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Local intra-articular injection of resveratrol delays cartilage degeneration in C57BL/6 mice by inducing autophagy via AMPK/mTOR pathway

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ABSTRACT

Autophagy is an essential cellular homeostasis mechanism that was found to be compromised in aging and osteoarthritis (OA) cartilage. Previous studies showed that resveratrol can effectively regulate autophagy in other cells. The purpose of this study was to determine whether the chondroprotective effect of resveratrol was related to chondrocyte autophagy and to elucidate underlying mechanisms. OA model was induced by destabilization of the medial meniscus (DMM) in 10-week-old male mice. OA mice were treated with resveratrol with/without 3-MA for 8 weeks beginning 4 weeks after surgery. The local intra-articular injection of resveratrol delayed articular cartilage degradation in DMM-induced OA by OARSI scoring systems and Safranin O-fast green. Resveratrol treatment increased Unc-51-like kinase1, Beclin1, microtubule-associated protein light chain 3, hypoxia inducible factor-1α, phosphorylated AMPK, collagen-2A1, Aggrecan expressions, but decreased hypoxia inducible factor-2α, phosphorylated mTOR, matrix metalloproteinases13 and a disintegrin and metalloproteinase with thrombospondin motifs 5 expressions. The effects of resveratrol were obviously blunted by 3-MA except HIF and AMPK. These findings indicate that resveratrol intra-articular injection delayed articular cartilage degeneration and promoted chondrocyte autophagy in an experimental model of surgical DMM-induced OA, in part via balancing HIF-1α and HIF-2α expressions and thereby regulating AMPK/mTOR signaling pathway.

1. Introduction

Osteoarthritis (OA) is the most common form of rheumatic disease, leading ultimately to chronic pain, restriction of joint mobility, and disability. At the cellular level, OA is characterized by a loss of tissue cellularity and extracellular matrix (ECM) damage.[1] Chondrocytes, the only cell population of the articular cartilage, are capable of responding to structural changes in the surrounding ECM by maintaining the dynamic equilibrium between production of the ECM and its enzymatic degradation. Therefore, maintaining the health of chondrocytes is an important factor for preventing cartilage degeneration.

Autophagy is a major physiologic mechanism that targets altered and dysfunctional cytosolic macromolecules, membranes, and organelles for delivery to lysosomes for degradation and recycling. Autophagy can be activated in response to various stress stimuli, including nutrient deprivation, growth factor depletion and hypoxia. Autophagy also exists in normal adult articular cartilage to maintain chondrocyte survival and biosynthetic function. The increased autophagy is an adaptive response to protect cells from stresses, and autophagy regulates OA-like gene expression changes through the modulation of apoptosis and reactive oxygen species.[1] Several studies have demonstrated that autophagy is involved in chondrocyte depletion during OA progression.[2–4]

Abbreviations: ADAMTS5, a disintegrin and metalloproteinase with thrombospondin motifs 5; AMPK, AMP activated protein kinase; DMM, destabilization of the medial meniscus; HIF-1, hypoxia inducible factor-1; LC3, microtubule-associated protein light chain 3; 3-MA, 3-methyladenine; mTOR, mammalian target of rapamycin; MMP13, Matrix metalloproteinases13; OA, osteoarthritis; ULK1, Unc-51−like kinase1.

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Autophagy is constitutively activated in normal cartilage, and compromised with aging and precedes cartilage cell death and structural damage.[5] Based on the extensive cell-protective functions of autophagy, it is plausible that autophagy plays a protective role in chondrocytes under stresses, and may prevent the articular cartilage degeneration in OA. Chondrocyte autophagy is stimulated by hypoxia inducible factor-1 (HIF-1) dependent AMP activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) because of the micro-environmental changes.[6–8] In addition, HIF-2α is a potent regulator of autophagy in maturing chondrocytes and probably acts as a brake on the autophagy-accelerator function of HIF-1α.[9] Taken together, manipulation of HIF-1α and HIF-2α could represent a promising approach to the treatment of OA.

Resveratrol (3,4′,5-trihydroxystilbene) is an active ingredient from our food sources, such as grapes, peanuts, which has long been used in traditional Chinese medicine. Resveratrol has been shown to have protective properties against age-related diseases, such as diabetes and heart disease, and has been described as a chemopreventive agent against cancer.[10] Moreover, resveratrol can trigger autophagy in cells from different organisms, extend life span in nematodes, and ameliorate the fitness of human cells undergoing metabolic stress.[11] Our previous studies show that resveratrol significantly prevents the destruction of OA cartilage by activating SIRT1 and suppressing the expression of HIF-2α and catabolic factors.[12] But it has been unclear whether resveratrol ameliorates the destruction of OA cartilage by regulating autophagy.

Therefore, the objective of this study was to examine the effect of local intra-articular injection of resveratrol on articular cartilage autophagy and to determine whether resveratrol modulates AMPK/mTOR signaling pathway in a murine model of OA. To further confirm our hypothesis, we employed 3-methyladenine (autophagy inhibitor, 3-MA) to inhibit the autophagy pathway. Our results confirmed that intra-articular injection of resveratrol protects articular cartilage after the destabilization of the medial meniscus (DMM) surgery through the induction of autophagy in chondrocytes by balancing HIF-1α and HIF-2α, and thereby regulating AMPK/mTOR signaling pathway.

2. Materials and methods

2.1. Animals and experimental design

All animal procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of the People's Republic of China. The protocol was approved by the Committee on Animal Research and Ethics of Xi'an Jiaotong University (Xi'an, China) (the committee's reference number: 2012009). Male C57BL/6 mice (18–22 g) were purchased from Sino-British SIPPR/BK Lab. Animal Ltd. Forty, 10-week-old, male C57BL/6 mice were divided into four groups as follows: OA with vehicle injection (OA group, n = 10); OA with resveratrol treatment (Res group, n = 10); OA with resveratrol and 3-MA treatment (Res+3-MA group, n = 10); sham surgery (sham group, n = 10).

After one week of acclimation, the mice were anesthetized via intraperitoneal injection of pentobarbiturate (0.5 mg/10 g body weight). Experimental OA was induced by DMM in the right knee.[13] Briefly, the joint capsule was opened with an incision just medial to the patellar tendon and the medial meniscotibial ligament was sectioned with microsurgical scissors on the right knee of the mice. In sham surgery group, surgery was performed on right knee joints, but the ligaments were visualized but not transected. All mice were allowed to move freely within their cages after surgery.

2.2. Resveratrol and 3-MA treatment

Resveratrol (Sigma Aldrich, St. Louis, USA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mg/ml to generate a stock solution, which was further diluted in PBS to a final concentration of 12.5 μg/μl 3-MA was dissolved and diluted with the same method at a final concentration of 20 mM. From the 5th week after surgery, Res and Res+3-MA treatment groups received a 10 μl intra-articular injection of resveratrol. Intra-articular injection through the patellar tendon was 95 performed using a micro needle (U-100 insulin syringe, 30 G; Becton Dickinson, Fukushima, 96 Japan), as described previously.[14] In addition, Mice in Res+3-MA group were intraperitoneally injected with 3-MA 30 mg/kg (Sigma Aldrich, St. Louis, USA). The sham and OA groups received an injection of 10 μl PBS into the right knee joint. All injections were applied twice a week over a period of 8 weeks by starting 4 weeks postoperatively. The dosage and frequency of resveratrol and 3-MA were selected based on previous studies.[15–18] Mice were sacrificed at 12 weeks after DMM surgery. The knee joints were harvested, and the joint tissue was subjected to histological evaluation, gene and protein expression analyses.

2.3. Histopathological evaluation for articular cartilage degeneration

Four right knee joint from each group were fixed in 10% neutral buffered formalin for 24 h, decalcified in TBD-2 for 48 h, followed by paraffin embedding. Serial sections (4 μm) were cut. Sections were stained with safranin O and then graded by three independent observers using the Osteoarthritis Research Society International (OARSI) scoring system.[19] The scores are defined as follows: 0: normal; 0.5: loss of toluidine blue without structural changes; 1: small fibrillations without loss of cartilage; 2: vertical clefts extending from the articular surface down to the layer immediately below the superficial tangential zone with some loss of surface lamina; 3: vertical clefts/erosion extending down to the calcified articular cartilage comprising <25% of the quadrant width; 4: vertical clefts/erosion extending down to the articular calcified cartilage comprising 25–50% of the quadrant width; 5: vertical clefts/erosion extending down to the calcified articular cartilage comprising 50–75% of the quadrant; and 6: vertical clefts/erosion extending down to the calcified articular cartilage comprising >75% of the quadrant width. Three non-consecutive sections were scored in each mouse and the scorers were blinded to treatment.

2.4. Western blotting analysis

Cartilage tissue from each group was cut into 1-mm–thin slices, and 200–1000 mg of frozen cartilage was pulverized in a liquid nitrogen–cooled freezer mill for 2 cycles of 1.5 min at the rate of maximum impact frequency (n = 3). Proteins in cartilage were isolated using a total protein extraction kit (Pierce) according to the manufacturer's instructions. The protein concentration was determined via the bicinchoninicacid assay (Pierce) using BSA as a standard. Protein (20 μg) of each sample was heated to 100 °C for 5 min and then resolved on a 4–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. The proteins were transferred to methanol-wetted polyvinylidene difluoride (PVDF) membranes in Tris/Glycine transfer buffer. Subsequently, the membranes were blocked for 1 h at room temperature in blocking buffer (5% skim milk powder, 0.5% Tween-20 in tris-buffered saline; TBS). Blots were incubated with antibodies of ULK1, Beclin-1, LC3-I/II, HIF-1α, HIF-2α, AMPKα, p-AMPKα (phospho T183/T172), mTOR, p-mTOR (phospho S2448), COL2A1, Aggrecan, MMP13, ADAMTS5 and GAPDH (ab128859, ab62557, ab62721,

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