Activation of Cytosolic Phospholipase A$_2$-α as a Novel Mechanism Regulating Endothelial Cell Cycle Progression and Angiogenesis*

Received for publication, September 19, 2008, and in revised form, December 17, 2008 Published, JBC Papers in Press, January 1, 2009, DOI 10.1074/jbc.M807282200

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Release of endothelial cells from contact-inhibition and cell cycle re-entry is required for the induction of new blood vessel formation by angiogenesis. Using a combination of chemical inhibition, loss of function, and gain of function approaches, we demonstrate that endothelial cell cycle re-entry, S phase progression, and subsequent angiogenic tubule formation are dependent upon the activity of cytosolic phospholipase A$_2$-α (cPLA$_2$-α). Inhibition of cPLA$_2$-α activity and small interfering RNA (siRNA)-mediated knockdown of endogenous cPLA$_2$-α reduced endothelial cell proliferation. In the absence of cPLA$_2$-α activity, endothelial cells exhibited retarded progression from G$_1$ through S phase, displayed reduced cyclin A/cdk2 expression, and generated less arachidonic acid. In quiescent endothelial cells, cPLA$_2$-α is inactivated upon its sequestration at the Golgi apparatus. Upon the stimulation of endothelial cell proliferation, activation of cPLA$_2$-α by release from the Golgi apparatus was critical to the induction of cyclin A expression and efficient cell cycle progression. Consequently, inhibition of cPLA$_2$-α was sufficient to block angiogenic tubule formation in vitro. Furthermore, the siRNA-mediated retardation of endothelial cell cycle re-entry and proliferation was reversed upon overexpression of an siRNA-resistant form of cPLA$_2$-α. Thus, activation of cPLA$_2$-α acts as a novel mechanism for the regulation of endothelial cell cycle re-entry, cell cycle progression, and angiogenesis.

The vascular endothelium consists of a monolayer of endothelial cells that lines the luminal surface of all blood vessels in vivo. The endothelium actively participates in a variety of key vascular processes such as the regulation of vascular tone and blood fluidity. In addition, the endothelium regulates the formation of new blood vessels by the process of angiogenesis in development, tissue repair, and tumor vascularization (1, 2). The mature endothelium consists of contact-inhibited confluent monolayers of cells that reside in the G$_0$ phase of quiescence. Upon loss of cell-cell contacts, endothelial cells re-enter the cell cycle and proliferate. This entry of endothelial cells into the cell cycle from G$_0$ is a critical component of the angiogenic response and the formation of new capillaries from pre-existing blood vessels (1, 2). Thus, the inhibition of endothelial cell proliferation has great potential for the treatment of diseases involving unwanted blood vessel formation.

The phospholipase A$_2$ (PLA$_2$) family of enzymes hydrolyze the sn-2 group of glycerophospholipids to concomitantly release free fatty acids and lysophospholipids (3). The PLA$_2$ family represents a diverse family of enzymes that can be divided into three main groups as follows: the group IV cytosolic PLA$_2$ (cPLA$_2$), the group VI Ca$^{2+}$-independent PLA$_2$ (iPLA$_2$), and the secretory PLA$_2$ enzymes (4). The cPLA$_2$ group of enzymes consists of at least six members (cPLA$_2$α, -β, -γ, -δ, -ε, and -ζ), of which cPLA$_2$α is the most extensively characterized. cPLA$_2$α is Ca$^{2+}$-sensitive and translocates to intracellular membranes upon agonist stimulation and cytosolic Ca$^{2+}$ elevation utilizing an N terminal Ca$^{2+}$-dependent lipid binding (C2) domain (5–7). Upon membrane binding, cPLA$_2$α preferentially cleaves phospholipids containing arachidonic acid (AA) at the sn-2 position to liberate free AA (3). As such, cPLA$_2$α is seen as the rate-limiting enzyme in receptor-mediated AA release (8). Proliferating, nonconfluent endothelial cells release much greater levels of arachidonic acid and prostaglandin than quiescent confluent cells (9–11), which has been attributed to elevated cPLA$_2$α activity. In quiescent confluent cells, cPLA$_2$α is inactivated upon sequestration at the Golgi apparatus and is subsequently released and activated in proliferating cells (11, 12). Despite this, the actual function of this differential regulation of cPLA$_2$α activity has not been defined.

Here we identify a novel role for cPLA$_2$α activation in the regulation of endothelial cell cycle progression. Upon the loss of cell-cell contacts and the induction of endothelial cell prolifer-
atation, activation of cPLA$_2$ is required for the induction of cyclin A expression and efficient progression through G$_1$ and S phases. Our work and work by others have previously shown that the activity of iPLA$_2$ also influences the progression of endothelial cells through S phase (13–15). Here we demonstrate that cPLA$_2$ and iPLA$_2$ work cooperatively to influence endothelial cell cycle progression with cPLA$_2$ providing a stimulation- and Ca$^{2+}$-dependent source of lipid metabolites required for controlling endothelial cell cycle progression in response to monolayer disruption or growth factor stimulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords as described previously (16, 17). Human dermal microvascular endothelial cells (HDMEC) were purchased from Promocell. Cells were cultured in endothelial cell basal medium supplemented with endothelial cell growth factor kit 2 (Promocell). All cells were grown on 0.1% (w/v) gelatin-coated cultureware and were not used in excess of four passages. The following antibodies were purchased: anti-αII-spectrin (C3; sc-48382) and anti-cPLA$_2$ (C20; Santa Cruz Biotechnology); anti-PCNA (610664), anti-cyclin A (611268), and anti-cdk2 (610145; BD Transduction Laboratories); anti-caspase-3, anti-caspase-7, anti-caspase-9, and poly(ADP-ribose) polymerase (Cell Signaling Technologies); horseradish peroxidase-conjugated secondary antibodies (Molecular Probes); and Alexafluor-conjugated secondary antibodies (Pierce) and goat IgG (1:3000) for 1 h, immunoreactive bands were visualized using a West Pico enhanced chemiluminescence (ECL) detection kit (Pierce). Images were captured on a Fuji Film Intelligent dark box II image reader. Band intensities were

**Proliferation ELISA**—HUVEC proliferation rates were assessed using a 5-bromo-2’-deoxyuridine (BrdUrd) incorporation-based ELISA (Roche Diagnostics). Cells were seeded at 1 × 10$^3$ cells per well (0.55 × 10$^3$ cells/cm$^2$) in 96-well plates, grown for 24 h, incubated for 16 h with inhibitors and BrdUrd, and then processed according to manufacturer’s instructions. Proliferation was also determined after 48 h of growth by assessing viable cell numbers using the MTS-based CellTiter$^\text{®}$ AQueous nonradioactive cell proliferation assay (Promega) according to the manufacturer’s instructions.

**Microscopy and Quantification**—Phase contrast images were acquired using an Olympus CK2 inverted microscope (10× lens) linked to an Olympus OM-1 camera. Deconvolution fluorescence microscopy was performed using an Olympus IX-70 inverted fluorescence microscope (63 × 1.5 oil immersion lens) and DeltaVision deconvolution system (Applied Precision Inc.). Individual optical sections of 0.2 μm were generated from 15 iterative cycles of deconvolution. Some images were collected using a Zeiss LSM510 META Axiovert 200 M confocal microscope. Multiple comparisons were performed using one-way analysis of variance and Tukey’s post-test analysis with GraphPad Prism software.

**Flow Cytometry**—Subconfluent HUVEC were cultured for 16 h in the presence or absence of inhibitor. Cells were then harvested, fixed in 70% ice-cold ethanol, and incubated with propidium iodide (50 μg/ml) and RNase A (20 μg/ml) for 3 h. DNA content was then assessed using a FACScalibur flow cytometer (BD Biosciences) and the percentage of cells in each phase of the cell cycle analyzed using Modfit software (Verity Software House). For some experiments G$_0$-synchronized confluent HUVECs, serum-starved overnight, were seeded at subconfluent density in the presence or absence of inhibitors for 14 h. Cells were then either chased in fresh media containing inhibitors for various time points prior to processing as above, or siRNA-treated cells were pulsed with media supplemented with 10 μM BrdUrd for 30 min prior to harvesting, fixation, and analysis for BrdUrd incorporation.

**Biochemistry**—Lysate preparation and Western analysis were performed as described previously (11). Immunoprecipitations were performed overnight at 4 °C using protein G-Sepharose (Upstate Biotechnology, Inc.), 3 μg of antibody, and 500 μg of total protein in 1% Nonidet P-40 lysis buffer (50 mM Tris/HCl, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM EGTA, 1% Nonidet P-40, pH 7.4). Samples (20 μg of protein or total bead volume) were resolved for 60 min at 30 mA/gel on 10% SDS-PAGE mini-gels using a discontinuous buffer system (18). For immunoblotting, protein was transferred onto nitrocellulose membranes for 3 h at 300 mA (19). Membranes were blocked in 5% (w/v) nonfat milk in PBS for 30 min and then incubated overnight with primary antibody (1:500) at room temperature. After incubation with horseradish peroxidase-conjugated anti-goat IgG (1:3000) for 1 h, immunoreactive bands were visualized using a West Pico enhanced chemiluminescence (ECL) detection kit (Pierce). Images were captured on a Fuji Film Intelligent dark box II image reader. Band intensities were
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FIGURE 1. cPLA₂α mediates endothelial cell proliferation. A, passive [³H]AA release from proliferating pre-labeled HUVECs was assessed by liquid scintillation counting following 18 h of growth in the presence of the indicated inhibitors. All treatments significantly reduce AA release, p < 0.05. B, A23187-induced [³H]AA release from subconfluent HUVECs following a 15-min preincubation with the indicated inhibitors was determined by liquid scintillation and expressed as a percentage of total [³H] incorporation. Results are expressed as a percentage of total [³H] incorporation (n = 3). Quantitation of HUVEC (C and D) and HDMEC (E) growth in the presence or absence of 2.5 μM pyrrolidine (C and E) or Wyeth-1 (D) was for 16 h (n = 6, ± S.E.). Proliferation was determined using a colorimetric ELISA based on BrdUrd (BrdUr) incorporation, as described under "Experimental Procedures," and was expressed as a percentage of proliferation in the presence of the inhibitor. F, quantification of VEGF-dependent HUVEC proliferation after 48 h in the presence of the indicated inhibitors was determined by MTS assay (n = 5). *, p < 0.01 versus control. **, p < 0.01 versus VEGF-stimulated control cells.

determined densitometrically using Aida (Advanced Image Data Analyzer) 2.11 software.

RNA Interference—HUVECs were transfected with either no siRNA (control), 10–50 nm nontargeting control siRNA (mock; D-001210-01; Dharmacon), or 10–50 nm annealed cPLA₂α siRNA (siRNA-1, 30774; or siRNA-2, 30953; Ambion) for 4 h using either Lipofectamine 2000 or RNAiMAX transfection reagent (Invitrogen). siRNA against iPLA₂-VIA has been described previously (15). Cells were recovered for 48 h prior to lysis. For cPLA₂α supplementation experiments, cells were transfected with siRNA-1 or scrambled sequence using RNAiMAX for 24 h before harvesting and transfection with mutant 774 cPLA₂ by electroporation as described previously (12). Cells were plated onto coverslips, recovered for 24 h, and processed for cyclin A, Ki67, and GFP expression analysis by immunofluorescence microscopy.

Immunofluorescence Microscopy—Immunofluorescence was performed as described previously (11). Briefly, cells grown on coverslips were fixed in 10% (v/v) formalin in neutral buffered saline (HT50-1-128; Sigma) for 5 min at 37 °C. After permeabilization with 0.1% (v/v) Triton X-100 for 5 min, cells were re-fixed (5 min), washed with PBS, and then incubated in 50 mM ammonium chloride for 10 min. Following PBS washes, nonspecific binding sites were blocked with 5% (v/v) donkey serum for 3 h. Cells were incubated overnight with primary antibody followed by the appropriate secondary antibodies. Finally, coverslips were mounted on microscope slides in Fluoromount-G mounting medium (Southern Biotech). For analysis of BrdUrd incorporation, cells were fixed in ice-cold 80% ethanol for 20 min followed by a 20-min fixative step in 100% ethanol on ice. Cells were then incubated with 2 N HCl, 0.5% Triton X-100 for 20 min at room temperature followed by a wash in 100 mM sodium tetraborohydrate. Cells were then processed as above with the indicated antibodies.

Differentiation and Migration Assays—To assess HUVEC differentiation, 1 × 10⁵ cells were seeded in 24-well dishes coated with 100 μl of Matrigel (BD Biosciences). Cells were incubated in the presence or absence of inhibitor for 16 h prior to imaging. Tube length was quantified using ImageJ software (rsb.info.nih). For migration assays, HUVECs (5 × 10⁵ cells) were seeded in serum-free media to the top chamber of 24-well modified Boyden chambers (3 μm pores; Transwell-Costar Corp.). Cells were allowed to migrate toward serum-containing media for 16 h in the presence of absence of inhibitor. Migrated cells were fixed, stained with DAPI, and then counted.

Angiogenesis Assay—Co-cultures of HUVECs seeded on a bed of human fibroblasts (TCS Cellworks) were cultured for 7 days in the presence or absence of inhibitor. Tubules were fixed, stained, and imaged by phase contrast microscopy. Tubule length was quantified using ImageJ software.

RESULTS

cPLA₂α Mediates Endothelial Cell Proliferation—We have previously demonstrated a requirement for both iPLA₂-V and cPLA₂α-mediated AA release in the regulation of HUVEC proliferation (11, 15). We sought to further examine the contribution of cPLA₂α to endothelial cell proliferation by measuring BrdUrd incorporation into DNA in the presence of concentrations of inhibitors that maximally block cPLA₂α activity. To initially define concentrations of inhibitors that maximally inhibited cPLA₂α activity, passive AA release generated by proliferating HUVECs was quantified following 18 h of incubation with varying concentrations of the cPLA₂α-specific inhibitors pyrrolidine and Wyeth-1 (Wy-1) (20, 21) or the cPLA₂α/iPLA₂ inhibitor AAOCPF₄ (22). Maximal inhibition of cPLA₂α-mediated AA release was achieved with between 2.5 and 5 μM pyrrolidine and 2.5 and 5 μM Wy-1 with no further increase in inhibition observed at higher concentrations (Fig. 1A).

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AACOCF$_3$ also maximally inhibited AA release at 2.5 $\mu$M, although the extent of this inhibition was much greater than with pyrrolidine or Wy-1 alone, consistent with the combined inhibition of both cPLA$_2$$\alpha$- and iPLA$_2$ activities, which contribute to the pool of released AA by proliferating endothelial cells. The modest reduction in long term AA release following cPLA$_2$$\alpha$ inhibition may reflect both the assay conditions, where radiolabeled AA may be produced by non-cPLA$_2$$\alpha$-dependent mechanisms that cannot be controlled for (such as normal membrane turnover) and the contribution of the cPLA$_2$$\alpha$-generated pool to this process.

However, ionophore treatment selectively liberates AA derived from pools accessible to the Ca$^{2+}$-dependent isoforms of PLA$_2$ and can thus be considered selective for the activation of cPLA$_2$$\alpha$ in HUVECs. Under these conditions, similar to the overnight release assay, short term (15 min) preincubation with 2.5 $\mu$M pyrrolidine or Wy-1 was also sufficient to maximally inhibit Ca$^{2+}$-induced AA release induced by the ionophore A23187 (Fig. 1B). AACOCF$_3$ appeared less effective at reducing stimulated AA release, and the varying extent of the inhibition by the various drugs may reflect the differing initial bioavailabilities of these structurally distinct compounds. A role for cPLA$_2$$\alpha$ in the regulation of endothelial cell proliferation was then confirmed upon incubation of HUVECs with 2.5 $\mu$M pyrrolidine or Wy-1 (Fig. 1, C and D). Maximal inhibition of cPLA$_2$$\alpha$ activity was sufficient to significantly retard HUVEC proliferation. Furthermore, incubation with 2.5 $\mu$M pyrrolidine also significantly inhibited HDMEC proliferation (Fig. 1E) suggesting that a role for cPLA$_2$$\alpha$ in the regulation of cellular proliferation is common to other endothelial cell types. The antiproliferative effect of cPLA$_2$$\alpha$ inhibition could not be attributed to increased cell death as the concentration of inhibitors used did not affect cell viability (as assessed by trypan blue exclusion and annexin V binding) or caspase-3 activation and did not promote the cleavage of αII-spectrin or poly(ADP-ribose) polymerase (data not shown). However, varying levels of inhibitor cytotoxicity were observed at doses exceeding those used in this study (data not shown).

Specific analysis of the vascular endothelial growth factor A (VEGF-A) pathway showed that cPLA$_2$$\alpha$ activity is required for the induction of cellular proliferation by this important angiogenic stimulus. Indeed, VEGF-A-mediated cell turnover was reduced by 40–50% in the presence of the various cPLA$_2$$\alpha$ inhibitors as determined by MTS-based assay of relative cell number after 48 h of growth (Fig. 1F). Furthermore, incubation with 5 $\mu$M AACOCF$_3$ and the combined inhibition of cPLA$_2$$\alpha$ and iPLA$_2$ reduced HUVEC proliferation to levels similar to those found in the absence of growth factor and serum, suggest-

### Figure 2. cPLA$_2$$\alpha$ mediates endothelial cell angiogenesis

A. phase contrast images of capillary-like tubules formed in co-culture assays over 7 days of growth in the presence or absence of 2.5 $\mu$M pyrrolidine or 2.5 $\mu$M Wyeth-1 (n = 3 ± S.E.) and quantification of total tubule lengths (n = 6 ± S.E.). B. quantification of HUVEC cell numbers over a 7-day growth period in the absence (empty bars) or presence (black bars) of 2.5 $\mu$M pyrrolidine (n = 3; ± S.E.) is shown. C. cells were grown in the presence of 2.5 $\mu$M pyrrolidine for 5 days and cell numbers quantified prior to inhibitor washout (control) and then after inhibitor washout and 8 days of recovery (recovery; n = 3 ± S.E.). D. quantification of HDMEC cell numbers over a 7-day growth period in the absence (empty bars) or presence (black bars) of 2.5 $\mu$M pyrrolidine (n = 3, ± S.E.) is shown. †, p < 0.05 versus uninhibited; ‡, p < 0.001 versus uninhibited; *, p < 0.01 versus control. E. quantification of serum-induced migration in the presence or absence of 2.5 $\mu$M pyrrolidine (n = 3 ± S.E.) is shown. F. phase contrast images of HUVECs incubated on Matrigel for 16 h in the presence or absence of 2.5 $\mu$M pyrrolidine or 2.5 $\mu$M Wy-1 (n = 3 ± S.E.) and quantification of total tubule lengths and total branch numbers (n = 6 ± S.E.) are shown. All results are representative of at least three separate experiments.
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FIGURE 3. Role of cPLA$_2$ activity in endothelial cell cycle progression. A and B, subconfluent HUVECs, grown in the presence or absence of 2.5 $\mu$m pyrrolidine for 16 h, were stained with propidium iodide and analyzed by flow cytometry to determine cellular DNA content. Cell cycle distribution was then determined using Modfit software and plotted ($n = 3$, $\pm$ S.E.). C, G$_0$-synchronized HUVECs were re-seeded subconfluently and allowed to re-enter the cell cycle for 14 h in the presence or absence of 2.5 $\mu$m pyrrolidine. Cells were then processed at various subsequent time points with or without inhibitor prior to staining with propidium iodide and analysis by flow cytometry as in A. The percentage of G$_0$-G$_1$ (left) and S phase cells (right) was determined using Modfit and expressed as a percentage of total cell number ($n = 4$; *, $p < 0.05$ versus respective control; **, $p < 0.01$ versus control 0 h). D, cells prepared as in C were incubated with media supplemented with BrdUrd (Brdu, 10 $\mu$m) for 45 min, 14 h after re-seeding. This was followed by ethanol fixation, staining with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody, and analysis by flow cytometry. BrdUrd incorporation was expressed as a percentage of total cells and compared with confluent monolayer uptake ($n = 4$; *, $p < 0.05$; **, $p < 0.01$). E, subconfluent HUVECs grown in the presence or absence of 2.5 $\mu$m pyrrolidine for 18 h were analyzed for Ki67 levels. Membranes were re-probed for GRASP55 to show equal loading. Relative immunoreactivities were determined using Aida densitometry software and plotted ($n = 3$, $\pm$ S.E.). F, subconfluent HUVECs grown in the presence or absence of 2.5 $\mu$m pyrrolidine for 24 h were analyzed for PCNA, cyclin A, and cdk2 levels by Western blotting. Relative immunoreactivities were determined using Aida densitometry software and plotted ($n = 3$, $\pm$ S.E.). All results are representative of at least three separate experiments. *, $p < 0.01$ versus control.

Signaling that cPLA$_2$ and iPLA$_2$ may function cooperatively during VEGF-induced endothelial cell proliferation.

cPLA$_2$α-mediated Proliferation Is Essential for Angiogenesis—Angiogenic tubule formation is a multistep process involving the proliferation, migration, and differentiation of endothelial cells (1, 2). Consequently, conversion of endothelial cells from quiescence to a proliferative state is vital to angiogenesis (1). As cPLA$_2$α activity is required for endothelial cell proliferation (Fig. 1), we hypothesized that cPLA$_2$α activity plays a key role in angiogenesis. Endothelial cell tubule formation can be assessed using co-culture assays in vitro. In this assay, HUVECs were seeded on a bed of human dermal fibroblasts and cultured for 7 days. Under these conditions endothelial cells form tubules with patent lumens, reminiscent of mature capillaries (23). Tubule formation in this assay involves the combined proliferation, migration, and differentiation of endothelial cells. Incubation with pyrrolidine reduced tubule length to 32.8 ± 4.8% of controls (Fig. 2A). Wy-1 also showed a similar ability to reduce tubule formation (Fig. 2A). Thus cPLA$_2$α activity regulates the formation of new blood capillaries by angiogenesis in in vitro assays.

To further examine the importance of cPLA$_2$α activity for endothelial cell proliferation during angiogenesis and the long term effects of pyrrolidine on cell viability, endothelial cell numbers were determined by counting over 7 days of culture (Fig. 2B). HUVECs seeded at a density of 3000 cells/cm$^2$ were given 24 h to settle and then were cultured for 7 days in the presence or absence of 2.5 $\mu$m pyrrolidine. At 24-h intervals, cells were harvested and counted (Fig. 2B). Control cell numbers increased as a function of time, whereas cells grown in the presence of pyrrolidine remained at a constant density. Inhibition of growth was not because of cell death as incubation with pyrrolidine for 7 days did not affect cell viability, and endothelial cell morphology was unaffected by cPLA$_2$α inhibition (data not shown). Furthermore, this block in cell proliferation was entirely reversible as cells grown in the presence of pyrrolidine for 5 days recovered upon washout of inhibitor, with a 24-fold increase in cell density after 8 days of recovery (Fig. 2C). Similarly, culture in the presence of pyrrolidine also significantly blocks HDMEC growth (Fig. 2D). Thus, long term inhibition of cPLA$_2$α had a profound effect on cell growth and the ability of endothelial cells to form angiogenic tubules.

We also assessed the role cPLA$_2$α in endothelial cell migration, and differentiation. HUVEC migration, as assayed using Boyden chambers (23), was not affected by incubation with pyrrolidine (Fig. 2E). In addition, pyrrolidine did not inhibit endothelial cell migration or differentiation, as studied using Matrigel assays (Fig. 2F). Here a fixed number of cells were grown on an appropriate substratum for 24 h. Sufficient cells are present
to allow migration and differentiation into tubes even in the absence of cell proliferation (23). In this assay, HUVEC tube formation, branching number, and branch length were not affected by inhibition of cPLA$_2$ with either pyrrolidine or Wy-1 (Fig. 2F). As cPLA$_2$ does not play a role in HUVEC migration or differentiation (Fig. 2, E and F), inhibition of endothelial cell proliferation caused by blocking cPLA$_2$ activity (Fig. 1, C and F) must be sufficient to block angiogenic tubule formation in coculture assays (Fig. 2A). The role of cPLA$_2$ in mediating this proliferation defect was examined in further detail below.

**cPLA$_2$ Modulates Endothelial Cell S Phase Progression and Cell Cycle Residence**—The role of cPLA$_2$ in the regulation of cell cycle progression was assessed by analyzing the cell cycle distribution of endothelial cells using flow cytometry. Proliferating endothelial cells were cultured in the presence or absence of 2.5 μM pyrrolidine for 16 h. Cells were then stained with propidium iodide, and cellular DNA content was analyzed by FACS. As determined by fluorescence-activated cell sorter, the number of cells in S phase was markedly reduced upon cPLA$_2$ inhibition (Fig. 3, A and B). As a result, significantly more cells resided in the G$_0$-G$_1$ phases of the cell cycle, suggesting that cPLA$_2$ modulates G$_1$ to S phase progression. To examine the rate of progression of HUVECs through the cell cycle upon cPLA$_2$ inhibition, serum-starved, quiescent confluent cells (G$_0$ synchronized) were trypsinized, re-seeded to subconfluent density, and allowed to re-enter the cell cycle for 14 h (optimized for maximal cell cycle re-entry, data not shown) in the presence or absence of pyrrolidine (2.5 μM). Cells were then incubated in fresh media with or without pyrrolidine for various time points (0, 3, 6, 9 h), harvested at the indicated times, ethanol-fixed, stained with propidium iodide, and analyzed by flow cytometry. As shown in Fig. 3C, treatment of cells with pyrrolidine resulted in a significant increase (~10%) in the number of cells in G$_0$-G$_1$ phase, despite a time-dependent reduction in the total number of cells in G$_0$-G$_1$. Importantly, the distribution of cells in S phase was also altered upon cPLA$_2$ inhibition over the 9-h chase period (Fig. 3C). Pyrrolidine-treated cells displayed both reduced numbers of cells in S phase (~5% reduction in total cell number) and a delay in reaching S phase peak (~6 h post-chase) compared with control cells (S phase peak reached ~3–4 h
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A. confluent HUVEC monolayers were wounded and allowed to recover for 16 h in the presence or absence of serum and growth factors. Cell lysates were prepared and subjected to immunoprecipitation (IP) of cPLA$_2$$\alpha$. Bound proteins were separated by SDS-PAGE and Western-blotted for phosphorylated serine 505 and total cPLA$_2$. Lysates were also assessed for phosphorylated and total ERK1/2.

B. quantification of phosphoserine 505 and phospho-ERK levels from six independent experiments. $p < 0.05$, n = 8. C and D, confluent HUVECs were loaded with [3H]AA overnight, wounded, and allowed to recover for 16 h in the presence or absence of 2.5 |M Wy-1. C, released [3H]AA was assayed by liquid scintillation. D, recovered cells were also stimulated with A23187 (5 |M, 15 min), and released [3H]AA was measured. *, $p < 0.05$, n = 3.

FIGURE 5. cPLA$_2$$\alpha$ is activated at the wound border. A, confluent HUVEC monolayers were wounded and allowed to recover for 16 h in the presence or absence of serum and growth factors. Cell lysates were prepared and subjected to immunoprecipitation (IP) of cPLA$_2$$\alpha$. Bound proteins were separated by SDS-PAGE and Western-blotted for phosphorylated serine 505 and total cPLA$_2$. Lysates were also assessed for phosphorylated and total ERK1/2. con, control. B, quantification of phosphoserine 505 and phospho-ERK levels from six independent experiments. $p < 0.05$, n = 6. C and D, confluent HUVECs were loaded with [3H]AA overnight, wounded, and allowed to recover for 16 h in the presence or absence of 2.5 $\mu$M Wy-1. C, released [3H]AA was assayed by liquid scintillation. D, recovered cells were also stimulated with A23187 (5 $\mu$M, 15 min), and released [3H]AA was measured. *, $p < 0.05$, n = 3.

post-chase). As the total number of cells in S phase under control conditions is quite small (10–15% total cell number), this reduction following cPLA$_2$$\alpha$ inhibition represents a significant decrease in proliferative potential. Additionally, the total number of BrdUrd-positive cells at time 0 (14 h after release from G0) was significantly reduced (~33% reduction) upon treatment with pyrrolidine (Fig. 3D). Taken together, these results indicate that cPLA$_2$$\alpha$ participates in the regulation of endothelial S phase progression.

Ki67 is a nuclear protein expressed in the G$_1$, S, G$_2$, and mitotic phases of the cell cycle but not in the G$_0$ phase of quiescence (24, 25). It is commonly used as a marker for identifying proliferating cells, and a decrease in its expression is indicative of reduced proliferative rates (26). Inhibition of cPLA$_2$$\alpha$ significantly reduced Ki67 expression in subconfluent HUVECs (Fig. 3E), indicating that fewer cells were in the G$_1$-M phase. Slower passage through the cell cycle may have led to the accumulation of quiescent cells displaying reduced Ki67 levels. Thus, cPLA$_2$$\alpha$ activity plays a key role in G$_1$ to S phase progression and maintenance of endothelial cell cycle residence.

Transition from G$_1$ to S phase requires the assembly and activation of the DNA replication complex to initiate DNA synthesis. S phase entry and replication complex formation can be monitored by assessing cellular levels of the replication clamp proliferating cell nuclear antigen (PCNA). PCNA expression is low throughout the cell cycle until early S phase and the initiation of replication complex formation (27, 28). PCNA expression was unaffected by cPLA$_2$$\alpha$ inhibition (Fig. 3F), suggesting that inhibition of S phase progression was after S phase entry and replication complex formation. Activation of pre-assembled replication complexes and initiation of DNA synthesis is mediated by the cyclin A-cdk2 complex in early S phase. Mammalian cells cannot synthesize DNA nor progress through S phase in the absence of cyclin A-cdk2 activity (29, 30). Cyclin A expression increases in early S phase in conjunction with the accumulation of cdk2 in the nucleus to form active cyclin A-cdk2 complexes.

Upon cPLA$_2$$\alpha$ inhibition, cyclin A levels were reduced to ~26% of controls, potentially accounting for the block in G$_1$-S phase progression and inhibition of DNA synthesis seen previously (Fig. 3F). Furthermore, inhibition of cPLA$_2$$\alpha$ also resulted in reduced cdk2 expression levels (~29% of control) as often occurs when S phase progression is artificially blocked (31, 32). Thus, in the absence of cPLA$_2$$\alpha$ activity endothelial cells enter S phase and express similar levels of PCNA relative to control cells; however, they are unable to proceed through S phase to G$_2$-M as efficiently, demonstrating that cPLA$_2$$\alpha$ modulates the progression of endothelial cells through S phase.

siRNA-mediated Knockdown of Endogenous cPLA$_2$$\alpha$—The previous experiments are consistent with the hypothesis that cPLA$_2$$\alpha$ plays a central role in the control of endothelial cell cycle residence and S phase progression. However, to exclude the possibility that the effects of cPLA$_2$$\alpha$ inhibitors were non-specific, we used RNA interference. Consistent with the inhibitor studies, knockdown of endogenous cPLA$_2$$\alpha$ by either 78 or 68% of control levels using two different cPLA$_2$$\alpha$-specific siRNA duplexes (Fig. 4A) significantly inhibited HUVEC pro-
liferation (Fig. 4B). Relative to nontargeting siRNA controls (mock), the rate of BrdUrd incorporation into HUVEC DNA was reduced by 44 or 43% upon transfection with siRNA-1 or siRNA-2, respectively (Fig. 4B). Furthermore, the siRNA-mediated knockdown of endogenous cPLA2α also resulted in reduced cellular levels of cyclin A and cdk2 while having no effect on PCNA expression (Fig. 4C). Additionally, cell cycle progression of siRNA-treated cells was examined by measurement of BrdUrd incorporation using flow cytometry. Subconfluent siRNA-transfected cells, released from G₀ 14 h previously, were pulsed with BrdUrd (10 μM) for 45 min prior to fixation, allowing an indication of S phase cell numbers to be established. Under these conditions, cPLA2α siRNA-treated cells showed a significant reduction (to ~20% of scrambled siRNA (mock)) in the total number of cells in S phase and in cells undergoing active cell cycle progression (Fig. 4D). Thus, cPLA2α activity plays a central role in the regulation of endothelial cell proliferation and S phase progression by modulating the expression of cell cycle proteins and influencing cell cycle residence time.

**Activation of cPLA2α by Release from the Golgi Is Critical to S Phase Progression**—We have previously demonstrated that cPLA2α activity is reduced in confluent monolayers of endothelial cells that are in the G₀ phase of quiescence (11), and we proposed that cPLA2α is inactivated in confluent endothelial cells upon its sequestration at the Golgi apparatus and exclusion from its intracellular membrane substrate (12). Wounding of confluent monolayers provided the stimulus for cPLA2α to be released from the Golgi apparatus and allow access its phospholipid substrate (11). To confirm that cPLA2α is activated at the wound border following release from the Golgi apparatus, confluent monolayers were scratch-wounded in a grid pattern and recovered for 18 h prior to lysis. Following immunoprecipitation, the phosphorylation status of cPLA2α on serine 505 was assessed by Western blotting as cPLA2α can be activated downstream of multiple signal transduction cascades upon phosphorylation of serine 505 by ERK1/2. Wounding and recovery of HUVECs resulted in the growth factor-dependent phosphorylation of cPLA2α consistent with the increased phosphorylation and activation of ERK1/2 (Fig. 5A and B). Serum-starved HUVECs exhibited both reduced phospho-ERK levels and reduced phosphorylation on the critical Ser-505 residue of cPLA2α. Importantly, the wounding and recovery of HUVEC monolayers also resulted in the increased release of AA during the recovery period (Fig. 5C). Recovered cells also displayed a dramatically increased capacity to release AA upon stimulation with the Ca²⁺ ionophore A23187; this release was blocked by inhibition of cPLA2α with Wyeth-1 (2.5 μM; Fig. 5D). These results are consistent with the release of cPLA2α from the Golgi apparatus being critical for the activation of the enzyme.

Furthermore, subconfluent, actively proliferating endothelial cells display elevated levels of activated cPLA2α compared with quiescent cells, as assessed by Western blotting of cPLA2α immunoprecipitates for phosphorylated serine 505 (Fig. 6A). Again, the elevated phosphorylation status of cPLA2α was dependent on the presence of growth factors in the media (Fig. 6, A and B). As reported previously (10), subconfluent endothelial cells also show a cPLA2α-dependent elevation in Ca²⁺-in-

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**FIGURE 6. The phosphorylation and activity of cPLA2α are elevated in subconfluent endothelial cells.** A, confluent or subconfluent HUVECs were incubated in full growth media (ff) or under serum-free conditions (sf) for 4 h prior to lysis. Lysates were immunoprecipitated (IP) with anti-cPLA2α antibody and bound proteins assessed by Western blotting for phosphorylated serine 505 or total cPLA2α. B, quantification of phosphorylated serine 505 levels from four independent levels is shown. p < 0.01, n = 4. C, A23187-stimulated (5 μM, 15 min) [³H]AA release from confluent and subconfluent cells was measured in the presence or absence or Wyeth-1 (2.5 μM, 30 min) and expressed as a percentage of total [³H] incorporated. *, p < 0.05; **, p < 0.01 versus control; ***, p < 0.001 versus control subconfluent levels; n = 3.
duced AA release compared with confluent HUVECs (Fig. 6C). Thus, cPLA$_2^\alpha$ is consistently activated in proliferating, but not quiescent, endothelial cells, and this correlates with AA release.

As cPLA$_2^\alpha$ modulates endothelial cell proliferation and S phase progression, we hypothesized that release from the Golgi and activation of cPLA$_2^\alpha$ would be required for re-entry of quiescent endothelial cells back into the cell cycle. Upon mechanical wounding of confluent monolayers, cells at the wound borders enter into the cell cycle from G0, proliferate, and migrate into the denuded areas. After 24 h of recovery, HUVECs at the wound border (indicated by the diagonal line) begin to express PCNA and cyclin A at high levels as they enter and progress through the cell cycle (Fig. 7, A and B). As reported previously (11), cells at the wound border underwent relocation of cPLA$_2^\alpha$ from the Golgi apparatus to the more diffuse staining of subconfluent HUVECs, indicating activation of cPLA$_2^\alpha$ (Fig. 7, A–D). This activation of cPLA$_2^\alpha$ would explain the elevated levels of AA released upon mechanical wounding of confluent endothelial cells shown in Fig. 6 (10). Importantly, release from the Golgi and activation of cPLA$_2^\alpha$ were critical to the induction of cyclin A expression (Fig. 7, A–D). In confluent HUVEC monolayers mechanically wounded and recovered for 24 h in the presence of 2.5 μM pyrrolidine, cPLA$_2^\alpha$ had relocated in cells at the wound border, similar to uninhibited cells (Fig. 7, A–D). Quantification of the number of cells expressing high levels of nuclear PCNA revealed that inhibition of cPLA$_2^\alpha$ activity had no effect on PCNA up-regulation upon wounding (Fig. 7E), consistent with replication complex formation. However, the number of cells expressing high levels of nuclear cyclin A at the wound border fell from ~49 to 7% (i.e. 7-fold) in the presence of pyrrolidine (Fig. 7F). Highly expressing cells were easily distinguishable as their cyclin A nuclear fluorescence intensity was consistently ~4-fold than low expressing cells. Upon cPLA$_2^\alpha$ inhibition, the number of cells expressing high levels of cyclin A at the wound border fell to the levels seen with quiescent confluent monolayers (Fig. 7, B and F).

A similar reduction in cyclin A-positive cells at the wound border was also found in cPLA$_2^\alpha$ siRNA- and Wyeth-1-treated cells (Fig. 8A). Furthermore, only cells at the wound border exhibited BrdUrd incorporation that was significantly inhibited by Wyeth-1 (2.5 μM) treatment (Fig. 8, B and C). We have previously demonstrated a role for iPLA$_2$ in the control of HUVEC proliferation and progression through S phase. Thus, we sought to examine whether there was a cooperative role for the two
phospholipases A2 in the control of S phase entry after monolayer wounding. BrdUrd incorporation was examined by immunofluorescence microscopy in wound-recovered HUVECs treated with siRNA to a scrambled sequence, cPLA2α (siRNA-1), iPLA2α (15), or cPLA2α and iPLA2α siRNA together. Consistent with previous findings, siRNA knockdown of cPLA2α reduced the number of BrdUrd-positive cells at the wound border (Fig. 8D). Treatment with the iPLA2 siRNA duplexes also resulted in an ~55% reduction in cells incorporating BrdUrd at the wound border. Importantly, the simultaneous knockdown of both cPLA2α and iPLA2α inhibited BrdUrd incorporation by ~80% in an additive manner (Fig. 8D). These results suggest that both Ca^{2+}-dependent and Ca^{2+}-independent phospholipase A2 isoforms may cooperate to coordinate the response of endothelial cells to various proliferative stimuli.

To demonstrate the specificity of the cPLA2α siRNA phenotype, the heterologous expression of an siRNA-resistant form of cPLA2α (774 cPLA2α) using a vector containing an internal ribosome entry site-GFP sequence was found to rescue the proliferation defect in siRNA-treated subconfluent HUVECs (Fig. 9A). A significant increase (~6-fold) in the number of cyclin A-positive cells was evident upon restoration of cPLA2α levels using the expression plasmid (Fig. 9B). Ki67 levels were also elevated under these conditions (Fig. 9A).

We have previously shown that the addition of exogenous AA to pyrrolidine-treated HUVECs was able to partially rescue the proliferation defect induced by cPLA2α inhibition (11). Here we sought to assess whether AA could rescue other alterations in the cell cycle. Although re-expression of cPLA2α was able to rescue the decrease in cyclin A and Ki67 levels induced by depletion of cPLA2α, we sought to examine whether the application of the cPLA2α metabolites, AA or L-α-lysophosphatidylcholine (LPC), could similarly rescue the expression of these key cell cycle proteins. Subconfluent proliferating HUVECs were treated with pyrrolidine together with either 10 μM AA, 20 μM AA, 20 μM LPC, 20 μM L-α-phosphatidylcholine (PC), or L-α-phosphatidylethanolamine for 48 h prior to analysis of cyclin A, cdk2, and Ki67 levels by Western blotting. As shown in Fig. 9C, AA unexpectedly reduced cyclin A expression while increasing the expression of cdk2 in control cells. However, addition of either PC, LPC, or L-α-phosphatidylethanolamine also increased the basal levels of cyclin A and cdk2, together with Ki67 indicating an increase in proliferation. Interestingly, only the addition of crude brain PC could completely reverse and/or prevent the cell cycle defects induced by cPLA2α inhibition, restoring both cyclin A and cdk2 levels (Fig. 9D). Similar findings were seen with siRNA-treated HUVECs (data not shown). Why neither AA nor LPC was able to rescue the expression of these key S phase proteins remains unclear, but it may implicate a crucial role for the cPLA2α protein itself or its modulation of cellular phospholipid content, as critical to the control of cell cycle progression.

Thus, under our experimental conditions, cell cycle re-entry of endothelial cells is at least partially dependent on cPLA2α activation. The Ca^{2+}-dependent translocation and activation displayed by cPLA2α may allow it to preferentially respond to stimuli such as vessel damage or growth factors. Indeed, the contribution of cPLA2α to regulating HUVEC proliferation appears more prominent in conditions where active detachment of cell-cell contacts is required by the cell prior to cell...
cycle re-entry (i.e. the ~19% decrease in BrdUrd positive cells evident upon trypsinization and subconfluent re-seeding in Fig. 4D versus the ~55% decrease evident following wounding in Fig. 8C). Thus, the activation of cPLA2 following monolayer disruption represents a novel mechanism mediating cyclin A-cdk2 expression and regulating S phase progression in endothelial cells. These results suggest that the Ca²⁺-dependent activation of cPLA₂α is an important step in mediating endothelial cell proliferation in damaged blood vessels as demonstrated by the disruption of angiogenic tubule formation upon inhibition of cPLA₂α.

**DISCUSSION**

This study shows that cPLA₂α activation helps to regulate endothelial cell S phase progression and cell cycle entry. First, after mechanical wounding of confluent monolayers, endothelial cells at the wound borders release cPLA₂α from the Golgi apparatus resulting in its activation. Second, cPLA₂α activation is required for the elevation of cyclin A and cdk2 levels and subsequent S phase progression. Without active cPLA₂α, the passage of endothelial cells through the early phases of the cell cycle (G₁ through S to early M) is retarded, which is sufficient to interfere with long term angiogenic tubule formation. Thus, cPLA₂α activation is a requirement for the efficient escape of quiescent endothelial cells from G₀ and entry into the cell cycle. Other studies have implicated arachidonic acid metabolites in the control of G₁ to S phase progression of cells (33–35); however, our findings provide the first evidence for the central role of cPLA₂α itself in cyclin A expression, S phase progression, and cell cycle entry. A role for the protein itself is further validated by the failure of AA or LPC, the two major products of cPLA₂α lipase function, to rescue cyclin A or cdk2 levels, despite previously being shown to partially rescue proliferation (11). Furthermore, in Chinese hamster ovary cells and neuroblastoma N2A cells, cPLA₂α activity is elevated during mid/late G₁ and following G₁ to S phase transition (36); and in rat thyroid cells, the cPLA₂α-dependent production of glycerophosphoinositol is required for
receptor-mediated proliferation, independent of AA metabolism pathways (37).

In addition, we found that inhibition of cPLA2α in other cell types (saphenous vein smooth muscle cells, MCF-7 carcinoma cells, and epithelial HeLa cells) dose-dependently blocked cellular proliferation (data not shown), suggesting that the role of cPLA2α activity in S phase progression is common to other cell types. However, the precise roles of AA and its downstream products as well as lysophospholipids in regulating proliferation still require clarification. This is partially due to technical issues with lipid stability and also cellular requirements for lipid by-products to be produced close to their sites of action for efficient downstream activity (i.e. AA and cyclooxygenase and lipoxygenase enzymes) (38–40). Despite this, inactivation of cPLA2α by sequestration at the Golgi apparatus is unique to endothelial cells with this phenomenon not evident in other cell types examined (e.g. HeLa, Madin-Darby canine kidney cells, A549, saphenous vein smooth muscle, and EA.hy.926 cells; data not shown).

Endothelial cells undergo the contact-inhibition of proliferation at confluence and become quiescent. As cPLA2α activity is a requirement for cell cycle re-entry, the existence of a readily releasable pool of cPLA2α in quiescent endothelial cells could be utilized to allow rapid cell cycle re-entry upon the loss of cell-cell contacts. The role that serine phosphorylation plays in regulating both cPLA2α activity and its release from the Golgi apparatus requires defining and could represent an important target for modulating phospholipid turnover. This is highlighted by the recent finding that phosphorylation of cPLA2α on serine 727 disrupted binding to p11 and annexin A2 allowing the enzyme to access its phospholipid substrate (41). Another unique function of endothelial cells is their ability to proliferate, migrate, and differentiate to form new capillaries during angiogenesis. The induction of this process is important in wound healing and critical for solid tumor growth, resulting in antiangiogenic drugs being one of the most promising avenues of anti-cancer therapies (42–45). The re-entry of quiescent endothelial cells into the cell cycle is critical to activate the angiogenesis program (1, 2), and we have shown that inhibition of cPLA2α was sufficient to prevent angiogenic tubule formation, due solely to defects in the endothelial cell proliferation machinery. As such, targeting of endothelial cPLA2α, either alone or in combination with anti-iPLA2-VIA therapy, could represent a new approach to inhibit tumor neovascularization. Indeed, cPLA2α activity and the generation of LPC were recently identified as crucial events in the protection of endothelial cells against radiation-induced apoptosis and may represent a new target for modulating the radiosensitivity of the endothelium (46).

We had previously demonstrated that inhibition of group VIA iPLA2 (iPLA2-VIA), but not iPLA2-VIB or secretory PLA2, also blocked endothelial cell S phase progression and cell proliferation (15). Our work now suggests that both cPLA2α and iPLA2-VIA play distinct roles in the regulation of endothelial cell cycle progression. Here we show that products of both enzymes are required for the efficient release of endothelial cells from contact inhibition and re-entry into the cell cycle. Thus, in vivo, cells could sense the products (i.e. AA, lysophospholipids, or as suggested by our results, a reduction in membrane phospholipids) of multiple PLA2s to determine their readiness for progression into S phase; this would provide a mechanism to coordinate the amount of phospholipid required by a cell to successfully progress though cellular division. Previous studies have linked phosphatidylcholine metabolism to cell cycle regulation, and its incorporation is an S phase-specific event (47–49). In a recent study, changes to membrane fluidity caused by alteration to iPLA2 activities were hypothesized to be responsible for the growth defects seen upon iPLA2 inhibition; inhibition of the Ca2+-independent isoform of PLA2 led to an increase in membrane PC and resulted in G1 phase arrest (13, 14). Furthermore, it has been reported that excess PC can stimulate iPLA2-VIA activity, providing a mechanism for the ability of PC to recover the proliferation effect induced by cPLA2α inhibition (50). Additionally, in contrast to cPLA2, the activity of iPLA2-VIA appears to be required for the migration of HUVECs as well as their proliferation, which represents an important difference between their cellular activities and represents an area of future study. Defining how changes in phospholipase activities and the subsequent defects in phospholipid metabolism impact upon the cell cycle is crucial to utilizing this pathway as a therapeutic target.

However, it remains unclear whether cPLA2α and iPLA2-VIA regulate cellular proliferation by distinct or overlapping coordinated mechanisms. Initial studies suggest that cPLA2α and iPLA2-VIA have a synergistic effect on cell proliferation, and this may reflect both their different cellular localization (i.e. Golgi versus cytoplasm, respectively) and sites of their preferred substrates (i.e. endoplasmic reticulum/perinucleus versus plasma membrane, respectively), as well as their varied modes of activation. These variations may allow a cell to subtly coordinate its proliferative response to intracellular and extracellular cues by manipulating cellular lipids. The challenge will be to define the precise mechanisms by which cPLA2α and other phospholipase A2 enzymes regulate cell cycle progression.

Acknowledgments—We thank M. H. Gelb for kindly providing the cPLA2-GFP construct, pyrrolidine, and Wyeth-1. We also thank G. J. Howell for assistance with all aspects of bioimaging.

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