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Protective effects of alogliptin against TNF-α-induced degradation of extracellular matrix in human chondrocytes

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ABSTRACT

Osteoarthritis (OA) is a common debilitating disease most prevalent among the elderly population worldwide. Excessive degradation of the articular extracellular matrix is a pivotal event in the development of OA. Preventative treatments against the destruction of type II collagen and aggrecan, the two main components of the articular extracellular matrix, may serve as a novel therapy against the progression of OA. In the current study, we investigated whether the DPP-4 inhibitor alogliptin could prevent degradation of the articular extracellular matrix in human primary chondrocytes. Pretreatment with alogliptin successfully prevented degradation of type II collagen and aggrecan in a dose-dependent manner by reducing increased expression of MMP-1, -3, and -13 as well as ADAMTS-4 and -5 induced by treatment with TNF-α. Furthermore, pretreatment with alogliptin also reduced TNF-α-induced expression of IKKα/β, IκBα and NF-κB in human primary chondrocytes. This suggests that DPP-4 inhibitors such as alogliptin may be used as an effective preventative therapy against continued destruction of the articular extracellular matrix in OA.

1. Introduction

Osteoarthritis (OA) is a major debilitating disease most prevalent among the elderly. While the exact mechanisms driving the development of OA remain poorly understood, excessive degradation of the proteoglycans type II collagen and aggrecan, the two main components of the articular extracellular matrix (ECM), leads to structural failure of the ECM and subsequent destruction of joints. Of these, type II collagen provides the “backbone” of the ECM while aggrecan gives cartilage its shock-absorptive property [1,2]. While aggrecan is continuously synthesized and degraded throughout a person's lifetime, type II collagen has a much lower capacity for turnover [2]. Due to this reduced capacity for turnover, degradation of type II collagen is a pivotal event in the progression of OA and excessive degradation is largely considered to be irreversible [3]. Therefore, it is of great importance to develop protective therapies against excessive degradation of the articular ECM.

Of the metalloproteinase (MMP) enzyme family, MMP-1, MMP-3 and MMP-13 have been reported to degrade type II collagen in articular cartilage [4]. A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) are another class of degradative enzymes, of which the aggrecanases ADAMTS-4 and ADAMTS-5 have been shown to degrade aggrecan in articular cartilage [5]. While expression of these enzymes is necessary to maintain normal cell turnover, an imbalance in the expression of any of them can disrupt the homeostatic state of the articular ECM, thereby triggering the development of OA. Increased expression of tumor necrosis factor-α (TNF-α) has been found in OA chondrocytes. TNF-α is a widely studied inflammatory cytokine, which has been shown to play a critical role in the progression of ECM degradation in OA cartilage [6–8]. TNF-α has also been shown to regulate the expression of MMPs and ADAMTSs [9]. Therefore, treatments targeting TNF-α are a potential therapy for the treatment and prevention of OA.

Alogliptin, a highly selective dipeptidyl-peptidase-4 (DPP-4) inhibitor, was approved by the FDA in 2013 for the treatment of type II diabetes mellitus [10]. DPP-4 inhibitors have been shown to inhibit the enzymatic breakdown of endogenous incretin hormones [11]. Therefore, we hypothesized that alogliptin may also prevent enzymatic breakdown of the articular ECM. In the present study, we found that pretreatment with alogliptin could attenuate excessive degradation of

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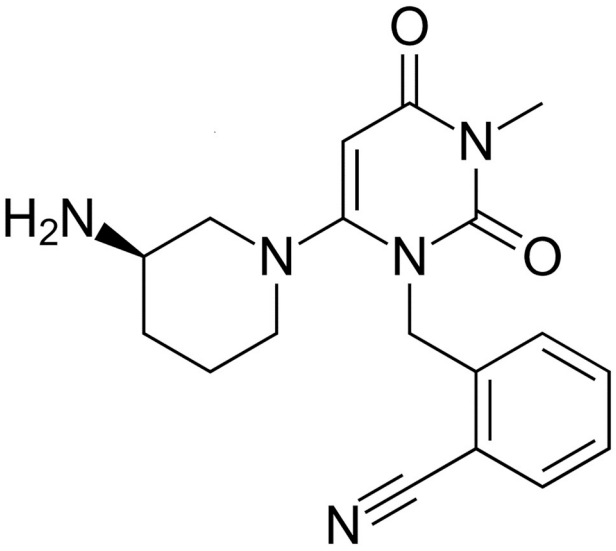

Fig. 1. Molecular structure of alogliptin.

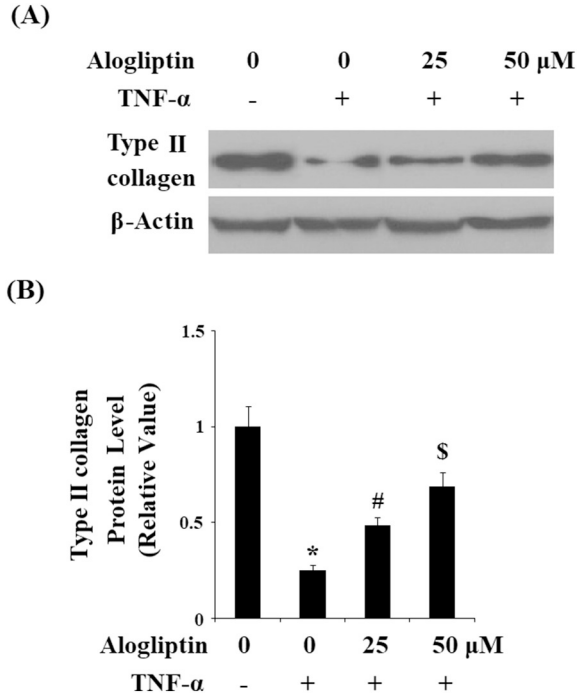

Fig. 2. Alogliptin attenuated TNF-α-induced degradation of type II collagen. Human primary chondrocytes were pretreated with alogliptin at the concentrations of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 24 h. (A). Representative western blot analysis results of type II collagen; (B). Quantification of type II collagen (*, #, $, $P < 0.01$ vs. previous group).

the ECM by downregulating TNF-α-induced expression of MMP-1, -3 and -13, ADAMT-4 and -5, phosphorylation of IκB kinase α/β (IKKα/β), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α (IκBα) and p38, nuclear translocation of p65, and subsequent activation of nuclear factor (NF)-κB.

2. Materials and methods

2.1. Cell culture, treatment, and transfection

Human primary chondrocytes were isolated from biopsy material designated to this study as previously reported [12]. The tissue was incubated with 0.2% type II collagenase (Life Technologies, USA) overnight at 37 °C. The isolated chondrocytes in alginate and monolayer were cultured in a 5% CO_2 incubator at 37 °C in standard DMEM/F12 feeding medium supplemented with 10% fetal bovine serum (FBS) and 50 μg gentamicin. The medium was changed every other day. At the time of confluence, the monolayer chondrocytes were liberated by trypsin digestion, washed three times in PBS and re-seeded into a 12-well plate for treatment. The cells were treated with or without TNF-α and varying concentrations of alogliptin.

2.2. Real-time polymerase chain reaction (PCR)

Total RNA was isolated from chondrocytes using a TRIzol RNA isolation kit (Thermo Fisher, USA) following the manufacturer's protocols. RNA concentration was determined using a Thermo Scientific NanoDrop 8000 spectophotometer. Two microgram RNA was translated to cDNA using a TaqMan™ Reverse Transcription Kit. qPCR was performed on a LightCycler® 96 Real-Time PCR System (Roche). GAPDH was used a reference gene. Relative quantitation was calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔCt symbolized the difference of Ct between sample and reference RNA.

2.3. Protein isolation and western blot analysis

After the necessary treatment, chondrocytes were harvested for protein isolation. Proteins from whole cells and nuclei were isolated using a Cytoplasmic and Nuclear Protein Extraction Kit (Fisher Scientific, USA) following the manufacturer's instructions. Concentrations of protein from whole cells or nuclei were measured using a BCA assay following the manufacturer's protocol (Thermo Fisher Scientific, USA). Twenty microgram total proteins and 30 μg nuclear proteins, respectively, were transferred into separate lanes on SDS polyacrylamide gels, and then transferred to PVDF membranes. After blocking with 5% milk, the membranes were incubated with primary antibody overnight in a cold, then washed three times and incubated with appropriate horseradish peroxidase (HRP)-conjugated IgG secondary antibody. Signals were visualized using an ECL chemiluminescence detection kit (GE Healthcare, USA). Antibodies directed against type II collagen (ab21291, Abcam), MMP-1 (sc-137044, Santa Cruz), MMP-3 (sc-400727, Santa Cruz), MMP-13 (sc-400626, Santa Cruz), aggrecan (AF1220, R&D Systems), ADAMTS-4 (MAB4307, R&D Systems), ADAMTS-5 (AF2198, R&D Systems), p-p38 (sc-101,759, Santa Cruz), p-IKKα/β (#2697, Cell Signaling Technology), total IKKα/β (#2682, Cell Signaling Technology), p65 (sc-71675, Santa Cruz), and β-actin (sc-1615, Santa Cruz) were used, and all secondary antibodies were purchased from Santa Cruz Biotechnology.

2.4. Promoter assay

The NF-κB promoter luciferase vector was obtained from Life Technologies, USA. Human chondrocytes were co-transfected with NF-κB promoter luciferase vector and a firefly luciferase promoter using Lipofectamine 2000 reagent (Life Technologies, USA). Human primary chondrocytes were pretreated with alogliptin at concentrations of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 24 h. Total cell lysates were prepared and the dual luciferase activities of renilla and firefly luciferase were measured. Renilla luciferase activity was used as an internal control.

2.5. Statistical analysis

All results are displayed as means ± SD from at least three independent experiments unless otherwise indicated. Statistical differences were determined by the independent group Student's *t*-test. Analyses were performed using GraphPad 5 (GraphPad Software, San Diego, CA, USA) or Image J software.

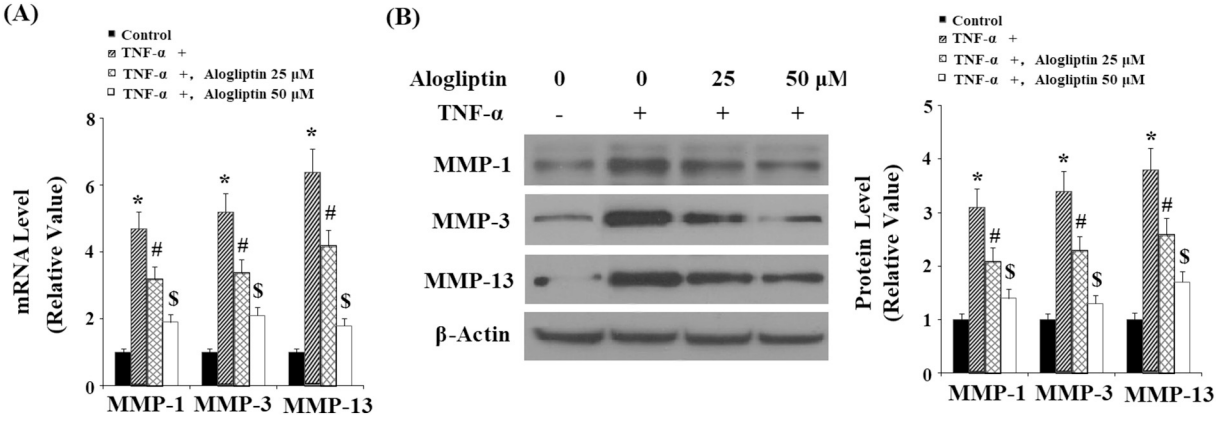

Fig. 3. Alogliptin reduced TNF-α-induced increase in expression of MMP-1, MMP-3, and MMP-13. Human primary chondrocytes were pretreated with alogliptin at the concentrations of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 24 h. (A). Gene levels of MMP-1, MMP-3, and MMP-13 determined by real-time PCR; (B). Protein levels of MMP-1, MMP-3, and MMP-13 determined by western blot analysis (*, #, $, P < 0.01 vs. previous group).

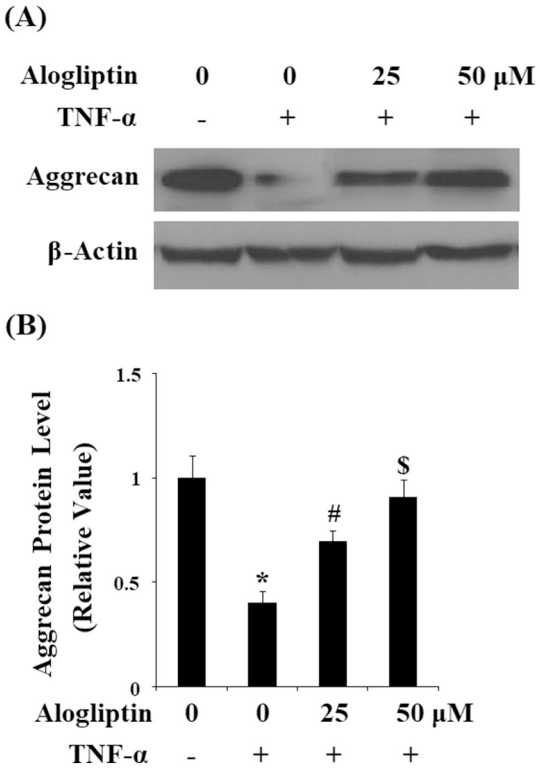

Fig. 4. Alogliptin attenuated TNF-α-induced degradation of aggrecan. Human primary chondrocytes were pretreated with alogliptin at the concentrations of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 24 h. (A). Representative western blot analysis results of aggrecan; (B). Quantification of aggrecan (*, #, $, P < 0.01 vs. previous group).

3. Results

The molecular structure of alogliptin is shown in Fig. 1. Firstly, we investigated whether pretreatment with alogliptin has a protective effect on human primary chondrocytes (HPCs) using β-actin as a control in each experiment. HPCs were pretreated with 25 or 50 μM alogliptin for 12 h, followed by treatment with TNF-α for 24 h. The results of western blot analysis shown in Fig. 2 demonstrate that alogliptin attenuated TNF-α-induced degradation of type II collagen in a dose-dependent manner. Next, we used real-time PCR and western blot analyses to determine the effects of pretreatment with alogliptin on TNF-α-induced expression of MMP-1, MMP-3, and MMP-13 at the gene (Fig. 3A) and protein (Fig. 3B) levels. Our results show that alogliptin did indeed reduce the expression of MMPs in HPCs induced by TNF-α in a dose-dependent manner.

Next, we set out to determine whether pretreatment with alogliptin at the concentrations of 25 and 50 μM could attenuate TNF-α-induced degradation of aggrecan using β-actin as a control. HPCs were incubated with alogliptin (25 and 50 μM) for 12 h followed by treatment with TNF-α for 24 h. The results of western blot analysis shown in Fig. 4 indicate that alogliptin could indeed attenuate degradation of aggrecan in a dose-dependent manner. Additionally, we also investigated the effects of pretreatment with alogliptin on the increase in expression of ADAMTS-4 and ADAMTS-5 induced by TNF-α. As shown in Fig. 5, the increase in expression of ADAMTS-4 and ADAMTS-5 induced by TNF-α was reduced in a dose-dependent manner by pretreatment with alogliptin for 12 h at the concentrations of 25 and 50 μM. This effect was confirmed at both the gene (Fig. 5A) and protein (Fig. 5B) levels by real-time PCR and western blot analyses, respectively.

The above findings indicate that pretreatment with alogliptin possesses a protective effect against TNF-α-induced degradation of ECM in chondrocytes in a "preventive" model. To examine whether alogliptin had a "therapeutic" effect against TNF-α-induced degradation of type II collagen and aggrecan, cells were treated with TNF-α for 12 h, and then alogliptin was added into the cell culture medium at a final concentration of 25 or 50 μM for 24 h. Western blot analysis results in Supplementary Fig. 1 demonstrate that treatment with alogliptin after TNF-α exposure could also prevent TNF-α-induced degradation of type II collagen and aggrecan. These findings suggest that alogliptin could exert both preventive and therapeutic actions against TNF-α-induced degradation f type II collagen and aggrecan.

Activation of p38 has been shown to be involved in TNF-α-induced inflammatory reactions in HPCs. We next investigated the effects of pretreatment with alogliptin on the activation of p38. HPCs were pretreated with alogliptin at the concentrations of 25 and 50 μM for 12 h followed by treatment with TNF-α for 1 h. As shown in Fig. 6, there was a significant decrease in the level of phosphorylated p38 induced by TNF-α after pretreatment with alogliptin. However, the level of total p38 remained constant. As shown in Fig. 7, we also found that pretreatment with alogliptin (25 and 50 μM) for 12 h reduced activation of IKKα/β and IκBα induced by treatment with TNF-α for 6 h. Additionally, we determined whether pretreatment with alogliptin influenced activation of NF-κB. HPCs were pretreated with 25 and 50 μM alogliptin for 12 h followed by treatment with TNF-α for 24 h. The results of western blot analysis shown in Fig. 8A demonstrate that alogliptin pretreatment significantly reduced nuclear levels of p65 in a dose-dependent manner. Furthermore, alogliptin also

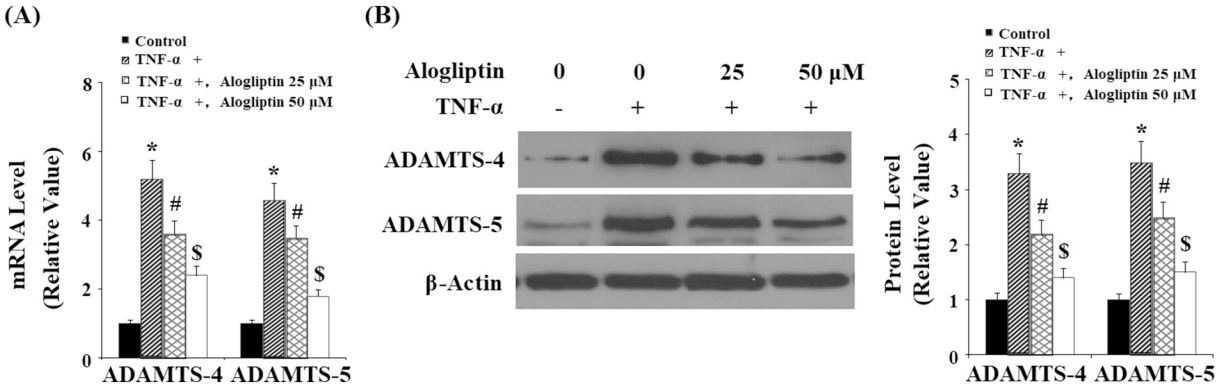

Fig. 5. Alogliptin reduced TNF-α-induced increase in ADAMTS-4 and ADAMTS-5. Human primary chondrocytes were pretreated with alogliptin at the concentration of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 24 h. (A). Gene levels of ADAMTS-4 and ADAMTS-5; (B). Protein levels of ADAMTS-4 and ADAMTS-5 (*, #, $, $P < 0.01$ vs. previous column group).

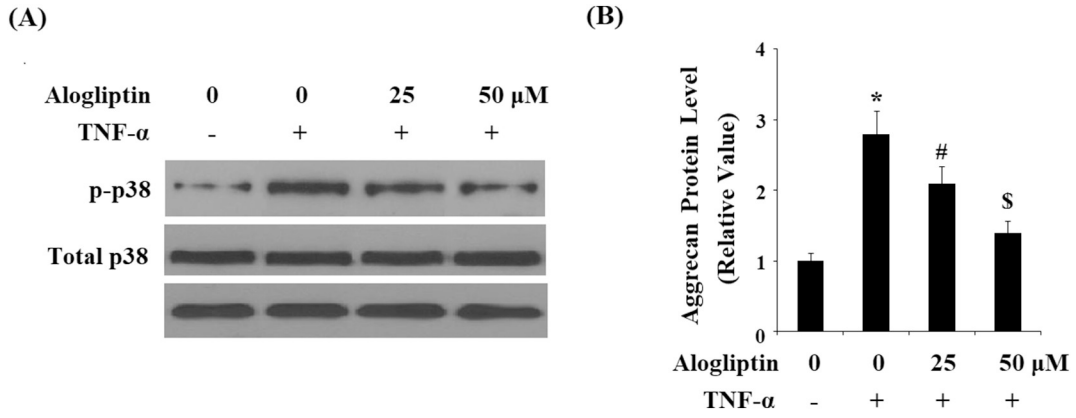

Fig. 6. Alogliptin reduced TNF-α-induced activation of p38. Human primary chondrocytes were pretreated with Alogliptin at the concentration of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 1 h. (A). Phosphorylated and total level of p38; (B). Quantitative analysis of p-p38/p38 (*, #, $, $P < 0.01$ vs. previous group).

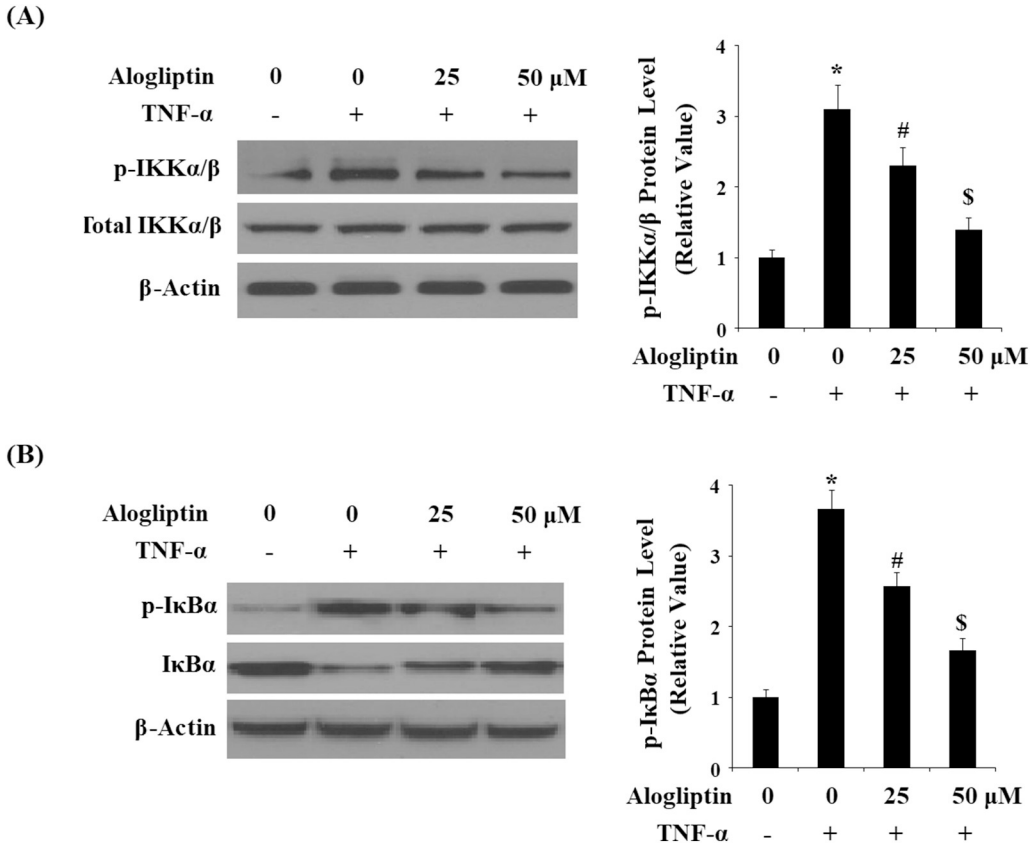

Fig. 7. Alogliptin reduced TNF-α-induced activation of IKKα/β and IκBα. Human primary chondrocytes were pretreated with alogliptin at the concentrations of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 6 h. (A). Phosphorylated and total levels of IKKα/β; (B). Phosphorylated and total levels of IκBα (*, #, $, $P < 0.01$ vs. previous group).

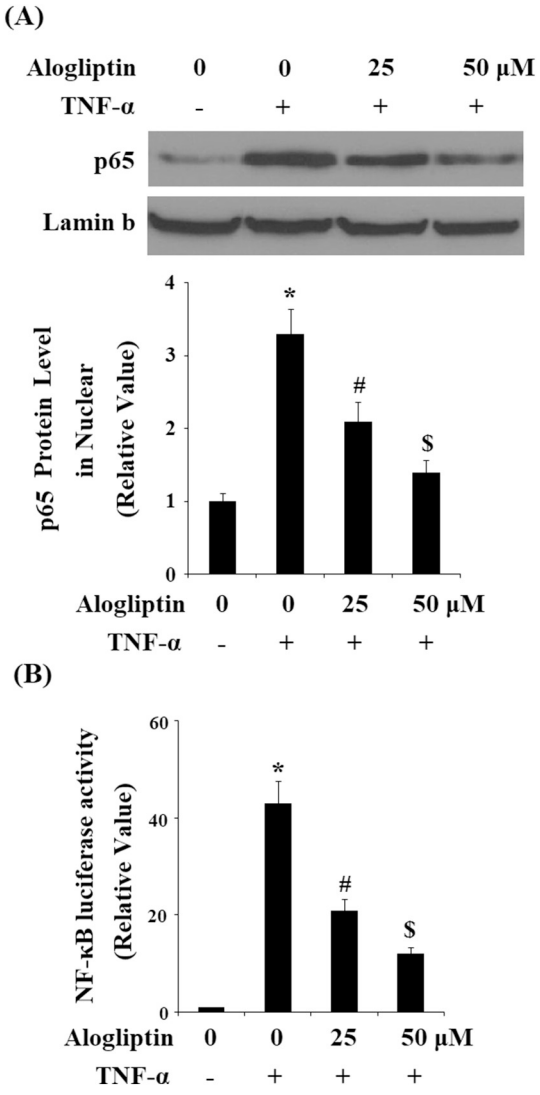

Fig. 8. Alogliptin reduced TNF-α-induced activation of NF-κB. Human primary chondrocytes were pretreated with alogliptin at the concentrations of 25 and 50 μM for 12 h, followed by treatment with TNF-α for 24 h. (A). Nuclear levels of p65; (B). NF-κB luciferase activity (*, #, $, P < 0.01 vs. previous group).

significantly reduced NF-κB luciferase activity in a dose-dependent manner (Fig. 8B).

4. Discussion

OA is characterized by excessive degradation of the articular ECM, which leads to destruction of joints and eventual loss of mobility. Currently, there are no existing therapies capable of halting the progression of OA, with the final means of treatment for late stage OA being arthroplasty, a highly invasive and burdensome solution [13]. While the exact mechanisms behind the development and progression of OA are complicated, therapies against the production of MMPs and ADAMTS have been suggested as a way to slow down or prevent degradation of the articular ECM [14–16]. MMPs, especially MMP-3 and -13, break down type II collagen in the articular ECM, while ADAMTS cleave proteoglycans and aggrecans, with ADAMTS-4 being the major enzyme responsible for cleavage of the Glu373–Ala374 bond in aggrecan [17]. Together, MMPs and ADAMTS degrade the primary components of the articular ECM and act as major contributors to the pathogenesis of OA. Expression of NF-κB has been shown to play an important role in the upregulation of MMPs and ADAMTS as seen in OA, and is widely known to play a causal role in a vast range of inflammatory diseases [18–20]. In the pathological progression of OA, the IKKα/IκBα/NF-κB inflammatory pathway is recognized as a central mediator of inflammation, cytokine production, cartilage degradation, cell proliferation, angiogenesis and pannus formation [21]. Under normal physiological conditions, inactive NF-κB exists in the cytoplasm as a heterodimer of the p50/p65 subunits. This inactive state is maintained by IκBα, a member of the IκB class of inhibitory proteins. However, in an oxidative stress environment, such as upon TNF-α stimulation, activation of the p38 mitogen activated protein kinase (MAPK) pathway leads to phsophorylation of IκBα by IKKα/β and subsequent nuclear translocation of p65, resulting in activation of NF-κB [22–24]. Thus, the p38 MAPK and IKKα/IκBα/NF-κB pathways may be good targets for therapies against the pathological progression of OA.

In the present study, we investigated the effects of pretreatment with the DPP-4 inhibitor alogliptin on these pathways in HPCs stimulated with TNF-α. Our results demonstrate that alogliptin could prevent degradation of the ECM by inhibiting activation of NF-κB via the p38/IKKα/IκBα signaling pathway, suggesting the potential for this drug as a novel OA treatment. Concordant with the present investigation, a contemporary study explored the effects of treatment with gemigliptin, another DPP-4 inhibitor, against excessive degradation of the articular ECM and found that gemigliptin could suppress expression of MMP-1, -3 and -13 and inhibit activation of NF-κB [22]. DPP-4 inhibitor has also been found to prevent activation of NF-κB in rat liver in vivo [25]. While DPP-4 inhibitors are known to inactivate various substrate hormones, chemokines, neuropeptides and growth factors, the mechanisms through which the different drugs in this class exert their effects remain poorly understood [26,27].

The main limitation of the current study is that we only examined the protective effects of alogliptin against TNF-α-induced degradation of ECM and inflammation in an in vitro primary chondrocytes culture model. It should be noticed that the pathological mechanism of OA is complicated and needs to be elucidated. A diversity of risk factors are reported to be involved in OA, including genetics, mechanical instability and joint injuries, ageing, obesity [28]. In addition to TNF-α, several other cytokines and inflammatory factors such as interferon [IFN]-γ, interleukins (ILs) have been implicated to play an important role in the pathophysiology of OA [29]. Animal experiment is one of the important ways to explore pathogenesis and treatment of OA. Future in vivo studies with animal models or clinical trials are necessary to verify the pharmacological function of alogliptin in OA. Besides chondrocytes, several other TNF-α-producing cells have been known to participate in the pathological progression of OA. For example, osteoblasts and osteoclasts act as key players during subchondral bone remodeling in OA [30]. Since OA is a disease of the whole joint, synovial cells and first of all synovial fibroblasts, play an important role in TNF-α-mediated processes of pathogenesis and perpetuation [31]. Cytokines and chemokines attract macrophages to the OA synovial tissue and induce the polarization of macrophages into a pro-inflammatory M1 macrophage phenotype to produce catabolic molecules such as IL-1β, TNF-α and pro-MMPs, which perpetuate cartilage degeneration [32]. Additionally, lymphocytes and Adipocytes are also involved in OA manifestation [33]. Further study is needed to clarify the efficacy of alogliptin in these cells.

The results of the present study demonstrate that pretreatment with alogliptin at regularly prescribed doses (25 or 50 μM) could reduce degradation of type II collagen and aggrecan in a dose-dependent manner by suppressing phosphorylation of p38 and activation of the IKKα/IκBα/NF-κB signaling pathway, thus showing the potential of this drug as a novel treatment for OA. Further study is needed to elucidate the mechanism through which alogliptin exerts its anti-inflammatory effects in chondrocytes.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.007.

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