Activation of the cytosolic calcium-independent phospholipase A₂ β isoform contributes to TRPC6 externalization *via* release of arachidonic acid

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During vascular interventions, oxidized low-density lipoprotein and lysophosphatidylcholine (lysoPC) accumulate at the site of arterial injury, inhibiting endothelial cell (EC) migration and arterial healing. LysoPC activates canonical transient receptor potential 6 (TRPC6) channels, leading to a prolonged increase in intracellular calcium ion concentration that inhibits EC migration. However, an initial increase in intracellular calcium ion concentration is required to activate TRPC6, and this mechanism remains elusive. We hypothesized that lysoPC activates the lipid-cleaving enzyme phospholipase A₂ (PLA₂), which releases arachidonic acid (AA) from the cellular membrane to open arachidonate-regulated calcium channels, allowing calcium influx that promotes externalization and activation of TRPC6 channels. The focus of this study was to identify the roles of calcium-dependent and/or calcium-independent PLA₂ in lysoPC-induced TRPC6 externalization. We show that lysoPC induced PLA₂ enzymatic activity and caused AA release in bovine aortic ECs. To identify the specific subgroup and the isoform(s) of PLA₂ involved in lysoPC-induced TRPC6 activation, transient knockdown studies were performed in the human endothelial cell line EA.hy926 using siRNA to inhibit the expression of genes encoding cPLA₂α, cPLA₂γ, iPLA₂β, or iPLA₂γ. Downregulation of the β isoform of iPLA₂ blocked lysoPC-induced release of AA from EC membranes and TRPC6 externalization, as well as preserved EC migration in the presence of lysoPC. We propose that blocking TRPC6 activation and promoting endothelial healing could improve the outcomes for patients undergoing cardiovascular interventions.

Endothelial cell (EC) healing is crucial for successful vascular interventions (1–3). Oxidized low-density lipoprotein (oxLDL) accumulates at the site of arterial injury caused by vascular interventions and inhibits EC migration. The major component of oxLDL that accounts for its antimigratory property is lysophosphatidylcholine (lysoPC) (4). We have previously shown that lysoPC inhibits EC migration *in vitro*
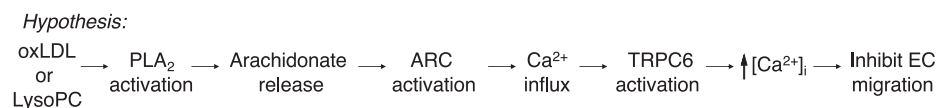
(5), and hypercholesterolemia inhibits EC healing of arterial injuries *in vivo* (6). One of the mechanisms involved in inhibition of EC migration/healing is the activation of canonical transient receptor potential (TRPC) channels, specifically TRPC6, and the subsequent prolonged increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) (7, 8). A transient increase in the $[Ca^{2+}]_i$ is essential to initiate EC migration (9, 10). However, the sustained increase in $[Ca^{2+}]_i$ specifically due to TRPC6 to TRPC5 channel activation cascade (11) disrupts EC focal adhesions and cytoskeleton that regulate cell movement, thus impeding EC migration essential for injury repair (5). In a mouse arterial injury model, a high-cholesterol diet significantly impairs endothelial healing in WT mice but is not inhibitory in TRPC6 null mice (6). This suggests that blocking lipid oxidation product(s)-induced TRPC6 activation could promote more rapid EC healing leading to improved outcomes after vascular interventions.

TRPC6 channel translocation (*i.e.*, externalization) to the plasma membrane is an essential step that proceeds TRPC6 channel activation. Our previous studies suggest that lysoPC causes an initial local increase in $[Ca^{2+}]_i$ that is essential to activate TRPC6 channels (8). However, the mechanism by which lysoPC activates TRPC6 and, more specifically, the mechanism of lysoPC-induced TRPC6 externalization to the plasma membrane still remain unclear. LysoPC can activate phospholipase A₂ (PLA₂) to release arachidonic acid (AA) from EC membranes (12, 13). This AA can activate arachidonate-regulated calcium channels in the plasma membrane (14), and the subsequent Ca²⁺ entry can provide the local increase in $[Ca^{2+}]_i$ required to externalize TRPC6 channels. Our working hypothesis is outlined in Figure 1. Currently, no TRPC6 inhibitors are available for clinical use, but inhibiting PLA₂ activity could potentially block the lysoPC-induced TRPC6 externalization and, therefore, block the activation pathway.

PLA₂ is a superfamily of at least 16 groups of enzymes responsible for the breakdown of glycerophospholipids into lysophospholipids and generation of AA required for eicosanoid and prostaglandin synthesis (15, 16). PLA₂ enzymes are broadly divided into secretory, cytosolic calcium-dependent

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Hypothesis:

oxLDL or LysoPC → PLA₂ activation → Arachidonate release → ARC activation → Ca²⁺ influx → TRPC6 activation → ↑[Ca²⁺]ᵢ → Inhibit EC migration

Figure 1. Schematic of our working hypothesis. We hypothesize that lysoPC activates phospholipase A₂ (PLA₂), which releases arachidonic acid (AA) from the cellular membrane to open arachidonate-regulated calcium (ARC) channels allowing calcium influx that causes TRPC6 channel externalization. The cascade of events after TRPC6 externalization in turn inhibits EC migration. EC, endothelial cell; lysoPC, lysophosphatidylcholine; oxLDL, oxidized low-density lipoprotein; TRPC, canonical transient receptor potential.

(group IV or cPLA₂), and cytosolic calcium-independent (group VI or iPLA₂) subgroups (16). cPLA₂ and iPLA₂ are the two subgroups most abundantly present in ECs (17). These two subgroups are further divided into various isoforms, including cPLA₂-α, cPLA₂-β, cPLA₂-γ, and cPLA₂-δ, and iPLA₂-β, iPLA₂-γ, -iPLA₂-ζ, -iPLA₂-η, and -iPLA₂-δ (15). The cPLA₂α-isoform (or group IVA) is the most abundant and well-studied cPLA₂ isoform, and it contains the characteristic calcium-binding C2 domain required for its activation. However, this calcium-binding C2 domain is lacking in the γ iso-form of cPLA₂ (group IVC), thus making cPLA₂γ the only known calcium-independent group IV cPLA₂ isoform (15). cPLA₂α is involved in EC proliferation and cell cycle progression (18, 19). cPLA₂γ, although present in the heart, skeletal muscle, and cultured synoviocytes, has unclear biological function (20). Among group VI iPLA₂, the well-described isoforms are iPLA₂β and iPLA₂γ, and both are involved in cell proliferation and membrane remodeling, among other functions (21).

The purpose of this study is to identify PLA₂ subgroup(s) and the specific isoform(s) that contribute to lysoPC-induced TRPC6 externalization and inhibition of EC migration. We show that iPLA₂β is the primary isoform involved in lysoPC-induced TRPC6 externalization. Inhibiting iPLA₂β blocks lysoPC-induced AA release from EC membranes, blocks TRPC6 externalization, and preserves EC migration.

Results

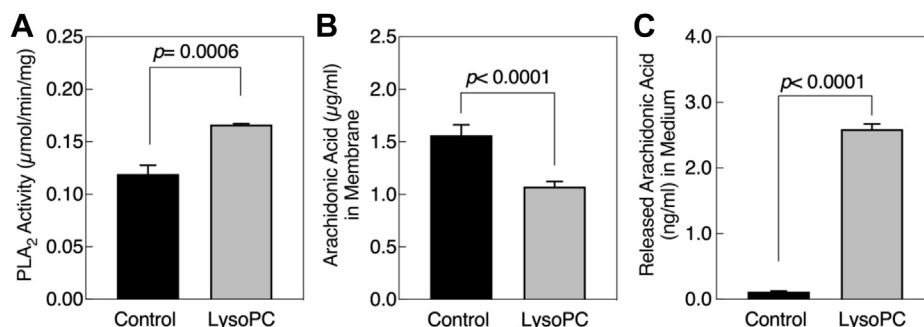### LysoPC activated phospholipase enzyme activity and caused AA release in bovine aortic ECs

To determine if lysoPC activated PLA₂, PLA₂ enzyme activity was assessed in bovine aortic ECs (BAECs). ECs were serum-starved for 18 h, and then, 12.5 μM lysoPC was added for 15 min. The cells were lysed, and supernatants were used to determine total PLA₂ activity with a synthetic substrate, arachidonoyl thio-PC. Under control conditions, PLA₂ activity was 0.119 ± 0.008 μmol/min/mg (Fig. 2A, control). LysoPC increased PLA₂ activity to 0.166 ± 0.001 μmol/min/mg (Fig. 2A, LysoPC), significantly higher than control conditions (n = 3, $p = 0.0006$).

Next, to determine if lysoPC induced release of AA, the AA content of the membrane fraction and the medium was measured. Serum-starved BAECs were incubated with or without 12.5 μM lysoPC for 15 min. The membrane fraction and the medium were isolated to determine the AA content. In the membrane fraction, the AA content was 1.56 ± 0.10 μg/ml in control cells but was reduced to 1.07 ± 0.048 μg/ml in cells incubated with lysoPC (n = 3, $p < 0.0001$, Fig. 2B). In contrast, the AA content in the medium was 0.11 ± 0.008 ng/ml in control cells, which increased to 2.59 ± 0.080 ng/ml in cells incubated with lysoPC (n = 3, $p < 0.0001$, Fig. 2C). The reduction in the membrane AA content suggested that lysoPC induced the release of AA from the BAEC membranes.

### Downregulation of iPLA₂β but not iPLA₂γ, cPLA₂α, or cPLA₂γ blocked lysoPC-induced TRPC6 externalization

To identify the role of cPLA₂ or iPLA₂ and the specific isoform(s) involved in lysoPC-induced TRPC6 externalization, siRNA-mediated downregulation of PLA₂ isoforms was undertaken. EA.hy926 cells, a human umbilical vein cell line, were transiently transfected with 25 nmol of cPLA₂α siRNA, cPLA₂γ siRNA, iPLA₂β siRNA, or iPLA₂γ siRNA. This resulted in a significant decrease in the mRNA levels of the respective isoforms in the siRNA-transfected cells compared with the

Figure 2. LysoPC activates phospholipase A₂ and induces arachidonic acid release from the membrane into the medium. A, confluent BAECs were serum-starved for 18 h and then incubated with or without lysoPC (12.5 μM) for 15 min. The cells were then lysed, and the supernatant was assessed for total PLA₂ enzyme activity. B and C, confluent BAECs were serum-starved for 18 h and then incubated with or without lysoPC (12.5 μM) for 15 min, and cells were lysed and the membrane fraction isolated. B, AA content of the membrane fraction was measured by ELISA. C, AA released into the medium was measured by LC/MS/MS. Values shown are the means ± SD (n = 3), analyzed with Student's t test and p values calculated. BAECs, bovine aortic ECs; lysoPC, lysophosphatidylcholine; PLA₂, phospholipase A₂.

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negative control siRNA (NsiRNA)-transfected cells (n = 3; Fig. 3, A–D). cPLA$_2$α and γ mRNA levels decreased by 96% (n = 3, p = 0.0003, Fig. 3A) and 91% (n = 3, p = 0.0001, Fig. 3B), respectively. iPLA$_2$β and γ mRNA levels decreased by 78% (n = 3, p = 0.0014, Fig. 3C) and 96% (n = 3, p = 0.0001, Fig. 3D), respectively. Downregulation of one isoform did not significantly affect the mRNA expression of the other isoform of the same subgroup (Fig. 3, A–D).
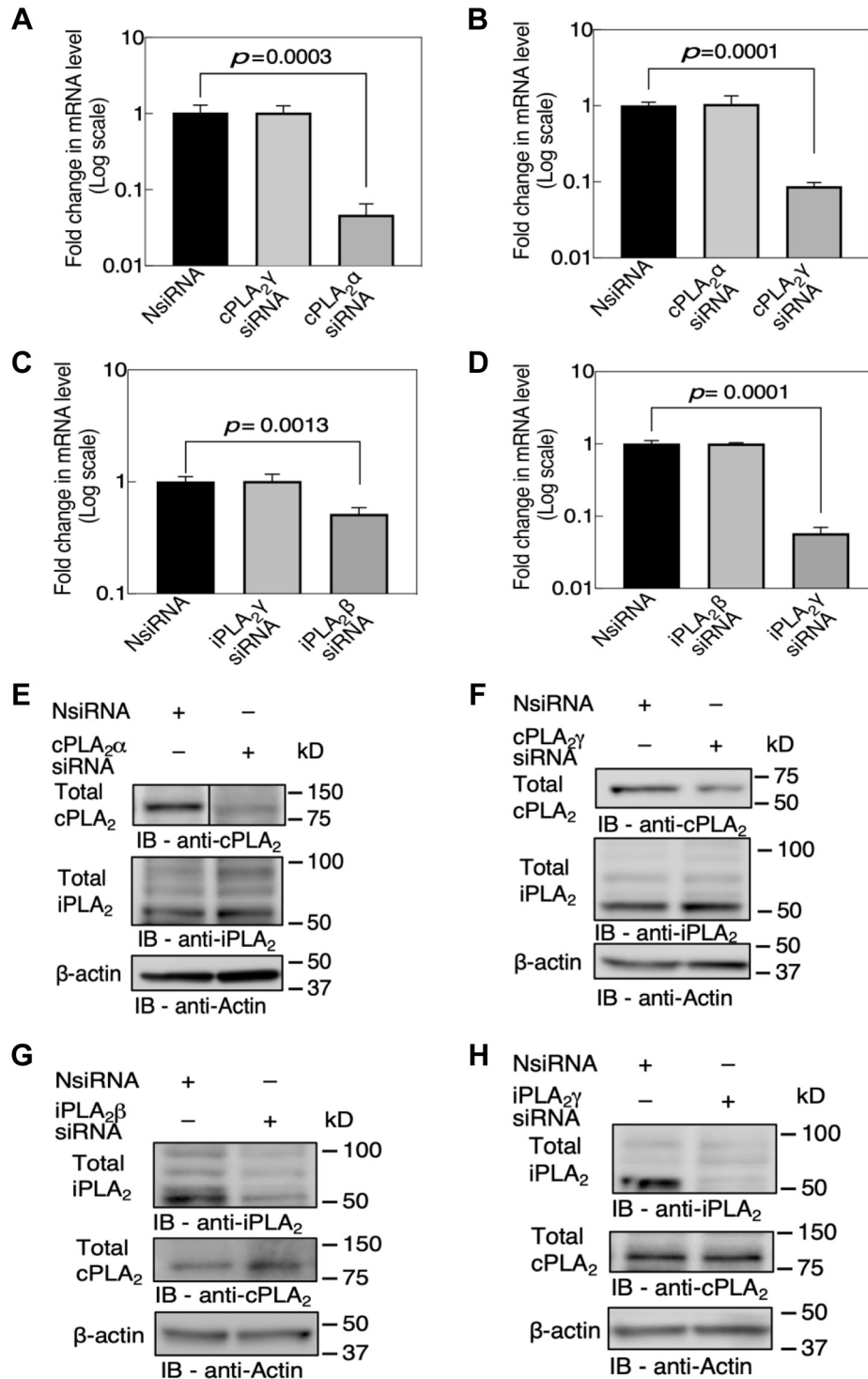
Figure 3. mRNA and protein expression in siRNA-mediated subgroup-specific PLA$_2$ isoform downregulation. EA.hy926 cells were transiently transfected with 25 nM of control siRNA (NsiRNA) or cPLA$_2$α, cPLA$_2$γ, iPLA$_2$β, or iPLA$_2$γ siRNA for 6 h in serum-free medium and then placed in medium with 10% FBS. A–D, siRNA-mediated downregulation of (A) cPLA$_2$α, (B) cPLA$_2$γ (C) iPLA$_2$β, or (D) iPLA$_2$γ was quantified using qRT-PCR at 48 h. Values shown are the means ± SD (n = 3), analyzed with one-way ANOVA using Tukey's multiple comparison test, and p values were calculated. *E* and *F*, representative immunoblots depict siRNA-mediated downregulation of PLA$_2$ isoforms (*E*) cPLA$_2$α, (*F*) cPLA$_2$γ (*G*) iPLA$_2$β, and (*H*) iPLA$_2$γ, detected using subgroup-specific antibody at 48 h after initiation of transfection (n = 3). *Line* in panel *E* indicates lanes rearranged from the same gel. FBS, fetal bovine serum; PLA$_2$, phospholipase A$_2$.

Role of iPLA$_2$ in TRPC6 externalization

The decrease in mRNA levels corresponded to a decrease in the protein levels (Fig. 3, E–H). cPLA$_2$ band at ∼90 kD, representing the α-isoform, was present in the NsiRNA-transfected cells and was significantly attenuated in the cPLA$_2$α siRNA-transfected cells (Fig. 3E). Similarly, cPLA$_2$ band at ∼60 kD, representing the cPLA$_2$γ isoform, was present in the NsiRNA-transfected cells and was significantly attenuated in the cPLA$_2$γ siRNA-transfected cells (Fig. 3F). Two distinct bands for iPLA$_2$, at ∼90 kDa and ∼63 kDa, were seen in the NsiRNA-transfected cells and were significantly attenuated in both iPLA$_2$β and iPLA$_2$γ siRNA-transfected cells (Fig. 3, G and H). Downregulation of one subgroup did not affect the protein expression of the other subgroup (Fig. 3, E–H).

The effect of cPLA$_2$α, cPLA$_2$γ, iPLA$_2$β, or iPLA$_2$γ downregulation on TRPC6 externalization was assessed by biotinylation assay in transfected EA.hy926 cells (Fig. 4, A–D). At baseline, externalized TRPC6 was comparable for control (NsiRNA), cPLA$_2$α, cPLA$_2$γ, iPLA$_2$β, or iPLA$_2$γ siRNA-transfected cells. Incubation with 10 μM lysoPC for 15 min increased TRPC6 externalization in ECs transfected with NsiRNA. Downregulation of cPLA$_2$α did not result in a significant decrease in lysoPC-induced TRPC6 externalization (n = 3; $p = 0.057$ compared with NsiRNA with lysoPC, Fig. 4A). Similarly, downregulation of cPLA$_2$γ isoform did not block lysoPC-induced TRPC6 externalization (n = 3; $p = 0.53$ compared with NsiRNA with lysoPC, Fig. 4B). Interestingly, the lysoPC-induced TRPC6 externalization was significantly attenuated in iPLA$_2$β downregulated cells compared with NsiRNA with lysoPC (n = 3, $p < 0.003$, Fig. 4C). Transient knockdown of iPLA$_2$γ, however, did not alter the lysoPC-induced TRPC6 externalization (n = 3; $p > 0.8$ compared with NsiRNA with lysoPC, Fig. 4D). These results suggested that lysoPC primarily activated iPLA$_2$β to promote externalization of TRPC6.

Downregulation of iPLA$_2$β and not iPLA$_2$γ, cPLA$_2$α, or cPLA$_2$γ blocked lysoPC-induced inhibition of EC migration

The effect of cPLA$_2$α, cPLA$_2$γ, iPLA$_2$β, or iPLA$_2$γ downregulation on lysoPC-induced inhibition of EC migration was assessed by razor scrape assay in EA.hy926 transfected cells (Fig. 5). Basal EC migration for NsiRNA, cPLA$_2$α siRNA, cPLA$_2$γ siRNA, iPLA$_2$β siRNA, or iPLA$_2$γ siRNA-transfected cells was similar (Fig. 5, A–D). In ECs transfected with NsiRNA, lysoPC reduced migration by ∼55 to 60% (n = 3; $p < 0.001$, Fig. 5, A–D). In cPLA$_2$α siRNA-transfected ECs, lysoPC inhibited migration by ∼70% (n = 3; $p = 0.44$, comparable with NsiRNA with lysoPC, Fig. 5A). Similarly, in cPLA$_2$γ siRNA-transfected ECs, lysoPC inhibited migration by ∼63% (n = 3; $p = 0.47$ comparable with NsiRNA with lysoPC, Fig. 5B). However, lysoPC inhibited migration by only 15% in iPLA$_2$β downregulated cells (n = 3; $p < 0.001$ compared with NsiRNA with lysoPC, Fig. 5C). LysoPC continued to inhibit migration in iPLA$_2$γ downregulated ECs, as it did in NsiRNA-transfected EC with lysoPC (n = 3; $p > 0.9$, Fig. 5D). Individual siRNAs were studied to determine if the effect on migration

was due to off-target effect of pooled iPLA$_2$β siRNA. The individual iPLA$_2$β siRNAs (35 nM) showed similar effects compared with the pooled iPLA$_2$β siRNA in preserving EC migration in lysoPC (Fig. S1, A and B). These results suggested that lysoPC activated iPLA$_2$β to inhibit EC migration in an *in vitro* migration assay.

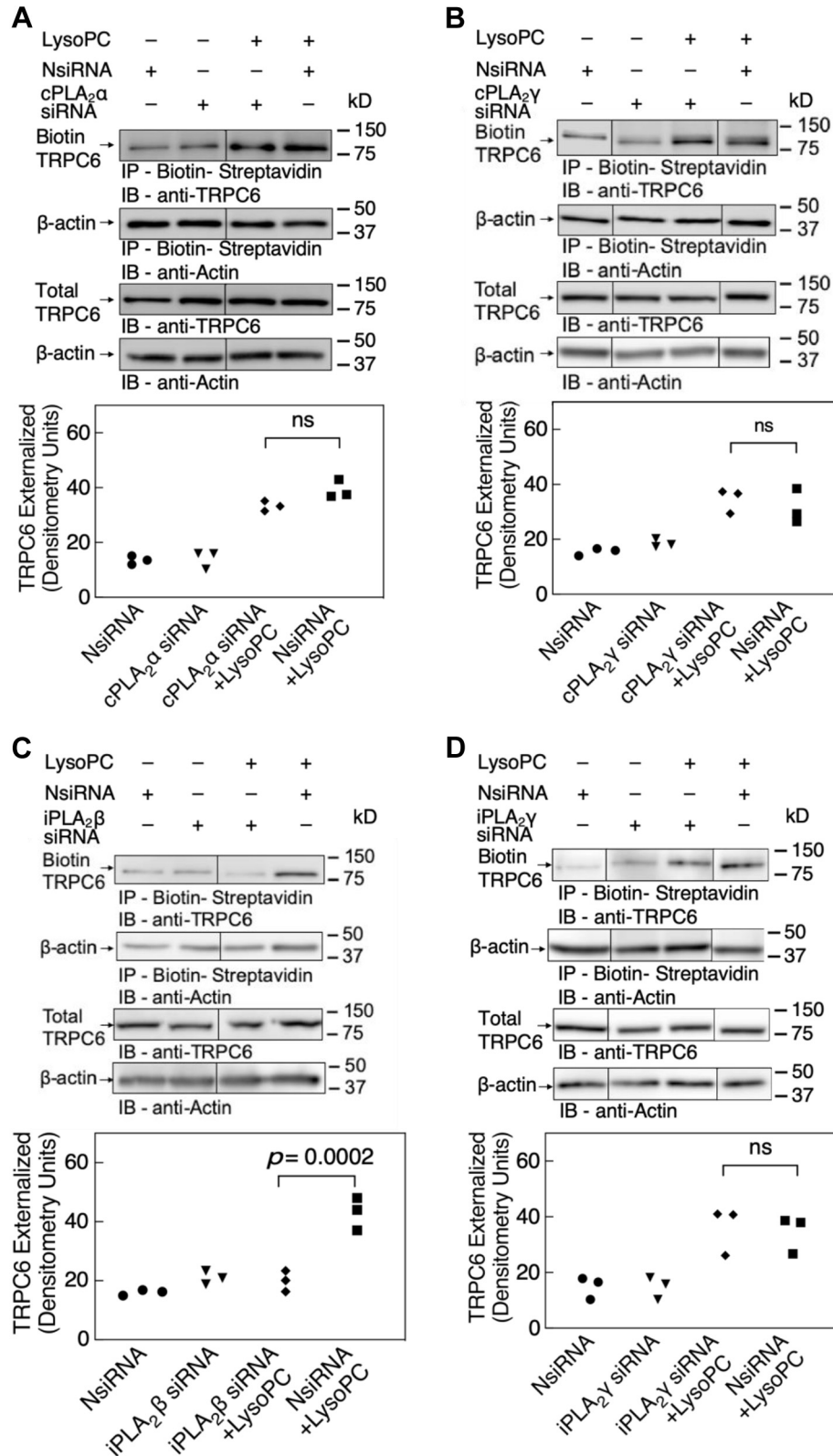### Downregulation of iPLA$_2$β inhibited lysoPC-induced release of AA

To ascertain if downregulation of iPLA$_2$β prevented the lysoPC-induced AA release from the membrane, AA ELISA assay was performed in transfected EA.hy926 cells. Cells were incubated with lysoPC for 15 min, then lysed, the membrane and medium fractions were isolated to assess the AA content (Fig. 6). AA content for NsiRNA and iPLA$_2$β siRNA transfected cell membranes was similar. LysoPC decreased the AA in the membrane in NsiRNA-transfected cells by 0.175 ± 0.041 μg/ml, but by only 0.0583 ± 0.050 μg/ml in iPLA$_2$β siRNA-transfected cells (n = 3, $p < 0.036$, Fig. 6A). This confirmed that iPLA$_2$β downregulation blocked lysoPC-induced release of AA from EC membranes. Similarly, lysoPC increased the AA content in the medium fraction of NsiRNA-transfected cells by 2.433 ± 0.305 ng/ml, but by only 1.4 ± 0.1 ng/ml in iPLA$_2$β siRNA-transfected cells (n = 3, $p < 0.005$, Fig. 6B). The prevention of lysoPC-induced AA release from EC membranes in iPLA$_2$β downregulated cells could contribute to the preservation of EC migration in the presence of lysoPC.

Downregulation of iPLA$_2$β inhibited lysoPC-induced increase in $[Ca^{2+}]_i$

We evaluated if downregulation of iPLA$_2$β prevented the lysoPC-induced increase in $[Ca^{2+}]_i$ using fluorometric assay. EA.hy926 cells transfected with NsiRNA or iPLA$_2$β siRNA were loaded with the FITC-conjugated fluorophore Calbryte 520 AM dye. The ECs were suspended and loaded into the sort chamber of a BD FACSMelody Cell Sorter maintained at 37 °C. After adjusting the baseline, lysoPC (10 μM) was added and the change in $[Ca^{2+}]_i$ was recorded. LysoPC increased $[Ca^{2+}]_i$ in NsiRNA-transfected cells to 1.38 times the baseline (representative graph, Fig. 7A), but only to 1.02 times in iPLA$_2$β siRNA-transfected cells (representative graph, Fig. 7B). iPLA$_2$β siRNA-transfected cells significantly attenuated lysoPC-induced increase in $[Ca^{2+}]_i$ (n = 3, $p < 0.029$, Fig. 7C). These results supported the role of iPLA$_2$β in lysoPC-induced increase in $[Ca^{2+}]_i$ required for TRPC6 externalization and inhibition of EC migration.

Discussion

OxLDL and lysoPC inhibit EC migration, and there is sufficient lysoPC in oxLDL to account for its antimigratory activity (4). LysoPC is one of the most potent antimigratory lysophospholipids, and our previous studies have shown that it inhibits EC migration at least in part by activating TRPC6, which leads to a cascade of events resulting in a prolonged increase in $[Ca^{2+}]_i$ that activates calpains and inhibits

Figure 4. Downregulation of iPLA$_2$β isoform blocks lysoPC-induced TRPC6 externalization. *A–D*, ECs were transiently transfected with NsiRNA or isoform-specific siRNA and serum-starved for 6 h. Then, lysoPC (10 μM) was added for 15 min, and externalized TRPC6 was detected by biotinylation assay. Total TRPC6 was detected in an aliquot of the cell lysate removed before biotinylation, and actin served as a loading control. Representative blots are shown in panel (*A*) cPLA$_2$α, (*B*) cPLA$_2$γ, (*C*) iPLA$_2$β, and (*D*) iPLA$_2$γ. *Lines* indicate lanes rearranged from the same gel. Densitometric measurements of externalized TRPC6 are represented in graphic form (n = 3), analyzed with one-way ANOVA using Tukey's multiple comparison test, and *p* values were calculated. NsiRNA (•); cPLA$_2$α, cPLA$_2$γ, iPLA$_2$β, or iPLA$_2$γ siRNA (▼); cPLA$_2$α, cPLA$_2$γ, iPLA$_2$β, or iPLA$_2$γ siRNA + lysoPC (◆); NsiRNA + lysoPC (■). iPLA$_2$, cytosolic calcium-independent PLA$_2$; lysoPC, lysophosphatidylcholine; ns, not significant; NsiRNA, negative control siRNA; PLA$_2$, phospholipase A$_2$; TRPC, canonical transient receptor potential.
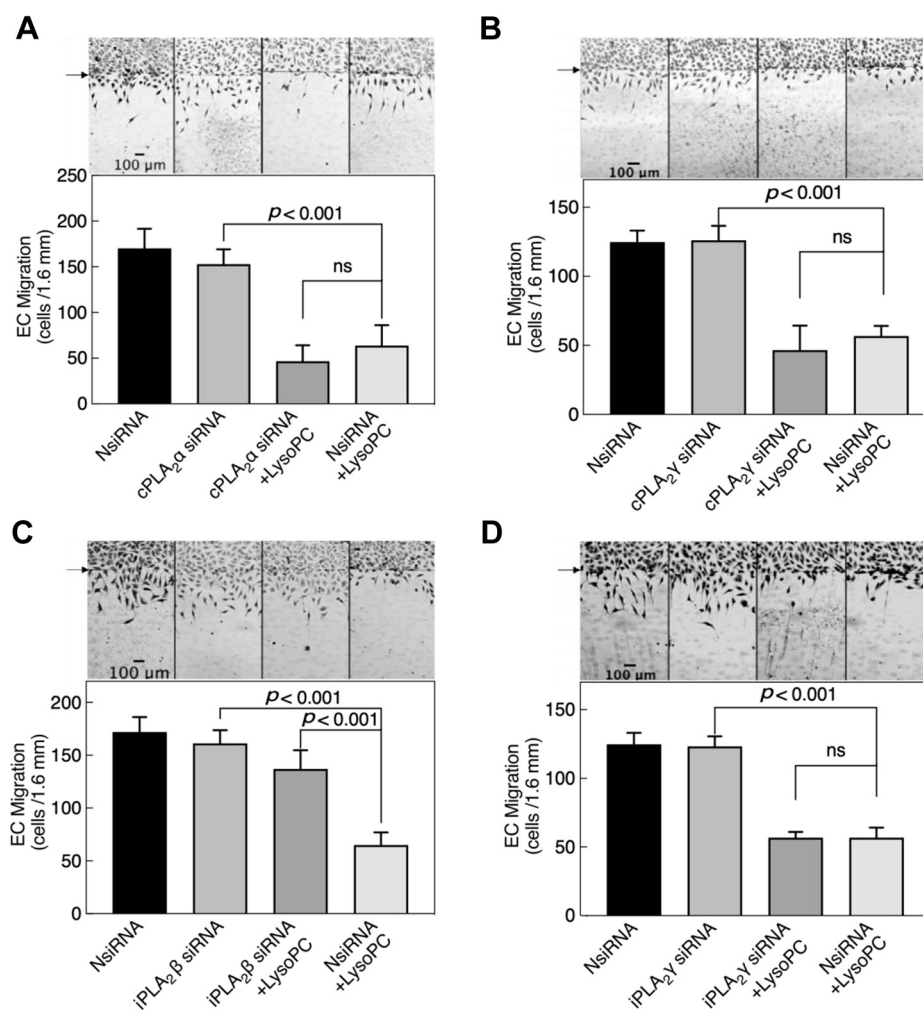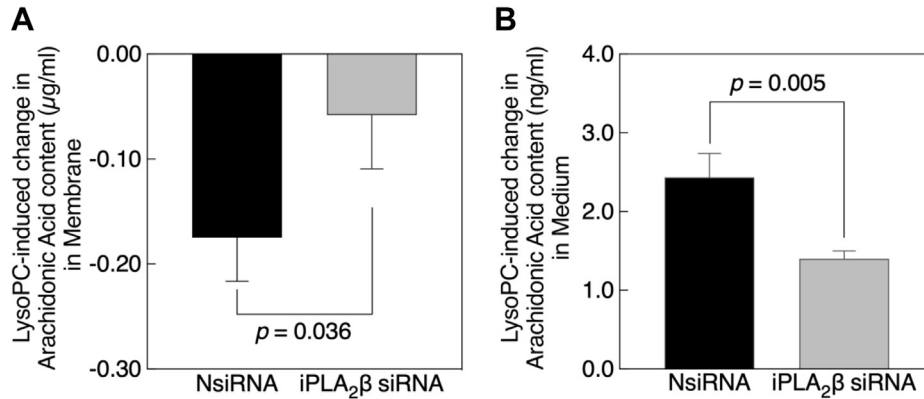
Figure 5. Downregulation of iPLA$_2$β isoform preserves EC migration in the presence of lysoPC. *A–D*, ECs were transiently transfected with NsiRNA or isoform-specific siRNA and then serum-starved for 6 h. The migration assay was initiated ± lysoPC (10 μM). Migration was quantified at 24 h. The *arrow* indicates the starting line of cell migration for assessment of the effect of (*A*) cPLA$_2$α, and (*B*) cPLA$_2$γ, (*C*) iPLA$_2$β, or (*D*) iPLA$_2$γ downregulation. The graphs represent mean ± SD (n = 4), analyzed with one-way ANOVA using Tukey's multiple comparison test, and *p* values were calculated. Representative images of four experiments are shown, 40× magnification; the scale bar represents 100 μm. cPLA$_2$, cytosolic calcium-dependent PLA$_2$; EC, endothelial cell; iPLA$_2$, cytosolic calcium-independent PLA$_2$; lysoPC, lysophosphatidylcholine; ns, not significant; NsiRNA, negative control siRNA.

cytoskeletal changes required for migration ([5], [11]). LysoPC-induced TRPC6 externalization requires a small, perhaps localized, increase in [Ca^{2+}]$_i$ ([8]); however, the source of the initial lysoPC-induced calcium flux in ECs is unclear. We postulate that lysoPC activates PLA$_2$ causing release of AA, which in turn opens arachidonate-regulated calcium channels leading to the localized increase in calcium. The goal of this study is to identify the PLA$_2$ involved in lysoPC-induced TRPC6 externalization and activation leading to inhibition of EC migration. The results presented here demonstrate that lysoPC activates PLA$_2$, in keeping with the findings of Lupo *et al.* ([22]) using rat brain ECs and oxLDLs. We also show that lysoPC releases AA from the cell membrane, which is in agreement with Wong *et al.* ([12]) who showed lysoPC induces AA release in human umbilical vein ECs. Interestingly, we show that iPLA$_2$, but not cPLA$_2$, mediates lysoPC-induced TRPC6 externalization. Using siRNA-mediated downregulation of specific isoforms, the β-isoform, but not the γ-isoform, of iPLA$_2$ appears to be responsible for

lysoPC-induced TRPC6 externalization. Downregulating iPLA$_2$β inhibits lysoPC-induced release of AA from the EC membrane, blocks the increase in [Ca^{2+}]$_i$, and preserves EC migration in the presence of lysoPC.

Earlier studies suggest that cPLA$_2$ is the major isoform involved in the AA release from membranes, whereas iPLA$_2$ is a housekeeping protein only involved in the incorporation of free AA into membranes ([23]–[25]). However, later studies show that in addition to its housekeeping function, iPLA$_2$ is involved in signal transduction pathways, as well as generation of AA and other lipid metabolites ([26], [27]). In fact, a role for iPLA$_2$ is suggested in agonist-induced AA release in aortic smooth muscle cells and RAW 264.7 macrophage cell line ([28], [29]). Furthermore, Balboa and Balsinde demonstrate the key role of iPLA$_2$ in the release of AA in human U937 cells during oxidative stress ([30], [31]). In addition, the role of iPLA$_2$β is associated with thrombin-induced AA release in human coronary artery ECs ([32]). Our data are consistent with these studies and suggest a role for iPLA$_2$, specifically the β isoform
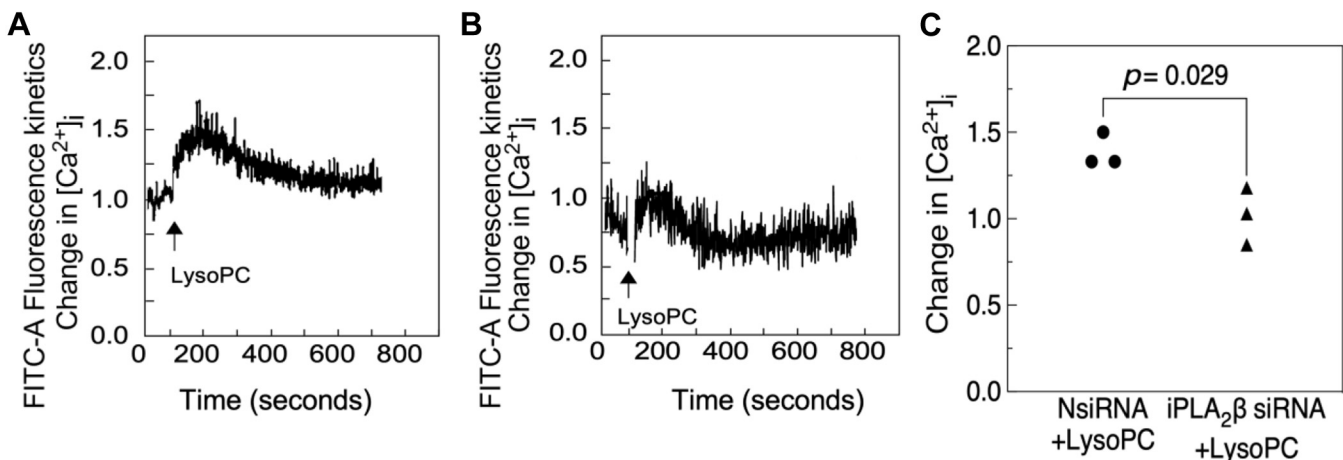
Figure 6. Downregulation of iPLA₂β isoform blocks lysoPC-induced arachidonic acid release from the membrane into medium. *A* and *B*, ECs transfected with NsiRNA or iPLA₂β siRNA were serum-starved for 6 h, then lysoPC (10 μM) added for 15 min. Cells were lysed, and the AA content of the membrane fraction was measured by ELISA, and the AA content in the medium was measured by LC/MS/MS. *A*, lysoPC-induced change in AA content in membrane fraction. *B*, lysoPC-induced change in AA content in the medium. Values shown are the means ± SD (n = 3), analyzed with Student's *t* test and *p* values calculated. EC, endothelial cell; iPLA₂, cytosolic calcium-independent PLA₂; lysoPC, lysophosphatidylcholine; NsiRNA, negative control siRNA.

of iPLA₂, in mediating lysoPC-induced TRPC6 externalization and activation in ECs.

Downregulating iPLA₂β modestly inhibits lysoPC-induced release of AA from the EC membrane (Fig. 6) but significantly preserves EC migration in the presence of lysoPC (Fig. 5*C*). LysoPC-induced AA release from the membrane is a localized event. Upon release, the free AA is either rapidly metabolized or incorporated back into phospholipid pool, or diffused into other cells (33). Wong *et al.* (12) show that AA release in ECs is both time and concentration dependent, maximal AA release being observed at 10 min with 50 μM lysoPC. In our AA release assay, 10 μM lysoPC is used to align with our functional assays and our previous studies. Hence, we observe the modest difference between lysoPC-induced AA release in cells transfected with NsiRNA compared with iPLA₂β siRNA. Furthermore, using the razor scrape assay, we demonstrate that this modest difference in

AA release translates into prevention of lysoPC-induced inhibition of EC migration in iPLA₂β-downregulated cells (Fig. 5*C*). AA release is measured minutes after incubation with lysoPC, while migration is measured at 24 h, allowing for a series of events to occur. The robust effect on migration in iPLA₂β downregulated cells may reflect the efficacy of AA release inhibition which is upstream in a cascade of events that eventually result in cytoskeletal changes that block migration.

iPLA₂β has been shown to be involved in the activation of other TRP channels such as TRPC5 (34) and TRPM8 (35). AL-Shawaf *et al.* (34) show that downregulation of iPLA₂β suppresses sphingosine 1-phosphate–induced, but not lysoPC-induced, TRPC5 channel activation in HEK cells containing conditional expression of TRPC5 (34). Our previous studies in ECs expressing both TRPC6 and TRPC5 show that lysoPC-induced TRPC6 activation precedes TRPC5 activation and

Figure 7. Downregulation of iPLA₂β isoform inhibits lysoPC-induced increase in $[Ca^{2+}]_i$. ECs transfected with NsiRNA and iPLA₂β siRNA were serum-starved for 6 h. ECs were loaded with the FITC-conjugated fluorophore Calbryte 520 AM dye. The ECs were suspended and loaded into the sort chamber of a BD FACSMelody Cell Sorter maintained at 37 °C. After adjusting the baseline, lysoPC (10 μM) was added. *A–C*, using the kinetic reading mode at Ex/Em 490/525 nm, relative changes in $[Ca^{2+}]_i$ after transfection with (*A*) NsiRNA or (*B*) iPLA₂β siRNA were determined. Representative graphs of three experiments are shown here. *C*, change in $[Ca^{2+}]_i$ measured by difference in mean $[Ca^{2+}]_i$ at baseline and after addition of lysoPC is presented in the graph. Values shown are the means ± SD (n = 3), analyzed with Student's *t* test and *p* values calculated. iPLA₂, cytosolic calcium-independent PLA₂; lysoPC, lysophosphatidylcholine; EC, endothelial cell; NsiRNA, negative control siRNA.

that downregulation of TRPC6 suppresses TRPC5 externalization in ECs incubated with lysoPC (11). In our present study, blocking iPLA$_2\beta$ inhibits lysoPC-induced TRPC6 externalization, which should result in decreased TRPC5 activation in cells expressing both channels. The difference in the role of iPLA$_2\beta$ in the TRPC activation in these studies may reflect the variety of mechanisms for TRPC5 activation, including activation by reactive oxygen species, changes in $[Ca^{2+}]_i$, or directly by lysoPC (36, 37).

Oxidized lipid products impede endothelial healing during vascular interventions. We have shown previously that lysoPC disrupts the delicate balance of $[Ca^{2+}]_i$ in ECs by activation of TRPC6 and *via* signal transduction pathways leading to TRPC5 activation and inhibition of EC migration (5, 7, 8, 11, 38). Our present study shows for the first time the role of iPLA$_2\beta$ in the externalization of TRPC6 and subsequent inhibition of EC migration by lysoPC. These results allow for selection of an isoform-specific pharmacological inhibitor, several of which are currently being used in clinical cancer therapy trials, and to test its efficacy to promote endothelial healing in an arterial injury model. Specifically blocking lysoPC-induced iPLA$_2\beta$ activation in ECs should prevent TRPC6 activation and preserve EC migration, thereby improving endothelial healing after interventions for cardiovascular diseases.

Experimental procedures

Cells and reagents

BAECs were isolated from adult bovine aortas by scraping after collagenase treatment (11). Assays involving BAECs were performed in replicates using cells from at least three different bovine aortas. BAECs between passages 4 and 9 were used for the assays. EAhy.926 cells, a primary human umbilical vein cell line, were purchased from the ATCC.

1-Palmitol-2-hydroxy-sn-glycero-3-phosphocholine (16:0 LysoPC) (catalog number (#): 855675p) was obtained from Avanti Polar Lipids, Inc AA (#90010) and heneicosapentaenoic acid (HPA) (#10670) were purchased from Cayman Chemical. The PLA$_2$ assay kits (#765021) were purchased from Cayman Chemical, and the AA ELISA kits (#MBS2608709 and #MBS267742) were purchased from MyBioSource. Antibodies for immunoblot analysis were purchased from Cell Signaling Technology and Santa Cruz Biotechnology as indicated below. siRNA for transfection studies were purchased from Dharmacon, Inc. ON-TARGETPlus siRNA SMARTpool [PLA2G4A: L-009886-00-0005, PLA2G4C: L-009663-00-0005, PLA2G6: L-009085-00-0005 (J-009085-12-0002 and J-009085-13-0002 individual siRNAs), PLA2G6B: L-010284-00-0005], and the NsiRNA were purchased from Santa Cruz Biotechnology (#sc-37007). RNA isolation kits were purchased from Qiagen (miRNeasy mini kit # 217004) and TaqMan assay kits for qRT-PCR were purchased from Thermo Fisher [PLA2G4A (Hs00233352_m1), PLA2G4C (Hs00234345_m1), PLA2G6A (Hs00899715_m1), PLA2G6B (HS00382272-m1), or GAPDH (Hs99999905_m1)].

EC culture

BAECs were cultured in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum (FBS, HyClone Laboratories #SH30541.03) and 1% antibiotic (penicillin/streptomycin). EA.hy926 were cultured in Eagle's Modified Essential Medium containing 10% (vol/vol) FBS.

Measurement of PLA$_2$ activity

Total PLA$_2$ activity in BAECs was measured using the cPLA$_2$ assay kit. The use of this assay kit without the specific purification procedure allowed measurement of total PLA$_2$ enzyme activity (39, 40). BAECs were grown in 60-mm dishes and serum-starved for 18 h. LysoPC (12.5 μM) was added for 15 min in appropriate dishes. Cells were then lysed in the lysis buffer (50 mM Hepes, 150 mM NaCl, 200 μM Na$_3$VO$_4$, 100 mM NaF, 1% Triton X-100, pH 7.4) containing protease inhibitors (cOmplete, Roche) for 30 min at 4 °C. Lysates were passed through needles, 20-gauge (20×) and 25-gauge (15×), and cleared by centrifugation at 12,000g for 15 min. PLA$_2$ assay was performed as per the manufacturer's protocol. Briefly, sample, blank, and positive control (bee venom) (10 μl) were added to a 96-well plate in triplicates. To initiate the reaction, arachidonoyl Thio-PC (200 μl) substrate was added to each well and mixed and incubated for 60 min at room temperature. DNTB/EGTA was then added to stop the enzymatic reaction and the absorbance read at 405 nm using a plate reader (SpectraMAX 190).

Downregulation of PLA$_2$

EA.hy926 cells at 70 to 80% confluency were incubated with 25 nM siRNA for 6 h using DharmaFECT reagent in serum-free medium according to the manufacturer's protocol, followed by full replacement of the medium supplemented with 10% FBS for the remainder of the 48 h. siRNA for PLA2G4A, PLA2G4C, PLA2G6A, and PLA2G6B (Dharmacon, Inc) and NsiRNA (Santa Cruz Biotechnology) were used. mRNA was isolated at 48 h with Qiagen miRNeasy mini kit and knockdown efficiency quantified with RT-qPCR using TaqMan assay kits. Samples were analyzed in triplicate, and target gene expression was normalized to GAPDH. Protein level knockdown was assessed at 48 h with immunoblot analysis.

Measurement of the AA content in the membrane and medium

BAECs or transfected EA.hy926 cells were serum-starved for 18 h or 6 h, respectively. LysoPC (12.5 μM or 10 μM) was then added for 15 min. Cells were processed as per the manufacturer's protocol using the Mem-PER Plus membrane extraction kit (Thermo Fisher) to obtain the membrane fraction. Briefly, cells were washed and centrifuged, the pellet was resuspended in the permeabilization buffer (350 μl), incubated for 10 min at 4 °C, and centrifuged at 16,000g for 15 min at 4 °C. The cytosolic fraction was carefully separated, the pellet was further resuspended in the solubilization buffer (250 μl) for 30 min at 4 °C, and centrifuged at 16,000g for 15 min to collect the membrane

fraction. The AA content of the membrane fraction was measured using an AA ELISA kit as per the manufacturer's protocol and the absorbance read at 450 nm using a plate reader (SpectraMAX 190).

For AA measurement, the medium was collected and processed using HPLC On-line LC/MS/MS. Briefly, the cell medium (500 µl) was mixed with methanol (50 µl) containing 2 µg/ml HPA as the internal standard and dried under N_2 flow. Methanol 75% (50 µl) was added to the dried sample, vortexed, and then filtered through a 0.22-µm membrane. A 5-µl aliquot was injected to the Vanquish HPLC and Quantiva triple quadrupole mass spectrometer (Thermo Fisher) (41). XCalibur software was used to process the data and obtain the peak areas of AA and HPA. The internal standard calibration curve was used to calculate the concentration of AA in the samples.

Immunoblot analysis

Immunoblot analysis was performed as previously described (11). Proteins of interest were detected using antibodies specific for rabbit TRPC6 (1:1000, Cell Signaling #16716S), rabbit cPLA₂ (1:1000, Cell Signaling #2832), mouse iPLA₂ (1:1000, Santa Cruz Biotechnology #sc-376563), and β-actin (1:2000, Santa Cruz Biotechnology #sc47778 HRP). Anti-rabbit (1:1000, antibodies-online #ABIN102010) or anti-mouse (1:1000, Santa Cruz Biotechnology #SC516102) antibodies were used for secondary antibodies.

TRPC6 externalization by biotinylation assay

Biotinylation of EC membrane surface proteins was performed as previously described (42). Briefly, transfected EA.hy926 cells were cultured in 60-mm dishes to 80% confluency and serum-starved for 6 h, and then lysoPC (10 µM) was added for 15 min. Externalized TRPC6 was detected by the biotinylation assay (42) and immunoblot analysis was performed.

EC migration

EC migration was assessed in a razor scrape assay in 12-well tissue culture plates as previously described (43). Briefly, transfected EA.hy926 cells were serum-starved for 6 h. The razor scrape was performed and cells allowed to migrate ± lysoPC (10 µM) for 24 h. Using a digital CCD camera mounted on a phase-contrast microscope, images were taken of three random fields, each corresponding to a starting line length of 1.6 mm, from three wells. Images were processed using NIH ImageJ analysis software (NIH, Bethesda, MD), and an observer blinded to the experimental conditions quantitated the migration.

Measurement of [Ca²⁺]ᵢ

ECs at 80 to 90% confluence were loaded with the FITC132 conjugated fluorophore Calbryte 520 AM dye (AAT Bioquest; Catalog No. 36310) following the manufacturer's protocol. After 35 min, the EC were suspended and loaded into the sort chamber of a BD FACSMelody Cell Sorter (BD Biosciences) maintained at 37 °C. After adjusting the baseline, 10 µM lysoPC was added and relative change in $[Ca^{2+}]_i$ was read using the kinetic reading mode at Ex/Em 490/525 nm. Kinetics data were analyzed using the FlowJo v10 software (BD Biosciences).

Statistics analysis

All experiments were performed at least in triplicate. Studies with BAECs used ECs isolated from at least three different animals. Values are presented as the mean ± SD. Data were analyzed by Student's *t* test or one-way ANOVA with appropriate post hoc analysis, and $p < 0.05$ was considered statistically significant.

Data availability

All the data described in the article are contained within the article.

Abbreviations—The abbreviations used are: $[Ca^{2+}]_i$, intracellular calcium ion concentration; AA, arachidonic acid; BAECs, bovine aortic ECs; cPLA₂, cytosolic calcium-dependent PLA₂; EC, endothelial cell; FBS, fetal bovine serum; HPA, heneicosapentaenoic acid; iPLA₂, cytosolic calcium-independent PLA₂; lysoPC, lysophosphatidylcholine; NsiRNA, negative control siRNA; oxLDL, oxidized

low-density lipoprotein; PLA$_2$, phospholipase A$_2$; TRPC, canonical transient receptor potential.

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