Original Paper

Globular Adiponectin Attenuated H₂O₂-Induced Apoptosis in Rat Chondrocytes by Inducing Autophagy Through the AMPK/mTOR Pathway

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Key Words

Adiponectin • Chondrocytes • Autophagy • Apoptosis • AMPK

Abstract

Background/Aims: Chondrocyte apoptosis is closely related to the development and progression of osteoarthritis. Global adiponectin (gAPN), secreted from adipose tissue, possesses potent anti-inflammatory and antiapoptotic properties in various cell types. This study aimed to investigate the role of autophagy induced by gAPN in the suppression of H₂O₂-induced apoptosis and the potential mechanism of gAPN-induced autophagy in chondrocytes.

Methods: H₂O₂ was used to induce apoptotic injury in rat chondrocytes. CCK-8 assay was performed to determine the viability of cells treated with different concentrations of gAPN with or without H₂O₂. Cell apoptosis was detected by flow cytometry and TUNEL staining. Mitochondrial membrane potential was examined using JC-1 fluorescence staining assay. The autophagy inhibitors 3-MA and Bafilomycin A1 were used to treat cells and then evaluate the effect of gAPN-induced autophagy. To determine the downstream pathway, chondrocytes were preincubated with the AMPK inhibitor Compound C. Beclin-1, LC3B, P62 and apoptosis-related proteins were identified by Western blot analysis. **Results:** H₂O₂ (400 μM)-induced chondrocytes apoptosis and caspase-3 activation were attenuated by gAPN (0.5 μg/mL). gAPN increased Bcl-2 expression and decreased Bax expression. The loss of mitochondrial membrane potential induced by H₂O₂ was also abolished by gAPN. Furthermore, the antiapoptotic effect of gAPN was related to gAPN-induced autophagy by increased formation of Beclin-1 and LC3B and P62 degradation. In particular, the inhibition of gAPN-induced autophagy by 3-MA prevented the protective effect of gAPN on apoptosis induced by H₂O₂. Moreover, gAPN increased p-AMPK expression and decreased p-mTOR expression. Compound C partly suppressed the expression of autophagy-related proteins and restored the expression of p-mTOR suppressed by gAPN. Thus, the AMPK/mTOR pathway played an important role

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in the induction of autophagy and protection of H_2O_2 -induced chondrocytes apoptosis by gAPN. **Conclusions:** gAPN protected chondrocytes from H_2O_2 -induced apoptosis by inducing autophagy possibly associated with AMPK/mTOR signal-pathway activation.

Introduction

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Osteoarthritis (OA) is a degenerative joint disease characterized by the progressive loss of articular cartilage, destruction of cartilage matrix, sclerosis of the subchondral bone, and formation of osteophyte [1]. OA occurs as the result of a variety of factors, including ageing, overload stress, and oxidative stress, and alters the physiological and biomechanical environment of joints [2, 3]. Reported studies have well demonstrated that cartilage matrix degradation and chondrocyte apoptosis induced by certain proteases and apoptotic factors are the two crucial pathogenic events occurring in OA [2-4].

Ageing-related oxidative stress has been considered to be a major causative factor for OA development [5, 6]. Hydrogen peroxide (H_2O_2) formed from a superoxide anion through superoxide dismutase, which produces reactive oxygen species (ROS), is closely associated with the induction of chondrocyte apoptosis *in vivo* [5] and *in vitro* [6]. H_2O_2 alters mitochondrial membrane permeability that allows for the release of cytochrome c into the cytoplasm [7] and is involved in the activation of caspase-3, one of the key mediators in apoptotic signaling pathways, thus ultimately results in cell apoptosis.

Autophagy, another type of self-destructive process from apoptosis, is a major cellular pathway essential for cell survival [8]. This process is crucial in the development and differentiation of cells and the maintenance of cytoplasmic organelle turnover [9]. Emerging evidence has shown that autophagy provides an efficient cellular defense mechanism and plays a cytoprotective role in various stressful conditions, including starvation, mitochondrial injury, and pathogen infection [10-12]. Growing evidence also highlights that the cytoprotective function of autophagy is mediated at least in part by the negative modulation of apoptosis [11-13]. For example, autophagy activation leads to the negative modulation of Bax [14, 15]. Beclin-1 mediated autophagy is also known to inhibit caspase-8 activity, thereby preventing apoptosis [16, 17]. Although autophagy is recognized as a critical role in cell death and survival, little is known about the mechanism of autophagy in OA. Paloma et al. found that the activation of autophagy significantly protected human chondrocytes against mitochondrial dysfunction. Caramés et al. [18] demonstrated that aged and OA articular cartilage were associated with the reduced expression of ULK1, Beclin-1, and LC3-II, suggesting that autophagy may play a protective role against chondrocyte death.

Adiponectin (APN) predominantly secreted from adipose tissue shows strong abilities in the regulation of various biological responses, including lipid and glucose metabolism [19] and also possesses potent anti-inflammatory, antiatherogenic, and antiapoptotic properties [20-22]. Apart from its well-characterized role in fat tissue metabolism and insulin resistance [23], APN played an important role in the survival and proliferation of the several types of cells. Much of the beneficial effect was attributed to the antiapoptotic actions of APN [24-26]. Meanwhile, a growing number of evidence suggests that autophagy negatively regulates the apoptotic process in various cellular conditions [11], adiponectin possesses potent antiapoptotic properties, and the effect of adiponectin on chondrocytes autophagy and its role in the suppression of H_2O_2 -induced chondrocytes apoptosis is not explored yet.

AMP-activated protein kinase (AMPK), a conserved energy sensor in eukaryotic cells, is well recognized as a key mediator of various biological responses induced by adiponectin [27]. In addition to the established role of AMPK in metabolism, the AMPK signaling pathway has been intensively studied in recent years because it coordinates metabolism with both apoptosis and autophagy [28]. AMPK controls autophagy through the mammalian target of rapamycin (mTOR) and Unc-51-like kinase 1 (ULK1) signaling [29]. mTOR, a conserved Ser/Thr protein kinase, is a potent inhibitor of autophagy. In addition, chondrocyte autophagy is known to be a constitutive homeostatic mechanism in articular cartilages [30], thus can be

promoted by AMPK signaling [31, 32] through mTOR suppression and the cartilage-specific genetic deletion of mTOR [33]. Therefore, we assumed that the underlying autophagic activation effect of APN on rat chondrocytes against chondrocyte apoptosis induced by H_2O_2 may be related to AMPK/mTOR signaling pathways.

Thus, we investigated the role of autophagy induced by globular adiponectin (gAPN) to better understand the mechanisms underlying the suppression of apoptosis by adiponectin in H_2O_2 -treated chondrocytes. In the present study, we demonstrated that gAPN activates the autophagy process in rat chondrocytes that is implicated in the suppression of H_2O_2 -induced apoptosis. Furthermore, we identified that AMPK/mTOR signaling pathway plays a critical role in the gAPN-induced expression of proteins related to autophagy.

Materials and Methods

Collection, isolation, and culture of rat articular chondrocytes

All experiments were approved by the Ethical Committee of Nanjing Medical University. The cartilage of 1-week-old Sprague-Dawley rats was harvested and minced before being digested with 0.25% trypsin (Gibco, USA). The trypsin was then removed, and the cartilage was washed with phosphate-buffered saline (PBS) (Gibco, USA) three times. Subsequently, 0.2% collagenase II (Gibco, USA) was added for digestion at 37 °C for 4–5 h. A 200 μ m mesh strainer was used to filter the above solution, and the cells were collected by centrifugation. The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1%–2% penicillin/streptomycin (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China) and incubated with 5% CO_2 at 37 °C. The F1 generation of chondrocytes was used for the experiments in this report.

Determination of experimental concentrations of H_2O_2 and gAPN by CCK-8 assay

1) The rat chondrocytes of the F1 generation were prepared into 1×10^5 /mL single-cell suspension that was seeded into 96-well plate, 10^4 cells in each well. After the cells adhered to the wall, the cells were starved for 24 h by adding 100 μ L serum-free culture medium. Five wells were randomly selected, and the culture media containing different concentrations of H_2O_2 (0, 200 μ M, 400 μ M, 600 μ M, 800 μ M) (Sea Derun Pharmaceutical, Beijing China) were added to treat the cells for 6 h. Subsequently, the cells were incubated at 37 °C for 30 min by the addition of 10 μ L CCK-8 solution. Absorbance was measured at 450 nm (BioTek, Vermont, USA).

2) The cells were starved for 24 h using the above-mentioned method. Then five wells were randomly selected. The gAPN (Biovision, USA) of different concentrations (0, 0.1, 0.5, 1, and 2 μ g/mL) was added into each well to treat the cells for 24 h, followed by 400 μ M H_2O_2 treatment for 6 h. One group was randomly selected as the control group in which no treatment was performed. Absorbance was measured by CCK-8 assay at 450 nm.

Cell apoptosis detection by TUNEL/DAPI staining

The chondrocytes of the F1 generation were seeded into 24-well plate. The cells in sub-aggregation state were starved for 24 h by using serum-free culture medium. After the cells in each treatment were dried, they were fixed in 4% paraformaldehyde (GuGe Biotechnology, Wuhan, China) for 1 h. Then, the cells were sealed for 10 min using the confining liquid (3% H_2O_2 , dissolved in methanol). After transparentization for 2 min using 0.1% Triton X-100 (Biosharp, Hefei, China), the cells were sealed for 1 h in TUNEL reaction mixture (Roche, Switzerland) at 37 °C in dark box. The cells were incubated with DAPI (Beyotime Biotechnology, China) for 5 min and were observed under an inverted fluorescence microscope (Olympus, Tokyo, Japan) at 2009 magnification. Randomly selected fields were photographed, and representative pictures were chosen to count apoptotic cells and total cells. The rate of TUNEL-positive cells in each field was calculated.

Detection of cell apoptosis rate by flow cytometry: Annexin V/PI double staining

The rat chondrocytes of the F1 generation were inoculated to six-well plate. After the cells adhered to the wall, they were divided into different treatment groups. When the treatment was over, the cells were collected and centrifuged for 5 min at 1500 r/min. The supernatant was discarded and then washed twice

with PBS buffer. The cells were resuspended in 500 μ L of binding buffer, and FITC-labeled Annexin V (20 μ g/mL) and PI (50 μ g/mL) (BD PharMingen, USA) were added, 5 μ L each. The reaction proceeded in the dark for 15 min, and cell apoptosis was detected by flow cytometry (BD FACS ARIA II, SORP, USA).

Measurement of mitochondrial membrane potential

The fluorescent probe, JC-1 (Beyotime Biotech, Nanjing, China), was employed to measure the mitochondrial membrane potential according to the manufacturer's instructions. After the indicated treatments, the cells cultured in 24-well plates (5×10^4 cells per well) were incubated with JC-1 staining solution (5 μ g/mL) for 20 min at 37 °C. Cells were then rinsed twice with JC-1 staining buffer, and fluorescence signals were measured by spectrofluorometry (Nikon Eclipse Ti, Japan) with 514 nm for excitation and 529 nm for the emission of green (monomer form) fluorescence, 585 nm for excitation, and 590 nm for the emission of red (aggregate form) fluorescence. The mitochondrial membrane potential of chondrocytes was calculated as the fluorescence ratio of red to green.

Western blot analysis

After each treatment, the cells were washed twice by PBS buffer. Pre-cooled cell lysis buffer was added. The reaction proceeded on ice for 5 min, and the cells were scraped off. The products of lysis were centrifuged for 15 min at 4 °C at 14000 g. The supernatant was collected to determine protein concentration by bicinchoninic acid (BCA) assay (Beyotime, Haimen, China). The equal volume of protein sample was subjected to SDS-PAGE (10% SDS). The proteins were transferred to the nitrocellulose membrane after electrophoresis, which was sealed with 5% defatted milk powder containing TBST (0.05% Tween-TBS) (Biosharp, Hefei, China) at room temperature for 1 h. Subsequently, goat anti-rabbit antibodies (p-AMPK, p-mTOR, Bcl-2, Bax, Cleaved caspase-3, Cleaved PARP, Beclin-1, LC3B, and P62) (Cell Signaling Technology, USA) were added to incubate the cells at 4 °C overnight. The cells were washed thrice with TBST, 15 min each time. Then, the cells reacted with HRP-labeled secondary antibodies (CST, USA) for 2 h, followed by cleaning thrice with TBST, 10 min each time. ECL (Thermo, Shanghai, China) development solution was added for imaging. The image was analyzed using Image Lab software (BioRad Laboratories, Hercules, CA, USA). The ratio of the absorbance of target band to that of GAPDH (CST, USA) band was used to measure the expression level of the proteins.

Immunofluorescence and LC3 staining

The chondrocytes of the F1 generation were seeded to a 24-well plate. The cells in sub-aggregation state were starved for 24 h by using serum-free culture medium. After the cells in each treatment were dried, they were fixed in 4% paraformaldehyde (GuGe Biotechnology, Wuhan, China) for 10 min at room temperature and blocked with PBS containing 10% goat serum, 0.3M glycine, 1% BSA, and 0.1% tween for 2 h at room temperature. The staining of the treated cells with LC3B antibody (Abcam, USA) (1 μ g/mL) was performed overnight at 4 °C in PBS containing 1% BSA and 0.1% tween. A DyLight 488 anti-rabbit polyclonal antibody (Abcam, USA) at 1/250 dilution was used as the secondary antibody. Nuclei were counterstained with DAPI and the fluorescence (Nikon Eclipse Ti, Japan) was observed.

Statistical analysis

Values are presented as the mean $\pm$ SEM of at least three experiments. Data were analyzed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests using GraphPad prism software version 5.01 (California, USA). Differences between groups were considered to be significant at $p < 0.05$.

Results

Effect of various concentrations of H_2O_2 on chondrocyte viability and effect of various concentrations of gAPN on H_2O_2 -treated chondrocytes

As demonstrated in Fig. 1A, subsequent to 6 h of H_2O_2 treatment, cell viability was significantly reduced compared with that of the control group in a dose-dependent manner by CCK-8 assays. Except for the 200 μ M group, the reduction of cell viability in all groups had a statistical significance compared to that in the control group ($P < 0.05$). Statistically

Fig. 1. Effect of different concentrations of H_2O_2 and gAPN + H_2O_2 treatment on chondrocyte viability. H_2O_2 treatment resulted in a reduction of cell viability compared with that of the control group in a dose-dependent manner. (A) significant reduction in cell viability was demonstrated in the treated groups ($P < 0.05$), except in the 200 μM group. (B) The cell viability of H_2O_2 model group was significantly reduced compared with that of the control group ($P < 0.05$). The cell viability significantly increased following gAPN treatment, compared with that of the H_2O_2 model group. # $P < 0.05$ versus the control group and * $P < 0.05$ versus the H_2O_2 group. Data are represented as the mean $\pm$ standard error of the mean obtained from three independent experiments ($n = 3$). H_2O_2 , hydrogen peroxide; gAPN, globular adiponectin.

Fig. 2. Effect of gAPN on H_2O_2 -induced apoptosis in chondrocytes. Chondrocytes were pretreated with 0.5 $\mu g/ml$ gAPN for 24 h then treated with or without 400 μM H_2O_2 for 6 h. (A) TUNEL labeling and DAPI staining, and (B) Annexin V/PI staining and flow cytometry assays were employed to assess chondrocyte apoptosis. # $P < 0.05$ versus the control group and * $P < 0.05$ versus the H_2O_2 group. Data are represented as the mean $\pm$ SEM ($n = 3$). H_2O_2 , hydrogen peroxide; gAPN, globular adiponectin. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; DAPI, 4',6-diamidino-2-phenylindole.

significant differences of cell viability did not occur in 400 μM , 600 μM , and 800 μM H_2O_2 -treated groups ($P > 0.05$), thus the 400 μM of H_2O_2 was chosen as the model concentration.

As shown in Fig. 1B, chondrocytes were pretreated with different concentrations of gAPN (0, 0.1, 0.5, 1, or 2 $\mu g/ml$) for 24 h followed by incubation with 400 μM H_2O_2 . Cell viability was significantly decreased by H_2O_2 , and H_2O_2 -induced cell damage was visibly reduced by the addition of gAPN with its concentration of 0.5 and 1 $\mu g/ml$ ($P < 0.05$). The protective effect on the two concentrations of gAPN had no statistically significant difference. Thus, 0.5 $\mu g/ml$ of gAPN was used in the following experiments.

gAPN protected chondrocytes from H₂O₂-induced apoptosis

To study whether the protective effect of gAPN on H₂O₂-induced cytotoxicity was mediated by apoptotic process, we used TUNEL staining and flow cytometry assays to assess chondrocyte apoptosis. Chondrocytes were pretreated with 0.5 µg/mL gAPN for 24 h then treated with or without 400 µM H₂O₂ for 6 h. For a negative control group without gAPN pretreatment, the cells were treated with H₂O₂ only. As shown in Fig. 2A, bright green apoptotic cells with condensed chromatin were observed using DAPI and TUNEL staining. Results showed that H₂O₂ significantly increased the percentage of TUNEL-positive cells compared to that of the control group ($P < 0.05$). Pretreatment with gAPN significantly reduced the percentage of H₂O₂-induced apoptotic chondrocytes ($P < 0.05$). Flow cytometry after Annexin V/PI staining was also used to detect chondrocyte apoptosis. The trend of the results was consistent with that of TUNEL staining. The percentage of apoptotic chondrocytes in the gAPN pretreated group was significantly decreased compared to that of the H₂O₂ group ($P < 0.05$) (Fig. 2B).

gAPN elicited antiapoptotic effect by inhibiting the mitochondrail-related intrinsic pathway

The balance of antiapoptotic protein Bcl-2 and pro-apoptotic protein Bax plays an important role in the regulation of mitochondrial integrity and cell survival [34]. To ascertain whether the mitochondrial-dependent apoptotic pathway was involved in the protective effect of gAPN on chondrocytes, the expression levels of Bcl-2 and Bax were detected. Western blot analysis revealed that H₂O₂ significantly decreased Bcl-2 and increased Bax expressions ($P < 0.05$). Compared to that of the H₂O₂ group, gAPN-pretreatment significantly increased the expression of Bcl-2 and decreased the expression of Bax ($P < 0.05$). Caspase-3, the major executor of apoptosis [35, 36] and the downstream effector protein of several apoptotic pathways was also examined. H₂O₂ significantly increased the expression of Cleaved-caspase3, and the pretreatment of gAPN significantly attenuated the effect of H₂O₂ in activating Caspase-3 ($P < 0.05$). Similar results were obtained for Cleaved-PARP, which facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. H₂O₂ increased the expression of Cleaved-PARP. However, the pretreatment of gAPN significantly decreased Cleaved-PARP expression ($P < 0.05$), as shown in Fig. 3A.

As the loss of mitochondrial membrane potential (MMP) is regarded as one of the inducers of mitochondrial-related apoptosis, whether gAPN influenced the loss of MMP in chondrocytes subjected to H₂O₂ was examined using JC-1 fluorescence staining assay. JC-1 exists as an aggregated form (red fluorescence) in the matrix of mitochondria with the normal MMP, and JC-1 is converted into the monomeric form (green fluorescence) with the loss of MMP. Consistent with the antiapoptosis effect, pretreatment with gAPN led to a strong protection from MMP loss induced by H₂O₂ as determined by the reduction of green fluorescence and increase of red fluorescence in gAPN pretreatment group compared to that in the H₂O₂ group. To quantify the changes of MMP, the red/green fluorescence intensity was assessed with a fluorescence plate reader. Compared to the control group, H₂O₂ induced a significant reduction of red/green fluorescence (0.55 ± 0.05 fold vs. control group, $P < 0.05$). Meanwhile, the addition of gAPN significantly reversed the change (0.74 ± 0.04 vs. 0.55 ± 0.05 in H₂O₂ group, $P < 0.05$), as shown in Fig. 3B.

gAPN-induced autophagy was correlated with the suppression of H₂O₂-induced apoptosis

To determine whether the antiapoptotic effect of gAPN on rat chondrocytes is related to cell autophagy, we measured the expression of Beclin-1, which is considered essential for the formation of autophagosomes (required for the initial stage of autophagy) [36], and microtubule-associated protein light chain 3B (LC3B), which is regarded as a major constituent of autophagosomes (essential for the relatively late stage of autophagy) [9]. LC3B consists of LC3-I and LC3-II, and upon autophagy activation LC3-I converts into LC3-II through lipidation and adheres to the membrane of phagophore for the formation of autophagosome. Therefore, the conversion of LC3-I into LC3-II is considered as a marker of autophagy activa-

Fig. 3. Effect of gAPN on changes in MMP and apoptosis-related proteins. Chondrocytes were pretreated with 0.5 $\mu\text{g}/\text{mL}$ gAPN for 24 h then treated with or without 400 μM H_2O_2 for 6 h. (A) Bcl-2, Bax, Caspase-3 and Cleaved PARP protein expression levels. GAPDH was used as a loading control. Results are presented as the ratio of Bcl-2 to Bax and Cleaved caspase-3 to total Caspase-3. (B) Representative images of each group photographed by fluorescent microscope. The red fluorescence represents JC-1 aggregates in the matrix of mitochondria with the normal MMP, whereas the green fluorescence represents JC-1 monomer appeared with the depolarization of mitochondrial membrane. The loss of MMP expressed a conversion of red to green. Data are represented as the mean $\pm$ SEM ($n=3$). $^{\#}P<0.05$ versus the control group and $^*P<0.05$ versus the H_2O_2 group. H_2O_2 , hydrogen peroxide; gAPN, globular adiponectin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

tion and autophagosome formation [37, 38]. As shown in Fig. 4A, gAPN pretreatment significantly increased the expressions of LC3-II and Beclin-1, indicating the effect of gAPN on autophagosome formation in H_2O_2 -treated chondrocytes. p62(SQSTM1/sequestosome 1) is implicated in various biological responses, including tumorigenesis, apoptosis, inflammation and autophagy. In particular, with regards to regulating autophagy, p62 delivers ubiquitin-bound protein complexes to the autophagosome and promotes degradation by autophagosome-lysosome fusion machinery. Meanwhile, p62 is also degraded along with the ubiquitinated substrates. Therefore, cellular p62 level is speculated to be diminished during the process of autophagy. H_2O_2 increased the expression of P62, whereas the decreased levels of p62 were observable after gAPN treatment, indicating gAPN increased autophagolysosomal degradation. To further prove gAPN induces autophagic flux in chondrocytes, cells were pretreated with Bafilomycin A1 (100

Fig. 4. Effects of H_2O_2 and gAPN on autophagosome formation in chondrocytes. Chondrocytes were pre-treated with 0.5 μ g/mL gAPN for 24 h then treated with or without 400 μ M H_2O_2 for 6 h. (A) Expressions of LC3B, Beclin-1 and P62 by Western blot. (B) LC3-II immunostaining. Significantly increased green bright puncta showed the formation of autophagosome. Results are presented as the ratio of LC3-II to LC3-I and P62/Beclin-1 to GAPDH. Data are represented as the mean $\pm$ SEM of three independent experiments. # $P < 0.05$ versus the H_2O_2 group, * $P < 0.05$ versus the gAPN group and $^sP < 0.05$ versus the gAPN+ H_2O_2 group. H_2O_2 , hydrogen peroxide; gAPN, globular adiponectin; BafA1, Bafilomycin A1, a specific autophagosome-lysosome inhibitor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

nM) (Sigma, USA), a specific autophagosome-lysosome inhibitor, followed by gAPN treatment. Interestingly, Bafilomycin treatment further enhanced p62 expression in the absence or presence of H_2O_2 . Unexpectedly, the treatment of H_2O_2 alone did not suppress the expression of LC3-II and Beclin-1 compared to that of the control group ($P > 0.05$). Immunofluorescence staining also showed that gAPN treatment resulted in increased bright LC3-II puncta compared with the untreated chondrocytes in Fig. 4B. These results suggested a negative correlation between gAPN-induced autophagy and H_2O_2 -induced apoptosis in chondrocytes.

gAPN-induced autophagy contributed to the suppression of H_2O_2 -induced apoptosis in chondrocytes

To further investigate the role of autophagy induced by gAPN in the suppression of H_2O_2 -induced apoptosis, we pretreated chondrocytes with 10 mM 3-MA (3-methyladenine,

Fig. 5. Role of autophagy induction by gAPN in the suppression of H_2O_2 -induced apoptosis in chondrocytes. Chondrocytes were treated with 0.5 μ g/mL gAPN or pretreated with 10 μ M 3-MA for 4 h before gAPN treatment followed by H_2O_2 . (A) Detection of cell apoptosis rate with Annexin V/PI staining and flow cytometry assays (B) Bcl-2, Bax, Cleaved PARP and Caspase-3 protein expression levels by Western blot. GAPDH was used as a loading control. Results are presented as the ratio of Bcl-2 to Bax and Cleaved caspase-3 to total Caspase-3. Data are represented as the mean $\pm$ SEM (n=3). *P<0.05 versus the H_2O_2 group and #P<0.05 versus the gAPN+ H_2O_2 group. H_2O_2 , hydrogen peroxide; 3-MA, 3-methyl adenine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

an inhibitor of autophagy (Sigma, USA) for 4 h before treatment with gAPN. Then, we used flow cytometry assays to assess chondrocyte apoptosis and measured the apoptosis-related protein Bcl-2/Bax, Cleaved caspase3, and Cleaved PARP expressions. Interestingly, the pretreatment of cells with 3-MA revealed a reduction ratio of Bcl-2/Bax and restored Caspase-3 activity suppressed by gAPN in H_2O_2 -treated cells (P<0.05), which ultimately increased Cleaved PARP expression. Flow cytometry result was consistent with that of the Western blot. The percentage of apoptotic chondrocytes in the 3-MA pretreated group was significantly increased compared to that in the gAPN+ H_2O_2 group (P<0.05) as depicted in Fig. 5. Taken together, these data provide vital evidence that autophagy induction by gAPN plays an important role in the suppression of H_2O_2 -induced apoptosis.

AMPK/mTOR signaling pathway is involved in gAPN-induced autophagy in H_2O_2 -treated chondrocytes

To investigate the molecular mechanism of autophagy induced by gAPN on H_2O_2 -treated chondrocytes, we examined the activation of AMPK signaling in gAPN treated chondrocytes because many beneficial biological responses induced by adiponectin are attributed to AMPK activation and its downstream transcriptional mediators [19, 39]. As depicted in Fig.

Fig. 6. Involvement of AMPK/mTOR signaling pathway in gAPN-induced autophagy in chondrocytes. Chondrocytes were treated with 0.5 μg/mL gAPN or pretreated with Compound C (10 μM) for 20 min before gAPN treatment prior to H₂O₂. The expression of phosphorylated and total AMPK and its downstream transcriptional mediator mTOR were assessed by Western blot. GAPDH was used as a loading control. Results are presented as the ratio of p-AMPK to AMPK and p-mTOR to mTOR. Data are represented as the mean ± SEM of three independent experiments. [#]P<0.05 versus the H₂O₂ group and ^{*}P<0.05 versus the gAPN+H₂O₂ group. H₂O₂, hydrogen peroxide; gAPN, globular adiponectin; Compound C, an inhibitor of AMPK; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

6, gAPN significantly increased the expression of the phosphorylation of AMPK (p-AMPK), accompanying with the increased expression of autophagy-related proteins LC3-II and Beclin-1 and P62 degradation in H₂O₂-treated cells, as shown in Fig. 7A. However, this effect was significantly inhibited by pretreatment with 10 μM Compound C (a potent, reversible, and selective AMPK inhibitor). To address how p-AMPK regulate autophagy in H₂O₂-treated chondrocytes, we examined the phosphorylation of the downstream regulator of AMPK. We found that gAPN inhibited the phosphorylation of mTOR (p-mTOR), whereas the use of Compound C restored the expression of p-mTOR (Fig. 6). The results of immunostaining was consistent with that of the Western blot shown in Fig. 7B. The inhibition of AMPK significantly decreased bright LC3-II puncta compared with uninhibited chondrocytes. These data suggested gAPN that mediated the induction of autophagy was associated with the AMPK/mTOR signaling pathway in H₂O₂-treated chondrocytes.

Discussion

We demonstrated for the first time that gAPN protects chondrocytes from H₂O₂-induced apoptosis through the activation of autophagy. Moreover, we have also presented that the AMPK/mTOR signaling pathway plays an important role in the gAPN-induced expression of proteins related to autophagy in chondrocytes and the prevention of H₂O₂-induced apoptosis by gAPN (Fig. 8).

OA is the most common chronic arthritis in the elderly, characterized by the gradual degradation of articular cartilage, synovial inflammation and pain, resulting in significant disability [1]. Reported studies have well demonstrated that cartilage matrix degradation and chondrocyte apoptosis induced by certain proteases and apoptotic factors are the two primary pathogenic events occurring in OA [3, 4]. Among all the reasons, the ageing-related

Fig. 7. Role of AMPK/mTOR signaling pathway in expression of autophagy-related proteins by gAPN in chondrocytes. Chondrocytes were treated with 0.5 μ g/mL gAPN or pretreated with Compound C (10 μ M) for 20 min before gAPN treatment prior to H_2O_2 . (A) Expressions of LC3B, P62 and Beclin-1 after inhibiting the p-AMPK with Compound C by Western blot. (B) Expression of LC3-II after inhibiting the p-AMPK by immunostaining. Results are presented as the ratio of LC3-II to LC3-I and p62, Beclin-1 to GAPDH. Data are represented as the mean $\pm$ SEM of three independent experiments. * P <0.05 versus the H_2O_2 group and * P <0.05 versus the gAPN+ H_2O_2 group. H_2O_2 , hydrogen peroxide; gAPN, globular adiponectin; Compound C, an inhibitor of AMPK; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

accumulation of reactive oxygen species (ROS) has been considered to be a major causative factor for chondrocyte apoptosis and OA development [5, 6]. Excess ROS increases the permeability of mitochondrial outer membrane, inducing the leakage of cytochrome C and apoptosis-inducing factors and hence cell apoptosis [7]. In this study, we used H_2O_2 to induce ROS-related apoptosis and found that cell viability was reduced in a dose-dependent manner.

Adiponectin is the most abundant adipokine secreted from adipose tissue. Aside from its well-characterized antiapoptotic and anti-inflammatory properties [20-22], adiponectin seems to have both catabolic and anabolic effects on the pathological changes of several tissues/cells involved in the initiation and progression of OA. Chen et al. [40] demonstrated the adiponectin up-regulated tissue inhibitor of metalloproteinase-2 (TIMP-2) and down-regulated IL-1b-induced MMP-13 at both mRNA and protein levels, ultimately alleviating the damage of articular cartilages. Furthermore, Ehling et al. found that adiponectin can stimulate the release of anti-inflammatory molecules, including IL-10 and IL-1 receptor antagonist [41, 42]. Additionally, Honsawek et al. suggested that the level of adiponectin in plasma and synovial fluid was negatively associated with the severity of OA in humans [43]. These results showed a protective effect of adiponectin in cartilage damage. Consistent with these research, our study found that gAPN (globular domain of APN) protected chondrocytes from H_2O_2 -induced apoptosis proved by the lower apoptosis rate of chondrocytes in gAPN pretreatment group than in the H_2O_2 group detected by flow cytometry and TUNEL staining. The balance of Bax and Bcl-2 expressions plays a key role in maintaining the integrity of

Fig. 8. Proposed model of autophagy activation by globular adiponectin and its protective effect against H_2O_2 -induced apoptosis in chondrocytes. H_2O_2 treatment causes chondrocyte apoptosis, which is modulated by the mitochondrial-related intrinsic pathway regulated by Bcl-2 family member Bax and ultimately leads to the activation of caspase-3, the executioner of apoptosis. AMPK/mTOR signaling pathway plays a key role in the gAPN-induced expression of proteins, including Beclin-1 and LC3B, involved in autophagy. Importantly, gAPN-induced autophagy plays an important role in the prevention of H_2O_2 -induced apoptosis in chondrocytes through the modulation of the mitochondrial-related intrinsic pathway of apoptosis. Detailed mechanisms underlying the inhibition of H_2O_2 -induced apoptosis by gAPN-induced autophagy remain to be determined. H_2O_2 , hydrogen peroxide; gAPN, globular adiponectin.

mitochondria and suppressing the mitochondrial apoptotic pathway [34]. The decrease of Bcl-2 and the increase of Bax led to the loss of MMP and the activation of caspase-3 [34]. In the present study, the pretreatment of gAPN significantly decreased the expression of Cleaved PARP and Cleaved caspase-3 and restored MMP suppressed by H_2O_2 , suggesting that gAPN elicited antiapoptotic effect through the inhibition of the mitochondrial-related intrinsic pathway. However, some research showed contrary results. The study of Lago demonstrated that adiponectin may have proinflammatory effects on chondrocytes by inducing the expression of NOS_2 and stimulating the release of proinflammatory cytokines, including IL-6, MMP-3, MMP-9, and monocyte chemoattractant protein-1 (MCP-1) [44]. Surprisingly, Filkova et al. reported that the increased serum levels of adiponectin in erosive OA was found compared with that in the non-erosive OA, suggesting that adiponectin may play a role in matrix degradations [45]. In addition, adiponectin facilitated joint inflammation and destruction by stimulating the syntheses of vascular endothelial growth factor (VEGF) and metalloproteases [46]. The contrasting results regarding the effect of adiponectin might be due to experimental conditions. In our study, we used H_2O_2 to establish cell apoptosis model and assessed the potential antiapoptotic effect of gAPN on the H_2O_2 -induced chondrocyte apoptosis *in vitro*. Because OA chondrocyte behavior and phenotypes can be affected by the surrounding matrix state, culture methods, and passage numbers [47, 48], these behavior and phenotypes might contribute to the difference of gAPN-induced responses in each study. Another possibility may be due to the different dosage of gAPN selected in experiments. In our study, we found that gAPN antagonized H_2O_2 -induced cytotoxicity in a dose-dependent manner at low concentrations. However, gAPN also showed cytotoxic property with increased dosage. Moreover, the different forms of adiponectin may have different biologic effects. Native adiponectin circulates in blood mainly in two types: globular adiponectin and full-length adiponectin. We used gAPN in our study, which is biologically active and easier to manufacture and administer [25, 49]. Meanwhile, full-length adiponectin, the most abundant isoforms of which are hexamers and high-molecular-weight forms, proved to have pro-inflammatory effects [50, 51].

Autophagy is a key mechanism for maintaining cell homeostasis by adjusting cell metabolism to nutrient supply and removing damaged organelles. Reports showed that dysfunctional cellular organelles and macromolecules increased remarkably in the degeneration cartilage, interrupting cell function. Meanwhile, autophagy can degrade these molecules, inhibiting chondrocyte apoptosis and blocking the degeneration of articular cartilages and OA development [52-54]. Additionally, evidence showed that ROS also interacted with autophagy during the degenerative process of cartilages [55, 56]. Wu

et al. [56] found that OA chondrocytes had lower autophagic level and higher level of ROS production compared with the normal chondrocytes. Cetrullo et al. [57] demonstrated that oxidative stress inhibited the expression of autophagy-related proteins in chondrocytes and promoted apoptosis. Even though a close (negative) relationship between autophagy and apoptosis has been previously suggested, the role of autophagy in the chondroprotective effect of adiponectin has not been explored. Among various autophagy-related (Atg) genes, Beclin-1 and LC3 play a critical role in the coordination of the cytoprotective function of autophagy while counteracting the apoptotic process [58]. Beclin-1 has been suggested to play a cardinal role in autophagosome formation through the localization of autophagy-related proteins to a pre-autophagosomal structure [58, 59]. LC3, an important constituent of autophagosomes, also plays an essential role in the fusion of autophagosomes with lysosomes for the degradation of damaged organelles by lysosomal enzymes [11]. In our study, we demonstrated for the first time that gAPN induced an increase in the expression of autophagy-related proteins LC3-II and Beclin-1, indicating gAPN induced autophagy of chondrocytes. However, simply detecting LC3-II formation does not provide information regarding the full process of autophagic flux. Importantly, we detected the expression of p62, an autophagy substrate known to recruit ubiquitinated proteins and gets degraded as autophagic flux progresses. We found gAPN reduced accumulation of p62 induced by H_2O_2 and autophagosome-lysosome inhibitor Bafilomycin A1 treatment further enhanced p62 expression, suggesting gAPN not only increases LC3-II protein levels but also activates autophagic flux in chondrocytes. All these data suggest that gAPN-mediated autophagy plays a critical role in the amelioration of H_2O_2 -induced chondrocyte apoptosis even though the treatment of H_2O_2 did not show a significant decrease of Atg proteins. To further prove these findings, we used 3-MA, a class III phosphoinositol 3-kinase inhibitor widely used as an inhibitor of autophagy, to block autophagy initiation. Results indicated that the antagonist capacity of gAPN against H_2O_2 -induced apoptosis was partly inhibited. This finding confirmed the importance of autophagy in the protective effect of gAPN against the apoptosis of chondrocytes.

AMPK acts as a sensor of cellular energy level and its activity is regulated by various cellular stresses, including hypoxia, glucose deprivation, and oxidative stress [28]. The activation of AMPK increased the catabolic process inside the cells, thus modulated numerous pathophysiological processes. Many beneficial biological responses induced by adiponectin are attributed to AMPK activation and its downstream transcriptional mediators [49, 60]. AMPK controls autophagy through the mammalian target of rapamycin (mTOR) and Unc-51-like kinase 1 (ULK1) signaling [29]. mTOR, a conserved Ser/Thr protein kinase, is a potent inhibitor of autophagy. AMPK can inhibit the activity of mTOR complex (mTORC1) either by directly phosphorylating Raptor, a regulatory component of mTORC1, or by phosphorylating tuberous sclerosis protein 2 (TSC2), which subsequently suppresses mTOR activity [29]. Recent studies have demonstrated that chondrocyte autophagy is a constitutive homeostatic mechanism in articular cartilages [30], which can be promoted by AMPK signaling [31, 32] through mTOR suppression. Furthermore, the cartilage-specific genetic deletion of mTOR [33] and the pharmacologic inhibition of mTOR signaling by rapamycin [61] upregulates autophagy and reduces the severity of experimental OA *in vivo*. Moreover, chondrocyte autophagy was reduced with a linked increase in apoptosis in human knee OA and mouse knee OA as for the phosphorylation of AMPK (p-AMPK) expression [18]. Based on these reports, we hypothesized that AMPK/mTOR signaling pathway is involved in the induction of autophagy by gAPN and plays a critical role in the prevention of H_2O_2 -induced apoptosis. In line with these previous results, we found that gAPN induced a high expression of p-AMPK accompanying with a high expression of Beclin-1 and LC3-II in chondrocytes treated with H_2O_2 , and down-regulated the expression of p-mTOR and P62 simultaneously. For further proof, the application of Compound C, an inhibitor of AMPK, significantly blocked the gAPN-induced autophagy in H_2O_2 -treated chondrocytes because the use of Compound C decreased the expression of Beclin-1 and LC3-II and restored H_2O_2 -induced expression of P62. Taken together, our results shown in the current study clearly demonstrate that the modulation of

AMPK/mTOR plays a key role in the regulation of autophagy in chondrocytes treated with gAPN and H₂O₂.

In conclusion, our data presented here demonstrated for the first time that gAPN protected chondrocytes from H₂O₂-induced apoptosis through the modulation of autophagy. This protective effect is mediated by AMPK/mTOR signaling pathway. These findings suggest that gAPN may be a novel survival factor for chondrocytes in the progress of OA. However, further studies are required to validate our findings in chondrocytes into normal articular cartilages and its role in the prevention of OA in an *in vivo* model.

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Disclosure Statement

All authors have no conflict of interest to state.

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