Ascorbic acid provides protection for human chondrocytes against oxidative stress

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Abstract. Oxidative stress is considered to be an important cause of dysfunction in chondrocytes and articular cartilage degradation, which leads to the pathogenesis of osteoarthritis (OA) and cartilage aging. The present study aimed to assess the effects of the widely applied antioxidant, ascorbic acid (AA), on human chondrocytes against hydrogen peroxide (H$_2$O$_2$) in vitro. Using annexin V-fluorescein isothiocyanate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and senescence-associated β-galactosidase assays, the present study identified that AA reduced apoptosis, reduced the loss of viability and markedly decreased H$_2$O$_2$-mediated senescence in cells treated with H$_2$O$_2$. Furthermore, AA not only stimulated the expression levels of collagens and proteoglycans, but also inhibited the differentiation of chondrocytes under conditions of oxidative stress. In addition, reverse transcription-quantitative polymerase chain reaction and western blotting demonstrated that AA decreased the activity of nrf2, NF-κB, AP1 and matrix metalloproteinase-3, which is stimulated by H$_2$O$_2$. In conclusion, AA efficiently protected human chondrocytes against damage induced by H$_2$O$_2$ by regulating multiple regulatory pathways.

Introduction

Chondrocyte dysfunction results in the cervical spondylosis associated with degeneration of the intervertebral disc, or the joint disorder osteoarthritis (OA), which is characterized by progressive breakdown of articular cartilage (1-3). Chondrocytes are sensitive to environmental and multiple cellular stresses, including inflammation, mechanical loading, stress to the endoplasmic reticulum and hypoxia (4-6). Reactive oxygen species (ROS), another major determinant of stress, including hydrogen peroxide (H$_2$O$_2$), hypochlorite ion, hydroxyl radical and superoxide anion, are involved in normal intracellular transduction and degenerative cellular processes. Increased ROS production is considered to be an important cause of chondrocyte dysfunction and articular cartilage degradation, which leads to the pathogenesis of OA and the aging of cartilage (7-10). Chondrocytes and cartilage tissues exhibit efficient protective strategies to minimize damage induced by increased ROS production, including H$_2$O$_2$ (7,11). Previous studies have investigated various antioxidant supplements, including resveratrol, quercetin, vitamin E, and other strategies (12-15). However, information available on the antioxidative status of chondrocytes is limited (16). Therefore, identifying available and promising antioxidative strategies or antioxidant drugs to combat oxidative stress in chondrocytes is required.

Ascorbic acid (AA) is widely used in clinical applications and is known for its role in bone formation, wound healing and the maintenance of healthy gums. AA is involved in protecting the immune system and combating infection and allergic reactions (17). AA is also regarded as the most important antioxidant, which provides protection against oxidative stress. It has been previously reported that AA may be a potential drug for therapeutic intervention and to slow the progression of age-associated diseases, including atherogenesis and Alzheimer’s disease (17,18). Previous studies have revealed that AA attenuates oxidative stress in diabetic aged rats (19,20). AA also exerts a role in chondroprotection: The application of AA and mechanical stimulation in combination was revealed to improve the mechanical characteristics of regenerated cartilage (21). AA was revealed to markedly affect the expression of proteins with specific functions in human articular chondrocytes under conditions of homogen-tic acid-induced stress, which may be mediated by protein oxidation (22). A combination of α-tocopherol (0.1-2.5 µM), AA (10-50 µM) and selenium (1-50 nM) provided a promising strategy to combat oxidative stress and cytokine-induced matrix degradation in chondrocytes (23). AA supplementation of chondrocytes following static loading was demonstrated to have the potential to reduce the morphological and biochemical degeneration of chondrocytes in vivo (24). These previous

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findings support the hypothesis that AA may be a promising drug or antioxidant to protect against the chondrocyte damage induced by oxidative stress. However, no reports in this research area currently exist.

The present study focused on the role of AA in combating oxidative stress and the crosstalk between AA and response transcription factors under conditions of oxidative stress in C28/I2 human chondrocytes.

Materials and methods

Cell culture and cell treatments. The human C28/I2 chondrocyte cell line was acquired from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultivated in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% fetal bovine serum (Gibco Life Technologies, Carlsbad, CA, USA) and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin; Invitrogen Life Technologies, Carlsbad, CA, USA), in 5% CO2 at 37°C. The cells were subcultured after reaching ~90% confluence. AA (Sigma-Aldrich, St. Louis, MO, USA) and H2O2 (Sigma-Aldrich) were diluted in serum-free DMEM. The cells were treated with H2O2 (100 µM) for 4 h following incubation with AA at 100 (AA100 group) or 200 µM (AA200 group) for 24 h after the C28/I2 cells had reached 80% confluence. Normal C28/I2 cells without treatment and C28/I2 cells treated with H2O2 (100 µM) were denoted as the N group and C group, respectively.

Analysis of apoptosis. The annexin-V-fluorescein isothiocyanate (FITC) apoptosis detection kit was used to analyze apoptosis in the cells, according to the manufacturer’s instructions (KeyGen Biotech. Co., Ltd., Nanjing, China). The cells were harvested following the indicated treatments and cell suspensions were fixed overnight with ice-cold 70% ethanol. The fixed cells were subsequently stained with propidium iodide or annexin V-FITC following centrifugation at 70 x g at 4˚C for 10 min. The resulting supernatants (nuclear fractions) were fractionated on 10% SDS-PAGE gels (Sigma-Aldrich) and reverse: 5'-TTAGTCCTTTACGCCCTTTCCAG-3'; COL2a1, forward: 5'-GACATTTCTCCGTCTACTGCC-3' and reverse: 5'-GTGTACGTGAACCTGTGTTG-3'; Ace1, forward: 5'-ACTGAGGAGAGGGTGGTTG-3' and reverse: 5'-CAACCGTAGATGCCAAGCT-3'; Nrf2, forward: 5'-TTGTAATGCTGCGGAAAAGCTG-3' and reverse: 5'-ATGTTGGAAGAGCGCCGATG-3'; GAPDH, forward: 5'-GCACCGTCAAGGCCAAGAAC-3' and reverse: 5'-ATGTTGGAAGAGCGCCGATG-3'.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The total RNA was isolated from the cells using an RNAeasy kit (Qiagen, Valencia, CA, USA). RT-qPCR was performed on an Applied Biosystems 7500 instrument with SYBR Green PCR kits (Applied Biosystems, Foster City, CA, USA). First-strand cDNA was synthesized using reverse transcription reagents (PrimeScript™ RT reagent kit; Takara Biotechnology Co., Ltd., Dalian, China). The reactions were conducted with the following thermocycling conditions: at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 56°C for 30 s and extension for 10 s at 72°C. The data were assessed using the 2^{-ΔΔCt} method for relative quantification (21,22). The GAPDH gene was used as the endogenous control for normalization. The primer sequences (synthesized by Sangon Biotech Co., Ltd., Beijing, China) were as follows: Col1a1, forward: 5'-CAAGATGTTGGGCTTACTAC-3' and reverse: 5'-TTGTAATGCTGCGGAAAAGCTG-3'; matrix metalloproteinase-3 (MMP-3), forward: 5'-CTGAGCTCCGAGACTCTCG-3' and reverse: 5'-CAACCGTAGATGCCAAGCT-3'; AP1, forward: 5'-TTGTAATGCTGCGGAAAAGCTG-3' and reverse: 5'-ATGTTGGAAGAGCGCCGATG-3'; GAPDH, forward: 5'-GCACCGTCAAGGCCAAGAAC-3' and reverse: 5'-ATGTTGGAAGAGCGCCGATG-3'.

Nuclear fraction preparation. The nuclear and cytosolic fractions were separated using a Nuclear and Cytoplasmic Protein Extraction kit obtained from the Beyotime Institute of Biotechnology (Jiangsu, China). The cell pellets were resuspended in 100 µl cytosolic extract A reagent, containing 1 mM PMSF and vortexed for 5 sec. Subsequently, 5 µl cytosolic extract B reagent was added to the lysates and the samples were vortexed for 5 sec. The supernatant (cytosolic fractions) was acquired following centrifugation at 13,000 x g at 4°C for 5 min, after which the pellets were resuspended in 30 µl nuclear extract reagent, containing 1 mM PMSF, and centrifuged again at 13,000 x g at 4°C for 10 min. The resulting supernatants (nuclear fraction) were extracted and subsequently analyzed to assess the protein expression of nrf2 in the nuclear fraction.

Immunoblotting. Western blotting was performed using a standard procedure, as described previously (6). Briefly, the C28/I2 chondrocytes were lysed using a lysis reagent (Promega Corporation, Madison, WI, USA) and the proteins were fractionated on 10% SDS-PAGE gels (Sigma-Aldrich) and indicated and following treatment, the cells were washed with phosphate-buffered saline (PBS), harvested and collected by centrifugation at 70 x g for 5 min at room temperature. The cells were treated with freshly prepared fluorimetric substrate for 2 h at 37°C in the dark. The fluorescence intensity of each reaction mixture was determined and quantified using Image J software (NIH, Bethesda, MD, USA). The average fluorescence intensity was analyzed from five fields for each treatment using ImageJ 1.38.
electrotransferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were incubated with primary antibodies for 2 h at 37˚C and then blocked with PBS containing 1% bovine serum albumin and 0.02% Tween 20 (Sigma-Aldrich). The signals were acquired using an enhanced chemiluminescence detection system (SuperSignal West Femto; Pierce Biotechnology, Inc., Rockford, IL, USA) following incubation with horseradish-peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:500; cat. no. G-21079; Pierce Biotechnology, Inc.). The primary antibodies used in the present study were as follows: Rabbit monoclonal anti-NF-κB (1:500; cat. no. 8242; Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit phosphorylated (p)-STAT3 (Tyr705; 1:500; p-NF-κB; cat. no. 3033; Cell Signaling Technology, Inc.), rabbit monoclonal anti-nrf2 (1:500; cat. no. SAB4501984; Sigma-Aldrich) and rabbit anti-β-actin antibody (1:1,000; cat. no. A2066; Sigma-Aldrich).

**Statistical analysis.** All experiments were performed, at least, in triplicate. All the data were expressed as the mean ± standard deviation or as the mean ± standard error. The PASW Statistics 18 software package (formerly SPSS Statistics) was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). Comparison among multiple samples was performed by Figure 1. Effect of AA on the apoptosis of C28/I2 cells in the presence of H_2O_2. The C28/I2 cells were preincubated with 100 or 200 µM AA (AA100 and AA200, respectively) for 24 h. The cells were subsequently treated with H_2O_2 for 4 h. (A-D) Apoptosis was measured using an annexin V-fluorescein isothiocyanate assay. All experiments were performed in triplicate. (E) The data were normalized and expressed as the mean ± standard deviation. Significant differences are denoted as *P<0.05 and **P<0.01. AA, ascorbic acid; N, normal C28/I2 cells; C, cells treated with 100 µM H_2O_2 for 4 h; AA100, cells preincubated with 100 µM AA for 24 h and treated with 100 µM H_2O_2 for 4 h; AA200, cells preincubated with 200 µM AA for 24 h and treated with 100 µM H_2O_2 for 4 h.

Figure 2. Measurement of cell viability and senescence. (A) Cell viability and (B) senescence were measured using MTT and β-gal assays, respectively. The data are presented as the mean ± standard deviation. Significant differences are denoted as *P<0.05. N, normal C28/I2 cells; C, cells treated with 100 µM H_2O_2 for 4 h; AA100, cells preincubated with 100 µM AA for 24 h and treated with 100 µM H_2O_2 for 4 h; AA200, cells preincubated with 200 µM AA for 24 h and treated with 100 µM H_2O_2 for 4 h.
one-way analysis of variance and Student's t-test was used to compare two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

AA reduces H$_2$O$_2$-mediated apoptosis. The C28/I2 human chondrocytes were treated with H$_2$O$_2$ to simulate pathophysiological oxidative stress as it occurs in vivo. The treatment groups were as follows: N group, normal C28/I2 cells; C group, cells treated with 100 µM H$_2$O$_2$ for 4 h; AA100 group, cells preincubated with 100 µM AA for 24 h and treated with 100 µM H$_2$O$_2$ for 4 h; AA200 group, cells preincubated with 200 µM AA for 24 h and treated with 100 µM H$_2$O$_2$ for 4 h. It was revealed that H$_2$O$_2$ significantly increased the levels of apoptosis when compared with the normal cells (N group, P<0.001), as shown in Fig. 1, notably, preincubation with 100 µM AA resulted in a significant decrease in the levels of apoptosis compared with the C group (P=0.034). An increased concentration of AA (200 µM) led to a modest decrease in the levels of apoptosis compared with the AA100 group. Significant differences in the levels of apoptosis between the AA groups and the N group were observed (P<0.001). These data suggested that AA markedly reduced H$_2$O$_2$-mediated apoptosis, however, preincubation with AA (100 and 200 µM) failed to completely abolish the effects of H$_2$O$_2$ on cellular apoptosis.

AA protects chondrocytes from H$_2$O$_2$-induced loss of viability and senescence. Cell viability and senescence are characterized by a loss of function and integrity, which contribute to the progressive degeneration of tissues (25,26). The cell viability and senescence of chondrocytes were determined under oxidative stress conditions mediated by H$_2$O$_2$ or AA pretreatment using an MTT assay or the SA-β-gal assay, as shown in Fig. 2. Incubation with H$_2$O$_2$ stimulated a loss in cell viability and promoted the senescence-like phenotype of chondrocytes when compared with the normal C28/I2 cells (P<0.001). Addition of 100 µM AA significantly reduced the loss of viability (P=0.023) and good agreement was obtained with the SA-β-gal activity assay used to measure senescence induced by H$_2$O$_2$ (P=0.026) compared with the C group. Following treatment with a higher concentration of AA (200 µM), the effects of AA were further enhanced (AA200 group vs. AA100 group, 56.48±9.17 vs. 53.17±10.40% with the MTT assay and 130.33±10.69 vs. 144.71±11.39% with the SA-β-gal assay; Fig. 2). These results clearly revealed that AA significantly protected chondrocytes from a loss of viability and from senescence induced by H$_2$O$_2$.

AA affects the differentiation and expression of proteoglycans and collagens in human chondrocytes exposed to H$_2$O$_2$. To investigate the role of AA in the expression and differentiation of collagens and proteoglycans in chondrocytes when exposed to oxidative stress, the expression levels of the major factors, Col1a1, Col2a1 and Agc1, were detected by RT-qPCR, as shown in Fig. 3A-C. Oxidative stress not only suppressed the expression levels of Col2a1 and Agc1 (P<0.001), but also increased the expression of Col1a1 (P<0.001) compared with the normal cells (N group). Preincubation with AA resulted in a significant increase in the relative expression levels of Col2a1 (AA100, P=0.005; AA200, P<0.001) and Agc1 (AA100, P=0.013; AA200, P=0.022), and significantly decreased the expression of Col1a1 (AA100, P=0.015; AA200, P=0.031) compared with the C group. The differentiation index (Fig. 3D) was calculated as the ratio of the expression levels between Col2a1 and Col1a1 (27). Addition of H$_2$O$_2$ decreased the differentiation index, whereas preincubation with AA...
increased the differentiation index (Fig. 3D). These findings revealed that AA not only stimulated the expression of collagen and proteoglycans, but also inhibited the differentiation of chondrocytes in the presence of oxidative stress.

AA contributes to the inhibition of multiple transcription factors in C28/I2 cells exposed to H₂O₂. In order to assess transcription factor(s), which are modulated by AA, certain known stress and toxicity responding factors (nrf2, NF-κB, AP1 and MMP-3) found in four different signaling pathways were investigated. H₂O₂ markedly upregulated the mRNA expression levels of nrf2, AP1 and MMP-3 (P<0.001), in addition to stimulating the activation of NF-κB when compared with the N group (Fig. 4). Preincubation with 100 µM AA attenuated the increase in the expression of nrf2 (P=0.022) and the protein expression of nrf2 induced by H₂O₂ (Figs. 4A and B). Furthermore, 100 µM AA decreased the activation of NF-κB compared with the C group (Fig. 4B). In addition, 100 µM AA decreased the mRNA expression levels of AP1 (P=0.032) and MMP-3 (P=0.006) compared with the C group (Figs. 4C and D). A higher concentration of AA (200 µM) elicited similar effects on the mRNA expression levels of the three transcription factors (nrf2, P=0.017; AP1, P=0.045; MMP-3, P=0.033) and on the activation of NF-κB (Fig. 4). These data suggested that AA may protect human chondrocytes from injuries induced by H₂O₂ by exerting regulatory roles in the antioxidant response via the protein kinase C (PKC)/nrf2, NF-κB and c-Jun N-terminal kinase (JNK)/AP1 signaling pathways, and in the inflammatory process. In other words, the effects of AA may be mediated via multiple regulatory pathways in C28/I2 cells when exposed to oxidative stress.

Discussion

Oxidative stress is regarded as a major cause of the degradation of chondrocytes and articular cartilage, which results in the pathogenesis of OA and the aging of cartilage (9,10,16). Several previous reports have focussed on the various strategies used to resist oxidative stress (14,28). Exploiting antioxidant treatment has been demonstrated as a promising strategy for the protection of chondrocytes against oxidative stress. Antioxidants were identified to exert cytoprotective effects in vitro and in vivo (29,30). AA, a widely used antioxidant, was revealed to delay the progression of age-associated diseases, which are highly susceptible to oxidative stress (19,21). However, it is unknown whether AA can provide any resistance to oxidative stress for the protection of chondrocytes. The present study hypothesized that AA may confer protective effects against chondrocyte damage, which is induced by H₂O₂. By examining the apoptotic rate, viability and senescence of chondrocytes, AA was revealed to effectively reduce H₂O₂-induced damage by attenuating the increase of apoptosis, loss of viability and increase of senescence (Fig. 1 and 2). Graeser et al (23) reported that administering a single dose of AA (10-50 µM) failed to protect chondrocytes from the damage induced by t-Butyl hydroperoxide. By contrast, Sharma et al (24) found that AA supplementation reduced the morphological and biochemical degeneration of chondrocytes in vivo. Notably, another previous report revealed that lower concentrations of AA did not protect against oxidative stress in retinal pigment epithelium cells until the concentration of AA reached 100 µM (31). The present study surmised that any

Figure 4. AA altered the expression levels of multiple transcription factors in C28/I2 cells under conditions of oxidative stress. The C28/I2 cells were treated accordingly and the expression of nrf2 was determined by (A) RT-qPCR and (B) western blotting. The total protein expression levels of NF-κB and pNF-κB were also measured by western blotting. β-actin was used as an endogenous control. The (C) API and (D) MMP-3 genes were detected by RT-qPCR. The 2−ΔΔCt method was used for relative quantification of the data and the data from three individual experiments were normalized, and statistically analyzed by analysis of variance. Significant differences were denoted as *P<0.05 and **P<0.01. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; p, phosphorylated; N, normal C28/I2 cells; C, cells treated with 100 µM H₂O₂ for 4 h; AA100, cells preincubated with 100 µM AA for 24 h and treated with 100 µM H₂O₂ for 4 h; AA200, cells preincubated with 200 µM AA for 24 h and treated with 100 µM H₂O₂ for 4 h.
resistance to oxidative stress exerted by AA was likely to be associated with the concentration of AA.

Collagens and proteoglycans (in chondrocytes, Col2a1 and Agc1 are expressed) are the predominant constituents of the extracellular matrix, which are important in chondrocytes competing against various stress factors (32,33). Previous studies confirmed that H$_2$O$_2$ reduces the level of proteoglycans in chondrocytes (15,34,35). In the present study, the data supported that of previous studies by identifying that Col2a1 and Agc1 are, respectively, the major collagen and proteoglycan expressed by chondrocytes (Fig. 3A-B). In addition, the present results have also demonstrated that the administration of AA efficiently counteracts the inhibition of Col2a1 and Agc1 by H$_2$O$_2$ in C28/12 cells. In normal cartilage, the differentiation index is high as a result of the large quantity of Col2a1 and low quantity of Col1a1 that are present. An augmented differentiation index is indicative of the active maintenance of the hyaline phenotype of chondrocytes (36,37). By contrast, an upregulated expression of Col1a1 or a decline in the differentiation index represented the formation of fibrocartilage rather than hyaline cartilage (37,38). The present study revealed that H$_2$O$_2$ not only upregulated the expression of the Col1a1 gene, but also inhibited the expression of Col2a1 (Figs. 3A, C and D). Furthermore, AA pretreatment inhibited the upregulation of the expression of the Col2a1 gene and downregulation of Col2a1 induced by H$_2$O$_2$ (Figs. 3A, C and D). Consequently, these findings demonstrated that AA not only stimulates the expression of collagens and proteoglycans, but also inhibits the differentiation of chondrocytes into fibrocartilage.

Several response transcription factors and signaling pathways, including MMP-3 (39), NF-κB (13), the PKC/Nrf2 pathway (40) and the JNK/AP1 signaling pathway (41,42), have been demonstrated to exert key effects on the protection against environmental and multiple cellular stresses in chondrocytes (13,39-42). Theoretically, the specific regulation of these response transcription factors can cause various changes in pathologies and physiologies. Previous studies have identified the crosstalk between AA and MMP-3 in non-Hodgkin's lymphoma (43), the AA-mediated inhibition of NF-kB leading to the suppression of liver fibrosis (44), the regulatory role of AA in the PKC/Nrf2 pathway (45) and a moderate AA-mediated attenuation of the elevated levels of the API1 gene induced by H$_2$O$_2$ in the retinal pigment epithelium (31). These findings demonstrate that AA may affect multiple signaling pathways and that the regulatory mechanism in which AA is involved is highly dependent on the micro-environment. In order to identify the transcription factor(s), which AA modulates, four response transcription factors were selected, including MMP-3, NF-κB, the PKC/Nrf2 pathway and the JNK/AP1 signaling pathway. AA was revealed to repress the elevated transcriptional activities of the response transcription factors induced by H$_2$O$_2$, lowering them to basal activities (Fig. 4). This demonstrated that AA can regulate multiple factors and signaling pathways. These findings also revealed how promising novel therapeutic methods, which lack any substantial untoward effects due to the inhibition of any given transcription factors may be acquired.

Overall, the present study demonstrated that AA protected cultured chondrocytes from apoptosis, loss of viability and an increase in senescence under conditions of H$_2$O$_2$-induced oxidative stress in vitro. In addition, AA not only stimulated the expression of collagens and proteoglycans, but also inhibited the differentiation of chondrocytes in the presence of oxidative stress. The effects of AA were mediated by multiple regulatory pathways (or factors). Future studies will focus on improving the efficacy of AA for alleviating damage to chondrocytes induced by oxidative stress, and an investigation of AA pretreatment in vivo.

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References


