BIOMOLECULES & THERAPEUTICS

Original Article
Biomol Ther 21(5), 364-370 (2013)

Resveratrol Inhibits Nitric Oxide-Induced Apoptosis via the NF-Kappa B Pathway in Rabbit Articular Chondrocytes

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Abstract

Resveratrol (trans-3,4'-trihydroxystillbene), a naturally occurring polyphenolic antioxidant found in grapes and red wine, elicits diverse biochemical responses and demonstrates anti-aging, anti-inflammatory, and anti-proliferative effects in several cell types. Previously, resveratrol was shown to regulate differentiation and inflammation in rabbit articular chondrocytes, while the direct production of nitric oxide (NO) in these cells by treatment with the NO donor sodium nitroprusside (SNP) led to apoptosis. In this study, the effect of resveratrol on NO-induced apoptosis in rabbit articular chondrocytes was investigated. Resveratrol dramatically reduced NO-induced apoptosis in chondrocytes, as determined by phase-contrast microscopy, the MTT assay, FACS analysis, and DAPI staining. Treatment with resveratrol inhibited the SNP-induced expression of p53 and p21 and reduced the expression of procaspase-3 in chondrocytes, as detected by western blot analysis. SNP-induced degradation of I-kappa B alpha (IκB-α) was rescued by resveratrol treatment, and the SN50 peptide-mediated inhibition of NF-kappa B (NF-κB) activity potently blocked SNP-induced caspase-3 activation and apoptosis. Our results suggest that resveratrol inhibits NO-induced apoptosis through the NF-κB pathway in articular chondrocytes.

Key Words: Resveratrol, Chondrocytes, Nitric oxide, Sodium nitroprusside, Apoptosis, NF-kappa B

INTRODUCTION

Resveratrol (trans-3, 4'-trihydroxystilbene) is a natural polyphenolic compound mainly found in several plants such as red grapes, mulberries, and peanuts (Marques *et al.*, 2009; Shakibaei *et al.*, 2009; Yu *et al.*, 2012). Resveratrol was first detected in the roots of hellebore (*Veratrum grandiflorum*) in 1940, and since then its powerful and diverse biological effects have been well documented: Resveratrol shows antioxidative, anti-inflammatory, anti-aging, and anti-cancer properties due to its anti-proliferative, cell cycle arrest-inducing, and anti-invasive effects (Shakibaei *et al.*, 2009; Mukherjee *et al.*, 2010). Recently, histological analysis *in vivo* has shown that injections of Resveratrol strongly protect against articular cartilage degradation in rabbit models for osteoarthritis (OA) and rheumatoid arthritis (RA) (Elmali *et al.*, 2005; Elmali *et al.*, 2007; Im *et al.*, 2012).

In human articular chondrocytes, anti-apoptotic and anti-inflammatory mechanisms regulated by Resveratrol have been elucidated (Shakibaei *et al.*, 2007; Csaki *et al.*, 2008). Moreover, Resveratrol inhibits the interleukin-1β (IL-1β)-mediated expression of inducible nitric oxide (NO) synthase in articular chondrocytes by activating SIRT1 and thereby suppressing Nuclear Factor kappa B (NF-κB) activity (Lei *et al.*, 2012).

Chondrocytes are differentiated from mesenchymal cells during embryonic development (Solursh, 1989). The phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of the cartilage-specific extracellular matrix (ECM) that ensures matrix integrity, which is compromised in degenerative diseases such as OA and RA (Choy and Panayi, 2001; Sandell and Aigner, 2001). Arthritis is characterized by structural and biochemical changes in chondrocytes and the cartilage, including the degradation of the cartilaginous matrix and insufficient synthesis of the ECM, which leads to a loss of the chondrocyte phenotype (Yoon *et al.*, 2007).

NO produced by inducible NO synthase in articular chondrocytes plays a central role in cartilaginous diseases such as OA and RA. NO triggers cartilage destruction by inducing apoptosis, dedifferentiation, and inflammatory responses

Open Access **http://dx.doi.org/10.4062/biomolther.2013.029**

Received Apr 16, 2013 **Revised** Jul 24, 2013 **Accepted** Jul 26, 2013

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such as cyclooxygenase (COX)-2 expression and prostaglandin E_2 (PGE_2) production in articular chondrocytes (Amin and Abramson, 1998; Abramson *et al.*, 2001; Sandell and Aigner, 2001). Production of NO in primary cultured articular chondrocytes by treatment with the NO donor sodium nitroprusside (SNP) leads to apoptosis, dedifferentiation, and COX-2 expression through a complex protein kinase signaling cascade that involves mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) (Abramson *et al.*, 2001; Kim *et al.*, 2002a; Kim *et al.*, 2002b; Kim *et al.*, 2003; Kim and Chun, 2003). However, the regulation of NO-induced apoptosis has not been clearly elucidated. This study investigated the effects of Resveratrol on the regulation of NO-induced apoptosis. Our results suggest that NF-κB signaling plays a role in the inhibition of by Resveratrol.

MATERIALS AND METHODS

Reagents and antibodies

The following reagents were from commercial sources: Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL; Gaithersburg, MD, USA); fetal bovine-calf serum (FCS) (Invitrogen Corp; Burlington, ON, Canada); Resveratrol, SNP, collagenase type II, ribonuclease A (RNase A), streptomycin, penicillin, dimethylsulfoxide (DMSO), propidium iodide (PI), 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI), Nonidet P-40 (NP-40), and Triton X-100 (Sigma-Aldrich; St. Louis, MO, USA). SN50, a peptide that inhibits the nuclear translocation of activated NFκB, was obtained from Biomol (Plymouth Meeting, PA, USA), and all antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) or Cell Signaling Technology (Danvers, MA, USA).

Cell cultures

Rabbit articular chondrocytes were isolated from the cartilage of 2-week-old New Zealand White rabbits as described (Yoon *et al.*, 2002). Cartilage slices were dissociated enzymatically for 6 h in 0.2% collagenase type II (381 units/ml) in DMEM. Individual cells were obtained by collecting the supernatant after brief centrifugation. The cells were suspended in DMEM supplemented with 10% (v/v) FCS, 50 μg/ml streptomycin, and 50 units/ml penicillin, and then plated in culture dishes at a density of 5×10^4 cells/cm^2. The medium was changed every 2 days after seeding, and cells reached confluence in approximately 5 days. After 3 days in culture, the cells were either treated with 1 mM SNP and 20 μM Resveratrol for 24 h, or pre-stimulated with 20 μM Resveratrol alone for 12 h before being co-treated with Resveratrol and 1 mM SNP for 24 h. SN50 was added 1 h before Resveratrol to inhibit the nuclear translocation of NF-κB. The study protocol was approved by the Institutional Review Board of Kongju National University (IRB No. 2011-2).

Cell proliferation assay

The MTT assay was used to quantify the proliferation of cells treated with Resveratrol and SNP. Cells were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured for 24 h before treating with Resveratrol and SNP. Next, 10 μl/well of MTT reagent1 (methylthiazole tetrazolium, 10 mg/ml) was added to the cells and the plates were incubated for 4 h at 37°C until the purple formazan crystals developed, after which 100 μl/well of MTT reagent 2 (solubilization buffer, 10% SDS with 0.01N HCl, DMSO) was added to the cells. After overnight incubation, the absorbance at 600 nm was measured with a spectrophotometer, and 4 wells were examined for each treatment.

Western blotting

For western blotting, chondrocyte proteins were extracted with a lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, phosphatase inhibitors (1 mM each NaF and Na_3VO_4) and the following protease inhibitors: 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF). The proteins were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, which were blocked with 5% non-fat dry milk for 1 h before incubating with primary antibodies overnight at 4°C. After washing, blots were developed using peroxidase-conjugated secondary antibodies and visualized using an ECL detection system. Actin and p53 were detected using antibodies purchased from Santa Cruz Biotechnology, and procaspase-3, I kappa B-α (IκB-α), and p21 with antibodies from Cell Signaling.

Flow cytometry

Cells were incubated with 1 mM SNP and 20 μM Resveratrol for 24 h, or pre-stimulated with 20 μM Resveratrol alone for 12 h before being co-treated with Resveratrol and SNP for 24 h before measuring apoptosis by flow cytometry. Briefly, after treatments with Resveratrol/SNP, cells were washed once with phosphate-buffered saline (PBS), fixed with ice-cold 70% ethanol, and then stored at 4°C. After washing once more with PBS, cells were suspended in 1 ml of a 50 μg/ml PI solution containing 50 μg/ml RNase A and 0.1% (v/v) NP-40, and then incubated in a 37°C water bath for an additional 20 min in the dark. Flow cytometric analyses were conducted using a cell sorter, and the Cell Quest software program (Partec, Munich, Germany) was used to determine the relative DNA content of cells based on blue fluorescence.

NF-κB -luciferase activity

Activation of NF-κB was determined indirectly by examining IκB-α degradation using Western blot analysis or directly using a reporter gene assay. For the luciferase reporter gene assay, chondrocytes were transfected with a plasmid containing the luciferase coding region and three tandem repeats of the serum response element. Following incubation in complete medium for 6 h, cells were left untreated or treated with the indicated pharmacological reagents and luciferase activity was determined using an assay kit (Promega, Madison, WI, USA).

DAPI staining

For DNA condensation studies, exponentially growing rabbit articular chondrocytes were cultured at a density of 2×10^4 cells/cm^2 in 12-well culture plates and treated with Resveratrol/SNP (as above) for 24 h. After washing with PBS, the chondrocytes were fixed using 3.5% paraformaldehyde in PBS for 15 min, permeabilized using 0.1% Triton X-100 for 10 min, and stained with DAPI (1 μg/ml) for 15 min. All steps were carried out at room temperature. Cells were then examined under a fluorescence microscope and photographed.

Data analyses and statistics

The results are expressed as the means ± SD. Values were calculated from the specified number of determinations. The data were subjected to an analysis of variance (ANOVA) using Tukey's test to analyze differences. A value of $p<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Resveratrol inhibits NO-induced apoptosis in rabbit articular chondrocytes

NO is a free radical that reacts with other radicals such as superoxide or molecular oxygen in tissues to generate highly damaging nitrosating species (Squadrito and Pryor, 1998; Bentz *et al.*, 2012). Exposure of cells *in vitro* and *in vivo* to NO-donor drugs can cause cell death and modulate the activity of mitochondrial enzymes, metalloproteinases (MMPs), and protein kinases through a nitrosylation process (Gu *et al.*, 2002; Gu *et al.*, 2010; Bentz *et al.*, 2012). Previous data have shown that Resveratrol treatment results in inhibition of growth in a wide variety of cancers (Fontecave *et al.*, 1998; Bai *et al.*, 2010). Also, we have confirmed Resveratrol inhibits cell growth in a dose- and time-dependent manner (*data not shown*).

To examine the anti-apoptotic effect of Resveratrol in NO-induced apoptosis, rabbit articular chondrocytes were treated with 20 μM Resveratrol and 1 mM SNP. Observation of cell morphology using a phase-contrast microscope showed that NO-induced apoptosis was inhibited by Resveratrol treatment (Fig. 1A). Resveratrol treatment also potently blocked NO-induced apoptotic death in articular chondrocytes, as measured by the MTT assay (Fig. 1B), similar to of its effect on cell morphology (Fig. 2A). NO-stimulated p53 and p21 expression also decreased under Resveratrol treatment as determined by western blot analysis and quantified by densitometric analysis, respectively (Fig. 1C, D).

Apoptotic cell death was suppressed in chondrocytes treated with SNP (1 mM) and Resveratrol (20 μM) for 24 h (Fig. 2A). The distribution of cells among the different phases of the cell cycle was calculated using the FloMax program (Fig. 2B): treatment with SNP increased the proportion of cells at the sub-G1 phase by approximately 36% relative to control, but when SNP was added together with Resveratrol, significantly fewer cells were found at the sub-G1 phase compared with SNP treatment alone. These results indicate that Resveratrol effectively reduced NO-triggered apoptosis in rabbit articular chondrocytes.

The effect of Resveratrol on IκB, caspases, and apoptosis

To determine the mechanism of Resveratrol-mediated inhibition of apoptosis and NF-κB activation, chondrocytes were treated with SNP (1 mM) and Resveratrol (20 μM) for 24 h. Western blotting showed that NO induced IκB-α degradation, Pro-caspase-3 and caspase-3 expression, and these effects were abrogated by Resveratrol (Fig. 3A). Quantification of the results using the Image J program showed in Fig. 3B. In accordance with the western blotting results (Fig. 3A), DAPI staining (Fig. 3C) showed Resveratrol potently blocked NO-induced apoptotic cell death. These findings suggest that Resveratrol prevents NO-induced apoptosis through NF-κB.

Resveratrol-induced inhibition of apoptosis occurs by a suppression of NF-κB activation

To address the role of NF-κB in the anti-apoptotic effect of Resveratrol, chondrocytes were treated with SN50, a peptide that inhibits the nuclear translocation of activated NF-κB.

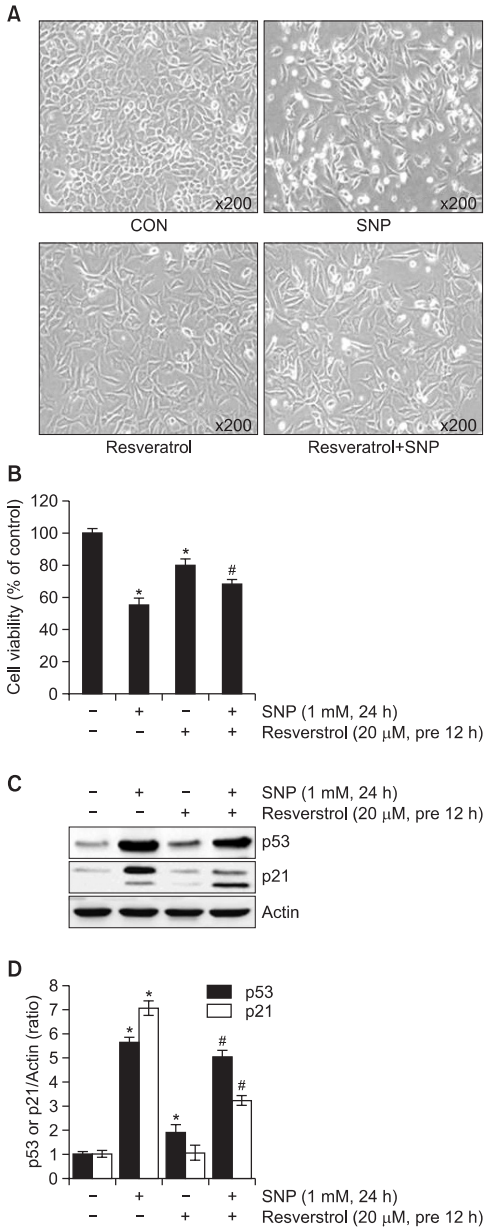

Fig. 1. Resveratrol inhibits NO-induced apoptosis in rabbit articular chondrocytes: cellular and biochemical analyses. (A) Articular chondrocytes were untreated or treated for 24 h with 20 μM Resveratrol with or without 1 mM SNP. Cells were viewed under a phase-contrast microscope and photographed (200× magnification). (B) Apoptosis was measured by the MTT assay. (C) Expression of p53, p21, and actin was examined by western blotting; actin was used as a loading control. (D) The relative amounts of p53 and p21 were quantified by densitometric measurements (Image J). Data are presented as the results of a typical experiment and as the means ± SD. (B, D) (n=4). *$p<0.05$ compared to the control and $^{\#}p<0.05$ compared to the SNP.

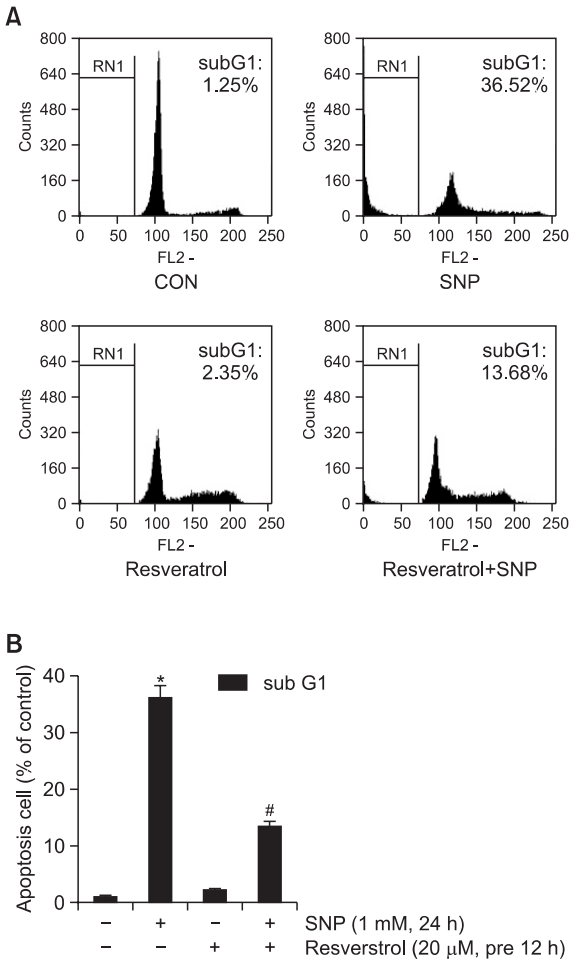

Fig. 2. Resveratrol inhibition of NO-induced apoptosis in rabbit articular chondrocytes: analysis by flow cytometry. (A) Articular chondrocytes were untreated (control) or treated for 24 h with 20 μM Resveratrol with or without 1 mM SNP. Apoptotic cells death was determined by FACS analysis; cells were fixed with 70% ethanol in PBS, and stained with PI. (B) The percentages of sub-G_1 cellular populations are shown. Data are presented as the results of a typical experiment and as the means ± SD. (B) (n=4). *p<0.05 compared to the control and $^{\#}p$<0.05 compared to the SNP.

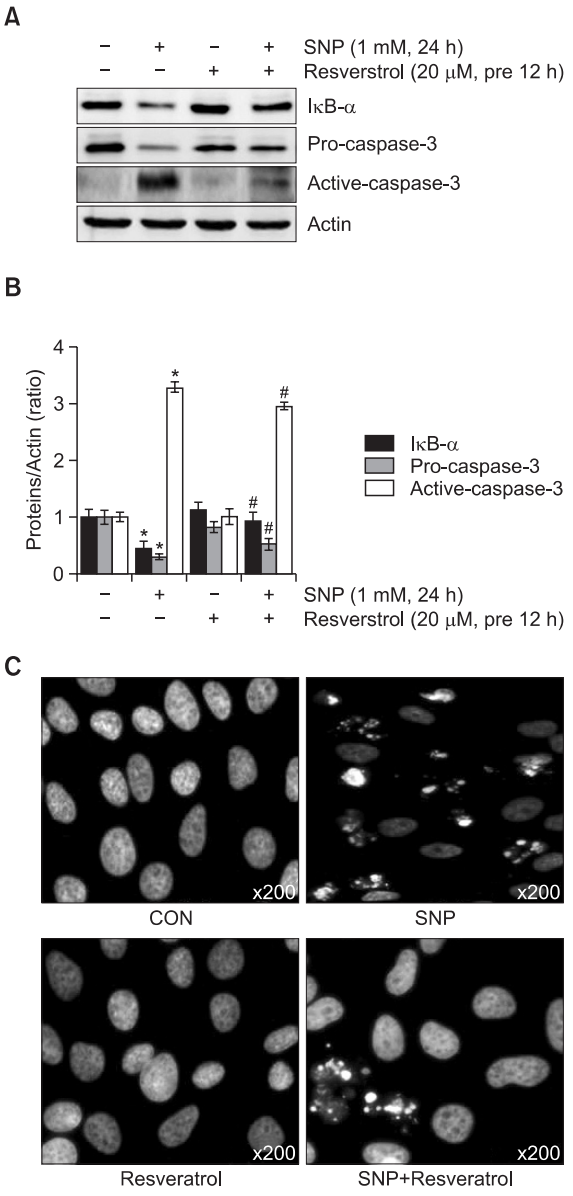

Fig. 3. Inhibition of SNP-induced apoptosis by the Resveratrol-associated NF-κB pathway. (A) Articular chondrocytes were treated with 20 μM Resveratrol for 12 h before stimulation with 1 mM SNP for 24 h. Expression of Iκ B-α and procaspase-3 was assessed by western blotting, with actin expression serving as the control. (B) The relative amounts of Iκ B-α, procaspase-3 and caspase-3 were quantified by densitometric measurements (image J). (C) DAPI-stained nuclei were observed under a fluorescence microscope. (A-C) Representative results and mean values with standard deviation are shown. *p<0.05 compared to the control and $^{\#}p$<0.05 compared to the SNP.

Phase contrast microscopy and MTT assays showed that the inhibition of NF-κB activity with the SN50 peptide enhanced Resveratrol-dependent suppression of apoptosis triggered by NO (Fig. 4A, B). In accord with the results of these cellular analyses, SN50 treatment bolstered the inhibition of NO-induced p53, p21, pro-caspase-3 and caspase-3 expression by Resveratrol (Fig. 4C). Chondrocytes that were pre-treated with SN50 for 1 h and the simulated with Resveratrol for 12 h then co-treated with SNP for 24 h. Inhibition of NF-κB activity with the SN50 peptide blocked NO-induced NF-κB activation by Resveratrol as using NF-κB-luciferase reporter assay (Fig. 4D). These findings suggest NO-induced apoptosis may involve the blockade of NF-κB activation.

DISCUSSION

NO plays a key signaling role in diverse physiological processes. NO regulates the survival of chondrocytes by inducing dedifferentiation and apoptosis (Li *et al.*, 2010; Bentz *et al.*, 2012; Cau *et al.*, 2012; Qureshi *et al.*, 2012). Recent studies from our laboratory have shown that Resveratrol inhibited cell

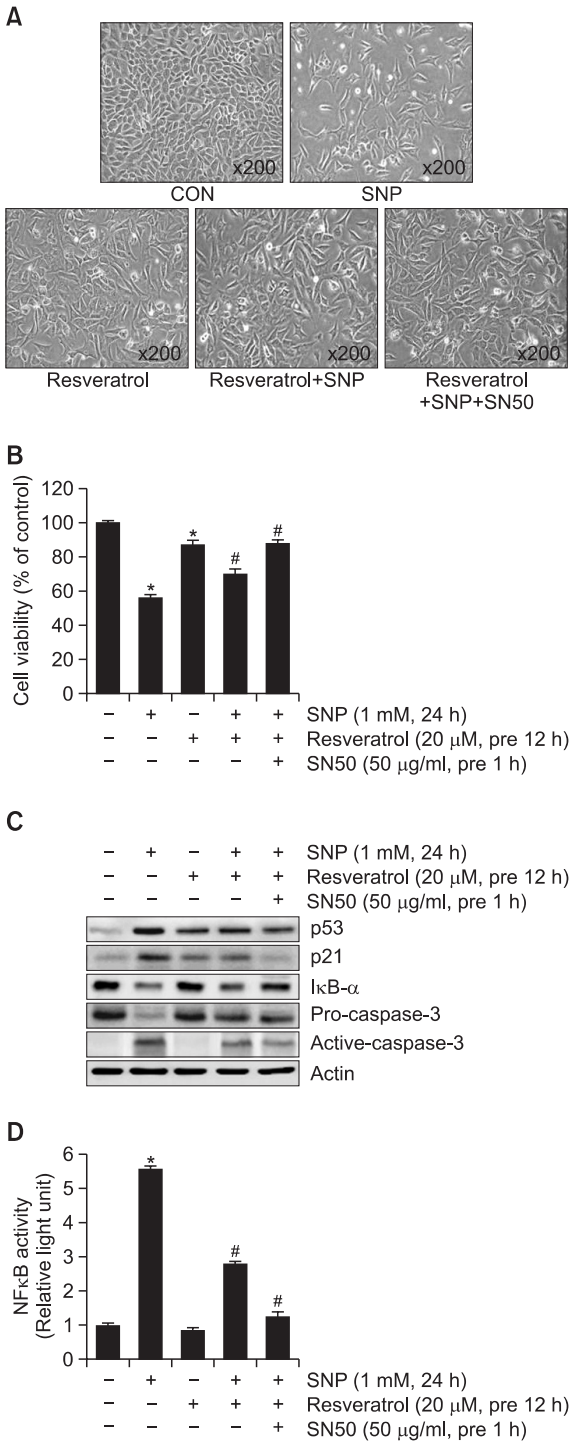

Fig. 4. Relationship between Resveratrol, NF-κB and NO-induced apoptosis: p53, p21 and procaspase-3. (A) Cells were either treated or not treated with 20 μM Resveratrol for 12 h prior to the addition of 1 mM SNP in the absence or presence of 50 μg/ml SN50 (NF-κB inhibitor); cells were photographed in phase contrast (200× magnification). (B) Cell viability was determined using the MTT assay. (C) Expression of p53, p21, procaspase-3, active-caspase-3, IκB-α, and actin was detected by western blotting; actin was used as the loading control. (D) NF-κB activity was determined by luciferase reporter assay. Data are presented as the results of a typical experiment and as the means ± SD. (B, D) (n=4). **p*<0.05 compared to the control and #*p*<0.05 compared to the SNP.

proliferation and regulated differentiation and COX-2 expression by ERK, p38, and Akt signaling (submitted). Moreover, our previous results showed that apoptosis in chondrocytes triggered by treatment with SNP (which generates NO) is regulated by the opposing actions of 2 MAPK subtypes, extracellular signal-regulated kinase-1/-2 (ERK-1/-2) and p38 kinase, coupled with an elevation of p53 protein levels, caspase-3 activation, and differentiation (Kim *et al.*, 2002a; Kim *et al.*, 2003). NO-induced activation of ERK-1/-2 induces differentiation and inhibits apoptosis, whereas activation of p38 kinase induces apoptosis and is responsible for the maintenance of differentiated phenotypes. Thus, inhibition of NO-induced apoptosis by the disruption of the actin cytoskeleton is consistent with the suppression of apoptotic signaling pathways such as the activation of p38 kinase, inhibition of PKC-α and ζ, NF-κB activation, p53 accumulation, and caspase-3 activation (Kim *et al.*, 2003; Kim *et al.*, 2002a; Kim *et al.*, 2002b; Kim *et al.*, 2002c).

NF-κB is an inducible factor that regulates various physiological processes including inflammatory responses and apoptosis. NF-κB plays a crucial role in arthritis, mediating important chondrocyte inflammatory responses that ultimately lead to cartilage degradation. Elevated NF-κB signaling in chondrocytes contributes to cartilage degradation in OA, and NF-κB binding activity is 2-fold higher in OA chondrocytes than in normal chondrocytes (Shakibaei *et al.*, 2008).

The known target genes of NF-κB include both apoptosis-protective genes such as Bcl-2 and p53 dependent pro-apoptotic genes such as NOXA, BAX and BID, suggesting that the cell type- and extracellular stimuli-dependent effects of NF-κB on apoptosis may be due to its specific effects on the expression of apoptosis-regulating genes. In a previous study, Kevin M. Ryan and Mary K. Ernst reported that induction of p53 causes an activation of NF-κB that correlates with the ability of p53 to induce apoptosis. Inhibition or loss of NF-κB activity abrogated p53-induced apoptosis, indicating that NF-κB is essential in p53-mediated cell death (Ryan *et al.*, 2000; Lin *et al.*, 2012).

Elmali *et al.* demonstrated that intra-articular injections of Resveratrol had a protective effect on the cartilage (Elmali *et al.*,2005; Elmali *et al.*,2007; Shakibaei *et al.*,2009). *In vitro* treatment with resveratrol of synoviocytes from rheumatoid arthritis patients demonstrated increased cysteine protease caspase-3 activity, proliferation of synoviocytes, and induction of cell apoptosis (Tang *et al.*,2006; Shakibaei *et al.*, 2009). It has been demonstrated *in vitro* that Resveratrol, unlike its apoptotic action in tumor cells, has an antiapoptotic effect on chondrocytes that is mediated through inhibition of IL-1β-induced stimulation of caspase-3 and cleavage of the DNA repair enzyme poly (ADP)ribose polymerase (PARP) in human articular chondrocytes (Shakibaei *et al.*, 2007; Shakibaei *et al.*, 2009). Furthermore, Resveratrol directly blocked caspase-3 and subsequent cleavage of PARP and reversed the IL-1β-induced upregulation of ROS in chondrocytes. Results from this study not only show the possible utility of Resveratrol in the prevention of osteoarthritis, but also provide interesting results concerning its function as an antioxidant. Moreover, it has been reported that Resveratrol induces p53 degradation through an ubiquitin-independent pathway and thus inhibits p53-dependent apoptosis (Csaki *et al.*, 2008; Shakibaei *et al.*, 2009).

This study investigated the effects of Resveratrol, a natu-

rally occurring phytoalexin and chemotherapeutic agent, on NO-induced NF-κB signaling and apoptosis in rabbit articular chondrocytes. The data presented in this paper provide convincing molecular evidence in support of the hypothesis that Resveratrol suppresses NO-induced apoptosis that occurs through the degradation of IκB-α in chondrocytes *in vitro*.

The findings of this study are the following: (1) Resveratrol inhibited NO-induced apoptosis as determined by phase-contrast microscopy, FACS, MTT analysis, and DAPI staining. (2) Resveratrol protected chondrocytes from SNP-stimulated p53 and p21 expression and reduced the levels of procaspase-3 and IκB-α as shown by western blotting. (3) The anti-apoptotic effects of Resveratrol were significantly strengthened by SN50, a specific inhibitor of NF-κB. Thus, blocking the degradation of IκB-α, or the nuclear translocation of NF-κB by treatment with SN50 decreased Resveratrol-dependent elevation in p53 and p21 expression. Moreover, SN50 accerelated Resveratrol-induced inhibition of NO-dependent increase in procaspase-3 levels in articular chondrocytes. Collectively, these data suggest that Resveratrol regulates NO-induced apoptosis via the blockage of NF-κB activation.

In conclusion, the apoptosis-preventing effect observed in *in vitro* systems as shown in this study may be protect of joint diseases such as arthritis. Although the significance of our findings should be validated in subsequent studies using *in vivo* animal models, the present study strongly suggests that Resveratrol might find use as a therapeutic agent in the treatment of osteoarthritis.

ACKNOWLEDGMENTS

This work was supported by a grant from the Korean Health Technology R&D project, Ministry of Health & Welfare, Republic of Korea (A120960-1201-0000300).

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