Regular Article

The Function of PPARγ/AMPK/SIRT-1 Pathway in Inflammatory Response of Human Articular Chondrocytes Stimulated by Advanced Glycation End Products

Hao Huang, Zhao-jun Wang, Hai-bin Zhang, Jian-xia Liang, Wen-dong Cao, Qi Wu, Chao-peng He, and Cheng Chen*

Department of Orthopedics, The 921st Hospital of the People's Liberation Army, The Second Affiliated Hospital of Hunan Normal University; Changsha, Hunan 410005, People's Republic of China.
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Accumulation of advanced glycation end products (AGEs) in the articular cartilage is a major risk factor for osteoarthritis (OA). To determine the mechanistic basis of AGE action in OA, we treated human articular chondrocytes with AGEs, and found that they not only up-regulated the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α, but also inhibited AMP-activated protein kinase (AMPK) phosphorylation and decreased sirtuin 1 (SIRT-1) levels in a concentration- and time-dependent manner. Pioglitazone, a peroxisome proliferator-activated receptor-γ (PPARγ) agonist restored the inhibited AMPK and SIRT-1 by AGEs. Pre-treatment of the cells with the agonists or antagonists of AMPK and SIRT-1 respectively abolished and augmented the inflammatory state induced by AGEs. Furthermore, AMPK agonist also restored the levels of SIRT-1 in the AGE-stimulated chondrocytes. Our findings indicate AGEs induce an inflammatory response in human articular chondrocytes *via* the PPARγ/AMPK/SIRT-1 pathway, which is therefore a potential target in OA therapy.

Key words AMP-activated protein kinase; advanced glycation end product; inflammatory; chondrocyte

INTRODUCTION

Osteoarthritis (OA) is a degenerative disease of the articular joints that can lead to disability in the advanced stages. A major risk factor for OA is aging,[1,2] specifically the accumulation of advanced glycation end products (AGEs) with age.[3] Since AGEs are degraded with routine protein recycling, they tend to accumulate in regions with low renewal rate, such as the articular cartilage.[4,5] AGEs cause degeneration of the articular cartilage by triggering autophagy[6] and apoptosis[7] in the chondrocytes, and by increasing matrix metalloproteinase (MMP) production,[8] which eventually lead to arthritic symptoms. Recent studies show that accumulation of AGEs is the pathological basis of OA.[9]

The relationship between inflammation and OA has been well established,[10] and the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α are directly linked to the progression of OA.[10] IL-1β and TNF-α levels were significantly elevated in the superficial zone of grade 2 and grade 3 arthritic cartilage, but undetectable in the normal cartilage specimens.[11] These cytokines inhibit the synthesis of extracellular matrix (ECM) components by blocking the anabolic pathways in chondrocytes.[12,13] They not only decrease proteoglycan and type II collagen synthesis[14,15] but also stimulate the chondrocytes to release MMP-1 and MMP-13, which further degrade the ECM.[16–18] Recent studies have linked the AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT-1) with OA. Both are crucial mediators of metabolic pathways.[19,20] AMPK is an conserved serine/threonine kinase which regulates cellular energy homeostasis, and is implicated in multiple age-related diseases.[21–23] SIRT-1, a member of the Sirtuin family, is downstream of AMPK and deacetylates proteins in response to changes in the nicotinamide adenine dinucleotide+/reduced nicotinamide adenine dinucleotide (NAD+/NADH) ratio.[24] AMPK and SIRT-1 levels are significantly lower in the osteoarthritic human and murine knee chondrocytes and cartilage, as well as in aged mouse knee cartilage.[25] In addition, chondrocytes deficient in AMPK and SIRT-1 are known to accelerate osteoarthritic progression.[26] On the other hand, activation of AMPK and SIRT-1 reverses homocysteine-induced oxidative stress in human chondrocytes.[27]

Peroxisome proliferator-activated receptor-γ (PPARγ) is a nuclear receptor that functions as a regulator of catabolism and inflammatory response.[28,29] It is down-regulated in OA cartilage,[30] and knockout of PPARγ leads to the development of OA, suggesting it is risk factor for OA.[31] Our previous research revealed that agonists of PPARγ had a protective effect on articular cartilage in animal models.[32] Furthermore, our previous results indicated AMPK and SIRT1 could be regulated by PPARγ, which was consistent with previous reports, Shen *et al.* and Chiang *et al.* reported that the inhibited AMPK and SIRT-1 could be up-regulated by PPARγ agonist in ethanol-fed mice and TNF-α-treated human neural stem cells (hNSCs).[33,34] Zhang *et al.* reported that activation of PPARγ has been shown to induce phosphorylation of AMPK and decrease inacticvation of SIRT-1 in the renal tissue of C57BL/6 mice.[35]

Pioglitazone, an anti-diabetic thiazolidinedione, increases insulin sensitivity and lowers blood sugar by binding to PPARγ. In our previous study, we found that pioglitazone reduced the AGEs-triggered high levels of IL-1β and TNF-α in chondrocytes in a concentration-dependent manner.[36] Based on these findings, we hypothesized that AGEs increase the levels of pro-inflammatory factors in the cartilage chondrocytes by inhibiting PPARγ/AMPK/SIRT-1.

* To whom correspondence should be addressed. e-mail: shifanhh@163.com

Table 1. Primers for Real-Time Fluorescent Quantitative PCR

Genes	Forward primer (5′→3′)	Reverse primer (5′→3′)
TNF-α	GTAGCCCATGTTGTAGCAAACC	CTGATGGTGTGGGTGAGGAG
IL-1β	AGGATATGGAGCAACAAGTGGT	AACACGCAGGACAGGTACAG
β-actin	TCATGAAGTGTGACGTGGACATC	CAGGAGGAGCAATGATCTTGATCT

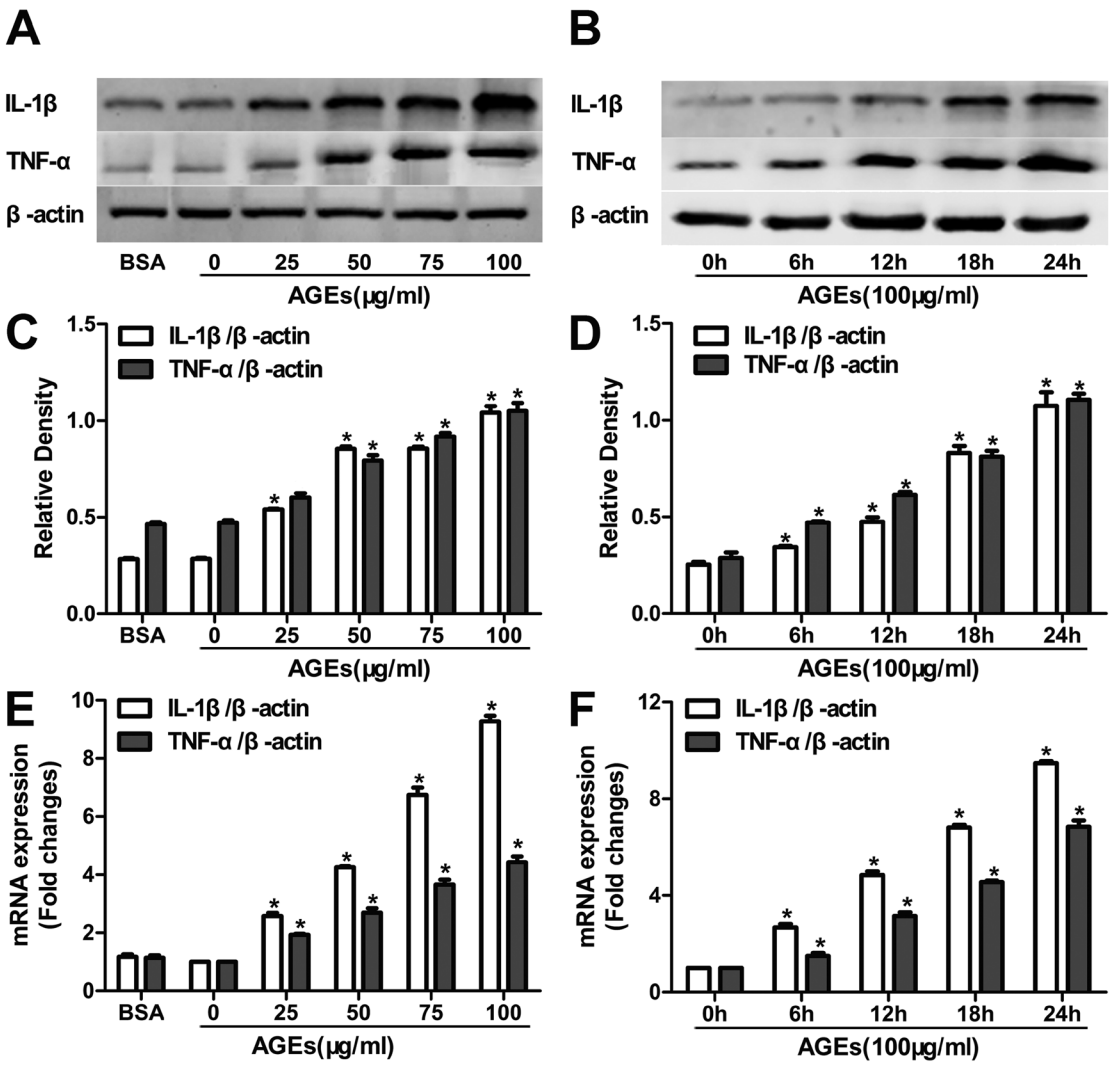

Fig. 1. Induction of Inflammatory Factors by AGEs in Human Articular Chondrocytes

(A, C and E). Cells were stimulated with varying doses of AGEs (0 to 100 μg/mL) for 24 h. Immunoblots and RT-PCR results showing levels of IL-1β and TNF-α proteins (A) and mRNA (E) levels, with the IL-1β/β-actin and TNF-α/β-actin ratios (C). $*p<0.05$ compared with control group (AGEs 0 μg/mL). (B, D and F). Cells were stimulated with 100 μg/mL AGEs for varying durations. Immunoblots and RT-PCR results showing levels of IL-1β and TNF-α proteins (B) and mRNA (F) levels, with the IL-1β/β-actin and TNF-α/β-actin ratios (D). $*p<0.05$ compared with control group (0h). The values are represented as mean ± standard deviation (S.D.) from three different experiments. β-Actin was used as a loading control.

MATERIALS AND METHODS

Chemicals Pioglitazone (a selective PPARγ agonist), A-769662 (a selective AMPK agonist), Dorsomorphin 2HCl (a selective AMPK inhibitor), SRT1720 (a selective SIRT-1 agonist) and EX 527 (a selective SIRT-1 inhibitor) were purchased from Selleck Chemicals (U.S.A.). Monoclonal antibodies specific for TNF-α, p-AMPK, AMPK, SIRT-1 and β-actin were purchased from CST Inc (U.S.) and rabbit polyclonal antibody against IL-1β from Bioss. AGE-BSA (a complex of N3-carboxymethyllysine (CML), pentosidine and other AGEs) was supplied by BioVision, Inc. (U.S.A.).

Chondrocytes Human articular chondrocytes were purchased from CHI-Scientific, and cells of generation ≤4 were used for the experiments. Chondrocytes were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C under 5% CO_2. The cells were stimulated with varying concentrations (0–100 μg/mL) of AGEs for 0, 6, 12, 18 and 24 h, and based on the experiment, pre-treated for 1h with Pioglitazone, A-769662, Dorsomorphin 2HCl, SRT1720 or EX 527.

Immunoblotting The chondrocytes were lysed, and the lysates were boiled at 100°C for 5 min with the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer to denature the proteins. Equal quantities

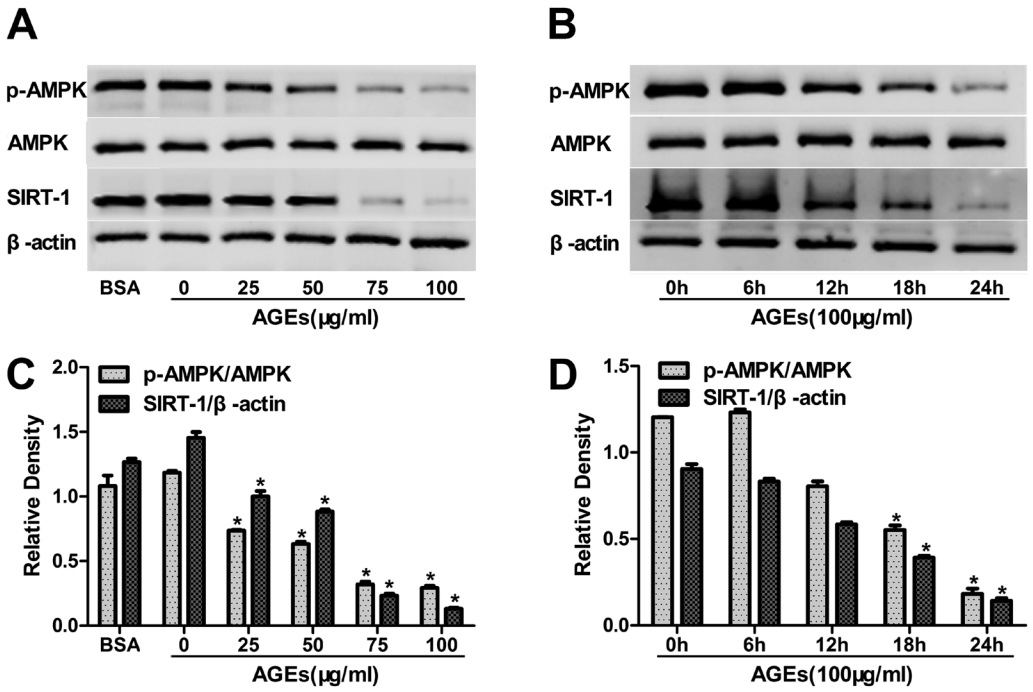

Fig. 2. Effect of AGEs on AMPK and SIRT-1 Levels in Human Articular Chondrocytes

(A, C). Cells were stimulated with varying doses of AGEs (0 to 100 μg/mL) for 24 h. Immunoblot showing AMPK and SIRT-1 protein levels (A), and the p-AMPK/AMPK and SIRT-1/β-actin ratios (C). *$p<0.05$ compared with control group (AGEs 0 μg/mL). (B, D). Cells were stimulated by 100 μg/mL AGEs for varying durations. Immunoblot showing AMPK and SIRT-1 protein levels (B), and the p-AMPK/AMPK and SIRT-1/β-actin ratios (D). *$p<0.05$ compared with control group (0 h). The values are represented as mean ± S.D. from three different experiments. β-Actin was used as a loading control for SIRT-1, and AMPK was used for p-AMPK.

of protein per sample were resolved on 10% SDS gels and electro-transferred to polyvinylidene difluoride membranes (Millipore, U.S.A.). The latter were blocked with 5% BSA, and then incubated overnight with primary antibodies at 4°C, followed by horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody. The signals were amplified by enhanced chemiluminescence (ECL) reagent and captured by Tanon 5500.

Quantitative RT-PCR RNA was extracted using TRIzol according to the manufacturer's instructions, and 1 μg RNA per sample was reversed transcribed into cDNA using Hiscript II Reverse transcription kit. The PCR reaction mix was prepared using SYBR® Green master mix, 50 μL template DNA, and 200 nM each of the sense and antisense primers (shown in Table 1). Real-time PCR was conducted on a thermal cycler (Bio-Rad Laboratories Inc., U.S.A.) with the following conditions: denaturation at 95°C for 5 min, and 40 cycles of 95°C for 10 s and 60°C for 30 s. The relative mRNA expression was calculated by the ΔΔCT method, and the fold changes were compared to the control and measured as $2^{(-\Delta\Delta CT)}$.

Statistical Analysis Data were reported as the mean with a 95% confidence interval (CI). Variances between groups were assessed by one-way ANOVA and Newman–Keuls multiple comparison test. Similarities between two groups were assessed using a Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

RESULTS

AGEs Induces Inflammatory Factors and Inhibits AMPK and SIRT-1 in the Human Articular Chondrocytes Human articular chondrocytes were stimulated *in vitro* with varying doses of AGEs (0–100 μg/mL) for 24 h or with 100 μg/mL AGEs for 0, 6, 12, 18 and 24 h. The AGEs significantly increased IL-1β and TNF-α protein (Figs. 1A–D) and mRNA (Figs. 1E, F) levels in a dose- and time-dependent manner. Based on the initial results, we used treated cells with 100 μg/mL AGEs for 24 h for the subsequent experiments. Since AMPK and SIRT-1 are involved in the inflammatory response in chondrocytes, we also analysed the effects of AGEs on their expression and activity levels. AGEs decreased the levels of p-AMPK and SIRT-1 in the chondrocytes in a concentration- and time-dependent manner (Fig. 2).

AGE-Induced Inflammation Is Regulated by AMPK and SIRT-1 To determine a potential mechanistic role of AMPK and/or SIRT-1 on the pro-inflammatory effects of AGEs, we treated the chondrocytes with the respective agonists and antagonists prior to AGE stimulation. As shown in Fig. 3 and Fig. 4, pre-treatment with either AMPK or SIRT-1 agonist abolished the AGE-induced increase in the levels of pro-inflammatory cytokines, whereas their respective antagonists further augmented the effect of AGEs. Taken together, AMPK and SIRT-1 inhibit AGE-mediated inflammation in human chondrocytes.

AMPK Blocks AGE-Mediated Inhibition of SIRT-1 To determine the relationship between AMPK and SIRT-1 in AGE-stimulated chondrocytes, we pre-treated the cells with different concentrations of the AMPK agonist. Cells pre-treated with AMPK agonist showed high levels of SIRT-1 even in the presence of AGEs, indicating that AMPK restored AGE-induced down-regulation of SIRT-1 (Fig. 5).

Pioglitazone Restores the Activity of AMPK and SIRT-1 Inhibited by AGEs To determine whether PPARγ affect the activity of AMPK and SIRT-1, Pioglitazone was used as PPARγ agonist in our experiments. We found that both of AMPK and SIRT-1 inhibited by AGEs were restored by Pio-

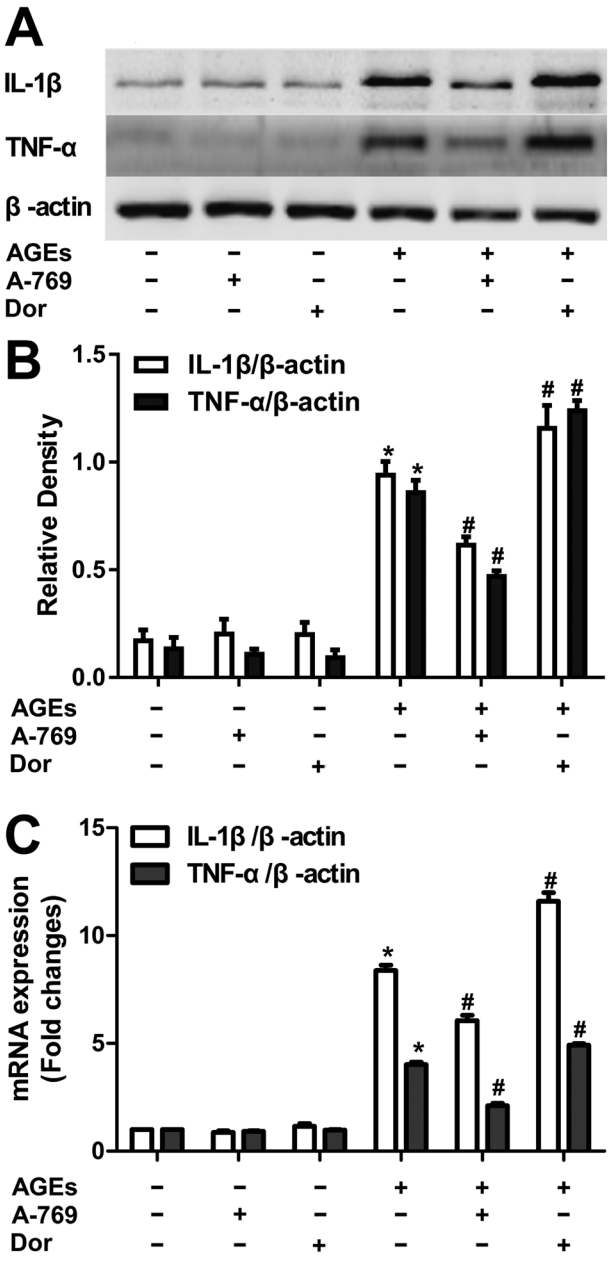

Fig. 3. Effect of AMPK Agonist and Inhibitor on Inflammatory Cytokine Expression

Human articular chondrocytes were pre-treated with A-769662 (0.15 mM) and Dorsomorphin 2HCl (10 μM) for 1h before stimulation with AGEs (100 μg/mL). Immunoblots and RT-PCR results showing levels of IL-1β and TNF-α proteins (A) and mRNA (C) levels, with the IL-1β/β-actin and TNF-α/β-actin ratios (B). A-769–A-769662; Dor, Dorsomorphin 2HCl; $^{*}p<0.05$ compared with control group (AGEs 0 μg/mL); $^{\#}p<0.05$ compared with AGEs 100 μg/mL group. The values are represented as mean ± S.D. from three different experiments. β-Actin was used as a loading control.

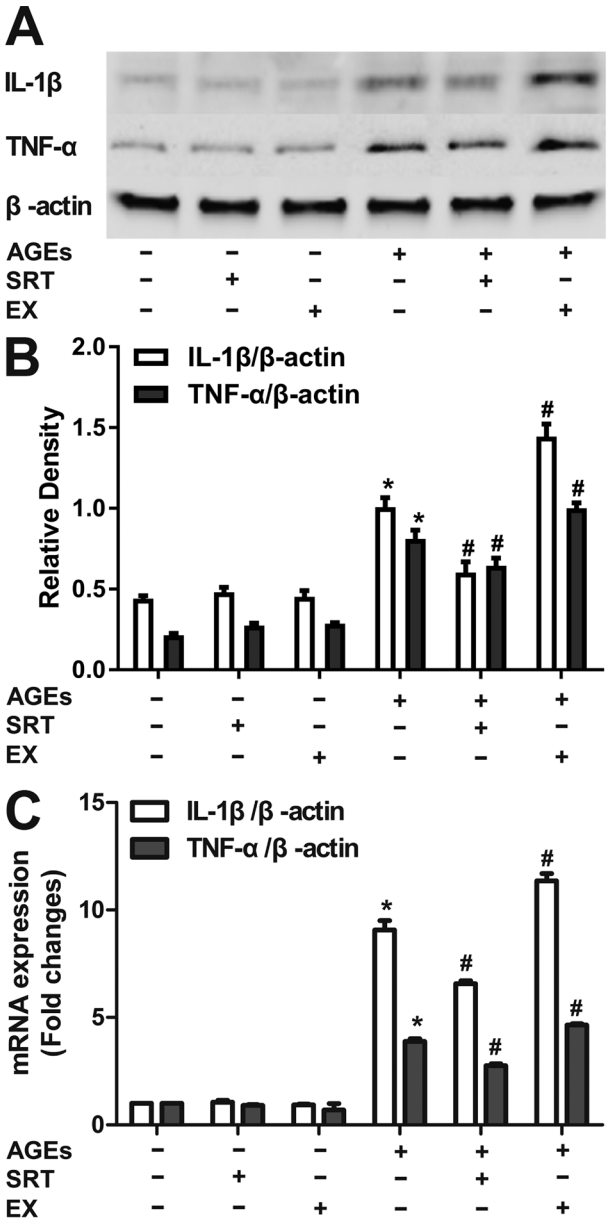

Fig. 4. Influence of SIRT-1 Agonist and Inhibitor on Inflammatory Cytokine Expression

Human articular chondrocytes were pre-incubated with SRT1720 (5 μM) and EX 527 (3 μM) for 1h before stimulation with AGEs (100 μg/mL). Immunoblots and RT-PCR results showing levels of IL-1β and TNF-α proteins (A) and mRNA (E) levels, with the IL-1β/β-actin and TNF-α/β-actin ratios (B). SRT, SRT1720; EX, EX 527; $^{*}p<0.05$ compared with control group (AGEs 0 μg/mL); $^{\#}p<0.05$ compared with AGEs 100 μg/mL group. The values are represented as mean ± S.D. from three different experiments. β-Actin was used as a loading control.

glitazone treatment in a dose-dependent manner (Fig. 6).

DISCUSSION

The prevalence of geriatric diseases is steadily increasing with a globally aging population. OA is a common disease among the elderly, and often leads to disability at the end stage.[37,38] There are no effective strategies at present for the prevention or treatment of OA.[38] The pathological basis of OA is cartilage degeneration,[39] which in turn is a result of the inhibition of cartilage ECM synthesis and increased production of proteolytic enzymes.[40] In addition, cartilage erosion is also associated with increased inflammation, and pro-inflammatory cytokines like IL-1β and TNF-α are elevated in OA,[41] which not only suppress ECM synthesis in the articular cartilage[14,15] but also degrade the ECM.[16–18]

AGEs refer to a large group of macromolecules, including proteins and lipids, that are glycated through a series of non-enzymatic reactions. AGEs are routinely formed and cleared in physiological conditions, but tend to accumulated with age due to impaired degradation mechanisms. Mahmoud and Elshazly demonstrated that AGE production and accumulation was significantly associated with the development and progression of OA.[42] In fact, AGEs are the molecular basis

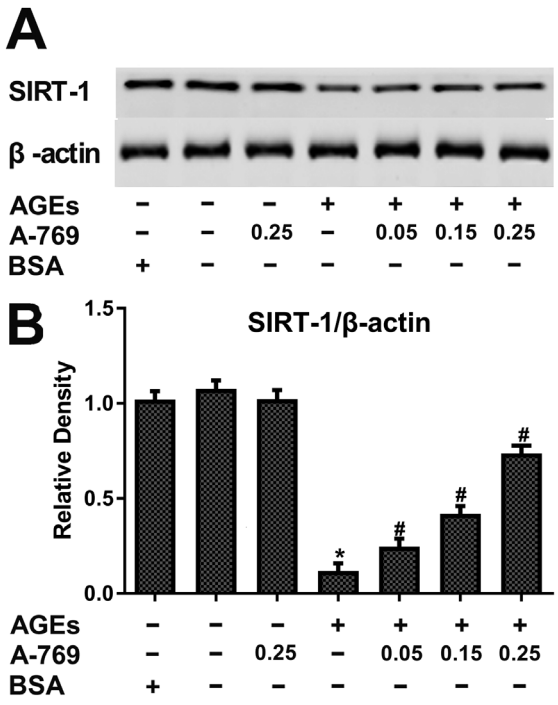

Fig. 5. Influence of AMPK Agonist on SIRT-1 Levels

Cells were pre-incubated with A-769662 (0, 0.15, 0.15, 0.25 mM) for 1 h before stimulation with AGEs (100 μg/mL). Immunoblot showing SIRT-1 protein levels (A), and the SIRT-1/β-actin ratios (B). A-769–A-769662; $^{*}p<0.05$ compared with control group (AGEs 0 μg/mL, BSA 0 μg/mL); $^{\#}p<0.05$ compared with AGEs 100 μg/mL group. The values are represented as mean ± S.D. from three different experiments. β-Actin was used as a loading control.

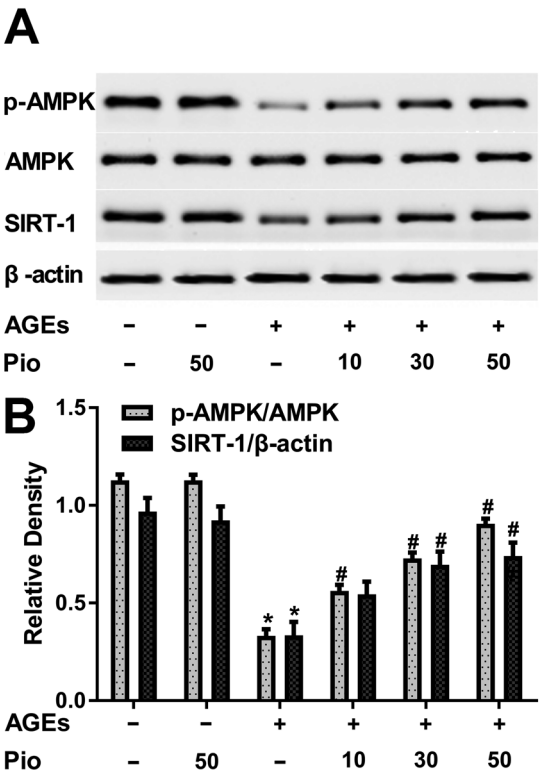

Fig. 6. Influence of PPARγ Agonist on AMPK and SIRT-1

Human articular chondrocytes were pre-incubated with Pioglitazone (0, 10, 30, 50 μM) for 1 h before stimulation with AGEs (100 μg/mL). Immunoblot showing AMPK and SIRT-1 protein levels (A), the p-AMPK/AMPK and SIRT-1/β-actin ratios (B). Pio, Pioglitazone; $^{*}p<0.05$ compared with control group (AGEs 0 μg/mL); $^{\#}p<0.05$ compared with AGEs 100 μg/mL group. The values are represented as mean ± S.D. from three different experiments. β-Actin was used as a loading control.

of age-related increase in OA risk.[43] However, the specific mechanism of its action in OA is still unknown.

AGEs are closely related to chronic inflammatory diseases. Upon binding to the specific RAGE (receptor for advanced glycation end products), the AGEs enhance the production of reactive oxygen species (ROS) and activate extracellular signal-regulated kinase 1/2 (ERK1/2) and nuclear factor kappa B (NF-κB) pathways in various diseases.[44] In this study, we found that AGEs increased the concentration of IL-1β and TNF-α in human chondrocytes *in vitro* in a concentration- and time-dependent manner. This is consistent with a previous study showing that AGEs drive OA by enhancing the inflammatory response in the articular chondrocytes.[36]

Recent studies indicate a pivotal role of the energy homeostasis regulator AMPK in the inflammatory response. Ovalbumin-induced eosinophil infiltration is more severe in the AMPK knockout mice,[45] and overexpression of constitutively-activated AMPK in the murine macrophages significantly inhibited inflammatory response, while AMPK inactivation had the opposite effect.[46] The anti-inflammatory action of AMPK is closely related to SIRT-1, an NAD+-dependent protein deacetylase that reduces the levels of acetylated NF-κB, AP-1 and histones, thereby inhibiting inflammatory pathways.[20,47–50] Alterations in AMPK activity correlated positively with SIRT-1 level and activity.[51] In streptozocin-induced diabetic mice, AMPK activators significantly increased SIRT-1 activity while enhancing retinal AMPK phosphorylation.[52] In glomerular mesangial cells, activation of AMPK increased the levels of SIRT-1.[53] Based on these findings, we hypothesized that AMPK and SIRT-1 attenuate AGE-induced inflammation in chondrocytes. Consistent with this, AGE-treated chondrocytes had significantly lower levels of p-AMPK, whereas an AMPK agonist not only restored the AMPK activity but also significantly reduced the levels of the inflammatory cytokines. In addition, AGEs also decreased the concentration of SIRT-1 in the chondrocytes, and a selective SIRT-1 agonist/inhibitor respectively inhibited and augmented AGEs-induced upregulation in inflammatory cytokines. SIRT-1 inactivates NF-κB by deacetylating the p65 subunit,[54] and in OA, inactivation of NF-κB inhibits the AGEs-induced inflammatory response in chondrocytes.[36] Furthermore, pre-treatment with the AMPK agonist restored SIRT-1 levels in the AGE-treated chondrocytes.

We showed an anti-inflammatory role of pioglitazone in chondrocytes, wherein it reduced the levels of TNF-α and IL-1β in the AGE-treated chondrocytes in a concentration-dependent manner.[36] Moreover, it is reported that AMPK and SIRT-1 could be regulated by PPARγ in ethanol-fed mice and TNF-α-treated hNSCs.[33,34] Therefore, we further speculated that pioglitazone can restore the p-AMPK and the SIRT-1 protein levels which were downregulated by AGEs. Consistent with this, AMPK and SIRT-1 inhibited by AGEs were restored by pioglitazone in chondrocytes.

Taken together, our findings demonstrate that AGEs induce an inflammatory response in chondrocytes by reducing the activity of AMPK and downregulating SIRT-1, which could be restored by activation of PPARγ, and the reactivation of the PPARγ/AMPK/SIRT-1 pathway alleviated the inflammatory state induced by AGEs. Therefore, the PPARγ/AMPK/SIRT-1

pathway is critical in cartilage degeneration, and a potential new target in OA treatment.

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Conflict of Interest The authors declare no conflict of interest.

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