Lysophosphatidylcholine Inhibits Endothelial Cell Migration by Increasing Intracellular Calcium and Activating Calpain

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Objective—Endothelial cell (EC) migration, essential for reestablishing arterial integrity after vascular injury, is inhibited by oxidized LDL (oxLDL) and lysophosphatidylcholine (lysoPC) that are present in the arterial wall. We tested the hypothesis that a mechanism responsible for lysoPC-induced inhibition is increased intracellular free calcium concentration ([Ca$^{2+}$]).

Methods and Results—LysoPC, at concentrations that inhibit in vitro EC migration to 35% of control, increased [Ca$^{2+}$], levels 3-fold. These effects of lysoPC were concentration dependent and reversible. LysoPC induced Ca$^{2+}$ influx within 10 minutes, and [Ca$^{2+}$], remained elevated for 2 hours. The calcium ionophore A23187 also increased [Ca$^{2+}$], and inhibited EC migration. Chelators of intracellular Ca$^{2+}$ (BAPTA/AM and EGTA/AM) and nonvoltage-sensitive channel blockers (lanthanum chloride and gadolinium chloride) blunted the lysoPC-induced [Ca$^{2+}$], rise and partially preserved EC migration. After lysoPC treatment, calpain, a calcium-dependent cysteine protease, was activated, and cytoskeletal changes occurred. Calpain inhibitors (calpastatin, MDL28170, and calpeptin) added before lysoPC prevented cytoskeletal protein cleavage and preserved EC migration at 60% of control levels.

Conclusions—LysoPC increases [Ca$^{2+}$]. In turn, activating calpains that can alter the cytoskeleton are activated and EC migration is inhibited. 

Key Words: endothelium ▪ cell migration ▪ calcium ▪ calpain

Endothelial cell (EC) migration plays a critical role in a variety of normal and pathological processes, including angiogenesis, tumor metastasis, and restoration of endothelial integrity after arterial injury. EC movement is altered by many factors, including oxidatively modified lipids and lipoproteins. Previous studies have shown that oxidized LDL (oxLDL), but not unmodified LDL, suppresses EC migration in vitro. In addition, lysophosphatidylcholine (lysoPC), a major lipid constituent formed during oxidation of LDL, inhibits EC movement in vitro. LysoPC is abundant in the plasma and accumulates in atherosclerotic lesions. Thus, lysoPC might adversely affect restoration of endothelial integrity after angioplasty of an atherosclerotic vessel. Despite the potential clinical importance of suppressed EC migration, the mechanism(s) by which oxLDL or lysoPC inhibits EC movement is poorly understood.

LysoPC integrates into cell membranes and can cause structural and functional alterations. Inhibition of EC migration by lysophospholipids is related to chain length and head-group size, suggesting that spatial disruption of lipid packing in the membrane affects membrane function and alters membrane fluidity. In addition, lysoPC can alter ion channels and increase the intracellular free calcium concentration ([Ca$^{2+}$]) in ECs.

Several studies have shown that changes in [Ca$^{2+}$], are important in EC migration. An increase in [Ca$^{2+}$], is required to initiate movement after an EC monolayer is wounded. On the other hand, [Ca$^{2+}$], is lower in migrating ECs, especially at the leading edge, suggesting that precise regulation of [Ca$^{2+}$], is essential for normal migration. Therefore, the lysoPC-induced increase in [Ca$^{2+}$], may contribute to the inhibition of migration by disrupting the time- or site-specific changes in [Ca$^{2+}$], required for cell movement.

Calcium influx activates a number of intracellular proteases and signaling pathways. One such group of proteases is the calpains. Calpains are intracellular, nonlysosomal, cysteine proteases that are activated by increased [Ca$^{2+}$]. Substrates for calpains include a variety of cytoskeletal proteins, tyrosine kinases, and phosphatases. Activated calpain is required for disruption of integrin-cytoskeletal linkages at the rear of the cell while lamellipodia protrusions and new adhesions and cytoskeletal attachments are forming at the leading edge of the cell.

In the current study, we explore the causal relation between lysoPC-induced increases in [Ca$^{2+}$], activation of calpain and inhibition of EC migration. We demonstrate that lysoPC, at concentrations that inhibit migration, increases [Ca$^{2+}$]. The increase in [Ca$^{2+}$], can be blunted by calcium chelators and...
channel blockers, and these agents also preserve EC migration. Calpain activation is induced by lysoPC, and calpain inhibitors partially preserve EC migration in the presence of lysoPC, suggesting that the mechanism by which lysoPC inhibits EC migration involves increased [Ca\(^{2+}\)], and calpain activation.

**Methods**

**Materials**

Fura-2-AM, A23187, BAPTA/AM, EGTA/AM, calpeptin, MDL28170, and calpastatin peptide were purchased from Calbiochem. 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lysoPC) was purchased from Avanti Polar Lipids. Monoclonal antibodies against calpain and calpastatin were purchased from Biomol (Plymouth Meeting, Pa). Monoclonal antibodies to talin, filamin, and actin were obtained from Chemicon (Temecula, Calif). Other chemicals were purchased from Sigma.

**EC Culture and Cell Migration Assay**

Bovine aortic ECs were isolated from fresh adult bovine aortas by gentle scraping after collagenase exposure. Cells between passages 4 and 10 were grown in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient mixture (1:1, vol/vol) containing 10% fetal calf serum (Hyclone Laboratories). EC migration was measured by the razor wound assay as previously described. In brief, 12-well tissue-culture plates containing a confluent monolayer of ECs were incubated overnight in serum-free DMEM containing 0.1% gelatin. A sterile razor blade was gently pressed into the plastic to mark a start line and then swept laterally to remove cells on one side of that line. ECs were allowed to migrate for 24 hours, then fixed, and stained with a modified Wright-Giemsa stain. An observer, blinded to the experimental conditions, counted cell migration by capturing images of 3 fields corresponding to a starting line length of 1.34 mm with the use of a digital CCD camera mounted on a phase-contrast microscope. Results of each experiment represent data from triplicate wells, expressed as mean±SD of the number of cells crossing 1.34 mm of the starting line. The effect of various compounds on EC migration was expressed as a percentage of migration in control wells.

**Measurement of [Ca\(^{2+}\)]**

The [Ca\(^{2+}\)] was estimated by using the Ca\(^{2+}\)-binding fluorophore fura-2 as previously described. In brief, ECs were cultured in 35-mm dishes designed for fluorescence microscopy (Bioptechs) and made quiescent overnight in serum-free DMEM. Cells were loaded with fura-2-AM (1 μM/L) for 30 minutes and then washed with Krebs-Ringer (KR) buffer (125 mmol/L NaCl, 5 mmol/L KCl, 1.2 mmol/L MgSO\(_4\), 11 mmol/L HEPES, 2.5 mmol/L CaCl\(_2\), and 25 mmol/L HEPES at pH 7.4) to remove excess fura-2. After the baseline [Ca\(^{2+}\)] was established, lysoPC (12.5 μmol/L) was added to cells (left arrow) and the effect on [Ca\(^{2+}\)] was monitored. After [Ca\(^{2+}\)] plateaued, lysoPC was removed by washing the cells with KR buffer, and readings were allowed to return to baseline. LysoPC (12.5 μmol/L) was again added (middle and right arrows) and the effect on [Ca\(^{2+}\)] was monitored. The trace is representative of 3 separate experiments (// denotes the wash period, during which no data were collected).

**Results**

**LysoPC-Induced Increase in [Ca\(^{2+}\)] and Inhibition of EC Migration**

In view of evidence that lysoPC increases [Ca\(^{2+}\)], and that [Ca\(^{2+}\)] is important in migration, our initial objective was to establish the correlation between a lysoPC-induced rise in [Ca\(^{2+}\)], and inhibition of EC migration. LysoPC (12.5 μmol/L) induced a significant increase in [Ca\(^{2+}\)], as determined by the calcium-sensitive fluorophore fura-2. Typically, the [Ca\(^{2+}\)] rose 10 minutes after addition of lysoPC. After [Ca\(^{2+}\)] was established, lysoPC (12.5 μmol/L) was not cytotoxic and maximum effect was noted at 30 minutes, cells were solubilized in cold lysis buffer containing protease inhibitors [20 mmol/L Tris-HCl, pH 7.2, with 150 mmol/L NaCl, 1% Triton X-100, 0.1% SDS, 1 mmol/L EDTA, 10 μg/mL aprotinin, and 0.01 mmol/L 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride]. Proteins were separated on a 4% to 12% gradient SDS–polyacrylamide electrophoresis gel, transferred to a polyvinylidene difluoride membrane, and probed with specific antibodies.
treatment (Figure 3). The diminished rise in [Ca$^{2+}$], was accompanied by partial preservation of EC migration. LaCl$_3$ maintained EC migration in the presence of lysoPC at 68% of control level (Figure 3). Similar results were observed with gadolinium chloride, another nonvoltage-sensitive channel blocker (data not shown). Bovine ECs apparently lack voltage-gated calcium channels, so, as expected, the voltage-sensitive channel blockers nifedipine and nitrendipine did not alter the rise in [Ca$^{2+}$], induced by lysoPC (data not shown). Our data suggested that after lysoPC treatment, calcium entered ECs through nonvoltage-gated channels and perhaps through additional channels not affected by nonvoltage- or voltage-sensitive channel blockers.

**Figure 2.** Concentration-dependent increase [Ca$^{2+}$], and inhibition of migration by lysoPC. ECs were loaded with fura-2. After the baseline [Ca$^{2+}$], was established, increasing concentrations of lysoPC were added to ECs as indicated (3 μmol/L, single closed arrow; 5 μmol/L, double closed arrow; 7 μmol/L, single open arrow; and 10 μmol/L, double open arrow). Between lysoPC exposures, cells were washed with KR buffer, and readings returned to baseline. The trace is representative of 8 separate experiments (//denotes the wash period, during which no data were collected). Insert: EC migration. The migration assay was started, and lysoPC (0, 5, or 10 μmol/L) was added as indicated. The horizontal arrow indicates the starting line. Migration was stopped after 24 hours. The pictures are representative of 2 separate experiments, each done in triplicate.

**Figure 3.** Nonvoltage-sensitive calcium channel blockers blunt lysoPC-induced inhibition of EC migration and [Ca$^{2+}$] rise. Confluent ECs were incubated with medium or LaCl$_3$ (250 μmol/L) for 10 minutes before initiation of migration. LysoPC (12.5 μmol/L) and LaCl$_3$ (250 μmol/L) were added as indicated, and the number of cells that had crossed the starting line at 24 hours was quantified by image analysis. Results are mean±SD (n=8; *P<0.0001 compared with control; †P<0.0001 compared with lysoPC). Insert: [Ca$^{2+}$] analysis. ECs were treated with fura-2, then treated with lysoPC (12.5 μmol/L, arrow), washed to remove lysoPC and to allow [Ca$^{2+}$] to return to baseline, and then incubated with LaCl$_3$ (250 μmol/L) for 10 minutes before treatment with lysoPC (12.5 μmol/L, double arrow). The trace is representative of 5 separate experiments (//denotes the wash period, during which no data were collected).

**Figure 4.** Calcium chelators blunt lysoPC-induced inhibition of EC migration and [Ca$^{2+}$] rise. Confluent ECs were incubated with medium or BAPTA/AM (25 μmol/L) for 30 minutes before initiation of the migration assay. LysoPC (12.5 μmol/L) and BAPTA/AM (25 μmol/L) were added as indicated during the migration assay, and migration was quantified at 24 hours. Results are depicted as mean±SD (n=9; *P<0.0001 compared with control; †P<0.0001 compared with lysoPC). Insert: [Ca$^{2+}$] analysis. ECs were treated with lysoPC (12.5 μmol/L, arrow), washed to remove lysoPC and to allow [Ca$^{2+}$] to return to baseline, and then incubated with BAPTA/AM (25 μmol/L) for 30 minutes before addition of lysoPC (12.5 μmol/L, double arrow). The trace is representative of 5 separate experiments (//denotes the wash period, during which no data were collected).

**Calcium Chelators Preserved EC Migration in the Presence of LysoPC**

The importance of calcium in lysoPC-induced inhibition of EC migration was further assessed by blocking the rise in [Ca$^{2+}$], by loading cells with intracellular calcium chelators. ECs that were loaded with BAPTA/AM (25 μmol/L) for 30 minutes and then treated with lysoPC had a blunted rise in [Ca$^{2+}$], (Figure 4). At the same time, BAPTA/AM loading preserved EC migration to 67% of control levels (Figure 4). Similar to the observation with the channel blockers, BAPTA/AM did not completely prevent the increase in [Ca$^{2+}$], and this was correlated with incomplete preservation of EC migration in the presence of lysoPC. Similar effects on [Ca$^{2+}$], and migration were observed with EGTA/AM (data not shown). The observation that calcium channel blockers and calcium chelators blunted the increase in [Ca$^{2+}$], and partially preserved migration supported the hypothesis that the lysoPC-induced rise in [Ca$^{2+}$], inhibited EC migration.

**Calcium Ionophore Inhibited EC Migration**

The role of calcium in the inhibition of EC migration was studied with an alternative mechanism to increase [Ca$^{2+}$].
A23187, a calcium ionophore, increased \([Ca^{2+}]\), in a pattern closely resembling that of lysoPC, with a rapid and sustained elevation in \([Ca^{2+}]\) (data not shown). A23187 (100 nmol/L) decreased EC migration to 22% of control levels (n=8, P<0.0001). As was observed with lysoPC, the presence of an intracellular calcium chelator, BAPTA/AM, partially preserved migration of A23187-treated ECs, restoring migration to 44% of control level, significantly improved compared with A23187 alone (P=0.0005). LysoPC and A23187 were strikingly similar in their abilities to induce a sustained increase in \([Ca^{2+}]\), and inhibit EC migration.

A prolonged increase in \([Ca^{2+}]\), inhibited EC migration, but a transient rise in calcium did not affect cell movement in the razor scrape assay. Bradykinin (100 nmol/L), a substance that induces a rapid release of calcium from intracellular stores, increased \([Ca^{2+}]\), to a similar level as lysoPC, but the increase lasted for only 2 minutes (data not shown). Bradykinin had no effect on EC migration. The number of ECs migrating across 1.34 mm of the starting line in control wells was 440±113 compared with 422±121, 424±115, and 419±117 in 100 nmol/L, 200 nmol/L, and 500 nmol/L bradykinin, respectively (P>0.5). The inability of a transient rise in \([Ca^{2+}]\), to alter migration suggested that a prolonged elevation of \([Ca^{2+}]\), is essential for inhibition of EC migration by lysoPC.

**LysoPC Increased Activation of Calpain in ECs**

Changes in cell morphology noted during EC migration assays paralleled the time course of \([Ca^{2+}]\) changes. One hour after initiation of the migration assay, control ECs were elongated and beginning to extend lamellipodia. LysoPC-treated ECs, on the other hand, became rounded and remained so for 2.5 hours. Then ECs began to resume a more normal shape and by 4 hours had regained a flattened, spread appearance (data not shown). This observation prompted investigation into pathways causing cytoskeletal alterations.

Increased \([Ca^{2+}]\), can induce calpain activation with autolysis of the calpain molecule and increased proteolytic activity. Calpain autolysis during EC incubation with lysoPC was assessed by immunoblot analysis. A marked increase in the 76-kDa cleavage product, relative to the intact 80-kDa protein, was detected after 1 hour of exposure to lysoPC and persisted for at least 4 hours (data not shown). The intensity of the 76-kDa band slowly declined but was still increased at 24 hours compared with baseline. The level of calpastatin, the natural inhibitor of calpain, was also assessed by immunoblot analysis, and no changes in calpastatin levels were detected during intervals ranging from 1 to 24 hours (data not shown). This suggested that calpain activity was increased.

To confirm that calpain proteolytic activity was increased in response to lysoPC, the appearance of talin and filamin degradation products in cell lysates was assessed by immunoblot analysis. LysoPC treatment of ECs resulted in increased calpain proteolytic activity within 1 hour, as indicated by a rise in the 47-kDa breakdown product of talin, and the cleavage product was elevated for at least 4 hours (Figure 5). The time course paralleled that in the immunoblot analysis of calpain autolysis. Similar results were observed for filamin degradation after lysoPC treatment of ECs (data not shown). Inhibitors of calpain, including calpastatin (1 μmol/L), Figure 6), calpeptin (1 μmol/L), and MDL28170 (1 μmol/L), inhibited the lysoPC-induced talin degradation. Interestingly, intracellular calcium chelators and the nonvoltage-sensitive channel blockers, BAPTA/AM and LaCl3, also prevented activation of calpain (data not shown), suggesting that the lysoPC-induced rise in \([Ca^{2+}]\), caused calpain activation.

**Calpain Inhibitors Preserved Migration in LysoPC-Treated ECs**

The role of calpain activation in the lysoPC-induced inhibition of EC migration was also assessed. Calpastatin peptide, at a concentration that prevented the degradation of talin,
Discussion

Cell migration involves multiple simultaneous processes. These include focal adhesion formation and insertion of membrane mass at the leading edge of the cell, intracellular force generation by actin polymerization, and focal adhesion disassembly and retrieval of membrane mass at the rear of the cell. These dynamic processes require complex intracellular signaling events including calcium influx. OxLDL and one of its major lipid components, lysoPC, inhibited EC migration, and both oxLDL and lysoPC increased intracellular [Ca\(^{2+}\)]\(i\). This study focused on the causal relation between lysoPC-mediated changes in [Ca\(^{2+}\)]\(i\), and the inhibition of EC migration.

The rise in [Ca\(^{2+}\)]\(i\), after addition of lysoPC requires influx of Ca\(^{2+}\) from the extracellular space through nonselective calcium channels. Inoue et al.\(^9\) suggest that lysoPC causes a biphasic increase in [Ca\(^{2+}\)]\(i\), with a rapid initial increase caused by release of calcium from intracellular stores followed by a sustained elevation in [Ca\(^{2+}\)]\(i\) due to influx of extracellular calcium. In our experiments, no early spike in [Ca\(^{2+}\)]\(i\), was seen after addition of lysoPC. One explanation for the discrepancy might be contamination of some commercial preparations of lysoPC with phospholipids that have platelet-activating factor (PAF) activity,\(^21\) and PAF causes a rapid increase in [Ca\(^{2+}\)]\(i\) in ECs. PAF-like activity does not appear to be responsible for our findings, because PAF receptor antagonists and pretreatment of the lysoPC preparations with phospholipase A\(_2\), which would remove sn-2 residues from PAF but not affect the structure of lysoPC, did not alter the effect on EC migration (data not shown).

Calcium flux plays a critical role in migration, and highly regulated [Ca\(^{2+}\)]\(i\), transients must occur for normal migration. A spike in [Ca\(^{2+}\)]\(i\), is required to initiate EC migration,\(^9\) and agents that inhibit calcium influx delay healing.\(^23\) On the other hand, a sustained increase of [Ca\(^{2+}\)]\(i\), by lysoPC, calcium ionophore, or thapsigargin in the presence of extracellular Ca\(^{2+}\) (data not shown) inhibits migration. Thus, both inhibition and excessive stimulation of calcium influx can suppress migration, perhaps by disturbing highly localized [Ca\(^{2+}\)]\(i\), transients that regulate cell attachment to the substratum at the leading edge and de-adhesion at the rear of the cell.

Precise site-specific regulation of calpain activation is also required for normal cell migration. Calpain is selectively activated at sites of integrin signaling complexes and can cleave proteins in these complexes, including integrin subunits and cytoskeletal proteins.\(^24\) Interruption of calpain activation can impair cell movement, as shown in calpain-deficient mouse fibroblasts and Chinese hamster ovary cells.\(^12,25\) Overexpression of calpastatin, the endogenous calpain inhibitor,\(^13\) or addition of exogenous calpain inhibitors\(^12\) impedes cell spreading and migration. These reports suggest that calpain is required for moving cells to detach from their substrate at their trailing edge and extend lamellipodia at their leading edge. On the other hand, our studies show that a generalized, sustained activation of calpain also inhibits EC migration. Transfected ECs that overexpress \(\mu\)-calpain have an overspread appearance, with increased stress fibers and focal adhesions.\(^26\) In our preliminary studies, these cells have very limited capacity to migrate (data not shown), and 5 \(\mu\)mol/L lysoPC disrupts focal adhesions, causing cells to lift from the substratum. These studies suggest that generalized inhibition or activation of calpain disrupts the time- and site-specific regulation required for normal cell migration and results in inhibition of movement.

Our studies show that calpain inhibitors (calpeptin, MDL28170, and calpastatin) partially preserve EC migration in lysoPC, although calpain inhibitors are reported to block cell spreading. This apparent contradiction is explained by our use of very low concentrations of these inhibitors (1 \(\mu\)mol/L), selected on the basis of preliminary studies that showed no effect on basal EC migration but prevention of lysoPC-induced calpain proteolytic activity as measured by talin breakdown. These concentrations are far below the 50 to 275 \(\mu\)mol/L concentrations reported to inhibit cell spreading.\(^13,26\) Most calpain inhibitors are not specific and can block cysteine and serine proteases;\(^27\) and calpeptin can inhibit protein tyrosine phosphatases.\(^28\) Calpastatin, however, is a specific inhibitor of calpain and does not inhibit other cysteine or serine proteases in vitro.\(^29\) The ability of calpastatin to preserve migration of lysoPC-treated ECs supports the hypothesis that diffuse, sustained calpain activation is a mechanism by which lysoPC impairs movement.

Our results suggest that lysoPC inhibits migration in part by inducing an increase in [Ca\(^{2+}\)]\(i\), that upregulates calpain activity. Calpain, in turn, can cleave cytoskeletal proteins, alter focal adhesion plaques, and activate tyrosine kinases and phosphatases.\(^11,24\) Blunting the increase in [Ca\(^{2+}\)]\(i\) partially preserves EC migration. The morphological changes of ECs after addition of lysoPC suggests that calpain impairs migration by excessive breakdown of cytoskeletal proteins. Although lysoPC also inhibits human EC migration in vitro (data not shown), assignment of clinical significance to these findings is premature. Further in vitro and in vivo studies are needed to better understand the mechanisms by which lipid oxidation products inhibit cell movement and to develop therapeutic agents to promote migration and restore endothelial integrity after angioplasty.

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References


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