Pitavastatin Inhibits Vascular Smooth Muscle Cell Proliferation by Inactivating Extracellular Signal-Regulated Kinases 1/2

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We recently reported that lysophosphatidylcholine (lysoPC) acts on vascular smooth muscle cells (VSMCs) to produce a mitogenic response through the activation of extracellular signal-regulated kinases 1/2 (ERK1/2). In this study, we examined the role of HMG-CoA reductase inhibitors on lysoPC-induced VSMC proliferation. Pitavastatin, a new HMG-CoA reductase inhibitor, suppressed lysoPC-induced DNA synthesis in primary cultured rat VSMCs. Since lysoPC-induced ERK1/2 activation contributes to smooth muscle cell proliferation, we explored the effect of pitavastatin on ERK1/2 activation. Pitavastatin inhibited lysoPC-induced ERK1/2 phosphorylation and ERK1/2 activation. The other HMG-CoA reductase inhibitors, atrovastatin and fluvastatin, also inhibited lysoPC-induced ERK1/2 phosphorylation. Pitavastatin also inhibited lysoPC-induced c-fos mRNA expression. To gain insight into the mechanism of the inhibitory effect of pitavastatin on ERK1/2 activation by lysoPC, we examined the role of the mevalonate pathways. Mevalonate and farnesylpyrophosphate reduced the inhibition of ERK1/2 phosphorylation by pitavastatin. These studies demonstrate that pitavastatin may inhibit lysoPC-induced VSMC proliferation, at least in part, by inactivating ERK1/2, which is linked to mevalonate metabolism. J Atheroscler Thromb, 2003; 10: 37-42.

Key words: Vascular smooth muscle cell, Lysophosphatidylcholine, ERK1/2, Pitavastatin

Introduction

Several lines of evidence have suggested that oxidized low-density lipoprotein (LDL) plays a key role in atherogenesis (1,2). Lysophosphatidylcholine (lysoPC) is one of the major phospholipid components which are increased during the oxidation of LDL (3). The concentration of lysoPC is elevated in atherosclerotic lesions in animals fed an atherogenic diet (4). LysoPC stimulates the proliferation and migration of vascular smooth muscle cells (VSMCs) (5), induces adhesion molecules in endothelial cells (6), and impairs endothelium-dependent vasorelaxation (7). Previously, we reported that lysoPC stimulated the activation of p42/44 mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) and the expression of the transcription factor c-Fos and c-Jun in cultured VSMCs (8). ERK1/2 are serine/threonine kinases activated by a variety of stimuli involved in cell proliferation and differentiation (9,10). Thus, lysoPC-induced ERK1/2 activation could be involved in the pathogenesis of atherosclerosis.

An elevated level of LDL-cholesterol has been recognized as the most important risk factor for coronary artery disease (CAD). The most effective method of LDL-
cholesterol lowering is the administration of inhibitors of HMG-CoA reductase, a rate-limiting enzyme in the cholesterol synthesis pathway (11). It is usually assumed that some beneficial effect of HMG-CoA reductase inhibitors statins on coronary events is linked to their hypocholesterolemic properties. However, because mevalonic acid (MVA), intracellularly synthesized by HMG-CoA reductase, is the precursor of numerous metabolites, the inhibition of HMG-CoA reductase can potentially result in pleiotropic effects (12-14); therefore, these effects may help to explain the anti-atherosclerotic properties of statins. In addition, many recent studies have involved the possible effects of HMG-CoA reductase inhibitors beyond their lipid-lowering actions (15).

Pitavastatin (+)-monocalcium bis (3R, 5S, 6E) -7- [2-cyclopropyl-4- (4-fluorophenyl) -3-quinolyl] -3,5-dihydroxy-6-heptenoate, CAS 147526-32-7), a novel HMG-CoA reductase inhibitor, pitavastatin, on lysoPC-induced ERK1/2 activation. We also determined whether pitavastatin inhibited VSMC proliferation and c-fos mRNA expression.

Materials and Methods

Dulbecco’s modified Eagle’s medium (DMEM), fetal calf serum (FCS), penicillin, and streptomycin were obtained from Life Technologies Inc. Polyclonal antibodies to Thr 202 and Tyr 204-phosphorylated p42/44 MAP kinase were purchased from New England Biolabs. Palmitoyl-lyso-PC (lyso-PC), which was 100% pure by HPLC and mass spectral analysis was purchased from Avanti Polar Lipids. Pitavastatin was from Kowa Co., Ltd.

Cell Culture

VSMCs were prepared from the thoracic aorta of 12-week-old Sprague-Dawley rats (Charles River Breeding Laboratories) by the explants method as previously described (18). Subcultured VSMCs from passages 3-15, were used in the experiments, showed >99% positive immunostaining of a smooth muscle α-actin antibody (Sigma) and were negative for mycoplasma infection by polymerase chain reaction (Stratagene). For subsequent experiments, cells at about 80% confluence in culture wells were used one day after serum depletion.

[Methyl-3H] Thymidine Incorporation

After serum starvation for 48 hours, cells in 24-well plates were incubated with lyso-PC (12.5 µM) and [Methyl-3H] thymidine (1 µCi/ml) for 24 hours. Cells were washed twice with PBS, followed by treatment with 5% trichloroacetic acid (TCA) which was added for 30 minutes at 4°C to precipitate proteins. TCA-precipitable radioactivity was counted in a scintillation counter.

Preparation of Cell Extracts and Western Blotting

VSMCs were stimulated with agonists for specified durations. After treatment, cells were washed with ice-cold PBS. Cells were lysed with ice-cold lysis buffer at pH 7.4, containing 500 mM HEPES, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, a mixture of protease inhibitors, and 1 mM sodium orthovanadate. Solubilized proteins were centrifuged at 14,000 g for 30 minutes, and supernatants were stored at –80°C. Proteins (25 µg) were separated on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis with the indicated antibodies using the ECL detection system (Amersham).

ERK1/2 Activity Assay

VSMCs grown on a 24-well plate were stimulated with agonists for the indicated period. The reaction was terminated by replacing the medium with ice-cold lysis buffer (10 mM Tris, pH 7.4, 20 mM NaCl, 2 mM EGTA, 2 mM diithiothreitol, and 1 mM orthovanadate with 10 µM leupeptin, and 10 µM aprotinin). After brief sonication, the samples were centrifuged for 5 minutes at 14,000 g, and the supernatant was assayed for ERK1/2 activity with a BIOTRAKT MAP kinase enzyme assay kit (Amersham) that measured the incorporation of [γ-33P] ATP into a specific ERK1/2 substrate (KRELVERPLTPAGEAPNQALLR) as previously described (8).

Northern blot analysis

Total RNA was isolated and Northern blot analysis was performed as described previously (8). Total RNA (20 µg) was size-separated by electrophoresis on 1% agarose/formaldehyde gels and then transferred to Hybond-N membranes (Amersham). The membranes were prehybridized at 2 hours at 65°C in 1 M NaCl, 10% Dextran, 1% SDS, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was carried out at 65°C overnight with the same solution and [α-32P] labeled with denatured c-fos. The membranes were washed with 2× SSC (1×, 150 mM NaCl and 15 mM sodium citrate) for 5 minutes at room temperature and then with 0.2× SSC, 0.1% SDS at 65°C for 30 minutes. The membranes were exposed to X-ray film.

Statistical analysis

Values were expressed as the mean±SD. Differences between data sets were evaluated by an unpaired Student’s t-test. A level of p<0.01 was accepted as statistically significant.
Inhibition of ERK1/2 by Pitavastatin

Results

Inhibition of lysoPC-induced DNA synthesis by pitavastatin

Previous studies have shown that lysoPC induced VSMC proliferation (5).

An increase in DNA synthesis was observed after exposure of the cells to lysoPC for 24 h (Fig. 1). Co-incubation with pitavastatin (100 µM) stopped the lysoPC-mediated increase in DNA synthesis but did not affect DNA synthesis in the absence of stimulation.

Inhibition of lysoPC-induced ERK1/2 phosphorylation by HMG-CoA reductase inhibitors

In response to lysoPC, there was an apparent phosphorylation of both ERK1 and ERK2, which are p44 and p42 MAPK, respectively, with peak activity at 7 minutes and then returning to the baseline as previously described (8).

To determine whether lysoPC-stimulated ERK1/2 phosphorylation is inhibited by pitavastatin, the effect of pitavastatin on lysoPC-induced ERK activation was examined. As shown in Fig. 2A, B pitavastatin (100 µM) markedly inhibited lysoPC-induced ERK1/2 phosphorylation and ERK1/2 activation. The inhibition by pitavastatin was concentration-dependent, starting at 10 µM and peaking at 100 µM (Fig. 2C). To clarify whether this effect of pitavastatin depends on HMG-CoA reductase inhibition, we examined the effects of other HMG-CoA reductase inhibitors, atrovastatin and fluvastatin. They all markedly inhibited lysoPC-induced ERK1/2 phosphorylation (Fig. 3).

Fig. 1. Inhibition of lysoPC-induced DNA synthesis by pitavastatin. After pretreatment with or without pitavastatin (100µM) for 48 hours, VSMCs were incubated with lysoPC (12.5µM) and [Methyl-3H] thymidine (1µCi/ml) for 24 hours. The total radioactivity incorporated into DNA was determined by liquid scintillation counting. **p<0.01 vs. DMSO-treated control.

Fig. 2. Effect of pitavastatin on ERK1/2 phosphorylation induced by lysoPC. After pretreatment with pitavastatin (100µM) for 48 hours, VSMCs were stimulated with lysoPC (12.5µM) for the indicated time. ERK1/2 phosphorylation (A) and activity (B) were determined. After pretreatment with pitavastatin for the indicated concentrations, VSMCs were stimulated with lysoPC (12.5µM) for 7 minutes (C). Values are means±SD. **p<0.01 vs. DMSO-treated control. Whole cell lysates were separated by SDS-PAGE and immunoblotted by the indicated antibodies. Results shown are representative of three separate experiments.

Fig. 3. Effects of atrovastatin and fluvastatin on ERK1/2 phosphorylation induced by lysoPC. After pretreatment with or without atrovastatin and fluvastatin with the indicated concentrations for 24 hours, VSMCs were stimulated with lysoPC for 7 minutes. Whole cell lysates were separated by SDS-PAGE and immunoblotted by the indicated antibodies. Results shown are representative of three separate experiments.
The effect of pitavastatin on c-fos mRNA expression
As previously reported (19), Northern blot analysis revealed that lysoPC-increased c-fos mRNA expression compared with the controls (Fig. 4). Pretreatment with pitavastatin markedly inhibited the c-fos mRNA expression.

The role of the mevalonate pathways
Ang II-induced ERK1/2 activation was inhibited with lovastatin and this inhibitory effect was completely prevented in the presence of mevalonate (20). Thus, to gain insight into the mechanism of the inhibitory effect of the pitavastatin on ERK1/2 activation by lysoPC, we examined the role of the mevalonate pathways. As described above, the incubation of VSMCs with pitavastatin nearly completely inhibited the ERK1/2 phosphorylation induced by lysoPC. The co-incubation of VSMCs with mevalonate partially stopped the inhibition of ERK1/2 phosphorylation by pitavastatin (Fig. 5A). Similarly, co-incubation of VSMCs with farnesyl pyrophosphate (FPP) partly prevented the effect of pitavastatin on ERK1/2 phosphorylation. The addition of mevalonate also restored the inhibitory effect of pitavastatin on Ang II-induced ERK1/2 phosphorylation.

Discussion
The non-lipid-related properties of statins may help to explain the early and significant cardiovascular event reduction reported in several clinical trials (21, 22). Therefore, we studied the effect of pitavastatin on ERK1/2 activation induced by lysoPC. The major findings of this study are 1) lysoPC-induced DNA synthesis was inhibited with pitavastatin. 2) lysoPC-induced ERK1/2 activation was inhibited with pitavastatin in VSMCs. 3) Induction of c-fos mRNA expression by lysoPC was also inhibited by pitavastatin. 4) These inhibitions were partially abrogated with mevalonate or FPP.

It was reported that fluvastatin, simvastatin, lovastatin, atorvastatin and cerivastatin, but not pravastatin, dose-dependently decreased smooth muscle cell (SMC) migration and proliferation (23). For example, lovastatin inhibited the Ang II induced-p21ras/MAP kinase pathway, which is linked to mevalonate metabolism in neonatal rat heart cells (20). The preincubation of mesangial cells with lovastatin inhibited the activation of MAP kinase stimulated by either FBS, PDGF, or EGF. Mevalonate acid and FPP, but not cholesterol or LDL, significantly prevented the lovastatin-induced inhibition of agonist-stimulated MAP kinase (24). Preincubation of VSMCs with cerivastatin (0.5 µM) prevented Ang II-induced ERK1/2 phosphorylation, NF-κB and AP-1 binding activity (25).

There is a significant difference in the potency needed to inhibit mevalonate synthesis between lipid soluble HMG-CoA reductase inhibitors, such as lovastatin and simvastatin (26, 27). Recently, a novel potent fully synthetic statin, pitavastatin was developed, however, there have been no studies clarifying whether pitavastatin also inhibits SMC proliferation and ERK1/2 activation. In addition, it remains unclear whether several statins also inhibit the lysoPC-induced ERK1/2 activation in VSMCs.

Since MAP kinase phosphorylation and activation occur through the ras signaling pathway, several studies have proposed that statins may interfere with ras signaling (24, 28). The postulated mechanisms involve ras farnesylation and ras cell membrane binding (24). Farnesylated p21 ras may be critical for cellular signaling. Inhibitors of HMG-CoA reductase inhibit the production of mevalonate and its metabolite, farnesol. This inhibition can be stopped by the simultaneous addition of either mevalonate or farnesol. These results suggest that the mechanism of the inhibitory effect of pitavastatin is partially mediated

![Fig. 4. Effect of pitavastatin on c-fos mRNA expression induced by lysoPC.](image)

After pretreatment with or without pitavastatin (50 µM) for 48 hours, VSMCs were stimulated with lysoPC for 60 minutes. Total RNA was extracted, and Northern blotting analysis was performed using c-fos as a probe. The filters were stained by methylene blue to check the relative loading of total RNA.

![Fig. 5. Effect of Mevalonic acid and FPP on ERK1/2 activation.](image)

After pretreatment with pitavastatin (50µM), Mevalonic acid (100µM) or FPP for 24 hours, VSMCs were stimulated with lysoPC (12.5µM) or AngII (10⁻⁷M) for 7 minutes.
Inhibition of ERK1/2 by Pitavastatin

References


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through the mevalonate pathway. However, the novel mechanisms of inhibition on ERK1/2 activation by pitavastatin remain to be investigated.

Upon activation, ERK1/2 translocate to the nucleus where they phosphorylate transcription factors, such as TCF/ELK-1, which are bound to the c-fos promoter. Increased c-Fos synthesis results in elevated AP-1 activity. AP-1 is a sequence-specific transcriptional activator composed of Jun and Fos subunits, that is involved in mitogenesis, differentiation, transformation and inflammation. The induction of transcription factors such as c-fos and c-jun has been linked to proliferation and activation (29, 30) which contributes to lesion formation during atherogenesis (31). Recently, a direct proportional association between c-fos mRNA expression and SMC proliferation was reported in the rat carotid artery after balloon injury (30, 32). Therefore, factors inhibiting c-fos may be of great interest in the treatment of atherosclerosis. The findings of this study showed that c-fos mRNA expression induced by lysoPC was eliminated by pitavastatin, suggesting that pitavastatin may inhibit VSMC proliferation mediated through the suppression of the ERK1/2-c-fos pathway in VSMCs.

There is compelling evidence to support the concept that lipids deposited in atherosclerotic lesions are derived mainly from modified LDL (31, 33, 34). Recent findings have suggested that SMC constitutes approximately 90-95% of the cellular component of an atherosclerotic lesion in a young adult and approximately 50% in advanced atherosclerosis plaque (33, 35). Thus, to prevent the progression of atherosclerosis, we need to reduce serum LDL levels and inhibit the oxidized LDL-induced proliferation of VSMCs. Large scale clinical trials have shown that HMG-CoA reductase inhibitors improve the clinical outcome of patients with atherosclerosis and coronary heart disease, exceeding the beneficial effects expected from plasma cholesterol lowering alone (36-38). However, the mechanisms underlying these beneficial effects are not clear. LysoPC-induced ERK1/2 activation is essential for the proliferation promoting signals in VSMCs. We found that pitavastatin in vitro inhibited lysoPC-induced ERK1/2 activation. Pitavastatin also attenuated the downstream c-fos activation in VSMCs. Blockade of the proliferation promoting signals by pitavastatin could at least in part, explain the beneficial impact of pitavastatin in vitro.

Since pitavastatin showed an inhibitory effect on ERK1/2 activation by lysoPC in addition to lowering the serum LDL levels, it might be a useful drug to prevent atherosclerosis. In conclusion, ERK1/2 activation is essential for the growth promoting signals activated by lysoPC in VSMCs. Pitavastatin might be a good drug for preventing atherosclerosis through the inhibition of ERK1/2 activation.

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