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European Journal of Pharmacology 410 (2000) 83–92

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Pravastatin down-regulates inflammatory mediators in human monocytes in vitro

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Received 26 June 2000; received in revised form 3 November 2000; accepted 10 November 2000

Abstract

There is experimental evidence that pravastatin, which is designed to inhibit the rate-limiting enzyme of cholesterol synthesis, can affect cell metabolism and proliferation. We therefore studied the effects of pravastatin on the generation of inflammatory mediators in non-stimulated and stimulated primary human monocytes in vitro. In our experimental model, pravastatin induced a dose-dependent inhibition of monocyte cholesterol synthesis (up to 67%), up-regulation of low density lipoprotein receptor mRNA (by about 35%) and reduction in intracellular cholesterol accumulation. In parallel, exposure of non-stimulated monocytes to various doses of pravastatin resulted in inhibition of monocyte chemoattractant protein-1 protein expression (up to 15-fold), reduction of tumour necrosis factor alpha (TNF-α) levels (up to 2.4-fold) and a total loss of metalloproteinase-9 activity in stimulated cells. Pravastatin at concentrations of 5, 100 and 500 μM caused an inhibition of TNF-α-induced cellular oxygen consumption from 2.4- to 5.5-fold. These data extend the findings of potential anti-inflammatory actions of statins and also suggest the possibility for pravastatin use in a broader spectrum of inflammatory situations.

Keywords: Pravastatin; Monocyte; Inflammation; Lipid metabolism; Pro-inflammatory molecule

1. Introduction

Clinical and experimental studies have shown that reduction in plasma cholesterol, particularly low density lipoprotein (LDL) cholesterol, by treatment with statins can induce regression of vascular atherosclerosis and reduction of cardiovascular-related morbidity (Desager and Horsmans, 1996; Corsini et al., 1998). Statins are competitive hydroxy-3-metyl glutaryl coenzyme A (HMG-CoA) reductase inhibitors that inhibit the synthesis of cholesterol from mevalonic acid by suppressing the conversion of HMG-CoA (Corsini et al., 1999). They also enhance the expression of LDL receptors, increase incorporation of LDL and reduce serum levels of cholesterol (Ma et al., 1986). Various studies have shown that specific statins can inhibit cholesterol accumulation in macrophages thereby reducing their activity (Weber et al., 1997; Bocan et al., 1994), can inhibit platelet aggregation leading to decreased platelet thrombus formation (Faggiotto and Paoletti, 1999; Carvalho et al., 1974) and influence vascular smooth muscle cell migration and proliferation (Weissberg et al., 1996). These diverse effects of statins on the biological processes contributing to atherosclerotic plaque development suggest that the benefit from treatment with statins cannot be only explained by a reduction in plasma total or LDL cholesterol levels (Wheeler, 1998; Kendall and Toescu, 1999).

Current knowledge suggest that atherosclerosis is an inflammatory process and that the initiation and progression of the lesions involves cellular migration, proliferation and production of a significant number of pro-inflammatory factors such as chemokines, cytokines, reactive oxygen species, and growth factors by all of the cell types within the artery wall (Plutzky, 1999). Monocytes are among the first cells to accumulate in early atherosclerotic lesions. Macrophage infiltration into the atherosclerotic lesion has been linked to activation of the transcription factor nuclear factor-κB (NF-κB) and synthesis of numerous NF-κB-dependent gene products including free radicals, cytokines, chemokines and metalloproteinases (Brand et al., 1996; Thurberg and Collins, 1998). Recent reports have shown that statins such as lovastatin and atorvastatin through the inhibition of NF-κB activity can reduce pro-

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00870-0

inflammatory cytokine and chemokine expression in smooth muscle cells and mononuclear cells and therefore play a role in the stabilisation of the lesion (Ortego et al., 1999).

Pravastatin is a hydrophilic, anionic drug and selective inhibitor of HMG-CoA reductase (Pan, 1991). The drug has been shown to reduce plasma LDL cholesterol concentrations in experimental animals as well as in humans (Koga et al., 1990; Parker et al., 1990). The inhibition by pravastatin of macrophage cholesterol synthesis, both in vivo and in vitro, was shown to be associated with enhanced cellular uptake of native LDL, but not the acetylated LDL or oxidized LDL (Keidar et al., 1994). Pravastatin was found to reduce macrophage content, calcification and new vessel formation in atherosclerotic plaques in cholesterol-fed rabbits and in atherosclerotic monkeys (Shiomi et al., 1995; Williams et al., 1998), suggesting that this drug can reduce inflammation and enhance plaque stability. It was also shown that pravastatin activates endothelial nitric oxide synthase (Kaesemeyer et al., 1999) and inhibits platelet aggregation and thrombus formation in an injured artery (Lacoste and Lam, 1996), in addition to its lipid-lowering effect. Results from the Long-term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group, showed that patients with a broad range of initial cholesterol levels (4.0–7.0 mM) had a reduced morbidity and mortality on treatment (The Lipid Study Group, 1998), thus high-lightning mechanisms of pravastatin additional to its lipid-lowering properties.

The aim of the present study was to correlate the effects of pravastatin on cellular lipid metabolism and generation of pro-inflammatory molecular species in non-stimulated and stimulated human monocytes in culture.

2. Materials and methods

Pravastatin sodium salt was provided by Bristol-Mayer Squibb (Bromma, Sweden). Concentrations of pravastatin were based on previous in vitro studies on human cell cultures (Kreuzer et al., 1991; Sakai et al., 1997; Kanno et al., 1999; Dunzendorf et al., 1997; Keidar et al., 1994). [1(2)-^{14}C] acetic acid and sodium salt (57 mCi mM^{-1}) were purchased from Amersham (Sweden). Tumor necrosis factor alpha (TNF-α), 4 beta-phorbol 12 beta-myristate 13 alpha-acetate (PMA), Oil Red O were obtained from Sigma. Human LDL was isolated by sequential ultracentrifugation as previously described (Janciauskiene et al., 1997). A narrow density range (1.034–1.054 kg l^{-1}) was used to prepare LDL.

2.1. Isolation and culture of monocytes

Human monocytes were isolated from buffy coats obtained from pooled plasma of different donors by the Ficoll-Hypaque procedure. Cell quality was analysed by autocounter (AC 900^{EO}, Swelab, Sweden). Monocytes were plated at a density of 4×10^6 cells ml^{-1} into plastic plates or dishes. After removal of non-adhering cells monocytes were cultured in RPMI 1640 (Gibco, Life Technologies, Paisley, Scotland) supplemented with 2 mM *N*-acetyl-L-alanyl-L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 1% nonessential amino acid, 2% sodium pyruvate and 20 mM HEPES (Fluka, Chemie) without serum at 37°C in 5% CO_2. Monocytes were activated with TNF-α (1 ng ml^{-1}) or PMA (1 μg ml^{-1}), for 45 min prior to experiments. Cells stimulated with PMA were washed with phosphate-buffered saline (PBS) and new medium containing no stimulus was added. All experiments were performed within 24 h using non-activated and activated monocytes alone or exposed to various concentrations of pravastatin. In some of the experiments, native LDL (80–100 μg ml^{-1}) was used.

2.2. Cholesterol synthesis assay

Cellular synthesis of cholesterol was estimated by measuring ^{14}C-acetate incorporation into sterols from cell extracts as described (Shah and Johnson, 1988). Pravastatin (5–500 μM) and LDL (100 μg ml^{-1}) separately or together were added to the monocyte cultures containing ^{14}C-acetate (1 μCi ml^{-1} media in 1.8 mM sodium acetate) and incubated for 24 h. After aspiration of the medium, cells were washed once with PBS and harvested in 1 ml cold medium containing 2 mM sodium acetate. Cells were centrifuged at 500 rpm for 5 min, resuspended in 1 ml 20 mM Tris buffer, pH 7.5 (cold) and 9 ml acetone:ethanol (1:1), precipitated on ice for 15 min and centrifuged (1000 rpm, 5 min). The supernatant (5 ml aliquots) was collected and 100 μl of cholesterol carrier (1 mg ml^{-1} cholesterol in acetone) and 2 ml of digitonin (5 mg ml^{-1} in 50% ethanol) were added and precipitated over night. Precipitates were washed twice with acetone:ether (1:1) and once with ether, dissolved in methanol and counted in a β-counter (Liquid Scintillation System TRI-CARB 300C).

2.3. RNA isolation

Total RNA from monocytes was isolated as outlined by Davis et al. (1986). Briefly, cold GT buffer (4 M guanidine thiocyanate; 3 M sodium acetate, pH 6 and 7% β-mercaptoethanol) was added directly to the cells in culture dishes. Sarkosyl was added to 2% and the lysate was layered onto a 4 ml 5.7 M CsCl cushion and centrifuged at 100,000 × *g* for 16 h. The pellet was washed with ethanol (95%), suspended in diethylpyrocarbonate-treated distilled water (dH_2O), precipitated in 95% ethanol at pH 5.0 and stored at −20°C.

2.4. Reverse-transcription polymerase chain reaction

LDL receptor mRNA was quantified by reverse-transcription polymerase chain reaction (RT-PCR) as de-

scribed previously (Janciauskiene and Lindgren, 1999). The oligonucleotides of 5′primer (5′-CAATGTCTCAC-CAAGCTCTG-3′) and 3′primer (5′-TCTGTCTCGAGGG-GTAGCTG-3′) were purchased from Pharmacia Biotech (Uppsala, Sweden). The amplification was performed with a Perkin Elmer Cetus thermocycler using the following cycle profile: denaturation at 95°C for 1 min, primer annealing and extension at 60°C for 1 min. The initial denaturation step was prolonged to 3 min, and after 32 cycles the reaction mixture was incubated at 72°C for 7 min and then cooled to 4°C.

2.5. Quantitative analysis of messenger RNA

Each PCR product (20 μl) was electrophoresed along with a DNA molecular weight marker (Pharmacia Biotech) in a 4% agarose-sieving gel (2:3 [wt/wt] NuSieve Agarose and 1:3 [wt/wt] SeaKem LE Agarose (In Vitro AB, Stockholm, Sweden) in TAE (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) running buffer at 90V for 3 to 4 h at +4°C. The gel was scanned in a FluorImager SI (Molecular Dynamics, Sunnyvale, CA) using an excitation wavelength of 488 nm (argon laser). Images were analyzed using ImageQuant software (Molecular Dynamics), and the signal intensity was calculated according to the vendor's instructions, giving semi-quantitative data on levels of mRNA for LDL receptor. The amount of LDL receptor PCR fragment was normalized to that of the internal standard. Values are expressed as percent of levels of LDL receptor mRNA in control monocytes.

2.6. Red oil staining

Monocytes were grown on cover slips in the presence of pravastatin (0–100 μM) and/or LDL (100 μg ml^{-1}) for 24 h. At the end of the incubation period, the cells were washed with PBS and fixed with 4% PBS-buffered formaldehyde for 15 min. In the next step, cells were rinsed with water, dipped for a few seconds in 60% isopropanol, stained in the Oil Red O for 15 min and rinsed again in 60% isopropanol to remove excess of stain. Cell nuclei were stained for a few seconds in the haematoxylin solution, washed with water and mounted with commercially available Mounting Medium (DAKO). Samples were analysed by microscope (Olympus B × 60) using the PC program Olympus MicroImage. Images were taken by digital camera (Sony, DKC-5000) at a magnification of 40 ×.

2.7. Interleukin-6, TNF-α, monocyte chemoattractant protein-1 and metalloproteinase-9 enzyme linked immunosorbent assays

Cell culture supernatants from monocytes treated with pravastatin (up to 1000 μM) for 24 h were analysed to determine human interleukin-6 (IL-6), TNF-α, metalloproteinase-9 (MMP-9), and monocyte chemoattractant protein-1 (MCP-1). A quantitative sandwich enzyme immunoassay (Quantikine™, R&D Systems, Minneapolis, USA) technique sensitive to pg ml^{-1} assay levels was used according to manufacturer's instructions.

2.8. Measurement of oxygen consumption

Freshly isolated monocytes, unstimulated or stimulated with TNF-α (1 ng ml^{-1}), were simultaneously treated with pravastatin (5, 100 and 500 μM). Oxygen consumption was measured polarographically with a Clark-type oxygen electrode (CB1-D3, Techtum Lab.) in a water-jacketed chamber connected to a circulating bath, at 37°C. The instrument was calibrated prior to each assay according to the manual instructions. Reaction mixtures containing 2×10^5 cells ml^{-1} in air saturated 0.5 ml monocyte culture medium were used.

2.9. [^{3}H] Thymidine incorporation assay

Cells were incubated with pravastatin (up to 2000 μM) for 20 h. [^{3}H] Thymidine (Amersham) was then added to the cells (0.2 μCi ml^{-1}) for a further 4-h incubation at 37°C. After the medium was aspirated, the cells were washed twice with 0.5 M NaCl and incubated for 5 min with 5% trichloroacetic acid. Cells were then washed with water, dissolved in 1 ml 0.5 M NaOH, neutralised with 200 μl HCl and radioactivity determined in a β-counter (Packard 300CD liquid scintillation spectrometer; Packard Instruments).

2.10. Statistical analysis

The differences in the means of experimental results were analysed for their statistical significance with independent-samples two-sided t test and/or one-way analysis of variance (ANOVA) combined with a multiple comparisons procedure (Scheffé multiple range test) with the overall significance level of $\alpha = 0.05$. Statistical Package for Social Sciences (SPSS for Windows, release 6.0) was used for the calculations (Norusis, 1993).

3. Results

3.1. Cholesterol synthesis and LDL receptor mRNA levels

The in vitro effect of pravastatin on monocyte cholesterol metabolism was assayed by measuring [^{14}C]-acetate incorporation into labelled, un-esterified cholesterol. Our data indicate that pravastatin added for 24 h to monocytes resulted in a dose-dependent inhibition of cholesterol synthesis (Fig. 1). A decrease of 43–67% in cholesterol

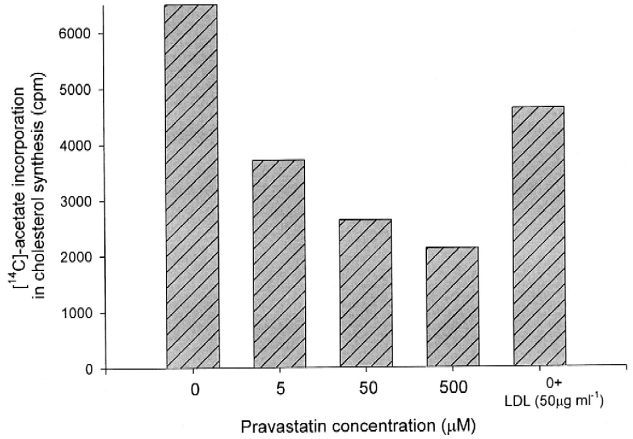

Fig. 1. The effect of pravastatin on cholesterol synthesis by monocytes. Monocytes were incubated alone, with native LDL (50 μg ml^{-1}) or with increasing concentrations of pravastatin for 24 h. Data show representative profiles of the pravastatin effects on monocyte cholesterol synthesis. Each bar represents the averages from two independent experiments.

synthesis was found in cells treated with 5 and 500 μM of pravastatin, respectively, whereas LDL (50 μg ml^{-1}) caused a 29% decrease in cholesterol synthesis under the same experimental conditions. This suppressive effect of pravastatin on new cholesterol synthesis coincided with enhanced LDL receptor mRNA synthesis of as much as $35 \pm 4\%$ ($P < 0.05$) in monocytes stimulated with concentrations of pravastatin from 5 to 100 μM. Whereas, a concentration of 500 μM pravastatin did not additionally increase cellular LDL receptor mRNA levels, suggesting a plateau is obtained at 100 μM (Fig. 2). Thymidine incorporation showed no cytotoxicity in the studied range of pravastatin.

3.2. Oil red O staining

Accumulation of lipids in monocytes treated with pravastatin and LDL alone or together was assessed qualitatively by Oil Red O staining. Monocytes treated with LDL (50 μg ml^{-1}) and pravastatin (100 μM) simultaneously showed much lower amounts of lipid droplets compared to monocytes treated with LDL alone, while control monocytes and those treated with pravastatin showed no lipid droplets (Fig. 3). The qualitative difference was semi-quantitatively expressed by counting the number of cells containing lipid droplets in a view-field of approximately 20 cells; control 0/18, (0%), pravastain 0/19, (0%), LDL 15/21, (71%), LDL and pravastatin 8/20 (40%). These data indicate that pravastatin inhibits lipid accumulation in human monocyte culture.

3.3. Pro-inflammatory cytokine release

We examined the levels of the pro-inflammatory cytokines IL-6 and TNF-α in medium from monocytes cultured with various amounts of pravastatin. Unstimulated cells were negative controls. Pravastatin had no effect on IL-6 levels, whereas decreases of about 27% in TNF-α levels were observed at concentrations ranging from 5 to 500 μM (Table 1). Cytokine production was also assayed on cells stimulated with PMA (1 μg ml^{-1}) for 45 min. Cytokines tested showed a large increase in response to PMA (Table 1). Addition of pravastatin resulted in a significant decrease in TNF-α levels (by about 2.4-fold, $P < 0.01$) relative to those observed in cells stimulated only with PMA, but had no effect on PMA induced IL-6 levels.

3.4. Monocyte chemoattractant protein-1 levels

Increased expression of MCP-1 has been indicated as a mechanism underlying monocyte infiltration during acute inflammatory processes (Rollins, 1996). To determine the effects of pravastatin on MCP-1 protein expression, monocytes were incubated for 24 h without and with addition of various concentrations of drug. As shown in Fig. 4, pravastatin inhibited MCP-1 expression up to 15.4-fold ($P < 0.01$) in unstimulated cells. These inhibitory effects on MCP-1 expression in monocytes were concentration dependent over a range from 5 to 500 μM of pravastatin.

3.5. Matrix metalloproteinase-9 levels

Cytokines IL-1 and TNF-α have been reported to induce production of MMP-9 in human monocytes (Saren et al., 1996). Since monocytes exposed to pravastatin for 24 h decrease pro-inflammatory cytokine release, we determined secretion of MMP-9 under these experimental con-

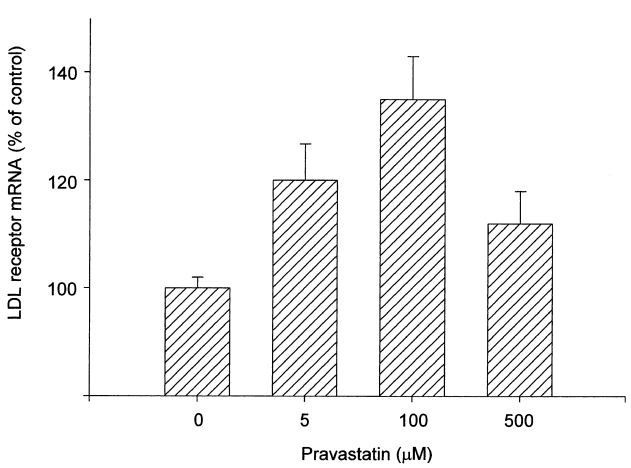

Fig. 2. The effects of pravastatin on the LDL receptor mRNA expression by monocytes. Each bar represents the mean ± SEM of three separate experiments. One-way ANOVA and the Scheffe multiple-comparison test ($\alpha = 0.05$) show that pravastatin at concentrations of 5 and 100 μM, but not at a concentration of 500 μM, significantly stimulate the LDL receptor mRNA expression.

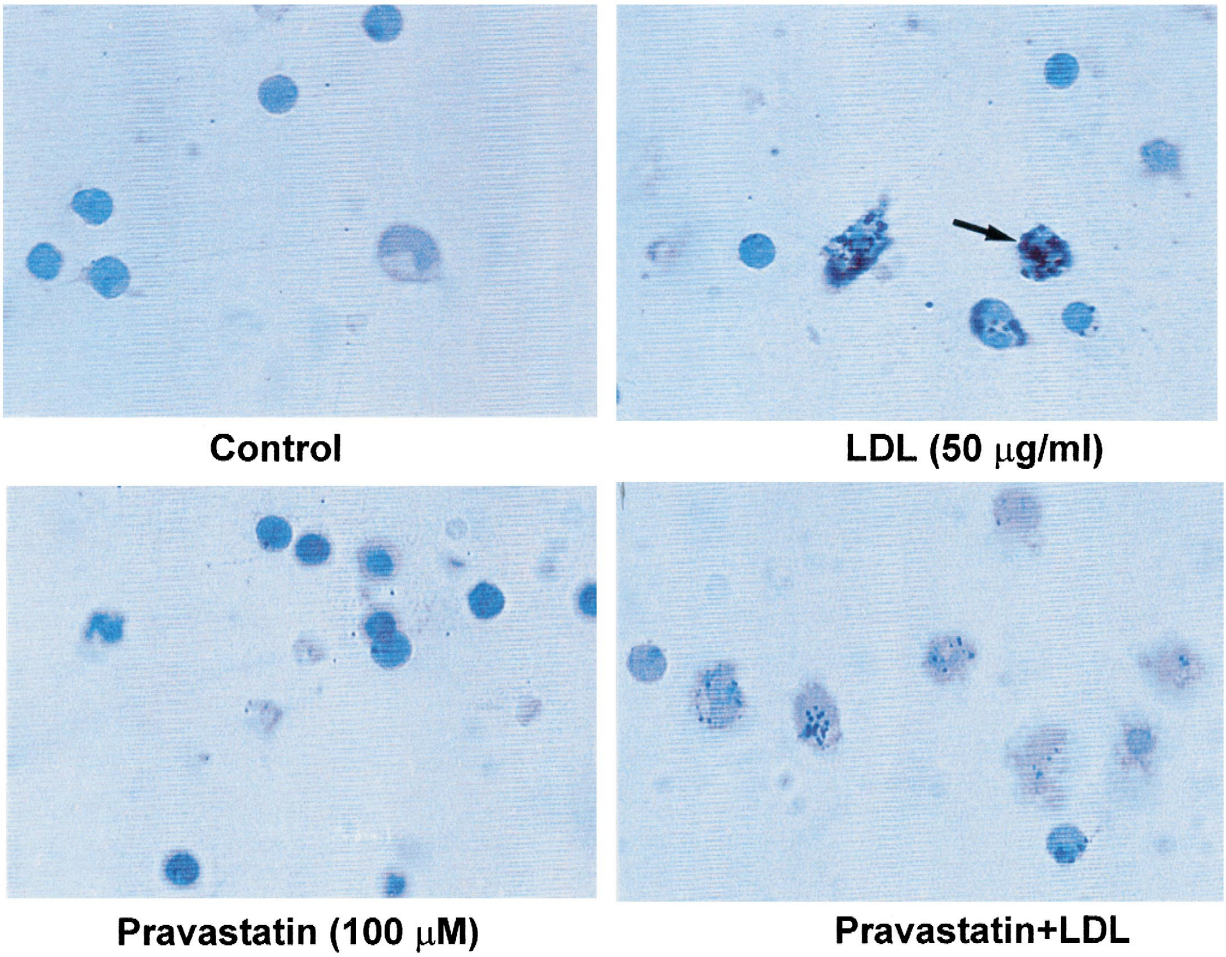

Fig. 3. The accumulation of lipids in monocytes visualised by staining with Oil Red O. Monocytes were incubated with LDL (50 μg ml^{-1}) and pravastatin (100 μM) separately or together for 24 h. The cells were fixed and stained with Oil Red (400 ×). The arrow indicates Oil Red O stained vacuoles.

ditions. As shown in Fig. 5, monocytes secreted detectable quantities of MMP-9 (0.13 ± 0.03 ng ml^{-1}), and this basal production was increased (by about 3.6-fold, $P < 0.05$) when cells were treated with TNF-α. Treatment of unstimulated cells with pravastatin resulted in a decrease in MMP-9 activity below the level of detectability. Exposing cells to a combination of TNF-α and various concentra-

Table 1
Cytokines produced by non-stimulated or PMA (1 μg ml^{-1}) stimulated monocytes, alone and with addition of various concentrations of pravastatin for 24 h. Mean and SD of two experiments

	IL-6 (pg ml^{-1})		TNF-α (pg ml^{-1})	
	Mean	SD	Mean	SD
Unstimulated cells				
Control	0.89	±0.001	2.02	±0.3
Pravastatin 5 μM	1.26	±0.1	1.91	±0.16
Pravastatin 100 μM	1.03	±0.002	1.47	±0.15
Pravastatin 500 μM	1.09	±0.09	1.47	±0.46
Stimulated cells				
Control	2.56	±0.19	67.3	±11.8
Pravastatin 5 μM	2.18	±0.43	64.9	±1.08
Pravastatin 100 μM	2.15	±0.48	55.4	±3.05
Pravastatin 500 μM	2.05	±3.19	23.1	±2.07

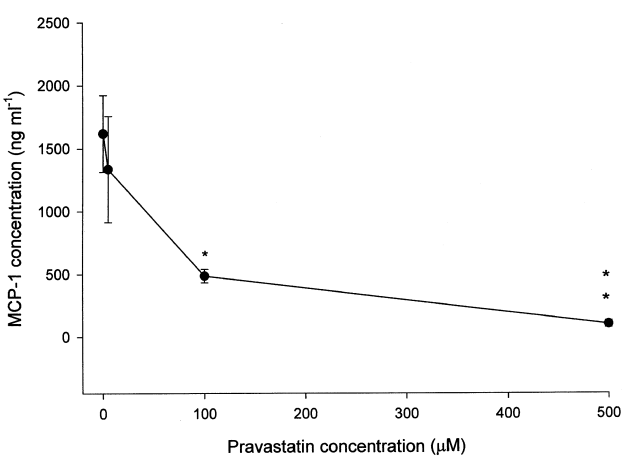

Fig. 4. MCP-1 protein expression by monocytes incubated with various concentrations of pravastatin for 24 h. MCP-1 protein levels were determined in monocyte culture medium as described under Methods. Each point represents the mean of two experiments ± SD.

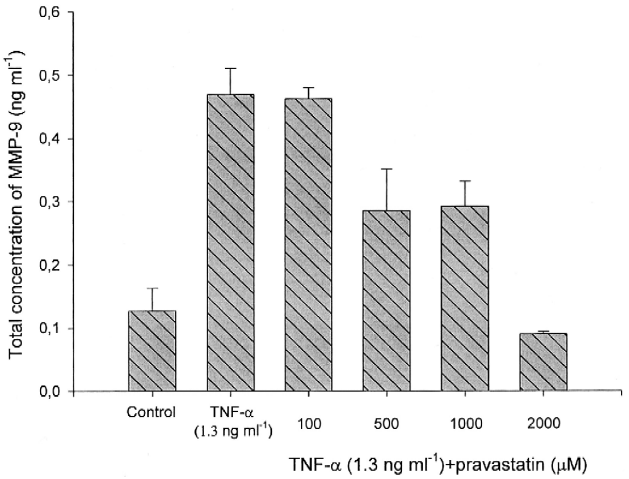

Fig. 5. MMP-9 release from the monocytes non-stimulated or stimulated for 24 h with TNF-α (1.3 ng ml^{-1}) alone or in the presence of various concentrations of pravastatin. Each bar represents the mean ± SD of four repeats. One-way ANOVA and the Scheffe multiple-comparison test ($\alpha = 0.05$) show that TNF-α significantly increases total MMP-9 levels. Pravastatin at concentrations of 500 and 1000 μM suppressed and at a concentration of 2000 μM totally abolished the stimulatory effect of TNF-α on MMP-9 release.

tions of pravastatin significantly decreased MMP-9 levels below those achieved using TNF-α alone (Fig. 5). Pravastatin at a concentration of 2000 μM totally abolished TNF-α-induced MMP-9 generation.

3.6. Cellular oxygen consumption

Cellular oxygen consumption was used to assess the effect of pravastatin on mitochondrial respiration in unstimulated and TNF-α-stimulated (1 ng ml^{-1}) monocytes. Results are presented from one representative experiment of three. As shown in Fig. 6, treatment of monocytes with TNF-α resulted in increased oxygen consumption by 2.2-fold compared to non-stimulated cells. Pravastatin at concentrations of 5 and 100 μM added simultaneously with TNF-α, abolished TNF-α effects on mitochondria respiration and the measured oxygen consumption levels were similar to those observed in control cells. Pravastatin used at a concentration of 500 μM reduced cellular oxygen consumption levels by 2.5-fold relative to controls (non-stimulated cells). Pravastatin added on non-stimulated cells did not affect oxygen consumption compared to controls (data not shown).

4. Discussion

The migration of monocytes to incipient atherogenic lesions and their activation and maturation to macrophages are strongly implicated in atherogenesis (Ross,1999). Monocyte-derived macrophages secrete cytokines, chemokines, growth-regulating molecules, metalloproteinases and other hydrolytic enzymes (Moreau et al., 1999), but the primary signals eliciting circulating monocyte migration and activation remain poorly understood. HMG-CoA reductase limits the rate of synthesis not only of cholesterol, but also of a range of other molecules involved in such functions as cellular respiration and cell–cell recognition. Therefore, it is reasonable to believe that statins may influence cellular processes other than cholesterol synthesis (Corsini et al., 1999). Statins have been shown to modify the atherogenic process in a number of ways, including reduction of the susceptibility of LDL to oxidation, inhibition of cholesterol synthesis in macrophages, correction of endothelial dysfunction and inhibition of cell proliferation (Aviram et al., 1992; Keidar et al., 1994; Hernandez-Perera et al., 1998; Corsini et al., 1993). These multiple biological activities of statins suggest that these drugs can be used to control multiple pathways of the inflammatory process.

We show here, consistent with work of other investigators, an inhibitory effect of pravastatin on cholesterol synthesis and a stimulatory effect on LDL receptor mRNA levels in monocytes. By using intracellular lipid staining with oil red O, we also demonstrate that monocytes treated with LDL and pravastatin simultaneously show lower amounts of lipid droplets as compared to monocytes treated with LDL alone. These data together indicate that pravastatin inhibits lipid accumulation in human monocyte culture. The ability of monocytes to respond to pravastatin's inhibitory effects on endogenous cholesterol synthesis has been proposed as being important in reducing macrophage

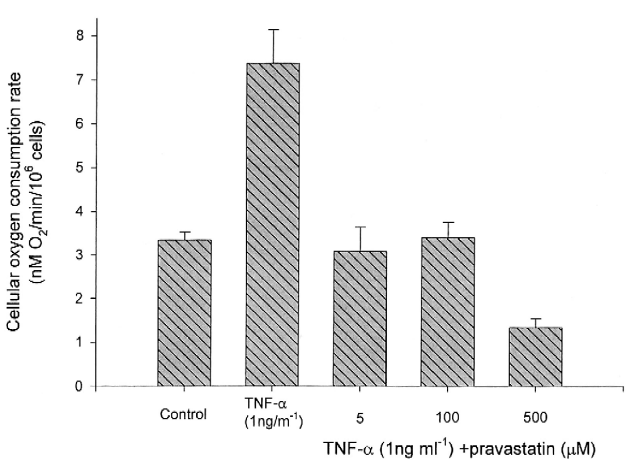

Fig. 6. Inhibition of TNF-α induced mitochondrial oxygen consumption by pravastatin in primary human monocytes. Cellular oxygen consumption was measured polarographically following stimulation of the cells with TNF-α alone or in the presence of indicated concentrations of pravastatin as described under Methods. Data from one representative experiment of three.

activation and foam-cell formation (Keidar et al., 1994). Possible mechanisms for pravastatin, other than as an inhibitor of HMG-CoA reductase, have been discussed, to our knowledge, only in one study regarding respiratory burst in neutrophils (Kanno et al., 1999). However, studies on non-lipid lowering effect of pravastatin in monocytes have shown that these effects could be blocked with mevalonate (Corsini et al., 1996; Sakai et al., 1997; Kreuzer et al., 1991; Dunzendorf et al., 1997). Therefore, it is likely that the effects seen are related to products of the mevalonate pathway, although not necessarily cholesterol.

In parallel experiments over the same concentration range, we examined non-lipid lowering effects. The experiments were designed to investigate the ability of pravastatin to reduce the inflammatory response by inhibiting monocyte pro-inflammatory activation. Recruitment of circulating monocytes into inflammatory sites is important in amplifying the local immune response, and when activated, monocytes up-regulates its expression of pro-inflammatory cytokines such as TNF-α and IL-6 (Delpedro et al., 1998). Pro-inflammatory cytokines dramatically increases the ability of monocytes to adhere to blood vessels by inducing adhesion molecules and their ligands on endothelial cells and leukocytes. Migration of cells into an inflammatory site is further stimulated by pro-inflammatory cytokine-induced production of chemotactic molecules (Roebuck, 1999). Chemokines are known to be involved in the pathogenesis of various inflammatory diseases through the promotion of directed migration of inflammatory cells (Mahalingam and Karupiah, 1999). MCP-1 is one such chemokine, and its increased expression has been associated with leukocyte recruitment in inflammatory conditions, including, initiation and development of atherosclerotic plaques, inflammatory bowel disease, chronic obstructive pulmonary disease and rheumatoid arthritis (Czaja et al., 1994; MacDermott et al., 1998; De Boer et al., 2000; Tucci et al., 1997). Expression and release of this chemokine was shown to be regulated by the generation of oxidative stress-related molecules (Marra et al., 1999).

We used non-stimulated and stimulated monocytes to examine the effects of pravastatin on cytokine production and found that even at the highest concentration used (500 μM), pravastatin had no effect on IL-6 production in both non-stimulated and stimulated cells, but in contrast, significantly inhibited PMA-induced TNF-α production. The origin of this large inhibitory effect of pravastatin on TNF-α, but not on IL-6 levels, is not known, but it may be linked to the degree of activation of monocytes and to the fact that synthesis and release of these two cytokines is known to be under independent control. Previously, it was shown that simvastatin had no effect on the production of pro-inflammatory cytokines IL-1, IL-6 and IL-8 in human monocytes (Fukuo et al., 1995). On the other hand, it has recently been found that another statin, lovastatin, inhibits cytokine (TNF-α, IL-1β and IL-6) production in rat primary astrocytes, microglia and macrophages (Pahan et al., 1997). These data together indicate that production of various pro-inflammatory cytokines is most likely mediated by several mechanisms having different sensitivity to the statins. We found that pravastatin is a highly potent suppresser of MCP-1 protein expression in monocyte cultures. Although the findings with a higher concentration of pravastatin may not be directly extrapolated to the in vivo situation, the inhibition of MCP-1 is likely to be an activity of pravastatin, since inhibitory effects were measurable at concentrations as low as 5 μM. Since expression of chemokines is largely dependent on the effects of oxidative stress, the observed inhibitory effect of pravastatin on MCP-1 levels suggests that this drug may also inhibit the production of other oxidative stress-related molecular species including cytokines.

MMP-9 (92-kD gelatinase/gelatinase B) is a zinc-dependent endopeptidase that degrades extracellular matrix proteins and is involved in inflammation, tissue remodelling, wound healing and processing of cytokines (Welgus et al., 1990). It is secreted by various cells, including monocytes, as a zymogen (pro-MMP) that is activated by a variety of proteinases. It was shown that increased production of MMP-9 by macrophages has a specific role for the complications of atherosclerosis, because of its contribution to matrix destruction which leads to plaque rupture (George, 1998). The inhibitory effects of statins, such as fluvastatin and simvastatin, on MMP-9 secretion were observed in cultured human monocytes, showing that these drugs can stabilise atherosclerotic plaques in addition to lowering lipids (Bellosta et al., 1998). Moreover, metalloproteinase levels have also been showed to be elevated in inflammatory bowel disease, rheumatoid arthritis, and chronic obstructive pulmonary disease (Baugh et al., 1999; Ahrens et al., 1996; Segura-Valdez et al., 2000), suggesting them to be of pathophysiological importance in these diseases. In this study, we have demonstrated that pravastatin reduces TNF-α-stimulated MMP-9 levels in monocyte cultures in a concentration-dependent manner, further expanding the range of anti-inflammatory activities of the statin class of drugs. Our findings that pravastatin inhibits release of TNF-α and generation of MMP-9 by activated monocytes confirm the potential of pravastatin to reduce inflammatory reactions and to prevent extracellular matrix from degradation induced by MMPs.

The inflammatory response involves the release of a number of factors including reactive oxygen species. The majority of reactive oxygen species produced as part of the inflammatory response comes from various phagocytic cells that, when activated, are capable of producing large amounts of superoxide and hydrogen peroxide (Bellavite, 1988; Fridovich, 1999). Activated phagocytic cells exhibit a marked increase in oxygen consumption in generating superoxide (Morel et al., 1991). Mitochondria produce reactive oxygen species as by-products of molecular oxygen consumption in the electron transport chain (Wei et

al., 1998). Since cytokines, especially TNF-α, are known to induce a respiratory burst, we stimulated monocytes with TNF-α alone and in the presence of pravastatin, and cellular respiration rates were determined polarographically, using a Clark-type oxygen electrode. We found that the pro-inflammatory cytokine TNF-α enhanced oxygen consumption in monocytes while an addition of increasing amounts of pravastatin rapidly and completely blocked the TNF-α-induced enhancement of oxygen consumption. Based on theses data, we suggest that pravastatin can inhibit the oxidative burst in activated monocytes and therefore prevent reactive oxygen species-generated tissue injury during the inflammatory response. Activated monocytes are known to promote the oxidation of LDL and the uptake of modified LDL resulting in the formation of foam cells (Kaplan and Aviram, 1999). The finding that pravastatin can prevent the oxidative burst in activated monocytes suggests that it can also inhibit the generation of oxidised LDL.

In this study, we demonstrate that pravastatin not only lowers cholesterol synthesis and lipid accumulation in human monocyte cultures, but also potently inhibits the generation of pro-inflammatory modulators and oxygen consumption in activated monocytes. Although much caution should be exercised in extrapolating such results to the clinical setting, these data extend the findings of potential anti-inflammatory actions of statins. They also suggest the possibility that pravastatin might be used in a broader spectrum of inflammatory situations where activated monocytes are strongly implicated, e.g. inflammatory bowel disease, rheumatoid arthritis or chronic obstructive pulmonary disease.

Acknowledgements

The authors are grateful to Birgitta Tenngart and Camilla Orbjörn for expert technical assistance with experiments. This work has been supported by grants from the Swedish Medical Research Council (13008, 1614) and the faculty of Medicine Lund University.

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