Resveratrol inhibit interleukin-1 β induced inflammatory response in human osteoarthritis chondrocytes through NF- κ B signaling pathway

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Abstract

Osteoarthritis (OA) is a common degenerative disease and affect millions of people. Resveratrol is a coumarin compound refined from traditional Chinese medicines with potential anti-inflammatory ability. This study aimed to evaluate protective anti-inflammatory effects of resveratrol in human OA chondrocytes. The chondrocytes were isolated from OA patients. CCK-8 method was used to explore the optimal dose of resveratrol for chondrocyte. Next, we used PCR and Western-blot method to assess the relative mRNA and protein expression in IL-1 β group. We further explored relevant mechanism of resveratrol for anti-inflammatory in human OA chondrocytes by immunofluorescence and Western-blot. The results showed that resveratrol blocked IL-1 β -stimulated production of NO and PGE2. In addition, resveratrol inhibited the expression of COX-2, iNOs, MMP-1, MMP-3, MMP-13, and increased the levels of aggrecan and collagen-II. Mechanistically, resveratrol suppressed IL-1 β -induced I κ B- α degradation and NF- κ B activation. In conclusion, our results demonstrate that resveratrol inhibits inflammation in OA via the regulation of NF- κ B signaling pathway, and suggested that resveratrol may be a potential therapeutic agent for OA.

Background

Osteoarthritis (OA) acts as a prevalent chronic age-related disease joint disease featuring cartilage degradation, osteophyte formation, and joint stiffness, which seriously affects the health of the elderly people^[1]. There is an increase in the incidence rate of OA with the aging of population, thus it is necessary to find an effective strategy for the treatment of OA^[2].

During the progression of OA, the increased expression of interleukin-1 β (IL-1 β) in the articular cartilage and synovium leads to the destruction of cartilage, downregulation of type II collagen^[3]. Furthermore, IL-1 β has the function of inducing the chondrocyte release of prostaglandin E2 (PGE2) and nitric oxide (NO). Thus, reducing the IL-1 β in articular cavity could delay the development of OA^[4].

Resveratrol is a common polyphenolic compound in the skin of red grapes^[5]. Resveratrol exhibits anti-ageing, anti-inflammatory, and anti-oxidant activities, and has cardio-, neuro-, and chondroprotective effects as proved by many studies^[6]. Nuclear factor-kappa B (NF- κ B) signaling pathway greatly affects the OA progress^[7]. NF- κ B is the central potential pathway that could regulating inflammatory factors. Normally, NF- κ B is combined with I κ B and located in the cytoplasm. Chondrocytes stimulated by IL-1 β would lead to the phosphorylation and degradation of I κ B, and then the NF- κ B p65 is dissociated from I κ B.

However, the effects of resveratrol in treatment of OA are still unknown. This study focuses on investigating whether Resveratrol has a beneficial role in inhibiting inflammatory response induced by IL-1 β in the human osteoarthritis chondrocyte. Besides, the underlying mechanisms of cell inflammatory response mediated by Resveratrol is also explored.

Methods

Reagents and antibodies

Resveratrol (purity>99%) was bought from the Sigma Chemical Co. (St. Louis, MO, USA). IL-1 β was purchased from PeproTech (Rocky Hill, NJ, USA). Primary antibodies against collagen-II, iNOs, and COX-2, and those against p65, p-p65, p-IkBa and IkBa were acquired from the Abcam (Cambridge, MA, USA) and the Cell Signaling Technology (Beverly, MA, USA), respectively. Cell Counting Kit-8 (CCK-8) was provided by the Boster Biotechnology (Wuhan, China).

Primary human chondrocyte culture

We isolated the human chondrocytes from articular cartilages of four knee OA patients (classified as Kellgren-Lawrence score IV). All patients underwent total knee arthroplasty surgery at the Second Affiliated Hospital of Soochow University. Briefly, cartilage tissues were firstly cut into small pieces, followed by being washed by Hank's for 3 times. Then, the small pieces underwent 30 min of digestion with 0.25 mg/mL trypsin, together with 8 h of digestion with 2 mg/mL collagenase type II at 37°C. Cells were first filtered by 180- μ m filter, and then collected and centrifuged at 100g for 5min. After washing by Hank's for three times, the chondrocytes were cultured in DMEM added with 1% penicillin and 10% FBS.

Cell viability

We used CCK-8 kit to measure chondrocytes viability following the instruction of manufacturers. In brief, we seeded human OA chondrocytes in 96-well plates (5×10^5 cells/ml) and treated them with or without resveratrol (6, 12, 24, 48 μ M) for 24h. After that, we added 10 μ L CCK-8 into the plates. All of the wells were incubated for 2–4 hours. Optical density of each wells was measured by Enzyme-linked immunodetector.

qRT-PCR

Total RNA from the human OA chondrocytes (1×10^7 cells) was extracted using a Trizol reagent (Life Technologies, USA) following the instruction of manufacturers. A spectrophotometer was employed to measure the concentration and integrity exhibited by the total RNA considering the A260/A280 ratio. The iScript cDNA synthesis kit (Bio-Rad, USA) helped to reversely transcribe RNA into cDNA in a 20 μ L reaction system. Forward primers and reverse primers are listed in *Table 1*. The $2^{-\Delta\Delta C_t}$ method was adopted to calculate the relative mRNA expression changes. Results are presented as the n-fold change compared with β -actin.

Western blot analysis

RIPA lysis buffer (Solarbio, Beijing, China) was used to lyse chondrocytes and the BCA Protein Quantitation Kit (Boster, Wuhan, China) helped to measure the concentration of the protein. The same amounts of protein from every group received SDS-PAGE treatment, followed by being transferred to nitrocellulose membranes. The membranes were blocked by using 5% skim milk, and received incubation with the primary antibodies against COX-2 (1:500), iNOS (1:1000), p-p65 (1:1000), p65 (1:1000), I κ B (1:1000), and p-I κ B (1:1000), followed by incubation with proper HRP-conjugated secondary antibodies. The bands on PVDF membranes were observed with the exposure machine Amersham Imager 600.

Immunofluorescence

The chondrocytes were seeded in confocal dish and treated as previously described. We used PBS to rinse the confocal dish for three times at a 5-minute interval. Subsequently, all samples of the cells were fixed by 4% paraformaldehyde for 15min. Then, PBS was used to wash these cells for 5 minutes for 3 times. Next, 0.5% BSA was used to block the chondrocytes for 30 minutes at 37°C. Then, chondrocytes were washed by PBS for three times. Then the samples underwent overnight incubation with rabbit anti-human collagen II antibody (1:500; Santacruz, USA) at 4°C. The chondrocytes were then received 30 min of incubation with FITC-labeled goat anti-rabbit secondary antibody (1:800; Boster, Wuhan, China) at room temperature. The nuclei were stained for one min with DAPI at room temperature and then washed by PBS for 3 times (5 min each time).

Statistical analysis

Data were analyzed with the SPSS 19.0 software (version 19.0, SPSS Corp., Chicago, American). Data are represented as the mean $\pm$ standard deviation (SD) of the three independent experiments. The one-way analysis of variance (ANOVA) was performed to analyze the statistical significance and Tukey's post-hoc test helped to compare different groups. GraphPad Prism software (Version 6.0; GraphPad Software, San Diego, CA, USA) was employed to graph. $P < 0.05$ was considered with a statistically significant difference.

Results

Resveratrol affects human OA chondrocyte viability

Chondrocytes was stimulated with different concentrations of resveratrol (6, 12, 24, 48 μ M) by CCK-8 method. According to **Figure. 1**, 48 μ M resveratrol remarkably lowered the viability of cell after 24h of treatment. There was no significant difference between the 6, 12, 24 μ M relative to the control group ($P > 0.05$).

Effect of resveratrol on NO, PGE₂, iNOS and COX-2 production in human OA chondrocytes

Based on **Figure. 2** and **Figure. 3**, IL-1 β could lead to an obvious increase in the PGE2, NO, COX-2 and iNOS production compared with the control group. Nevertheless, following resveratrol administration at higher concentrations (24 μ M), the PGE2, NO, COX-2 and iNOS production was significantly decreased.

Resveratrol affects the expression of MMP-1, MMP-3 and MMP-13 in human OA chondrocytes

Figure. 4, displays an obvious increase in the expression of MMP-1, MMP-3 and MMP-13 mRNA due to IL-1 β . Resveratrol significantly reduced the MMP-1, MMP-3 and MMP-13 due to IL-1 β , and the optimal dose was 24 μ M.

Resveratrol affects the expression of aggrecan and collagen-II in human OA chondrocytes

Immunofluorescence results were presented in **Figure. 5**. We found that IL-1 β could remarkably decrease the collagen-II expression. The expression of collagen-II presented an obvious uptrend in resveratrol group than IL-1 β group. We used RT-PCR to further confirm our immunofluorescence results. We measured Aggrecan and collagen-II relative expression in these groups, finding the obvious decrease in the expression of collagen-II and Aggrecan due to IL-1 β . Resveratrol could rescue the degradation of Aggrecan and collagen-II in part (**Figure. 6**).

Resveratrol affects the activation of NF- κ B in human OA chondrocytes

As demonstrated in Figure. 7A and B, treatment with IL-1 β in chondrocytes increased the expression of p-I κ B or p-p65. Nevertheless, groups under the co-treatment with resveratrol still exhibited a lower level of p-I κ B or p-p65 relative to the IL-1 β group. The total I κ B and p65 presented an unchangeable expression in different treatment groups respectively. As demonstrated by these data, resveratrol could restore the activation level exhibited by p-I κ B and p-p65 signaling cascades (**Figure. 7**).

Discussion

We firstly identify the optimal dose of resveratrol in inhibiting inflammation of OA. Resveratrol significantly decreased the overproduction of NO, PGE2, COX-2, MMP-1, MMP-3, MMP-13 and iNOS stimulated by IL-1 β . These effects was mainly regulated the NF- κ B signaling pathway.

Resveratrol was extracted from the root of *Veratrum grandiflorum* in 1940 for the first time by a Japanese^[8]. Possessing a stilbene structure, resveratrol acts as a non-flavonoid polyphenol compound. It has been proven that resveratrol can make plants more resistant to pathogens as well as improve environment deterioration^[6]. Wei et al.^[6] also revealed that resveratrol could improve the inflammatory damage as well as protect against OA in an OA rat model. Xu et al.^[5] revealed that resveratrol could inhibit TLR4 through activating PI3K/Akt signaling pathways in obesity-related osteoarthritis, thus exerting anti-osteoarthritic effects. Hussain et al.^[9] conducted a pilot interventional study and found that resveratrol was superior in terms of safety and efficacy to meloxicam alone for the treatment of pain and improvement of physical function in knee OA patients.

Firstly, we found that the optimal dose of resveratrol by CCK-8 methods. We found that when the dose of resveratrol larger than 48 μM , the cells viability was down with statistically significantly than control group and other dose of resveratrol. Kang et al.^[10] revealed that resveratrol had no cytotoxic effect at 1 to 100 μM . Reason may be that they used rabbit chondrocyte and performed lactate dehydrogenase (LDH) assay to assess toxic effect.

It is known that articular chondrocytes-generated IL-1 β has the function of stimulating the development of osteoarthritis. As reported, IL-1 β could remarkably enhance the expressions exhibited by pro-inflammatory cytokines and MMPs in the chondrocyte; therefore, it enjoys a wide application in the stimulation of inflammatory response in the chondrocyte to simulate OA in vitro^[11]. When we used IL-1 β stimulate the chondrocytes, we found an obviously up-regulated MMP-1, MMP-3 and MMP-13^[12]. MMPs serve as the major enzymes regulating the remodeling of tissue as well as the degradation of extracellular matrix like collagens and aggrecan. Above results conformed previous findings^[13]. Gu et al.^[14] reported that resveratrol had the function of suppressing the MMP-13 and IL-6 expression in human articular chondrocytes induced by IL-1 β through signaling cascades dependent and independent of TLR4/MyD88. Therefore, it is speculated that resveratrol could make MMPs less produced and activated during the progress of OA, thus exerting the anti-inflammation effect.

NF- κB signaling serves as an essential pathway related to the OA pathogenesis. Rigoglou et al.^[15] proposed that any strategies interfering NF- κB signaling pathway activation could provide novel potential treatment options for OA. When the NF- κB signaling pathway was activated, downstream protein such as NOS, COX-2 and MMPs were overexpression^[16,17]. In normal cartilage, NF- κB is located in the cytoplasm and bonded with I κB . Stimulating with IL-1 β and other stimulations would contribute to the phosphorylation and degradation of I κB , and then NF- κB p65 could be dissociated from I κB ^[18], followed by being translocated into nucleus for activating genes related to inflammatory. Conforming the previously published study results, this study found the ability of IL-1 β to facilitate p65 phosphorylation and I $\kappa\text{B}\alpha$ degradation in the chondrocyte. Also, treating with resveratrol could weaken the NF- κB activation.

Moreover, resveratrol potently inhibited MMP-1, MMP-3 and MMP-13 expressions and aggrecan and collagen-II degradation, and the extracellular matrix was degraded. Besides, resveratrol exerted an obvious suppressing impact on the phosphorylation of NF- κB stimulated by IL-1 β in human OA chondrocytes.

Conclusion

To sum up, according to the current study, resveratrol weakens the inflammatory response induced by IL-1 β in human OA chondrocytes. As proved by other studies regarding the potential mechanism, inhibiting the activation of NF- κB could enhance the protective effect imposed by resveratrol. The study result confirmed eriodictyol as a novel preventative agent specific to OA. Nevertheless, the currently obtained

results with regard to the in vitro situation might not be applicable for the in vivo situation, thus direct tests are needed.

Declarations

Acknowledgment

Not Applicable.

Availability of data and material

Requests for the raw data may be sent to the corresponding author.

Abbreviations

OA = Osteoarthritis; IL-1 β = interleukin-1 β ; CCK-8 = cell counting kit-8; COX-2 = cyclooxygenase-2; MMP-1 = matrix metalloproteinase-1; MMP-3 = matrix metalloproteinase-3; MMP-13 = matrix metalloproteinase-13; NF- κ B = nuclear factor-kappa B; PGE2 = prostaglandin E2; NO = nitric oxide;

Conflicts of Interest

The authors declare that there are no conflicts of interest in the paper.

Competing interests

The authors declare that they have no competing interests.

Author contribution

XFS, YC and QRD conducted experiments; MQZ analyzed data; QRD and XFS wrote manuscript; YC revised manuscript; XFS and YC designed the experiment and edited manuscript.

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Consent for publication

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Not applicable.

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Table

Gene	Forward primer	Reverse primer
MMP-1	5'-GGGAATAAGTACTGGGCTGTTTCAG-3'	5'-CCTCAGAAAGAGCAGCATCGATATG-3'
MMP-3	5'-CTGGCCTGCTGGCTCATGCTT-3'	5'-GCAGGGTCCTTGGAGTGGTCA-3'
MMP-13	5'-CCAGAACTTCCCAACCAT-3'	5'-ACCCTCCATAATGTCATACC-3'
GAPDH	5'-CGACAGTCAGCCGCATCTT-3'	5'-CCAATACGACCAAATCCGTTG-3'

Table 1: Primers used in this study.

Figures

Figure 2

How resveratrol affects the viability of human OA chondrocyte. The cells received 24 h of treatment with resveratrol (0, 6, 12, 24, 48 μM). CCK-8 assay helped to determine the viability exhibited by cell. The values are represented as the mean ± SD of 3 separate experiments. *p < 0.05 in comparison to the 0 μM group.

Figure 4

Effect of resveratrol on IL-1 β -induced Nitrite (A) and PGE-2 (B) expression in human OA chondrocytes induced by IL-1 β . Chondrocytes received pretreatment with resveratrol at different concentrates (6, 12, 24 μ M), followed by 24 h of treatment with or without IL-1 β (10 ng/ml). # $p < 0.05$ in comparison to the control group, * $p < 0.05$, ** $p < 0.01$ in comparison to the IL-1 β group.

Figure 6

Effects of the resveratrol on the expression of iNOS and COX-2 in human OA chondrocytes induced by IL-1 β .

Figure 8

Effects of resveratrol on the expression of MMP-1 (A), MMP-3 (B) and MMP-13 (C) in human OA chondrocytes induced by IL-1 β . #p<0.05 relative to the control group, *p < 0.05, **p < 0.01 relative to the IL-1 β group.

Figure 10

Effects of the resveratrol on the expression of collagen-II in human OA chondrocytes induced by IL-1 β .

Figure 12

Effects of resveratrol on the expression of aggrecan and collagen-II in human OA chondrocytes induced by IL-1 β . #p<0.05 in comparison to the control group, *p < 0.05, **p < 0.01 in comparison to the IL-1 β group.

Figure 14

Effects of resveratrol affects the activation of NF- κ B in human OA chondrocytes.