Resveratrol attenuated TNF- α -induced MMP-3 expression in human nucleus pulposus cells by activating autophagy via AMPK/SIRT1 signaling pathway

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Abstract

Resveratrol (RSV) is known to play a role of anti-TNF- α in a number of cell types. However, whether RSV modulates the effects of TNF- α on human nucleus pulposus (NP) cells is unknown. The purpose of this study is to investigate whether RSV regulates TNF- α -induced matrix metalloproteinase-3 (MMP-3) expression. Via quantitative real-time polymerase chain reaction (qRT-PCR) analysis, we found that MMP-3 expression induced by TNF- α was inhibited by RSV treatment. Depending on Western blot and qRT-PCR assay, we found that RSV induced autophagy in human NP cells, whereas inhibition of autophagy remarkably abolished the restraining role of RSV in the TNF- α -mediated up-regulation of MMP-3. Furthermore, RSV increased SIRT1 expression and SIRT1 knockdown significantly suppressed RSV-induced autophagy in NP cells. RSV also activated AMP-activated protein kinase (AMPK), while inhibition of AMPK notably abolished RSV-induced SIRT1 expression. Our data showed that RSV attenuated TNF- α -induced MMP-3 expression in human NP cells by activating autophagy via AMPK/SIRT1 signaling pathway. This new finding suggested that RSV might act as a novel preventive and therapeutic role in intervertebral disc degeneration.

Keywords: Resveratrol, TNF- α , nucleus pulposus cell, MMP-3, autophagy, SIRT1, AMPK

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Introduction

An estimated 60–80% of adults are known to be affected by low back pain (LBP) at least once in their lifetime.^{1–3} In addition to being a significant health hazard, LBP imposes a considerable economic burden on the affected families and the society at large. Intervertebral disc (IVD) degeneration is thought to be the leading contributor to LBP.⁴

Studies have implicated an increased expression of multiple inflammatory cytokines in the causation of IVD degeneration. Among these, TNF- α , a potent proinflammatory cytokine, was demonstrated as having an increased expression in the resident nucleus pulposus (NP) and annulus fibrosus (AF) cells of patients with IVD degeneration and herniation.^{5,6} TNF- α participates in IVD degeneration in several ways: by increasing the levels of other cytokines, by promoting inflammatory hyperalgesia, by inducing expression of a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS4), ADAMTS5, and matrix metalloproteinases (MMPs), and by inhibiting collagen and proteoglycan synthesis.^{6–10}

Resveratrol (RSV, trans-3,5,4'-trihydroxy-trans-stilbene) is a natural phytoalexin found in peanuts, grapes, and

several other plants. Recently, the anti-inflammatory, anti-aging, anticancer, cardioprotective, and articular cartilage protective effect of RSV have been demonstrated in different cell types.^{11–19} IVD degeneration is characterized by disc inflammation and cell senescence. Nucleus pulposus cells share several common characteristics with chondrocytes. There is sufficient rationale for assessing potential therapeutic use of RSV for the treatment of IVD.

Recently, RSV was shown to inhibit apoptosis of NP cells through activation of SIRT1, Akt, and autophagy.^{20,21} These findings indicate a potential role of RSV in the prevention and treatment of IVD degeneration. As TNF- α is known to be a initial driving factor of IVD degeneration, the aim of this study was to assess whether RSV has any inhibitory effect on TNF- α -induced catabolic processes in NP cells.

Material and methods

Materials

Recombinant human TNF- α was purchased from Peprotech (Rocky Hill, NJ, USA). The cell counting kit-8 (CCK-8) was obtained from Dojindo (Tokyo, Japan). The anti-light chain

(LC) 3, anti-Beclin-1, anti-phospho-AMPK, anti-AMPK, and anti- β -actin antibodies were obtained from Cell Signaling (Beverly, MA, USA). The anti-sirtuin 1 antibody was purchased from Santa Cruz (Dallas, TX, USA).

Human NP cell culture and treatment

Human NP cells and NP cell medium were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). NP cells were cultured and treated with TNF- α as described previously.²² For inhibitor treatment experiments, cells were pretreated with sirtinol, nicotinamide (nico), or compound C for 1 h prior to RSV treatment.

siRNA transfection

Small interfering RNA (siRNA) for human SIRT1 gene silencing was purchased from Santa Cruz (Dallas). Human NP Cells were transfected with SIRT1 siRNA using Lipofectamine 2000, according to the manufacturer's instructions. Six hours post transfection, the medium was replaced with NP cell culture medium (without antibiotics).

Quantitative real-time PCR

Gene expression of MMP-3 was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). The isolation of total RNA, cDNA synthesis by reverse transcription, and qRT-PCR were performed as described previously.²² Real-time PCR primer sequences are as follows: human MMP-3 (F: 5' TTCCGCCTGTCTCAAGATGATAT 3', R: 5' AAAGGACAAAGCAGGATCACAGTT 3'), human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (F: 5' ATGGGAAGGTGAAGGTCG 3', R: 5' TAA AAGCAGCCCTGGTGACC 3').

Protein extraction and Western blotting

After treatments, plates were placed on ice immediately and washed with ice-cold phosphate-buffered saline. Cells were lysed by ice-cold radioimmunoprecipitation assay buffer including 1 $\times$ protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA) and 1 $\times$ phosphatase inhibitor cocktail (Roche Applied Science). Total proteins were separated on 8–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred by electroblotting onto polyvinylidene difluoride membranes (EMD Millipore Corporation, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween (50 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 0.1% Tween-20) for 2 h and then incubated overnight at 4°C with the previously mentioned antibodies all at a dilution of 1:1000. Proteins were visualized using Super Signal West Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA).

Statistical analysis

Data are presented as mean $\pm$ standard error of mean (SEM). Intergroup differences were analyzed by one-way analysis of variance (ANOVA) test using SPSS version 17.0, (SPSS, Chicago, IL, USA). $p < 0.05$ was considered

statistically significant. All experiments were repeated at least three times independently.

Results

RSV attenuated TNF- α -induced MMP-3 expression through stimulating autophagy in human NP cells

Human NP cells were first treated with TNF- α (100 ng/mL) in the presence and absence of RSV (24 μ M) for 24 h. MMP-3 mRNA expression was studied by qRT-PCR. As shown in Figure 1(a), MMP-3 expression was significantly

Figure 1 RSV attenuated TNF- α -induced MMP-3 expression by stimulating autophagy in human NP cells. NP cells were treated with both TNF- α (100 ng/mL) and RSV (6, 12, and 24 μ M) in the presence or absence of 3-MA (5 and 10 mM) for 24 h. (a) TNF- α (100 ng/mL) caused a significant increase in MMP-3 expression in human NP cells ($^a p < 0.01$ vs. group 1). RSV (24 μ M) significantly attenuated TNF- α -induced MMP-3 expression ($^b p < 0.01$ vs. group 2). (b) NP cells were treated with both TNF- α and RSV for 24 h; the expression of LC3 and Beclin-1 were detected by Western blot. RSV significantly upregulated the expression ratio of LC3-II/I and Beclin-1/ β -actin in a dose-dependent manner. (c) Western blot analysis showing that 3-MA significantly inhibited RSV-induced upregulation of the ratio of LC3-II/I. (d) Quantitative real-time polymerase chain reaction results showing significant decrease in TNF- α -induced MMP-3 expression caused by RSV (24 μ M) ($^c p < 0.01$ vs. group 5). 3-MA (10 mM) significantly reversed the effect of RSV on the expression of MMP-3 ($^d p < 0.01$ vs. group 6). 3-MA: 3-methyladenine; LC3: light chain 3; LC3II/I: light chain 3 II/I; MMP-3: matrix metalloproteinase-3; NP: nucleus pulposus; RSV: resveratrol

upregulated by TNF- α , while RSV significantly attenuated the TNF- α -induced MMP-3 expression.

RSV was shown as a common activator of autophagy.²³ In addition, autophagy and inflammation are closely related.²⁴ Thus, we speculated that RSV exerted its anti-TNF- α effect by stimulating autophagy. We first examined the impact of RSV treatment on autophagy in TNF- α -treated human NP cells. NP cells were treated with RSV (0, 6, 12, and 24 μ M) in the presence of TNF- α (100 ng/mL) for 24 h. Western blot analysis showed that both the expression of Beclin-1 and the ratio of microtubule-associated protein 1 light chain 3 II/I (LC3-II/I) were upregulated by RSV in the TNF- α -treated human NP cells, in a dose-dependent manner (Figure 1b).

Further, we used 3-methyladenine (3-MA) to inhibit RSV-induced autophagy. We found that in the RSV-treated human NP cells, 3-MA treatment resulted in a decrease in the ratio of LC3-II/I (Figure 1c). Further, RSV-induced

inhibition of MMP-3 expression was remarkably blocked by 3-MA in TNF- α -treated NP cells (Figure 1d). These results suggest that inhibition of autophagy significantly countered the restraining role of RSV in the TNF- α -mediated upregulation of MMP-3.

RSV stimulated autophagy by activating SIRT1 expression in TNF- α -treated human NP cells

We explored the mechanism by which RSV stimulated autophagy in TNF- α -treated human NP cells. RSV is an activator of sirtuin 1 (SIRT1), which in turn is an important regulator of autophagy.²⁵ Western blot analysis showed that SIRT1 was significantly upregulated by RSV treatment in a dose-dependent manner in TNF- α -treated NP cells (Figure 2a). In order to inhibit the RSV-induced SIRT1 activation, we pretreated NP cells with sirtinol or nico, both of which are commonly used inhibitors of SIRT1. On Western blot

Figure 2 RSV stimulated autophagy by activating SIRT1 expression in TNF- α -treated human NP cells. (a) Cells were treated with TNF- α and RSV for 24 h. Western blot analysis showing increased expression of SIRT1 caused by RSV in a dose-dependent manner. (b) Cells were treated with sirtinol (10 μ M) or nico (5 mM) before treatment with TNF- α and RSV. Both sirtinol and nico inhibited the SIRT1 expression and caused upregulation of LC3-II/I ratio as induced by RSV. (c) SIRT1 was knocked down by SIRT1 siRNA. RSV-induced upregulation of LC3-II/I ratio was reversed by SIRT1 siRNA knockdown. (d) RSV-induced inhibition of MMP-3 expression ($^a p < 0.05$ vs. group 2) was reversed by pretreatment with sirtinol ($^b p < 0.05$ vs. group 3) or nico ($^c p < 0.05$ vs. group 3). (e) SIRT1 siRNA reversed the RSV-induced inhibition of MMP-3 expression ($^d p < 0.05$ vs. group 7; $^e p < 0.01$ vs. group 8). LC3II/I: light chain 3 II/I; MMP-3: matrix metalloproteinase-3; Nico: nicotinamide; RSV: resveratrol; siRNA: small interfering RNA; SIRT1: silent mating type information regulator 2 homolog 1

analysis, both sirtinol and nico inhibited RSV-induced SIRT1 activation (Figure 2b). Moreover, the two inhibitors decreased the ratio of LC3-II/I (Figure 2b).

To further confirm the role of SIRT1, we knocked down SIRT1 in NP cells by transfection with SIRT1 siRNA (Figure 2c). SIRT1 knockdown also resulted in a decrease in the ratio of LC3-II/I (Figure 2c). Moreover, RSV-induced downregulation of TNF- α -mediated MMP-3 expression was inhibited by SIRT1 inhibitors (Figure 2d) or by SIRT1 knockdown (Figure 2e). Together, these results appear to suggest that RSV stimulated autophagy via regulating SIRT1; and that RSV attenuated TNF- α -induced MMP-3 expression, through activating SIRT1/autophagy in TNF- α -treated NP cells.

RSV upregulated SIRT1 expression by activating AMPK in TNF- α -treated human NP cells

We investigated the mechanism of RSV regulated SIRT1 expression in TNF- α -treated human NP cells. RSV has earlier been shown to regulate SIRT1 via activating AMP-activated protein kinase (AMPK).²⁶ Western blot analysis showed that the ratio of phosphorylated AMPK proteins/total AMPK proteins were notably upregulated by RSV treatment of human NP cells for 2 h (Figure 3a), suggesting that AMPK was activated by RSV. Further, we used compound C, a widely used inhibitor for AMPK, to inhibit RSV-induced AMPK activation. As shown in Figure 3(b), RSV-induced phosphorylation of AMPK was inhibited by compound C. Meanwhile, RSV-induced upregulation of SIRT1 was markedly inhibited by compound C in the TNF- α -treated human NP cells (Figure 3c). These results suggest that RSV upregulated SIRT1 expression by activating AMPK (Figure 4).

Discussion

IVD degeneration is a global health problem. Proinflammatory cytokine TNF- α is known to play a vital role in its pathogenesis and progression. Recent studies have demonstrated a protective role of RSV against inflammation in other tissues. However, whether RSV has an anti-TNF- α effect in NP cells is not known. In this study, we demonstrated for the first time, that RSV attenuated TNF- α -induced MMP-3 expression by stimulating autophagy in human NP cells through the AMPK/SIRT1 signaling pathway (Figure 4).

The degradation of the extracellular matrix (ECM) is one of the most prominent features of IVD degeneration. MMPs are the main force of the degradation of ECM during IVD degeneration and MMP-3 is the most important one in the family of MMPs. MMP-3 can not only efficiently degrade

Figure 4 Putative signal transduction pathway: RSV attenuated TNF- α -induced MMP-3 expression in human nucleus pulposus cells by activating autophagy via the AMPK/SIRT1 signaling pathway. AMPK: AMP-activated protein kinase; MMP-3: matrix metalloproteinase-3; RSV: resveratrol

Figure 3 RSV upregulated SIRT1 expression by activating AMPK in TNF- α -treated human NP cells. (a) Cells were treated with RSV (24 μ M) for 2 h. Total AMPK and phosphorylated AMPK were detected by western blot. RSV remarkably upregulated the ratio of phosphorylated AMPK/total AMPK. (b) cC, an inhibitor of AMPK, significantly inhibited RSV-induced phosphorylation of AMPK. (c) Pretreatment of compound C significantly inhibited RSV-induced SIRT1 expression. AMPK: AMP-activated protein kinase; cC: compound C; NP: nucleus pulposus; RSV: resveratrol

components of ECM, including aggrecan, collagen, and fibronectin,^{27–29} but also activate several other MMPs indirectly.^{30,31} Accordingly, MMP-3 is considered as the key enzyme in the regulation of matrix degradation in the process of IVD degeneration.^{32,33} As mentioned earlier, TNF- α upregulates expression of MMPs in NP cells. Furthermore, TNF- α has its highest induction on MMP-3 expression compared to other MMPs.³⁴ Therefore, we selected MMP-3 as a marker to investigate the anti-TNF- α effect of RSV on NP cells. Our results demonstrated that RSV significantly reduced TNF- α -induced MMP-3 expression.

Subsequently, we investigated the mechanism by which RSV inhibits TNF- α -induced MMP-3 expression. We observed that RSV stimulated autophagy in human NP cells. When autophagy was inhibited by 3-MA, RSV failed to attenuate TNF- α -induced MMP-3 expression. The findings suggested that RSV inhibited TNF- α -induced MMP-3 expression by stimulating autophagy. To the best of our knowledge, this is the first time such a correlation between RSV-induced autophagy and catabolic effect of TNF- α is demonstrated in NP cells.

Autophagy is a self-protective mechanism for maintaining homeostasis under stressful conditions.³⁵ Autophagy and inflammation are thought to be closely related as the former is known to play an important role in regulating inflammation.²⁴ The anti-TNF- α effect of RSV, a natural activator of autophagy, is well documented.^{36–38} Studies have shown that RSV inhibits inflammatory response by regulating nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signaling pathways.^{38–41} In the present study, we found that RSV did not effect the phosphorylation of these pathways (data not shown). Consistent with other studies, we too found that RSV restrained inflammatory response by activating autophagy.

We further studied the mechanism by which RSV stimulated autophagy in NP cells. Jiang *et al.*²¹ found that RSV stimulated autophagy by activating SIRT1 in NP cells. The phosphorylation of AMPK has been shown to control the SIRT1 activity by increasing cellular NAD⁺ levels.⁴² In the neuronal cells and human umbilical endothelial vein cells, RSV-induced activation of autophagy was shown to be modulated by AMPK/SIRT1 pathway.^{26,43} In human NP cells, we found that treatment of RSV for 2 h induced phosphorylation of AMPK.

Pretreatment of NP cells with compound C inhibited RSV-induced phosphorylation of AMPK. In addition, RSV-mediated upregulation of SIRT1 was prevented by pretreatment with compound C. These findings suggest that the stimulation of autophagy by RSV was mediated by activation of AMPK/SIRT1 signaling pathway.

In conclusion, we showed that RSV-induced autophagy attenuated the catabolic effect of TNF- α by downregulation of MMP-3 in human NP cells. And RSV was shown to activate autophagy through AMPK/SIRT1 signaling pathway. The prospects for use of RSV as a novel preventive and therapeutic approach to IVD degeneration appear promising.

Author contributions: X-HW designed the experiments. X-HW and LZ conducted the experiments and wrote the manuscript; XH, Y-TW, J-PB, X-HX, LL and FW performed part of the experiments; and X-TW directed the designation of the experiments, evaluated the results, and supervised this study. X-HW and LZ contributed equally to this work.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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