Zafirlukast attenuates advanced glycation end-products (AGEs)-induced degradation of articular extracellular matrix (ECM)

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ABSTRACT

Zafirlukast, a leukotriene receptor antagonist, has been shown to exert a wide range of effects including anti-asthmatic, anti-inflammatory and oral anti-bacterial. Osteoarthritis is one of the most prevalent age-related public health burdens in the modern world. In the present study, we applied zafirlukast in the treatment of human primary chondrocytes and found that it exerts potent anti-osteoarthritic effects. Zafirlukast inhibited AGEs-induced degradation of the articular extracellular matrix by suppressing expression of MMPs, ADAMTS, NOX-4, generation of ROS, and activation of NF-κB via the IκBα/JNK/AP-1 pathway through targeted inhibition of CysLTR1. These findings suggest that zafirlukast possesses a protective effect against AGEs- induced damage and dysfunction in human chondrocytes.

1. Introduction

Osteoarthritis (OA) is a global public health problem and the number of people living with OA is estimated to drastically increase over the next several decades [1]. The causes of OA are variable and include such things as mechanical overloading, injury, obesity, accompanying inflammatory diseases, and namely, the natural ageing process. However, the exact mechanisms driving the development and progression of OA remain poorly understood. Advanced glycation end-products (AGEs) resulting from non-enzymatic protein glycation accumulate in tissues over time due to their antidegradative properties [2]. Excessive accumulation of AGEs has a deleterious effect on various physiological processes, including cartilage regeneration. Receptor of AGEs (RAGE) is expressed in articular chondrocytes and has been found to stimulate OA chondrocytes to increase production of MMP-3, MMP-13, and TNF-α. This further induces the release of MMPs and leads to sustained MMP-induced degradation of type II collagen [3].

The articular extracellular matrix (ECM) is mainly composed of type II collagen and aggrecan, which give cartilage its rigid and shock-absorptive properties, respectively. Excessive degradation of these two components leads to eventual physical destruction of the joint [2,4]. While regular cell turnover is required to maintain homeostasis, any imbalance in this degradative and regenerative process can result in a pathological cascade of events. Overexpression of MMPs and ADAMTS, which break down type II collagen and aggrecan, respectively, induced by AGEs is a pivotal event in the development and progression of age-related OA. Of these, MMP-3 and MMP-13 are recognized as the main collagen-degrading enzymes, while ADAMTS-4 and ADAMTS-5 are the leading aggrecanases [5]. Additionally, activation of NF-κB is recognized as a major contributing factor in a number of chronic inflammatory diseases. Exacerbation of NF-κB activation further contributes to the pathological development of OA.

Chondrocytes were first found to express the type 1 cysteine leukotriene receptor (CysLTR1) in 2009. Although originally and most commonly used as a therapeutic treatment for chronic asthma and allergies, blockage of CysLTR1 has been shown to play a role in

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regulating inflammatory pain, fracture repair, chondrocyte proliferation and senescence, among other things [6–8]. It has recently been shown to promote insulin secretion and exert an antibacterial effect in oral infections [9,10]. Zafirlukast is a selective CysLTR1 antagonist that was approved by the FDA in 1996 and has shown good tolerability post-marketing [11]. In the present study, we exposed human primary chondrocytes to AGEs in the presence or absence of zafirlukast to investigate whether zafirlukast has a protective impact against AGEs-induced type II collagen and aggrecan degradation in human chondrocytes.

2. Materials and methods

2.1. Cell culture and treatment

Human primary chondrocytes were commercially purchased from Lonza (Cat#: CC-2550). Human primary chondrocytes were cultured with chondrocyte growth basal medium (Cat#: CC-3217, Lonza) diluted with the contents of a CGMTM chondrocyte growth BulletKit (Cat#: CC-3216, Lonza). All cells were used at 90% confluence. Primary human chondrocytes were treated with 100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 24 h.

2.2. RNA isolation and real time-quantitative PCR

Total RNA was isolated from cells using a PicoPure RNA Isolation Kit (Cat#: KIT0204, Thermo Fisher Scientific) following the manufacturer's instructions. cDNA was synthesized using a TaqMan MicroRNA Reverse Transcription Kit (Cat#: 4366596, Thermo Fisher Scientific) from 2 μg total mRNA. RNA expression levels were determined using real-time quantitative PCR with SYBR Green mixture reagent (Qiagen, USA) on an ABI PRISM 7900HT detection system (Applied Biosystems, USA). GAPDH was used as an internal control and gene expressions were assessed using the $2^{-\Delta\Delta Ct}$ method.

2.3. Western blot analysis

Total protein was extracted from cells using RIPA lysis and extraction buffer (Cat#: 89900, Thermo, USA). After briefly washing with DPBS, cells were treated with 500 μL cold RIPA buffer on ice for 20 min, and then separated into 1.7 ml centrifuge tubes. The resulting liquid supernatants were harvested by centrifugation at 13,500 rpm for 10 min. Protein concentration was determined using a Pierce Rapid Gold BCA Protein Assay Kit (Thermo, USA). For western blots, 20–50 μg protein samples were separated by 8%–12% SDS-PAGE (BioRad, Canada) and transferred onto PVDF membranes (Millipore, USA). The membranes were blocked in 5% slim milk before incubation with specific primary antibodies at 4 °C overnight. On the following day, the membranes were incubated with appropriated HRP-conjugated secondary antibody. After washing, protein bands were visualized using Super ECL Detection Reagent (Yeasen Biotech Co., Ltd., Shanghai, China) and the data was analyzed using ImageJ software.
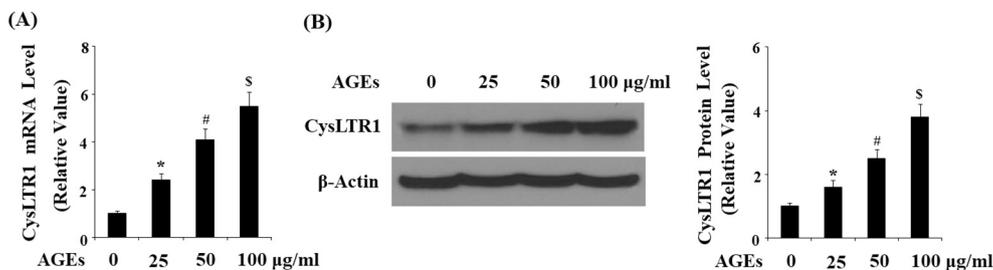
2.4. Reactive oxygen species (ROS) generation assay

Primary human chondrocytes were treated with 100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 24 h. Intracellular ROS in chondrocytes was measured using a 2,7-di-chlorofuorescin diacetate (DCFH-DA) staining assay. Briefly, after the necessary treatment, cells were loaded with 1 μM DCFH-DA (Sigma-Aldrich, USA) in culture medium without FBS and maintained in darkness for 15 min. Then cells were washed 3 times with PBS. Green fluorescence was captured by a fluorescence microscope DM500 (Leica Microsystems, Germany).

2.5. Luciferase reporter gene assay

One μg reporter gene (either pNF-κB-luc, AP-1-luc or negative control) (Qiagen) was transfected into chondrocytes using Lipofectamine 2000 (Life Technologies, USA). Transfection efficiency was controlled by measuring β-galactosidase activity. After 24 h, cells were stimulated with 100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 24 h. Luciferase and β-gal activities were evaluated using commercial kits from Promega, USA. Transcriptional activity was indexed by luciferase activity normalized to β-galactosidase activity and compared with un-treated controls.

2.6. Data analysis

All data are presented as means ± standard error of the mean (SEM). The changes for each treatment group were compared using summary measure analysis. Group means were compared using the Student's t-test or ANOVA. A P value < 0.05 was considered statistically significant.

3. Results

3.1. AGEs increase expression of CysLTR1

CysLTR1 has been shown to be expressed on the surface of chondrocytes. We first treated human primary chondrocytes (HPCs) with AGEs at the concentrations of 25, 50, and 100 μg/ml for 24 h to determine whether exposure to AGEs increases expression of CysLTR1. As shown in Fig. 1, the results of real-time PCR and western blot analysis show that expression of CysLTR1 is indeed increased upon exposure of chondrocytes to AGEs at both the mRNA and protein levels in vitro. This suggests that CysLTR1 may play a role in the effect of AGEs on chondrocytes.

3.2. Zafirlukast ameliorates AGEs-induced expression of NOX-4 and production of ROS

AGEs have been shown to increase expression of NOX-4 and generation of ROS by chondrocytes. HPCs were treated with 100 μg/ml AGEs in the presence or absence of 2.5 or 5 μM zafirlukast for 24 h. As shown in Fig. 2A and B, western blot analysis and DCFH-DA assay

(A)

(B)

Fig. 1. Advanced glycation end products (AGEs) treatment increased the expression of the type 1 cysteinyl leukotriene receptor 1 (CysLTR1) in a dose dependent manner. Chondrocytes were treated with the indicated dose of AGEs (25, 50, 100 μg/ml) for 24 h. (A). Real-time PCR analysis of CysLTR1 at the mRNA level; (B). Western blot analysis of CysLTR1 at the protein level. "Relative value" represents normalization to control group without AGEs treatment (*, #, $, P < 0.01 vs. previous group, n = 7–8).
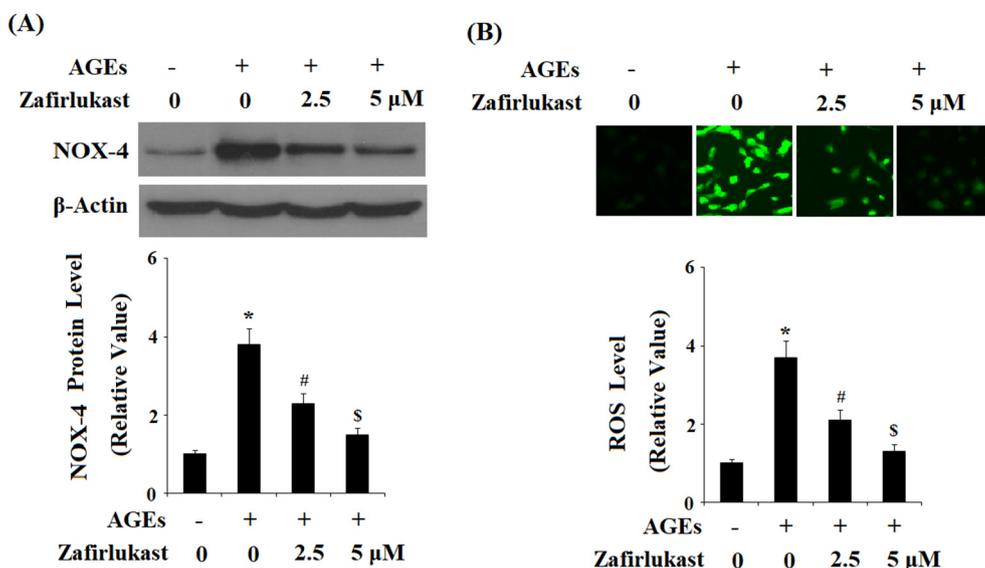
(A)

(B)

respectively revealed that zafirlukast successfully ameliorated AGEs-induced expression of NOX-4 and inhibited generation of ROS in a dose-dependent manner.

3.3. Zafirlukast suppresses AGEs-induced degradation of aggrecan and type II collagen

AGEs are well recognized as catalysts of degradation of the articular ECM. To determine the effects of zafirlukast treatment on degradation of aggrecan and type II collagen, the main components of articular cartilage, we exposed HPCs to 100 μg/ml AGEs in the presence or absence of zafirlukast at the concentrations of 2.5 and 5 μM for 24 h. As shown in Fig. 3, western blot analysis revealed that zafirlukast suppressed AGEs-induced degradation of aggrecan and type II collagen in a dose-dependent manner.

3.4. Zafirlukast suppressed AGEs-induced expression of MMP-3 and MMP-13

To further elucidate the mechanism by which zafirlukast suppressed degradation of type II collagen in HPCs, we investigated the effect of 2.5 and 5 μM zafirlukast on the expression of MMP-3 and MMP-13 induced by exposure to AGEs. The results of real-time PCR and western blot analysis in Fig. 4 show that upon exposure to 100 μg/ml AGEs for 24 h, 2.5 and 5 μM zafirlukast suppressed AGEs-induced expression of MMP-3 and MMP-13 at both the mRNA and protein levels in a dose-dependent
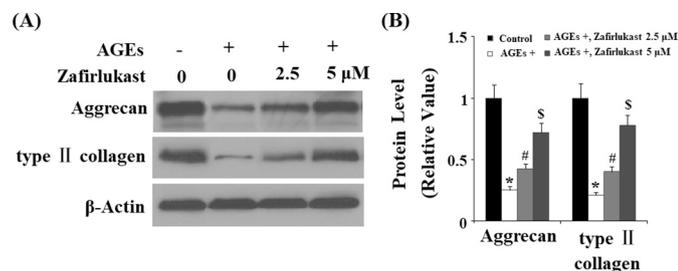
manner.

3.5. Zafirlukast suppressed AGEs-induced expression of ADAMTS-4 and ADAMTS-5

ADAMTS-4 and ADAMTS-5 are the main aggrecanases responsible for cleaving aggrecan in articular cartilage. To determine whether the antidegradative effect of zafirlukast on aggrecan was mediated by suppression of ADAMTS-4 and ADAMTS-5, we exposed HPCs to 100 μg/ml AGEs for 24 h in the presence or absence of 2.5 and 5 μM zafirlukast. As shown in Fig. 5, the results of real-time PCR and western blot analysis confirmed that zafirlukast successfully suppressed the expression of ADAMTS-4 and ADAMTS-5 at both the mRNA and protein levels in a dose-dependent manner.

3.6. Zafirlukast suppressed AGEs-induced activation of JNK

To investigate whether the MAPK pathway is involved in the effects of zafirlukast on degradation of the articular ECM, we measured the activation of the ERK1/2, p38, and JNK kinases by measuring phosphorylated and total levels of ERK1/2, p38, and JNK via western blot analysis. As shown in Fig. 6, HPCs treated with 100 μg/ml AGEs in the presence or absence of 2.5 or 5 μM zafirlukast for 2 h had significantly increased phosphorylation of ERK1/2, p38, and JNK with AGEs treatment alone, while 2.5 and 5 μM zafirlukast suppressed AGEs-induced activation of these kinases in a dose-dependent manner.

3.7. Zafirlukast suppressed AGEs-induced activation of AP-1

Activation of the MAPK pathway especially JNK is closely linked to AP-1 signaling. To determine whether the activity of zafirlukast also affects AGEs-induced activation of AP-1, we treated HPCs with 100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 24 h. As shown in Fig. 7, activation of the two primary components of AP-1, c-Jun and c-fos, was significantly increased by AGEs, which was ameliorated by zafirlukast in a dose-dependent manner.

3.8. Zafirlukast suppressed AGEs-induced phosphorylation and degradation of IκBα

AGEs have been shown to induce phosphorylation of IκBα, which leads to nuclear translocation of p65 protein and subsequent activation of NF-κB. To determine the effects of zafirlukast on AGEs-induced phosphorylation and degradation of IκBα, we treated HPCs with

(A)

(B)

Fig. 3. Zafirlukast suppresses AGEs- induced degradation of aggrecan and type II collagen. Primary human chondrocytes were treated with 100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 24 h. (A). Western blot analysis of aggrecan and type II collagen; (B). Quantification of aggrecan and type II collagen. "Relative value" represents normalization to control group without AGEs and Zafirlukast treatment (*, #, $, P < 0.01 vs. previous group, n = 8).
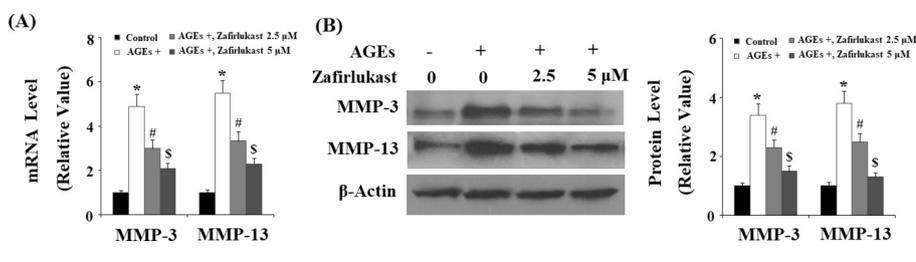
Fig. 4. Zafirlukast suppressed AGEs-induced expression of MMP-3 and MMP-13. Primary human chondrocytes were treated with 100 µg/ml AGEs in the presence or absence of 2.5 and 5 µM zafirlukast for 24 h. (A). Real-time analysis of MMP-3 and MMP-13 at the mRNA levels; (B). Western blot analysis of MMP-3 and MMP-13 at the protein levels. "Relative value" represents normalization to control group without AGEs and Zafirlukast treatment (*, #, $, P < 0.01 vs. previous group, n = 7–8).
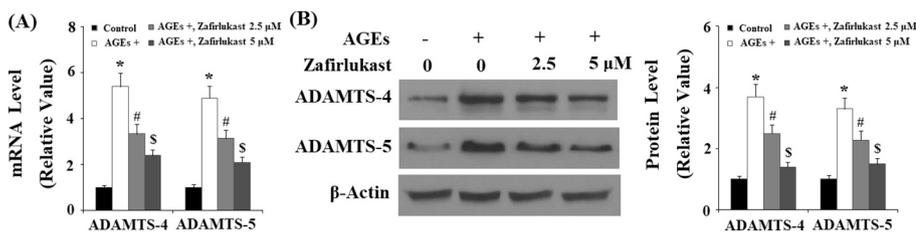

Fig. 5. Zafirlukast suppressed AGEs-induced expression of ADAMTS-4 and ADAMTS-5. Primary human chondrocytes were treated with 100 µg/ml AGEs in the presence or absence of 2.5 and 5 µM zafirlukast for 24 h. (A). Real-time analysis of ADAMTS-4 and ADAMTS-5 at the mRNA levels; (B). Western blot analysis of ADAMTS-4 and ADAMTS-5 at the protein levels. "Relative value" represents normalization to control group without AGEs and Zafirlukast treatment (*, #, $, P < 0.01 vs. previous group, n = 7–8).
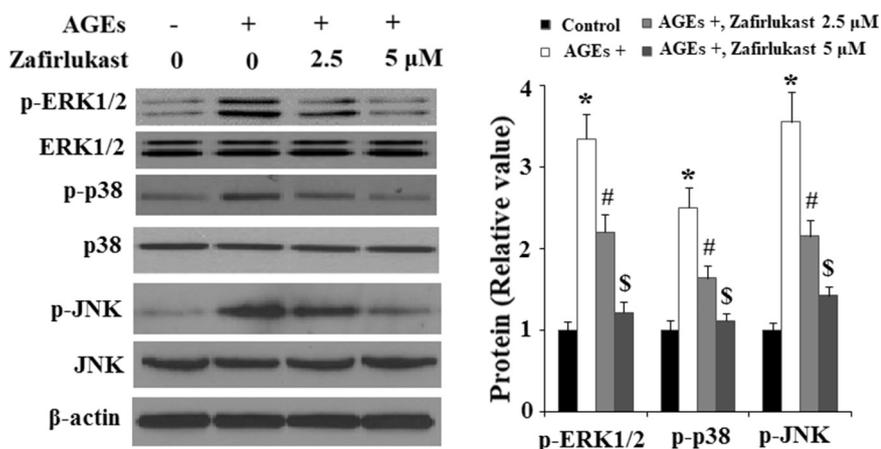

Fig. 6. Zafirlukast suppressed AGEs-induced activation of ERK1/2, p38, and JNK. Primary human chondrocytes were treated with 100 µg/ml AGEs in the presence or absence of 2.5 and 5 µM zafirlukast for 2 h. Phosphorylated and total levels of ERK1/2, p38, and JNK were determined by western blot analysis (*, #, $, P < 0.01 vs. previous group). "Relative value" represents normalization to control group without AGEs and Zafirlukast treatment (*, #, $, P < 0.01 vs. previous group, n = 7–8).
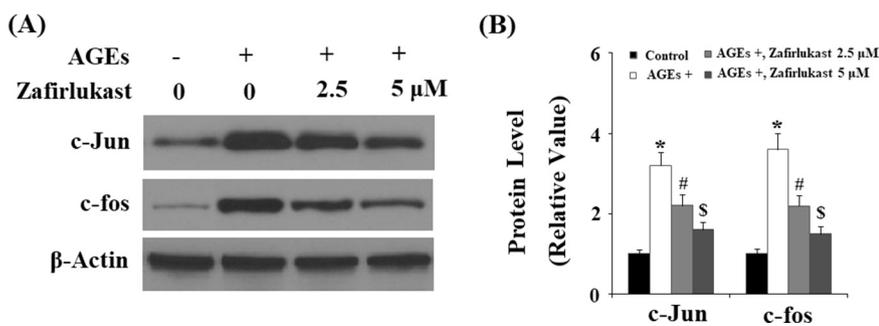

Fig. 7. Zafirlukast suppressed AGEs-induced activation of AP-1. Primary human chondrocytes were treated with 100 µg/ml AGEs in the presence or absence of 2.5 and 5 µM zafirlukast for 24 h. (A). The components of AP-1, c-Jun and c-fos, were determined by western blot analysis; (B). Luciferase activity of AP-1. "Relative value" represents normalization to control group without AGEs and Zafirlukast treatment (*, #, $, P < 0.01 vs. previous group, n = 8).
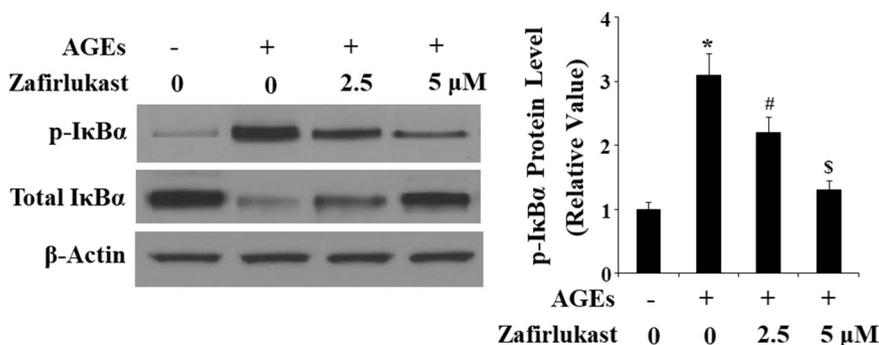

Fig. 8. Zafirlukast suppressed AGEs-induced phosphorylation and degradation of IκBα. Primary human chondrocytes were treated with 100 µg/ml AGEs in the presence or absence of 2.5 and 5 µM zafirlukast for 2 h. Phosphorylated and total levels of IκBα were determined by western blot analysis. "Relative value" represents normalization to control group without AGEs and Zafirlukast treatment (*, #, $, P < 0.01 vs. previous group, n = 8).

(A)

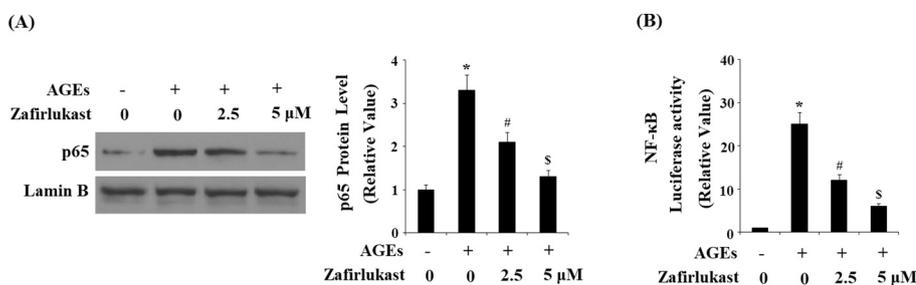|			
AGEs	-	+	+	+
Zafirlukast	0	0	2.5	5 μM

p65

Lamin B

(B)

Fig. 9. Zafirlukast suppressed AGEs-induced activation of NF-κB. Primary human chondrocytes were treated with 100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 24 h. (A). Nuclear level of p65 was determined by western blot analysis; Lamin B was used as a negative control; (B). Luciferase activity of NF-κB promoter. "Relative value" represents normalization to control group without AGEs and Zafirlukast treatment (*, #, $, $P < 0.01$ vs. previous group, n = 7–8).
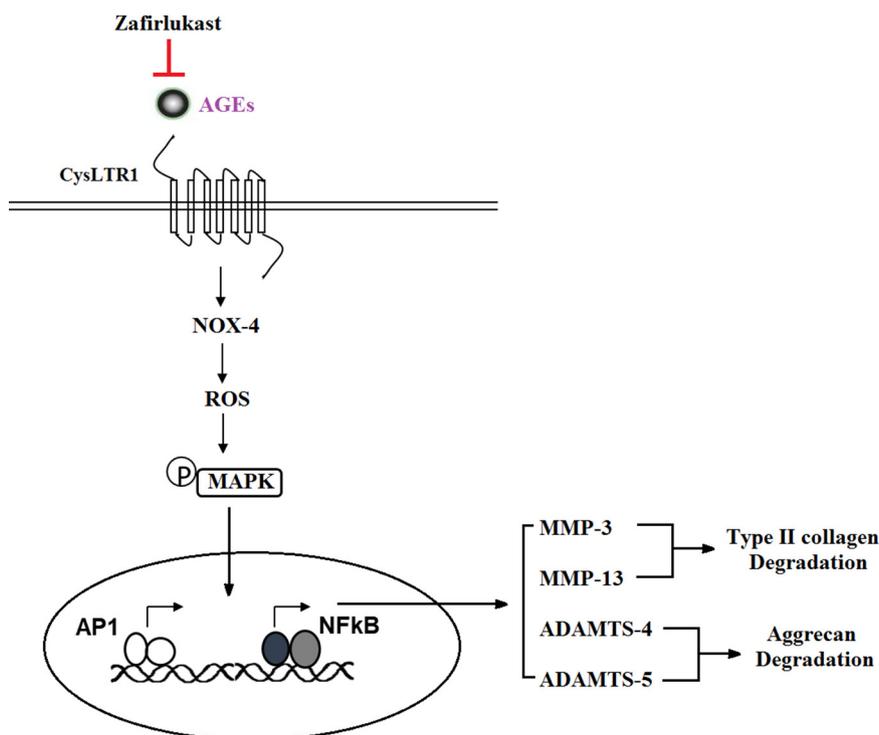

Fig. 10. A graphic summary of this study.

100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 2 h. As shown in Fig. 8, zafirlukast suppressed AGEs-induced phosphorylation and degradation of IκBα in a dose-dependent manner, indicating that the effects of zafirlukast involve the IκBα pathway.

3.9. Zafirlukast suppressed AGEs-induced activation of NF-κB

To confirm that zafirlukast suppresses AGEs-induced activation of NF-κB via the IκBα pathway, HPCs treated with 100 μg/ml AGEs in the presence or absence of 2.5 and 5 μM zafirlukast for 24 h were subjected to western blot analysis and luciferase assay. As shown in Fig. 9, the results of western blot analysis indicate that zafirlukast reduced the nuclear level of p65 in a dose-dependent manner. Concordantly, the results of luciferase reporter assay show that zafirlukast suppressed AGEs-induced activation of NF-κB in a dose-dependent manner.

4. Discussion

Although the public health burden of OA is already great and expected to increase considerably in coming years, there are few reliable treatments against the development and progression of the disease. The G protein-coupled receptor CysLTR1, but not CysLTR2, has been shown to be expressed on chondrocytes and to be increased in response to TNF-α [12]. Increased expression of CysLTR1 has also been found in various types of cancers [13]. Activation of CysLTR1 by the CysLTs

LTC4, LTD4 and LTE4 has been shown to play a role in a variety of inflammatory diseases [14]. The drug family of leukotriene receptor antagonists, which includes montelukast, zafirlukast and pranlukast, has been widely used in the treatment of asthma for over a decade. Among these, zafirlukast is a selective CysLTR1 antagonist [15]. Recently, research has been conducted to investigate the effects of "leukasts" in other disease states. A study from 2018 showed that montelukast suppressed chondrocyte senescence [12]. Another recent study reported that montelukast plays a beneficial role in treating mechanical overload [16]. Thus, we chose to investigate the effects of its relative zafirlukast on chondrocytes exposed to AGEs insult to gain an understanding of the potential role of this drug in the treatment of OA.

In the development and progression of OA, excessive degradation of the articular ECM by MMPs and ADAMTS is the result of a myriad of molecular processes. AGEs have been shown to up-regulate NOX-4, which functions as the catalytic subunit of the NADPH oxidase complex and acts as an oxygen sensor and catalyzes the reduction of molecular oxygen to various ROS [17,18]. Excessive production of ROS could initiate a variety of intracellular signaling pathways including the MAPK signaling pathway, which has been linked with the activation of the transcriptional factors including AP1 and NF-κB [19]. Both of AP1 and NF-κB are important for the expression of MMPs and ADAMTS. Our findings demonstrate that treatment with zafirlukast could prevent this process. A graphic summary of this study is shown in Fig. 10.

As shown by the results of this study, exposure of chondrocytes to

AGEs caused a significant dose-dependent increase in expression of CysLTR1, which was ameliorated by treatment with 2.5 and 5 µM zafirlukast in a dose-dependent manner (Fig. 1). Additionally, as shown in Fig. 2A and B, exposure to 100 ng/ml AGEs potently induced expression of NOX-4 and generation of ROS as demonstrated by western blot analysis and DCFA-DH assay, respectively. Remarkably, treatment with 2.5 and 5 µM zafirlukast significantly inhibited expression of NOX-4 and nearly abolished AGEs-induced generation of ROS. This shows that zafirlukast may ameliorate the pro-inflammatory effects of AGEs in chondrocytes. Next, we investigated whether zafirlukast had a similar beneficial effect on AGEs-induced degradation of type II collagen and the proteoglycan aggrecan in chondrocytes. As shown in Fig. 3, treatment of chondrocytes exposed to 100 ng/ml AGEs with zafirlukast (2.5 and 5 µM) ameliorated AGEs-induced degradation of type II collagen and aggrecan in a dose-dependent manner, with 5 µM almost completely rescuing this degradation. To determine whether this outcome involved expression of MMPs and ADAMTS mediated by CysLTR1, we again exposed chondrocytes to 100 ng/ml AGEs in the presence or absence of 2.5 and 5 µM zafirlukast for 24 h. As shown in Figs. 4 and 5, zafirlukast inhibited expression of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 induced by AGEs in a dose-dependent manner. Notably, of these, MMP-3 showed the greatest inhibition, with 5 µM zafirlukast almost completely inhibiting the increase in expression induced by AGEs.

The MAPK pathway has been shown to be involved in OA. Phosphorylation of ERK1/2, p38, and JNK decreases proteoglycan synthesis and increases expression of MMP-13 [20]. Increased phosphorylation of JNK promotes activation of the transcriptional factor AP-1 through phosphorylation of its heterodimer, c-fos and c-Jun. In addition to chondrocyte dysfunction in OA, both AP-1 and NF-κB have been shown to mediate a wide range of inflammatory reactions [21]. As shown in Fig. 6, treatment with 2.5 and 5 µM significantly reduced the level of phosphorylated JNK induced by AGEs, while total JNK remained constant. Notably, treatment with 5 µM zafirlukast almost completely abolished phosphorylation of JNK induced by AGEs. Additionally, we found that treatment with 2.5 and 5 µM zafirlukast inhibited AGE-induced activation of AP-1 by inhibiting phosphorylation of c-fos and c-Jun in a dose-dependent manner. Here, the level of phosphorylated c-fos showed the strongest inhibition upon treatment with 5 µM zafirlukast (Figure7). Phosphorylation of IκBα by IKKα/β and subsequent nuclear translocation of p65 results in activation of NF-κB, which has been implicated as playing a major role in a wide variety of chronic inflammatory diseases such as OA [22–24]. As shown in Fig. 8, treatment with 100 ng/ml AGEs resulted in a significant increase in phosphorylated IκBα as well as a significant decrease in total IκBα. These effects were ameliorated by treatment with 2.5 and 5 µM zafirlukast for 2 h. Cells treated with 5 µM zafirlukast showed almost complete recovery to basal levels of phosphorylated and total IκBα. Next, we investigated the effects of zafirlukast on AGEs-induced activation of NF-κB. As shown in Fig. 9, zafirlukast successfully attenuated nuclear translocation of p65 protein and subsequent activation of NF-κB induced by treatment with 100 ng/ml AGEs in a dose-dependent manner. Notably, the dose of 5 µM zafirlukast once again resulted in almost complete rescue of nuclear levels of p65.

Taken together, the results of this study indicate that CysLTR1 expressed on chondrocytes may play a role in the molecular mechanisms behind the development and progression of OA. The selective CysLTR1 antagonist zafirlukast may have potential as a novel therapy for OA by inhibiting expression of MMPs, ADAMTS, generation of ROS, and activation of JNK, AP-1 and NF-κB. Further study is required to fully elucidate the role of CysLTR1 in OA.

Acknowledgements

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