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Simvastatin Decreases IL-6 and IL-8 Production in Epithelial Cells

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Abstract

Many cardiovascular studies have suggested that 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors (statins) have anti-inflammatory effects independent of cholesterol lowering. As a chronic inflammatory disease, periodontitis shares some mechanisms with atherosclerosis. Since oral epithelial cells participate importantly in periodontal inflammation, we measured simvastatin effects on interleukin-6 and interleukin-8 production by cultured human epithelial cell line (KB cells) in response to interleukin-1 α . Simvastatin decreased production, an effect reversed by adding mevalonate or geranylgeranyl pyrophosphate, but not farnesyl pyrophosphate. Simvastatin was found to reduce NF- κ B and AP-1 promoter activity in KB cells. Dominant-negative Rac1 severely inhibited interleukin-1 α -induced NF- κ B and AP-1 promoter activity. Our results may indicate an anti-inflammatory effect of simvastatin on human oral epithelial cells, apparently involving Rac1 GTPase inhibition.

Keywords

simvastatin (3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitor); pleiotropic effects; inflammatory cytokines; periodontitis; human oral epithelial cell

INTRODUCTION

Chronic inflammatory periodontal disease is highly prevalent, especially in late middle age, when cardiovascular disease is also common. In periodontitis, production of pro-inflammatory cytokines and tissue-degradative enzymes is initiated and advanced by oral bacterial infection, ultimately resulting in destruction of periodontal tissue. Interleukin (IL)-1 is of particular interest, since it is a multifunctional cytokine that mediates inflammatory tissue destruction (Stashenko *et al.*, 1991). Furthermore, oral epithelial cells participate actively in inflammatory responses (Suchett-Kaye *et al.*, 1998).

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Statins such as simvastatin are pharmacologic 3-hydroxy-3-methylglutaryl co-enzyme A (HMG-CoA) reductase inhibitors that inhibit synthesis of cholesterol, which is important in the development of atherosclerosis. Statin administration significantly reduces the risk of cardiovascular disease in susceptible patients, largely by lowering plasma lipid concentration. In addition, statins have been found to exert anti-inflammatory and immunomodulatory actions (Blanco-Colio *et al.*, 2003), as well as alleviating endothelial dysfunction (O'Driscoll *et al.*, 1997), and reducing thrombotic tendencies in the blood (Rauch *et al.*, 2000).

Distinct from cardiovascular disease, the actions of statins in oral tissues still are poorly understood. We therefore studied the anti-inflammatory effect of statins in oral tissues, since cardiovascular disease and periodontitis both represent chronic, progressive inflammatory states. Specifically, we investigated the effect of simvastatin on a human epithelial cell line KB, which has been extensively used as a model for the study of gingival epithelial cells (Tada *et al.*, 2003), stimulated by IL-1 α .

MATERIALS & METHODS

Cell Culture and Treatment

The human epithelial cell line KB was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). KB cells were cultured in minimum essential medium (MEM; Invitrogen, Groningen, The Netherlands) supplemented with 10% fetal bovine serum (FBS; Moregate Biotech, Bulimba, Australia). Cells were seeded in culture plates at a density of $7.5 \times 10^4/\text{cm}^2$, and cultured overnight in MEM supplemented with 10% FBS. Then the medium was replaced with MEM supplemented with 1% FBS, to minimize any serum-induced effect on the cells. After 24 hrs, KB cells were treated with 1.0 ng/mL of recombinant human IL-1 α (Pepro Tech Ec, London, UK) with or without exposure for 5 hr with specified concentrations of simvastatin (Merck, Rahway, NJ, USA), mevalonate (Sigma Chemical, St. Louis, MO, USA), geranylgeranyl pyrophosphate (GGPP; Sigma), and farnesyl pyrophosphate (FPP; Sigma).

Cytotoxicity

The cytotoxicity of simvastatin for KB cells was evaluated by the release of a representative cytoplasmic enzyme, lactate dehydrogenase (LDH), into culture supernatants. These were sampled and subjected to assay with a LDH detection kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions.

Cytokine Measurement

To investigate production of inflammatory cytokines by KB cells, we collected supernatant from each culture. Production of cytokines [IL-1 β , IL-6, IL-8, IL-10, IL-12p70, and tumor necrosis factor (TNF)- α] was evaluated by a cytometric bead array (BD Pharmingen, San Diego, CA, USA) that combines the principles of the "sandwich" immunoassay with the capability of flow cytometry for simultaneous measurements.

Plasmids

The following plasmids were used: a mammalian nuclear factor kappa B (NF- κ B)-responsive luciferase reporter vector (pNF- κ B-Luc; Stratagene, San Diego, CA, USA); a mammalian activating protein-1 (AP-1)-responsive luciferase reporter vector (pAP-1-Luc; Stratagene); myc-tagged N19RhoA, N17Rac1, and N17Cdc42 (Takemoto *et al.*, 2001); and a mammalian β -galactosidase expression vector (pCMV- β gal).

Transient Transfection and Reporter Gene Assays

Nearly confluent KB cells in wells of a 24-well plate were transfected with plasmids with the use of LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. These plasmids included: N19RhoA, N17Rac1, or N17Cdc42 (0.5 µg each); pNF-κB-Luc or pAP-1-Luc (0.5 µg); and pCMV-βgal (0.2 µg). Approximately 48 hrs after transfection, KB cells were treated as indicated above (Cell Culture and Treatment) for 5 hrs. Luciferase activity was normalized to β-galactosidase activity for each sample. Results are expressed as a ratio relative to control activity (n-fold induction).

Statistical Analysis

Data are expressed as the mean ± standard deviation. Statistical significance of differences between groups was analyzed by one-way analyses of variance (ANOVA) and Bonferroni/Dunn tests.

RESULTS

Effect of Simvastatin on IL-1α-induced Inflammatory Cytokines in KB Cells

To determine whether treatment with simvastatin regulated IL-1α-induced inflammatory cytokine production in KB cells, we calculated the concentration of inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α) by cytometric bead array. Comparison of 0 M and 10⁻⁸–10⁻⁶ M simvastatin showed dose-dependent down-regulation of IL-1α-induced IL-6 and IL-8 production by KB cells (Fig. 1A). In the present study, little production of IL-1β, IL-10, IL-12p70, or TNF-α was observed, even in the absence of simvastatin (data not shown).

Toxicity of Simvastatin to KB Cells

A 24-hour exposure to 10⁻⁸–10⁻⁶ M simvastatin had no appreciable effect on LDH release from KB cells (Fig. 1B). At a simvastatin concentration of 10⁻⁵ M, cytotoxicity was evident (36% cytotoxicity vs. 5% at 10⁻⁶ M).

Mevalonate Reverses Inhibition of IL-6 and IL-8 Production by Simvastatin

Because statins are inhibitors of HMG-CoA reductase, incubation of cells with these compounds results in depletion of mevalonate. To test whether simvastatin-mediated inhibition of IL-6 and IL-8 production was specific and dependent on mevalonate depletion, we incubated KB cells with simvastatin in the presence or absence of mevalonate. Supplementation with mevalonate blocked inhibition by simvastatin of IL-6 and IL-8 production by IL-1α-stimulated KB cells (Fig. 2A).

GGPP Reverses Inhibition of IL-6 and IL-8 Production by Simvastatin

FPP and GGPP are important for post-translational modification of small GTPases of the Ras/Rho family. Prenylation is a prerequisite for the activation of these proteins. Ras proteins are predominantly farnesylated, while Rho proteins are mainly geranylgeranylated. To test whether Ras or Rho proteins are involved in the simvastatin-dependent reduction of IL-6 and IL-8 expression, we incubated KB cells with simvastatin in the presence of an isoprenoid intermediate, either FPP or GGPP. GGPP almost completely blocked simvastatin-mediated inhibition of IL-6 and IL-8 production by IL-1α-stimulated KB cells (Fig. 2A). In contrast, FPP was ineffective.

Effects of Simvastatin on NF- κ B and AP-1 Promoter Activity

Since NF- κ B and AP-1 are essential for IL-1 α -stimulated IL-6 and IL-8 expression, we examined whether simvastatin down-regulated NF- κ B and AP-1 promoter activity in IL-1 α -stimulated KB cells, and observed suppression by simvastatin of both promoters (Fig. 3).

Inhibition of Rho Family GTPases as a Mechanism Suppressing IL-1 α -induced NF- κ B and AP-1 Promoter Activity

We examined the function of each Rho family GTPase (Rac1, Cdc42, or RhoA) with respect to IL-1 α -induced NF- κ B and AP-1 promoter activity in KB cells that had been transiently transfected with a dominant-negative form of each Rho family GTPase. Introduction of the dominant-negative form of Rac (N17Rac1) significantly reduced IL-1 α -induced NF- κ B and AP-1 promoter activity. The dominant-negative form of Cdc42 (N17Cdc42) and the dominant-negative form of RhoA (N19RhoA) also reduced IL-1 α -induced NF- κ B and AP-1 promoter activity, albeit less effectively (Fig. 4).

DISCUSSION

In this study, we provide evidence for the first time that simvastatin reduces IL-1 α -induced production of inflammatory cytokines such as IL-6 and IL-8 by human oral epithelial cells, as well as evidence suggesting that the inhibitory action of simvastatin could be mediated by the prevention of Rac prenylation. In turn, this interference would reduce NF- κ B and AP-1 promoter activation.

When KB cells were treated with simvastatin at or below 10^{-6} M, LDH release into culture medium was not significantly increased (Fig. 1C). This essentially ruled out cytotoxicity as a reason for reduced expression of IL-6 and IL-8. Instead, this effect resulted from specific inhibition of HMG-CoA reductase, since inhibition was reversed by the addition of mevalonate, the product of the HMG-CoA reductase reaction (Fig. 2A). This indicates that the mevalonate pathway was involved in the regulation of inflammatory cytokine expression. Next, we observed that reduction of IL-6 and IL-8 production by simvastatin in IL-1 α -stimulated KB cells was reversed completely by the addition of GGPP but not by FPP (Fig. 2A). Similar observations have been reported concerning pleiotropic effects of simvastatin in other types of cells, such as endothelial cells, cardiac myocytes, and macrophages (Takemoto and Liao, 2001). Small GTPases are known to mediate a variety of cellular events, including proliferation, migration, and responses to extracellular stimuli (Etienne-Manneville and Hall, 2002). Since FPP and GGPP are responsible for prenylation of Ras and Rho GTPases, respectively, our observation indicates that prenylation of Rho GTPases is involved in IL-1 α -stimulated inflammation in KB cells. Participation of Rho GTPases such as RhoA, Rac1, and Cdc42 in signal transduction cascades preceding extracellular stimuli to the cell nucleus has been described in previous reports. For example, several groups have reported that Rac can activate NF- κ B when transfected into cells (Jefferies and O'Neill, 2000; Jefferies *et al.*, 2001). Our results also showed that Rac1 is a key regulator of IL-1 α -induced NF- κ B and AP-1 activity in KB cells, and suggested that a decrease in geranylgeranylation of Rac1 was a key step in the inhibition of cytokine production by simvastatin. Since NF- κ B and AP-1 coordinate expression of a wide variety of genes that control immune responses and are involved in many inflammatory diseases (Georganas *et al.*, 2000), statins might have beneficial effects in periodontal disease as well as in cardiovascular disease.

Periodontitis has been found to be a risk marker for cardiovascular disease caused by atherosclerosis (Beck and Offenbacher, 2001; D'Aiuto *et al.*, 2004), while severe

periodontitis is more prevalent in patients with than in those without hyperlipidemia (Pohl *et al.*, 1995).

In addition to more direct mechanisms, administration of statins to cardiovascular patients conceivably might suppress atherosclerosis, in part, by decreasing circulating inflammatory mediators arising from oral tissue. In addition to the anti-inflammatory effect of simvastatin in KB cells, we have made some similar observations in human gingival fibroblasts (data not shown). Our findings of inhibition of IL-1 α -induced inflammatory cytokine expression *in vitro* may have broad clinical impact.

Pharmacokinetic analyses of simvastatin have shown approximate plasma concentrations of 10^{-9} to 10^{-7} M in volunteers taking clinical doses (Yang *et al.*, 2003). Generally, concentrations of medications in gingival crevicular fluid tend to be 10 or 100 times those in plasma; accordingly, clinical doses would be expected to exert anti-inflammatory effects upon oral tissues.

In conclusion, our results indicated that simvastatin has an anti-inflammatory effect on human oral epithelial cells involving mechanisms independent of cholesterol-lowering. Whether these actions also suppress other constituents of inflammation—such as matrix metalloproteinases, cellular adhesion molecules, and chemokines—remains to be investigated.

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Figure 1.

Dose-dependent effects of simvastatin on KB cells. **(A)** Cellular expression of IL-6 and IL-8 is shown in cultures after treatment with the indicated concentrations of simvastatin for 7 hrs, and/or IL-1 α (1 ng/mL) for the last 5 of these hrs. Data are expressed as means $\pm$ SD (n = 4). * P < 0.01 and ** P < 0.001 vs. control (treated with IL-1 α). **(B)** Evaluation of cytotoxicity from simvastatin according to release of LDH into medium was carried out after 24 hrs of culture. LC, low control (medium alone); HC, high control (1% Triton X100).

Figure 2.

Reversal of inhibitory effect of simvastatin on KB cells by co-treatment with downstream metabolites of HMG-CoA reductase. **(A)** Inhibitory effects of simvastatin (10^{-6} M) on KB cells were reversed by co-treatment with mevalonate (10^{-4} M) or GGPP (5×10^{-6} M), but not with FPP (5×10^{-6} M). Data are expressed as means $\pm$ SD ($n = 4$). * $P < 0.001$ vs. control (treated with IL-1 α). **(B)** Schematic representation of the mevalonate pathway. Statins block conversion of HMG-CoA to mevalonate. This leads to reduced synthesis of cholesterol and decreased prenylation of proteins such as small GTPases. Isopentenyl-PP, isopentenyl pyrophosphate; Geranyl-PP, geranyl pyrophosphate.

Figure 3.

Suppression of NF-κB and AP-1 activity in IL-1α-stimulated KB cells by simvastatin (10^{-6} M). KB cells were transiently co-transfected with pNF-κB-luc or pAP-1-luc, together with pCMV-κgal. The cells were analyzed 48 hrs later, with five-hour stimulation with IL-1α (1 ng/mL). All results were normalized for transfection efficiency using expression of β-galactosidase. Data are expressed as means $\pm$ SD ($n = 4$). * $P < 0.001$ vs. control (treated with IL-1α).

Figure 4.

Effects of Rho family GTPases on IL-1 α -mediated transactivation of NF- κ B and AP-1. **(A)** IL-1 α -induced NF- κ B and AP-1 promoter activity of the transient transfectants of N17Rac1 was greatly inhibited, and IL-1 α -induced NF- κ B and AP-1 promoter activities of transiently transfected cells (N17Cdc42 and N19RhoA) also were inhibited. Mock, transient transfectants of empty vector. Data represent means $\pm$ SD ($n = 4$). * $P < 0.01$ and ** $P < 0.001$ vs. control (mock with IL-1 α). **(B)** Model depicting contributions of Rho family GTPases to NF- κ B and AP-1 activation by IL-1. Rho, Rho family GTPases; GG-Rho, geranylgeranylated Rho family GTPases.