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H_2O_2 INDUCES APOPTOSIS OF RABBIT CHONDROCYTES VIA BOTH THE EXTRINSIC AND THE CASPASE-INDEPENDENT INTRINSIC PATHWAYS

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Osteoarthritis (OA), one of the most common joint diseases with unknown etiology, is characterized by the progressive destruction of articular cartilage and the apoptosis of chondrocytes. The purpose of this study is to elucidate the molecular mechanisms of H_2O_2-mediated rabbit chondrocytes apoptosis. CCK-8 assay showed that H_2O_2 treatment induced a remarkable reduction of cell viability, which was further verified by the remarkable phosphatidylserine externalization after H_2O_2 treatment for 1 h, the typical characteristics of apoptosis. H_2O_2 treatment induced a significant dysfunction of mitochondrial membrane potential ($\Delta\Psi m$), but did not induce casapse-9 activation, indicating that H_2O_2 treatment induced caspase-independent intrinsic apoptosis that was further verified by the fact that silencing of AIF but not inhibiting caspase-9 potently prevented H_2O_2-induced apoptosis. H_2O_2 treatment induced a significant increase of caspase-8 and -3 activation, and inhibition of caspase-8 or -3 significantly prevented H_2O_2-induced apoptosis, suggesting that the extrinsic pathway played an important role. Collectively, our findings demonstrate that H_2O_2 induces apoptosis via both the casapse-8-mediated extrinsic and the caspase-independent intrinsic apoptosis pathways in rabbit chondrocytes.

Keywords: Chondrocytes; apoptosis; H_2O_2; caspases; AIF.

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1. Introduction

Osteoarthritis (OA), a common degenerative disease of human articular cartilage, is characterized by extracellular matrix damage and an important loss in tissue cellularity.[1] As the only cell-type resident in normal mature cartilage, chondrocytes play a key role in the synthesis and maintenance of articular cartilage extracellular matrix.[2] Although the prevalence of OA is increased in aged individuals and a direct correlation between chondrocyte apoptosis and cartilage degradation secondary to OA has been demonstrated,[3] the molecular mechanism underlying the apoptosis of chondrocytes is not well defined.

There are two independent apoptotic pathways for the induction of chondrocyte apoptosis: the Fas and the NO pathways.[4] It is reported that expression levels of both Fas and FasL appears to be elevated in synovial fluid from patients with OA, and Fas expression was elevated in OA cartilage.[5] Similarly, the expression of caspase-3 and -8 has been shown to be increased in human osteoarthritic cartilage and animal model in molecular studies.[6,7]

There is growing evidence that overproduction of reactive oxygen species (ROS) plays a pivotal role in promoting articular cartilage dysfunction and degeneration. In response to various stimuli, chondrocytes have the ability to produce all kinds of ROS, including hydrogen peroxide (H_2O_2), superoxide anion and hydroxyl radicals. As the predominant site for intracellular ROS production and a prime target for oxidative damage, mitochondria are considered to be involved in the apoptosis of chondrocytes.[8] Moreover, recent studies demonstrated that chondrocytes in OA, as compared with normal chondrocytes, showed a significant decrease in activity of the mitochondrial respiratory chain complexes as well as a reduction in the mitochondrial membrane potential ($\Delta\Psi m$).[9,10] In addition, H_2O_2 mediates the inflammatory response involved in the matrix metalloproteinases degradation, thereby inhibiting the synthesis of collagen and proteoglycans by the NO pathways.[11,12]

To determine whether H_2O_2 is an important factor involved in the pathology of OA, this report is designed to explore the molecular mechanism of H_2O_2-induced chondrocyte apoptosis. Our findings demonstrate that the caspase-8-mediated extrinsic and the AIF-mediated caspase-independent intrinsic pathways play an important role in H_2O_2-induced chondrocyte apoptosis.

2. Material and Methods

2.1. *Cell culture and plasmid transfection*

Chondrocytes were isolated from 6 weeks old New Zealand rabbits weighing approximately 2 kg (Experimental Animal Center of Guangzhou province, China) as described.[13] Cartilage collected aseptically from the bilateral joints of the knees, hips and shoulders were minced into small pieces, treated with 0.015% trypsin (Gibco BRL, Gaithersburg, MD) for 30 min, and subsequently digested with 0.025% collagenase type II (Invitrogen, California, USA) in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, USA) in an orbital shaker at 37°C for 4−6 h. The digested tissues were centrifuged at 3000 rpm for 5 min. The supernatant layer produced following centrifugation was discarded and the precipitates were resuspended in DMEM with 10% fetal bovine serum (FBS; Sijiqing, Hangzhou, China), then the cell suspension was filtered through a 300 mm metal mesh filter to remove matrix debris. After centrifuged, the resulting cell pellet was cultured in DMEM supplemented with 10% FBS. Cells were cultured in this growth medium as monolayer cultures at 37°C and 5% CO_2. The growth medium was changed every other day. Subcultures were performed with trypsin, and cells in the first to second passage were used.

2.2. *Materials*

H_2O_2 was from Hengjian (Guangdong, China). Z-VAD-fmk (a pan-caspase inhibitor), Z-DQMD-fmk (caspase-3 specific inhibitor), Z-IETD-fmk (caspase 8 specific inhibitor) and Z-LEHD-fmk (caspase-9 specific inhibitor) were obtained from BioVision (San Francisco, USA). N-acetylcysteine (NAC), Hoechst 33258, RNase A and propidium iodide (PI) were obtained from Sigma (St. Louis, USA). Lysotracker was purchased from Invitrogen (California, USA). Antibodies of caspase-8, cas-pase-9, Bid, FasL, Cytoc and β-actin were all from Cell Signaling Technology (Cell Signaling, Beverly, Massachusetts). All the secondary antibodies were supplied by Molecular Probes (Eugene, Oregon). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, USA).

2.3. *Assay of cell viability and apoptosis*

Cell viability was assessed by Cell Counting Kit-8 (CCK-8, Dojindo, Japan) assay as described

previously.[14] All experiments were performed in quadruple occasions. Cell apoptosis detection was performed by flow cytometry (FCM) analysis using Annexin V-FITC/PI apoptosis detection kit (Bender Medsystems, Vienna, Austria) as previously described[13] and 5000 events were recorded for each FCM analysis. Apoptotic cells are those stained with Annexin V+/PI− (early apoptotic) plus Annexin V+/PI+ (late apoptotic cell).

2.4. *Measurement of mitochondrial membrane potential ($\Delta\Psi m$)*

Rhodamine 123 (Rho 123; Sigma, St. Louis, USA) was used to analyze $\Delta\Psi m$ by FCM as previously described.[15] Briefly, cells were harvested and stained with 10 μM Rho 123 for 30 min at 37°C in the dark, and then washed with PBS twice and subsequently assayed by FCM. Results were expressed as the proportion of cells with low Rho 123 fluorescence indicating the loss of $\Delta\Psi m$. Cells strained with Rho 123 and tetramethylrhodamine methyl ester (TMRM; Sigma, St. Louis, USA) were treated with H_2O_2 and imaged by the confocal laser scanning microscope system (LSM510-ConfoCor2) (Carl Zeiss, German) to assess the series changes in $\Delta\Psi m$. All the quantitative analysis of the fluorescence images was performed by Zeiss Rel3.2 image processing software (Zeiss, Jena, Germany). The. excitation wavelengths were 488 nm for Rho 123, 543 nm for TMRM. The emission detection filters were band pass 505−570 nm for Rho 123 band pass 565−615 IR for TMRM.

2.5. *Fluorometric determination of caspase enzymatic activation*

Activities of caspase-8, -9 or -3 were measured using Ac-DEVD-AFC, Ac-IETD-AFC and Ac-LEHD-AFC (Alexis, Switzerland), respectively, according to the manufacturer's instructions. Collected cells were washed twice with cold PBS, and were lysed in lysis buffer (25 mM HEPES (pH 7.4), 0.1% TritonX-l00, 10% glycerol, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin and 10 mg/ml leupeptin). The extract was transferred to a microlon ELISA plate with 100 μl/well. Proluminescence caspase-8, -9 or -3 substrates were added to extract in each well of a microlon ELISA plate at 100 μM final concentration at room temperature. Caspases activity was measured continuously by monitoring the release of fluorogenic AFC at 37°C. In the presence of caspase-8, -9 or -3 aminoluciferin was liberated from the proluminescence substance and utilized as a substrate for the luciferase reaction. The resultant luminescence in relative light units was measured by using auto microplate reader (infinite M200, Tecan, Austria). The excitation wavelength of AFC was 405 nm and the emission detection channel was 478−535 nm. The reaction mixture without protein was referred to the background and was subtracted from samples.

2.6. *Western blotting analysis*

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton-100, 1 mM PMSF and protease inhibitor cocktail set I). After removing insoluble material by centrifugation for 5 min at 12, 000×g, the protein concentration was estimated in the supernatant using the Bio-Rad protein assay (Bio-Rad, Munich, Germany) according to the manufacturer's protocol. Protein was separated by SDS-PAGE under reducing conditions before transferring onto nitrocellulose membranes (Millipore, Billerica, USA). Blots were blocked in TBST buffer containing 5% non-fat dry milk for 1 h at room temperature. The membrane was incubated overnight at 4°C with the respective primary antibodies. After repeated washings with TBST, the membranes were incubated with the secondary antibody for 1 h at room temperature before continuing to wash with TBST. Detection was performed using the Odyssey Infrared Imaging System (LI-COR Biosciences, Nebraska, USA). Equal loading was verified by antibodies against β-Actin.

2.7. *Statistical analysis*

Results were expressed as mean ± standard deviation (SD). ANOVA was used to compare the mean differences between samples using the statistical software SPSS version 16.0 (SPSS, Chicago). Throughout the work, p values less than 0.05 were considered to be statistically significant.

3. Result

3.1. *H_2O_2 triggers chondrocytes apoptosis*

Exposure of chondrocytes to different concentrations of H_2O_2 for 2 h induced a dose-dependent cytotoxicity, especially treatment with 0.3 mM of

H_2O_2 for 2 h induced a remarkable decrease of cell viability [see Fig. 1(a)]. We next treated chondrocytes with 0.3 mM of H_2O_2 for 0, 30 and 60 min, and CCK-8 assay showed that compared with control, the inhibition of chondrocytes for 30 and 60 min both nearly reached 64% [see Fig. 1(b)]. Approximately, 0.3 mM of H_2O_2 was adopted in the following experiments without indicated concentration. Hoechst 33258, a sensitive fluorochrome to DNA, was used to assess the changes on the H_2O_2-induced nuclear morphology. Normal cells exhibited diffused staining of the chromatin, while the cells after exposure to H_2O_2 for 30 min underwent nuclear concentration and DNA fragmentation [see Fig. 1(c)], the typical characteristics of apoptosis.

To further confirm whether H_2O_2-induced cytotoxicity is associated with apoptosis, we used FCM to examine the integrity of cell membrane and the externalization of phosphatidylserine (PS) by using Annexin V/PI staining assay. Our data showed that the percentage of cells with PS externalization were 16.8% and 25% at 30 and 60 min after H_2O_2 treatment [see Fig. 1(d)], which was significantly inhibited by NAC pretreatment, implying that H_2O_2 treatment induced cell death dominantly via an apoptotic manner.

3.2. *H_2O_2 induces a loss of mitochondrial membrane potential ($\Delta\Psi m$)*

To ascertain whether the intrinsic pathway is involved in H_2O_2-induced apoptosis, FCM analysis was used to monitor the loss of $\Delta\Psi m$ by detecting the reduction of Rho 123 fluorescence. As showed in Fig. 2(a), H_2O_2 induced an increase of percentage of cells with low Rho 123 fluorescence intensity from 3.4% (Control) to 45.9% (H_2O_2), and pretreatment with NAC significantly inhibited the H_2O_2-induced loss of $\Delta\Psi m$.

Real-time dynamical fluorescence images were also performed to assess the dynamical changes in $\Delta\Psi m$ after H_2O_2 treatment using confocal fluorescence scanning microscope. The mitochondria distributed evenly in cytoplasm are clearly visualized as green streaks for control cells, but H_2O_2

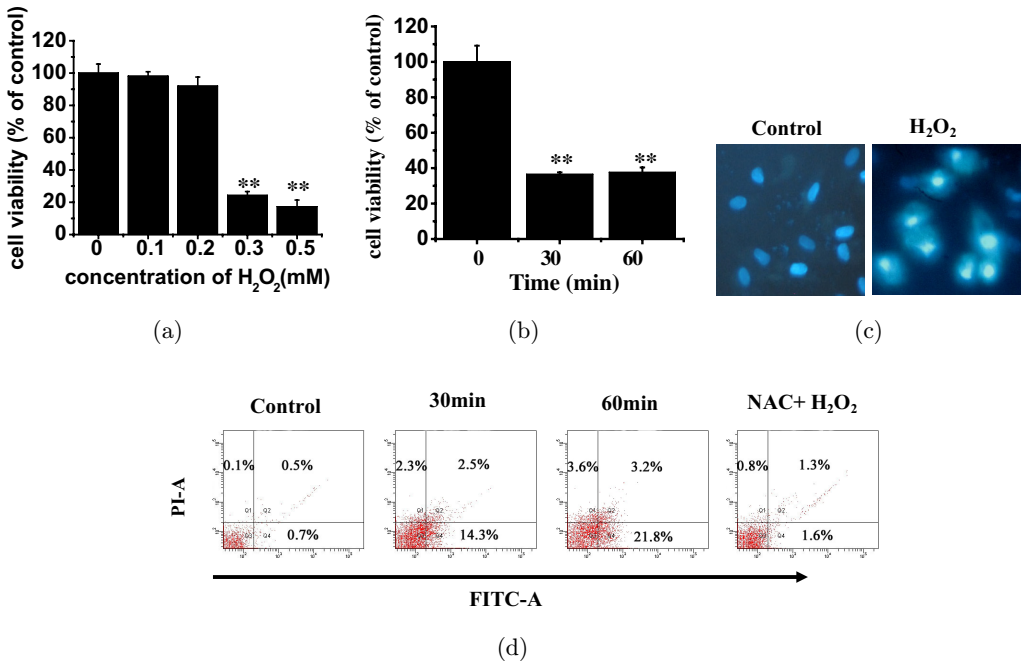

Fig. 1. H_2O_2 induces apoptosis in rabbit chondrocytes. (a) H_2O_2 induced concentration-dependent cytotoxicity assessed by CCK-8 assay. Cells were plated and treated with various concentration of H_2O_2 for 2 h. Data are mean ± SD ($n = 3$; ANOVA: $^{**}p < 0.01$, compared with control cells). (b) H_2O_2 induced time-dependent cytotoxicity assessed by CCK-8 assay. Cells were treated with 0.3 mM H_2O_2 for 0, 30 and 60 min, respectively. Data are mean ± SD ($n = 3$; ANOVA: $^{**}p < 0.01$, compared with control cells). (c) H_2O_2 induced nuclear concentration of cells stained with Hoechst 33258. The cells were treated with 0.3 mM of H_2O_2 for 30 min, and the nuclear morphology was detected by Hoechst 33258 staining and examined by fluorescence microscope. Magnification × 400. (d) FCM analysis of H_2O_2-induced apoptosis by fluorescence-activated sorting analysis of Annexin V-FITC/PI. The cells treated with different conditions were stained with 5 μl of Annexin V-FITC and 10 μl of PI (10 μg/ml), respectively before being analyzed.

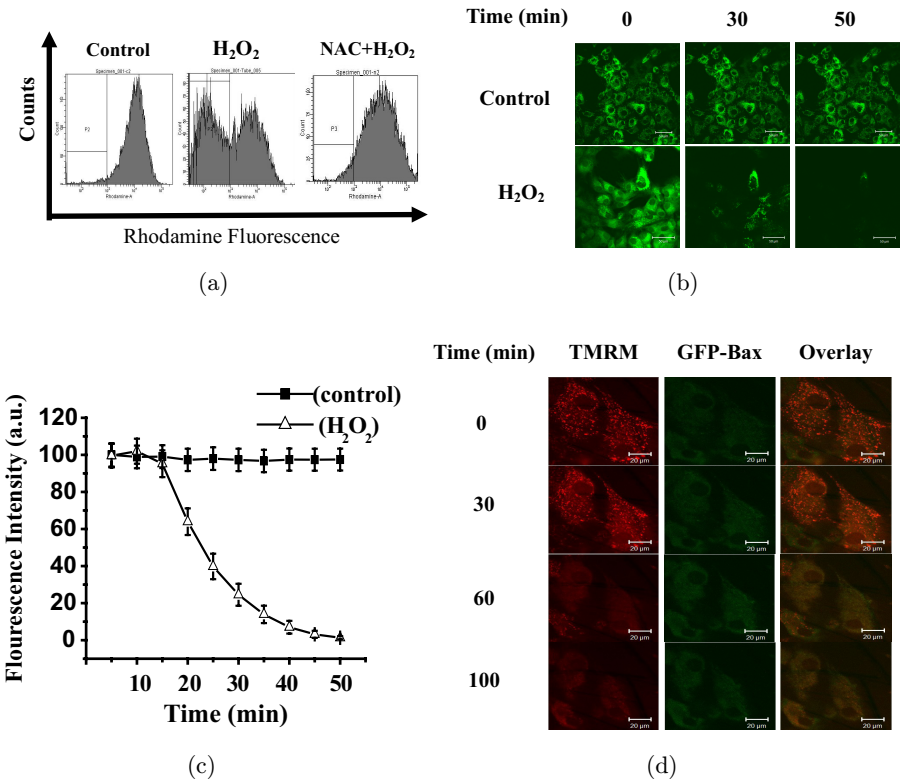

Fig. 2. H_2O_2 induces a loss of mitochondrial membrane potential. (a) FCM analysis of H_2O_2-induced loss of mitochondrial membrane potential ($\Delta\Psi m$). The cells stained with 10 μM Rho 123 for 30 min were treated with H_2O_2 in the presence or absence of NAC for 30 min. The number in left quadrant represents the percentage of cells with reduction in $\Delta\Psi m$. (b) Dynamics of fluorescence images of cells stained with Rho 123 by confocal time-lapse fluorescence microscopy. Scale Bar: 50 μm. (c) Dynamics of fluorescence intensities of Rho 123 inside live cells corresponding to (b). (d) Spatio-temporal distribution of Bax and mitochondria inside live cells in control and H_2O_2-treated cells, respectively by confocal microscopy imaging. Cells were transfected with GFP-Bax and stained with TMRM to fluorescently label mitochondria and Bax, respectively. Scale Bar: 20 μm.

treatment induced obvious fluorescence reduction [see Fig. 2(b)] and the corresponding dynamics of $\Delta\Psi m$ after H_2O_2 treatment was showed in Fig. 2(c) ($n = 5$), further demonstrating that the intrinsic pathway was involved in H_2O_2-induced apoptosis. To assess whether proapoptotic protein Bax was involved in the H_2O_2-triggered intrinsic pathway, we used time-lapse confocal dual channels fluorescence microscope to monitor the spatio-temporal distribution of both Bax and mitochondria in live chondrocytes after H_2O_2 treatment. The chondrocytes expressing Bax-GFP was stained with TMRM to probe mitochondria. Our data showed that although exposure of chondrocytes to H_2O_2 for 60 and 100 min induced serious damage to mitochondria, Bax is still diffused in the whole cell the same as that in healthy cells [see Fig. 2(d)], suggesting that Bax was not involved in the H_2O_2-induced apoptosis.

3.3. *Caspase-3 and -8 but not -9 are involved in H_2O_2-induced apoptosis*

To determine whether caspase-8, -9 and -3 were involved in H_2O_2-induced apoptosis of chondrocytes, fluorometric substrate assay was used to assess the activities of caspase-8, -9 and -3 during H_2O_2-induced apoptosis. As showed in Fig. 3(a), H_2O_2 treatment induced a significant increase in the activation levels of caspase-3 and -8 but not caspase-9. In addition, pretreatment with pan caspase inhibitors or caspase-8/3 but not -9 inhibitor for 1 h significantly prevented H_2O_2-induced apoptosis [see Fig. 3(b)], suggesting that caspase-8 and -3 but not -9 were involved in H_2O_2-induced apoptosis. Western blotting analysis showed that H_2O_2 treatment significantly down-regulated the expression level of caspase-3 and -8 but not caspase-9 [see Figs. 3(c) and 3(d)]. In addition, H_2O_2

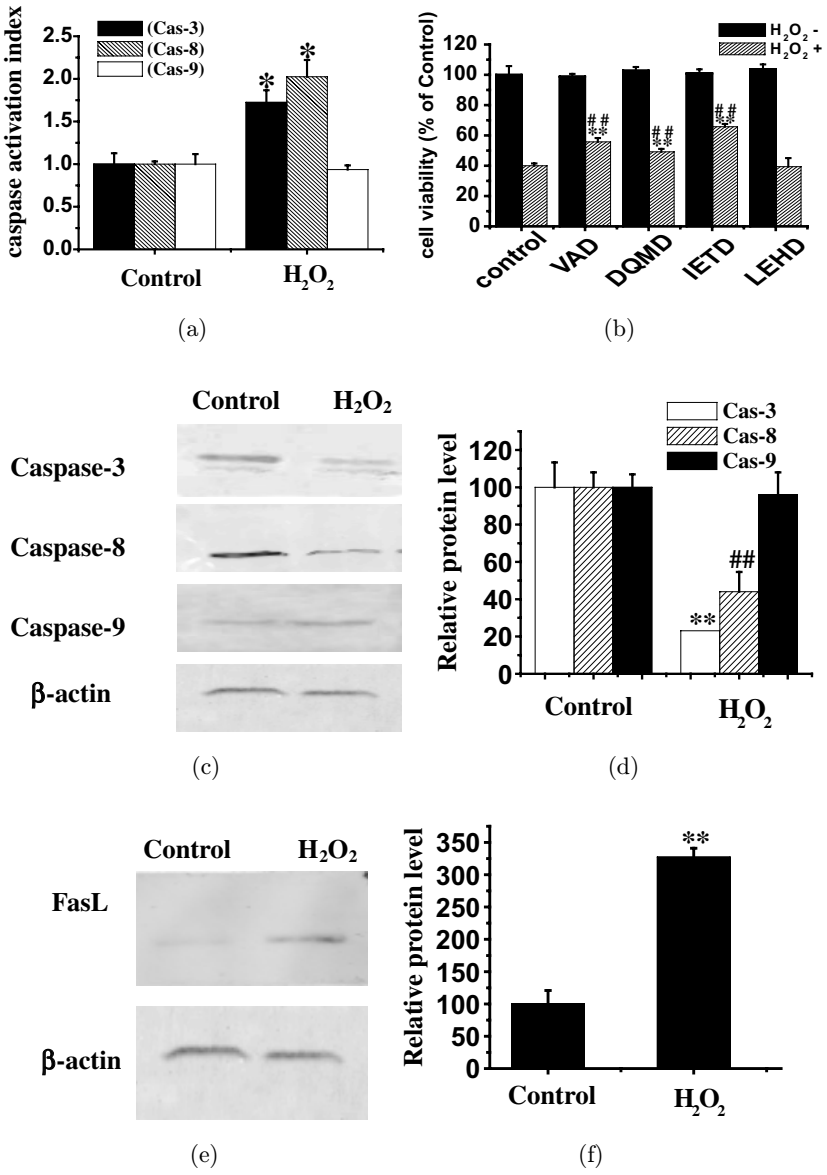

Fig. 3. Caspase-8 and -3 but not caspase-9 is involved in H_2O_2 induced chondrocytes apoptosis. (a) H_2O_2 treatment induced the activation of caspase-3 and -8 but not caspase-9 by fluorometric determination of caspase enzymatic activation. Data are mean ± SD ($n = 3$; ANOVA: $^*p < 0.05$, compared with control cells). (b) H_2O_2-induced caspase-8- and -3-dependent cytotoxicity assessed by CCK-8 assay. Cells were treated with 0.3 mM H_2O_2 for 2 h in the presence or absence of 5 mM NAC, zVAD-fmk, zDQMD-fmk, zIETD-fmk and zLEHD-fmk, respectively. Data are mean ± SD ($n = 3$; ANOVA: $^{**}p < 0.01$, compared with blank control cells; $\#p < 0.05$ and $\#\#p < 0.01$, compared with the H_2O_2-treated cells). (c) Western Blotting analysis of the expression levels of caspase-3, -8 and -9 for the control and H_2O_2-treated cells. (d) Quantitative analysis of caspase-3, -8 and -9 levels. Data are mean ± SD ($n = 3$; ANOVA: $^{**}p < 0.01$, $^{\#\#}p < 0.01$, compared with control cells). (e) H_2O_2 potently up-regulated the expression level of FasL determined by Western blotting analysis. β-actin served as the loading control. (f) Quantitative analysis of FasL level. Data are mean ± SD ($n = 3$; ANOVA: $^{**}p < 0.01$, compared with control cells).

treatment significantly up-regulated the expression level of FasL [Figs. 3(e) and 3(f)]. These data demonstrate that the caspase-8-mediated extrinsic pathway plays an important role in H_2O_2-induced apoptosis.

3.4. *H_2O_2 induces chondrocytes apoptosis via an AIF-mediated intrinsic pathway*

To ascertain the role of AIF in H_2O_2-induced apoptosis, we used CCK-8 assay to assess the effect of

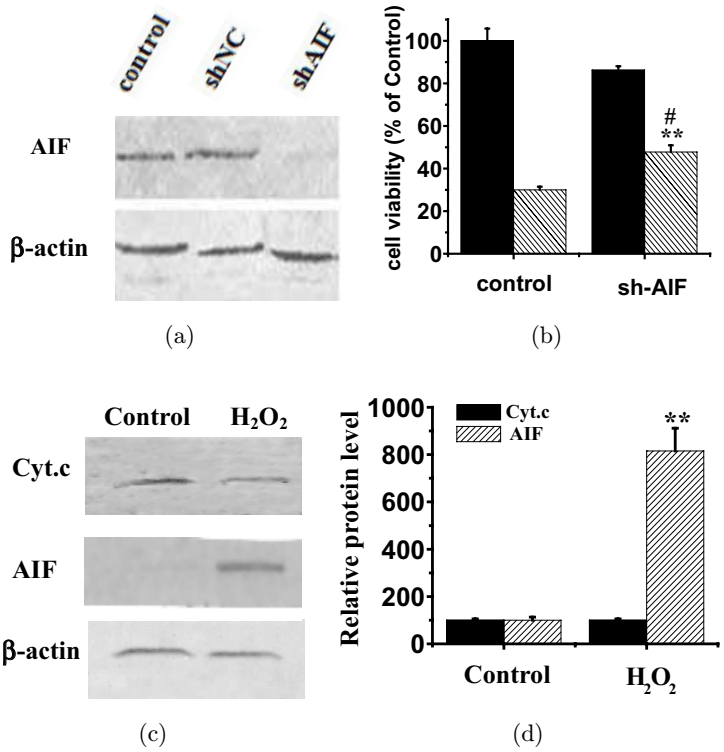

Fig. 4. AIF plays an important role in H_2O_2-induced apoptosis. (a) The effect of silencing AIF (shAIF) verified by Western blotting analysis. (b) Silencing AIF largely attenuated H_2O_2-induced cell death assessed by CCK-8 assay. The shAIF was used as a negative control. Data are mean ± SD ($n = 3$; ANOVA: $^{**}p < 0.01$, compared with control cells, $\#p < 0.01$, compared with H_2O_2 treatment alone cells). (c) H_2O_2 induced the up-regulation expression levels of AIF but not Cyt.c by Western blotting analysis. β-actin served as the loading control. (d) Quantitative analysis of Cyt.c and AIF expression levels. Data are mean ± SD ($n = 3$; ANOVA: $^{**}p < 0.01$, compared with control cells).

silencing AIF by shAIF on H_2O_2-induced cytotoxicity. Western blotting analysis demonstrated that shAIF potently down-regulated the expression level of AIF [see Fig. 4(a)], and that silencing AIF largely attenuated H_2O_2-induced cell death compared with the cells treated with H_2O_2 alone [see Fig. 4(b)]. Western blotting analysis showed that H_2O_2 treatment remarkably up-regulated the expression of AIF but not cytochrome c [see Figs. 4(c) and 4(d)], further confirming the important role of AIF but not cytochrome c in H_2O_2-induced apoptosis.

4. Discussion

OA is the most common aged-related cartilage and joint disease leading to joint instability, chronic pain and disability that may require lifelong therapy. Growing evidences suggest that chondrocytes apoptosis plays important roles in the degradation of articular cartilage in human OA.[2,16] H_2O_2, a membrane-permeable reagent physiologically produced in large amounts by chondrocytes, has been widely used to assess the effects of ROS on chondrocytes *in vitro*. Herein, we demonstrate that H_2O_2 induces chondrocytes apoptosis via the caspase-8-mediated extrinsic and AIF-mediated caspase-independent intrinsic pathways.

Accumulation of intracellular ROS is generally caused by increasing ROS generation and decreasing ROS degradation. Besides the endogenous generation of ROS, many environmental stimuli including cytokines, ultraviolet (UV) radiation, chemotherapeutic agents, hyperthermia and even growth factors have the ability to induce a generation of high levels of ROS that can perturb the normal redox balance and disrupt the cartilage homeostasis. Compared to young adult rats, increased levels of ROS were detected in cartilage from old.[17] Accumulating evidences also suggest that the levels of antioxidant enzymes, including enzymatic scavengers SOD, catalase and glutathione peroxidase, are present at lower levels with aging[17,18] and in OA cartilage.[19] These may be reasons for the incidence of OA in the elderly which is more than young people.[16]

Our data that H_2O_2 less than 0.3 mM nearly has no influence on chondrocytes survival, but 0.3 mM or higher H_2O_2 almost completely induced chondrocytes apoptosis within 30 min [see Fig. 1(a)] demonstrate that a small quantity of ROS does not create serious damage to chondrocytes due to the ability of oxygen radical scavenging in cells. As long as the levels of ROS is under the control of the cellular antioxidant defenses system, ROS can be considered as regulatory factors for proper functioning of cellular processes. However, when ROS accumulated to a certain extent that exceeds the antioxidant capacities of the cell, an oxidative damage occurs that triggers a cascade of harmful events including DNA damage, protein nitration, lipid peroxidation and activation of matrix metalloproteinases (MMPs), contributing to the structural and functional cartilage damages. Damaged mitochondria in ROS overloading cells are thought to release more ROS and set in motion a vicious cycle of increasing DNA damage leading to increased ROS production that in turn leads to more DNA damage. The various inflammatory mediators have been found to be increased in OA, including IL-1, IL-6, TNF-α and other cytokines can all stimulate the further production of ROS and ROS in turn can be involved in the increased production of these cytokins.[20] Therefore, the accumulation of ROS and apoptosis of chondrocytes may play important roles in the development of OA.[21]

Our observations that H_2O_2 treatment remarkably activate caspase-3 and -8 but not caspase-9 [see Fig. 3 (a)], and pretreatment with zVAD-fmk, zDQMD-fmk, especially zIETD-fmk but not zLEHD-fmk significantly prevent chondrocytes apoptosis [see Fig. 3 (b)] demonstrate that caspase-8/3 mediate H_2O_2-induced chondrocytes apoptosis. In combination with the data that H_2O_2 induces a significant activation of FasL [see Fig. 3(e)], we convince that the caspase-8-mediated extrinsic pathway plays an important role in H_2O_2-induced chondrocytes apoptosis. It is well known that caspase-8-mediated cleavage of the BH3-only protein Bid into a truncated protein (tBid) and subsequent translocation of tBid to mitochondria lies at the nexus of extrinsic and intrinsic pathway.[22] However, the spatio-temporal dynamical distribution of Bid inside live chondrocytes expressing Bid-GFP during H_2O_2-induced apoptosis (data not shown) showed that H_2O_2 treatment did not induce Bid cleavage and after translocation to the mitochondria, indicating that Bid does not participate in the H_2O_2-induced intrinsic pathway.

Our observations that H_2O_2 treatment induced remarkable loss of the mitochondrial membrane potential (see Fig. 2) and did not induce caspase-9 activation and cytochrome c release from mitochondria indicate that H_2O_2 treatment may trigger a caspase-independent intrinsic pathway. The fact that silencing AIF by shRNA largely prevented the H_2O_2-induced apoptosis (see Fig. 4) suggests that AIF plays an important role in H_2O_2-induced apoptosis. It has been reported that H_2O_2 treatment enhanced intracellular Ca^{2+} and preincubation with the intracellular Ca^{2+} chelator protected chondrocyte against H_2O_2-induced apoptosis.[23,24] The elevation of Ca^{2+} caused calpain activation and subsequent release of AIF from mitochondria. In addition to inducing an increase of intracellular Ca^{2+} concentration, ROS can also lead to oxidative modification of AIF.[25−27] Therefore, it is reasonable to convince that H_2O_2 triggers an AIF-mediated caspase-independent intrinsic pathway to mediate apoptosis.

In conclusion, our data strongly demonstrate that H_2O_2 induces apoptosis of rabbit chondrocytes via the caspase-8-mediated extrinsic and AIF-mediated caspase-independent intrinsic pathways. However, the molecular mechanism by which H_2O_2 neutralizes the interaction between pro-apoptosis and anti-apoptosis proteins is unknown, which will be the task of future investigations. Understanding the basic mechanisms by which excessive ROS affect cell function at the molecular level may provide the knowledge needed to develop novel preventative treatments for OA.

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