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Original article

Phase 2 enzyme inducer sulphoraphane blocks prostaglandin and nitric oxide synthesis in human articular chondrocytes and inhibits cartilage matrix degradation

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Abstract

Objective. We explored the inhibitory effect of sulphoraphane (SFN), a potent inducer of Phase 2 enzymes, on cytokine-induced prostaglandin E_2 (PGE_2) and nitric oxide (NO) production and cartilage degradation in articular chondrocytes. The regulatory mechanism of SFN on nuclear factor (NF)-κB was investigated.

Methods. Chondrocytes were obtained from patients with knee OA. Chondrocytes were stimulated with IL-1β or TNF-α with or without pre-incubation with SFN. Production of PGE_2 and NO was evaluated by the Griess reaction and an ELISA. The expression of microsomal PGE synthase (mPGES), cyclo-oxygenase (COX)-2 and inducible NO synthase (iNOS) was evaluated by real-time RT–PCR and western blot analysis. The regulation of NF-κB activity was explored using luciferase and chromatin immunoprecipitation assays as well as a western blot for phosphorylated IκB kinase (IKK), IκB and the degradation of IκB. Proteoglycan and type II collagen degradation products released from explant cultures were analysed using the dimethylmethylene blue assay and an ELISA for C-terminal telopeptides of type II collagen.

Results. SFN inhibited the production of PGE_2 and NO induced by IL-1β and TNF-α. At a concentration as low as 5 μM, SFN completely inhibited mPGES, COX-2 and iNOS at the mRNA and protein levels, and proteoglycan and type II collagen degradation product release in explant culture. Various signalling pathways required for the NF-κB activation were affected by SFN.

Conclusion. SFN inhibited a broad range of catabolic mechanisms in articular chondrocytes. SFN may be a safe and effective candidate drug for the inhibition of cartilage degradation in arthritic diseases.

Key words: chondrocyte, sulphoraphane, PGE_2, nitric oxide, osteoarthritis.

BASIC SCIENCE

Introduction

OA, a leading cause of disability among the elderly, is associated with substantial economic and societal costs. Degradation of the extracellular matrix (ECM) in articular cartilage is a central event leading to joint destruction in OA. Since current treatments for OA act only on the symptoms and do not prevent or cure OA, studies have attempted to find effective agents that actually inhibit the degeneration of articular cartilage. As OA is a slowly progressing, indolent disease, minimal toxicity is a prerequisite for any disease-modifying anti-OA drug that is proposed.

The Phase 2 enzymes, including NADP(H):quinone oxidoreductase 1, haem oxygenase-1 and γ-glutamylcysteine ligase have traditionally been recognized as those enzymes converting carcinogens to inactive metabolites, thus conferring protection from carcinogenesis [1]. Epidemiological data linking the dietary intake of cruciferous vegetables and a reduced risk of different types of malignancies are

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attributed to the Phase 2 enzyme-inducing constituents, especially the isothiocyanates (ITCs) [2].

Sulphoraphane [SFN; 1-isothiocyanato-4-(methylsulphinyl)-butane], a naturally occurring ITC obtained through the consumption of broccoli, is one of the most potent inducers of the Phase 2 enzymes implicated in carcinogen detoxification [3]. In addition to chemopreventive and cytoprotective activity, Phase 2 enzyme inducers have recently been found to have anti-inflammatory activity, such as the ability to inhibit inducible nitric oxide synthase (iNOS) and cyclo-oxygenase (COX)-2 in IFN-γ-stimulated mouse macrophages [4].

Previously we reported that SFN effectively suppressed the up-regulation of MMP-1, -3 and -13 induced by pro-inflammatory cytokines in articular chondrocytes [5]. Although MMPs play a major role in the progression of cartilage degradation, other mediators also have important catabolic functions. Proteins involved in prostanoid synthesis, such as COX-2 and microsomal prostaglandin E synthase (mPGES), are up-regulated in a surgically induced rat OA model and OA-affected cartilage, suggesting a catabolic role of prostanoids [6–8]. Although very low levels of PGE_2 may have anabolic functions in cartilage, nanomolar to micromolar concentrations of it produced by OA tissues are predominantly catabolic, leading to an inhibition of proteoglycan synthesis, increased MMP-13 and increased type II collagen degradation [9]. Another important player is nitric oxide (NO), which is produced in human OA cartilage and has several roles, including the inhibition of cartilage matrix synthesis, up-regulation of MMPs and induction of chondrocyte apoptosis in combination with reactive oxygen species [10–12]. Treatment with a selective inhibitor of iNOS in a canine OA model resulted in decreased production of MMPs, peroxynitrite and IL-1β in articular cartilage, indicating the therapeutic potential of modulating NO in the treatment of OA [13].

In this study we explored the inhibitory effect of SFN on cytokine-induced PGE_2 and NO production in articular chondrocytes and cartilage matrix degradation in explant cartilage. Because the activation/suppression of p65 and nuclear factor (NF)-κB signalling pathway is one of the most important regulatory mechanisms for inflammation and catabolism of cartilage in response to diverse stimuli such as pro-inflammatory mediators and biomechanical signals [14], the inhibitory mechanism of SFN on the regulation of NF-κB activation was also investigated.

Methods

Reagents

SFN was purchased from LKT Laboratories (St Paul, MN, USA); human recombinant IL-1β, TNF-α, oncostatin M (OSM) and an ELISA assay kit for assaying PGE_2 were obtained from R&D Systems (Minneapolis, MN, USA); nitrate/nitrite colorimetric assay kits, along with rabbit anti-human mPGES and mouse anti-human COX-2 were purchased from Cayman Chemical (Ann Arbor, MI, USA); mouse anti-human iNOS was acquired from Millipore (Billerica, MA, USA); rabbit anti-human-IκB kinase (IKK)β, rabbit anti-human phospho-IKKαβ, rabbit anti-human IκB-α and rabbit anti-human phospho IκB-α were purchased from Cell Signaling (Danvers, MA, USA); and mouse anti-human β-actin was acquired from Sigma (St Louis, MO, USA). All other reagents were from Sigma unless otherwise specified.

Chondrocyte monolayer and explant cultures

Cartilage samples were obtained from the femoral condyle and tibial plateau of the knee from patients with OA at the time of joint replacement surgery [$n = 36$, female 94.5%, mean age 69.5 (6.9) years]. The collection and use of human samples were reviewed and approved by the institutional review board of Hallym University Sacred Heart Hospital (Anyang, Korea). Written informed consent was obtained from all donors.

Full-thickness cartilage slices were obtained from above the subchondral bone from a relatively lesion-free area. Chondrocytes were cultured in monolayers and in explants as described previously [15].

Briefly, for monolayer cultures, slices were minced and incubated sequentially with pronase and collagenase, and released cells were seeded at a density of 5×10^6/plate in 10-cm culture plates in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 1% Fungizone (Gibco, Grand Island, NY, USA) and penicillin/streptomycin (150 U/ml and 50 mg/ml, respectively). After ~7 days, confluent chondrocytes were split once and seeded at high density; these first-passage chondrocytes were used within 2 days in subsequent experiments.

For the explant culture, full-thickness slices were obtained from the femoral condyle. Each slice was cut further, and a piece of ~2-mm width × 5-mm length × full thickness was weighed and cultured at 200 μl/well in a 48-well culture plate in the same medium as described above for the monolayer culture. Monolayer and explant cultures were incubated with DMEM containing 0.5% fetal calf serum for 16 h prior to treatment with pro-inflammatory cytokines. For the blocking experiments, SFN was dissolved in distilled water just before the initiation of an experiment and was added to the chondrocytes at various concentrations 1 and 24 h in monolayer and explant cultures, respectively, before stimulation with pro-inflammatory cytokines.

ELISA

The culture supernatants of chondrocytes were harvested after a 24-h (monolayer) and 72-h (explants) incubation with IL-1β or TNF-α and stored frozen at −70°C. PGE_2 was quantified in cell supernatants by ELISA using a PGE_2 immunoassay kit according to the manufacturer's protocol (R&D Systems). The detection limit was 13.4 pg/ml. The number of chondrocytes in each treatment condition was quantified using the 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously [16], and ELISA results were adjusted by the chondrocyte number under each treatment condition.

Nitrate/nitrite quantification

The culture medium of the chondrocytes was harvested as for the PGE_2 assay and analysed with a nitrate/nitrite colorimetric assay kit, as recommended by the manufacturer (Cayman Chemical). Briefly, nitrate was converted to nitrite using nitrate reductase and then the Griess reagents were added to convert nitrite into a deep-purple azo compound. The absorbance of the azo chromophore was measured to determine the nitrite concentration at 540 nm using a plate reader. The detection limit of the assay was 1 μm nitrite. The results were adjusted by the chondrocyte number under each treatment condition.

Real-time RT–PCR

First-passage chondrocytes were seeded (500 000/well) in 6-well culture plates and serum-starved before treatment with SFN, IL-1β or TNF-α. Total RNA was isolated 4 h after stimulation with IL-1β or TNF-α using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA was treated with RNase-free DNase I (Ambion, Austin, TX, USA) to remove contaminating DNA. Total RNA (600 ng) was reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's guidelines. Real-time RT–PCR analysis of COX-2, mPGES and iNOS was performed in a total volume of 20 μl containing one-tenth of the reverse transcriptase reaction, 100 nM sense and antisense primers, and 2 μl of Lightcycler FastStart DNA Master SYBR Green I (Roche, Mannheim, Germany). The following sense and antisense primers for each molecule were used: for COX-2, 5′-TGGGAAGCCTTCTCTAACCTC-3′ (sense) and 5′-TCAGGAAGCTGCTTTTTACCTT-3′ (antisense); for mPGES, 5′-GGAACGACATGGAGACCATC-3′ (sense) and 5′-GGAAGACCAGGAAGTGCATC-3′ (antisense); for iNOS, 5′-ACATTGATGAGAAGCTGTCCCAC-3′ (sense) and 5′-CAAAGGCTGTGAGTCCTGCAC-3′ (antisense); and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-TGATGACATCAAGAAGGTGGTGAAG-3′ (sense) and 5′-TCCTTGGAGGCCATGTGGGCCAT-3′ (antisense). After incubation at 95°C for 10 min, the mixtures were subjected to 40 amplification cycles [10 s at 95°C for denaturation, 5 s at 61°C (59°C for COX-2) for annealing and 15 s at 72°C for extension]. Incorporation of SYBR Green dye into PCR products was monitored in real time using a LightCycler 2.0 Instrument (Roche), allowing determination of the threshold cycle (C_T) at which exponential amplification of PCR products begins. Relative mRNA amount was calculated by 1/2 C_T of a specific gene/1/2 C_T of GAPDH. The specificity of PCR products was determined by melting curve analysis and agarose gel electrophoresis. Each PCR generated only the expected specific amplicon, as shown by the melting-temperature profiles of the final product and by gel electrophoresis to test PCRs. Each PCR was performed in duplicate for three independent cartilage donors.

Western blot

Cellular protein was extracted in lysis buffer containing 50 mM sodium acetate (pH 5.8), 10% v/v SDS, 1 mM Na_2–EDTA, 1 mM phenylmethylsulphonyl fluoride, 1 μg/ml aprotinin, 1 mM sodium fluoride and 1 mM sodium orthovanadate at 4°C. For evaluation of mPGES expression, microsomal proteins were prepared by homogenization in a buffer containing 0.1 M potassium phosphate (pH 7.4), 0.25 M sucrose, 2 mM EDTA and complete protease inhibitor mixture. The sonicated cells were centrifuged (600 *g* for 5 min) and the supernatant was again centrifuged (12 000 *g* for 10 min). The supernatant was removed and centrifuged a third time (170 000 *g* for 1 h) to separate the microsomal fraction (pellet) from the cytosolic fraction (supernatant). Protein concentrations were measured using the bicinchoninic acid (BCA) method with BSA as the standard. Proteins were separated on a 12% SDS–polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Blots were blocked with Tris-buffered saline containing 5% non-fat milk at room temperature for 1 h and then incubated with the respective antibodies overnight at 4°C. The blots were then incubated with 1 : 5000 peroxidase-conjugated secondary antibody for 1 h. Bound immunoglobulin was detected using an enhanced chemiluminescence kit (Amersham Corp., Buckinghamshire, UK).

Luciferase assay

Chondrocytes were transiently transfected with 1 μg plasmid containing an NF-κB promoter element upstream of luciferase or a control vector. The expression vector was introduced to cells using the CarriGene reagent (Kinovate Life Sciences, Oceanside, CA, USA) following the manufacturer's protocol. The transfected cells, which were then cultured in complete medium for 24 h, were used in further assays. Following incubation with IL-1β or TNF-α with or without SFN pre-treatment, luciferase activity was measured using a TD 20/20 luminometer. Luciferase activity was normalized to the activity of *Renilla*. The change in luciferase activity in response to SFN pre-treatment was expressed relative to SFN-untreated controls.

Chromatin immunoprecipitation assay

Subconfluent chondrocytes were serum-starved and treated with IL-1β or TNF-α for 1 h with or without 1-h SFN pre-treatment. Following treatment, the pre-cleared chromatin was immunoprecipitated for 16 h with a specific antibody against NF-κB p65 (Abcam, Cambridge, UK). For PCR amplification, 35–40 PCR cycles were performed using the following primers: sense 5′-GAAAAGACATCTG GCGGAAA-3′ and antisense 5′-TCCCTCCTCTCCCCTTA AAA-3′ (COX-2 promoter −436 to −235 bp), and sense 5′-TACTGCCCCTGAATCTTGC-3′ and antisense 5′-GGC ACTTTGCTGCGTTTTCT-3′ (iNOS promoter −5765 to −5576 bp). Human IgG served as the negative control. The amplification products were analysed on a 2% agarose gel and visualized by ethidium bromide staining. The bands detected were of the correct sizes for the selected fragment of the COX-2 and iNOS.

Proteoglycan and type II collagen degradation product measurement

Cartilage explants were incubated in media containing IL-1β or TNF-α in the presence or absence of SFN, and the media were collected on Days 3, 6 and 9 for the measurement of proteoglycan degradation. The amount of sulphated GAGs, reflecting the amount of proteoglycans released in the culture medium, was determined using a commercial kit (Biocolor, Belfast, UK) according to the manufacturer's recommendations. Briefly, collected media were mixed with dye reagent containing 1,9-dimethylene blue for 30 min and then the GAG–dye complex was separated by centrifugation. The dye pellet was dissociated with propan-1-ol solution and absorbance was read at 656 nm with a plate reader. For type II collagen degradation, cartilage explants were incubated in media containing 5 ng/ml IL-1β plus 50 ng/ml OSM in the presence or absence of SFN, and media were collected on Days 3 and 6. The Urine CartiLaps ELISA Kit measuring C-terminal telopeptides of type II collagen (CTX-II; Nordic Bioscience Diagnostics, Herlev, Denmark) was used according to the manufacturer's recommendations.

Data analysis

Data are expressed as the means (s.d.). Differences between treatment groups were tested using the Mann–Whitney U-test (GraphPad Prism, version 3; GraphPad Software, San Diego, CA, USA). Significance was established at the 95% CI ($P < 0.05$).

Results

Inhibition of PGE_2 induced by pro-inflammatory cytokines with SFN

We determined whether SFN inhibited PGE_2 induced by pro-inflammatory cytokines in articular chondrocytes. A 1-h pre-treatment with SFN led to significant inhibition of PGE_2 induced by either IL-1β or TNF-α in monolayer cultured articular chondrocytes (Fig. 1A). The inhibition was concentration dependent and universally significant at concentrations >2 μM. Next, we determined whether SFN also suppressed PGE_2 induction in cartilage explants. Explants were treated with SFN for 24 h before stimulation with IL-1β or TNF-α. After 72 h of cytokine treatment, culture supernatants were collected and the levels of PGE_2 were measured. In explants, SFN also significantly inhibited PGE_2 production induced by IL-1β or TNF-α (Fig. 1B). These results show that SFN was effective in inhibiting one of the catabolic mediators in articular cartilage, PGE_2, not only in monolayer cultured chondrocytes, but also in chondrocytes embedded in their natural ECM. We then performed a time-course analysis to assess whether the suppression of PGE_2 by SFN was effective even after addition of pro-inflammatory cytokines. As shown in Fig. 1C, SFN effectively inhibited PGE_2 release even when it was added 4 h after the addition of each cytokine. SFN did not induce significant cell death under any of the treatment conditions tested.

Fig. 1 Inhibition of PGE_2 up-regulation by SFN. SFN was added at the indicated concentrations 1 h [monolayer (**A**)] or 24 h [explants (**B**)] before stimulation with IL-1β or TNF-α. PGE_2 production was measured 24 h (monolayer) or 72 h (explant) after IL-1β or TNF-α treatment from the conditioned media. (**C**) SFN (10 μm) was added to the monolayer chondrocytes at the indicated times relative to the addition of IL-1β or TNF-α. PGE_2 production was measured 24 h after IL-1β or TNF-α treatment by ELISA using a PGE_2 immunoassay kit according to the manufacturer's protocol. Results are given as the mean (s.d.) of the mean percentage of IL-1β or TNF-α treatment alone, which was set at 100%. The data represent duplicate experiments from non-pooled culture supernatants from four (monolayer) and three (explants) donors, respectively. *$P < 0.05$, compared with IL-1β or TNF-α treatment alone.

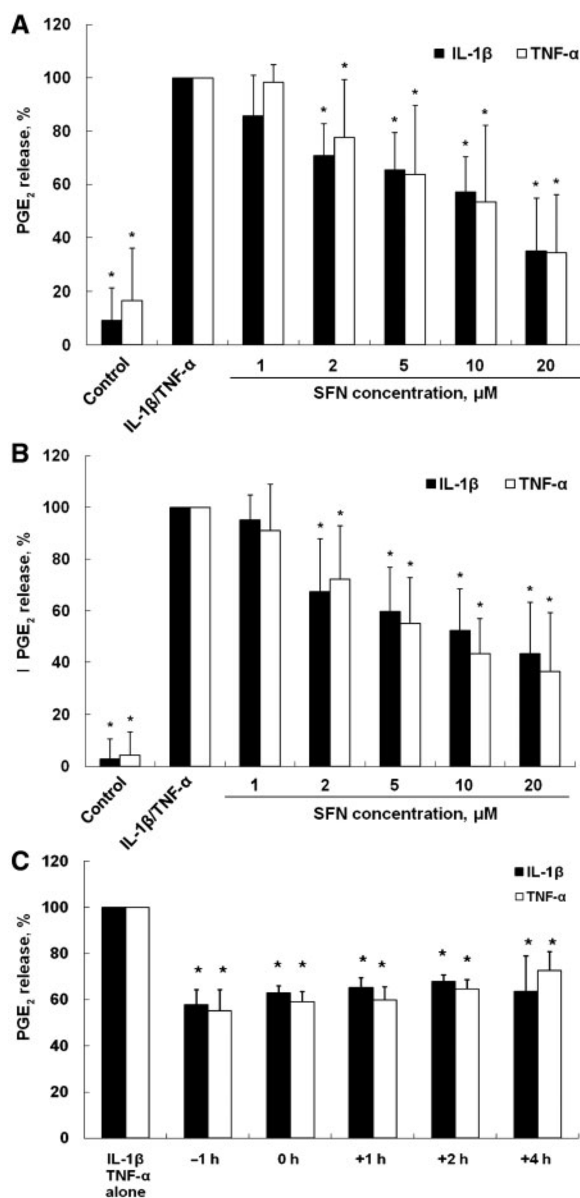
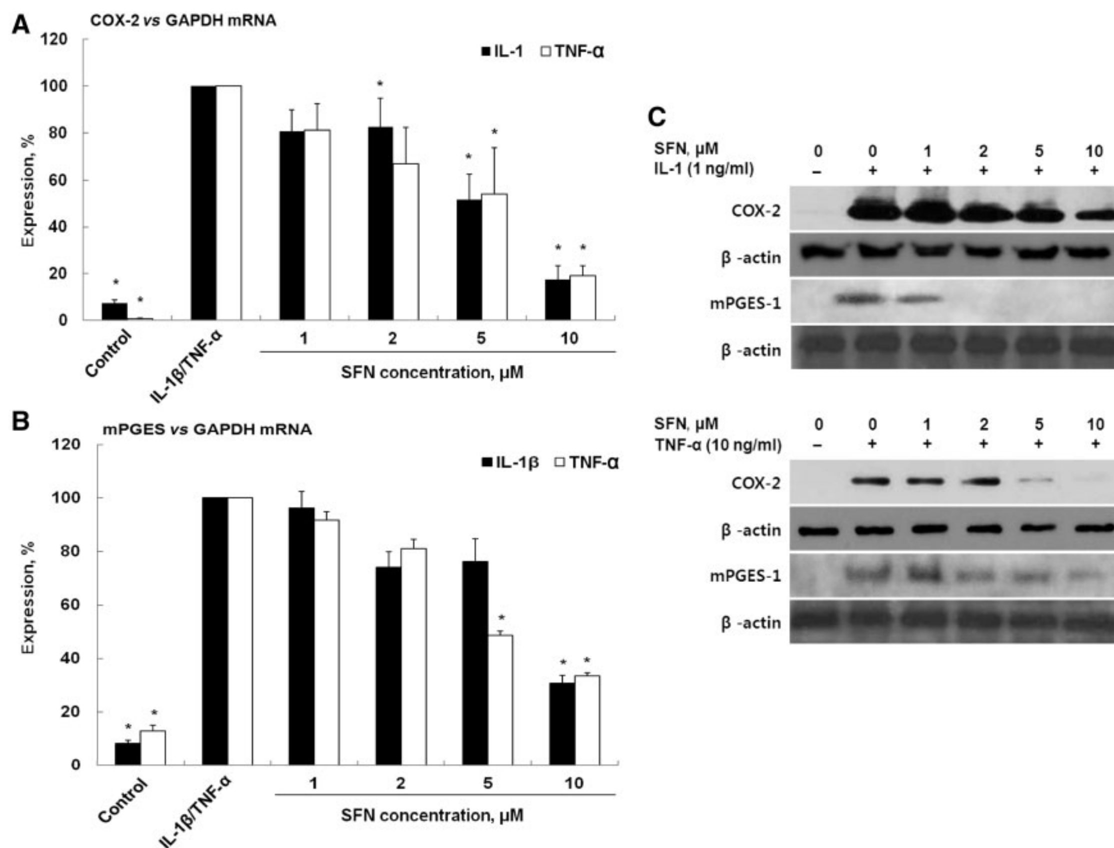


Fig. 2 Inhibition of COX-2 and mPGES up-regulation by SFN. (**A** and **B**) SFN was added to the chondrocytes 1 h before stimulation with IL-1β or TNF-α. Total RNA was isolated 4 h after cytokine stimulation and real-time RT-PCR was performed using COX-2 (**A**), mPGES (**B**) and GAPDH primer sets. The mRNA expression level of IL-1β or TNF-α alone was set at 100%. The data represent duplicate samples from non-pooled RNA from three different donors. *$P < 0.05$ compared with IL-1β or TNF-α treatment alone. (**C**) Inhibition of COX-2 and mPGES protein up-regulation by SFN. SFN was added to the chondrocytes 1 h before stimulation with IL-1β or TNF-α and protein (microsomal protein for mPGES) was extracted 24 h after cytokine stimulation. Twenty micrograms (COX-2) and 5 μg (mPGES) of each protein sample was separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). The expression of COX-2 and mPGES was analysed by western blotting. The data represent non-pooled samples from five (COX-2) and four (mPGES) different donors.



Regulation of COX-2 and mPGES by SFN

Next, we determined whether SFN suppressed PGE_2 induction by suppressing COX-2 or mPGES mRNA levels by real-time RT–PCR analysis. After 1 h of incubation with 10 μM SFN, the observed expression of COX-2 was 17 and 19% for IL-1β and TNF-α, respectively, compared with the control, set at 100% (Fig. 2A). The mPGES mRNA concentration also decreased significantly with a 1-h SFN pre-treatment, from 5 μM for TNF-α and from 10 μM for IL-1β (Fig. 2B). Western blot analysis revealed that SFN significantly inhibited up-regulation of COX-2 and mPGES protein induced by IL-1β or TNF-α in articular chondrocytes (Fig. 2C). These results show that suppression of PGE_2 by SFN involves the down-regulation of COX-2 and mPGES.

Inhibition of NO induced by pro-inflammatory cytokines with SFN

We next determined whether SFN inhibited another mediator of cartilage degradation, i.e. NO induced by pro-inflammatory cytokines in articular chondrocytes. A 1-h pre-treatment with SFN led to significant inhibition of NO induced by either IL-1β or TNF-α in monolayer cultured articular chondrocytes (Fig. 3A). The inhibition was significant at 1 μM and concentration dependent. Next, we examined whether SFN also suppressed NO production in cartilage explants. Explants were treated with SFN for 24 h before stimulation with IL-1β or TNF-α. After 72 h, culture supernatants were collected and levels of NO were measured. SFN significantly inhibited NO production induced by IL-1β or TNF-α, starting at 5 and 2 μM for IL-1β and

Fig. 3 Inhibition of NO by SFN. SFN was added at the indicated concentrations 1 h [monolayer (**A**)] or 24 h [explants (**B**)] before stimulation with IL-1β or TNF-α. NO production was measured 24 h (monolayer) or 72 h (explant) after IL-1β or TNF-α treatment from the conditioned media. (**C**) SFN (10 μM) was added to the monolayer chondrocytes at the indicated times relative to the addition of IL-1β or TNF-α. NO production was measured 24 h after IL-1β or TNF-α treatment with a nitrate/nitrite colorimetric assay kit. Results are given as the mean (s.d.) of the percentage of IL-1β or TNF-α treatment alone, which were set at 100%. The data represent duplicate experiments from non-pooled culture supernatants from four donors each for monolayer and explant culture. *$P < 0.05$, compared with IL-1β or TNF-α treatment alone.

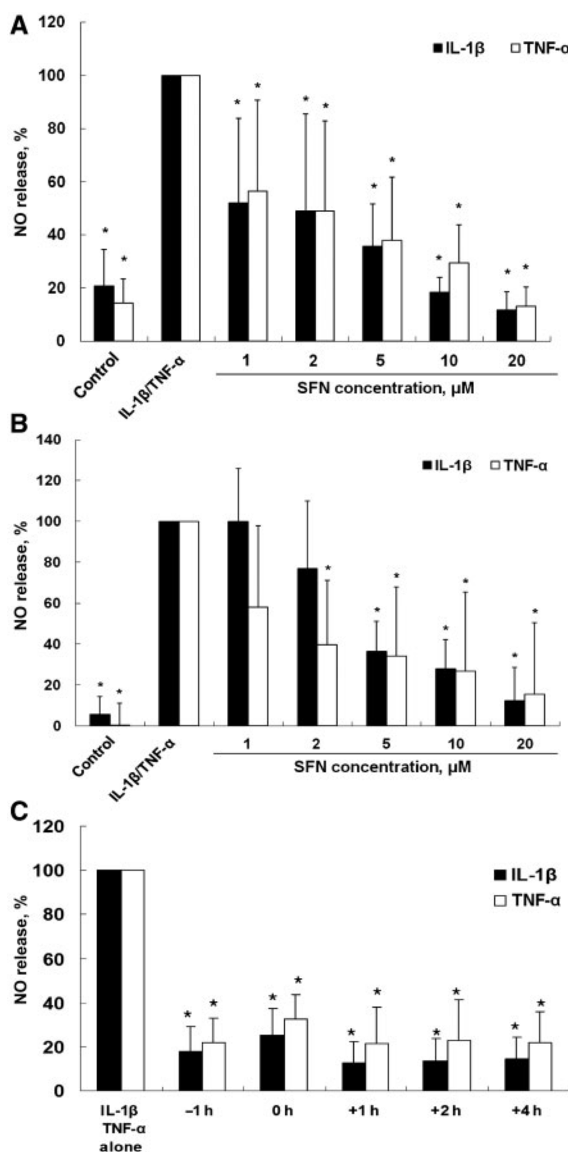
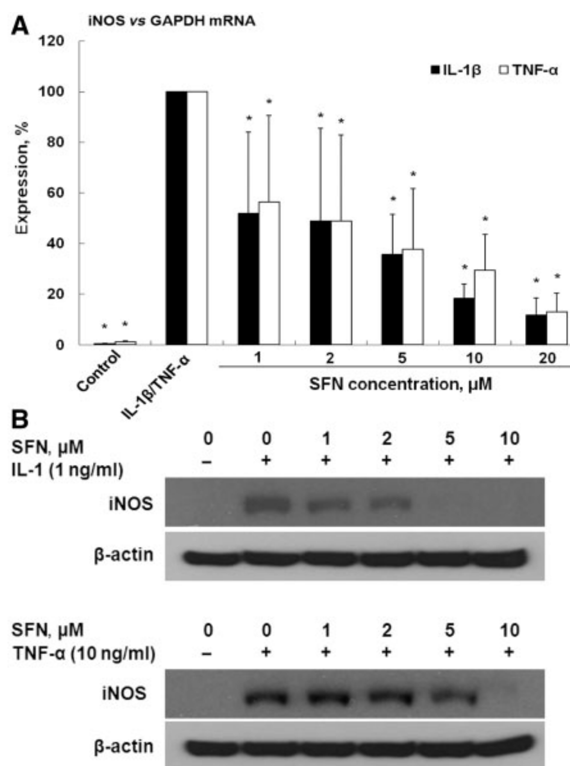


Fig. 4 Inhibition of iNOS mRNA and protein up-regulation induced with IL-1β or TNF-α by SFN. (**A**) SFN was added to the chondrocytes 1 h before stimulation with IL-1β or TNF-α. Total RNA was isolated 4 h after cytokine stimulation and real-time RT–PCR was performed using iNOS and GAPDH primer sets. The mRNA expression level of IL-1β or TNF-α alone was set at 100%. The data represent duplicate samples from non-pooled RNA from three different donors. *$P < 0.05$ compared with IL-1β or TNF-α treatment alone. (**B**) SFN was added to the chondrocytes 1 h before stimulation with IL-1β or TNF-α, and protein was extracted 24 h after cytokine stimulation. Twenty micrograms of proteins were loaded onto SDS–PAGE and analysed by western blotting. The data represent three non-pooled protein samples from different donors.

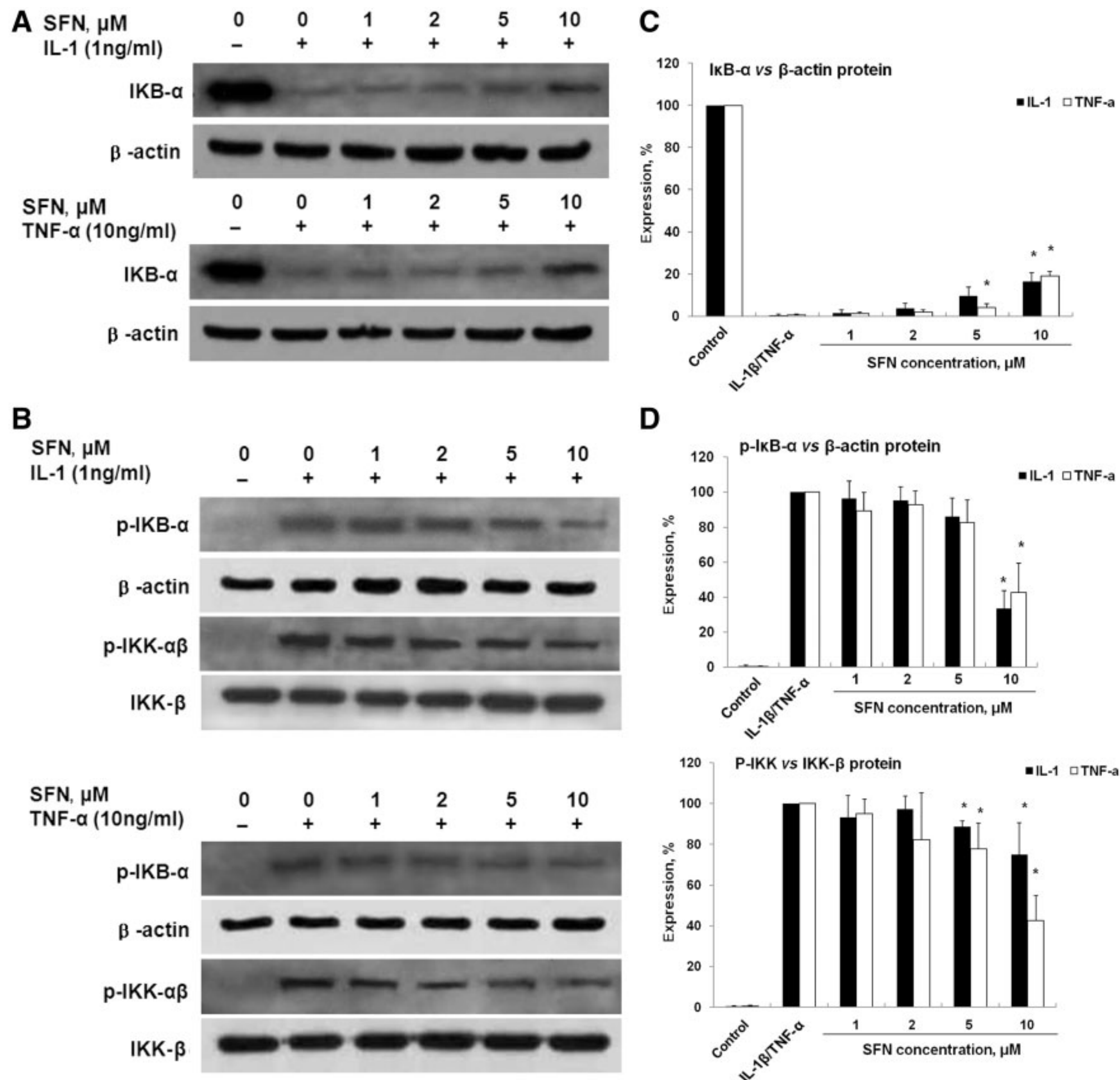


TNF-α, respectively (Fig. 3B). A time-course analysis showed that the suppression of NO by SFN was effective 4 h after the addition of pro-inflammatory cytokines (Fig. 3C). These results showed that SFN was effective in inhibiting NO production in both monolayer cultured chondrocytes and in explant cultured chondrocytes, suggesting its potential as a chondroprotective agent.

Regulation of iNOS by SFN

Next, we determined whether SFN suppressed NO induction by regulating iNOS at the mRNA and protein level. SFN significantly inhibited up-regulation of iNOS mRNA and protein induced by IL-1β or TNF-α in articular chondrocytes (Fig. 4). iNOS mRNA and protein induced by IL-1 was significantly inhibited by SFN starting from 1 μM

Fig. 5 Inhibition of IκB-α degradation and IκB-α and IKK-αβ phosphorylation by SFN. Monolayer cultured human articular chondrocytes were treated with 1–10 μM SFN 1 h before the addition of IL-1β or TNF-α. Proteins were extracted 15 min after cytokine treatment from the chondrocytes, and 20 μg of each protein sample was separated by 12% SDS–PAGE. IκB-α degradation (**A**) and IκB-α and IKK-αβ phosphorylation (**B**) were analysed by western blotting. Blots were stripped and reprobed for β-actin or non-phospho-specific antibodies. The data represent four non-pooled samples from different donors. Densitometry analysis of western blot for IκB-α degradation (**C**) and IκB-α and IKK-αβ phosphorylation (**D**). Normalization for loading differences was achieved by dividing the densitometry values for individual bands by the densitometry values for β-actin (**C**) or β-actin and IKK-β (**D**) in the same lane. For IκB-α, band density of control samples was set at 100%. For IκB-α and IKK-αβ phosphorylation, band density of cytokine treatment alone was set at 100%. $^{*}P < 0.05$ compared with IL-1β or TNF-α treatment alone.

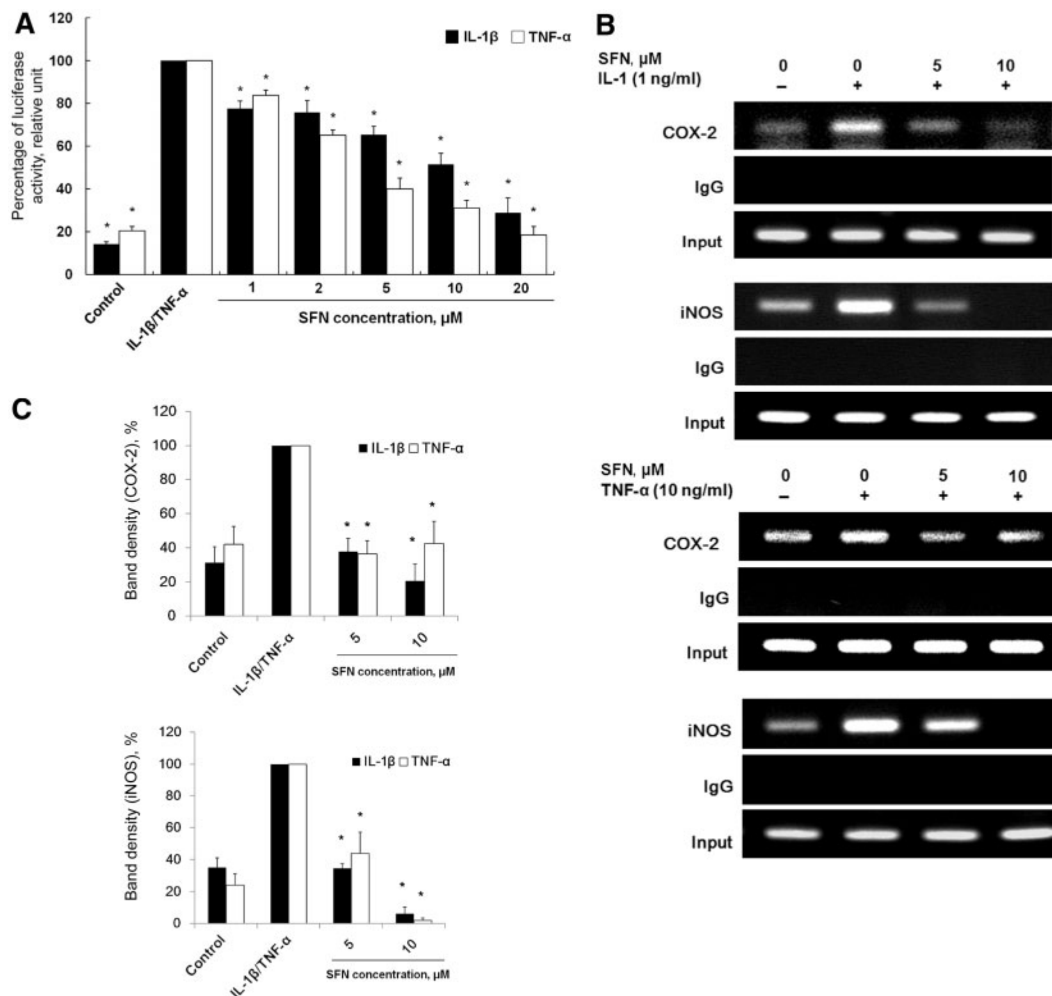


concentration. Whereas iNOS mRNA induced by TNF-α was significantly inhibited by 1 μM SFN, it was only at 5 μM that a significant of inhibition of protein expression was observed.

Regulation of NF-κB activation by SFN

Previously we demonstrated that SFN inhibited NF-κB nuclear translocation in pro-inflammatory cytokine-stimulated articular chondrocytes using an electrophoretic mobility shift assay [5]. To further examine the mechanism of NF-κB activation, we observed the pattern of IκB degradation, IKK phosphorylation and IκB phosphorylation (Fig. 5A and B). IκB degradation, induced with IL-1β and TNF-α, was significantly inhibited by 10 μM SFN. At the same concentration of SFN, inhibition of IκB-α and IKK phosphorylation induced by both cytokines was

Fig. 6 Inhibition of NF-κB promoter activity and NF-κB subunit p65 binding with the COX-2 and iNOS promoter by SFN. (**A**) Chondrocytes were transiently transfected with 1 μg of plasmid containing the NF-κB promoter element upstream of luciferase or a control vector. Following incubation with IL-1β or TNF-α with or without SFN pre-treatment, luciferase activity was measured. The change in luciferase activity in response to SFN pre-treatment is expressed relative to SFN-untreated controls. (**B**) Subconfluent chondrocytes were serum starved and treated with IL-1β or TNF-α for 1 h with or without 1-h SFN pre-treatment. Following cytokine treatment, the pre-cleared chromatin was immunoprecipitated for 16 h with a specific antibody against NF-κB p65 and then amplified with PCR. The amplification products were analysed on a 2% agarose gel and visualized by ethidium bromide staining. The data represent three non-pooled samples from different donors. (**C**) Densitometry data for chromatin immunoprecipitation assay. Band density of cytokine treatment alone was set at 100%. $^{*}P < 0.05$ compared with IL-1β or TNF-α treatment alone.

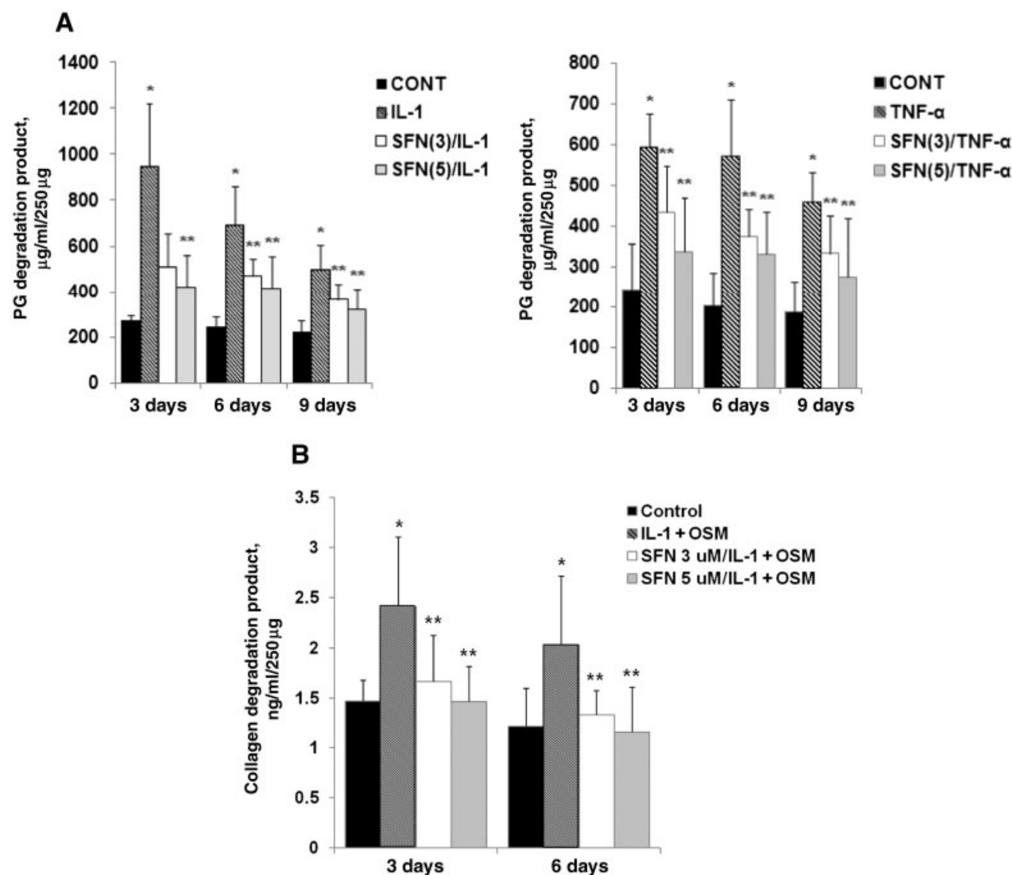


observed. Then we performed luciferase assays and the results showed that SFN significantly reduced NF-κB promoter luciferase activity induced by IL-1β or TNF-α dose dependently (Fig. 6A). Finally, we determined whether SFN regulated the binding of NF-κB subunit p65 with the regulatory sequences of COX-2 and iNOS promoters by standard chromatin immunoprecipitation procedures, based on the fact that both are NF-κB-induced genes that regulate pro-inflammatory signalling. In chondrocytes, p65 was detectable at the COX-2 and iNOS promoters at 1 h after IL-1β stimulation. This was inhibited by pre-treatment with SFN. The binding of p65 stimulated by TNF-α was also inhibited by SFN pre-treatment (Fig. 6B).

Suppression of proteoglycan and type II collagen degradation by SFN pre-treatment

To confirm the inhibitory effect of SFN on cartilage matrix catabolism, we cultured cartilage explants with pro-inflammatory cytokines and observed the modulation of proteoglycan or type II collagen degradation products released into the culture media. Treatment with IL-1β or TNF-α led to increased release of proteoglycan

Fig. 7 Suppression of the release of proteoglycans and type II collagen degradation products by SFN. (**A**) Cartilage slices were cultured in explants, incubated in media containing 1 ng/ml IL-1β (left panel) or 10 ng/ml TNF-α (right panel) with or without SFN for 3–9 days. The medium was changed every 3 days. The amount of sulphated GAGs was determined. Results are given as the mean (S.D.) of the mean amount of GAGs (μg) released into the culture medium per 250 μg cartilage (representative of duplicate experiments from three donors). *$P < 0.05$ compared with the control. **$P < 0.05$ compared with IL-1β or TNF-α alone. (**B**) Cartilage slices were incubated in medium containing 5 ng/ml IL-1β and 50 ng/ml OSM with or without SFN for 3 and 6 days. The medium was changed every 3 days. Type II collagen degradation was analysed using a Urine CartiLaps ELISA kit. Results are given as the mean (S.D.) of the mean amount of CTX-II (ng) released in the culture medium per 250 μg cartilage. The data represent duplicate experiments from non-pooled culture supernatants from three donors. *$P < 0.05$ compared with the control. **$P < 0.05$ compared with IL-1β or TNF-α alone.



degradation products after 3 days of culture, which was effectively inhibited by SFN co-treatment (Fig. 7). The degree of inhibition was significant at 3 μM SFN, and the inhibitory effect persisted after 9 days of culture. Treatment of cartilage explants with IL-1β or TNF-α resulted in slow breakdown of type II collagen and thus necessitated prolonged culture of the explants (data not shown). Thus we treated cartilage explants with 5 ng/ml IL-1β and 50 ng/ml OSM to hasten the process of collagen degradation. Under these conditions, type II collagen degradation increased significantly after 3 days. As in the case with proteoglycan, co-treatment with SFN led to significant down-regulation of type II collagen degradation in explant cultured cartilage stimulated with IL-1β and OSM. The inhibition was significant after 3 and 6 days. After 9 days, the type II collagen degradation products no longer increased in the IL-1β and OSM-stimulated explants (data not shown).

Discussion

In this study we showed that the Phase 2 enzyme inducer SFN potently inhibited PGE_2 and NO production induced by two prototypical inflammatory cytokines, IL-1β and TNF-α, in human OA chondrocytes. Inhibition of PGE_2 and NO by SFN was observed in both monolayer and explant cultures, and was accompanied by significant down-regulation of matrix degradation in pro-inflammatory cytokine-stimulated cartilage explants. SFN affected a wide range of intracellular signalling pathways required for the NF-κB activation.

SFN has been extensively investigated for its effects in protection against carcinogenesis. Although significant overlap exists in the mechanisms underlying malignancy and inflammation, the anti-inflammatory effect of SFN was investigated only recently. SFN was shown to inhibit IL-8 production by airway epithelial cells stimulated with diesel extract [17]. A study in rats showed that broccoli sprouts containing high levels of glucoraphanin, which is metabolized to SFN, significantly reduced oxidative stress and inflammation in the kidneys and in the cardiovascular system [18]. SFN has also been reported to influence diverse organ systems and disease models, ranging from the protection of human skin from the inflammation caused by exposure to ultraviolet light to decreased brain damage caused by intracranial haemorrhage [19, 20].

The therapeutic implications of a Phase 2 enzyme inducer on rheumatic disease have also been suggested in previous studies. The Phase 2 enzyme inducer D3T was shown to down-regulate shear-activated COX-2 protein and PGE_2 release in chondrocytes, and this result suggested that Phase 2 enzyme inducers may represent a safe alternative to COX-2 inhibitors [21]. In our previous study using human OA chondrocytes, we found that SFN potently inhibited production of three MMPs important in the degradation of cartilage matrix induced by IL-1β and TNF-α [5]. We also demonstrated that SFN attenuated the course of CIA and antibody-induced arthritis, two mouse models of human RA [22]. SFN exhibited profound anti-inflammatory and immune modulatory effects, including inhibition of synovial hyperplasia, induction of synoviocyte apoptosis, inhibition of activated T-cell proliferation, and inhibition of production of IL-17 and TNF-α by RA T cells. Antigen-specific T-cell proliferation and autoantibody production were also inhibited. Notably, a recent report showed that low micromolar SFN concentrations exerted prosurvival and anti-apoptotic actions in chondrocyte cell lines and OA chondrocytes treated with a variety of death stimuli, such as TNF-α/cycloheximide, hydrogen peroxide and growth-related oncogene-α [23]. While the report by Facchini *et al*. [23] showed that the number of cells decreased significantly without inducing cell death after 1 day of incubation with 5 μM SFN, we did not observe a significant decrease in cell number, quantified with MTT, at up to 20 μM SFN [5]. This difference may have been the result of the different cell types used (C28/I2 chondrocytes *vs* human OA primary chondrocytes). The anti-apoptotic effect on chondrocytes contrasts with the proapoptotic effect on synovial fibroblasts and suggests that SFN may work in different directions between synoviocytes and chondrocytes in terms of regulation of cell death, thus offering a potentially beneficial therapeutic approach for the treatment of arthritis.

In rats, the plasma concentration of SFN can peak at 20 μM 4 h after an oral dose of 50 μmol of SFN, and this increase in plasma concentration of SFN is accompanied by the induction of genes that are important in cellular defence mechanisms and cell-cycle regulation [24]. Ingestion of 200 μmol of broccoli sprout ITCs leads to peak plasma concentration of 3.4 μM in humans [25, 26]. Thus the micromolar concentrations of SFN used in our study are achievable *in vivo*.

The direct targets and cellular receptors involved in the anti-inflammatory effect of SFN remain elusive, although the effects on NF-κB have been most extensively reported. SFN has been shown to impair NF-κB activation induced by lipopolysaccharide (LPS) in RAW 264.7 macrophages and to decrease NF-κB 65-kDa protein expression in a human malignant glioblastoma cell line [27, 28]. While our results also showed that SFN inhibited NF-κB activation in IL-1β- or TNF-α-treated chondrocytes by down-regulating IKK activation, IκB phosphorylation and IκB degradation, others have reported that SFN did not interfere with LPS-induced degradation of IκB or NF-κB nuclear translocation in RAW 264.7 cells, but instead reduced the DNA-binding ability of NF-κB directly [29]. In our study, SFN also down-regulated the promoter-binding ability of p65 induced with IL-1β and TNF-α in chondrocytes. The mechanism of inhibitory effect on NF-κB–DNA binding by SFN include formation of dithiocarbamate and binding to essential Cys residues of NF-κB subunits and inhibition of the thioredoxin/thioredoxin reductase system, a key redox mechanism regulating NF-κB DNA binding [30].

In our study, we used human OA chondrocytes exclusively instead of normal chondrocytes, because of the difficulty of obtaining enough normal human cartilage for the experiment. It would have been interesting to observe whether the pattern of suppression by SFN was any different between normal and OA chondrocytes, and caution would be needed to extrapolate our data into normal chondrocytes.

In conclusion, SFN was found to inhibit PGE_2 and NO production, and matrix degradation in pro-inflammatory cytokine-stimulated chondrocytes. Delineation of the biochemical mechanisms regulating cartilage catabolism by SFN may lead to the identification of safe and effective therapeutic targets for inhibiting cartilage degradation in arthritic diseases.

Rheumatology key messages

- SFN suppresses PGE_2 or NO production from articular chondrocytes.
- SFN inhibits proteoglycan and type II collagen degradation product release in cartilage explant culture.
- SFN may be a candidate therapeutic agent for treatment of cartilage degradation in arthritides.

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