

Carvacrol ameliorates inflammatory response in interleukin 1 β -stimulated human chondrocytes

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Abstract. Carvacrol, a monoterpenic phenol present in *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme), possesses anti-inflammatory effects; however, little is known about the effects and underlying mechanism of carvacrol on chondrocytes in osteoarthritis (OA). The present study aimed to investigate the protective effects of carvacrol against inflammation in interleukin 1 β (IL-1 β)-stimulated human chondrocytes. The results indicated that carvacrol inhibited nitric oxide (NO) and prostaglandin E2 (PGE2) production, and decreased the expression of inducible NO synthase (iNOS) and cyclooxygenase (COX-2). Carvacrol also suppressed the protein expression levels of matrix metalloproteinase (MMP)-3 and MMP-13 in IL-1 β -stimulated human OA chondrocytes. Furthermore, carvacrol suppressed the activation of nuclear factor (NF)- κ B signaling pathway in IL-1 β -induced human chondrocytes. In conclusion, the present results demonstrated that carvacrol was able to inhibit IL-1 β -induced NO and PGE2 production, as well as iNOS, COX-2 and MMPs expression in human chondrocytes by suppressing the activation of NF- κ B signaling pathway. Thus, carvacrol may have potential therapeutic functions for the treatment of OA.

Introduction

Osteoarthritis (OA) is a common chronic degenerative joint disease and is a leading cause of pain and disability in the adult population. In Asian countries, the incidence of OA in individuals aged >65 years, may increase from 6.8% in 2008 to 16.2% in 2040 (1). Risk factors of OA may be divided into person-level factors (age, gender, obesity, genetics and diet) and joint-level factors (injury, malalignment and abnormal loading of the joints), which interact in a complex manner (2). It is characterized by bone remodeling, synovium inflammation and cartilage loss (3). Although the management of OA has been diverse, including pharmacological therapy treatment

options, surgical interventions and orthopedic procedures (4,5), there are no effective drug treatments that are able to reverse disease progression (6,7). Therefore, the development of new therapeutic strategies that are effective and safe for OA treatment are required.

OA pathogenesis is complex and involves the interaction of numerous factors, and an increasing number of studies have suggested that inflammation serves a key role in the pathogenesis of OA (8-10). Chondrocytes secrete pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), that may contribute to the progression of OA (11). IL-1 β was reported to enhance the production of matrix metalloproteinases (MMPs) and to inhibit the synthesis of extracellular matrix (ECM), thus contributing the progression of OA (12,13).

Carvacrol is a monoterpenic phenol that is present in *Origanum vulgare* (oregano) and *Thymus vulgaris* (thyme), which has been demonstrated to possess a spectrum of pharmacological activities, including antioxidative, analgesic, antihepatotoxic, antimicrobial and antitumoral (14,15). In addition, a previous study confirmed its anti-inflammatory property. For example, carvacrol was reported to inhibit the levels of inflammatory cytokines and the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in ischemic cortical tissues (16). However, the effects and underlying mechanism of carvacrol on chondrocytes in OA remain unknown. The present study aimed to investigate the protective effects of carvacrol against inflammation in IL-1 β -stimulated human chondrocytes, and the results demonstrated that carvacrol pretreatment inhibited IL-1 β -induced nitric oxide (NO) and prostaglandin E2 (PGE2) production, and reduced the expression levels of iNOS, COX-2 and MMPs in human OA chondrocytes by suppressing the activation of the NF- κ B signaling pathway. Thus, carvacrol may provide a potential therapeutic function for the treatment of OA.

Materials and methods

Normal human articular cartilage chondrocyte culture and treatment. Normal human articular cartilage was obtained from eight patients (4 men, 4 women; age 24-41 years) after death or from trauma in Tianjin Hospital (Tianjin, China). The normal donors were significantly younger whose articular cartilage had no degenerative changes. Written informed consent was

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obtained from the patients according to the terms of the Ethics Committee of Tianjin Hospital. Chondrocytes were isolated from cartilage as previously described (17). Briefly, cartilage fragments were digested with 0.25% trypsin for 15 min and incubated with 0.2% (v/v) collagenase for 4 h at 37°C. The resulting cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from HyClone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37°C and 5% CO₂ in a humidified incubator.

Human chondrocytes (1x10⁵ cells/well) were pretreated with various concentrations of carvacrol (0, 1, 5 and 10 µg/ml; Sigma-Aldrich; Merck KGaA) for 2 h and then co-incubated in the absence or presence of IL-1β (10 ng/ml; Sigma-Aldrich; Merck KGaA) for 24 h at 37°C.

Cell viability assay. Cell viability was measured by the MTT assay. Briefly, following treatments, human chondrocytes (1x10⁵ cells/well) were incubated with MTT solution (5 mg/ml; Sigma-Aldrich; Merck KGaA) at 37°C for 4 h; subsequently, the purple formazan crystals were dissolved using dimethyl sulfoxide by shaking at room temperature for 10 min. Spectrophotometric absorbance was measured at 570 nm using a multifunctional microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All experiments were repeated at least three times.

Measurement of NO and PGE2 levels. Nitrite levels in the culture medium were detected by the Griess reaction as previously described (18). The level of PGE2 was evaluated using a ELISA kit (cat no. KHL1701; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol. All experiments were repeated at least three times.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from human chondrocytes (1x10⁶ cells/well) using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA (1 µg) using the PrimeScript RT Reagent kit with gDNA Eraser (Takara Bio, Inc., Otsu, Japan). Subsequently, a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to conduct RT-qPCR using SYBR Premix Ex Taq II (Takara Bio, Inc.). Primers used in qPCR are listed as follows: iNOS forward, 5'-TTTCCAAGACACTTCACCA-3' and reverse, 5'-ATCTCCTTTGTTACC GCTTCC-3'; COX-2 forward, 5'-GAGAGATGTATCCTC CCACAGTCA-3' and reverse, 5'-GACCAGGCACCAGAC CAAAG-3'; MMP-3 forward, 5'-GCATTGGCTGAGTGA AAGAGACTGTATC-3' and reverse, 5'-ATGATGAACGAT GGACAGATGA-3'; MMP-13 forward, 5'-AGTAGTTCCAAA GGCTACAACCTGTTT-3' and reverse, 5'-GGAGTGGTC AAGCCCTAAGGA-3'; GAPDH forward, 5'-CTGGGGTAC ACTGAGCA-3' and reverse, 5'-AAGTGGTTCGTTGAGG GCAATG-3'. GAPDH was used as a reference gene. The PCR amplification cycles were performed as follows: 30 sec at 95°C, followed by 40 cycles of 5 sec at 95°C and 30 sec at 60°C. The 2^{-ΔΔCt} method (19) was used to calculate relative

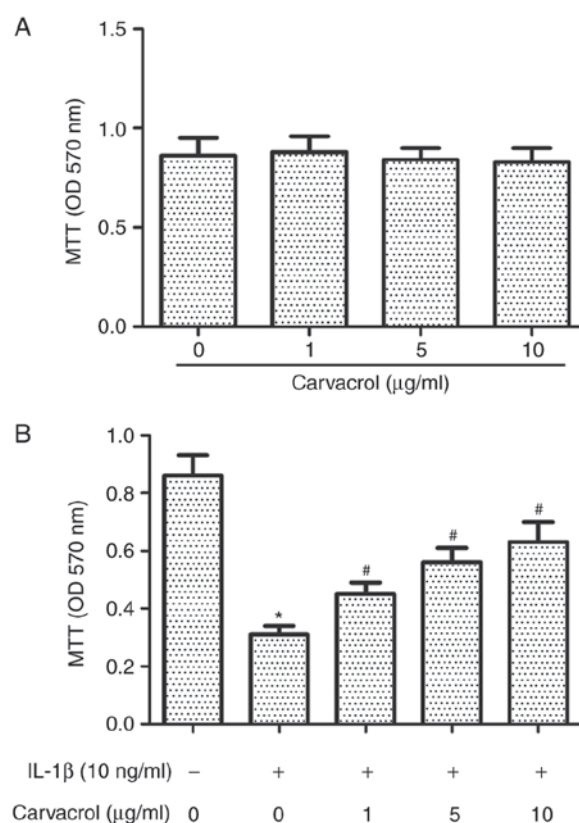


Figure 1. Effects of carvacrol on human osteoarthritis chondrocyte viability. (A) Human chondrocytes (1x10⁵ cells/well) were cultured with various concentrations of carvacrol (0, 1, 5 and 10 µg/ml) for 24 h. Cell viability was detected by the MTT assay. (B) Human chondrocytes (1x10⁵ cells/well) were pretreated with various concentrations (0, 1, 5 10 µg/ml) of carvacrol for 2 h, followed by stimulation with or without IL-1β (10 ng/ml) for 24 h. Cell viability was detected by the MTT assay. All experiments were repeated at least three times. Data are presented as the mean ± standard deviation; *P<0.05 vs. control group; #P<0.05 vs. IL-1β group. IL, interleukin; OD, optical density.

changes in gene expression. All experiments were repeated at least three times.

Western blot analysis. Human chondrocytes (1x10⁶ cells/well) were lysed in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitor mixtures (Sigma-Aldrich; Merck KGaA). Protein concentrations were determined using a Bradford assay (Bio-Rad Laboratories, Inc.). Protein lysates (30 µg) were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes (both from Bio-Rad Laboratories, Inc.). Membranes were blocked in 5% non-fat milk for 2 h at room temperature and then incubated with rabbit anti-human iNOS (1:500; ab3523), COX-2 (1:500; ab52237), MMP-3 (1:500; ab53015), MMP-13 (1:3,000; ab39012), NF-κB p65 (1:50,000; ab32536) and IκBα primary antibodies (1:1,000; ab32518; all from Abcam, Cambridge, UK) overnight at 4°C. Following washing with TBS containing 0.1% Tween-20 (TBST; Sigma-Aldrich; Merck KGaA), membranes were incubated with horseradish peroxidase-conjugated secondary goat anti-rabbit immunoglobulin G antibodies (1:1,000; sc-2922; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 1 h. Membranes were

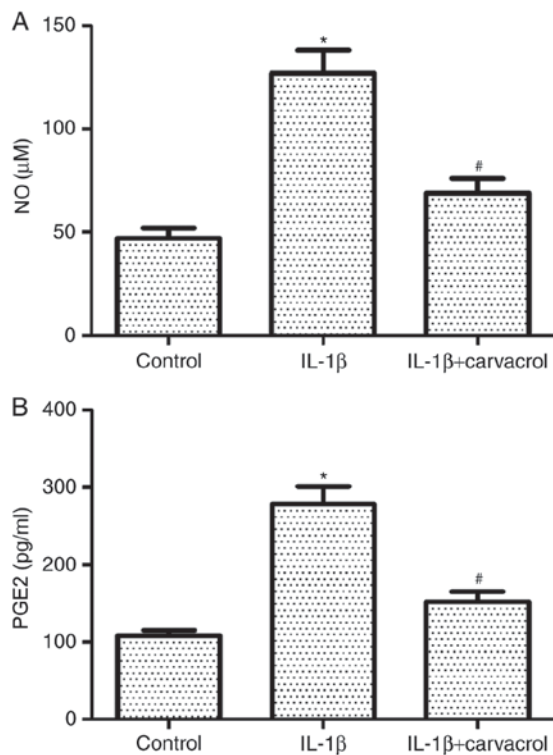


Figure 2. Carvacrol inhibits IL-1 β -induced NO and PGE2 production in osteoarthritis chondrocytes. Human chondrocytes at (1×10^5 cells/well) were pretreated with or without carvacrol (10μ g/ml) for 2 h, followed by stimulation with IL-1 β (10 ng/ml) for 24 h. (A) NO production was determined using the Griess reagent. (B) PGE2 production was determined using a commercial ELISA kit. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation; * $P < 0.05$ vs. control group; # $P < 0.05$ vs. IL-1 β group. IL, interleukin; NO, nitric oxide; PGE2, prostaglandin E2.

washed with TBST buffer, and immunoreactivity was detected with Enhanced Chemiluminescence reagent (GE Healthcare Life Sciences) and quantified by the Quantity One (Bio-Rad Laboratories, Inc.) version 5.2 software. β -actin was used as the internal control. All experiments were repeated at least three times.

Statistical analysis. Statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean \pm standard deviation. One-way analysis of variance followed by Newman-Keuls post-hoc test was used for the statistical comparison of multiple groups. Results from two groups were evaluated using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of carvacrol on human OA chondrocyte viability. Carvacrol cytotoxicity on chondrocyte viability was examined by MTT assay. Compared with untreated chondrocytes, the various treatments with carvacrol at concentrations between 1 and 10μ g/ml did not significantly affect cell viability (Fig. 1A). Treatment with IL-1 β (10 ng/ml) significantly reduced cell viability (Fig. 1B); whereas, pretreatment with carvacrol reversed the effects of IL-1 β in a concentration-dependent

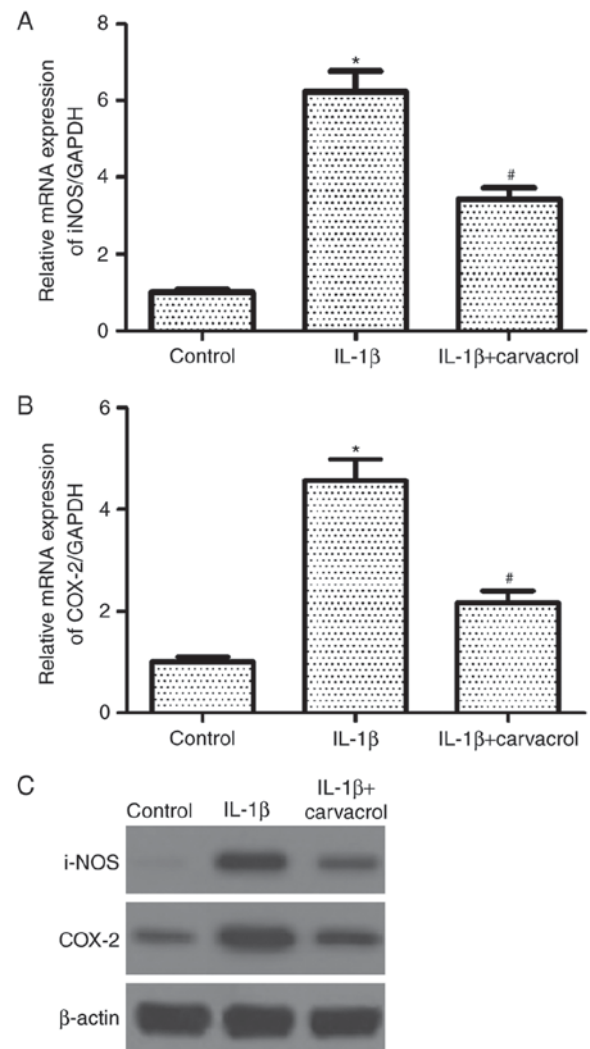


Figure 3. Carvacrol inhibits IL-1 β -induced iNOS and COX-2 expression in OA chondrocytes. Human chondrocytes (1×10^5 cells/well) were pretreated with or without carvacrol (10μ g/ml) for 2 h, followed by stimulation with IL-1 β (10 ng/ml) for 24 h. mRNA expression levels of (A) iNOS and (B) COX-2 were evaluated by reverse transcription-quantitative polymerase chain reaction. (C) Protein expression levels of iNOS and COX-2 were evaluated by western blotting. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation; * $P < 0.05$ vs. control group; # $P < 0.05$ vs. IL-1 β group. COX, cyclooxygenase; IL, interleukin; iNOS, inducible NO synthase.

manner (Fig. 1B). The highest inhibition was observed with 10μ g/ml carvacrol treatment. This concentration of carvacrol was used in the following experiment.

Carvacrol inhibits IL-1 β -induced NO and PGE2 production in OA chondrocytes. The effects of carvacrol on NO and PGE2 production in IL-1 β -induced chondrocytes were also investigated. IL-1 β treatment significantly induced the production of NO and PGE2 in OA chondrocytes (Fig. 2), and these increased levels of expression were significantly inhibited in cells co-treated with carvacrol.

Carvacrol inhibits IL-1 β -induced iNOS and COX-2 expression in OA chondrocytes. Western blot analysis was used to determine the effects of carvacrol on iNOS and COX-2 expression in human chondrocytes stimulated with IL-1 β . The

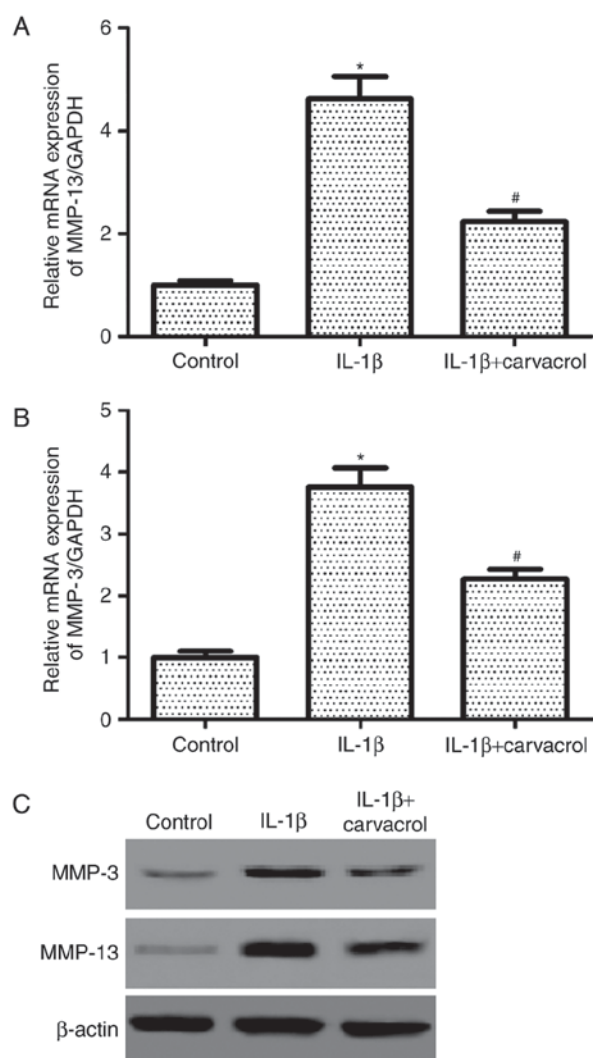


Figure 4. Carvacrol inhibits IL-1 β -induced MMP-3 and MMP-13 expression in OA chondrocytes. Human chondrocytes (1×10^5 cells/well) were pretreated with or without carvacrol (10 μ g/ml) for 2 h, followed by stimulation with IL-1 β (10 ng/ml) for 24 h. mRNA expression levels of (A) MMP-13 and (B) MMP-3 were evaluated by reverse transcription-quantitative polymerase chain reaction. (C) The protein expression levels of MMP-3 and MMP-13 were evaluated by western blotting. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation; * $P < 0.05$ vs. control group; # $P < 0.05$ vs. IL-1 β group. IL, interleukin; MMP, matrix metalloproteinase.

mRNA and protein expression levels of iNOS and COX-2 were markedly increased following IL-1 β incubation compared with untreated controls (Fig. 3). By contrast, chondrocytes that were co-treated with carvacrol exhibited a notable decrease in iNOS and COX-2 expressions compared with IL-1 β -treated OA chondrocytes.

Carvacrol inhibits IL-1 β -induced MMP-3 and MMP-13 expression in OA chondrocytes. A number of studies have demonstrated that MMPs serve crucial roles in the initiation and progression of OA (20-22). Therefore, the effects of carvacrol on MMP-3 and MMP-13 expression in IL-1 β -induced chondrocytes were examined. RT-qPCR analysis results demonstrated that the mRNA expression levels of MMP-13 and MMP-3 generated in IL-1 β -induced chondrocytes

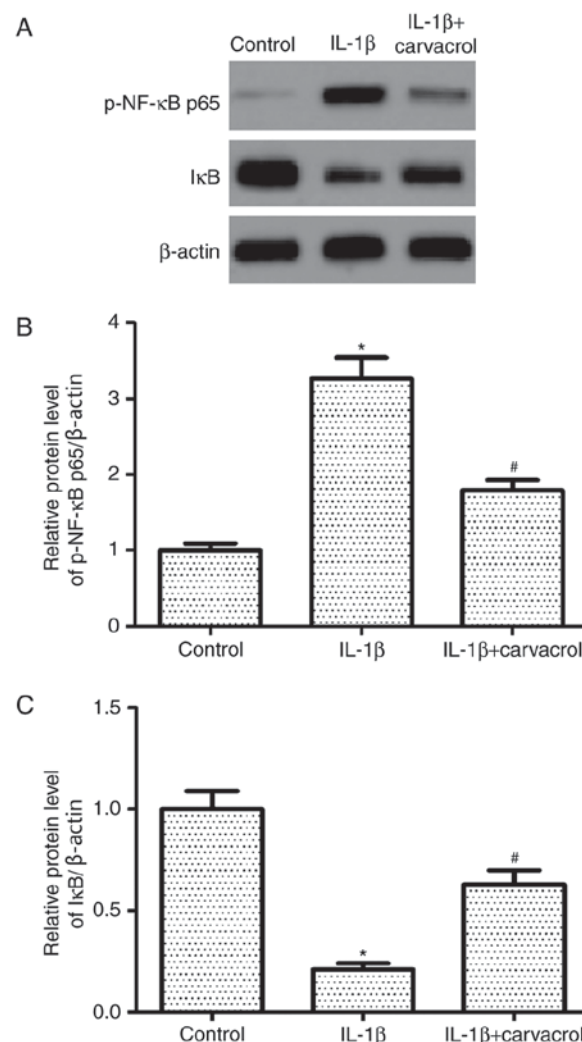


Figure 5. Carvacrol inhibits the activation of NF- κ B signaling pathway in chondrocytes. Human chondrocytes (1×10^5 cells/well) were pretreated with or without carvacrol (10 μ g/ml) for 2 h, followed by stimulation with IL-1 β (10 ng/ml) for 24 h. (A) p-NF- κ B p65 and I κ B α protein expression levels were evaluated by western blotting. Quantification of protein expression levels was performed by calculating the band density ratio of (B) p-NF- κ B p65/ β -actin or (C) I κ B α / β -actin. All experiments were repeated at least three times. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. control group; # $P < 0.05$ vs. IL-1 β group. I κ B α , NF- κ B inhibitor α ; IL, interleukin; NF, nuclear factor; p, phosphorylated.

increased significantly compared with controls (Fig. 4A and B, respectively). However, pretreatment with carvacrol greatly inhibited these IL-1 β -induced effects. Similarly, western blot analysis demonstrated that carvacrol was able to suppress IL-1 β -induced MMP-3 and MMP-13 protein expression in OA chondrocytes (Fig. 4C).

Carvacrol inhibits the activation of nuclear factor (NF)- κ B signaling pathway in chondrocytes. Activation of NF- κ B signaling pathway has been reported to participate in inflammation in OA (23). Therefore, the effects of carvacrol on NF- κ B activation in human chondrocytes stimulated with IL-1 β were investigated. Western blot analysis data revealed that IL-1 β treatment significantly increased the protein expression level of phosphorylated-NF- κ B p65 and reduced the protein expression level of I κ B α in chondrocytes compared with untreated

chondrocytes (Fig. 5). Notably, co-treatment with carvacrol significantly decreased the IL-1 β -induced expression of NF- κ B in chondrocytes and increased the protein expression level of I κ B α .

Discussion

A number of previous studies have reported that carvacrol possesses anti-inflammatory effects (24-27). Results from the present study indicated that carvacrol inhibited NO and PGE2 production, as well as decreased iNOS and COX-2 expression. Carvacrol was also demonstrated to suppress the protein expression levels of MMP-3 and MMP-13 in IL-1 β -stimulated human OA chondrocytes. Furthermore, carvacrol suppressed the activation of NF- κ B signaling pathway in IL-1 β -induced human chondrocytes.

IL-1 β treatment has been widely used to mimic the micro-environment of OA in *in vitro* studies (28-30); in the present study, IL-1 β -induced human OA chondrocytes were used as a model to investigate the protective effects of carvacrol on human chondrocytes, and the results suggested that pretreatment with carvacrol was able to reverse IL-1 β -reduced cell viability.

NO has been demonstrated to serve a pivotal role in the development of OA (31). It is produced by iNOS in several types of cells, including chondrocytes (32). PGE2 is an inflammatory mediator that is elevated by COX-2 (33). In addition, previous studies have reported that IL-1 β was able to induce iNOS and COX-2 expression in chondrocytes, which led to elevated production of NO and PGE2, respectively (34,35). The present study observed that carvacrol treatment inhibited NO and PGE2 production, as well as decreased iNOS and COX-2 expression in IL-1 β -stimulated human OA chondrocytes. These results are in agreement with previous studies, which reported that carvacrol significantly downregulated the expression levels of TNF- α , IL-6, iNOS and COX-2 in D-galactosamine-induced hepatotoxic rats (36).

An increasing number of studies have indicated that MMPs may also be involved in the progression of OA (22,37,38). For example, MMP-3 was reported to induce inflammation by activating various pro-MMPs and the cleavage of extracellular components (39). MMP-13 serves a crucial role in the degradation of collagens, proteoglycans and other ECM macromolecules in cartilage (40). Additional studies have demonstrated that IL-1 β was able to upregulate the expression of MMPs in chondrocytes (41-43). The present study observed that carvacrol co-treatment suppressed IL-1 β -induced MMP-3 and MMP-13 protein expression in OA chondrocytes. These results suggested that carvacrol exhibited chondroprotective activity by downregulating MMP expression *in vitro*.

The NF- κ B signaling pathway serves an important role in OA pathogenesis (44-46). Stimulation by inflammatory mediators such as IL-1 β leads to the phosphorylation and degradation of the inhibitory subunit, which allows the active NF- κ B complex to translocate into nucleus and induced the expression of various inflammation-related genes that regulate the synthesis of cytokines, chemokines and adhesion molecules (47). It was reported previously that the NF- κ B inhibitor, pyrrolidine dithiocarbamate, decreased IL-1 β -induced MMP-3 and MMP-13 production in human

chondrocytes (44). A recent study using ischemic cortical tissues confirmed that carvacrol treatment was able to suppress the ischemia/reperfusion-induced increase in nuclear NF- κ B p65 protein expression in (16). Similarly, results from the present study revealed that pretreatment with carvacrol significantly inhibited IL-1 β -induced NF- κ B activation in OA chondrocytes. These data suggested that carvacrol may inhibit IL-1 β -induced inflammation in chondrocytes by suppressing the activation of the NF- κ B signaling pathway.

In conclusion, the present results demonstrated that carvacrol pretreatment was able to inhibit IL-1 β -induced NO and PGE2 production, as well as reduced the expression levels of iNOS, COX-2, MMPs in human OA chondrocytes by suppressing the activation of NF- κ B signaling pathway. Thus, carvacrol may provide a potential therapeutic function for the treatment of OA.

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