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ORIGINAL ARTICLE

Knee Joint Injection Resveratrol Amelioration Inflammation in Collagen Antibody Induced Arthritis

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Background: Resveratrol has been considered to possess anti-inflammatory properties. Here, we propose that intra-articular resveratrol injection might ameliorate acute inflammation in a single knee joint in mice with collagen antibody-induced arthritis (CAIA). **Methods:** The clinical expression of CAIA was analyzed by two investigators. Resveratrol (30 mg/kg in 30 μL dimethyl sulfoxide [DMSO]) was injected into the knee joint of BALB/c mice from days 0 to 9. Footpad thickness was photographed and calculated. On day 10, the joints were examined histopathologically. Serum cytokine levels were measured by enzyme-linked immunosorbent assay. mRNA levels in paws were measured by real-time polymerase chain reaction (PCR). **Results:** In CAIA, acute inflammation was induced on day 7 and continued until day 10. In the resveratrol-treated mice, the severity of inflammation was obviously reduced. Expression levels of interleukin-1β, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase were decreased in the treated mice; however, tumor necrosis factor-α (TNF-α) levels did not change significantly after treatment. **Conclusion:** The anti-inflammatory mechanism of resveratrol is associated with NF-κB inhibition. Further investigations of the clinical applications of resveratrol are required.

Key words: Resveratrol, experimental arthritis, anti-inflammation, knee joint injection

INTRODUCTION

Resveratrol, a constituent of red wine, has long been considered to possess anti-inflammatory properties. It has been shown to inhibit inflammatory cytokine response and reactive oxygen species (ROS) in neutrophils, monocytes, and macrophages and to suppress osteoarthritis (OA) development in a rabbit model.[1] We postulate that resveratrol can prevent acute inflammation in mice with collagen antibody (Ab)-induced arthritis (CAIA).

Rheumatoid arthritis (RA) is an inflammatory disorder that affects 1% of the adults worldwide. RA's hallmark is leukocyte infiltration of the synovium.[2] This inflammatory process stimulates the proliferation of apoptosis-resistant fibroblast-like synoviocytes (FLS), leading to pannus formation and joint destruction.[3,4] Intra-articular expression of pro-inflammatory cytokines, particularly that of TNF-α and interleukin (IL)-1β, plays key roles in RA pathogenesis.[5]

Anti-type II collagen Ab (anti-CII Ab) is an autoAb known to be present in RA patients.[6] CAIA is commonly used as an RA model for screening antirheumatic drugs because of its similarity with human RA.[7] In the CAIA model, anti-CII Ab plays an important role; it induces arthritis in mice by passive transfer.[7] An arthritis model using a mixture of four monoclonal Abs (mAbs) (monoclonal anti-CII Abs) has been established.[8] This model can be prepared in constant yields by using various strains of mice, independent of major histocompatibility complex haplotypes.[8] However, to date, how arthritis develops remains unclear. There is considerable evidence demonstrating resveratrol's anti-inflammatory properties, including ROS inhibition in neutrophils,[9] monocytes,[10] and macrophages.[11] It has been shown that resveratrol inhibits the release of various cytokines from

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macrophages and lymphocytes, such as IL-6, IFN-γ, IL-2, TNF-α, and IL-12.[12-14] In stimulated macrophages, the expression of inducible nitric oxide synthase (iNOS) and the release of nitric oxide are reduced by resveratrol.[13,15,16] Decreased cyclooxygenase (COX)-1 and COX-2 expression and activity induced by resveratrol are also apparent.[17,18] Other reports have also demonstrated that resveratrol inhibits matrix metalloproteinase-9 expression.[19,20] Moreover, resveratrol can increase cell apoptosis by decreasing Bcl-2 expression and by increasing caspase substrate levels and Bax expression along with reducing cell proliferation.[21-25] The underlying mechanisms for the inhibitory effects of resveratrol mentioned above could be due to its ability to inhibit factors involved in gene transcription, such as MAPK, c-JNK, AP-1, and NF-κB.[26] Indeed, recently, the effect of resveratrol on NF-κB has been in focus. It has been suggested that resveratrol prevents the translocation of NF-κB into the nucleus by inhibiting Iκ-B kinase.[27] Alternatively, resveratrol acts on NF-κB through its sirtuin-like activity, which deacetylates NF-κB.[28,29]

Our study aimed to reveal the anti-inflammatory effects of resveratrol in a murine experimental arthritis model and to develop a potential therapy for RA in future.

MATERIALS AND METHODS

Materials

Resveratrol (trans-3,5,4′-trihydroxystilbene) (Sigma Aldrich, USA) was prepared in DMSO and stored at −20°C. Resveratrol (30 mg/kg in 30 μL DMSO) was injected into the knee joint of BALB/c mice from days 0 to 9. The control group was administered a knee injection with 30 μL DMSO.

Mice

Male BALB/cByJNarl (BALB/c) mice aged 6 weeks were purchased from the National Laboratory Animal Center (Taiwan) and subsequently bred and maintained under specific pathogen-free conditions in the Laboratory Animal Center of the National Defense Medical Center (Taipei, Taiwan).

Induction of arthritis in mice

An arthritogenic mAb cocktail and lipopolysaccharide (LPS) were purchased from Immuno-Biological Laboratories (Chemicon International, Inc., Temecula, CA, USA). Polyethylene glycol (PG200) was purchased from Sigma Aldrich (USA). Arthritis was induced by the method of Terato *et al*. by using an arthritogenic mAb cocktail.[8] The mAb cocktail contained four mAbs (F10, A2, D8, and D1) in equal amounts. Three clones (F10, A2, and D8) were type IgG2a and 1 clone (D1) was type IgG2b. For inducing arthritis, each mouse was intravenously injected with 2 mg anti-CII Ab, and 3 days later, 50 μg (BALB/c background) of LPS (*Escherichia coli* 0111:B4) was injected intraperitoneally.

Clinical assessment of arthritis

Arthritis development was monitored in all the four limbs by using a macroscopic scoring system. Briefly, each swollen or red toe was given 1 point; each swollen joint (metatarsal phalangeal joints, metacarpal phalangeal joints, proximal interphalangeal joints, and distal interphalangeal joints), 1 point; and a swollen ankle (maximum score per limb, 15; maximum score per mouse, 60), 5 points.[30] The mice were examined on days 0, 3, 7, and 10 after Ab injection. The thickness of each paw was measured in a noncontact manner (Simplified Geometry Measurement System, Advanced Design Research Technology Co. Ltd, UK). For each mouse, the footpad swelling was imaged three times by two investigators. Footpad thickness was measured from the distal part to the tip of the ankle (3 mm) and at the base of the first toe. A trained researcher independently performed a blinded evaluation of the imaging data.[31]

Histologic examination

Knee and ankle joints were fixed in 10% formalin, decalcified, trimmed, and embedded in paraffin. Sections were prepared from the tissue blocks and stained with hematoxylin and eosin. Histopathological scoring was performed as described below. The joints of arthritic mice were assigned inflammatory scores of 0–5 for inflammation, according to the following criteria: 0, normal; 1, minimal infiltration of inflammatory cells in the periarticular area; 2, mild infiltration; 3, moderate infiltration; 4, marked infiltration; and 5, severe infiltration.[32] Each slide was scored by three independent observers, and the average score was used.

Cytokine assay

An enzyme-linked immunosorbent assay for IL-1β was performed using purified mAb-coated plates. All procedures followed the standard protocols recommended by R and D Systems. Cytokine concentrations were measured using an MRX microplate reader (Dynex Technologies Inc., Chantilly, VA) at 450 nm (reference, 540 nm).

RNA isolation, reverse transcription, and real-time polymerase chain reaction

Total RNA was isolated using the Trizol method, with homogenization of the samples in Trizol lysis buffer, followed by chloroform extraction (Invitrogen, Life Technology, Carlsbad, CA, USA). The RNA was dissolved in 20 μL RNase-free water and quantified using a spectrophotometer. The optical density 260/280 nm ratios were determined. For cDNA synthesis, 5 μg

total RNA was reverse transcribed at 50°C for 60 min, using 200 units Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The primer sequence was as follows: IL-1β, forward primer (5′-CCAGCAGGTTATCATCATCATCC), reverse primer (5′-CTCGCAGCAGCACATCAAC); COX-2, forward primer (5′-GAGTCATTCACCAGACAGATTG), reverse primer (5′-CTTGTACAGCAATTGGCACA); TNF-α, forward primer (5′-GGGCCACCACGCTCTTCTGTCT), reverse primer (5′-GCCACTCCAGCTGCTCCTCCAC); iNOS, forward primer (5′-CAGCTGGGCTGTACAAACCTT), reverse primer (5′-CATTGGAAGTGAAGCGTTTCG); and GAPDH, forward primer (5′-TGGCAAAGTGGAGATTGTTGCC), reverse primer (5′-AAGATGGTGATGGGCTTCCCG). An SYBR Green master mix kit (Bio-Rad, Hercules, CA) was used for each real-time PCR. Briefly, PCR was performed as follows: 94°C for 2 min; followed by 40 cycles of denaturation, annealing, and extension at 94°C for 15 s, 64°C for 30 s, and 72°C for 45 s, respectively; and a final extension at 72°C for 10 min. For all samples and the GAPDH control, PCR was performed in triplicate. The ratios for each product/GAPDH mRNA were calculated for each sample. Data were expressed as the fold increase or fold decrease in mRNA. The products of all real-time PCR assays were run on a gel to confirm the presence of a single band.

Cell culture

RAW 264.7 cells were cultured in RPMI-1640 supplemented with 10% endotoxin-free, heat-inactivated, fetal calf serum (Gibco, Thermo Fisher, USA). Macrophage cells were seeded (6×10^5) in 6-well plates and incubated overnight. After incubation, the cells were pretreated with 30 or 60 μM resveratrol in 1% PEG200 for 4 h. Then, LPS (1 μg/mL) was added for 1 h in real-time PCR and for 4 h in the protein study.

Protein extraction and Western Blotting

Protein was extracted from cells by homogenizing in 0.2 ml of PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Gyeonggi-do, Korea) and then incubated on ice for 20–30 min to induce cell lyses. After centrifugation at 13,000 rpm for 5 min at 4°C, the supernatant was transferred to a new Eppendorf tube. Protein concentration was determined using the BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL, USA), and BSA was used as the standard. Twenty micrograms of the protein samples was separated on 10% SDS-PAGE, followed by transferring to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% BSA at room temperature for 2 h and then incubated with rabbit anti-phosphorylated p65 Ab (1:1000; cell signaling) or rabbit anti-p65 Ab (1:1000; cell signaling) for 2 h. After washing with phosphate-buffered saline with Tween (PBST) (0.05% Tween 20 in PBS) three times, the membrane was incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG Ab (1:5000; Santa Cruz Biotechnology Inc., Santa Cruz , CA, USA) for 1 h. The membrane was subsequently washed with PBST three times. After incubating with chemiluminescent HRP substrate (Millipore Corp., Bedford, MA, USA) for 1 min, the signals were detected by the LAS-3000 imaging system (Fujifilm, Tokyo, Japan).

Statistical analysis

Statistical significance was determined using the Mann–Whitney U-test. Values were indicated as mean ± standard error of mean. $P < 0.05$ was considered statistically significant.

RESULTS

Footpad thickness after resveratrol or dimethyl sulfoxide knee injection

There was no definite difference between the groups with regard to footpad thickness ($n = 5$). In both groups, footpad thickness dramatically increased from days 7 to 10 [Figure 1] as compared to the baseline; the footpad thickness on day 7 showed an average increase of 0.468 ± 0.127 mm (resveratrol in DMSO group) and that on day 10 showed an average increase of 0.457 ± 0.133 mm ($n = 5$). The footpad thickness of the resveratrol in DMSO group was lower than that of the DMSO group until 7 experimental days. However, there was no significant difference.

Body weight after resveratrol or dimethyl sulfoxide knee injection

The body weight fluctuation after CAIA induction is shown

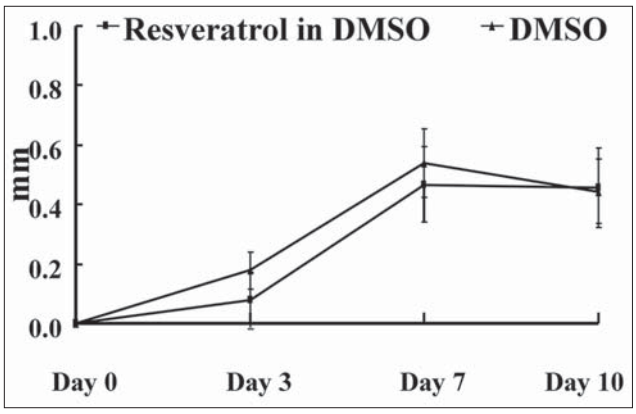

Figure 1: Footpad thickness changes. BALB/c mice were administered a knee joint injection of either resveratrol in dimethyl sulfoxide or dimethyl sulfoxide alone. The relative changes in the footpad thickness of the two groups were recorded, and the data are represented as mean ± standard error of mean

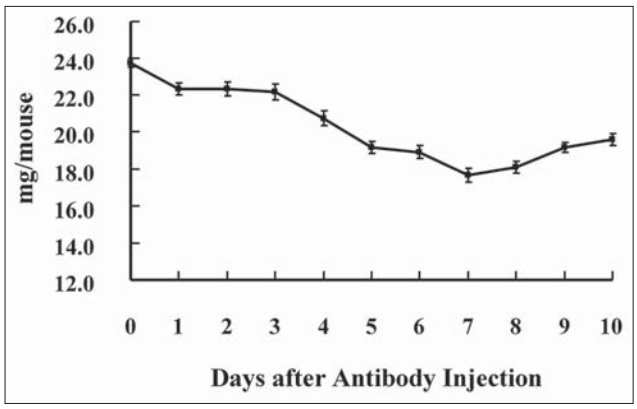

Figure 2: Body weight fluctuation. BALB/c mice were administered anti-collagen II antibody on day 0 for collagen antibody-induced arthritis induction. Lipopolysaccharide was injected on day 3. The data are represented as mean ± standard error of mean

in Figure 2. The body weight changed every day. The most significant body weight loss was observed around day 7. The inflammatory severity of CAIA was the strongest around the same time. The body weight decreased progressively up to day 7 and gained slowly thereafter, until day 10.

Interleukin-1β concentration in the irrigation fluid of knee joints injected with resveratrol (left knee) and dimethyl sulfoxide (right knee)

The IL-1β level in the knee joint irrigation fluid was measured on day 10, and it was found to be significantly higher in the DMSO-injected knee (82.76 ± 12.38 pg/mL) than in the resveratrol-injected knee (62.39 ± 8.3 pg/mL) [Figure 3].

Inflammatory score

Inflammatory score was reduced in the resveratrol-injected knees, but there was no difference between the inflammation in the left and right ankle joints ($P = 0.0215$)

The experimental period was unremarkable with no infections. The knee and ankle joints were harvested on day 10. The inflammatory score of the DMSO-injected knee group was 3.95 ± 0.23, whereas that of the resveratrol-injected knee group was 2.2 ± 0.58 ($n = 5$, $P = 0.0215$). Changes in inflammation were observed in both the right and left ankle joints (inflammatory scores: 4.4 ± 0.29 and 4.4 ± 0.29, respectively) [Figure 4]. However, there was no difference between the right and left ankle joints with regard to inflammatory scores.

Anti-inflammatory effect of resveratrol in RAW 264.7 cells treated with lipopolysaccharide

The relative expression level of IL-1β in RAW 264.7 cells injected with LPS was reduced in the groups pretreated with

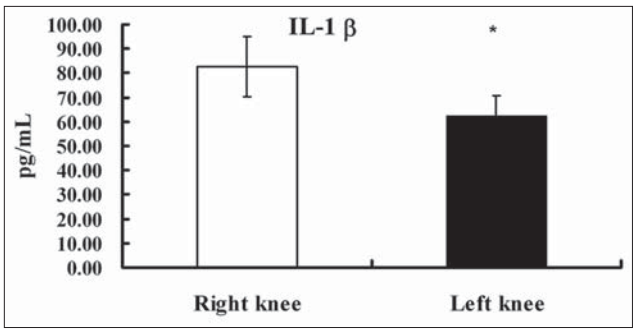

Figure 3: Interleukin-1β concentration in the knee joint irrigation fluid. The right knee was treated with dimethyl sulfoxide, and the left knee was treated with resveratrol in dimethyl sulfoxide. The concentration of IL-1β in the irrigation fluid was measured using enzyme-linked immunosorbent assay, and the data are represented as mean ± standard error of mean ($n = 5$, $^*P = 0.043$)

resveratrol ($^*P < 0.05$). The relative expression level of IL-1β in the 60-μM group was significantly different from that in the 30 μM group ($^*P < 0.05$). These data show that resveratrol reduces IL-1β expression in a dose-dependent manner in RAW 264.7 cells treated with LPS [Figure 5]. The relative expression levels of COX-2 and iNOS also showed the same pattern as that of IL-1β, but that of TNF-α showed contrary results.

Inhibitory effects of resveratrol on the lipopolysaccharide-induced nuclear translocation of p65

In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor, inhibitory kappa B-α (IκB-α), which upon LPS stimulation is phosphorylated by its inhibitor IκB kinases, ubiquitinated, and then rapidly degraded via 26S proteasome, thus releasing NF-κB. NF-κB is a dimer composed of p65 and p50 or p52 subunits. The p65 subunit of NF-κB translocates from the cytosol to the nucleus after its release from IκBs and gets subsequently phosphorylated by protein kinase A and MSK1 at Ser276 which allows for increased interaction with the transcriptional co-activator p300/CBP to further enhance the transcriptional activity of this transcription factor. We examined the effect of resveratrol on LPS-induced activation of p65, using Western blotting. In case of pretreatment with resveratrol, Western blotting showed that the phosphorylated p65 level was decreased [Figure 6].

DISCUSSION

The results presented in this study demonstrate that intra-articular administration of resveratrol (30 mg/kg in 30 μL DMSO) through the acute stage of CAIA, starting with the anti-collagen Ab injection, ameliorates the inflammation in CAIA mice. The CAIA course followed by inflammation depends on the production of many inflammatory proteins. An important source

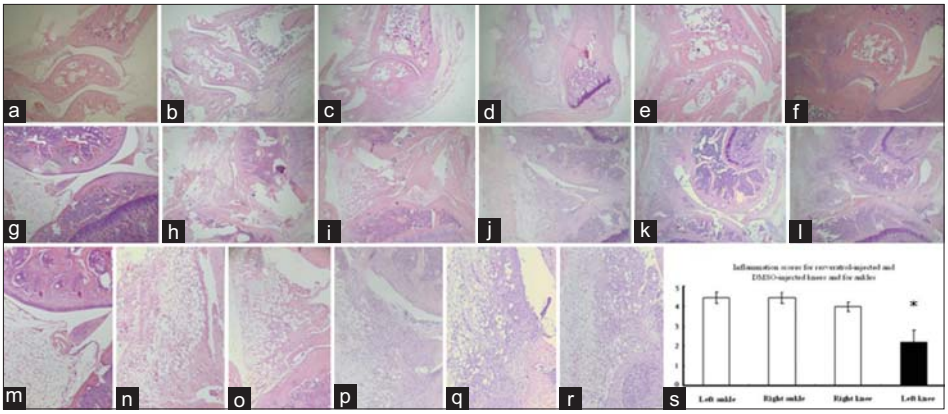

Figure 4: Pathological changes in the knee and ankle joints. The photographs of the typical histology of inflammation from scores 0 to 5 are shown. (a-f) Representative histology of ankle inflammation from scores 0 to 5. (g-l) Representative histology of knee inflammation from scores 0 to 5. (m-r) Representative infiltrations of inflammatory cells in the synovium from scores 0 to 5. (s) The left knee joints were injected with resveratrol in dimethyl sulfoxide, and the right knee joints were injected with dimethyl sulfoxide alone. There was a significant difference between the left and right knees (**P* < 0.05)

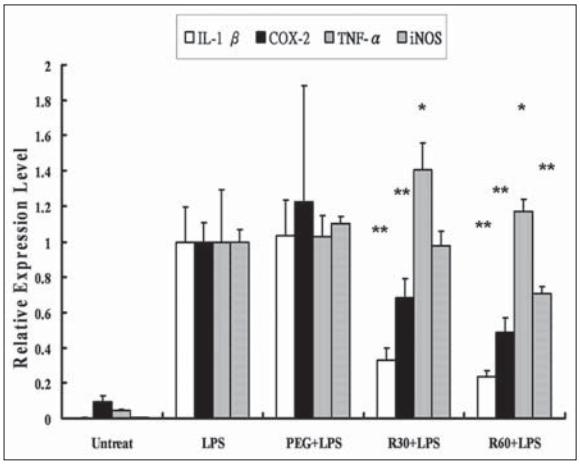

Figure 5: The relative expression levels of inflammatory cytokines in RAW 264.7 cells. There was a significant decrease in interleukin-1β expression in lipopolysaccharide + resveratrol groups (30 or 60 μM/L resveratrol groups) (*n* = 3, experiment performed in triplicate). The results showed a similar pattern as interleukin-1β in relative expression of COX-2 and iNOS mRNA. Significant TNF-α expression was noted 1 h after lipopolysaccharide stimulation, and a significant increase was noted in cells pretreated with 30 or 60 μM/L resveratrol. (**P* < 0.05; ***P* < 0.01)

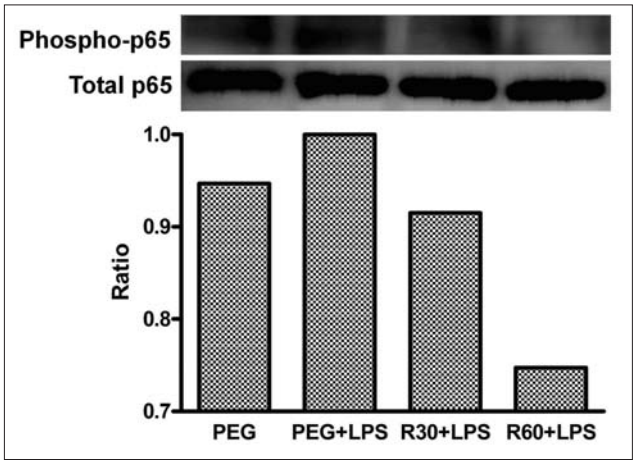

Figure 6: Resveratrol inhibited lipopolysaccharide-induced activation of nuclear factor-κB. The activation of nuclear factor-κB was evaluated by the phosphorylation of nuclear factor-κB subunit p65. Phosphorylated p65 and total p65 were detected by Western blotting. Phosphorylated p65 was normalized with total p65 to produce a ratio which was used to compare the relative amount of activated p65. The data shown are representatives of two independent experiments

for producing these mediators is the macrophages. Macrophages activated by LPS produce many inflammatory cytokines, including IL-1β and TNF-α.[33] IL-1β and TNF-α are strong regulatory proteins that induce the expression of several genes believed to participate in tissue destruction, infection, inflammation, and shock.[34-36] The synthesis of cytokines such as TNF-α, IL-1β, IL-6, and IL-8 is mediated by NF-κB as is the expression of iNOS and COX -2.[37-41] NF-κB, therefore, represents a potential target for strategies that aim to inhibit pro-inflammatory cytokine expression to treat a variety of inflammatory disorders such as RA and OA. Resveratrol suppresses TNF-induced NF-κB activation in myeloid (U-937), lymphoid (Jurkat), and epithelial (HeLa and H4) cells.[26] Resveratrol also blocks NF-κB activation induced by phorbol myristate acetate, LPS, H_2O_2, okadaic acid, and ceramide.[26] Treatment of J774.2 macrophages with kaempferol, apigenin, and resveratrol significantly suppresses TNF-α gene expression. However, IL-1β gene expression is inhibited only by treatment with kaempferol and apigenin.[42] Some studies have shown that the anti-inflammatory effect of resveratrol is due to its inhibitory activity on ROS and prostaglandin production.[11,43,44] Our results indicated that the anti-inflammatory effect of resveratrol is associated with the suppression of IL-1β, COX-2, and iNOS but not TNF-α.

Elmali *et al*. reported that resveratrol has anti-inflammatory effects on inflammatory arthritis induced by repeated intra-articular injection of high-dose LPS.[45] This model of arthritis, first established by Idogawa *et al*., was a monoarthritis model.[46] They suggested that this arthritis model in rabbits can be used to screen anti-rheumatic drugs. Comparatively, the CAIA was a model representing systemic inflammatory experimental arthritis, induced by a monoclonal anti-collagen II Ab adjuvant with LPS. Integrating all the results, the anti-inflammatory effect of resveratrol was effective not only in experimental monoarthritis, but also in systemic arthritis. However, the concentration of resveratrol (molecular weight: 228.24) used in our study (30 mg/kg) was 13.2 times than that used in Elmali's study (10 μmol/kg).

Elmali *et al*. also reported the anti-inflammatory effect of resveratrol in DMSO in an OA model by unilateral anterior cruciate ligament transection.[1] There was significant amelioration of cartilage destruction and synovium inflammation. Chondrocyte protection from IL-1β-induced catabolic effects was also noted.[47] Our findings were consistent with these data.

DMSO is a solvent commonly used in experiments to dissolve hydrophobic substances. Soler *et al*. reported that DMSO significantly reduced the inflammatory process in protamine sulfate-induced cystitis, but instilling with 50% DMSO in normal rats provoked mild inflammation in the normal mucosa.[48] The DMSO disturbed the cell studies than PEG200 as a solvent of our choice in cell studies. Ackland *et al*. reported that low-molecular-weight PEG may potentially play a role in the therapy of systemic inflammation and sepsis.[49] It could be the reason why the phospho-p65 expression is significantly reduced in the R60+LPS group, even more decreased than in the PEG only control group. In our study, there was a statistical difference between the DMSO and resveratrol in DMSO groups.

In this study, we did not investigate the relationship between IL-1β and NF-κB. Some studies have revealed the possible applications of resveratrol in treating RA: (1) Resveratrol activates caspase-8, which in turn modulates the mitochondrial apoptotic machinery to promote the apoptosis of RA FLS[50] and (2) Resveratrol seems to be an effective *in vitro* anti-inflammatory agent and exerts chondroprotective effects via the suppression of IL-1β, ROS, and tumor suppressor protein p53 production.[51]

CONCLUSIONS

This is the first study to demonstrate that knee injection of resveratrol can also ameliorate inflammation and reduce IL-1β, iNOS, and COX-2 expression in mice with CAIA. The anti-inflammatory mechanism of resveratrol is through, at least in part, the inhibition of NF-κB.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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