, Cell Signaling Technologies, USA); secondary anti-rabbit IgG antibody (1: 1000, #7074, Cell Signaling Technologies, USA).

Quantitative real-time polymerase chain reaction

Total intracellular RNA of human chondrocytes was prepared from cells using Trizol reagent. Isolated RNA was then purified using the TURBO DNA free system (Thermo Fisher Scientific, USA). The RNA concentration was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). RNA was reverse-transcribed into cDNA using SuperScript II reverse transcriptase (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Real-time PCR was carried out with the fluorescent dye SYBR Green master mix (Thermo Fisher Scientific, Inc.) on ABI PRISM7700 sequence detection system. Relative quantification of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized against that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Senescence-associated β-galactosidase (SA-β -gal) activity

SA-β-gal activity was evaluated using a β-galactosidase staining kit (Beyotime Institute of Biotechnology, China). After the indicated treatment, primary chondrocytes were fixed for 30 min at RT, followed by incubation with the staining solution overnight at 37°C, the absolute numbers of SA-β-gal+ cells were quantified from six random fields each sample.

Cell cycle assay

To evaluate the effects of CB1 activation on IL-1β arrested cell cycle progression, flow cytometric analyses were performed. Briefly, human primary chondrocytes were fixed with 70% ethanol at 4°C overnight. After washing three times with PBS, chondrocytes were loaded with 50 mmol/l propidium iodide (#P1304MP, Thermo Fisher

Scientific, USA) for 30 min and subjected to flow cytometric analyses. The proportion of cells in the G0/G1, S and G2/M phases was calculated.

Statistical analysis

Experimental data collected from four to six independent experiments were used to calculate means. Results are presented as means ± SD. Statistical analysis was performed with the SPSS statistical software 16.0. Two-way ANOVA with *post hoc* Tukey's test for multiple group comparisons was used to study statistical significance. Statistical significance was assumed if $P<0.05$.

Results

To examine the effects of CB1 activation on senescent features of OA chondrocytes, the marker SA-β-Gal was assessed. IL-1β treatment (Figure 1(a)) significantly increased the percentage of cells positive for SA-β-Gal, which was significantly attenuated by the selective CB1 agonist ACEA. Conversely, AM251, a CB1 antagonist, abolished the inhibitory effects of ACEA on β-galactosidase activity. The levels of β-galactosidase staining were quantified by fluorometry in a microplate reader. As shown in Figure 1(b), the presence of ACEA significantly reduced the level of SA-β-Gal of chondrocytes induced by IL-1β. Consistently, AM251 abolished this effect. We then investigated whether the selective CB1 agonist ACEA had an influence on cell cycle. As shown in Figure 2, our results indicate that IL-1β (10 ng/ml) treatment arrested primary chondrocytes in the G0/G1 phase (67.8% in IL-1β treatment group vs. 51.6% in vehicle group). As expected, the

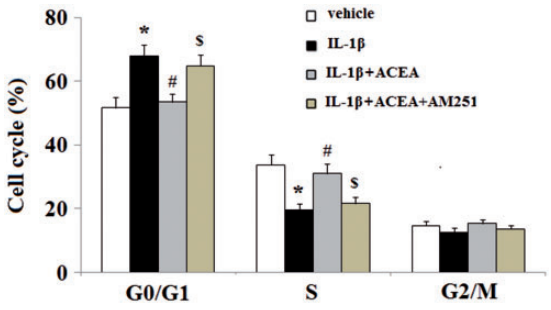

Figure 2. The selective cannabinoid receptor 1 (CB1) agonist ACEA rescued the arresting effects of IL-1β on the cell cycle of human primary chondrocytes. Human primary chondrocytes were treated with 10 ng/ml IL-1β in the presence or absence of ACEA or the selective CB1 antagonist AM251 for 24 h. Cell cycle analysis was performed by FACS (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β treatment group; $, $P < 0.01$ vs. IL-1β+ACEA group, $n = 3$–4). (A color version of this figure is available in the online journal.)

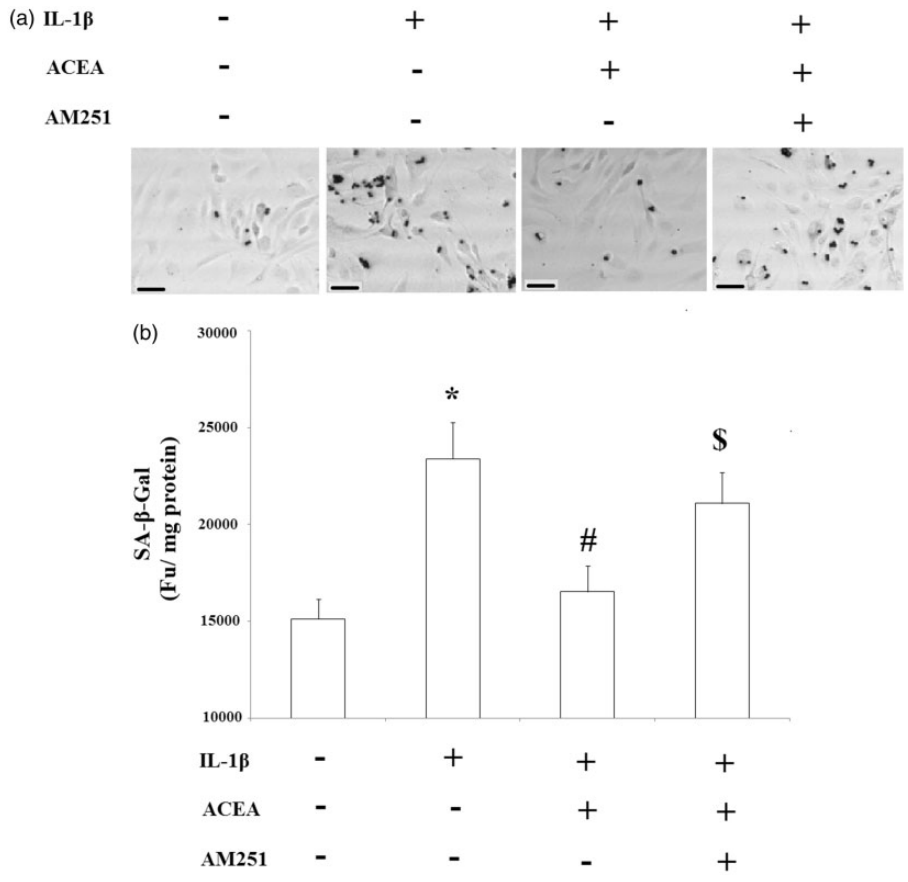

Figure 1. Activation of cannabinoid receptor 1 (CB1) by the selective CB1 agonist ACEA abolished IL-1β-induced senescence in human primary chondrocytes. Human primary chondrocytes were treated with 10 ng/ml IL-1β in the presence or absence of ACEA or the selective CB1 antagonist AM251 for 24 h. (a) Representative light microscopy-images of cells stained with the senescent marker SA-β-Gal in monolayer cultures of human primary chondrocytes; Scale bars, 50 μM; 200×; (b) SA-β-Gal was quantified by fluorometry (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β treatment group; $, $P < 0.01$ vs. IL-1β+ACEA group, $n = 3$–4).

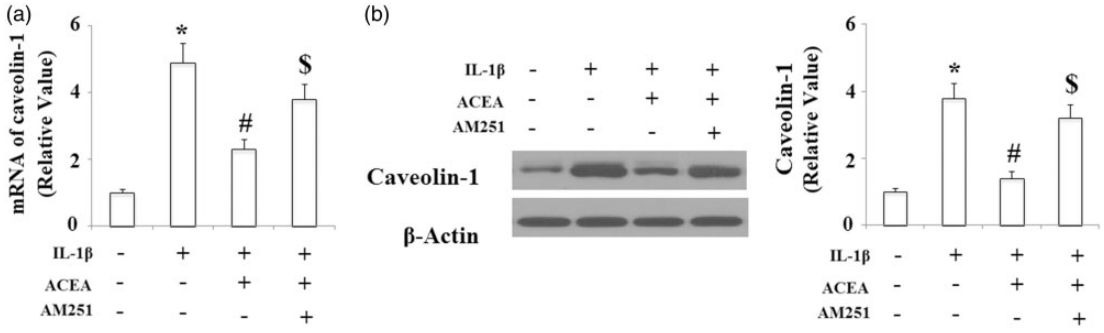

Figure 3. Effects of the selective cannabinoid receptor 1 (CB1) agonist ACEA on caveolin-1. Human primary chondrocytes were treated with 10 ng/ml IL-1β in the presence or absence of ACEA or the selective CB1 antagonist AM251 for 24 h. (a) The expression of caveolin-1 at the mRNA level was determined by real-time PCR analysis; (b) Protein levels of caveolin-1 were determined by Western blot analysis (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β treatment group; $, $P < 0.01$ vs. IL-1β+ACEA group, $n = 3$–4).

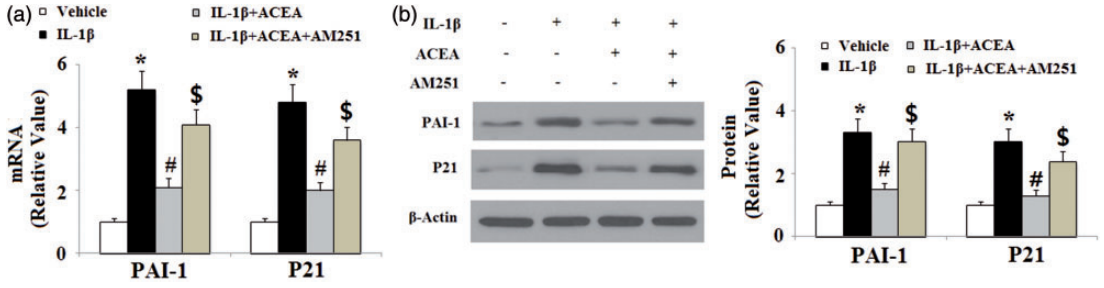

Figure 4. Effect of the selective cannabinoid receptor 1 (CB1) agonist ACEA on the expressions of PAI-1 and p21. Human primary chondrocytes were treated with 10 ng/ml IL-1β in the presence or absence of ACEA or the selective CB1 antagonist AM251 for 24 h. (a) The expression of PAI-1 and P21 at the mRNA levels was determined by real-time PCR analysis; (b) Protein levels of PAI-1 and P21 were determined by Western blot analysis (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β treatment group; $, $P < 0.01$ vs. IL-1β+ACEA group, $n = 3$–4). (A color version of this figure is available in the online journal.)

selective CB1 agonist ACEA abolished the effects of IL-1β by reducing the proportion of chondrocytes in the G0/G1 phase to 53.5%.

To characterize the molecular mechanisms of ACEA that protect against senescence induced by IL-1β, we investigated whether ACEA regulated the expression of key senescence-associated genes. Caveolin-1 has been considered an important regulator of stress (oxidative stress and IL-1β-induced cellular senescence).[22] As shown in Figure 3 (a), real-time PCR results revealed that IL-1β treatment enhanced the expression of caveolin-1 at the mRNA levels, which were significantly attenuated by ACEA. However, the CB1 antagonist AM251 abolished this effect. Consistently, we confirmed the effects of ACEA and AM251 on the expression of caveolin-1 at the protein level (Figure 3(b)).

PAI-1 and p21 are the two critical biomarkers and mediators of cellular senescence and aging. The effects of ACEA and AM251 on the expressions of PAI-1 and p21 were measured. IL-1β treatment significantly increased the gene expression of PAI-1 and p21, which were obviously inhibited by ACEA (Figure 4(a)). Conversely, the presence of AM251 prevented the inhibitory effects of ACEA on both the expression of PAI-1 and p21. These effects were verified at the protein level (Figure 4(b)). To elucidate whether a sirtuin 1 (sirt1)-mediated pathway is involved in the effects of CB1 on IL-1β-induced senescence in chondrocytes, we next investigated the contribution of sirt1. The Western blot results in Figure 5 demonstrate that IL-1β treatment reduced the level of sirt1, which was reversed by ACEA. To confirm the involvement of sirt1 in the effects of ACEA on IL-1β-induced senescence, human primary chondrocytes were treated with ACEA with or without the selective sirt1 inhibitor NAM. As the quantitative results shown in Figure 6(a) demonstrate, NAM abolished the decrease in SA-β-gal-positive cells inferred by ACEA treatment. Notably, Western blot analysis revealed that NAM treatment also attenuated the reduction in expression of caveolin-1, PAI-1, and p21 inferred by ACEA (Figure 6 (b)). These findings implicate that activation of CB1 by ACEA suppressed senescence in human primary chondrocytes by increasing the deacetylase activity of sirt1.

To further confirm the effects of ACEA and AM251 on cellular senescence and the related signaling pathways in human chondrocytes, we examined the basal levels of caveolin-1, PAI, p21, and sirt1 in response to ACEA or AM251 without IL-1β. The results in Supplementary Figure 1(a) indicate that ACEA treatment significantly reduced the expression of caveolin-1, PAI, and p21 but increased the expression of sirt1. Additionally, AM251 treatment significantly increased the expression of caveolin-1, PAI, and p21 but reduced the expression of sirt1.

Discussion

It is well documented that a low degree of inflammation plays a crucial role in the pathological development of

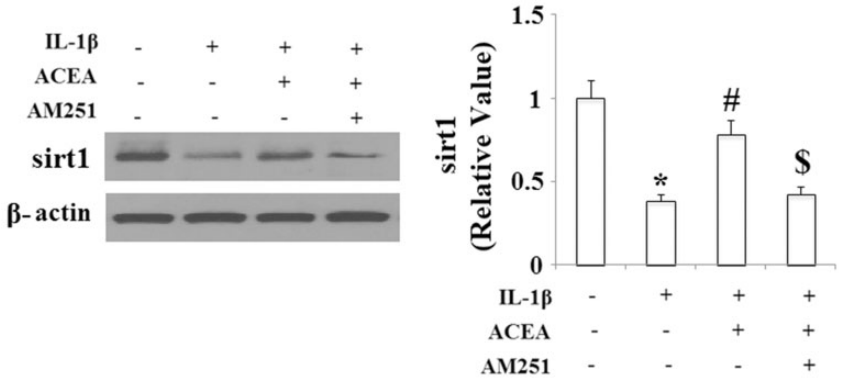

Figure 5. Effects of the selective cannabinoid receptor 1 (CB1) agonist ACEA on the expression of sirt1. Human primary chondrocytes were treated with 10 ng/ml IL-1β in the presence or absence of ACEA or the selective CB1 antagonist AM251 for 24 h. Protein levels of sirt1 were determined by Western blot analysis (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β treatment group; $, $P < 0.01$ vs. IL-1β+ACEA group, $n = 3$–4).

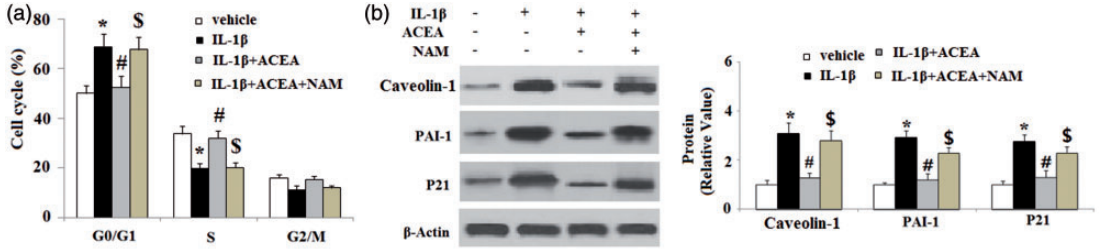

Figure 6. The effects of the selective cannabinoid receptor 1 (CB1) agonist ACEA on inhibiting senescence in human primary chondrocytes are mediated by sirt. Human primary chondrocytes were treated with 10 ng/ml IL-1β in the presence or absence of ACEA or the selective sirt1 inhibitor NAM for 24 h. (a) Percentages of SA-ß-Gal-positive human primary chondrocytes in the different groups; (b) Expression of caveolin-1, PAI-1 and p21 at the protein level was determined by Western blot analysis (*, $P < 0.01$ vs. vehicle group; #, $P < 0.01$ vs. IL-1β treatment group; $, $P < 0.01$ vs. IL-1β+ACEA group, $n = 3$–4). (A color version of this figure is available in the online journal.)

articular cartilage degeneration. The pro-inflammatory cytokine IL-1β is important for cartilage degradation and acts as an inhibitor of ECM synthesis.[23] Senescent chondrocytes generated excessive matrix metalloproteinase 13 (MMP-13) and aggrecanases, resulting in the degradation of collagen II and aggrecan.[24] Excessive secretion of IL-1β causes the overproduction of MMPs and aggrecanases in chondrocytes. Therefore, it is speculated that IL-1β-induced cellular senescence is associated with cartilage degradation. There is a more extensive loss of chondrocytes in cartilages from OA patients as compared to that of age-matched healthy subjects. However, the extent of chondrocyte cell death is debatable.[25] Extracellular and intracellular factors affecting chondrocyte aging might cooperate together to disrupt cartilage homeostasis.[26] In the present study, we utilized an IL-1β-induced senescent model *in vitro* with human primary chondrocytes to investigate the protective effects of the selective CB1 agonist ACEA against cellular senescence. Our findings demonstrate that ACEA can delay senescence of human primary chondrocytes. Additionally, the inhibitory effects of ACEA on cellular senescence are prevented by the selective CB1 agonist AM251, suggesting the involvement of CB1. Since information on the effects of ACEA in cellular senescence is little, our findings displaying that delayed senescence of human primary chondrocytes promoted by the selective CB1 agonist ACEA may serve as an effective prevention of OA.

There are two types of senescence: intrinsic telomere-dependent replicative senescence and extrinsic stress-induced senescence.[7] Aging has been linked with the onset of OA as chondrocytes undergo "extrinsic senescence." Blockage of chondrocyte senescence has been considered as an effective strategy for OA treatment.[27–29] IL-1β-induced senescence in chondrocytes resulted in the alteration of homeostasis in the joint, thereby decreasing the capacity of cells to maintain and repair the tissue.[30] Interestingly, we found that ACEA suppressed IL-1β-induced expression of caveolin-1, an important mediator of IL-1β-induced cellular senescence in articular chondrocytes.[31] Multiple lines of evidence have shown that caveolin-1-rich regions of plasma membrane are associated with receptors and molecules, which play an important role in degradative pathways, such as MMP synthesis and activation.[31] Our results showing that activation of CB1 by ACEA decreased IL-1β-induced cellular senescence and caveolin-1 expression suggested the potential roles of ACEA in maintaining the integrity of articular cartilage. Up-regulation of p21 mediated by caveolin-1 has been considered as a reliable marker of cumulative DNA damage and senescence in chondrocytes.[32] Notably, ACEA reduced the expression of PAI-1 and p21 induced by IL-1β. These results implicate that ACEA treatment attenuated IL-1β-accelerated senescence in human primary chondrocytes and may be effective for prevention of OA.

Sirt1 is an important NAD-dependent deacetylase which has been involved in ageing and age-related diseases. Recent studies have implicated the roles of sirt1 in the pathogenesis of OA. Expression of sirt1 was ubiquitously observed in osteoarthritic chondrocytes. Loss of sirt1 in chondrocytes led to the accelerated progression development of OA in mice, suggesting a preventive role of sirt1 against the development of OA.[33] It has been reported that sirt1 downregulated apoptosis-related gene expression in chondrocytes in OA.[34] Additionally, overexpression of sirt1 inhibited expression of osteoarthritic genes such as MMP-13 in chondrocytes.[35] Sirt1 has been involved in several physiological activities, including cell cycle, senescence, and apoptosis. Importantly, sirt1 has a capacity for protecting cells from premature senescence.[36] Notably, we found that administration of the selective CB1 agonist ACEA increased the basal level of sirt1 but reduced the basal levels of caveolin-1, PAI, and p21 in the absence of IL-1β. In contrast, the CB1 antagonist AM251 treatment decreased the basal level of sirt1 but increased the basal levels of caveolin-1, PAI, and p21. These findings suggest that these chemicals do not necessarily suppress IL-1β-induced cellular senescence. Also, the selective CB1 agonist ACEA restored IL-1β- induced decrease in the expression of sirt1, which implies that activation of CB1 by ACEA might be capable of regulating other intracellular biological functions through sirt1 and its signaling cascades.

The relevance of these findings to human diseases is validated by the results showing that ACEA ameliorated IL-1β-induced cellular senescence in human chondrocytes. Consistently, it has been reported that the two main endocannabinoids AEA and 2-AG were found in the OA synovia, but not in normal controls.[18] Our results suggest an association of the endocannabinoid receptor system with OA onset and a new mechanism by which cannabinoids may block cellular senescence in OA. However, there are several limitations in our study: (1) Only *in vitro* experiments were performed in the current study. It has been noticed that animal models are more important for developmental and regenerative studies. Therefore, it is necessary to perform *in vivo* experiments using animal models to confirm the role of ACEA in the progression of OA; (2) In this study, we used IL-1β-stimulated human chondrocytes as a cell model. Stimulation with IL-1β could promote degradation of the cartilage matrix by increasing the expressions of MMPs, aggrecanases, and other catabolic enzymes in OA.[37,38] However, the OA disease process is complex to the extent that a single treatment of IL-1β may not completely imitate the pathological mechanisms of OA. (3) We should select more time points in order to conduct a more detailed investigation of the molecular mechanisms and signaling pathways involved. Future investigations will provide us with a complete picture of the pathological process of OA.

Authors' contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript. Dawei Zhang wrote the paper and prepared images; Zongyu Li and Bingsheng Li wrote and edited the paper; Gang Zhang prepared samples, Zongyu Li and Bingsheng Li obtained the funding.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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