Curcumin Inhibits Advanced Glycation End Products-Induced Mitochondrial Dysfunction in Chondrocyte Via Upregulaed AMPKα/PGC-1a Pathway

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Research Article

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Abstract

Aims: Aging is considered a hallmark of cartilage degradation and OA pathogenesis. Formation of advanced glycation end products (AGEs) contributes to prominent features of osteoarthritis, which might be damaged the chondrocyte mitochondrial function demonstrated in our previous study. AMP-activated protein kinase (AMPK) and peroxisome proliferator–activated receptor γ coactivator 1α (PGC-1α) are two critical bioenergy sensors to maintain cartilage homeostasis.The study was undertaken to test whether curcumin, a well-known polyphenolic compound, inhibited AGEs-induced chondrocyte mitochondrial dysfunction and the mechanism involved the AMPKa-PGC-1α pathway.

Methods and Results: We knocked down AMPKa and PGC-1α by small interfering RNA. We assessed mitochondrial mitochondrial potential, mitochondrial DNA (mtDNA) content and ATP production by JC-1 method, rt-pcr and assay kit, respectively. Our results showed that curcumin could significantly increased the mitochondrial biogenesis capacity in both cartilage explants and primary cultured chondrocyte induced by AGEs, correlated with concomitant induction of phosphorylation of AMPKa and PGC-1a. In parallel, curcumin significantly increased the expression of NRF2 and TFAM decreased by AGEs. In addition, curcumin attenuated AGEs-induced mitochondrial ROS generation, increased SOD2 expression, attenuated chondrocyte catabolic responses to AGEs such as release of NO, MMP-3 and MMP-13. However, curcumin had decreased capacity to increase each of those same effect readouts in AGEs treated AMPKa -siRNA or PGC-1α-siRNA chondrocyte.

Conclusions: In conclusion, curcumin might maintain AGEs-decreased mitochondrial function via AMPKa-PGC-1α pathway to limit oxidative stress, thus leading to protecting cartilage matrix from degradation.

Introduction

Osteoarthritis (OA) is an aging-related chronic inflammatory joint disease, primarily characterized by excessive degradation of the components of the extracellular matrix [1–2]. Mitochondrial dysfunction of human articular chondrocytes is considered a hallmark of cartilage degradation and OA pathogenesis, even though chondrocytes are not enriched in mitochondria[3, 4]. OA chondrocytes demonstrate decreases in mitochondrial biogenesis, OXPHOS and cellular ATP levels, and increases in mitochondria-mediated oxidative stress and apoptosis, reduced antioxidant capacity, and enhanced catabolic responses to inflammatory cytokines[5]. However the reason for the age-related mitochondrial dysfunction in OA was unknown exactly. As we know, the most dramatic age-related change was the accumulation of AGEs[6]. Once AGEs are formed, they remain in the tissue until the protein involved is degraded, which renders articular cartilage tissue increasingly brittle and thus more prone to mechanical damage. In addition to affecting the mechanical properties of tissue, increased AGEs decrease the synthesis of proteoglycans and collagens in articular cartilage chondrocytes [7–9]. In primary rabbit chondrocytes, we have found that AGEs could induced chondrocyte apoptosis and decrease the levels of mitochondrial $\triangle\Psi$and ATP production[10]. These results indicated that AGEs could induce chondrocyte

mitochondrial dysfunction, thus leading to the increased oxidative stress, inflammation and matrix catabolism. This fact may help to design a new therapeutic strategy based on targeting of mitochondrial dysfunction for a large number of patients who suffer from OA.

Curcumin is the main component of turmeric, also known as the Curcuma longa, which belongs to the ginger family, Zingiberaceae[11].Over recent decades, curcumin has been demonstrated to be potential as a treatment agent for osteoarthritis. Its efficacy in reducing pain, physical function, and quality of life has been demonstrated in many clinical trials and animal models [12–14]. Vitro studies demonstrated that curcumin could prevent the apoptosis of chondrocytes, suppress the release of proteoglycans and metal metalloproteases and expression of cyclooxygenase and inflammatory cytokines in chondrocytes. These were achieved by blocking the activation of NF-κB system and oxidative stress in the chondrocytes[15, 16]. Furthermore, some research groups have independently demonstrated that curcumin is able to elicit mitochondrial biogenesis in different mammalian tissues mainly through the induction of the AMPK/PGC-1a-related signaling pathway[17]. Data obtained from those studies would be reinforced by the quantification of mtDNA, as well as the investigation regarding the involvement of other regulators of mitochondrial biogenesis, such as the NRF1 and TFAM. In view of the revealed relationship between curcumin and AMPKa, mitochondrial biogenesis, the beneficial effects of the agent curcumin tested in the OA clinical trials and animal models are conceivably in part due to AMPKa activation, thus promoting the mitochondrial biogenesis in chondrocyte, which warrant further investigation.

In our previous study, we have demonstrated that curcumin could inhibit AGEs-induced upregulation of TNF-a and MMP-13 in rabbit chondrocytes[18]. However, the precise mechanism was not exactly clear. In this paper, we tested whether curcumin could protect the AGEs-induced mitochondrial dysfunction via upregulating AMPK/PGC-1α-related signaling pathway, thus leading to inhibit the catabolic responses.

Materials And Methods

Cell and Materials

Normal human chondrocyte were purchased from iCell Bioscience Inc(Shanghai, CHINA). AGEs were purchased from BioVision Inc.(Milpitas CA, USA).Curcumin, the selective AMPK activator AICAR and AMPK inhibitor Compound C were purchased from Sigma (St Louis, MO, USA). Recombinant human MMP-3, MMP-13 quantikine enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems, Inc. (Minneapolis, MN). Human small interfering RNAs (siRNAs) for AMPKa, PGC-1α and the control siRNA were from Invitrogen. JC-1, ATP production assay kit and NO release assay kit were from Beyotime Institute of Biotechnology, China. All other reagents were of highest purity available.

Rabbit cartilage explants preparation

Rabbit articular cartilage from the metacarpophalangeal joints of 5-week-old male rabbits was dissected into 25 cm^3 discs. The cartilage was then incubated in DMEM(supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin) for 30 min at 37°C and 5% CO_2 in a petri dish. After that, the

cartilage was incubated in refreshed medium for 24h to ensure sterility. Then 30-35 mg portions of cartilage were placed into wells of 24 well-plates and cultured at 37°C and 5% CO^2 in DMEM. The study was approved by the Ethics Committee of Gan Su Province Hospital(No.2020092).

Hematoxylin and eosin (H&E)

Cartilage samples were fxed in 4% paraformaldehyde and embedded in wax, then were then cut into 5 mm thick sections perpendicular to the articular cartilage surface. The sections were evaluated for tissue morphology by staining with Hematoxylin and eosin (H&E).

ATP Bioluminescence Assay

The ATP levels were evaluated using an ATP bioluminescence assay kit. This technique is well established and uses the ATP dependence of the light omitting luciferase-catalyzed oxidation of luciferin for the measurement of extremely low concentrations of ATP.

Determination of mitochondrial membrane potential

Mitochondrial membrane potential was assessed by using JC-1. The decline in mitochondrial membrane potential will lead to leakage of JC-1 from mitochondria. JC-1was added to cell cultures for 30 min at room temperature, and then the cells were washed twice with phosphate-buffered saline. The fluorescence of JC-1 was observed using a confocal laser scanning microscope (Leica TCS SP2, German) with excitation at 488nm and emission at 510nm.

Measurement of the release of NO, MMP-3、MMP-13 and ROS production

Levels of NO and MMP-3 and MMP-13 in conditioned media were assayed using the Griess reaction method and ELISA, respectively. The determination of ROS was based on the oxidation of 2', 7'-dichlorofluorescein diacetate (DCFH-DA) by peroxide. In brief, cells were washed and incubated with DCFH-DA for 20 min at 37 °C in the dark. Cells were then washed twice and harvested in PBS. The fluorescence of DCFH was detected with a flow cytometer (FAC-SCalibur; BD Biosciences, San Jose, CA, USA) with excitation at 488 nm and emission at 530 nm.

SiRNA of PGC-1α and AMPKα in chondrocytes

Small interfering RNA (siRNA) construction and transfection PGC-1α and AMPKα siRNA assays were performed using Silencer Select Predesigned siRNA. Chondrocytes were grown to 30% confluence before transfection, according to the manufacturer's protocol. Transfection complexes were prepared in Opti-MEM serum-free medium by mixing 1.5 mL of Oligofectamine and 2 mM of siRNA. Forty-eight hours after siRNA transfection, cells were subjected to AGEs or curcumin, and subsequently analyzed for the expression of the related indexes. Level of expression of PGC-1α or AMPKα was examined by western blot analysis. Densitometry was performed using the Image J program (National Institutes of Health).

Western Blot Analysis

After the indicated treatments, cell extracts were prepared in phosphate-buffered saline that contained 25 μl of protease inhibitor cocktail. Aliquots of the cell extract were separated by sodium dodecyl sulfate polyacrylamide gel electro-phoresis, and western blot analyses were carried out using the indicated antibodies. Antibody binding was detected by enhanced chemiluminescence. The bands were scanned and densitometrically analyzed using an automatic image analysis system (Alpha Innotech Corp., San Leandro, Calif., USA). These quantitative analyses were normalized to GAPDH.

Statistical analysis

All values are expressed as mean ± SD. Data were analyzed using a one-way or two-way ANOVA followed by Newman-Student's t-test. $P<0.05$ was considered significant.

Results

Curcumin attenuates AGEs-induced mitochondrial dysfunction in cartilage explants and primary cultured chondrocyte

We first examined the mitochondria membrane potential as it related to the capacity of cells to generate ATP by oxidative phosphorylation. We treated the cultivated rabbit cartilage with AGEs(300μg/ml) for 7d and then stained with H&E.The H&E staining analysis demonstrated that in the normal control group, the cartilage matrix was uniform, intact and jelly-like, showed middle or depth degree staining; the chondrocytes were small and flatted; and the matrix in the AGEs-treated cartilage group showed fibrosis-like changes in different degrees. In addition, the chondrocytes were apoptosis in varying degrees identified by nuclear fragmentation and karyolysis. In the curcumin(20μM) group, the matrix fibrosis was much more lightened, and there were less small fissures and vacuoles after cartilage matrix absorption(Figure 1A). The articular chondrocytes of each group were isolated and extracted, and the mitochondrial membrane potential was detected by JC-1 staining. The red/green fluorescence ratio was calculated for the mitochondria membrane potential, and the results showed that mitochondria membrane potential(Figure1B), mitochondrial DNA content(Figure1C) and ATP production(Figure1D) were significantly decreased treated with AGEs compared with the control, however which were significantly reversed by pretreated curcumin before added AGEs.

We confirmed the protective effect of curcumin in primary cultured human chondrocyte. As shown in Figure2, treatment of chondrocytes with AGEs significantly reduced the mitochondria membrane potential, mitochondrial DNA contentand ATP production, increased the chondrocyte apoptosis assayed by TUNEL, as well as, all of these were reversed by curcumin(5,10, 20μM)(Figure2A,B,C,D). In addition, the promotion effect of curcumin on AGEs-decreased mitochondria membrane potential, ATP production and mitochondrial DNA content and increased-apoptosis were much less in either AGEs-treated PGC-1α or AMPKa siRNA chondrocyte(Figure2A,B,C,D).

Effect of curcumin on AGEs induced AMPKα and PGC-1α expression in chondrocyte

Duing to the revrsed effect of siRNA AMPKα/PGC-1α in the protective effect of curcumin on AGEs-induced mitochondrial dysfunction, next we directly evaluated the effect of curcumin on AGEs induced PGC-1α and AMPKα expression. Western blot analysis indicated that both curcumin and AICAR(a highly selective AMPK chemical activator) clearly increased the protein level of phosphorylated AMPKα (Figure 3A) but not the total AMPK(data were not shown here). Since AMPKα appears to be involved in PGC-1α expression in many cells including chondrocyte, next the effect of curcumin on AGEs induced PGC-1α activation was detected. The results showed that curcumin (20μM) increased PGC-1α protein level response to AGEs (300 μg/ml)(Figure3B). To further confirm the role of AMPKα activation in mediating the promotion effect of curcumin on PGC-1α expression, next we used Compound C (an AMPK specific inhibitor) to explore the underlying relationship. The results showed that both curcumin (20μM) and AICAR(10μM) could up-regulate the protein levels of AGEs induced PGC-1α (Figure3B), however, the promotion effects of curcumin on the protein levels of AGEs induced PGC-1α were partially counteracted in pretreated with Compound C(50μM)(Figure 3C).

In order to explore the downstream mechanism involved in the PGC-1α pathway on mitochondrial biogenesis, next we detect the effect of curcumin on AGEs induced protein level of transcription factor A (TFAM) and nuclear respiratory factors 2(NRF2). The results showed that both expression of TFAM and NRF2 were significantly reduced (Figure 3D-I) in AGEs treated chondrocytes, but increased by curcumin (20μM) pretreated before incubation with AGEs. In addition, either PGC-1α siRNA or AMPKα siRNA reduced the promotion effect of curcumin on TFAM and NRF2 expression (Figure 3D-I).

Curcumin attenuates AGEs-induced ROS generation via AMPKa/PGC-1α

It is well-known that mitochondria consume most of the cellular oxygen and produce reactive oxygen species (ROS) as by products. Various studies have shown that downregulation of superoxide dismutase 2 (SOD2) and upregulation of ROS following mitochondrial dysfunction contribute to the pathogenesis of OA. In addition to mitochondrial biogenesis, we also examined the ROS production. As shown in Figure 4, expression of SOD2 was significantly reduced (Figure 4B) in AGEs treated chondrocytes followed by elevating levels of ROS production (Figure 4A); but increased by curcumin (20μM) pretreated before incubation with AGEs. As expected, either PGC-1α siRNA or AMPKα siRNA reduced the promotion effect of curcumin on SOD2 expression (Figure 4B) and the elimination effect of ROS (Figure 4A).

Curcumin attenuates chondrocyte catabolic responses to AGEs via AMPKa/PGC-1α

The results showed the release of NO (Figure 4E), MMP-3 (Figure 4C) and MMP-13 (Figure 4D) induced by AGEs were significantly decreased in curcumin (20μM) treated chondrocyte. In addition, when blocked AMPKa or PGC-1α with siRNA before curcumin treatment, the protective effect of curcumin in chondrocyte was significantly dismissed(Figure 4D-E).

Discussion

In the present study, we found that curcumin significantly promoted mitochondrial biogenesis capacity reduced by AGEs in both chondrocyte and rabbit cartilage explants, in addition, correlated with concomitant induction of phosphorylation of AMPKa and PGC-1a.

Mitochondrial biogenesis, *i.e.* the generation of new mitochondria, is a complex event depending on both mitochondrial and nuclear genomes to occur in mammalian cells including chondrocyte. We found that mitochondrial biogenesis capacity is significantly reduced in AGEs-treated chondrocyte, indicated by deceased mitochondrial DNA content and reduced intracellular ATP level, all of which were improved by curcumin. A growing body of evidence indicates that curcumin triggers mitochondrial biogenesis in both vitro and vivo experimental models. It have found that curcumin induced an increase in the number of mitochondria in 3T3-L1 and primary white adipocytes[19]. In vivo experimental models, studies have found that curcumin alone potentiated the effects of training regarding the upregulation of the components of the respiratory chain. Curcumin also amplified the effects of exercise training upon mitochondrial DNA (mtDNA) in both muscles, demonstrating the ability to induce mitochondrial biogenesis in vivo[20]. However, the exact mechanism by which curcumin exerts this effect remains to be completely understood. Mitochondrial biogenesis is stimulated under increased energetic needs by a signaling pathway involving PGC-1a as a major modulator. PGC-1a is a target of the NAD+-dependent deacetylase sirtuin 1 (SIRT1) during the control of mitochondrial biogenesis[21, 22]. Furthermore, AMPK is able to modulate the levels of NAD+, causing SIRT1 activation, a protein that activates PGC-1a through deacetylation[23]. In this context, the AMPK/PGC-1a signaling pathway orchestrates mitochondrial function and dynamics and also participates in the maintenance of the redox environment in mammalian cells.

Next, we directly detect the effect of AMPKa/PGC-1α pathway on the mitochondrial function. In the present study, we demonstrated that curcumin could up-regulated the expression of AMPKa and PGC-1α stimulated with AGEs in chondrocyte. Moreover, AMPKa activation was required for the promotion effect of curcumin on AGEs-induced PGC-1α expression. In addition, AMPKa-mediated PGC-1a pathway was participated in the chondroprotection of curcumin. However, the underlying mechanism of the promotion effect of curcumin on AMPKa activity in chondrocyte was exactly unknown in the present study. A previous study has put forward that curcumin could increases cyclic adenosine monophosphate (cAMP), which activates PKA and increase activation of AMPK in skeletal muscle and subsequently improve mitochondrial biogenesis [24]. Thus, the cAMP signaling pathway might play an important role in the regulation effect of curcumin on AMPKa, which needed to further explore.

In order to further understand how AMPK/PGC-1a contributed curcumin chondrocyte-protective effect, we detected the downstream targets of AMPKa /PGC-1a. PGC-1a acts upstream of NRF1 and NRF2, activating these transcription factors, in addition to upregulating the estrogen-related receptora, leading to augmented expression of nuclear DNA codifying mitochondrial proteins. The expression of the regulators known as TFAM and mitochondrial transcription factors B1 and B2 is augmented, triggering the expression of specific mitochondrial RNA associated with mitochondrial biogenesis. TFAM is involved in the transcription and replication of mitochondrial DNA (mtDNA) and also participates in the maintenance

of mtDNA homeostasis[25, 26]. Thus, next we detected the expression of NRF2 and TFAM, which are responsible for mitochondrial biogenesis and maintenance of mtDNA copy number, respectively. Our results showed that curcumin could promoted the expression of NRF2 and TFAM, however, had decreased capacity to increase each of these same readouts in either AMPKa or PGC-1α knockdown chondrocytes.

Mitochondria dysfunction leads to elevated levels of ROS, which promotes cartilage degradation directly by cleaving collagen and aggrecan and indirectly by activating matrix metalloproteinases [27].Next, we further studied the role of AMPKa/ PGC-1α pathway in anti-catabolic and antioxidant properties of curcumin in chondrocyte. Nrf2 has been reported to play a significant role in regulating inflammation and antioxidative stress via the HO-1-SOD2 signaling axis[28]. Furthermore, Nrf2 is an important factor involved in reducing inflammation and oxidative stress in many inflammation-related diseases, including cancer and arthritis, more importantly, previous studies have found that Nrf2 activation could up-regulated Nrf2-dependent ARE factors including SOD2 in temporomandibular joint chondrocytes[28]. These results indicated that in this study, the promoted effect of curcumin on SOD2 could be due to the upregulated expression of Nrf2 in AGEs-treated chondrocyte. mitochondrial dysfunction and reduced activity of SOD2 are associated with an increase in mitochondrial-derived ROS and are in part responsible for the increase in chondrocyte ROS. Excess levels of these ROS not only cause oxidative-damage but, perhaps more importantly, cause a disruption in cell signaling pathways that are redox-regulated, including Akt and MAP kinase signaling that plays a role in cartilage degradation as well as chondrocyte cell death[27, 29–30]. In previous studies found that Nrf2 silencing distinctly abolished the curcumin induced suppression of inflammatory mediators and the enhancement of cartilage anabolic factor expression in human temporomandibular joint chondrocytes. However, it need to perform experimental designs involving knockdown of Nrf2 in order to investigate whether this transcription factor is mediating mitochondrial biogenesis control in chondrocyte for curcumin. In parallel, in our results, Our results showed curcumin could attenuate AGEs-induced ROS generation and increased SOD2 expression; in addition, curcumin could attenuate chondrocyte catabolic responses to AGEs such as release of NO, mmp-3 and mmp-13, however, these effects of curcumin were significantly reversed by AMPKa or PGC-1α siRNA, theses results suggested that AMPKa/PGC-1α were involved in the inhibiton effect for curcumin of cartilage degradation induced by AGEs.

Conclusions

In conclusion, our researches have independently demonstrated that curcumin is able to elicit mitochondrial biogenesis decreased by AGEs in chondrocyte mainly through the induction of the AMPKa/PGC-1a-related signaling pathway(Fig. 4F). As we know, mitochondrial dysfunction is the main culprit in a myriad of diseases including OA. This fact opens a new therapeutic window based on targeting mitochondrial dysfunction for treatment of OA. Our results provided better understand the role of curcumin as an inducer of mitochondrial biogenesis for the treatment of OA.

Declarations

Data availability statement

All the data are available upon reasonable request.

Statement of Ethics

This study was approved by the Ethics Committee of Gan Su Province Hospital (No.2020092), and performed according to the guidelines of the animal ethical committee for use of experimental animals in China.

Disclosure Statement

The authors state that there is no conflict of interest.

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Author Contributions

S.W. and Q.Y. designed experiments. Q.Y. ,T.J. and Y.S. performed experiments and edited the manuscript. Z.C. analyzed experimental data. S.W. reviewed the manuscript. All authors read and approved the manuscript.

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Figures

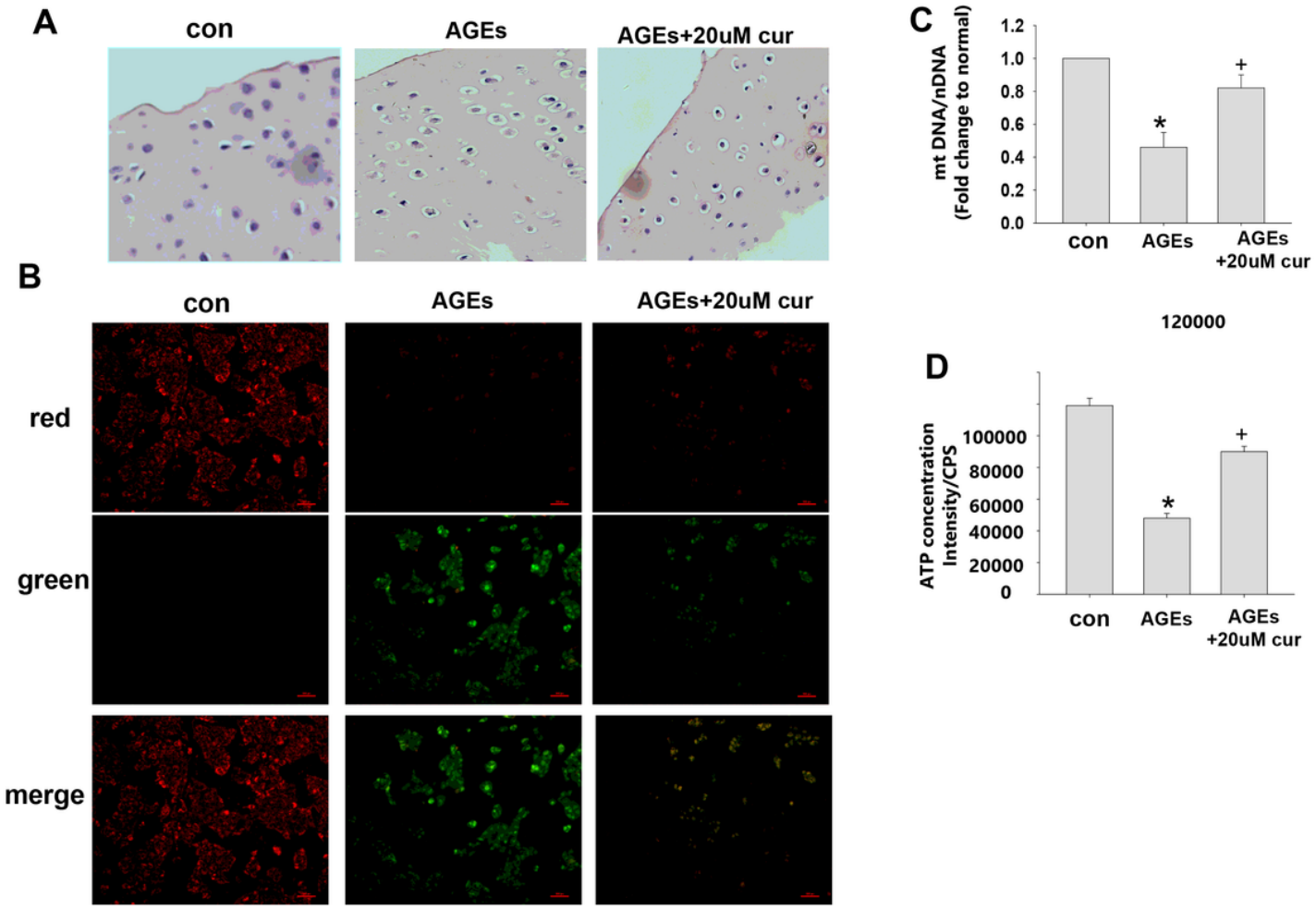

Figure 1

Figure 1

Curcumin attenuates AGEs-induced mitochondrial dysfunction in cartilage explants and isolated chondrocyte.The cartilage discs (30-35 mg) were co-treated with 300μg/ml of AGEs and for 7days. The articular chondrocytes of each group were isolated and extracted. A Cartilage sections were stained with H&E staining(×400). B The mitochondrial membrane potential was detected by JC-1 staining in isolated chondrocyte. C Mitochondrial DNA content was determined by qPCR. D Assayed mitochondrial ATP production. Data are the mean±SD. *p<0.05 compared with the control, +p<0.05 compared with AGEs treratment; n =3.

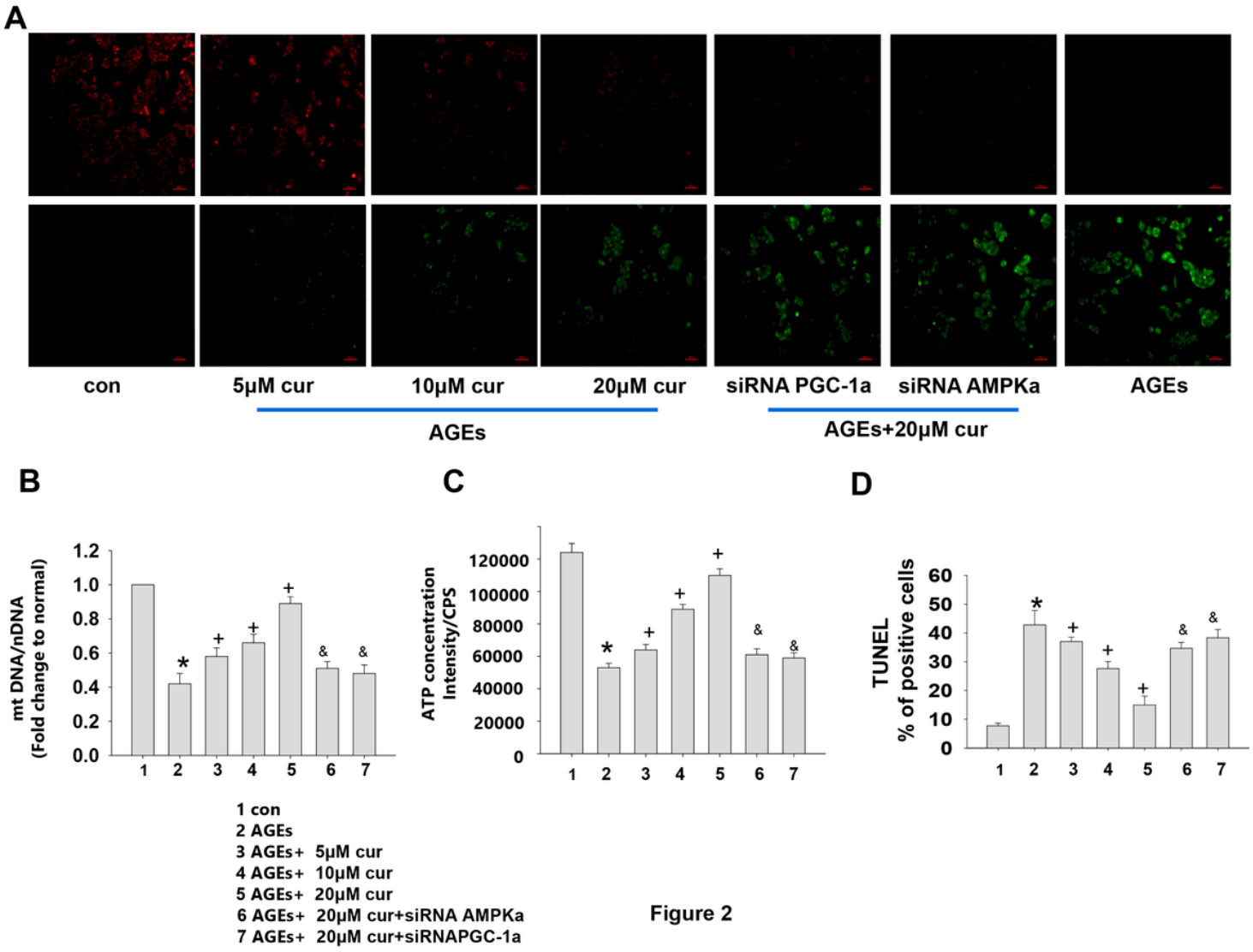

Figure 2

Curcumin attenuates AGEs-induced mitochondrial dysfunction in primary cultured chondrocytes. Cells were transfected with AMPKa siRNA or PGC-1α siRNA and the nontarget control for 48h, and then treated with different concentration curcumin (5,10,20μM) for an additional 2h before stimulation with AGEs (300μg/ml) for 24h. A Mitochondrial membrane potential (△Ψm) was stained by the JC-1. B Mitochondrial DNA content was determined by qPCR. C Assayed mitochondrial ATP production. D The chondrocyte apoptosis were assayed by TUNEL. Data are the mean±SD. *p<0.05 compared with the control, +p<0.05 compared with AGEs treratment; &p<0.05 compared with AGEs plus 20μM curcumin treatment. n =3.

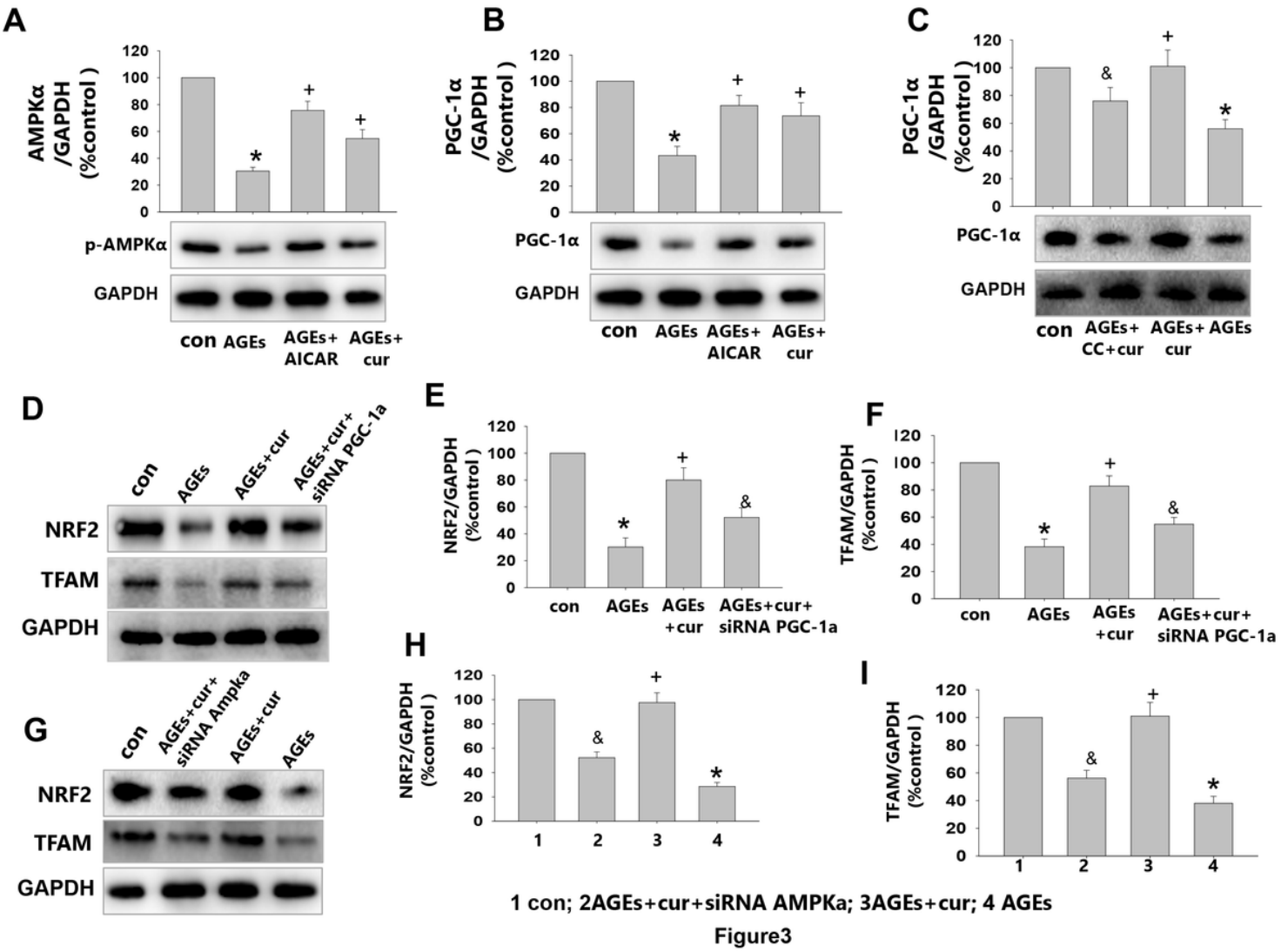

Figure 3

Effect of curcumin on AGEs induced AMPKα and PGC-1α expression in chondrocyte. Cultured chondrocytes were transfected with AMPKα siRNA or PGC-1α siRNA and the nontarget control for 48h, and then treated with AICAR(10μM) or 20μM curcumin for an additional 2h before stimulation with AGEs (300μg/ml) for 24h. Cultured chondrocytes were pretreated with Compound C(50μM) for 0.5h and then treated with 20μM curcumin for an additional 2h before stimulation with AGEs (300μg/ml) for 24h. A Westernblot of the expression of p-AMPKa. B,C expression of PGC-1α assayed by westernblot. D-I Westernblot of the expression of NRF2 and TFAM. Data are the mean±SD. *$p<0.05$ compared with the control, +$p<0.05$ compared with AGEs treratment; &$p<0.05$ compared with AGEs plus 20μM curcumin treatment. n =3.

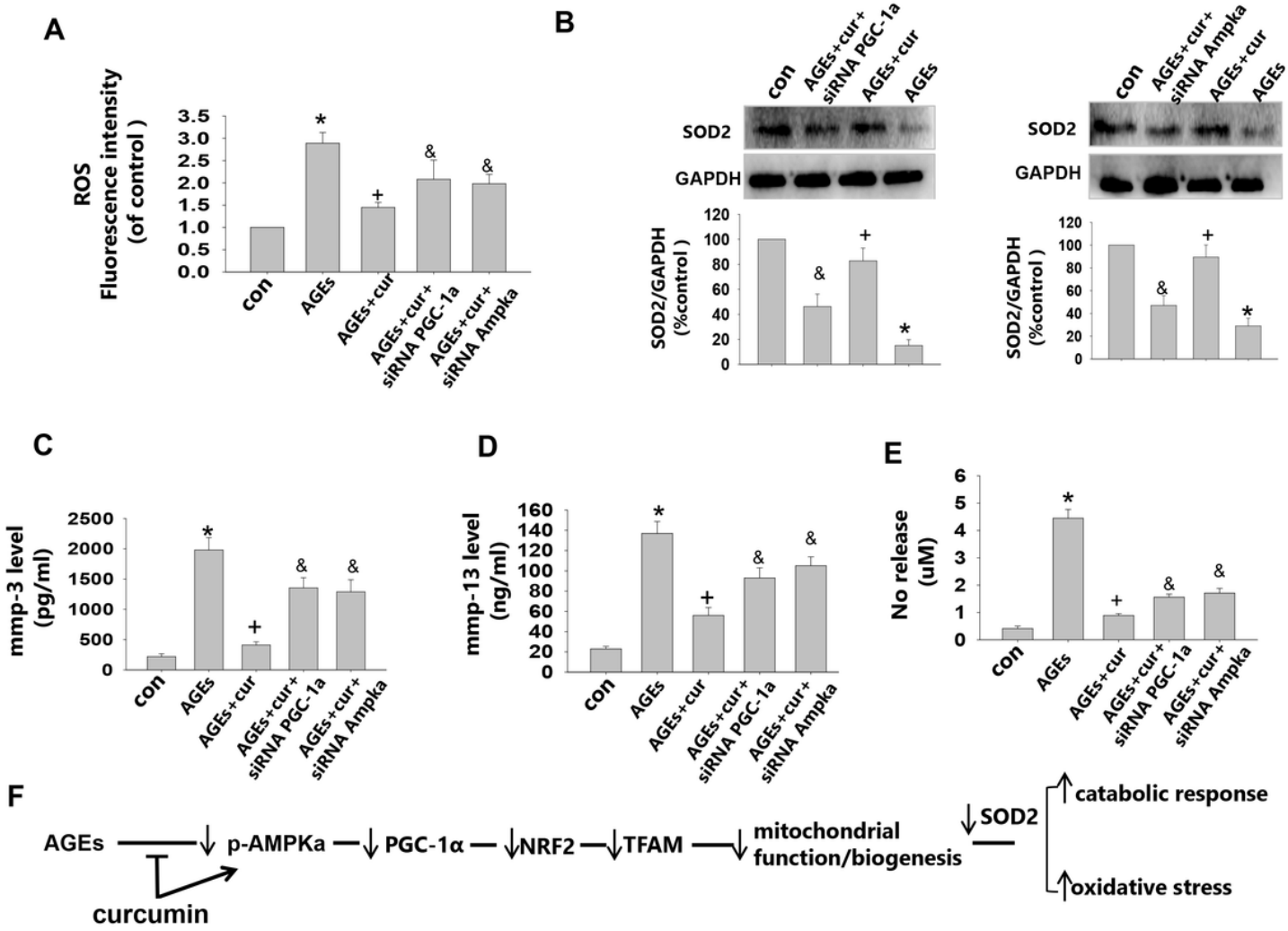

Figure 4

Figure 4

Curcumin attenuates AGEs-induced ROS generation and chondrocyte catabolic responses to AGEs via AMPKa/PGC-1α. Effect of curcumin on AGEs induced AMPKα and PGC-1α expression in chondrocyte. Cultured chondrocytes were transfected with AMPKα siRNA or PGC-1α siRNA and the nontarget control for 48h, and then treated with 20μM curcumin for an additional 2h before stimulation with AGEs (300μg/ml) for 24h. A Fluorescence intensity was expressed as a percentage of increased intensity to quantify ROS levels. B Western blot of the expression of SOD2. C Release of nitric oxide (NO), matrix metalloproteinase 3 (MMP-3)(D) and MMP-13(E)were assayed by ELISA kits. F Outlining of proposed mechanism for AMPKa/PGC-1α pathway mediating the chondroprotection of curcumin in AGEs-treated chondrocyte. Data are the mean±SD. *p<0.05 compared with the control, +p<0.05 compared with AGEs treratment; &p<0.05 compared with AGEs plus 20μM curcumin treatment. n =3.