Aminoguanidine inhibits IL-1β-induced protein expression of iNOS and COX-2 by blocking the NF-κB signaling pathway in rat articular chondrocytes

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Received July 7, 2019; Accepted April 29, 2020

DOI: 10.3892/etm.2020.9021

Abstract. Osteoarthritis is a chronic joint disease which has a serious impact on the health and quality of life of affected humans and animals. As an inhibitor of inducible nitric oxide synthase (iNOS), aminoguanidine (AG) displays anti-inflammatory effects. The purpose of the present study was to investigate the effect of AG on the expression of iNOS and cyclooxygenase-2 (COX-2), and the activity of the NF-κB signaling pathway in rat chondrocytes stimulated by interleukin-1β (IL-1β). The viability of chondrocytes treated with AG (0.3, 1 or 3 mM) alone was determined using a Cell Counting Kit-8 assay. Subsequently, the chondrocytes were treated with either 10 ng/ml IL-1β alone, or co-treated with increasing concentrations of AG (0.3, 1 or 3 mM) and 10 ng/ml IL-1β. The protein levels of COX-2, iNOS, phosphorylated (p)-p65, p65, p-NF-κβ inhibitor α (IκBα), IκBα, p-inhibitor of NF-κβ-β (IKKβ) and IKKβ were evaluated by western blotting. NF-κB translocation was determined by immunofluorescence analysis. Western blotting and reverse transcription-quantitative PCR were used to detect expression levels of relevant proteins/genes. The results suggested that the inhibitory effect of AG on the protein and gene expression levels of iNOS and COX-2 in IL-1β-treated chondrocytes was dose-dependent. In addition, AG decreased the level of phosphorylation of IKKβ, IκBα and NF-κB p65, the degradation of IKKβ, IκBα and p65, and the translocation of NF-κB in IL-1β-stimulated chondrocytes. The most significant inhibitory effect of AG was observed at a concentration of 1 mM. Therefore, the present study suggested that AG may serve as a potential agent to reduce the inflammatory response of chondrocytes stimulated by IL-1β.

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Key words: aminoguanidine, inducible nitric oxide synthase, cyclooxygenase-2, NF-κB, interleukin-1β, chondrocytes

Introduction

Osteoarthritis (OA) is a common chronic and irreversible joint disease (1,2) that causes alterations to the morphology, structure and function of chondrocytes (3). Overexpression of inflammatory mediators, including cytokines [interleukin (IL)-1β], reactive oxygen species and matrix-degrading enzymes, leads to progressive deterioration of cartilage, synovium and subchondral bone (4). At present, no effective treatments to prevent or reverse progressive joint injury are available (5).

AG is an inducible nitric oxide synthase (iNOS) inhibitor, which can regulate the activity and expression levels of iNOS (6). iNOS inhibitors have been used to alleviate articular cartilage injury, pain and inflammation in a surgical model of OA (7). In addition, AG has various pharmacological effects, including anti-inflammatory effects that can modulate OA and articular inflammation (8). Additionally, it has been reported that AG protects against colonic inflammation by inhibiting the expression of NF-κB/p65 (9). In a previous study, AG treatment reduced the protein expression levels of iNOS and p65 in the liver of an animal model of diabetes (10). Furthermore, AG can reduce the level of osteocyte apoptosis during non-traumatic osteonecrosis (11). Although the pharmacological effects of AG have been studied in numerous cell types (12-17), it remains unclear whether AG can influence iNOS and cyclooxygenase-2 (COX-2) expression, and the NF-κB signaling pathway in rat chondrocytes.

The pathogenesis of OA is related to the activation of a number of different molecular pathways, such as the NF-κB and MAPK pathways (18). Activation of the NF-κB signaling pathway directly affects the pathogenesis and development of OA (19). Activated NF-κB molecules trigger increases in the levels of other proinflammatory cytokines, for example IL-1β, tumor necrosis factor α and IL-6, leading to increased extracellular degradation and reduced synthesis of collagen and proteoglycans, which further contribute to the onset of OA (20). The activation of NF-κB has been hypothesized

to be closely associated with IL-1β-stimulated OA (20). Furthermore, it has been hypothesized that the proinflammatory responses stimulated by IL-1β stimulate chondrocytes during OA, leading to increases in COX-2 and iNOS expression by activating NF-κB (21-23). In addition, NF-κB promotes articular cartilage breakdown by inducing the expression of matrix metalloproteinases and ADAMTs in OA chondrocytes (20,24). Elevated levels of NF-κB in OA chondrocytes lead to the overexpression of COX2 and iNOS, which further contribute to cartilage inflammation (25,26) and degeneration of articular cartilage (24).

Targeted therapies that interfere with NF-κB signaling may serve as a novel therapeutic strategy for the treatment of OA. In the present study, IL-1β-treated articular chondrocytes were used to investigate whether AG inhibited iNOS and COX-2 expression, and the NF-κB signaling pathway.

Materials and methods

Chondrocyte isolation and culture. Sprague-Dawley rats (age, 2-3 weeks; weight, 41±3.5 g; sex, male:female, 1:1) obtained from The Second Affiliated Hospital of Harbin Medical University (Harbin, China) were anaesthetized with isoflurane and sacrificed by cervical dislocation. Articular cartilage from the femoral heads and knees was isolated, and ground into small pieces under sterile conditions. All experiments were approved by the Departmental Animal Care and Use Committee at Northeast Agricultural University.

As previously described (27), cartilage debris was isolated with trypsin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C for 30 min, and subsequently rinsed with PBS containing penicillin and streptomycin solution. The tissue was digested with collagenase Type II (Gibco; Thermo Fisher Scientific, Inc.) in PBS at 37˚C for 4 h. Subsequently, the supernatant was collected and centrifuged at 500 x g, 27˚C for 7 min. The harvested articular chondrocytes were placed in 25 m^2 culture flasks containing DMEM supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin, and were incubated at 37˚C with 5% CO_2. When the monolayer on the bottom of the culture flask reached 80-90% confluence, the cells were passaged and the second-generation chondrocyte cells were isolated for subsequent experiments.

Chondrocyte treatment. To assess cell viability, chondrocytes were seeded in 96-well plates ($5x10^4$/ml, 100 μl/well), treated with 0, 0.3, 1, or 3 mM AG (Sigma-Aldrich; Merck KGaA) for 24, 48 and 72 h at 37˚C and evaluated using a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.), as described below. For the evaluation of iNOS and COX-2 expression, chondrocyte cells were exposed to the following conditions for 24 h at 37˚C: i) 10 ng/ml IL-1β (PeproTech, Inc.) alone or ii) co-treatment with different concentrations of AG (0.3, 1, 3 mM) and 10 ng/ml IL-1β for 24 h, after treatment with 10 ng/ml IL-1β for 24 h alone. The activity of the NF-κB signaling pathway was assessed in chondrocytes treated with either 10 ng/ml IL-1β alone for 0.5 h or co-treated with AG (0.3, 1 or 3 mM) and 10 ng/ml IL-1β at 37˚C for 2 h. Rat chondrocytes cultured without AG and IL-1β were used as controls.

Cell viability assay. The cytotoxicity of AG was determined using a CCK-8 assay. The assay was conducted on chondrocytes treated with varying concentrations of AG (0.3, 1 or 3 mM) for 24, 48, and 72 h, as aforementioned. Cells were incubated with 10 μl CCK-8 reagent at 37˚C for 2 h. Subsequently, the absorbance at a wavelength of 450 nm was determined using a microplate reader.

Western blot analysis. AG-treated chondrocyte cells were washed with PBS and collected with cell scrapers. Total protein was extracted from AG-treated chondrocyte cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) by centrifugation at 10,000 x g for 15 min at 4˚C. Total protein was quantified using a bicinchoninic acid assay. Protein (15 μl/lane) was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Subsequently, the membranes were blocked with 5% skim-milk at room temperature for 1 h. The membranes were incubated at 4˚C overnight with primary antibodies targeted against: p65 (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc.), NF-κβ inhibitor α (IκBα; cat. no. 9242; 1:1,000; Cell Signaling Technology, Inc.), inhibitor of NF-κβ-β (IKKβ; cat. no. ab124957; 1:1,000; Abcam), phosphorylated (p)-p65 (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc.), p-IκBα (cat. no. 9246; 1:1,000; Cell Signaling Technology, Inc.), p-IKKβ (cat. no. ab59195; 1:1,000; Abcam), iNOS (cat. no. WL0992a; 1:500; Wanleibio Co., Ltd.), COX-2 (cat. no. WL01750; 1:500; Wanleibio Co., Ltd.) and GAPDH (cat. no. TA-08; 1:3,000; OriGene Technologies, Inc.). Subsequently, the membranes were washed with TBST. Following primary incubation, the membranes were incubated for 2 h at 37˚C with horseradish peroxidase-conjugated secondary antibody goat anti-mouse IgG and horseradish peroxidase-conjugated secondary antibody goat anti-rabbit IgG (cat. nos. ZB2305 and ZB-2301, respectively; 1:1,000; ZSGB-BIO). Protein bands were visualized using an ECL kit (Beyotime Institute of Biotechnology) and the signals were analyzed using a Tanon detection system (Tanon Science and Technology Co., Ltd.). GAPDH was used as the loading control. The densities of bands were analyzed using ImageJ (version 1.51; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from the chondrocyte cells using the BioSci™ Tissue/Cultured Cell Total RNA Extraction kit (Dakewe Bioengineering Co., Ltd.), according to the manufacturer's instructions. Total RNA (1 μg), dNTPs (cat. no. AD101-12; Beijing TransGen Biotech Co., Ltd.), primer (cat. no. AH101-02; Beijing TransGen Biotech Co., Ltd.) and TransScript®II reverse transcriptase (cat. no. AH101-02; Beijing TransGen Biotech Co., Ltd.) were used to synthesize cDNA. The temperature protocol was as follows: 50˚C for 30 min and 85˚C for 5 sec. Quantitative PCR (qPCR) was performed using SuperReal PreMix Plus (SYBR Green; cat. no. FP205; Tiangen Biotech Co., Ltd.) according to the manufacturer's protocols. The following thermocycling conditions used for qPCR: 15 min at 95˚C for initial denaturation; followed by 40 cycles at 95˚C for 15 sec, 60˚C for 32 sec, and 72˚C for 5 min. The following primer pairs were used for qPCR: GAPDH forward, 5'-GATGCCCCCATGTTTGTGAT-3' and reverse, 5'-GGCATGGACTGTGGTCATGAG-3'; iNOS forward, 5'-GAGACGCACAGG

CAGAGGTTG-3' and reverse, 5'-AGCAGGCACACGCAA TGATGG-3'; and COX-2 forward, 5'-AGAAGCGAGGAC CTGGGTTCA C-3' and reverse, 5'-ACACCTCTCCACCGA TGACCTG-3'. Protein levels were quantified using the $2^{-\Delta\Delta Cq}$ method (28) and normalized to the internal reference gene GAPDH.

Immunofluorescence assay. After treating with 10% FBS, Second-generation chondrocytes cultured with DMEM supplemented with 0.05% FBS were treated with 10 ng/ml IL-1β, or co-incubated with 10 ng/ml IL-1β and 1 mM AG for 2 h at 37˚C. Subsequently, the cells were washed with PBS and fixed with 4% paraformaldehyde for 1 h at 37˚C. The cells were washed with PBS, blocked with 10% goat serum (cat. no. AR1009; 0.3% Triton, 1:10; Boster Biological Technology) for 1 h at room temperature and rinsed with PBS. Subsequently, the cells were incubated with primary antibodies against p-p65 (cat. no. 3033; 1:100; Cell Signaling Technology, Inc.) overnight at 4˚C. Following primary incubation, the cells were gently washed and then incubated with a horseradish peroxidase-conjugated secondary antibody goat anti-rabbit IgG (cat. no. ZB-2301; 1:250; OriGene Technologies, Inc.) for 2 h at 37˚C. Subsequently, the cells were stained with DAPI (Beyotime Institute of Biotechnology) at room temperature for 15 min. Cells were rinsed and observed under a fluorescence microscope in six randomly-selected fields (magnification, x400).

Statistical analysis. Statistical analyses were performed using SPSS software (version 18.0; SPSS, Inc.). Data are presented as the mean ± standard deviation. And all experiments were performed at least three times. Differences were assessed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of AG on cell viability. To determine whether different concentrations of AG were cytotoxic to rat chondrocytes, cells were treated with AG at varying concentrations (0.3, 1 or 3 mM) for 24, 48, and 72 h. The results suggested that treatment with AG did not significantly alter the viability of the cells, as measured by a CCK-8 assay ($P>0.05$; Fig. 1).

Effect of AG on IL-1β-induced expression levels of iNOS and COX2 in chondrocytes. Chondrocytes treated with IL-1β displayed significantly increased COX-2 and iNOS expression levels compared with the control cells (Fig. 2A-E). Compared with cells stimulated with IL-1β alone, the protein expression levels of iNOS and COX-2 were decreased in a dose-dependent manner by AG ($P<0.05$; Fig. 2A-C). The gene expression levels of iNOS and COX-2 were also decreased in a dose-dependent manner by AG ($P<0.05$; Fig. 2D and E).

Effect of AG on the activity of NF-κB in chondrocytes induced by IL-1β. Western blot analysis suggested that the levels of p-IKKβ and p-IκBα were significantly increased in IL-1β-stimulated chondrocytes compared with the control cells ($P<0.05$; Fig. 3A, C and E). Moreover, the protein levels

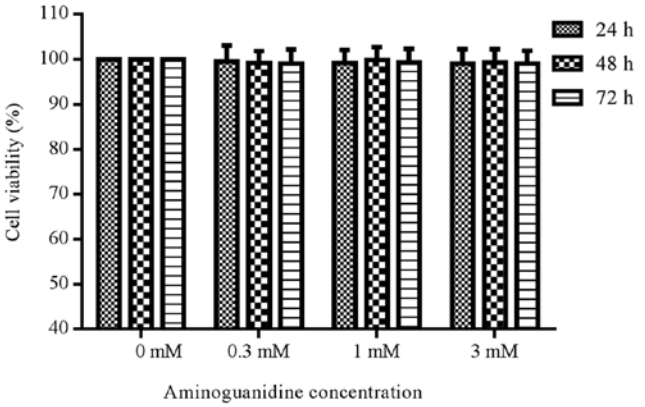

Figure 1. Cytotoxicity of different concentrations of AG on chondrocytes. AG did not alter the viability of rat chondrocytes. AG, aminoguanidine.

of p-IKKβ and p-IκBα were significantly reduced in chondrocytes co-treated with AG and IL-1β ($P<0.05$; Fig. 3C and E). Interestingly, the lowest p-IKKβ and p-IκBα protein expression levels were observed in chondrocytes treated with 1 mM AG (Fig. 3C and E). Chondrocyte treatment with IL-1β significantly reduced the protein expression levels of IκBα ($P<0.05$; Fig. 3D) and IKKβ levels ($P<0.05$; Fig. 3B) compared with the control cells. Chondrocytes co-cultured with AG and IL-1β displayed decreased expression levels of IKKβ and IκBα compared with the IL-1β group (Fig. 3B and D). Similar to the results obtained for p-IKKβ/IKKβ and p-IκBα/IκBα, the highest inhibitory effect of AG on IL-1β-induced p-IKKβ/IKKβ and p-IκBα/IκBα alterations was observed in cells treated with 1 mM AG (Fig. 3C and D).

Treatment with IL-1β significantly reduced the protein expression levels of NF-κB p65 in chondrocytes compared with the control cells (Fig. 3F). By contrast, co-treatment with AG and IL-1β resulted in significantly higher expression levels of p65 compared with cells stimulated with IL-1β alone ($P<0.05$; Fig. 3F). Compared with cells stimulated with IL-1β alone, co-treatment with 0.3, 1 or 3 mM AG and IL-1β significantly reduced the p-p65/p65 ($P<0.05$; Fig. 3G). Importantly, the greatest decrease in p-p65 levels was observed in the group treated with 1 mM AG (Fig. 3G). Taken together, the results suggested that AG inhibited the activity of NF-κB in chondrocytes activated by IL-1β and that AG displayed the strongest inhibitory effect at a concentration of 1 mM.

Effect of AG on the nuclear translocation of NF-κB p65. No p65 staining was observed in the nuclei of untreated control cells, indicated by no green fluorescence inside the nucleus (Fig. 4). However, IL-1β-stimulated chondrocytes displayed p65 staining in the nucleus, but not in the cytoplasm (Fig. 4). However, IL-1β-stimulated chondrocytes displayed p-p65 staining in the nucleus. Therefore, strong green fluorescence was observed in the nucleus (Fig. 4). In cells co-treated with 1 mM AG and 10 ng/ml IL-1β, the intensity of green fluorescence in the nucleus decreased. The levels of cytoplasmic staining were also low in this group (Fig. 4). Therefore, these results suggested that the entry of p-p65 into the nucleus was limited in cells co-treated with AG and IL-1β.

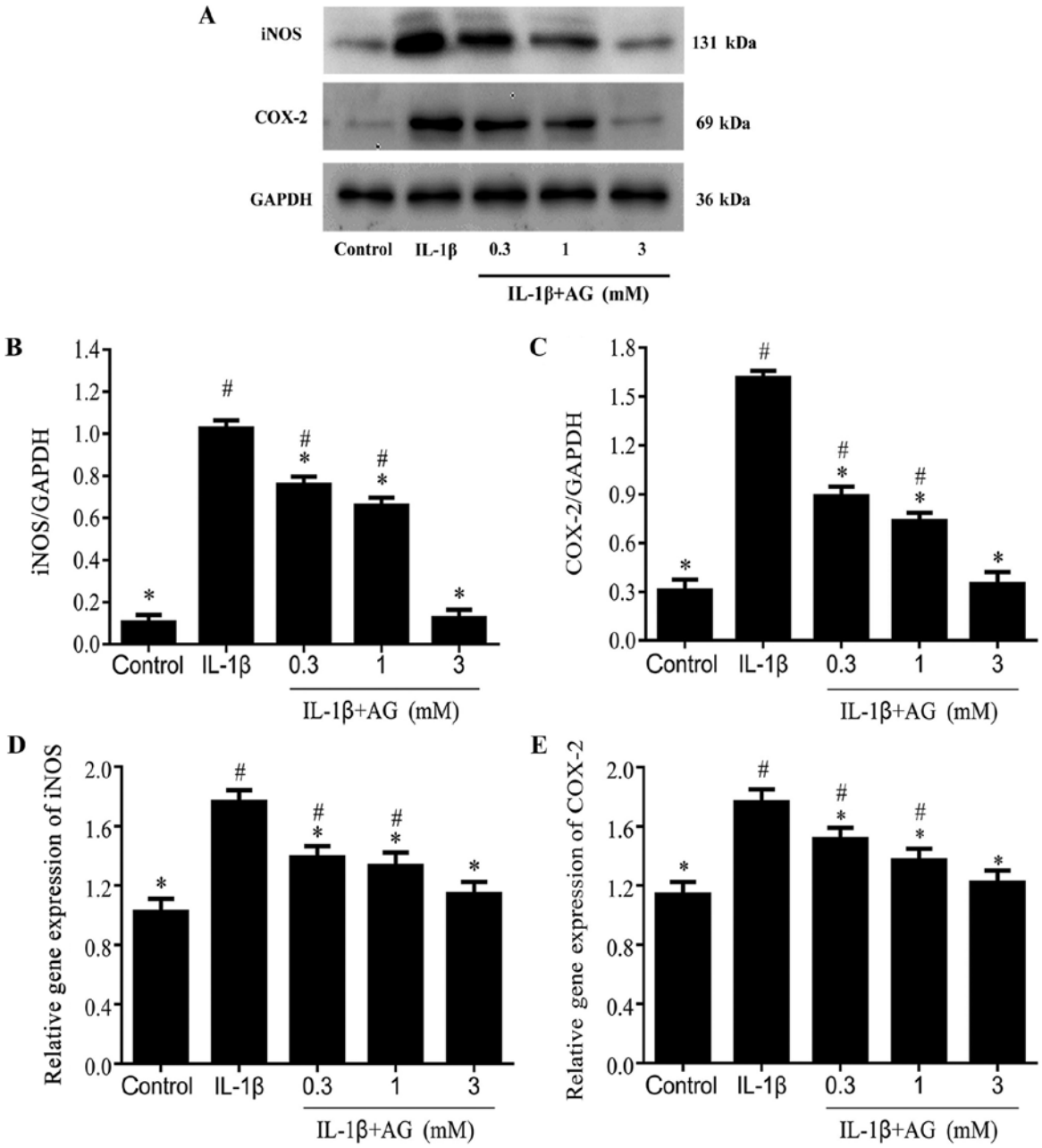

Figure 2. Protein and mRNA expression levels of iNOS and COX-2 in the different treatment groups. (A) Western blotting was used to determine the protein expression levels of iNOS and COX-2. Quantification of western blotting for (B) iNOS and (C) COX-2. mRNA expression levels of (D) iNOS and (E) COX-2. #P<0.05 vs. the control group. *P<0.05 vs. the IL-1β group. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; IL, interleukin; AG, aminoguanidine.

Discussion

OA is a chronic joint disease (1) and at present, no effective preventive or therapeutic drugs are available for the treatment of the disease (2). Therefore, the development of effective compounds for the treatment of OA is important. In the present study, the potential of AG, a compound that inhibits iNOS, to prevent or delay the progression of OA was investigated.

The roles of AG as an inhibitor of oxidation (29), apoptosis (30,31) and inflammation (32-34) have been widely recognized and accepted. In addition to these well-recognized roles, AG can inhibit the generation of isoproterenol-induced reactive oxygen species, and restore levels of antioxidant superoxide dismutase, glutathione and catalase in the heart (35). AG can also suppress the production of nitric oxide and the expression of iNOS in osteocytes, leading to the release of cytochrome C and the induction of osteocyte apoptosis (11). Moreover, AG can decrease the accumulation of glycosylation products, which can lead to endoplasmic reticulum stress-induced cell apoptosis (36). Furthermore, AG mediates articular inflammatory processes by downregulating IL-1β production in human osteoarthritic synovial tissue and cartilage (8). Therefore, in the present study, the possible associations between the inhibitory effect of AG on inflammation and the NF-κB signaling pathway were investigated.

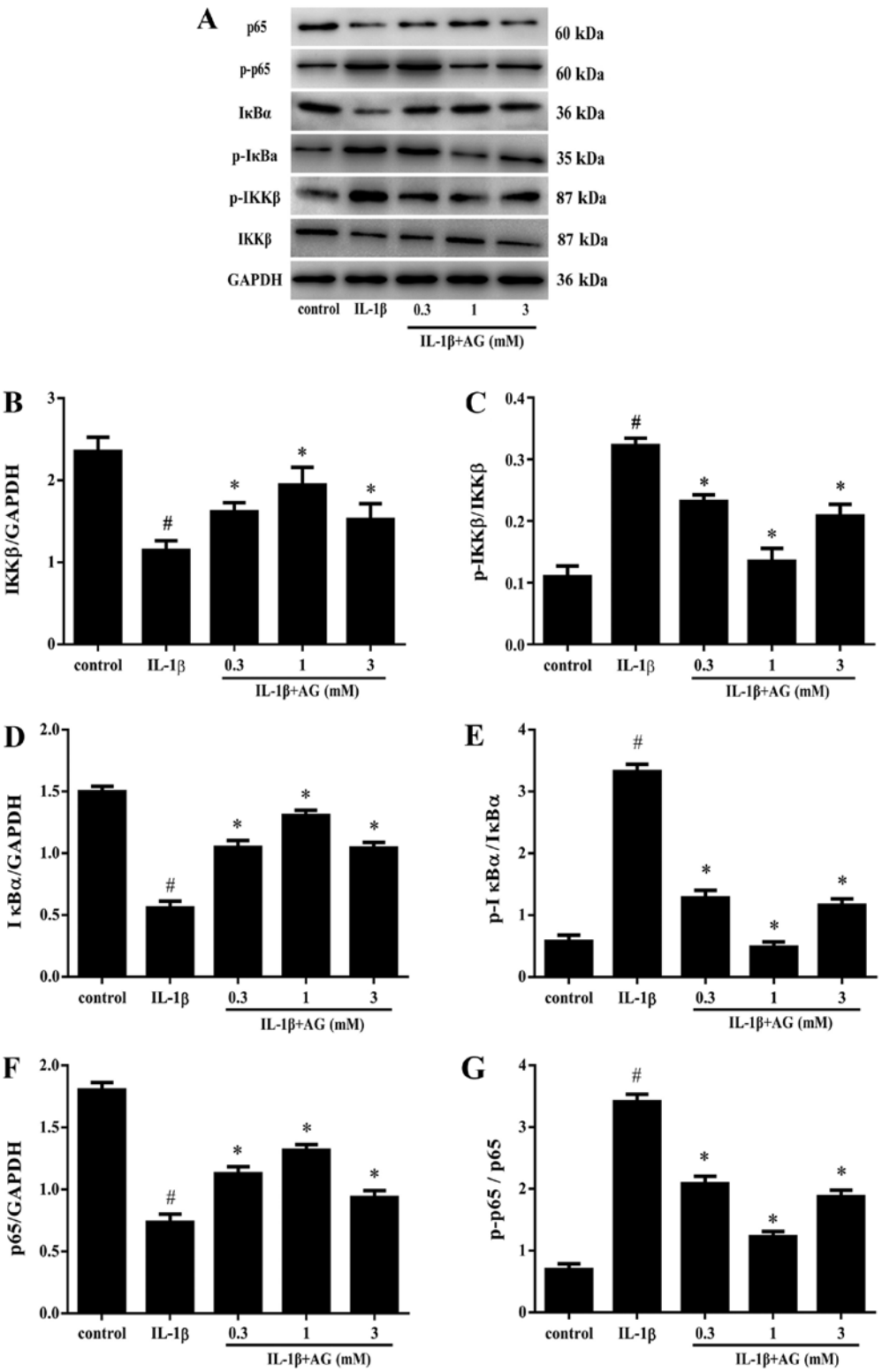

Figure 3. Protein levels of IKKβ, p-IKKβ, IκBα, p-IκBα, p65 and p-p65 in the different treatment groups. (A) Western blotting was performed to assess the protein expression levels of p-IKKβ, p-IκBα, IKKβ, IκBα, p65, and p-p65. Densitometric analysis of western blotting for (B) IKKβ, (C) p-IKKβ/IKKβ, (D) IκBα, (E) p-IκBα/IκBα, (F) p65 and (G) p-p65/p65. #P<0.05 vs. the control group. *P<0.05 vs. the IL-1β group. IKKβ, inhibitor of NF-κβ-β; p, phosphorylated; IκBα, NF-κβ inhibitor α; IL, interleukin; AG, aminoguanidine.

IL-1β is involved in the pathogenesis of OA and it may result in marked alterations to the cartilage, including matrix degradation, inflammation, chondrocyte phenotypic changes and chondrocyte apoptosis (26,37-39). Previous studies have reported that 10 ng/ml IL-1β induces an inflammatory response in chondrocytes (27,40); therefore, 10 ng/ml was used as the working concentration of IL-1β in the present study. IL-1β induces iNOS and COX-2 expression, promotes the synthesis of inflammatory mediators, including prostaglandin E2 and nitric oxide, and stimulates

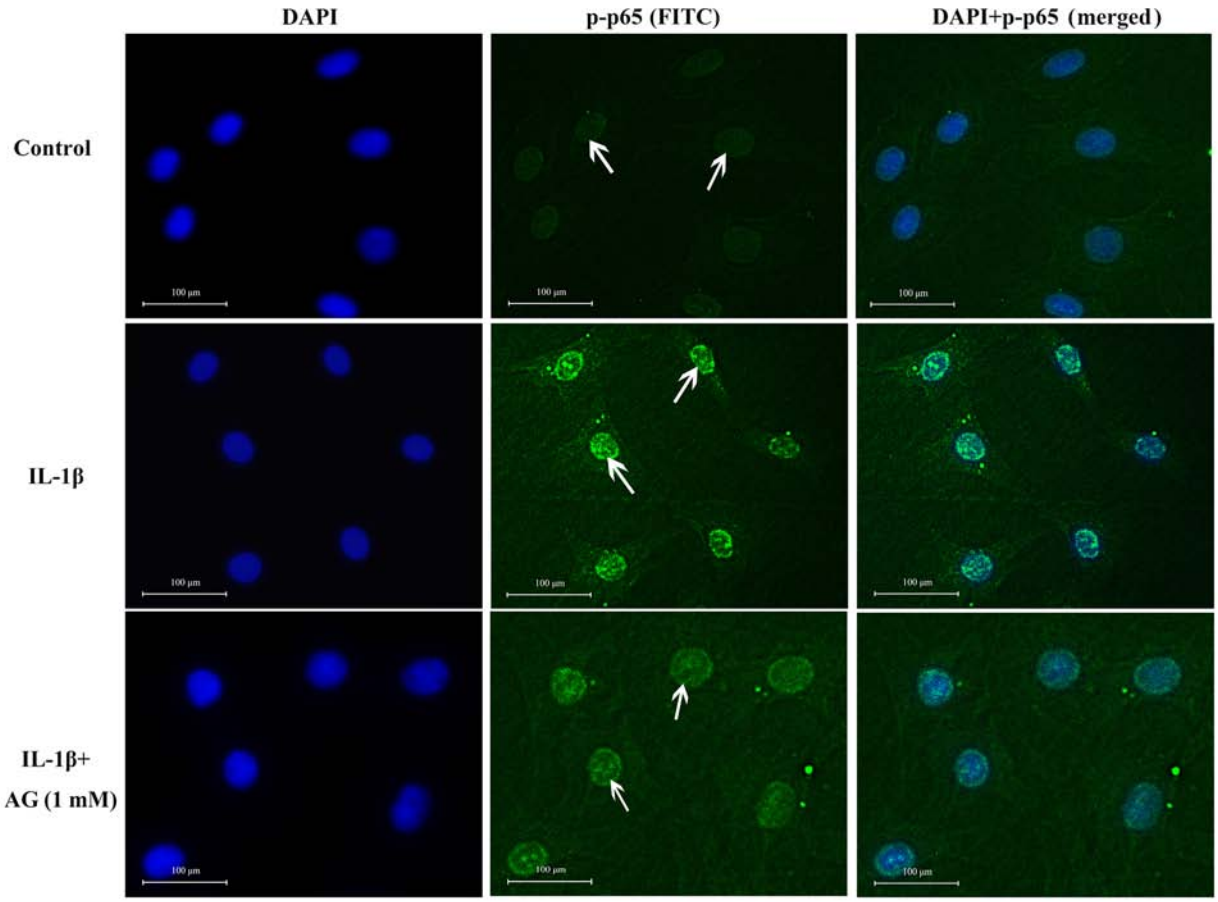

Figure 4. Immunofluorescence assay for the nuclear translocation of NF-κB p65. The control group displayed no intense green fluorescence in the nucleus. Chondrocytes treated with IL-1β displayed intense green fluorescence in the nucleus. The green fluorescence inside the nucleus was reduced in chondrocytes co-treated with IL-1β and AG. The white arrows indicate intense green fluorescence. IL, interleukin; AG, aminoguanidine; p, phosphorylated.

articular chondrocytes to produce high levels of NF-κB (26). Therefore, in the present study, chondrocytes were stimulated with IL-1β to mimic the pathophysiology of OA. The results of the present study were consistent with previous studies, suggesting that IL-1β induced increased iNOS and COX-2 expression (37,41,42).

iNOS is not only an inflammatory mediator, but is also essential for the initiation and promotion of the inflammatory response (43). iNOS can significantly increase the production of nitric oxide, which leads to destruction of articular cartilage and chondrocyte apoptosis (44). In addition, iNOS can regulate other inflammatory processes, for example, cortisol can interact with or induce iNOS to increase the extent of DNA damage, and the formation of reactive oxygen/nitrogen species (45). Increased gene and protein expression levels of iNOS and COX-2 contribute to pain and joint inflammation in patients with OA (46). Moreover, current OA treatment strategies focus on the use of anti-inflammatory drugs that reduce COX-2 levels, which can alleviate joint pain and swelling (47). Therefore, reducing the expression of iNOS and COX-2 in chondrocytes could potentially ameliorate joint inflammation and the degeneration of articular cartilage in patients with OA. In a recent study, treatment with kaempferol reduced the expression levels of iNOS and COX-2 in a dose-dependent manner in IL-1β-stimulated rat chondrocytes (23). Consistently, the results of the present study indicated that AG decreased the gene and protein expression levels of iNOS and COX-2 in IL-1β-stimulated rat articular chondrocytes. Therefore, the present study suggested that AG might reduce inflammatory responses.

Furthermore, the present study suggested that the expression of COX-2 and iNOS may be closely associated with the activation of the NF-κB signaling pathway. In a study conducted by Lianxu *et al* (39), an small interfering RNA targeted against NF-κB p65 reduced iNOS and COX-2 expression levels in rat chondrocytes stimulated by IL-1β. Additionally, IL-1β-mediated iNOS expression was reduced following suppression of NF-κB activity in rat chondrocytes (48). The aforementioned studies suggested that increased iNOS and COX-2 protein expression levels in IL-1β-stimulated rat chondrocytes are dependent upon the activation of NF-κB. Furthermore, it has been reported that the protein expression level of IκBα and the activity of NF-κB are decreased by IKKβ phosphorylation (49). Activated NF-κB translocates into the nucleus to induce the expression of iNOS and COX-2 (50) and other proinflammatory cytokines, such as NO and PGE2 (26), which further aggravate OA symptoms. The results reported in the aforementioned studies were consistent with the results of the present study, which indicated that IL-1β successfully induced an inflammatory response in chondrocytes. Therefore, suppressing NF-κB activity might serve as a novel therapeutic option for OA (11,25,36,46,47). In the present

study, AG inhibited IKKβ phosphorylation, IκBα degradation and NF-κB/p65 activation. Similar effects have been reported with pomegranate fruit extract (51). Pomegranate fruit extract inhibited IL-6 production, the expression of IKKβ mRNA, the degradation of IκBα and the nuclear translocation of NF-κB/p65 in OA chondrocytes. Furthermore, AG inhibited expression of COX-2 and iNOS, and similar effects have been observed with chrysin (52), suggesting that there is a common mechanism of action among these drugs. The present study suggested that AG inhibited NF-κB activation and suppressed the inflammatory response in IL-1β-stimulated chondrocytes. Therefore, it was hypothesized that AG inhibited nuclear translocation of NF-κB/p65 by inhibiting the phosphorylation of IκBα and IKKβ, thereby reducing the expression of iNOS and COX-2, and suppressing the inflammatory response. Collectively, these results indicated that AG has the potential to protect articular chondrocytes. Further investigation into the clinical application of AG is required.

In conclusion, AG downregulated iNOS and COX-2 expression by blocking the NF-κB signaling pathway; therefore, AG may protect chondrocytes. Additionally, 1 mM AG was the most effective concentration for the inhibition of inflammation. Furthermore, the present study indicated that AG may serve as a potential therapeutic for OA, therefore, providing the theoretical basis for future clinical studies.

Acknowledgements

Not applicable.

Funding

The present study was supported by The National Key R&D Program of China (grant no. 2017YFD0502200) and the Major National Science and Technology Special and Key Research Projects-Provincial Funding Projects (grant no. GX18B023).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YM designed the study and prepared the manuscript. LG designed the study and produced the final draft of manuscript before submitting. YM, TM and XS analyzed the data. YM, ZZ, HB, YL, HH, RY and YW performed the experiments and processed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Departmental Animal Care and Use Committee at Northeast Agricultural University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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