

Influence of simvastatin on the production of pro-inflammatory cytokines and nitric oxide by activated human chondrocytes

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Abstract

Objective

In addition to their cholesterol-lowering action, statins have been suggested to exert anti-inflammatory activities. In this study we evaluate whether simvastatin could influence the production of pro-inflammatory cytokines (interleukin (IL)-6 and IL-8) and nitric oxide (NO) by activated human chondrocytes.

Methods

Human isolated chondrocytes and cartilage explants were pre-incubated with simvastatin (0.5, 5, 10 and 50 $\mu\text{mol/L}$) for 48 h. Then the cultures were stimulated with a mixture of IL-1 β and TNF- α (10 ng/mL) and co-incubated with simvastatin for an additional 48 h. A flow cytometric microsphere-based immunoassay was performed to detect cytokine secretion in the supernatants. NO production was quantified using the Griess assay.

Results

Simvastatin demonstrated significant dose-dependent inhibition of IL-6 and IL-8 production of isolated chondrocytes and cartilage explants up to 99% for IL-6 and up to 88% for IL-8 ($p < 0.01$). At the higher concentrations simvastatin decreased NO production by both isolated chondrocytes (up to 43%, $p < 0.01$) and cartilage explants (up to 30%, $p < 0.01$).

Conclusion

This study demonstrates anti-inflammatory properties of simvastatin in chondrocytes in vitro, suggesting a potential cartilage-protective role for statins in arthritis.

Key words

Rheumatoid arthritis, chondrocytes, statins, interleukin-6, interleukin-8, nitric oxide.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by inflammatory synovitis and articular destruction. It is generally accepted that the pro-inflammatory cytokines interleukin (IL) -1 and tumor necrosis factor alpha (TNF- α) play a pivotal role in the pathogenesis of RA. In response to these cytokines, chondrocytes are able to produce inflammatory mediators such as nitric oxide (NO) (1) and pro-inflammatory cytokines (2, 3) which lead to further persistent inflammation and finally joint destruction. IL-6 has been linked to the IL-1 induced inhibition of proteoglycan synthesis in chondrocytes (4, 5). Furthermore, IL-6 stimulates osteoclastogenesis and bone resorption, implicating it in a pathogenic role in joint destruction in patients with RA (6, 7). On the other hand, IL-8 has been shown to contribute to the pathogenesis of RA by recruiting leukocytes into the inflamed joint (8, 9) with subsequent neutrophil-mediated cartilage damage (10). NO also plays an important role in cartilage degeneration and the effects of NO on chondrocytes include inhibition of matrix synthesis (11, 12), modulation of metalloproteases (13), increased susceptibility to injury by other oxidants, and the induction of apoptosis (14, 15).

In addition, it has been shown that RA is associated with increased cardiovascular mortality and morbidity (16, 17). 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or statins, are potent inhibitors of cholesterol biosynthesis and have been shown to reduce cardiovascular morbidity and mortality (18, 19). Although statins work in part through lipid modulation, recent studies indicate that statins also have anti-inflammatory and immunomodulatory effects (20, 21). It has been hypothesized that most of these pleiotropic effects of statins might result from blocking the synthesis of isoprenoid intermediates in the cholesterol biosynthetic pathway in a way comparable to the amino-bisphosphonates (22). As a consequence, the administration of statins in RA might result in a two-fold clinical effect. In addition to lowering the cardiovascular

risk, these drugs might contribute to reducing inflammation (23). Inhibitory effects of various statins on pro-inflammatory cytokines and NO in different cell types such as vascular smooth muscle cells, monocytes, microglia, astrocytes endothelial cells, and adipocytes have been demonstrated (24-31). However, other studies showed no effect or an enhancement of these mediators in several cell types (32-36). These discrepancies might partially be attributed to the different types of statins, different species or cell types, and different experimental protocols used. The present study was designed to assess the potential anti-inflammatory effects of simvastatin on chondrocytes. For this purpose, the effect of simvastatin on the production of pro-inflammatory mediators (IL-6, IL-8 and NO) by activated human isolated chondrocytes, as well as cartilage explants, was evaluated.

Methods

Culture of isolated chondrocytes

Normal human chondrocytes ($n = 10$) were obtained from organ donors ($n = 4$), from patients undergoing joint replacement for hip fracture ($n = 5$), or from autopsies ($n = 1$). Cartilage obtained from patients with rheumatoid arthritis, destructive osteoarthritis, active infection or malignancy, or from patients who had undergone treatment with statins, bisphosphonates or glucocorticoids was excluded. The median age of the donors was 66 years (range 44-79).

Chondrocytes were isolated using hyaluronidase (Roche, Germany), protease (Sigma, St. Louis, USA) and collagenase type 1A (Sigma), as described elsewhere (3). After isolation, the chondrocytes were concentrated at 10^6 cells/mL and cultured in a monolayer in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, UK) supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin (Invitrogen) and 10% foetal bovine serum (FBS, Sigma) (DMEM+10% FBS) for 24 h.

Culture of cartilage explants

Normal human cartilage explants ($n = 10$) were obtained from organ donors ($n = 6$) or from patients undergoing

Competing interests: none declared.

total joint replacement for hip fracture ($n = 4$). The same exclusion criteria as for the isolated chondrocyte cultures were used. The median age of the donors was 58 years (range 34–85). Full-thickness cartilage discs of equal size were obtained using a 4 mm biopsy punch (PFM, Köln, Germany). The explants were pooled and cultured in DMEM+10% FBS. Viability, assessed by calcein AM (Molecular Probes, Invitrogen)/propidium iodide (PI, Sigma) staining of the cells/cartilage explants, always exceeded 80% at the start of the experiments (3, 37).

Pre-incubation with simvastatin

Chondrocyte/cartilage cultures were incubated for 48 h with different concentrations (0.5, 5, 10, 50 $\mu\text{mol/L}$) of simvastatin (Calbiochem, UK), or in DMEM+10% FBS alone as a control. This time point was chosen based on a previous study (38).

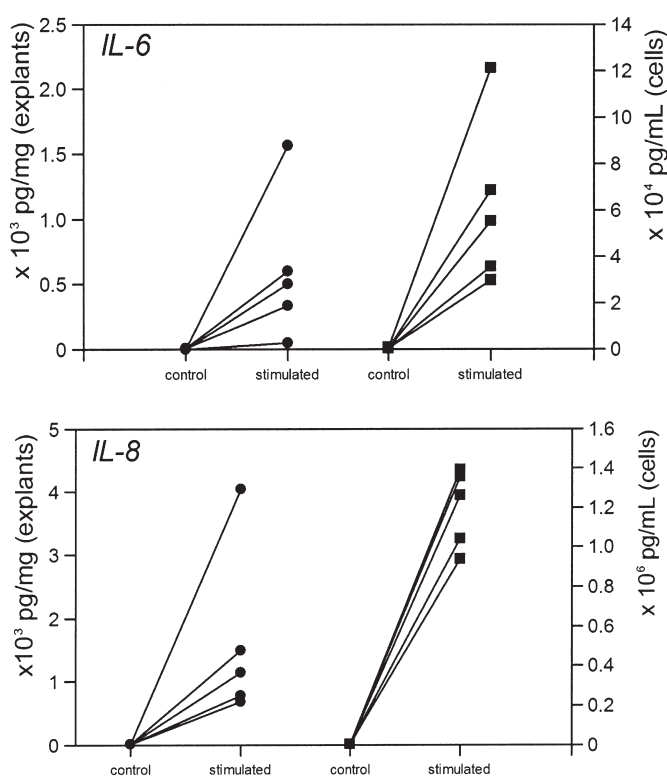
Stimulation of cartilage and chondrocyte culture

The medium was replaced with DMEM without FBS and with or without simvastatin. The chondrocytes and cartilage explants were then stimulated with a mixture of human IL-1 β (PeproTech House, London, UK) and human TNF- α (PeproTech House), each in a concentration of 10 ng/mL, for 48 h. Both the concentration and the stimulation period were chosen based on previous work by our group (1, 3, 39). After stimulation, supernatants were collected and stored at -20°C until further analysis. In line with a recent study (40) and previous work by us (38), no effect of simvastatin on the viability of chondrocytes was detected.

Flow cytometric microsphere-based immunoassay

Cytokine production was measured as described elsewhere (3, 41). Briefly, a flow cytometric microsphere-based immunoassay (Cytometric Bead Array, CBA, BD Biosciences, Erembodegem, Belgium) was used to detect the pro-inflammatory cytokines IL-6 and IL-8 and the regulatory cytokines IL-10 and IL12p70 in the supernatants. The instrument was calibrated using Calibrite

Fig. 1. IL-6 and IL-8 production of isolated chondrocytes (squares) and cartilage explants (circles) before and after stimulation with IL-1 β and TNF- α (10 ng/mL) ($n = 5$).



beads (BD) and cytometer set up beads (BD), in accordance with the manufacturer's instructions.

Three thousand events were measured and analysed. A monomeric microsphere population was gated out on forward and sideward scatter. Mean Fluorescence Intensity values (MFI) were measured and a standard curve was constructed using known concentrations of cytokine and a 4-parameter logistic function. The assay sensitivity ranged from 1.9 to 3.6 pg/mL depending on the cytokine. The results of cytokines measured in the culture medium were expressed in pg/mL for the isolated chondrocyte cultures. Cartilage explants were tapped dry and weighed, and results were expressed in pg/mg.

Nitric oxide measurement

Nitric oxide was measured as nitrite, a stable product of NO, using a spectrophotometric method based upon the Griess assay (1). Results of the isolated chondrocyte cultures were expressed in $\mu\text{mol/L}$ nitrite and results of the cartilage explants in $\mu\text{mol/mg}$ nitrite.

Statistics

All statistical analyses were performed

using SPSS 12.0. Data were logarithmically transformed to obtain a normal distribution. Repeated measures ANOVA was used, followed by a Bonferroni post-test where appropriate. A p -value < 0.05 was considered significant.

Results

Cytokine production

Stimulation for 48 h with a mixture of IL-1 β and TNF- α (10 ng/mL) induced an approximately 10^4 increase in IL-6 production compared to unstimulated controls (for the isolated chondrocytes, range 3.0×10^4 to 12.1×10^4 pg/mL, and for the cartilage explants, range 0.05×10^3 to 1.6×10^3 pg/mg) (Fig. 1). In the isolated chondrocytes, IL-8 production was 2×10^4 enhanced after stimulation (range 0.9×10^6 – 1.4×10^6 pg/mL). Stimulation of the cartilage explants enhanced IL-8 production approximately 5×10^3 times (0.7×10^3 – 4.0×10^3 pg/mg). Pre-incubation of isolated chondrocytes and cartilage explants with simvastatin resulted in a dose-dependent decrease in cytokine production ($p < 0.01$) of up to 99% for IL-6 and up to 86% for IL-8 (Figs. 2 and 3). No production of IL-12p70 was observed, while the production of IL-10 was around the detection

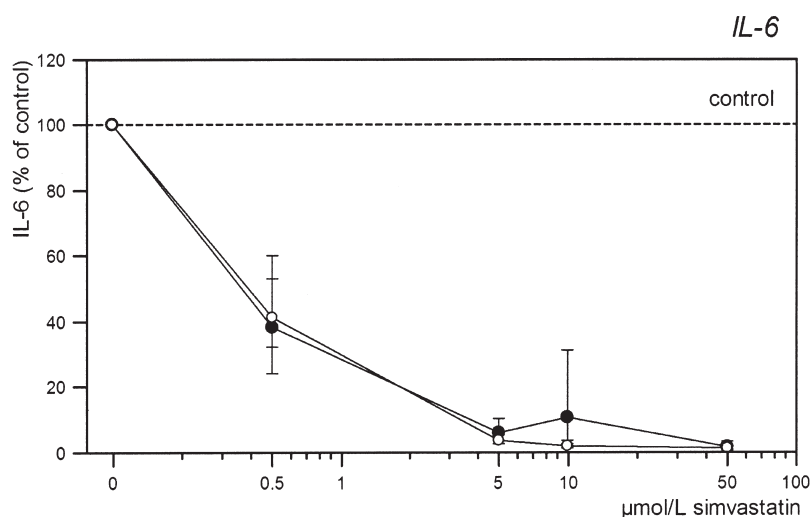


Fig. 2. Effect of different concentrations of simvastatin on IL-6 production by isolated chondrocytes (open circles, $p < 0.01$) and cartilage explants (closed circles, $p < 0.01$) ($n = 5$) stimulated with IL-1 β and TNF- α (10 ng/mL). Results are expressed as the percentage of cytokine production (geometric mean \pm SEM).

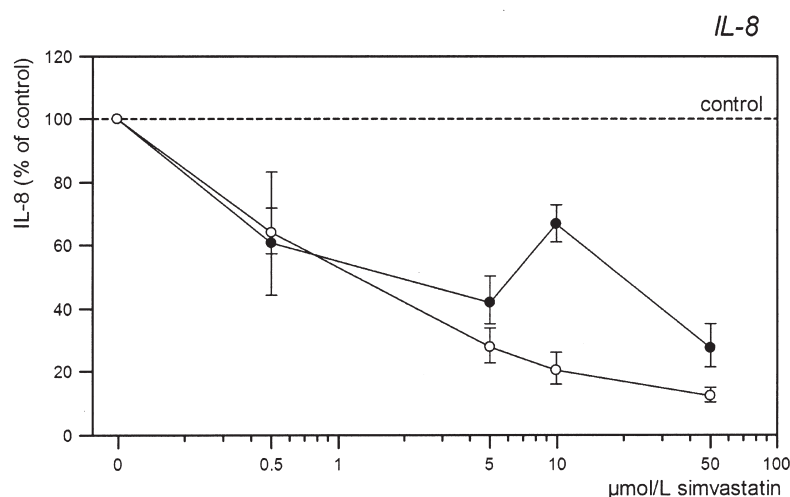


Fig. 3. Effect of different concentrations of simvastatin on IL-8 production by isolated chondrocytes (open circles, $p < 0.01$) and cartilage explants (closed circles, $p < 0.01$) ($n = 5$) stimulated with IL-1 β and TNF- α (10 ng/mL). Results are expressed as the percentage of cytokine production (geometric mean \pm SEM).

limit, implying that these values could not be accurately analysed.

Nitric oxide production

Stimulation with IL-1 β and TNF- α (10 ng/mL) resulted in an approximately 10^3 increase in NO production of the isolated chondrocytes (range 18.8 – 75.4 μ mol/L). Stimulation of the cartilage explants enhanced NO production by 5 $\times 10^3$ fold (range 1.3 – 3.6 μ mol/mg). In the cartilage explants, a significant linear association was found for the effect of simvastatin on NO production ($p < 0.01$) and inhibition of up to 30% was found (Fig. 4). For isolated chondro-

cytes, a significant quadratic association between NO production and the different concentrations of simvastatin was found ($p < 0.01$). A post-test evaluation with Bonferroni correction in order to compare the concentrations separately showed that the lowest concentration of simvastatin (0.5 μ mol/L) significantly enhanced NO production by 17% ($p < 0.01$), whereas the highest concentration (50 μ mol/L) inhibited NO production by 43% ($p < 0.01$).

Discussion

Increasing clinical and experimental data indicate that statins, independent

of their lipid-lowering activity, possess anti-inflammatory and immunomodulatory effects (recently reviewed by Greenwood *et al.* (42)). These properties point to a possible role of statins in chronic inflammatory, immune-mediated diseases like RA (42). In an early double-blind, placebo-controlled clinical trial, a modest but significant decrease in disease activity, as well as a significant decrease in the markers of systemic inflammation (including ESR, CRP, fibrinogen and IL-6) was demonstrated in RA patients taking atorvastatin compared to the placebo group (43).

However, little is known about the effect of statins on chondrocytes. In the RA joint pro-inflammatory cytokines such as IL-1 and TNF- α are secreted and transform chondrocytes into cells that contribute to the breakdown of the cartilage matrix. The main purpose of our study was to investigate the potential effects of simvastatin, a widely used lipophilic statin, on the production of the pro-inflammatory mediators IL-6, IL-8 and NO by activated human isolated chondrocytes and cartilage explants. IL-6 and IL-8 production of unstimulated chondrocytes was very low, whereas upon activation with IL-1 β and TNF- α a clear enhancement in these cytokines production was observed. This study demonstrates that simvastatin is able to inhibit production of the pro-inflammatory cytokines IL-6 and IL-8 both by activated isolated chondrocytes and by cartilage explants.

This finding is important as both cytokines play a role in the degradation of RA affected joints (4, 5, 8, 10, 44). Our data are in line with the observations of Barsante *et al.*, who found that atorvastatin decreased the leukocyte influx and local pro-inflammatory cytokine production in joints in an animal model of RA. Moreover, these authors demonstrated that the administration of atorvastatin reduced cartilage and bone destruction in this model (45). In addition to cytokines, elevated nitrite levels are found in the synovial fluid of RA patients (46) and chondrocytes are known to be a major source of NO during inflammation of the joint (47). We found decreased NO production after

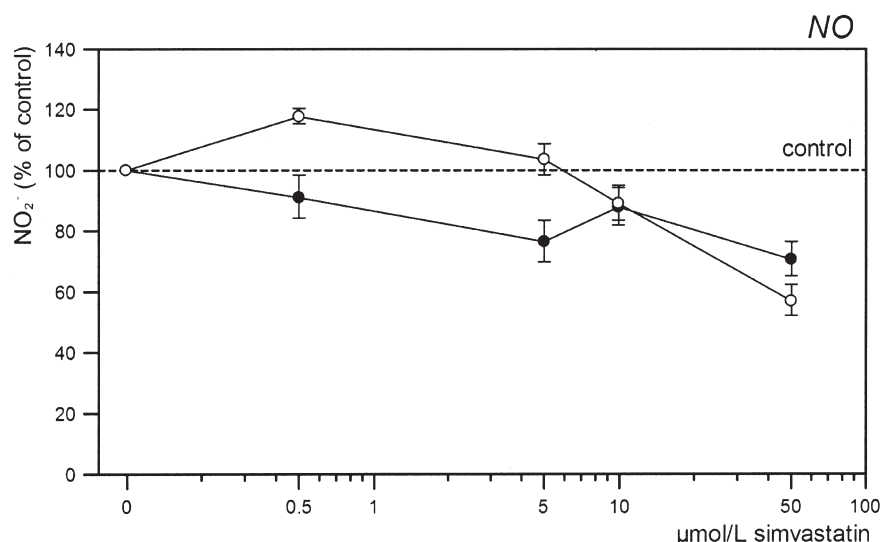


Fig. 4. Effect of different concentrations of simvastatin on NO production by isolated chondrocytes (open circles, $p < 0.01$) and cartilage explants (closed circles, $p < 0.01$) ($n = 10$) stimulated with IL-1 β and TNF- α (10 ng/mL). Results are expressed as the percentage of NO production (geometric mean \pm SEM).

the addition of higher concentrations of simvastatin in both cartilage explants and isolated chondrocytes. The initial increase in NO production after addition of the lowest concentration of simvastatin, which was detected only in the isolated chondrocyte cultures, remains unclear. Although dual effects of statins on angiogenesis have been described, with lower concentrations having proangiogenic effects but higher concentrations having angiostatic effects (48, 49), this has not been reported for NO production. Furthermore, an initial elevation in NO production was not found for the cartilage explants, which resemble most closely the *in vivo* situation, as it is generally accepted that the extracellular matrix is important for the chondrocyte metabolism (50).

Our data, together with a recent study performed by Lazzerini *et al.* which demonstrated an inhibitory effect of simvastatin on the chondrocytic production of matrix metalloproteinase-3 (40), provide evidence for cartilage-protective effects of simvastatin on chondrocytes without apparently inducing apoptosis.

The most likely explanation of the anti-inflammatory effects found in this study is that statins, by inhibiting the synthesis of L-mevalonic-acid, also reduce the synthesis of several important isoprenoid intermediates, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) in the cholesterol biosynthetic pathway (51).

FPP and GGPP play an important role in protein prenylation, such as the prenylation of the Ras and Rho families of small GTPases, which are implicated in multiple cell functions, including nuclear factor- κ (NF- κ B) signal transduction (52, 53). Moreover, recent data demonstrates that statins downregulate the activation of NF- κ B (54-56), which is a major factor for regulating the expression of diverse pro-inflammatory mediators (57). The observed reduction in NO, IL-6 and IL-8 production in our study might be due to an inhibition of the NF- κ B pathway and/or other signalling pathways.

In conclusion, this study demonstrates anti-inflammatory properties for simvastatin in chondrocytes *in vitro*, suggesting a potential cartilage-protective role for statins in arthritis.

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