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# Inhibition of the Expression of Matrix Metalloproteinases in Articular Chondrocytes by Resveratrol through Affecting Nuclear Factor-Kappa B Signaling Pathway

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## Abstract

In the present study, we tried to examine whether resveratrol regulates the expression of matrix metalloproteinases (MMPs) through affecting nuclear factor-kappa B (NF-κB) in articular chondrocytes. Rabbit articular chondrocytes were cultured in a monolayer, and reverse transcription-polymerase chain reaction (RT-PCR) was used to measure interleukin-1β (IL-1β)-induced gene expression of MMP-3, MMP-1, MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs-4 (ADAMTS-4), ADAMTS-5 and type II collagen. Effect of resveratrol on IL-1β-induced secretion of MMP-3 was investigated in rabbit articular chondrocytes using western blot analysis. To elucidate the action mechanism of resveratrol, effect of resveratrol on IL-1β-induced NF-κB signaling pathway was investigated in SW1353, a human chondrosarcoma cell line, by western blot analysis. The results were as follows: (1) resveratrol inhibited the gene expression of MMP-3, MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5, but increased the gene expression of type II collagen; (2) resveratrol reduced the secretion of MMP-3; (3) resveratrol inhibited IL-1β-induced activation (phosphorylation) of inhibitory kappa B kinase (IKK), and thus phosphorylation and degradation of inhibitory kappa Bα (IκBα); (4) resveratrol inhibited IL-1β-induced phosphorylation and nuclear translocation of NF-κB p65. This, in turn, led to the down-regulation of gene expression of MMPs in SW1353 cells. These results suggest that resveratrol can regulate the expression of MMPs through affecting NF-κB by directly acting on articular chondrocytes.

**Key Words:** Resveratrol, Chondrocytes, MMPs, Osteoarthritis

## INTRODUCTION

Osteoarthritis has been reported to be a common, chronic degenerative articular disorder and many elderly people suffered from this disease. Progressive cartilage loss, osteophytes formation, subchondral bone remodeling, and synovitis are the major pathophysiologic features of the symptoms of osteoarthritis and destruction of the equilibrium between degradation and synthesis of articular cartilage is the major cause of osteoarthritis (Mankin, 1982; Aigner and McKenna, 2002).

In the pathophysiology of osteoarthritis, inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) play a pivotal role. Articular chondrocytes stimulated by IL-1β produce the proteins related to inflammation response such as matrix-degrading enzymes including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS). The activation of these degradative enzymes provokes the degradation of collagen and proteoglycans in articular cartilage, and MMPs play an important role in the destruction of osteoarthritic articular cartilage (Dean *et al*., 1989; Kullich *et al*., 2007).

Among various MMPs including MMP-1, -2, -3, -7, -8, -9, -10, -11, and -13, MMP-3 activates procollagenase and decomposes proteoglycans, in articular cartilage (Birkedal-Han-

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sen *et al*., 1993; Garnero *et al*., 2000; Lin *et al*., 2004; Burrage *et al*., 2006). On the other hand, MMP-1 and MMP-13 also play significant roles in the degradation of cartilage in osteoarthritis. Another degradative enzyme, ADAMTS-4, is a major aggrecanase in cartilage and ADAMTS-5 is important in matrix destruction of osteoarthritic cartilage (Freemont *et al*., 1997; Goupille *et al*., 1998; Kanyama *et al*., 2000; Yoshihara *et al*., 2000; Neuhold *et al*., 2001; Jo *et al*., 2003; Stanton *et al*., 2005; Echtermeyer *et al*., 2009; Little *et al*., 2009).

Therefore, we suggest that exploration of the mechanisms through which the expressions of MMPs are regulated by natural products isolated from medicinal plants used as arthritis remedies in folk medicine would back up both the effective treatment of osteoarthritis and the development of novel therapeutic strategies. We tried to examine the potential activity of some natural products on the expression of MMPs in articular chondrocytes and reported that multiple natural products affected the gene expression, secretion (production) and proteolytic activity of MMP-3, *in vitro* and *in vivo* (Kang *et al*., 2014; Park *et al*., 2015; Nam *et al*., 2016; Park *et al*., 2016).

As claimed by a number of reports, resveratrol, a natural product isolated from *Polygonum cuspidatum*, a medicinal plant used for controlling various inflammatory diseases in traditional oriental medicine, showed the diverse biological activities including anti-inflammatory and anti-oxidative effects (Xiao *et al*., 2000; Buhrmann *et al*., 2017; Pan *et al*., 2017; Daverey and Agrawal, 2018; Wiedemann *et al*., 2018).

However, to the best of our knowledge, there has been no report about the effect of resveratrol on the expression of multiple MMPs including MMP-3 in primary cultured rabbit articular chondrocytes and its potential effect on NF-κB signaling pathway in human articular chondrocytes.

Therefore, in the present study, to evaluate the chondroprotective activity of resveratrol, we investigated its effects on IL-1β-induced expression of MMPs in primary cultured rabbit articular chondrocytes and on IL-1β-induced transduction of NF-κB signaling involved in the expression of MMPs in SW1353, human articular chondrocytes.

## MATERIALS AND METHODS

### Materials

All the chemicals and reagents used in this experiment, including resveratrol (purity: 98.0%) (Fig. 1), were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco-BRL (Grand Island, NY, USA) and recombinant human IL-1β was purchased from R&D Systems (Minneapolis, MN, USA). Anti-NF-κB p65 (sc-8008), anti-IκBα (sc-371), anti-actin (sc-8432), anti-p84 (sc-98783), anti-TRAF2 (sc-7187), anti-TRADD (sc-7868) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-RIP1 antibody (#610459) was purchased from BD biosciences (San Jose, CA, USA). Phospho–specific anti-p65 (serine 536, #3036S), phospho-specific anti-IκBα (serine 32/36, #9246), anti-phospho-IKKα/β (Ser176/180, #2687) antibodies were purchased from Cell signaling Technology Inc (Danvers, MA, USA). A Goat Anti-rabbit IgG (#401315) or Goat Anti-mouse IgG (#401215) was used as the secondary antibody (Calbiochem, Carlsbad, CA, USA).

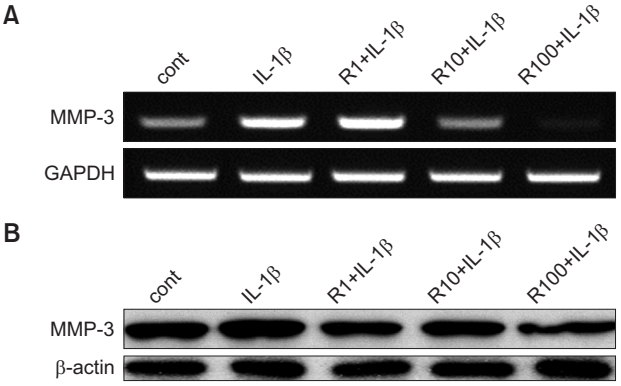


**Fig. 1.** Effect of resveratrol on MMP-3 gene expression and secretion in rabbit articular chondrocytes. Primary cultured rabbit articular chondrocytes were pretreated with varying concentrations (1, 10, and 100 μM) of resveratrol for 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. MMP-3 gene expression level was measured by RT-PCR (A). Culture supernatants were collected for measurement of the levels of produced and secreted MMP-3 by western blot analysis (B). Three independent experiments were performed and the representative data were shown. cont, control; R, resveratrol. Concentration unit is μM.

### Primary cultures of chondrocytes from rabbit articular cartilage

Male New Zealand White rabbits were obtained from Daehan Biolink (Seoul, Korea) at 2 weeks of age. Animals were housed one animal per cage, provided with distilled water and food *ad libitum*, and kept under a 12 h light/dark cycle (lights on from 08:00-20:00) at constant temperature (22.5°C) and humidity (55%). Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, and care was regulated by Chungnam National University (the approval number of animal experiment: CNU-00795) (Daejeon, Korea). Rabbit articular chondrocytes were isolated from the tibial plateau and femoral condyle in cartilage of the knee joint. Cartilage was washed in phosphate-buffered saline (PBS) and minced into pieces measuring 2 $mm^3$, approximately. Cartilage tissue was digested for 4 h with 0.2% type II collagenase at 37°C. After collection of individual cells by brief centrifugation, the cells were transferred to 100 mm culture dishes (seeding density: $10^5$ cells/$cm^2$) in 12 mL Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), in the presence of penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were cultured at 37°C in a humidified, 5% $CO_2$/95% air, water-jacketed incubator, and medium was replaced every other day (Moon *et al*., 2011).

### Treatment of primary cultured chondrocytes with resveratrol

Chondrocytes were seeded on 6-well culture plates (for RT-PCR) or 60 mm culture dishes (for western blotting) at a density of $10^5$ cells/$cm^2$. After 2 days in monolayer culture, the cells were incubated for 2 h in growth medium with 1, 10, or 100 μM of resveratrol followed by incubation in the presence or absence of IL-1β (10 ng/mL) for 24 h. Resveratrol was dissolved in dimethylsulfoxide, diluted in PBS, and administered in culture medium (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulf-

oxide in medium did not affect the gene expression of MMPs or secretion of MMP-3, in primary cultured chondrocytes. The supernatant was collected and centrifuged, and cell and supernatant fractions were stored at –80°C until use.

### Isolation of total RNA from primary cultured chondrocytes and RT-PCR

Total RNA was isolated from chondrocytes using the Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc., Gyeonggi, Korea), and reverse transcribed using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. About 2 μg of total RNA was primed with 1 μg of oligo (dT) in a final reaction volume of 30 μL. Two μL of RT reaction product was amplified in 20 μL using Thermoprime Plus DNA Polymerase (ABgene, Rochester, NY, USA). PCR was performed with the following primers: MMP-3 (5′ATG GAC CTT CTT CAG CAA 3′, 5′TCA TTA TGT CAG CCT CTC 3′), MMP-13 (5′AGG AGC ATG GCG ACT TCT AC 3′, 5′TAA AAA CAG CTC CGC ATC AA 3′), MMP-1 (5′TCA GTT CGT CCT CAC TCC AG 3′, 5′TTG GTC CAC CTG TCA TCT TC 3′), ADAMTS-4 (5′CAA GGT CCC ATG TGC AAC GT 3′, 5′CAT CTG CCA CCA CCA GTG TCT 3′), ADAMTS-5 (5′TGT CCT GCCAGC GGATGT 3′; 5′ACG GAA TTA CTG TAC GGC CTA CA 3′), and type II collagen (5′AAC ACT GCC AAC GTC CAG AT 3′, 5′CTG ACG CAC GGT ATA GGT GA 3′). GAPDH (5′ACT GGC GTC TTC ACC ACC AT 3′; 5′AAG GCC ATG CCA GTG AGC TT 3′) was used as a quantitative control. The PCR products increased as the concentration of RNA increased. The amplified fragment sizes were 350 base pairs (bp) for MMP-3, 458 bp for MMP-13, 300 bp for MMP-1, 90 bp for ADAMTS-4, 110 bp for ADAMTS-5, 220 bp for type II collagen, and 400 bp for GAPDH. After PCR, 15 μL of PCR products were subjected to 2% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator (Moon *et al*., 2011).

### Western blot analysis for measuring secretion level of MMP-3 in culture supernatant

Chondrocytes (confluent in 60 mm culture dish) were incubated for 2 h in growth medium with 1, 10, or 100 μM of resveratrol followed by incubation in the presence or absence of IL-1β (10 ng/mL) for 24 h. After the treatment, the supernatant was collected and the cells were harvested using 3 x trypsin-EDTA solution and then centrifuged in a microcentrifuge (1,200 rpm, 3 min, 4°C). The Bradford assay was used to measure protein concentrations in culture supernatants to ensure consistent weight of protein samples subjected to electrophoresis. Culture supernatant samples containing MMP-3 protein (50 μg each) were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene difluoride (PVDF) membrane. Blots were blocked using 5% skim milk in Tris-buffered saline/Tween 20 (TBS-T), and probed overnight with MMP-3 antibody in blocking buffer at 4°C. Antibody against MMP-3 was purchased from Santa Cruz Biotechnology. Membranes were washed with TBS-T and probed for 1 h with a secondary antibody conjugated with horseradish peroxidase (Calbiochem). After 4 washes with TBS-T, immunoreactive bands were detected using an enhanced chemiluminescence kit (Pierce ECL western blotting substrate, Thermo Scientific, Waltham, MA, USA).

### SW1353 cell cultures

SW1353 cells, a human articular chondrosarcoma cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL), streptomycin (100 μg/mL) and HEPES (25 mM) at 37°C in a humidified, 5% $CO_2$/95% air, water-jacketed incubator.

### Treatment of SW1353 cells with resveratrol

SW1353 cells were seeded on 6-well culture plates (for RT-PCR) or 100 mm culture dishes (for western blotting) at a density of $10^5$ cells/cm$^2$. After 24 h of serum deprivation, cells were pretreated with 1, 10, 50, or 100 μM of resveratrol for 2 h and treated with IL-1β (10 ng/mL) for 24 h in serum-free DMEM. Resveratrol was dissolved in dimethylsulfoxide, diluted in PBS, and administered in culture medium (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulfoxide in medium did not affect the gene expression of MMPs in SW1353 cells. After 24 h, the total RNA was extracted for measuring the expression of MMPs (in 6-well culture plate) by using RT-PCR.

### Total RNA isolation from SW1353 cells and RT-PCR

Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc.) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation) according to the manufacturer's instructions. Two μg of total RNA was primed with 1 μg of oligo (dT) in a final volume of 50 μL (RT reaction). Two μL of RT reaction product was PCR amplified in a 25 μL by using Thermorprime Plus DNA Polymerase (ABgene). PCR was performed with the following primers: MMP-3 (5'-GGT GTG GAG TTC CTG ATG TT-3', 3'-TGG TCC CTG TTG TAT CCT TTG-5'), MMP-13 (5'-AGC ATC TGG AGT AAC CGT ATT G-3', 3'-CCA GCC ACG CAT AGT CAT ATA G-5'), MMP-1 (5'-TCT CTT GGA CTC TCC CAT TCT-3', 3'-AAT AAG TAC TGG GCT GTT CAG G-5'), ADAMTS-4 (5'-CAG ACA GCC CTC CAT CTA AAC-3', 3'-ATA AGT GGT GTG TGT ATG CGT-5'), and ADAMTS-5 (5'-TAA TAA CCC TGC TCC CAG AAA C-3', 3'-CAT ACT CCG CAC TTG TCA TAC T-5'). GAPDH (5'-ACA TCA TCC CTG CCT CTA CT-3', 3'-CTC TCT TCC TCT TGT GCT CTT G) was used as a quantitative control. The PCR products increased as the concentration of RNA increased. After PCR, 15 μL of PCR products were subjected to 2% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator (Moon *et al*., 2011).

### Preparation of nuclear and cytosolic extracts

SW1353 cells (confluent in 100 mm culture dish) were pretreated for 2 h at 37°C with 1, 10, 50, or 100 μM of resveratrol and then stimulated with IL-1β (10 ng/mL) for 24 h. After the treatment of the cells with resveratrol, the cells were harvested using 3 x trypsin-EDTA solution and then centrifuged in a microcentrifuge (1,200 rpm, 3 min, 4°C). The supernatant was discarded and the cell pellet was washed by suspending in PBS. The cytoplasmic and nuclear protein fractions were extracted using NE-PER® nuclear and cytoplasmic extraction reagent (Thermo Scientific) according to the manufacturer's instructions. Both extracts were stored at –20°C. Protein content in extract was determined by Bradford method.

### Preparation of whole cell extract

After the treatment of the cells with resveratrol as mentioned above, media were aspirated and the cells were washed with cold PBS. The cells were collected by scraping and centrifuged at 3,000 rpm for 5 min. The supernatant was discarded. The cells were mixed with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min with continuous agitation. The lysate was centrifuged in a microcentrifuge at 14,000 rpm for 15 min at 4°C. The supernatant was used or immediately stored at –80°C. Protein content in extract was determined by Bradford method.

### Detection of proteins by western blot analysis

Cytosolic, nuclear and whole cell extracts containing proteins (each 50 μg as protein) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto the polyvinylidene difluoride (PVDF) membrane. The blots were blocked using 5% skim milk and probed with appropriate primary antibody in blocking buffer overnight at 4°C. The membrane was washed with PBS and then probed with the secondary antibody conjugated with horseradish peroxidase. Immunoreactive bands were detected by an enhanced chemiluminescence kit (Pierce ECL western blotting substrate, Thermo Scientific).

### Statistics

Means of individual group were converted to percent control and expressed as mean ± SEM. The difference between groups was assessed using one-way ANOVA and Holm-Sidak test as a post-hoc test. $p<0.05$ was considered as significantly different.

## RESULTS

### Effect of resveratrol on MMP-3 gene expression in rabbit articular chondrocytes

To investigate the potential activity of resveratrol on the gene expression of MMP-3, the key MMP involved in destruction of articular cartilage, MMP-3 gene expression was measured after pretreatment of resveratrol. As can be seen in Fig. 1A, resveratrol inhibited IL-1β-induced MMP-3 gene expression. Cytotoxcity to primary cultured chondrocytes was checked by lactate dehydrogenase (LDH) assay and there was no cytotoxic effect of reveratrol, at 1 to 100 μM (data were not shown).

### Effect of resveratrol on IL-1β-induced secretion of MMP-3 from rabbit articular chondrocytes

If resveratrol can affect the MMP-3 gene expression at the transcriptional level, it should be investigated whether resveratrol affects IL-1β-induced secretion of MMP-3 proteins from rabbit articular chondrocytes. As can be seen in Fig. 1B, stimulation with IL-1β (10 ng/mL) increased secretion of MMP-3 from chondrocytes. Resveratrol reduced the effect of IL-1β on MMP-3 secretion. This result means that resveratrol can control the steps of protein synthesis and secretion of MMP-3.

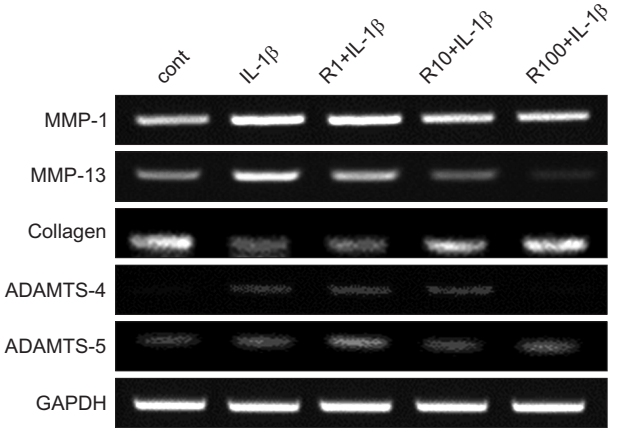


**Fig. 2.** Effect of resveratrol on the gene expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, or collagen type II in rabbit articular chondrocytes. Primary cultured rabbit articular chondrocytes were pretreated with varying concentrations (1, 10, and 100 μM) of resveratrol for 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. The gene expression level of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, or collagen type II was measured by RT-PCR. Three independent experiments were performed and the representative data were shown. cont, control; R, resveratrol. Concentration unit is μM.

### Effect of resveratrol on the gene expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5 or type II collagen in rabbit articular chondrocytes

If resveratrol can affect the gene expression of MMP-3, the key matrix metalloproteinase involved in destruction of articular cartilage, it should be investigated whether resveratrol affects the gene expression of MMP-1, MMP-13, ADAMTS-4 or ADAMTS-5, the other degradative enzymes related to destruction of articular cartilage, and type II collagen, in rabbit chondrocytes. As can be seen in Fig. 2, resveratrol showed the suppression of IL-1β-induced gene expression of MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5, in rabbit chondrocytes. Furthermore, resveratrol showed an additional chondroprotective effect by restoring the compromised gene expression of type II collagen by IL-1β, in rabbit chondrocytes.

### Effect of resveratrol on the gene expression of MMP-1, MMP-3, MMP-13, ADAMTS-4 or ADAMTS-5 in SW1353 cells

As can be seen in Fig. 3A, resveratrol inhibited IL-1β-induced gene expression of MMP-1, MMP-3, MMP-13, ADAMTS-4, or ADAMTS-5, in SW1353 human articular chondrocytes, as did in rabbit articular chondrocytes.

### Effect of resveratrol on IL-1β-induced secretion of MMP-3 from SW1353 cells

As can be seen in Fig. 3B, stimulation with IL-1β (10 ng/mL) increased secretion of MMP-3 from SW1353 cells. Resveratrol reduced the effect of IL-1β on MMP-3 secretion. This result means that resveratrol can control the steps of protein synthesis and secretion of MMP-3, in SW1353 cells.

### Effect of resveratrol on IL-1β-induced phosphorylation and translocation of NF-κB p65

As shown in Fig. 4A, nuclear translocation of NF-κB p65

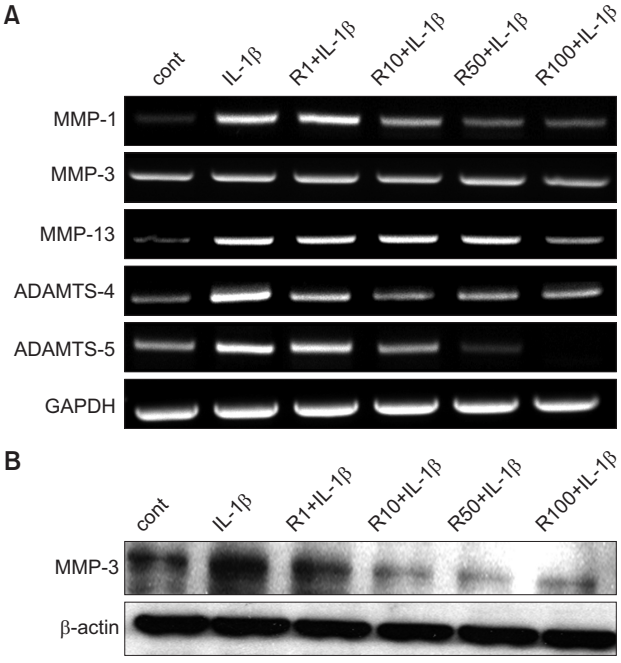


**Fig. 3.** Effect of resveratrol on the gene expression of MMP-3, MMP-1, MMP-13, ADAMTS-4, or ADAMTS-5 and MMP-3 secretion, in SW1353 cells. SW1353 cells were pretreated with varying concentrations (1, 10, 50, and 100 μM) of resveratrol for 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. The gene expression level of MMP-3, MMP-1, MMP-13, ADAMTS-4, or ADAMTS-5 was measured by RT-PCR (A). Culture supernatants were collected for measurement of the levels of produced and secreted MMP-3 by western blot analysis (B). Three independent experiments were performed and the representative data were shown. cont, control; R, resveratrol. Concentration unit is μM.

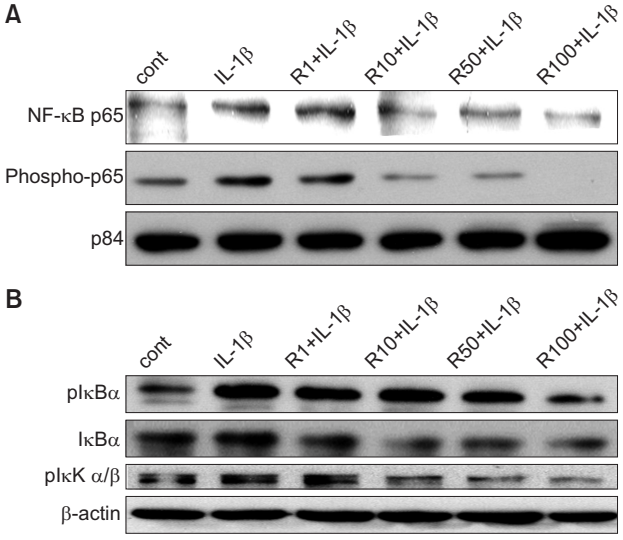


**Fig. 4.** Effect of resveratrol on IL-1β-induced phosphorylation and translocation of NF-κB p65, phosphorylation of IκBα, degradation of IκBα and phosphorylation of IKKα/β, in SW1353 cells. SW1353 cells were incubated with varying concentrations of resveratrol for 2 h and treated with 10 ng/mL IL-1β for 24 h. Nuclear protein extracts were prepared and subjected to western blot analysis using antibodies against p65 and phospho-p65. The result shown is a representative of three independent experiments. As a loading control, p84 levels were analyzed (A). Cytoplasmic extracts were fractionated and then subjected to western blot analysis using phospho-specific IκBα (Ser 32/36) antibody or antibody against anti-IκBα. Whole cell lysates (100 μg) were prepared and then subjected to western blot analysis using phospho-specific IKKα/β (Ser 176/180) antibody. The result shown is a representative of three independent experiments. Equal protein loading was evaluated by β-actin levels (B). cont, control; R, resveratrol. Concentration unit is μM.

by IL-1β was inhibited by pretreatment with resveratrol, dose-dependently. In the nuclear fraction of the cells treated with IL-1β only, there was an increase in nuclear translocation of p65. In the cells treated with resveratrol plus IL-1β, the level of p65 was gradually decreased as compared to the cells treated with IL-1β only. Transcriptional activity of p65 largely depends on its phosphorylation. IL-1β-induced phosphorylation of p65 was reached optimal level at 30 min. However, resveratrol suppressed the phosphorylation of p65.

### Effect of resveratrol on IL-1β-induced IκBα phosphorylation, IκBα degradation, and IKK phosphorylation

As shown in Fig. 4B, IL-1β increased the phosphorylation of IκBα. Preincubation of SW 1353 cells with resveratrol prior to IL-1β treatment inhibited the phosphorylation of IκBα. IL-1β also induced degradation of IκBα. IκBα degradation was inhibited by pretreatment of resveratrol. Activation of IKK, which phosphorylates IκBα, depends on its phosphorylation. Therefore, we investigated whether resveratrol inhibits the IL-1β-induced phosphorylation of IKKα/β. IL-1β activated the IKKα/β, although resveratrol suppressed its activation by regulating the serine 176/180 phosphorylation of IKKα/β.

### Effect of resveratrol on adaptor proteins of TNF receptor (TNFR)1 signaling

IL-1β was reported to stimulate NF-κB signaling pathway (Chen *et al*., 2015; Ji *et al*., 2017). Also, IL-1β augmented TNF signaling through the upregulation of TNF secretion and TNFR1 cell surface expression (Jayaraman *et al*., 2013). TNF-α binding to TNFR1 provokes receptor trimerization and recruitment of several downstream signaling proteins to their cytoplasmic domains (Hsu *et al*., 1995). TNFR1 interacts with the signaling protein TNFR1-associated death domain protein (TRADD), which recruits the adaptor protein, TNF receptor-associated factor 2 (TRAF2) (Hsu *et al*., 1996), receptor interacting protein (RIP1) (Stanger *et al*., 1995) and Fas associated protein with death domain (FADD) (Chinnaiyan *et al*., 1995). Subsequently, RIP1 is mainly polyubiquitinated and induces the recruitment to the TNFR1 and activation of IKK complex. For this reason, we investigated whether resveratrol affects the adaptor protein expression of TNFR1 signaling pathway. As shown in Fig. 5, resveratrol showed the tendency of affecting the expression of RIP1.

## DISCUSSION

Developing a pharmacological tool to reset the broken equilibrium between destruction and synthesis of osteoarthritic articular cartilage might be a promising strategy for the regulation of osteoarthritis. In osteoarthritis, onset and progression of disease are due to various inflammatory cytokines, such

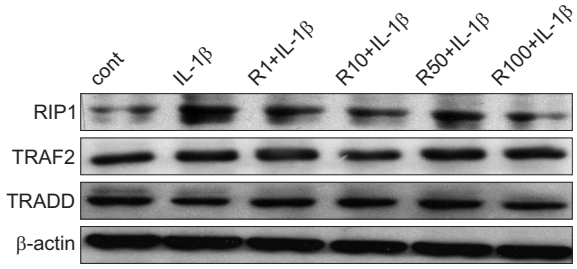


**Fig. 5.** Effect of resveratrol on adaptor proteins of TNF receptor (TNFR) 1 signaling, in SW1353 cells. SW1353 cells, either untreated or pretreated with varying concentrations of resveratrol for 2 h, were treated with 10 ng/mL IL-1β for 24 h. Whole cell lysates were prepared and analyzed by western blotting using antibodies against RIP1, TRAF2 and TRADD. The results shown are representative of three independent experiments. Equal protein loading was evaluated by β-actin. cont, control; R, resveratrol. Concentration unit is μM.

as IL-1β and TNF-α, in articular tissues and fluids that are produced by chondrocytes and/or interact with chondrocytes, as well as to low-grade inflammation in intra-articular regions (Bonnet and Walsh, 2005; Kobayashi *et al*., 2005; Loeser, 2006; Goldring *et al*., 2008).

IL-1β produced by articular chondrocytes is known to stimulate the progression of osteoarthritis. Its mode of action in the initiation and progression of degradation of articular cartilage has been reported to inhibit the synthesis of collagen and stimulate MMPs expression (Aida *et al*., 2005; Kobayashi *et al*., 2005; Pantsulaia *et al*., 2010). Especially, MMP-3 plays a pivotal role in pathogenesis of osteoarthritis by degrading components of the extracellular matrix, such as proteoglycans. MMP-3 levels were increased more than MMP-1 levels in knee joints of osteoarthritis patients, compared to the control group (Garnero *et al*., 2000; Lijnen, 2002).

The expression of MMPs stimulated by IL-1β has been reported to be associated with the NF-κB signaling pathway. Natural products isolated from anti-inflammatory medicinal plants, coptisine, taraxasterol, matrine, and another anti-inflammatory medicinal plant, Schisandrae Fructus, were reported to suppress the expression of MMPs through inhibition of NF-κB activation in chondrocytes (Lu *et al*., 2015; Piao *et al*., 2015; Jeong *et al*., 2015; Zhou *et al*., 2016). Among the intracellular signaling pathways initiated by IL-1β, NF-κB pathway is of importance. NF-κB, a heterodimer composed of p65, p50 and IκBα, is present in the cytoplasm as an inactive state. Responding to diverse stimuli, the IκBα subunit is phosphorylated, degraded, and provoked the translocation of p50-p65 heterodimer from cytoplasm to the nucleus. The p50-p65 acts as a transcription factor regulating the expression of numerous genes including MMP-3 (Jeong *et al*., 2015).

Resveratrol has been reported to regulate the diverse intracellular signaling pathways. Shakibaei *et al*. reported that resveratrol suppressed IL-1β-induced expression of COX-2, VEGF, MMP-9 and MMP-3 in human articular chondrocytes. They showed that resveratrol regulated NF-κB signaling pathway via inhibition of degradation of IκBα without affecting IKK activation (Shakibaei *et al*., 2008). Gu and colleagues also reported that resveratrol inhibited the IL-1β-induced expression of MMP-13 and IL-6 in human articular chondrocytes through TLR4/MyD88-dependent and -independent signaling cascades (Liu *et al*., 2014; Gu *et al*., 2017). In human tenocytes, resveratrol suppressed IL-1β-induced expression of MMP-1, MMP-9, MMP-13 and COX-2, via affecting NF-κB signaling pathway by inhibition of IKK activation, IκBα phosphorylation and nuclear translocation of NF-κB (Busch *et al*., 2012).

Based on the information from above reports, in this study, we designed the experiments to show the definite activity of resveratrol on the expression of MMP-3, MMP-13, MMP-1, ADAMTS-4, ADAMTS-5 and type II collagen, in both primary cultured articular chondrocytes and SW 1353, a human chondrosarcoma cell line. Besides, in order to explicitly determine the effect of resveratrol on NF-κB activation followed by IL-1β treatment, we designed the experiment to check whether resveratrol suppresses IL-1β-induced nuclear translocation of NF-κB p65, phosphorylation and degradation of IκBα , phosphorylation of IKKs, and TNF receptor signaling, in SW1353 cells.

We demonstrated that resveratrol regulated the gene expression of MMPs, by directly acting on rabbit and human articular chondrocytes. Resveratrol inhibited IL-1β-induced gene expression of MMP-3, MMP-13, MMP-1, ADAMTS-4, and ADAMTS-5 and normalized the compromised gene expression of type II collagen by IL-1β, in rabbit articular chondrocytes (Fig. 1A, 2). Therefore, the chondroprotective effect of resveratrol is due to its regulation of the gene expression of multiple proteases involved in the degradation of articular cartilage, as well as by its restoration of the compromised gene expression of type II collagen, at the transcriptional level. This effect of resveratrol, was also reported by Maepa and his colleagues, which resveratrol augmented the expression of collagen type II proteins in porcine articular cartilage (Maepa *et al*., 2016). Also, IL-1β-stimulated secretion of MMP-3 from rabbit articular chondrocytes were reduced by resveratrol (Fig. 1B). In SW1353 cells, resveratrol also suppressed both IL-1β-induced gene expression of MMP-1, MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5 and IL-1β-stimulated secretion of MMP-3 (Fig. 3A, 3B). This result means that resveratrol can regulate the step of protein synthesis and secretion of MMP-3 in tissues of osteoarthritic articular cartilage.

To determine the effect of resveratrol on NF-κB activation followed by IL-1β treatment, we investigated whether resveratrol suppresses IL-1β-induced nuclear translocation of NF-κB p65, in SW1353 cells. As shown in Fig. 4A, nuclear translocation of NF-κB p65 by IL-1β was inhibited by pretreatment with resveratrol. In the nuclear fraction of the IL-1β only-treated cells, there was an increase in nuclear translocation of p65. In the cells treated with resveratrol plus IL-1β, the level of p65 was gradually decreased as compared to the IL-1β only-treated cells. NF-κB activation involves the phosphorylation of IκBα by IKKs, resulting in IκBα degradation. As a result, NF-κB is released and translocates freely into the nucleus. Therefore, effect of resveratrol on the phosphorylation and degradation of IκBα was examined. Resveratrol appears to affect the phosphorylation and degradation of IκBa which is required for NF-κB dimerization and maximal activation of transcription. As shown in Fig. 4B, IL-1β increased the phosphorylation of IκBa. Preincubation of SW1353 cells with resveratrol prior to IL-1β exposure suppressed the phosphorylation of IκBa. IκBa degradation is required for the activation of NF-κB. Therefore, we checked whether resveratrol inhibits IL-1β-induced NF-κB activation by inhibition of IκBa degradation. As shown in Fig. 4B, IL-1β showed the induction of IκBa degradation. Preincu-

bation of SW1353 cells with resveratrol prior to IL-1β exposure inhibited the degradation of IκBa.

The degradation of IκB was reported to be mediated by proteaseome. The key regulatory step in this pathway involves the activation of IκB kinase (IKK) complex. Activation of IKK depends on phosphorylation. Consequently, we examined whether resveratrol inhibits the IL-1β-induced activity of IKKα/β. IL-1β activated the IKKα/β, although resveratrol suppressed its activation by regulating the serine 176/180 phosphorylation of IKKα/β (Fig. 4B).

As aforementioned above, IL-1β augmented TNF signaling through the upregulation of TNF secretion and TNFR1 cell surface expression. It has been reported that TNF-α binding to TNFR1 provokes receptor trimerization and recruitment of several downstream signaling proteins to their cytoplasmic domains. TNFR1 interacts with the signaling protein TNFR1-associated death domain protein (TRADD), which recruits the adaptor protein, TNF receptor-associated factor 2 (TRAF2), receptor interacting protein (RIP1). Subsequently, RIP1 induces the recruitment to the TNFR1 and activation of IKK complex. Based on this information, we investigated whether resveratrol affects the adaptor protein expression of TNFR1 signaling pathway. As shown in Fig. 5, resveratrol showed the tendency of affecting the expression of RIP1.

In summary, the inhibitory action of resveratrol on the expression of MMPs in articular chondrocytes might be mediated by, at least in part, IL-1β-induced degradation of IκBa and nuclear translocation of NF-κB p65, and explains the traditional use of *Polygonum cuspidatum*, as an anti-inflammatory agent for diverse inflammatory diseases, in folk medicine. We suggest it is valuable to find the natural products that have specific suppressive effects on the expression of MMPs - in view of both basic and clinical sciences - and the result from this study suggests a possibility of developing resveratrol as a candidate for novel agent controlling cartilage damage in osteoarthritis, although further studies are required.

## CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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