Inhibitory effect of pitavastatin (NK-104) on the C-reactive-protein-induced interleukin-8 production in human aortic endothelial cells

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ABSTRACT

Recent data have indicated that CRP (C-reactive protein) plays a role in atherosclerosis, in addition to being a marker for inflammatory diseases. IL-8 (interleukin-8), a CXC chemokine, is present in human coronary atheroma and promotes monocyte–endothelial cell adhesion. In the present study, we examined the effect of pitavastatin (NK-104), a synthetic statin (3-hydroxy-3-methylglutaryl CoA reductase inhibitor), on IL-8 production induced by CRP in human AoEC (aortic endothelial cells). We also investigated whether CRP can induce IL-8 production and if the activation of signalling pathways are functionally related. The concentrations of IL-8 in the media after stimulation with CRP were measured by ELISA, and the expression of IL-8 mRNA was assessed by Northern blot. The phosphorylation of MAPKs (mitogen-activated protein kinases) was determined by Western blot. The production of IL-8 induced by CRP (10 µg/ml) was enhanced significantly and was inhibited by pitavastatin. The expression of IL-8 mRNA was increased in a dose-dependent manner after stimulation with CRP (1–100 µg/ml), whereas expression of IL-8 mRNA induced by CRP (50 µg/ml) was significantly diminished by 5 µM pitavastatin. Furthermore, specific MAPK inhibitors (PD98059, SB203580 and SP600125) inhibited the expression of IL-8 mRNA induced by CRP (50 µg/ml). The phosphorylation of all three MAPKs [ERK (extracellular-signal-regulated kinase), p38 MAPK and JNK (c-Jun N-terminal kinase)] induced by CRP (10 µg/ml) was also significantly inhibited by pitavastatin. Our results suggest that CRP may play a role in atherosclerosis via IL-8 production and pitavastatin may prevent the progression of atherosclerosis not only by lowering plasma low-density lipoprotein cholesterol levels, but also by suppressing IL-8 production in endothelial cells through the inhibition of MAPK (ERK, p38 MAPK and JNK) pathways.

INTRODUCTION

CRP (C-reactive protein) is an acute inflammatory marker that is mainly produced by hepatocytes [1], and plays a role in host defence against bacterial infection. Several recent studies have provided evidence that the inflammatory response plays an important role in the onset, development and evolution of atherosclerotic lesions

Key words: aortic endothelial cell, atherosclerosis, C-reactive protein, inflammation, interleukin-8, mitogen-activated protein kinase, statin.

Abbreviations: AoEC, aortic endothelial cells; CRP, C-reactive protein; ERK, extracellular-signal-regulated kinase; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA; IL-8, interleukin-8; JNK, c-Jun N-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; statin, HMG-CoA reductase inhibitor; TBST, Tris-buffered saline containing 0.1% Tween-20.

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[2,3], and the acute-phase reactant CRP is an important risk factor for atherosclerosis and coronary heart disease [4,5]. A correlation between serum CRP levels and the onset of acute coronary syndromes has also been demonstrated [6–10].

The basic mechanisms responsible for this correlation are not clear. Several investigations have shown that CRP induces the production of inflammatory cytokines in monocytes and promotes monocyte chemotaxis [11]. In endothelial cells, CRP increases the expression of cell adhesion molecules, MCP-1 (monocyte-chemoattractant protein-1) and IL-8 (interleukin-8) [12,13]. CRP also mediates modified LDL (low-density lipoprotein) uptake by macrophages [14]. All these data suggest that CRP may be involved in the progression of atherosclerosis.

IL-8 is a chemokine of the CXC family that is present in macrophage-rich areas of human coronary atheroma [15–17]. IL-8 can induce the proliferation and migration of smooth muscle cells [18]. Mice lacking IL-8 receptors are less susceptible to atherosclerosis than those with IL-8 receptors [19]. Recently, IL-8 has been shown to be a powerful trigger for the adhesion of monocytes to the endothelium [20]. These data appear to indicate that IL-8 plays a role in the formation of atherosclerotic lesions.

On the other hand, statins [HMG-CoA (3-hydroxy-3-methylglutaryl CoA) reductase inhibitors] are generally used as therapeutic agents for the treatment of hypercholesterolaemia. These drugs exert an inhibitory effect on cholesterol synthesis mainly in the liver by inhibiting the rate-limiting step of in vivo cholesterol synthesis [21]. Several clinical trials [22–26] have demonstrated that statins can ameliorate atherosclerosis and reduce cardiovascular-related morbidity and mortality. The preventative effect of statins in coronary artery disease has also been observed in patients with diabetes mellitus [27]. In recent years, the pleiotropic effects of statins on vascular cells, apart from their lipid-lowering properties, have been focused upon [28]. We have reported previously that pravastatin suppresses thrombin-induced IL-8 production in human AoEC (aortic endothelial cells) [29].

In the present study, we examined the effect of pitavastatin, a new potent synthetic statin, on IL-8 production induced by CRP in AoEC in an attempt to elucidate the possible beneficial effects of pitavastatin on the prevention of atherosclerotic lesions associated with high levels of CRP.

METHODS

Cell culture
Human AoEC (Kurabou) at passages 5–10 were cultured in 10-cm diameter culture dishes coated with type I collagen (Iwaki) in MCDB131 (Sigma) supplemented with 10% (v/v) FCS (fetal calf serum), 2 mM L-glutamine, 20 mM HEPES, 60 μg/ml heparin, 20 μg/ml bovine pituitary extracts (Kyokutou), 1.25 μg/ml fungizone, 100 units/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO2 atmosphere. For the experiments, AoEC were cultured in media in the absence or presence of pitavastatin (NK-104; Kowa Company Ltd) for 24 h.

Measurement of IL-8 in the media
AoEC were cultured in medium containing pitavastatin in 24-well culture plates coated with type I collagen. Following stimulation of AoEC with highly purified (>99%) recombinant human CRP (10 μg/ml; Calbiochem), the medium was collected and the concentration of IL-8 was measured by ELISA as reported previously [30].

Northern blot analysis
Northern blot analysis was performed according to the method described previously [31]. Briefly, AoEC were cultured in medium in the absence or presence of pitavastatin in 10-cm diameter culture dishes coated with type I collagen for 24 h. After stimulation with CRP (50 μg/ml) for 4 h, RNA from AoEC was extracted with ISOGEN (Nippon Gene). A portion (20 μg) of the total RNA was electrophoresed on a 1% (w/v) agarose gel containing 6.6% (v/v) formaldehyde at 45 V, and then blotted on to a nylon filter membrane (Amersham). The membrane was prehybridized, hybridized, washed and analysed according to the method recommended by the manufacturer. A digoxigenin-labelled probe for human IL-8 cDNA with dUTP by the random priming method (Roche Diagnostics) was used for the hybridization. The intensity of the bands was analysed with NIH image.

Western blot analysis
The phosphorylation of MAPKs (mitogen-activated protein kinases) was analysed by a non-radioactive method using a commercially available kit (Cell Signaling Technology). Briefly, cell lysates were prepared using lysis buffer [30 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% (w/v) Triton X-100, 12 mM sodium deoxycholic acid, 1 mM sodium orthovanadate, 160 mM sodium fluoride, 150 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM PMSF (pH 7.5)]. The cell lysates were subjected to SDS/PAGE, and then transferred on to a membrane (Millipore) at 100 V for 1 h. The membrane was washed with TBST [Tris-buffered saline (23 mM Tris/HCl, 137 mM NaCl and 23 mM KCl, pH 7.4) containing 0.1% Tween 20] and blocked with blocking buffer [TBST containing 5% (w/v) non-fat dry milk] for 1 h at room temperature. The blots were then incubated with primary antibodies [phospho-specific antibodies recognizing Thr222 and Tyr244 of ERK (extracellular-signal-regulated protein kinase; p44/42 MAPK), Thr180 and Tyr182 of p38 MAPK and Thr183 and Tyr185 of JNK (c-Jun N-terminal kinase; p54 JNK), and antibodies against ERK...
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RESULTS

Effect of CRP on IL-8 production in AoEC

Initially, we examined whether CRP stimulated IL-8 release in AoEC. CRP at 10 µg/ml induced a significant 3.32 ± 0.94-fold increase (P < 0.01) in IL-8 release into the culture medium compared with control cells. In our preliminary experiments, we confirmed that polymyxin B, which binds to and inactivates Gram-negative endotoxin [32], did not abrogate the effect of CRP (results not shown). Subsequently, we examined the effect of CRP on IL-8 mRNA expression in AoEC using Northern blot analysis. As shown in Figure 1, IL-8 mRNA expression induced by CRP was enhanced in a dose-dependent manner. These data demonstrate that CRP stimulates IL-8 production at both the protein and mRNA levels in AoEC, which is consistent with a previous report [13].

Effect of pitavastatin on IL-8 production induced by CRP in AoEC

As shown in Figure 2(a), IL-8 release induced by 10 µg/ml CRP was significantly inhibited by pitavastatin in a dose-dependent manner. To confirm that pitavastatin had no cytotoxic effects, the viability of AoEC cultured without or with pitavastatin (0.5, 1, 5 and 10 µM respectively) for 24 h was evaluated by measuring LDH (lactate dehydrogenase) levels in the culture medium. No significant differences in LDH levels were observed among the samples examined (results not shown), consistent with previous reports [33–35]. The apoptosis of AoEC cultured without or with pitavastatin (0.5, 1, 5 and 10 µM respectively) for 24 h was also evaluated by measuring caspase 3/7 activity (caspase-3/7 assay; Promega). There were no significant differences in the levels of the caspase 3/7 activity among any of the samples (result not shown). We then examined the effect of pitavastatin on...
Effect of pitavastatin on MAPK phosphorylation induced by CRP in human AoEC

AoEC were cultured without or with the indicated concentration of pitavastatin (NK-104; 0.5 to 10 µM) for 24 h respectively. Cells were then cultured in serum-free medium for 12 h and stimulated with CRP (10 µg/ml) for 10 min. The phosphorylation of MAPK induced by CRP was analysed by Western blotting, as described in the Methods section. The intensity of the band of each phospho-ERK (a), phospho-p38 MAPK (b) and phospho-JNK (c) was corrected with that of total ERK, p38 MAPK and JNK respectively. The data are representative of three experiments (means ± S.D.). *P < 0.05 compared with control; **P < 0.05 compared with 10 µg/ml CRP.

Figure 3 Effect of pitavastatin on MAPK phosphorylation induced by CRP in human AoEC

As it has been reported that the expression of IL-8 mRNA is regulated by ERK, p38 MAPK and JNK, we examined the effect of pitavastatin on the activity of these kinases in AoEC. As shown in Figure 3(a), phosphorylation of ERK induced by CRP was inhibited by pitavastatin in a dose-dependent manner. The phosphorylation of p38 MAPK (Figure 3b) and JNK (Figure 3c) induced by CRP was also inhibited by 10 µM pitavastatin.

Effect of ERK, p38 MAPK and JNK inhibitors on IL-8 mRNA expression induced by CRP in AoEC

The effect of MAPK inhibitors PD98059 [a MEK (MAPK/ERK kinase) inhibitor], SB203580 (a p38 MAPK inhibitor) and SP600125 (a JNK inhibitor) on IL-8 mRNA expression in AoEC was investigated. As shown in Figure 4, all of the inhibitors reduced the expression of IL-8 mRNA induced by CRP in AoEC. These data suggest that the production of IL-8 induced by CRP is dependent on three different pathways (ERK, p38 MAPK and JNK pathways) in AoEC, and that pitavastatin...
DISCUSSION

In the present study, we report for the first time that pitavastatin, a new potent synthetic statin, inhibits IL-8 mRNA expression induced by CRP in human AoEC through the inhibition of three different MAPK pathways.

The large body of epidemiological data indicates that statins are useful in preventing the progression of atherosclerosis in patients with hypercholesterolaemia by reducing their serum cholesterol levels [22–27]. Recently, the direct effect of statins on endothelial cells, aside from their cholesterol-lowering effect, has been reported [28]. Statins reduce not only cholesterol, but also intracellular pools of farnesyl and geranylgeranyl pyrophosphates, which are the metabolites of mevalonate [21]. It has been demonstrated that many proteins, including small G-proteins such as Ras, Rho and Rac, are modified by isoprenoids. This modification is necessary for the proper cellular localization and function of the proteins [36,37]. Indeed, the inhibition of the Ras/MAPK pathway by statins has been reported in AoEC [29], aortic smooth muscle cells [38] and cardiac myocytes [39,40]. We have reported previously that pravastatin inhibits thrombin-induced IL-8 production through the Ras/ERK pathway in endothelial cells [29]. Morikawa et al. [41] reported that pitavastatin inhibited IL-8 mRNA levels in HUVEC (human umbilical vein endothelial cells) without stimulation. Our present study showed that pitavastatin inhibited CRP-induced IL-8 production in AoEC via the three pathways examined (ERK, p38 MAPK and JNK). As shown in Figure 2, pitavastatin inhibited IL-8 production induced by CRP in AoEC at both the protein and mRNA levels. We also confirmed that the treatment of AoEC with pitavastatin inhibited the ERK, p38 MAPK and JNK pathways respectively (Figure 3). Furthermore, PD98059, an inhibitor of MEK, the upstream activator of ERK, SB203580, an inhibitor of p38 MAPK, and SP600125, an inhibitor of JNK, inhibited the expression of IL-8 mRNA induced by CRP (Figure 4). Therefore we postulate that pitavastatin may inhibit the isoprenylation of small GTP-binding proteins such as Ras and Rho which, in turn, may affect their ability to interact with the plasma membrane. This inhibition of the MAPK pathways would lead to the reduced IL-8 production in AoEC. Indeed, Hiraoka et al. [42] reported the inhibition of ERK and RhoA by pitavastatin in THP-1 cells, and Masamura et al. [33] reported that mevalonate reversed the induction of thrombomodulin expression mediated by pitavastatin in endothelial cells.

IL-8 is one of the chemokines produced mainly by macrophages but also by endothelial cells and is a potent chemo-attractant that is important for mediating monocyte–endothelial cell adhesion. Furthermore, monocyte–endothelial cell interactions in the presence of hyperglycaemia are mediated by the induction of IL-8 synthesis in AoEC, suggesting that IL-8 may play a major role in diabetes by promoting monocyte adhesion to endothelial cells [29–31,43]. Therefore the inhibition of IL-8 production in endothelial cells may play an important role in the prevention of atherosclerosis by pitavastatin particularly in diabetic patients, and the administration of pitavastatin to diabetic patients could be useful for the prevention of atherosclerotic diseases. The effect of pitavastatin on atherosclerotic diseases associated with high levels of CRP, such as ischaemic heart disease and stroke, in primary and/or secondary prevention needs to be demonstrated in further clinical studies.

In conclusion, our results suggest that CRP may play a role in atherosclerosis and pitavastatin, a new potent synthetic statin, may prevent the progression of atherosclerosis not only by lowering plasma LDL-cholesterol levels, but also by suppressing IL-8 production in endothelial cells by the inhibition of the MAPK (ERK, p38 MAPK and JNK) pathways. To our knowledge, this is the first report showing that pitavastatin may prevent the progression of atherosclerosis by regulating the inflammatory response and cytokine production from vascular endothelial cells.
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