Resveratrol suppresses interleukin-1β-induced inflammatory signaling and apoptosis in human articular chondrocytes: Potential for use as a novel nutraceutical for the treatment of osteoarthritis

Mehdi Shakibaei [a,*], *Constanze Csaki* [a,1], *Simone Nebrich* [a,1], *Ali Mobasheri* [b]

[a] *Ludwig-Maximilians-University Munich, Faculty of Medicine, Institute of Anatomy, Musculoskeletal Research Group, Pettenkoferstrasse 11, D-80336 Munich, Germany*

[b] *Division of Veterinary Medicine, The School of Veterinary Medicine and Science, The University of Nottingham, Sutton Bonington Campus, United Kingdom*

[*] *Corresponding author*. Tel.: +49 89 5160 4827; fax: +49 89 5160 4828.
E-mail address: mehdi.shakibaei@med.uni-muenchen.de (M. Shakibaei).

[1] These authors equally contributed to this work.

1. Introduction

Osteoarthritis (OA) is a progressive and debilitating inflammatory disease of synovial joints associated with the ageing process. It is characterized by loss of articular cartilage, synovial inflammation and subchondral bone alterations that result in abnormal articulation, joint instability, pain and loss of function [1]. OA in humans and animals is exacerbated and perpetuated by the hyperplasia of synoviocytes and the local production and release of pro-inflammatory cytokines (interleukin-1β, interleukin-6, tumour necrosis factor-α) and enzymes responsible for the synthesis of inflammatory pain mediators (e.g. cyclooxygenase-2) [2]. At the molecular level OA is characterized by an imbalance between cartilage anabolism and catabolism [1]. Disruption of chondrocytes homeostasis results in metabolic changes, loss of adhesion molecules, elevation of matrix metalloproteinases and aggrecanases, and degradation of major extracellular matrix (ECM) components including collagen type II and cartilage-specific proteoglycans. The expression and biological activity of all the catabolic factors listed above is regulated by the transcription factor nuclear factor-κB (NF-κB). NF-κB is a ubiquitous transcription factor that resides in the cytoplasm but, when activated, is translocated to the nucleus, where it induces gene transcription. NF-κB may be switched on by free radicals, inflammatory agents and endotoxins. When this activation takes place, NF-κB induces the expression of more than 400 genes some of which are intimately involved in regulating apoptosis, proliferation and inflammation [3].

The aim of treating patients with OA (or RA) is to control pain and joint swelling, delay disease progression, and improve quality of life. At present OA is treated with symptom modifying analgesics such as NSAIDs, and intra-articular therapies such as glucocorticoids and hyaluronic acid. There are at present no effective structure-modifying drugs capable of reversing the changes that occur in OA. With the exception of anti-inflammatory corticosteroids and non-steroidal anti-inflammatory drugs which inhibit cyclooxygenase-2, the enzyme responsible for prostaglandin biosynthesis in inflammation, no specific therapy based on fundamental intra-cellular pathways of chondrocytes and synoviocytes exists for the medical management of OA. In addition, anti-rheumatic drugs (DMARDs) are used to modify the disease, for example sulfasalazine, leflunomide, hydroxychloroquine and newer therapies such as anti-TNF-α therapy (etanercept, infliximab and adalimumab), anti-CD20 therapy (rituximab) and abatacept. However, all of these agents are associated with numerous side effects which can be quite adverse. Pro-inflammatory cytokines, inflammatory enzymes such as COX-2, matrix metalloproteinases are produced by activated synoviocytes and articular chondrocytes and play a pivotal role in the pathogenesis of OA [4–9]. It has been reported and shown that the majority of the pro-inflammatory proteins linked to arthritis are regulated by NF-κB [10].

In recent years dietary phytochemicals and herbal remedies have been introduced for OA therapy. These include stinging nettle, Jucurba, catechins such as epigallocatechin, Trypterygium wilfordii hook F, resveratrol and curcumin [11–17]. Some of these are known to inhibit NF-κB in chondrocytes and other cell types [13,17–31]. Resveratrol (*trans*-3,5,4′-trihydroxystilbene) was isolated for the first time in 1940 as a constituent of the roots of white hellebore, but has since been found in various plants including grapes, cranberries and peanuts [26,32]. In plants, resveratrol functions microbiologically as a defence phytoalexin which protects against fungal and bacterial infections [33]. Resveratrol can inhibit the activation of NF-κB and thus down-regulate NF-κB-regulated pro-inflammatory gene products such as COX-2, IL-1β, and IL-6, which play an important role in the pathogenesis of OA [18,26,31]. Recent results from our laboratories have shown that resveratrol is capable of exerting anti-apoptotic (programmed cell death), anti-oxidative (inhibits reactive oxygen intermediates), anti-tumour suppressor protein p53, and anti-inflammatory functions in chondrocytes [34,35]. These chemopreventive properties of resveratrol have been associated with the inhibition of NF-κB. Although the mechanism by which resveratrol interferes with the activation of NF-κB in chondrocytes is not clear, it seems that inhibition of its degradation is the principal target [16,36] probably by inhibition of IκB kinase activity [37]. Resveratrol blocked NF-κB activation induced by TNF-α, IL-1β, PMA, LPS, H_2O_2, okadaic acid, and ceramide in a dose- and time-dependent manner [26,38]. Particularly, the TNF-induced phosphorylation and nuclear translocation of the p65 subunit of NF-κB and NF-κB-dependent reporter gene transcription was inhibited. The suppression of NF-κB activation coincided with inhibition of AP-1 activation. Resveratrol also abolished TNF-induced activation of mitogen-activated kinase kinase, c-Jun terminal kinase and abrogated TNF-induced cytotoxicity and caspase-activation. Both ROI generation and lipid peroxidation induced by TNF were suppressed by resveratrol [26]. In cancer cells resveratrol was shown to inhibit cell proliferation and arrested the cells in the S-phase or G2-phase of the cell cycle, thus preventing their progression through the cell cycle. Resveratrol leads to apoptotic cell death in several cell lines since induction of activated caspase-3, accumulation of p53 and p21 and cleavage of the DNA repair enzyme poly (ADP-ribose) polymerase could be demonstrated [38]. The effect of resveratrol on chondrocyte survival has not been investigated yet.

Although resveratrol is a potent inhibitor of NF-κB, its effects on articular chondrocytes have not yet been investigated at the cellular or molecular levels. Accordingly, the aim of this study was to exploit an *in vitro* model of osteoarthritis to study the effects of resveratrol on IL-1β signaling and determine whether resveratrol can suppress NF-κB-activation and NF-κB-regulated gene products.

2. Materials and methods

2.1. *Antibodies*

Secondary antibodies were purchased from Chemicon International and Dianova (Temecula, CA, USA; Hamburg, Germany). Antibodies to β-actin (A5316) were from Sigma (Munich, Germany). Antibodies raised against MMP-9 (MAB911), MMP-3 (MAB905) were purchased from R&D

Systems (Abingdon, UK). Cyclooxygenase-2 (160-112) antibody was obtained from Cayman Chemical (Ann Arbor, MI, USA). Antibodies against p65 and against pan-IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phospho-specific IκBα (Ser 32/36) and against anti-phospho-specific p65 (Ser536) were obtained from Cell Technology (Beverly, MA). Anti-IκBα kinase (IKK)-α and anti-IKK-β were obtained from Imgenex (Germany). Anti-vascular endothelial growth factor (VEGF) antibody was purchased from NeoMarkers (Fremont, CA). Monoclonal anti-PARP [poly (ADP-ribose) polymerase] (7D3-6) antibodies were purchased from Becton Dickinson (Heidelberg, Germany). R&D System, Inc., (Heidelberg, Germany) provided the polyclonal anti-active caspase-3 (AF835) antibody. Peptide aldehydes, a specific proteosome inhibitor N-Ac-Leu-Leu-norleucinal (ALLN) was obtained from Boehringer Mannheim (Mannheim, Germany). All antibodies were used at concentrations and dilutions recommended by the manufacturer (dilutions ranged from 1:100 for immunomorphological experiments and 1:10000 for Western blot analysis).

2.2. Growth medium and chemicals

Growth medium (Ham's F-12/Dulbecco's modified Eagle's medium (50/50) containing 10% fetal calf serum (FCS), 25 μg/ml ascorbic acid, 50 IU/ml streptomycin, 50 IU/ml penicillin, 2.5 μg/ml amphotericin B, essential amino acids and L-glutamine) was obtained from Seromed (Munich, Germany). Trypsin/EDTA (EC 3.4.21.4) were purchased from Sigma (Munich, Germany). Epon was obtained from Plano (Marburg, Germany). The APAAP-Kit derived from Dako (Carpinteria, CA, USA). Resveratrol was purchased from Sigma (Munich, Germany). Resveratrol was prepared as a 50 mg/ml solution in ethanol and then further diluted in cell culture medium. IL-1β was obtained from Strathman Biotech GmbH (Hannover, Germany).

2.3. Chondrocyte isolation and culture

Cartilage explants from healthy femoral head articular cartilage obtained during joint replacement surgery for femoral neck fractures were used to isolate primary human articular chondrocytes [39]. Cartilage slices were digested primarily with 1% pronase for 2 h at 37 °C and subsequently with 0.2% (v/v) collagenase for 4 h at 37 °C. Primary chondrocytes were cultured at 0.1×10^6 cells/ml in petri dishes in monolayer culture for a period of 24 h at 37 °C with 5% CO_2. Cartilage samples were derived from human patients with full informed consent and local ethics committee approval.

2.4. Apoptotic assay

To obtain ligation of pro-inflammatory cytokines in the absence of any other stimulation caused by serum growth factor, primary human articular chondrocytes were washed three times with serum-starved medium and incubated for 1 h with serum-starved medium (0.5% FCS). Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β alone for 0, 1, 2, 4, 8, 16 and 32 h or pre-stimulated with 100 μM resveratrol or 100 μM ALLN alone for 4 h and then co-treated with IL-1β (10 ng/ml) for 1, 2, 4, 8, 16 and 32 h. Cells were analysed with an electron microscope (TEM 10, Zeiss, Jena, Germany). For statistical analysis, ultrathin sections of the samples were prepared and evaluated with an electron microscope (Zeiss TEM 10). The number of cells with morphological features of apoptotic cell death was determined by scoring 100 cells from 20 different microscopic fields.

2.5. Immunolocalization of NF-κB

The effect of resveratrol on the IL-1β-induced nuclear translocation of p65 was examined by an immunocytochemical method (APAAP method) as described previously [40]. Chondrocyte cultures were treated either with 10 ng/ml IL-1β for 0, 5, 15 and 30 min alone or the cells were pre-treated with resveratrol 100 μM for 4 h and then co-treated with 10 ng/ml IL-1β for 0, 5, 15 and 30 min.

For investigation of NF-κB translocation and IκBα-phosphorylation, chondrocyte cultures were treated either with 10 ng/ml IL-1β for 0, 5, 15, 30 and 60 min alone or the cells were pre-treated with resveratrol 100 μM for 4 h and then co-treated with 10 ng/ml IL-1β for 0, 5, 10, 15, 20, 30, 40 and 60 min and nuclear and cytoplasmic extracts were prepared. These experiments were performed in triplicate and the results are provided as mean values from three independent experiments.

2.6. Alkaline phosphatase anti-alkaline phosphatase (APAAP) technique

Chondrocytes seeded on glass plates, were fixed for 10 min in ice-cold methanol, washed twice (5 min) in TBS [0.05 M Tris in 0.15 M NaCl (pH 7.6)] at ambient temperature (AT) and then pre-incubated with normal serum (diluted 1:20 in TBS), for 10 min at AT. The cells were incubated with the primary antibodies (anti-p65, diluted 1:100 in TBS), in a humidified chamber overnight at 4 °C. Cells were then rinsed twice with TBS. Since rabbit polyclonal primary antibodies were used, cells were additionally incubated with mouse anti-rabbit IgG antibodies (diluted 1:50 in TBS) for 30 min at AT. After washing again, incubation with the dual-system bridge antibodies (diluted 1:50 in TBS) was performed and cells were treated with the dual-system APAAP complex (diluted 1:50 in TBS) for 30 min at AT. Cells were thoroughly rinsed with TBS and counter-stained with new fuchsin for 30 min at AT. Finally, cells were washed, air dried and mounted in Kaisers' glycerol gelatin prior to examination in an Axiophot 100 light microscope (Zeiss, Jena, Germany).

2.7. Transmission electron microscopy

Cells were fixed 1 h with Karnovsky-fixative followed by post-fixation in 1% OsO_4 solution (0.1 M phosphate buffer). Monolayer cell pellets were rinsed and dehydrated in an ascending alcohol series before embedded in Epon and cut on a Reichert-Jung Ultracut E (Germany). Ultrathin sections were contrasted with 2% uranyl acetate/lead citrate. A transmission electron microscope (TEM 10, Zeiss, Jena, Germany) was used to examine the cultures.

2.8. Isolation of chondrocyte nuclei

Chondrocytes were trypsinized and washed twice in 1 ml ice-cold PBS. The supernatant was carefully removed. The cell pellet was re-suspended in 400 μl hypotonic lysis buffer containing protease inhibitors and incubated on ice for 15 min. 12.5 μl of 10% NP-40 were added and the cell suspension vigorously mixed for 15 s. The extracts were centrifuged for 1.5 min. The supernatants (cytoplasmic extracts) were frozen at −70 °C. 25 μl ice-cold nuclear extraction buffer were added to the pellets and incubated for 30 min with intermittent mixing. Extracts were centrifuged and the supernatant (nuclear extracts) transferred to pre-chilled tubes for storage at −70 °C.

2.9. Western blot analysis

Western blot analysis was performed as described earlier [34,35]. Briefly, to determine the effect of resveratrol on IL-1β-dependent IκBα-phosphorylation, IκBα degradation and p65 translocation, whole cell lysates, cytoplasmic and nuclear extracts of chondrocyte monolayers were washed three times with Hanks solution and whole cell proteins were extracted by incubation with lysis buffer (50 mM Tris/HCl, pH 7.2, 150 mM NaCl, l% (v/v) Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.01% (v/v) aprotinin, 4 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF) on ice for 30 min, and cell debris was removed by centrifugation. Supernatants were stored at −70 °C until use. Total protein concentration of whole cell, nuclear and cytoplasmic extracts was determined according to the bicinchoninic acid system (Uptima, Interchim, Montlucon, France) using BSA as a standard. After adjusting the equal amounts of total protein (50 μg of protein per lane), proteins were separated by SDS-PAGE (5%, 7.5%, 12% gels) under reducing conditions. The separated proteins were transferred onto nitrocellulose membranes. Membranes were pre-incubated in blocking buffer (5% (w/v) skimmed milk powder in PBS/0.1% Tween 20) for 30 min, and incubated with primary antibodies against p65, IκBα, p-IκBα, VEGF, COX-2, MMP-3, -9, caspase-3 and PARP (overnight, 4 °C). Membranes were washed three times with blocking buffer, and incubated with alkaline phosphatase conjugated secondary antibodies for 30 min. They were finally washed three times in 0.1 M Tris pH 9.5 containing 0.05 M $MgCl_2$ and 0.1 M NaCl. Nitro blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (p-toluidine salt; Pierce, Rockford, IL, USA) were used as substrates to reveal alkaline phosphatase conjugated specific antigen-antibody complexes. Specific binding was quantified by densitometry using ''Quantity one'' (Bio-Rad Laboratories Inc. CA, USA).

2.10. Immune complex kinase assay

To test the effect of resveratrol on IL-1β-induced IKK activation, immune complex kinase assays were performed. The IKK complex was immunoprecipitated from whole cell lysates with antibodies against IKK-α and IKK-β and subsequently incubated with protein A/G-agarose beads (Pierce, Germany). After a 2 h incubation, the beads were washed with lysis buffer and resuspended in a kinase assay solution containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM dithiothreitol, 10 μM unlabeled ATP and 2 mg substrate GST-IκBα (amino acid 1–54) and incubated at 30 °C for 30 min. This was followed by boiling in SDS-PAGE sample buffer for 5 min. The proteins were transferred to a nitrocellulose membrane after SDS-polyacrylamide gel electrophoresis under reducing conditions as described above. Phosphorylation of GST-IκBα was assessed using a specific antibody against phospho-specific IκBα (Ser 32/36). To demonstrate the total amounts of IKK-α and IKK-β in each sample, whole-cell lysates were transferred to a nitrocellulose membrane after SDS-polyacrylamide gel electrophoresis under reducing conditions as described above. Detection of IKK-α and IKK-β was performed by immunoblotting with either anti-IKK-α or anti-IKK-β antibodies.

2.11. Statistical analysis

The results are expressed as the means ± S.D. of a representative experiment performed in triplicate. The means were compared using Student's *t*-test assuming equal variances. $p < 0.05$ was considered statistically significant.

3. Results

This study was undertaken to investigate the effect of resveratrol on the signaling pathway leading to the activation of the transcription factor NF-κB signaling pathway and on NF-κB-regulated gene products in chondrocytes during osteoarthritis. Chondrocytes treated with 100 μM resveratrol showed no signs of cytotoxic effects or any negative effect on the viability of cells, as determined by the Trypan blue dye exclusion test (data not shown) at the light microscopic and ultrastructural level. To examine the effect of resveratrol on the NF-κB activation pathway, we used IL-1β because the pathway activated by this cytokine is relatively well understood.

3.1. Resveratrol suppresses IL-1β-induced NF-κB-dependent pro-inflammatory and matrix degradation gene products

COX-2 is an enzyme that catalyzes the production of prostaglandin E2 (PGE2) from arachidonic acid, which is an important inflammatory mediator that has been linked to the pathogenesis of OA [46]. Vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) play an important role in the pathogenesis of arthritis by promoting angiogenesis in the synovial joint and facilitating infiltration of inflammatory cells in the synovial joint by virtue of its property to degrade extracellular matrix [47–49]. Hence, we examined whether resveratrol could modulate IL-1β-induced expression of VEGF, MMP-3, -9, and COX-2 proteins. As shown in Fig. 1A, IL-1β-induced the expression of VEGF, COX-2, MMP-3, -9 gene products in a time-dependent manner, and resveratrol significantly inhibited their expression. Densitometric analysis of typical Western blot experiments performed in triplicate (Fig. 1B) showed that VEGF-, MMP-3-, -9-, and COX-2-expression increased in IL-1β treated chondrocytes compared to untreated chondrocytes.

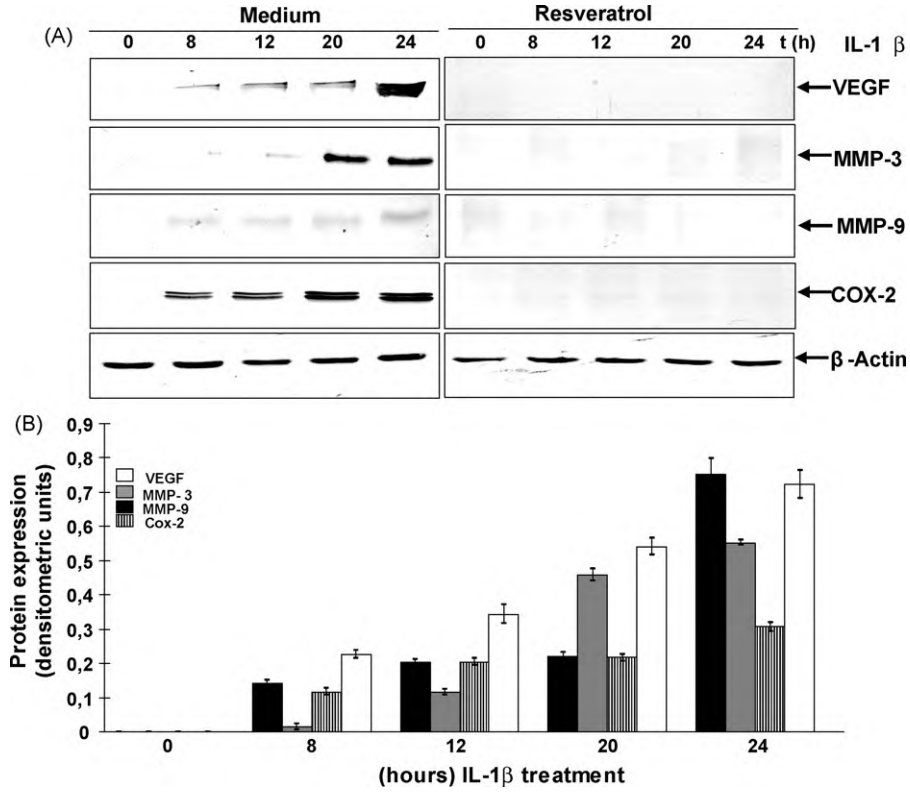

Fig. 1 – Resveratrol inhibits IL-1β-induced, NF-κB-dependent expression of pro-inflammatory and matrix degrading gene products. Chondrocyte cultures were either treated with 10 ng/ml IL-1β for 0, 8, 12, 20 and 24 h alone or the cells were pre-treated with resveratrol 100 μM for 4 h and then co-treated with 10 ng/ml IL-1β for the same time periods indicated above. (A) Whole cell extracts were prepared and cell lysates were resolved by SDS-PAGE, electro-transferred to nitrocellulose membrane and then probed for expression of VEGF, MMP-3, MMP-9, COX-2 and β-actin by Western blot analysis using antibodies to these proteins, β-actin as an internal control. IL-1β-treated and IL-1β co-treated with resveratrol samples were run under identical conditions and probed with the same immunoblotting solutions. Densitometric quantification (B) demonstrates that IL-1β-induced the expression of VEGF, MMP-3, -9, COX-2 gene products in a time-dependent manner compared to control cultures. Values are means ± S.D. of a representative experiment performed in triplicate. Data shown are representative of three independent experiments.

3.2. *Resveratrol blocks IL-1β-induced nuclear-translocation of NF-κB as revealed by APAAP*

Chondrocytes were either left untreated (Fig. 2A) or treated with 10 ng/ml IL-1β alone for 5, 15 and 30 min (Fig. 2B–D) or pre-treated with 100 μM resveratrol for 4 h followed by co-treatment with 10 ng/ml IL-1β and 100 μM resveratrol for the same time periods (Fig. 2E–G). Control chondrocytes revealed only cytoplasmic labelling of NF-κB (Fig. 2A). After 15 min of treatment, IL-1β-stimulated chondrocytes showed a clear and positive labelling for activated NF-κB in nuclei and to a lesser extent in the cytoplasm of chondrocytes (Fig. 2B–D). Chondrocytes that were pre-treated with resveratrol (4 h) and then co-treated with IL-1β and 100 μM resveratrol showed positive staining in the cytoplasm and clearly decreased, nuclear NF-κB staining (Fig. 2E–G).

3.3. *Resveratrol inhibits NF-κB activation in a dose- and time-dependent manner*

To examine whether resveratrol inhibits the IL-1β-induced activation of NF-κB, nuclear protein extracts of serum-starved chondrocytes were probed for the phosphorylated p65 NF-κB-subunit after pre-treatment with the indicated concentrations (Fig. 3) of resveratrol for 4 h followed by co-treatment with 10 ng/ml IL-1β and resveratrol for 30 min. Furthermore, chondrocytes were pre-incubated with 100 μM resveratrol for the indicated times then treated with 10 ng/ml IL-1β for 30 min. The Western blotting results confirmed that resveratrol alone had no effect on NF-κB activation. However, resveratrol inhibited IL-1β-induced NF-κB activation in a dose-dependent manner (Fig. 3A). The inhibition of NF-κB activation by resveratrol was also found to be time-dependent (Fig. 3B). Taken together, these findings indicate that the inhibition of NF-κB activation by resveratrol is dose- as well as time-dependent.

3.4. *Effects of resveratrol on the IκBα by IL-1β in chondrocytes*

3.4.1. *Resveratrol inhibits IL-1β-induced IκBα degradation in chondrocytes*

As shown above, resveratrol inhibits IL-1β-induced activation of NF-κB and its translocation to the chondrocyte nucleus. Therefore, we examined more precisely the upstream kinetics

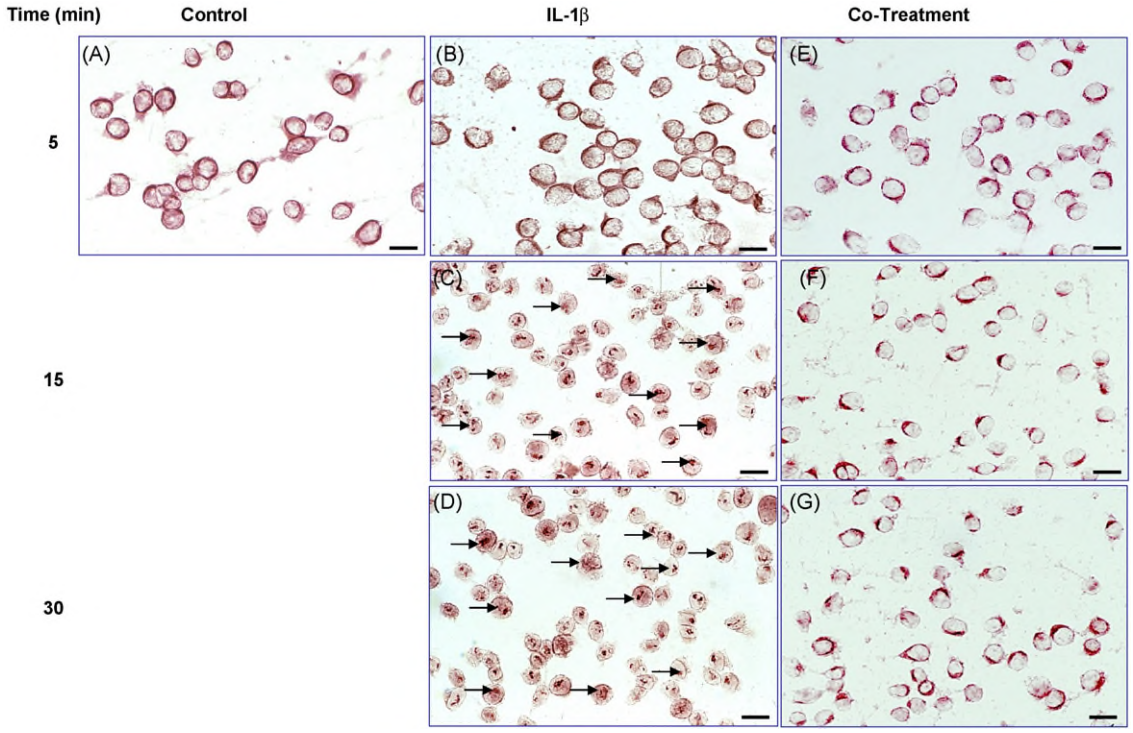

Fig. 2 – Resveratrol mediated inhibition of IL-1β-induced phospho p65 nuclear translocation revealed by APAAP. Chondrocyte cultures either served as controls (A, not treated) or were treated either with 10 ng/ml IL-1β for 0, 5, 15 and 30 min alone or the cells were pre-treated with resveratrol 100 μM for 4 h and then co-treated with 10 ng/ml IL-1β for 0, 5, 15 and 30 min before immunolabelling with phospho p65 antibodies. In control cells anti-phospho p65 labelling was restricted to the cytoplasm (A). Cells treated with IL-1β alone revealed nuclear translocation of phospho p65 (B–D) that was partly inhibited by co-treatment with resveratrol (E–G). Data shown are representative of three independent experiments. A–G: ×160, bars = 50 μm.

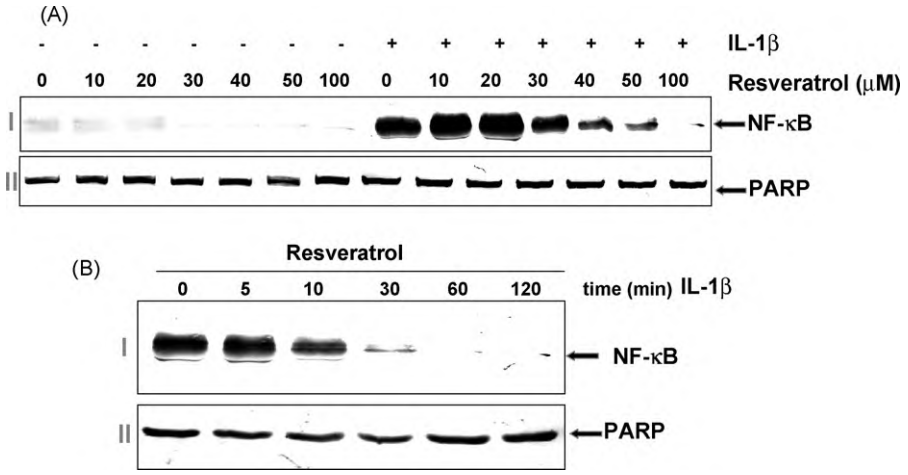

Fig. 3 – Resveratrol inhibits IL-1β-induced phosphorylation and translocation of p65 in nuclear extracts of chondrocytes. (A) Western blot analysis with IL-1β-treated chondrocyte nuclear extracts. Serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-incubated with the indicated concentrations of resveratrol for 4 h and then co-treated with 10 ng/ml IL-1β for 30 min. The nuclear extracts were probed for phospho p65 by Western blot analysis using antibodies to phospho-specific p65 (I) and PARP (II) (control). (B) Serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-incubated with 100 μM resveratrol for the indicated times, co-treated with 10 ng/ml IL-1β for 30 min and then probed for phospho p65 by Western blot analysis using antibodies to phospho-specific p65 and PARP (control). The inhibition of NF-κB activation by resveratrol was dose- as well as time-dependent (I). Synthesis of PARP was unaffected in nuclear extracts (II).

of NF-κB activation by IL-1β in chondrocytes. It is well known that an important prerequisite for the activation of NF-κB is the phosphorylation and degradation of IκBα, the natural blocker of NF-κB [43,44].

To examine whether inhibition of IL-1β-induced NF-κB activation occurs through inhibition of IκBα degradation, we treated some chondrocyte cultures with 10 ng/ml IL-1β for the indicated times (Fig. 4A) and other chondrocyte cultures first with 100 μM resveratrol for 4 h followed by co-treatment with 10 ng/ml IL-1β for the indicated time periods. The activation of IκBα and p-IκBα in the cytoplasm of chondrocytes was determined by Western blot analysis using anti-IκBα and anti-β-actin (control) antibodies. IL-1β induced IκBα degradation in untreated cultures as early as 10 min and reached a maximum at 30 min, but IL-1β could not induce IκBα degradation in resveratrol pre-treated chondrocytes (Fig. 4A I). Taken together, these results suggest that resveratrol blocks IL-1β-induced IκBα degradation.

3.4.2. *Resveratrol accumulates IL-1β-induced IκBα-phosphorylation in chondrocytes*

Furthermore, IL-1β induced IκBα-phosphorylation in control cells within 10 min. Interestingly, in chondrocytes pre-treated with resveratrol, the phosphorylation of IκBα persisted in the interval times (Fig. 4A II). To confirm this result, we used a specific proteasome inhibitor N-Ac-Leu-Leu-norleucinal (ALLN) [45] which inhibits the degradation of IκBα by 26S proteasome. As shown in Fig. 4B I, in chondrocytes treated with resveratrol and/or with the inhibitor (ALLN), IL-1β-induced IκBα-phosphorylation was increased (Fig. 4B I). Taken together, resveratrol accumulated IL-1β-induced phosphorylation of IκBα by inhibition of proteasome function.

3.4.3. *Resveratrol accumulates IL-1β-induced ubiquitinated IκBα*

It is well known, that IκBα-phosphorylation by pro-inflammatory cytokines leads to ubiquitination and degradation of IκBα [43]. To test that resveratrol influences IL-1β-induced IκBα-ubiquitination, serum starved chondrocytes were treated with resveratrol or IL-1β, or pre-treated with resveratrol or ALLN and then co-treated with IL-1β. Chondrocytes treatment with IL-1β alone induced low ubiquitination of IκBα. In opposite when the chondrocytes were pre-treated with resveratrol or ALLN and co-treated with IL-1β, high-molecular-mass bands were seen, indicating the ubiquitination of IκBα was clearly stabilized (Fig. 4C). Treatment with resveratrol alone did not affect IκBα-ubiquitination. These results clearly show that resveratrol affects, like ALLN, the phosphorylation and ubiquitination of IκBα by inhibition of proteasome function.

3.4.4. *Resveratrol does not affect IL-1β-induced IKK activation*

Because resveratrol sustained IL-1β-induced phosphorylation of IκBα by inhibition of proteasome function, we determined the effect of resveratrol on IL-1β-induced IKK activation, which is required for pro-inflammatory cytokine-induced phosphorylation of IκBα [43]. As shown in Fig. 4D I by immune complex kinase assay, IL-1β activated IKK as early as 4 min after IL-1β treatment, but resveratrol did not inhibit IL-1β-induced activation of IKK. IL-1β or resveratrol had no direct effect on the expression of IKKα or IKKβ proteins (Fig. 4D II and III).

3.5. *Resveratrol suppresses IL-1β-induced apoptosis in chondrocytes*

The effect of resveratrol on IL-1β-induced apoptosis in chondrocytes was examined by the electron microscopy assay. Serum starved primary isolated chondrocytes were either treated with 10 ng/ml IL-1β for 0, 1, 2, 4 and 8 h or pre-treated with 100 μM resveratrol for 4 h and being co-treated with 10 ng/ml IL-1β and 100 μM resveratrol for the same time periods. Control monolayer chondrocytes showed a rounded or flattened shape with small cytoplasmic processes, a large mostly euchromatic nucleus with nucleoli and a well-structured cytoplasm (Fig. 5A). IL-1β-treated chondrocytes showed degenerative changes such as swelling of rough ER and clustering of swollen mitochondria and degeneration of other cell organelles. After longer incubation periods (8 h) more severe features of cellular degeneration were seen in response to IL-1β treatment. These include areas of condensed heterochromatin in the cell nuclei. The flattened monolayer chondrocytes became more and more rounded, lost their microvilli-like processes and became apoptotic (Fig. 5B–E). Chondrocytes that were pre-treated with resveratrol (4 h) and then co-treated with IL-1β and 100 μM resveratrol showed less severe cellular degeneration on the ultrastructural level as early as 1 h after co-treatment (Fig. 5F–I). The chondrocytes regained a flattened shape and numerous microvilli-like cytoplasmic processes. To test whether this process is a proteasome-dependent pathway, serum starved chondrocytes were pre-stimulated with 100 μM ALLN for 4 h and co-treated with 10 ng/ml IL-1β and 100 μM ALLN for the same time periods. The results clearly showed a time-dependent decrease in the morphological degenerative features comparable to resveratrol pre-treated chondrocytes (Fig. 5J–M). Statistical evaluation of the data clearly highlighted changes in the number of cells with apoptotic features before and after IL-1β-treatment. Co-treatment with resveratrol or the proteasome inhibitor ALLN clearly decreased the number of cells with apoptotic features (Fig. 6).

3.6. *Resveratrol suppresses IL-1β-induced NF-κB-dependent apoptotic gene products*

In light of the above results, we propose that resveratrol inhibits not only the nuclear translocation of NF-κB, but further inhibits NF-κB-dependent apoptotic gene products. NF-κB regulates the expression of the pro-apoptotic proteins such as PARP and caspase-3. They play a major role in apoptosis and prolonging the survival of cells [41,42]. We tested the hypothesis that resveratrol modulates IL-1β-induced expression of these genes. We treated chondrocyte cultures with 10 ng/ml IL-1β for the indicated times. Other chondrocyte cultures were first treated with 100 μM resveratrol for 4 h followed by co-treatment with 10 ng/ml IL-1β for the indicated time periods. As shown in Fig. 7A resveratrol significantly inhibited IL-1β-induced expression of these pro-apoptotic proteins in a time-dependent manner. To test,

whether this process is a proteasome-dependent pathway, we performed the same experiment with ALLN. Serum starved chondrocytes were pre-treated with 100 μM ALLN for 4 h, then co-treated with 10 ng/ml IL-1β and 100 μM ALLN for the same time periods. The obtained results were similar to those of chondrocytes treated with resveratrol and IL-1β (Fig. 7A). Densitometric analysis of typical Western blot experiments performed in triplicate showed that caspase 3- (Fig. 7B) and

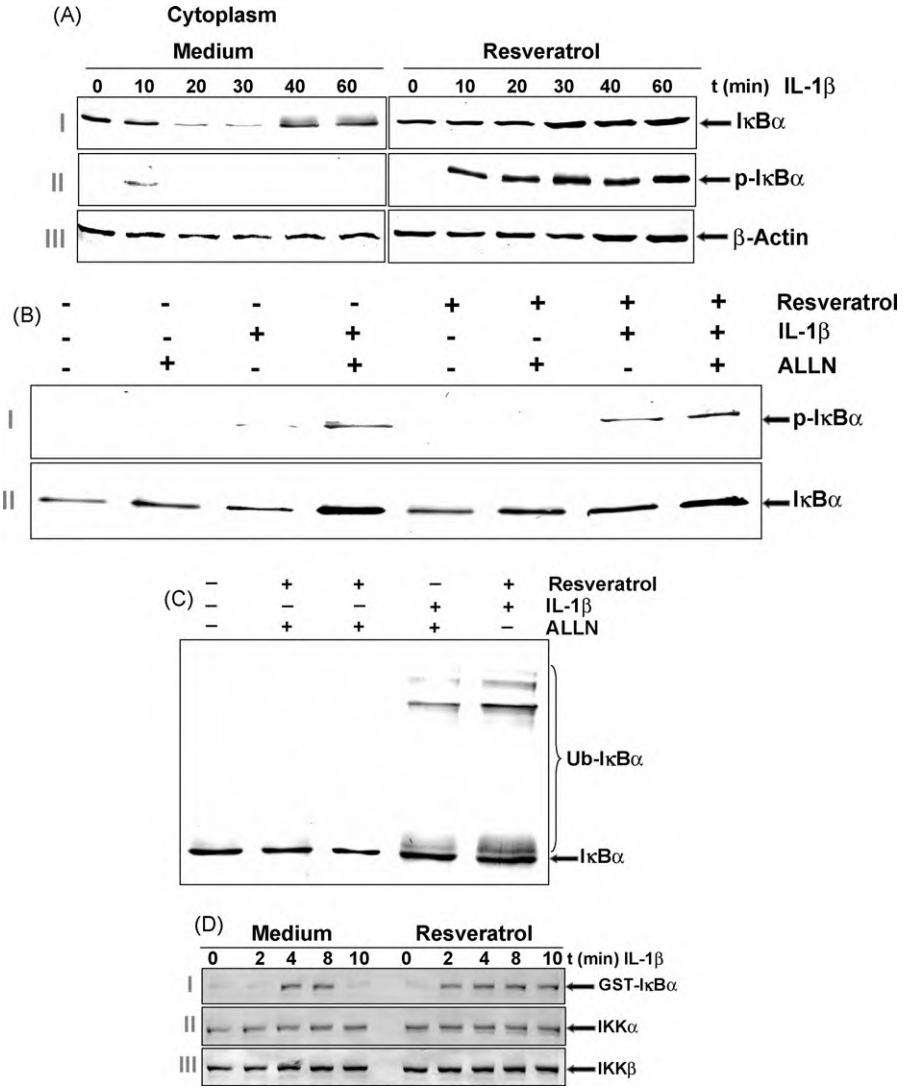

Fig. 4 – Effects of resveratrol on the IκBα by IL-1β in chondrocytes. (A) Resveratrol inhibits IL-1β-dependent IκBα degradation: serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-incubated with 100 μM resveratrol for 4 h and then co-treated with 10 ng/ml IL-1β for the indicated times or left untreated. The cytoplasmic extracts were prepared, fractionated on SDS-PAGE, and electro-transferred to nitrocellulose membranes. Western blot analysis was performed with anti-IκBα and anti-phospho-specific anti-IκBα and anti-β-actin (control). (B) Effect of resveratrol on the phosphorylation of IκBα by IL-1β: serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-incubated with 100 μM resveratrol or 100 μg/ml ALLN for 4 h and then treated with 10 ng/ml IL-1β for 30 min. The cytoplasmic extracts were prepared and Western blot analysis was performed using anti-IκBα and -p-IκBα antibodies. Treatment of chondrocytes with 10 ng/ml IL-1β revealed an increase in the phosphorylated IκBα-form in cytoplasmic extracts. Resveratrol significantly increased phosphorylation of IκBα in the presence or absence of the inhibitor. (C) Effect of resveratrol on IL-1ß-induced ubiquitination of IκBα: serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-treated with 100 μM resveratrol or ALLN for 4 h and then co-treated with 10 ng/ml IL-1β for 30 min. Whole cell extracts were prepared followed by Western blot analysis using antibodies to IκBα. Treatment with IL-1β alone induced minor ubiquitination of IκBα, but in chondrocytes co-treated with resveratrol or ALLN and IL-1β, ubiquitination of IκBα was significantly increased. Resveratrol, like ALLN inhibited the IL-1β-induced ubiquitination/degradation of IκBα. Data shown are representative of three independent experiments. (D) Resveratrol inhibits IL-1ß-induced IκBα kinase activity: serum-starved chondrocytes (0.1×10^6 cells/ml) were pre-treated with 100 μM resveratrol for 4 h and then co-treated with 10 ng/ml IL-1β for the indicated time. Whole cell extracts were immunoprecipitated with antibody against IKKα and analysed by an immune complex kinase assay. Data shown are representative of three independent experiments.

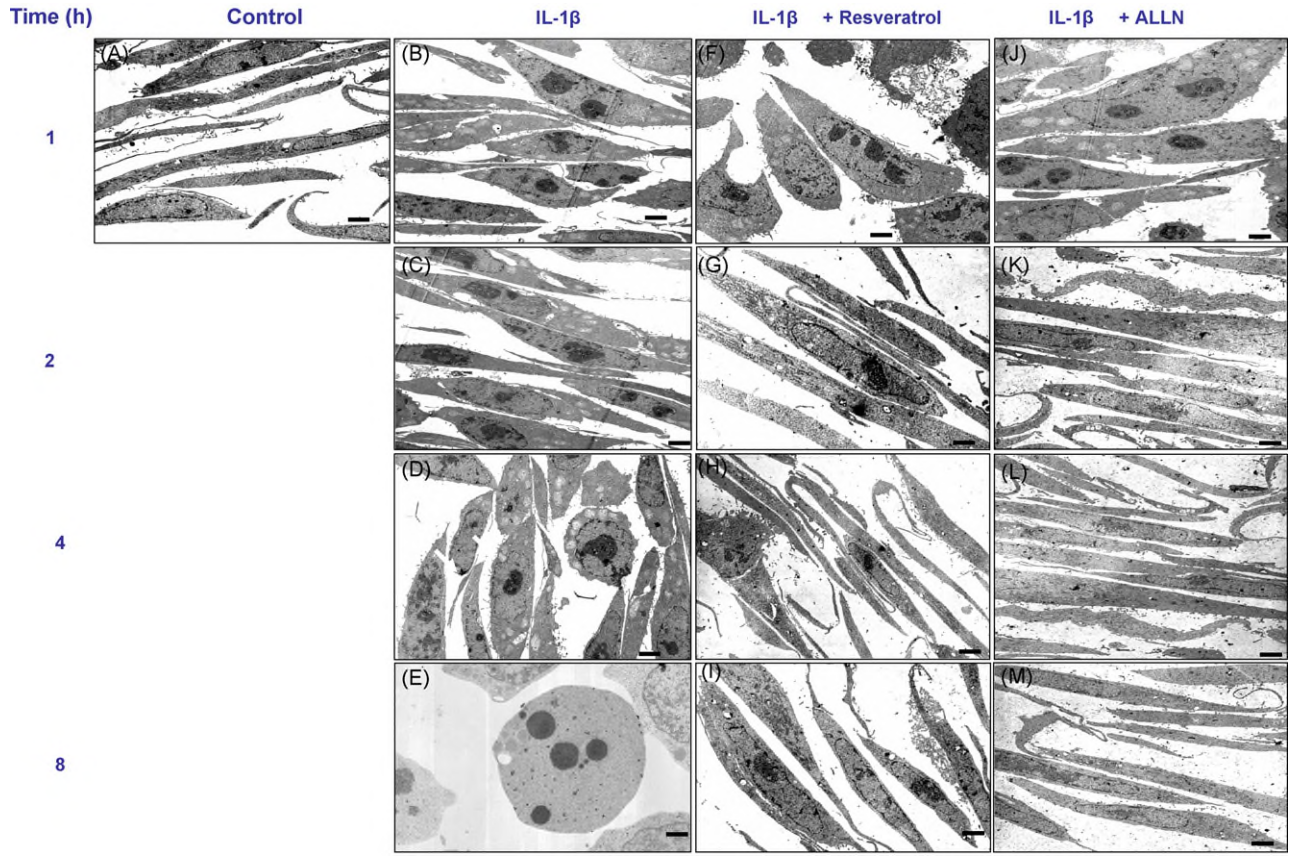

Fig. 5 – Effect of resveratrol on IL-1β-induced apoptosis in chondrocytes *in vitro*. Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β alone for 0, 1, 2, 4 and 8 h or pre-stimulated with 100 μM resveratrol or with 100 μM ALLN alone for 4 h and then co-treated with IL-1β (10 ng/ml) for 1, 2, 4 and 8 h and evaluated with a transmission electron microscope. Chondrocytes, treated with 10 ng/ml IL-1β exhibited characteristic features of degeneration: annular chromatin condensation at the nuclear envelope and swelling of mitochondria and rough ER in a time-dependent manner (B–E). Chondrocytes that were pre-treated with resveratrol or ALLN and then co-treated with IL-1β and 100 μM resveratrol (F–I) or 100 μM ALLN (J–M) showed less severe cell degeneration at the ultrastructural level. In control cultures no adverse ultrastructural changes were observed (A). A–M: ×5000; bar = 1 μM.

PARP-expression (Fig. 7C) increased in IL-1β treated chondrocytes compared to untreated chondrocytes.

The schematic shown in Fig. 8 illustrates the molecular mechanisms we propose to be involved in the resveratrol mediated inhibition of IL-1β-induced gene expression and its anti-apoptotic effects.

4. Discussion

The present study was undertaken to investigate the effects of resveratrol, a naturally occurring phytoalexin and chemotherapeutic agent on IL-1β-induced transcription factor nuclear factor-κB (NF-κB) signaling and apoptosis in chondrocytes. The data presented in this paper provides convincing molecular evidence to support the hypothesis that resveratrol suppresses IL-1β-induced apoptosis through down-regulation of NF-κB and NF-κB-regulated gene products by inhibition of IκBα degradation and proteasome function in human chondrocytes *in vitro*.

We have made the following novel observations: (1) resveratrol suppressed IL-1β-induced, NF-κB-dependent pro-inflammatory and matrix degrading gene products. (2) The activation and translocation of p65 from cytoplasm to the nucleus could be demonstrated immunohistochemically following IL-1β treatment of human chondrocytes. (3) Resveratrol inhibited NF-κB activation in a dose- and time-dependent manner. (4) Resveratrol inhibited IL-1β-induced IκBα degradation and consequently accumulated IL-1β-induced IκBα-phosphorylation. (5) IL-1β-induced morphological alterations, swollen mitochondria and dilated endoplasmic reticulum and apoptosis; these changes were abolished through co-treatment with resveratrol as documented by electron microscopy. (6) Finally, Resveratrol suppressed IL-1β-induced NF-κB-dependent expression of apoptosis-related gene products. We have shown that inhibition of NF-κB activation by resveratrol occurs through the accumulation of phosphorylated IκBα, ubiquitinated IκBα and inhibition of proteasome activity. These molecular events may be accounted for by inhibition of IL-1β-induced NF-κB-dependent apoptosis, a process we believe to be mediated by resveratrol.

Resveratrol is a dietary phytoalexin used by plants as a host defence mechanism against microbial infections (including

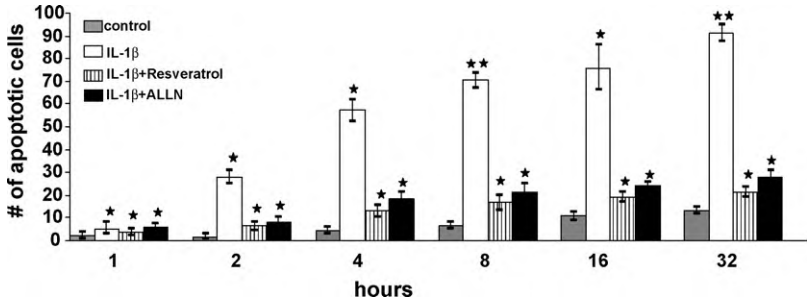

Fig. 6 – Effect of resveratrol on IL-1β-induced apoptosis in chondrocytes *in vitro*. Serum-starved human articular chondrocytes were exposed to 10 ng/ml IL-1β alone for 0, 2, 4, 8, 16 and 32 h or pre-stimulated with 100 μM resveratrol or with 100 μM ALLN alone for 4 h and then co-treated with IL-1β (10 ng/ml) for 1, 2, 4 and 8 h. The number of cells with apoptotic features was determined by scoring 100 cells from 20 different microscopic fields in ultrathin sections evaluated with an electron microscope (Zeiss EM 10). This assay was performed in triplicate and the results are provided as mean values with standard deviations from three independent experiments. Values were compared to the control and statistically significant values with $p < 0.05$ were designated by an asterisk (*).

defence against fungi, protozoa and other pathogens) or UV irradiation [33,50]. Resveratrol has been found in various vegetables and fruits including red grapes, peanuts, mulberries and in the root extract of the oriental medicinal plant *Polygonum cuspidatum*. The potent anti-inflammatory properties of foods containing resveratrol have their favourite ingredients in Asian herbal medicine [33,51]. In recent years, it has been reported that resveratrol possesses anti-inflammatory, immuno-modulatory, antioxidant, cardio-protective and chemopreventive effects [52–54].

Recent studies from our laboratory have shown that resveratrol blocks the IL-1β-induced apoptosis in cultured human chondrocytes by inhibiting caspase-3, cleavage of PARP and p53 activation [34]. In contrast, other studies have demonstrated that resveratrol promotes apoptotic cell death in several tumour cell lines, through the induction of activated

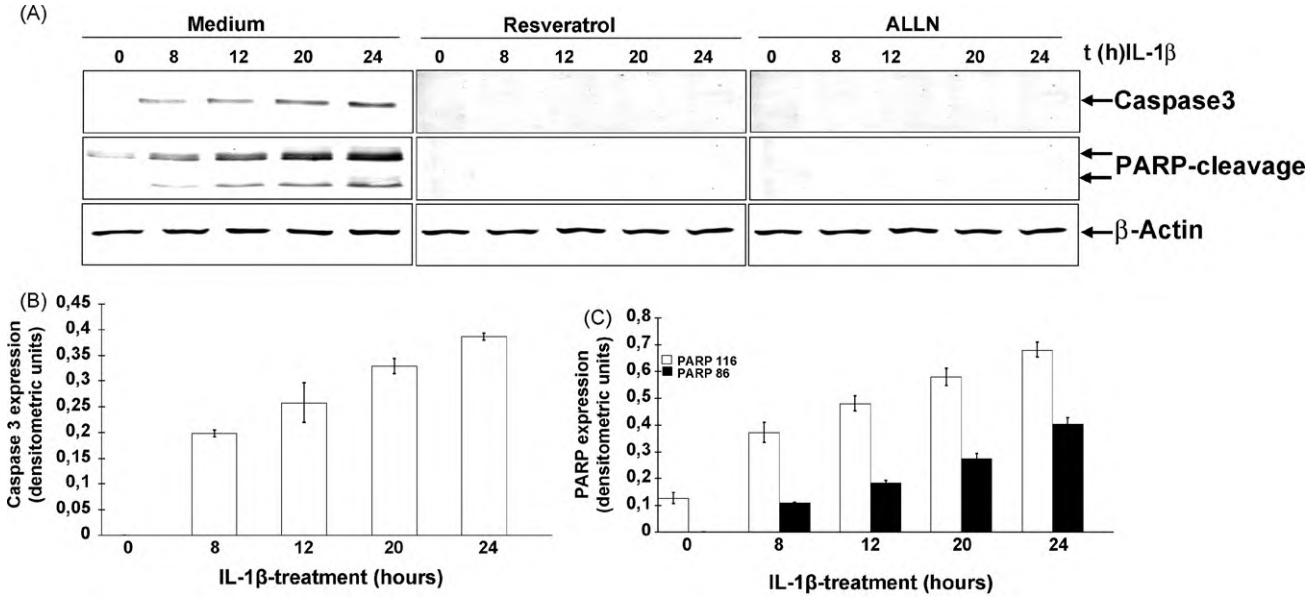

Fig. 7 – Resveratrol suppresses IL-1β-induced NF-κB-dependent expression of apoptosis-related gene products. Chondrocyte cultures were either treated with 10 ng/ml IL-1β for 0, 8, 12, 20 and 24 h alone or the cells were pre-treated with resveratrol 100 μM or ALLN 100 μM for 4 h and then co-treated with 10 ng/ml IL-1β for the same time period indicated above. Whole cell extracts (A) were prepared and cell lysates were resolved by SDS-PAGE, electro-transferred to nitrocellulose membrane and then probed for expression of PARP and caspase-3 by Western blot analysis using antibodies to these proteins. β-actin served as an internal control. IL-1β-treated and IL-1β co-treated with resveratrol or with ALLN samples were run under identical conditions and probed with the same immunoblotting solutions. Densitometric quatitation demonstrates that IL-1β-induced the expression of caspase-3 (B) and PARP cleavage (C) in a time-dependent manner compared to control. Values are means ± S.D. of a representative experiment performed in triplicate. Data shown are representative of three independent experiments.

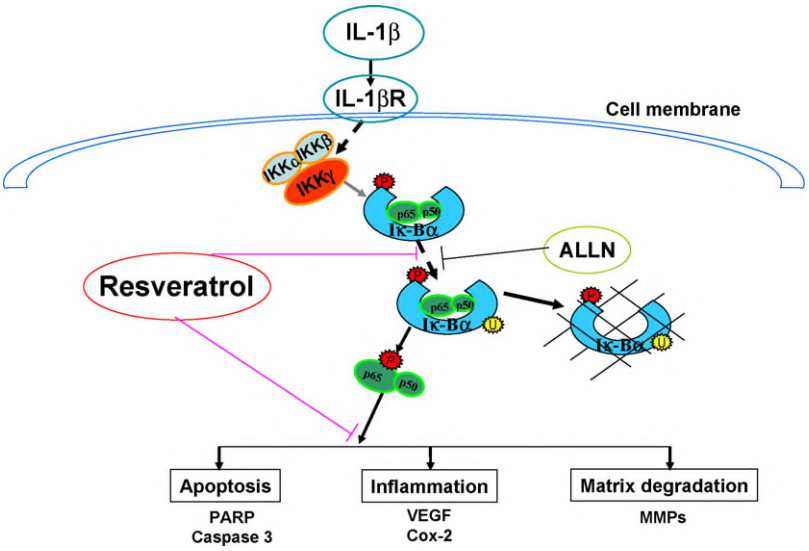

Fig. 8 – Working model for the inhibitory effect of resveratrol on IL-1β-induced NF-κB activation and apoptosis in chondrocytes *in vitro*.

caspase-3, accumulation of p53 and p21 and cleavage of PARP [38]. The mechanism by which resveratrol blocks the apoptotic effects of IL-1β may involve suppression of the transcription factor NF-κB and its downstream gene targets. NF-κB plays an important role in normal physiological and immune mediated functions. However, inappropriate regulation of NF-κB activity has been implicated in the pathogenesis of several diseases including RA and OA [55,56]. Furthermore, it has been shown that both subunits (p50 and p65) of NF-κB are abundant in RA, OA and in synovitis [57]. In reality, pro-inflammatory cytokines such as IL-1β and TNF-α are known to activate NF-κB [58], which in cartilage stimulates further pro-inflammatory cytokine expression and leads to further cartilage degradation [59].

We found that resveratrol inhibits IL-1β-induced apoptosis and NF-κB activation in human chondrocytes. Our studies are the first to our knowledge to demonstrate that resveratrol does not block the IL-1β-induced IKK activation in cultured human chondrocytes. Furthermore, we have also demonstrated that this inhibition is mediated by accumulation of IL-1β-induced phospho-IκBα and ubiquitinated-IκBα degradation and these are through inhibition of proteasome function. Indeed, it is well known that IκBα-phosphorylation by pro-inflammatory cytokines leads to ubiquitination and degradation of IκBα [43]. To confirm that this occurs in chondrocytes, the cells were treated with a specific proteasome inhibitor N-Ac-Leu-Leu-norleucinal (ALLN) [45], which inhibits the degradation of IκBα by 26S proteasome. As shown here, resveratrol and ALLN sustained IL-1β-induced phosphorylation of IκBα by inhibition of proteasome function. These results further indicate that inhibition of NF-κB activation through resveratrol is associated with inhibition of proteosome function.

In addition to these findings *in vitro*, our results also showed that resveratrol may have therapeutic effects *in vivo*. Resveratrol inhibited the expression of several important NF-κB-regulated proteins. First, resveratrol alone completely suppressed NF-κB activation and translocation to the nucleus, a finding which is consistent with the immunohistochemical analysis of the NF-κB subunit p65. Second, Western blot analysis showed that resveratrol down-regulated the expression of MMPs, which are intimately linked with cartilage matrix degradation in OA [24,60,61]. Third, the expression of VEGF and COX-2, which are linked with angiogenesis and inflammation in cartilage [47–49,62,63], was significantly decreased by resveratrol. Finally, resveratrol clearly down-regulated NF-κB-regulated pro-apoptotic proteins, including caspase-3 and PARP in human chondrocytes. Thus, use of resveratrol might provide a novel approach to the treatment of osteoarthritis and rheumatoid arthritis.

In recent years, novel and modern therapeutic strategies have focussed on specific inhibition of the NF-κB signaling pathway, especially its inappropriate activation; these approaches may prove to be important in the development of innovative arthritis treatments [64–67]. Interestingly, it has been reported that proteasome function inhibitors and peptides that block nuclear translocation and nuclear localization of NF-κB have been used to suppress the NF-κB signaling pathway *in vivo* [68,69]. It has been reported that treatment with proteasome inhibitors (i.e. bortezomib) significantly decreases NF-κB activation and this is associated with decreasing quantities of IL-1β in synovial cells derived from RA patients [70]. Although NF-κB has anti-apoptotic effects [71,72], it has been reported that NF-κB may also play an important role in chondrocyte apoptosis during OA [11]. Kuhn et al. [73] have shown that NF-κB activation is linked to the anti-apoptotic effects of IL-1β against the death receptor CD-95 (FAS/APO-1). They have demonstrated that NF-κB-dependent mechanisms antagonize the CD-95-stimulated apoptosis through caspase-3 activation in human chondrocytes [74]. Other studies and our own results have shown that NF-κB is involved in apoptotic events in chondrocytes [59]. Although the signaling pathways involved in NF-κB-mediated apoptosis are quite complex and are not yet fully elucidated, the results of this study show that activation of NF-κB by IL-1β leads to

apoptosis of chondrocytes; a process which is blocked by inhibition of IκBα degradation and translocation of NF-κB to the nucleus by resveratrol. Indeed, several other agents have also been reported to inhibit NF-κB including curcumin in different cell types [13,59,75], flavonoids [75], thalidomide/leflunomide [76], lactacystin [77], glucosamine [11], diacehrein [78] and vitamin C [79].

Overall, NF-κB plays a crucial role in inflammatory processes such as RA and OA, leading to cartilage degradation and joint disease. NF-κB is therefore an attractive therapeutic target in the clinical treatment of RA and OA. This study shows that resveratrol has potential as a naturally occurring anti-inflammatory agent for treating OA through suppression of NF-κB and of NF-κB-regulated gene products. The results presented in this study provide a novel and mechanistic explanation for resveratrol's actions on the NF-κB pathway as a proteasome inhibitor. Although ongoing laboratory research will shed more light on the biochemical action of resveratrol, randomized double-blind and placebo controlled clinical trials are necessary to confirm our *in vitro* findings in patients with OA and RA.

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