HMG-CoA Reductase Inhibitors Prevent Migration of Human Coronary Smooth Muscle Cells Through Suppression of Increase in Oxidative Stress

Kenichi Yasunari, Kensaku Maeda, Mieko Minami, Junichi Yoshikawa

Abstract—In vitro and in vivo evidence of a decrease in vascular smooth muscle cell (SMC) migration induced by 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors has been reported. When added to SMC cultures for 6 hours, the HMG-CoA reductase inhibitors fluvastatin, simvastatin, and pravastatin at 1 μmol/L resulted in a 48%, 50%, and 16% suppression, respectively, of human coronary SMC migration; these reductions mirrored the suppression in oxidative stress induced by 1 μmol/L lysophosphatidylcholine (lyso-PC) of 50%, 53% and 19%, respectively. The hydroxylated metabolites of fluvastatin, M₂ and M₃, at 1 μmol/L also suppressed the enhancement of SMC migration by 58% and 45% and the increase in oxidative stress induced by lyso-PC of 58% and 49%, respectively. Lyso-PC activated phospholipase D and protein kinase C (PKC), and this activation was also suppressed by HMG-CoA reductase inhibitors. The inhibition of phospholipase D and PKC was reversed by 100 μmol/L mevalonate, its isoprenoid derivative, farnesol, and geranylgeraniol but not by 10 μmol/L squalene. Antisense oligodeoxynucleotides at 5 μmol/L to PKC-α, but not those to the PKC-β isoform, suppressed the lyso-PC–mediated increases in SMC migration and oxidative stress. These findings suggest that HMG-CoA reductase inhibitors have direct antimigratory effects on the vascular wall beyond their effects on plasma lipids and that they might exert such antimigratory effects via suppression of the phospholipase D– and PKC (possibly PKC-α)-induced increase in oxidative stress, which might in turn prevent significant coronary artery disease. (Arterioscler Thromb Vasc Biol. 2001;21:937-942.)

Key Words: lipids • atherosclerosis • smooth muscle • coronary disease

Clinical and experimental studies have shown that a reduction of plasma cholesterol, particularly LDL cholesterol, reduces the risk of cardiovascular events in both primary and secondary prevention. The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which are cholesterol-reducing drugs, can achieve a relatively large reduction in plasma cholesterol. The beneficial effects of HMG-CoA reductase inhibitors are usually assumed to result from their ability to reduce cholesterol synthesis. However, a variety of experimental findings suggest that statins can interfere with major events involved in the formation of atherosclerotic lesions, independent of their hypocholesterolemic properties.

Vascular smooth muscle cell (SMC) migration in the arterial wall is an important mechanism in atherogenesis and is a possible determinant of restenosis after angioplasty. In addition, increased oxidative stress is reported to play an important role in SMC migration, suggesting a relation between oxidative stress and SMC migration. Because there exists in vitro and in vivo evidence of decreased SMC proliferation after administration of fluvastatin, simvastatin, or lovastatin, it is possible that statins have the potential to decrease oxidative stress and SMC migration.

Therefore, we examined whether suppression of lysophosphatidylcholine (lyso-PC), a major component of oxidized LDL–mediated SMC migration and oxidative stress, could be assessed after administration of therapeutic concentrations (1 μmol/L) of statins. We also examined the effects of the hydroxylated metabolites of fluvastatin, 5-hydroxyfluvastatin (M₂) and 6-hydroxyfluvastatin (M₃), which exist in plasma, on the increase in SMC migration and oxidative stress induced by lyso-PC.

Methods

Cell Culture
Human coronary artery SMCs were cultured in smooth muscle basal medium (SMBM, Clonetics) containing human epidermal growth factor (0.5 ng/mL), human fibroblast growth factor (2 ng/mL), 5% fetal calf serum, 50 mg/mL gentamicin sulfate, and 50 mg/mL amphotericin B, as previously described. Subconfluent SMCs between the fourth and eighth passages were used for experiments.

Migration Assay
Migration of SMCs was assayed by a modification of the Boyden chamber method with the use of microchemotaxis chambers (Neuro Probe Inc) and polycarbonate filters (Nucleopore Corp) with pores.
12 μm in diameter, as previously reported. A 200-μL SMC suspension (~3.0×10⁶ cells) was placed in the upper chamber, and 40 μL of SMBM containing a migration factor (such as lyso-PC) in the presence or absence of statins, a phospholipase D (PLD) inhibitor, or a protein kinase C (PKC) inhibitor was placed in the lower chamber. The chamber was incubated at 37°C under 5% CO₂ in air for 6 hours. Migration activity was expressed as the number of cells that had migrated in 4 high-power fields (HPF; ×400).

**Assay of Intracellular Oxidative Stress**

Intracellular oxidative stress was measured with a fluorescent dye, carboxydiethylfluorescein diacetate-bis-AM ester (Molecular Probe Corp), a nonpolar compound that is converted to a nonfluorescent polar derivative by cellular esterases after its incorporation into cells. After 5 minutes of incubation at room temperature, the fluorescence intensity of each point was measured by flow cytometry as previously reported.

**HMG-CoA Reductase Assay**

The cell pellet was homogenized at 4°C in a Dounce homogenizer in 10 mmol/L potassium phosphate buffer (pH 7.4), 1 mmol/L EDTA, and 30 mmol/L nicotinamide. Aliquots of the 15 000g supernatant were used for the HMG-CoA reductase assay according to the method of Brown and Goldstein for cultured cells. The enzyme reaction was linear with time up to 60 minutes and for protein concentrations up to 0.5 mg. Protein content was measured by the Bradford method.

**PLD Activity Measured by Ethanolamine Release**

SMCs in 35-mm dishes were cultured in medium containing [³H]-ethanolamine (5 μCi/mL per dish) for 24 hours (the latter half of the 48-hour period with 0.1% fetal calf serum) to label cellular phosphatidylethanolamine. After a 0.5- to 1-hour incubation, the reaction was terminated by adding 0.75 mL methanol. The cells were harvested by gentle scraping. Fractionation of ethanolamine metabolites from the aqueous phase was performed on Dowex 50W (H⁺) packed columns as previously described.

**Cell Fractionation and Assay of PKC**

SMCs were cultured in medium with 0.1% fetal calf serum for 48 hours. Cell fractionation was performed as previously reported. PKC activity was measured by a modification of a method with the Amersham PKC assay system (Amersham Japan Corp).

**Fluorescence Microscopy**

SMCs were visualized through a fluorescence microscope (Olympus IX70, ×400 water-immersion objective lens) equipped with a camera (Olympus PM-C 35DX). An automatic gain-control mode in the camera allowed suitable transmission images to be obtained with low-level light. To elicit fluorescent images, the preparation was illuminated with a 200-W mercury lamp. The light was passed through a quartz collector, heat filter, and an excitation filter to epi-illuminate the preparation. Fluorescence emission from the sample was passed through a band-pass filter (515 nm) and into the camera.

**Antisense Oligonucleotides**

Phosphothioate-modified oligodeoxynucleotides for the PK-C-α and PK-C-β isoforms were designed and reported by Li et al. and purified by high-performance liquid chromatography by Japan Bio Service Co: antisense PK-C-α, 5'-CCG CGT GGA GTC GTC GCC CG-3'; sense PK-C-α, 5'-CCG GCA AGC ACT CCA CGG CG-3'; antisense PK-C-β, 5'-AGC GCA CGG TGC TCT CCT CG-3'; and sense PK-C-β, 5'-CCG GGA GAC CAC CGT CGG CT-3'. These oligodeoxynucleotides were added at a concentration of 5 μmol/L to serum-free SMBM 24 hours before the start of cell stimulation with platelet-derived growth factor-BB (PDGF-BB), with transfection by use of a cationic compound. Lipofectin reagent ( Gibco BRL) and oligonucleotides were effectively taken up by SMCs.

**Immunoblotting**

SMCs grown on a 6-well plate were stimulated with agonists at 37°C in serum-free SMBM for specified durations. After brief sonication (5 seconds), the samples were boiled for 5 minutes at 95°C and centrifuged (14 000g, 5 minutes) at 4°C, and the supernatant (25 μL) was subjected to SDS–polyacrylamide gel electrophoresis. Proteins in the gel were transferred to a polyvinylidene difluoride membrane by electroblotting. The membrane was treated with rabbit polyclonal antibodies that detect the PKC-α, PKC-β, or PKC-βII isoform. After incubation with secondary anti-rabbit antibodies, immunoreactive proteins were detected by the CDP-Star chemiluminescence system (New England Biolabs Inc).

**Statistical Methods**

Statistical analysis was performed by ANOVA and Schefee’s modified t test. Values of P<0.05 were considered significant.

**Results**

**Effects of HMG-CoA Reductase Inhibitors on SMC Migration**

Fluvastatin dose-dependently suppressed the 1 μmol/L lyso-PC–mediated increase in SMC migration (control, 19.1±1.8 cells/4 HPF; lyso-PC, 42.7±1.8 cells/4 HPF; lyso-PC and 0.1 μmol/L fluvastatin, 35.5±1.8 cells/4 HPF; lyso-PC and 0.5 μmol/L fluvastatin, 26.4±1.4 cells/4 HPF; lyso-PC plus 1 μmol/L fluvastatin, 18.4±1.8 cells/4 HPF, n=8). The values in all lyso-PC/fluvastatin groups were significantly different from those in the lyso-PC–only group. Fluvastatin, simvastatin, and pravastatin, each at 1 μmol/L, suppressed the lyso-PC–induced migration of SMCs by 48%, 50%, and 16%, respectively (Table 1), but did not affect the basal level of migration at 1 μmol/L (control, 20.2±2.4 cells/4 HPF; fluvastatin, 18.4±2.2 cells/4 HPF; simvastatin, 18.0±2.6 cells/4 HPF; and pravastatin, 19.4±2.4 cells/4 HPF; n=8). The M₂ and M₃ hydroxylated metabolites of fluvastatin, each at 1 μmol/L, also suppressed the lyso-PC–induced migration of SMCs, by 58% and 45%, respectively (Table 1). Fluvastatin at 1 μmol/L also suppressed SMC migration mediated by 5 ng/mL PDGF-BB or 10 ng/mL basic fibroblast growth factor (bFGF) (control, 15.0±2.2 cells/4 HPF; PDGF-BB, 40.2±4.4 cells/4 HPF; bFGF, 38.6±4.2 cells/4 HPF; PDGF-BB and fluvastatin, 22.4±3.4 cells/4 HPF; and bFGF and fluvastatin, 27.6±4.4 cells/4 HPF, n=8). Values for the growth factor with fluvastatin groups were significantly different from the values for the respective growth factor alone: P<0.05 compared with either PDGF-BB alone or with bFGF alone.

**Effects of HMG-CoA Reductase Inhibitors on SMC Oxidative Stress**

Fluvastatin dose-dependently suppressed the 1 μmol/L lyso-PC–mediated increase in oxidative stress (control, 59.9±10.0; lyso-PC, 133.2±6.6; lyso-PC and 0.5 μmol/L fluvastatin, 83.3±6.6; lyso-PC and 1 μmol/L fluvastatin, 65.0±6.0; all values in arbitrary units, n=8). The values in all lyso-PC/fluvastatin groups were significantly different from those of lyso-PC alone. Fluvastatin, simvastatin, and pravastatin, each at 1 μmol/L, suppressed the lyso-PC–induced increase in SMC oxidative stress, by 50%, 53%, and 19%, respectively (Table 1), although these compounds did not affect the basal level of SMC oxidative stress. The M₂ and M₃ hydroxylated metabolites of fluvastatin, each at 1 μmol/L,
also suppressed the lyso-PC–induced increase in SMC oxidative stress, by 58% and 49%, respectively (Table 1). Representative flow cytometric findings and results of fluorescence microscopy for SMC oxidative stress are shown in Figure 1.

Effects of HMG-CoA Reductase Inhibitors on the HMG-CoA Reductase Activity of Lyso-PC–Stimulated SMCs

Fluvastatin, simvastatin, and pravastatin, each at 1 μmol/L, suppressed the lyso-PC–induced HMG-CoA reductase activities of SMCs by 39%, 52% and 19%, respectively. However, the hydroxylated metabolites of fluvastatin M2 and M3, each also at 1 μmol/L, did not suppress the lyso-PC–induced HMG-CoA reductase activities of SMCs (Table 1).

Involvement of PLD, PKC, and Tyrosine Kinase in the Lyso-PC–Mediated Increase in Oxidative Stress

To assess the involvement of PLD and PKC in the lyso-PC–mediated increase in migration and oxidative stress, PLD activity and membrane-bound PKC activity were measured, because it has been reported that lyso-PC stimulates superoxide production via PKC19 and that oxidative stress in human SMCs is mediated by PLD.20 Lyso-PC increased both PLD and PKC activities, which had been suppressed by the HMG-CoA reductase inhibitors fluvastatin, including its hydroxylated metabolites M2 and M3, simvastatin, and pravastatin (Table 2).

To examine the mechanisms by which lyso-PC increases oxidative stress and migration, the effects of the specific PLD, PKC, and tyrosine kinase inhibitors suramin, calphostin C, and genistein on the lyso-PC–mediated increases in oxidative stress and migration were tested. Suramin at 100 μmol/L, calphostin C at 1 μmol/L, and genistein at 100 μmol/L, each of which completely suppressed the lyso-PC–induced increase in PLD and PKC activities (Table 2), also suppressed the 1 μmol/L lyso-PC–induced increases in SMC migration (control, 16.0±0.8 cells/4 HPF; lyso-PC, 32.2±1.2, cells/4 HPF; lyso-PC plus suramin, 17.0±1.0 cells/4 HPF; lyso-PC plus calphostin C, 15.4±1.4 cells/4 HPF).

**Figure 1.** Representative fluorescence microscopic (A) and flow cytometric (B) findings for effects of 1 μmol/L fluvastatin (Flu) on 1 μmol/L lyso-PC–mediated increase in oxidative stress. The area below the flow cytometric curve is the same (3000 cells). Therefore, a decrease in number at low fluorescence intensity indicates an increase in oxidative stress. Relative fluorescence intensity was measured by flow cytometry as follows: fluorescence intensity=[(fluorescence of each channel)×(cell number of the channel)]/total cell number.

**TABLE 1.** Effects of Statins on Migration, Oxidative Stress, and HMG-CoA Reductase Activity of Lyso-PC–Treated SMCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Migration Activity, Cells/4 HPF</th>
<th>Oxidative Stress, AU</th>
<th>HMG-CoA Reductase Activity, pmol Mevalonate Formed/mg Protein per Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lyso-PC, 1 μmol/L</td>
<td>37.7±2.3</td>
<td>143.2±6.6</td>
<td>49.6±4.4</td>
</tr>
<tr>
<td>+ Flu 1 μmol/L</td>
<td>19.5±2.3*</td>
<td>71.6±5.0*</td>
<td>30.2±3.6*</td>
</tr>
<tr>
<td>+ M2 1 μmol/L</td>
<td>15.9±2.5*</td>
<td>60.0±6.6*</td>
<td>45.2±3.6</td>
</tr>
<tr>
<td>+ M3 1 μmol/L</td>
<td>20.9±2.7*</td>
<td>73.3±6.0*</td>
<td>44.6±3.8</td>
</tr>
<tr>
<td>+ Sim 1 μmol/L</td>
<td>19.0±2.4*</td>
<td>66.6±3.4*</td>
<td>23.6±2.8*</td>
</tr>
<tr>
<td>+ Pra 1 μmol/L</td>
<td>31.8±1.8*</td>
<td>116.6±5.0*</td>
<td>40.0±4.0*</td>
</tr>
</tbody>
</table>

AU indicates arbitrary unit. Values shown are the effects of fluvastatin (Flu), its hydroxylated metabolites M2 and M3, simvastatin (Sim), and pravastatin (Pra) on the induction of migration, oxidative stress, and HMG-CoA reductase activity by administration of 1 μmol/L lyso-PC for 6 hours. Migration activities are expressed as the number of cells in 4 HPF and are mean±SD of 8 measurements. Oxidative stress was measured by flow cytometry and expressed as fluorescence intensity (mean±SD, n=8). HMG-CoA reductase activities were measured by mevalonate formed per mg protein per min (mean±SD, n=8).

*P<0.05 vs. the 1 μmol/L lyso-PC group.
The effects of the antioxidant α-tocopherol on the lyso-PC–induced increase in SMC oxidative stress and migration were also studied. α-Tocopherol also suppressed the lyso-PC–induced increase in SMC oxidative stress and migration from those of lyso-PC only, and the lyso-PC plus fluvastatin values were significantly different from those of lyso-PC alone.

Effects of Antioxidants on Lyso-PC–Induced Increases in SMC Oxidative Stress and Migration

The effects of the antioxidant α-tocopherol on the lyso-PC–induced increase in SMC oxidative stress and migration were also studied. α-Tocopherol also suppressed the lyso-PC–induced increase in SMC migration (control, 16.0±1.2 cells/4 HPF; and lyso-PC, 32.2±1.2 cells/4 HPF; and lyso-PC plus α-tocopherol, 18.0±1.4 cells/4 HPF) and oxidative stress (Figure 2A). Again, the lyso-PC–only values were significantly different from control, and the lyso-PC plus α-tocopherol values were significantly different from those of lyso-PC alone.

Inhibition of PKC-α or PKC-β Isoform Activation by Antisense Oligonucleotide

To determine whether activation of the PKC-α or PKC-β isoform is associated with the lyso-PC–induced increase in SMC migration and oxidative stress, the effects of antisense oligonucleotides to PKC-α or PKC-β isoform, which significantly blocked the expression of PKC-α, PKC-βI, or PKC-βII (Figure I), were examined. Antisense oligonucleotide to PKC-α but not of those to the PKC-β isoform at 5 μmol/L significantly suppressed the lyso-PC–induced changes (Table 3). Sense oligonucleotides to neither the
PKC-α isoform nor the PKC-β isoform had any effects on these lyso-PC–induced changes at 5 μmol/L (Table 3).

**Discussion**

The ability of HMG-CoA reductase inhibitors to suppress oxidative stress and migration of SMCs (Table 1) at reported therapeutic concentrations (1 μmol/L)10 may be of clinical interest, given that interference with these processes has been considered a possible basis for antiatherosclerotic therapy.7 Greater effects on suppression of SMC oxidative stress and migration (Table 1) were observed for lipophilic compounds (simvastatin and fluvastatin),21 consistent with previous observations.6,8

Although the specific mechanisms by which lyso-PC acts as a chemotactic factor for coronary SMCs remain to be determined, the present findings suggest that lyso-PC may affect PLD activity, leading to the activation of membrane-bound PKC (Table 2), which in turn results in increased oxidative stress (Figure 2A). Lyso-PC has in fact been reported to activate PLD in coronary endothelial cells22 and PKC in SMCs.23 Evidence of activation of PKC has been observed in SMCs in atherosclerotic lesion at autopsy.24 In addition, PKC activation due to lyso-PC is reported to affect PLD activity, leading to the activation of membrane-bound PKC.23 Furthermore, it has been reported that PKC activation increases oxidative stress in SMCs.20 Thus, it is also possible that activation of PLD via PKC-α activation increases oxidative stress in SMCs. Increased oxidative stress is reported to play an important role in SMC migration.8 We have also confirmed that tyrosine kinase may be involved in PLD activation by lyso-PC in SMCs (Table 2), which was already shown for oxidized LDL in SMCs,26 and that tyrosine kinase may be involved in migration and oxidative stress (Figure 2A), a concept that is consistent with findings in neutrophils.27

We found in the present study that the HMG-CoA reductase inhibitors fluvastatin, simvastatin, and pravastatin directly suppressed an increase in oxidative stress, suggesting that the mechanism common to these drugs may underlie their effects on regulating oxidative stress in SMCs. The addition of mevalonate to HMG-CoA reductase inhibitor–treated SMCs restored not only their ability to activate PLD and PKC in SMCs (Table 1) but alsoler their ability to suppress SMC migration and oxidative stress (Figure 2B). This effect of HMG-CoA reductase inhibitors appears to be related to their inhibition of prenylation of heterotrimeric and low-molecular-weight guanosine triphosphate–binding proteins that are involved in signal transduction.28 Although the rather small effect of pravastatin on HMG-CoA reductase activity (≈20%) does not necessarily explain the 20% reduction in isoprenylation, this may be a mechanism by which HMG-CoA reductase inhibitors at least partially inhibit PLD activity, because mevalonate, farnesol, and geranylgeraniol but not squalene restored the PLD activity that had been suppressed by the HMG-CoA reductase inhibitor fluvastatin (Table 2). In fact, it has been reported that the βγ subunits of heterometric guanosine triphosphate–binding protein and the low-molecular-weight guanosine triphosphate–binding protein Rho mediate PLD activation in SMCs29 and that HMG-CoA reductase inhibitors induce isoprenylation in SMCs.30

In the present study, we also demonstrated for the first time that the hydroxylated metabolites of fluvastatin, M1 and M3, directly decreased the increase in SMC oxidative stress induced by lyso-PC through PLD and PKC suppression (Table 2). However, superoxide anion–scavenging properties of fluvastatin and its metabolites have been reported.31 Because PLD and PKC inhibitors completely blocked the increase in oxidative stress and SMC migration induced by lyso-PC, the predominant mechanism of the antioxidative and antimigratory effects of statins may be through PLD and PKC suppression rather than by direct scavenging properties. However, fluvastatin and its metabolites, which have less HMG-CoA reductase inhibitory action than does simvastatin, have antioxidation and antimigration effects equivalent to those of simvastatin (Table 1). This suggests that 2 different mechanisms (suppression of mevalonate pathway–mediated PLD activation and direct scavenging caused by 2 metabolically linked compounds) may coincidentally result in qualitatively and quantitatively similar effects: suppression of SMC oxidative stress and migration due to the action of fluvastatin.
In conclusion, our findings show that HMG-CoA reductase inhibitors may, at least in experimental models, affect the early events of atherogenesis by suppressing increased SMC oxidative stress and migration, possibly through local suppression of PLD and PKC activities.

Acknowledgments
The authors would like to thank Keiko Hirata and Sayuri Takagi for secretarial assistance.

References
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doi: 10.1161/01.ATV.21.6.937

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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