The Confluence-dependent Interaction of Cytosolic Phospholipase A₂-α with Annexin A1 Regulates Endothelial Cell Prostaglandin E₂ Generation

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The regulated generation of prostaglandins from endothelial cells is critical to vascular function. Here we identify a novel mechanism for the regulation of endothelial cell prostaglandin generation. Cytosolic phospholipase A₂-α (cPLA₂) cleaves phospholipids in a Ca²⁺-dependent manner to yield free arachidonic acid and lysophospholipid. Arachidonic acid is then converted into prostaglandins by the action of cyclooxygenase enzymes and downstream synthases. By previously undefined mechanisms, nonconfluent endothelial cells generate greater levels of prostaglandins than confluent cells. Here we demonstrate that Ca²⁺-independent association of cPLA₂ with the Golgi apparatus of confluent endothelial cells correlates with decreased prostaglandin synthesis. Golgi association blocks arachidonic acid release and prevents functional coupling between cPLA₂ and COX-mediated prostaglandin synthesis. When inactivated at the Golgi apparatus of confluent endothelial cells, cPLA₂ is associated with the phospholipid-binding protein annexin A1. Furthermore, the siRNA-mediated knockdown of endogenous annexin A1 significantly reverses the inhibitory effect of confluence on endothelial cell prostaglandin generation. Thus the confluence-dependent interaction of cPLA₂ and annexin A1 at the Golgi acts as a novel molecular switch controlling cPLA₂ activity and endothelial cell prostaglandin generation.

Cytosolic phospholipase A₂-α (cPLA₂) is an 85-kDa, Ca²⁺-sensitive member of the phospholipase A₂ (PLA₂) family of enzymes (1, 2) which includes the Ca²⁺-independent (iPLA₂) and secretory phospholipases A₂ (3). The PLA₂ enzymes hydrolyze the sn-2 fatty acyl bond of phospholipids to simultaneously generate free fatty acid and lysophospholipids (4). Upon agonist stimulation and cytosolic Ca²⁺ elevation, cPLA₂ translocates to intracellular membranes utilizing an N-terminal Ca²⁺-dependent lipid binding (CalB) domain (5–7). Upon membrane binding, cPLA₂ preferentially cleaves phospholipids containing arachidonic acid (AA) at the sn-2 position to liberate free AA (4). Consequently, cPLA₂ is seen as the rate-limiting enzyme in receptor-mediated AA release (8).

Ca²⁺ elevation can induce relocation of cPLA₂ to the specific intracellular membranes in which the downstream AA-metabolizing cyclooxygenase (COX) enzymes are also located (2). There are two isoforms of COX that have been extensively characterized (COX-1 and -2) (9), and more recently an alternative COX-1 splice variant, COX-3, has also been cloned (10). The spatiotemporal co-localization of cPLA₂ with COX can couple these enzymes to facilitate efficient conversion of AA into prostaglandins (2, 11). Chimeric cPLA₂ mutants specifically targeted to intracellular membranes in which COX does not reside do not couple with COX and drastically reduce prostaglandin production (11). Thus, the subcellular targeting of cPLA₂ to specific intracellular membranes is essential for the regulation of both AA and prostaglandin production. Despite this, the subcellular targeting of cPLA₂ in endothelial cells and its functional coupling with downstream COX enzymes has received little attention.

Release of prostaglandins by endothelial cells, the cells lining the luminal surface of all blood vessels, is essential to the control of vascular tone and thrombus formation (12, 13). Therefore, regulation of endothelial prostaglandin generation is critical to the maintenance of normal vascular function. Nonconfluent endothelial cells generate much greater levels of AA and prostaglandin than confluent cells (14–16), which has been attributed to elevated cPLA₂ activity. Despite this, the actual mechanism of this differential regulation of cPLA₂ activity has not been defined.

Inhibition of cPLA₂ activity by the phospholipid-binding protein, annexin A1, and the resulting block in AA metabolite release is a mechanism by which glucocorticoids exert their anti-inflammatory action (17–19). Annexin A1 is known to inhibit cPLA₂ activity upon interaction with the CalB domain of cPLA₂ in vitro (20, 21); however, the relevance of this interaction to processes other than inflammation remains unclear.
Here we demonstrate that annexin A1 acts as a novel regulator of endothelial cell AA and prostaglandin generation upon interaction with cPLA₂α at the Golgi apparatus of confluent cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords as previously described (22, 23). Human dermal microvascular endothelial cells 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 from Sigma or Invitrogen unless otherwise stated. cPLA₂α (BEL) were purchased from BioMol. All other reagents were used.

**Endothelial Cell AA and Prostaglandin Generation**—This technique was performed as previously described (16). Briefly, HUVECs were labeled for 24 h with 1 μCi/ml [³H]AA, washed with phosphate-buffered saline, and then incubated with 2.5 μM Fluo3-AM in serum-free media (plus 0.3% (w/v) fatty acid-free bovine serum albumin) for 30 min. Cells were then incubated with 5 μM A23187 in serum-free media for 60 min. Isolated fractions were analyzed by SDS-PAGE and immunoblotting. Iodixanol gradients were performed essentially as described by Yang et al. (27), except a 10°–30% gradient was utilized. Differential centrifugation enrichment of membrane fractions was performed as described previously by Lamour et al. (28).

**Microscopy and Quantitation**—Deconvolution fluorescence microscopy was performed using an Olympus IX-70 inverted fluorescence microscope (63 x 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. Quantification of co-localization was determined using the IMARIS software suite (Bitplane AG). Gray scale values below 10% of the maximum pixel intensity were eliminated as background. Co-localized pixels were expressed as percentages of the total pixels selected. Some images were captured using an inverted Zeiss LSM 510 META Axioper 200M confocal microscope.

**Determination of Cytosolic Ca²⁺ Concentration**—HUVECs cultured on glass bottom dishes were washed with HEPES/Tyrode's buffer and incubated with 2.5 μM Fluo3-AM (Molecular Probes) for 30 min. Subsequently, cells were washed and then incubated with HEPES/Tyrode's buffer (plus 1 mM Ca²⁺) for 20 min. Cells were placed in a heated chamber (37 °C) above an Olympus IX-70 inverted fluorescence microscope. Fluctuations in cytosolic Ca²⁺ were monitored by acquisition of fluorescence images.

**AA Release**—This technique was performed as previously (16). Briefly, HUVECs were labeled for 24 h with 1 μCi/ml [³H]AA, washed with phosphate-buffered saline, and then incubated with 10 μM BEL for 30 min to inhibit background iPLA₂ activity. Cells were then stimulated with 5 μM A23187 in serum-free media (plus 0.3% (w/v) fatty acid-free bovine serum albumin). Aliquots of media and cell lysate were counted by liquid scintillation for radioactivity.
cPLA₂α and Endothelial Cell Prostaglandin Generation

Prostaglandin E₂ Generation—HUVECs were cultured to the required cell density in 6-well culture dishes. Cells were washed and then in some cases incubated with 50 μM A23187 and/or 10 μM BEL for 30 min prior to treatment with 5 μM A23187 in HEPES/Tyrode’s buffer with 1 mM CaCl₂ for 15 min. Aliquots of media were assayed for prostaglandin E₂ (PGE₂) content using a high sensitivity ELISA (Assay Designs).

RNA Interference—HUVECs were transfected with either no siRNA (control), 50 nM nontargeting control siRNA (mock; D-001201-01; Dharmaco), or 50 nM annealed annexin A1 siRNA (siRNA; 593139; Ambion) for 4 h using the Lipofectamine2000 transfection reagent (Invitrogen). Cells were recovered for 48 h prior to lysis.

RESULTS

Cytosolic Ca²⁺ Elevation Targets cPLA₂α to Intracellular Membranes in Subconfluent Endothelial Cells—cPLA₂α activity is greater in subconfluent endothelial cells than in quiescent, confluent endothelial cells (15, 16). We have previously shown that subconfluent and confluent endothelial cells (see supplemental Fig. 1) express equal amounts of cPLA₂α (16), indicating that mechanisms other than control of cPLA₂α expression are responsible for confluence-dependent changes in its activity. In response to elevated cytosolic Ca²⁺, cPLA₂α is activated by relocation to intracellular membranes. Recruitment to specific membranes is required for the regulation of cPLA₂α activity (2, 11); however, the precise membranes to which cPLA₂α relocates in primary endothelial cells have not been defined. Therefore, we investigated the Ca²⁺-induced relocation of cPLA₂α.

In HUVECs, cPLA₂α was detectable as a 110 kDa band by Western blotting using a well characterized affinity-purified antibody specific to the C-terminal region of cPLA₂α (16, 31). Additionally, the immunoreactivity was removed by preabsorption of the antibody with the antigenic peptide (supplemental Fig. 2A). By immunofluorescence microscopy, cPLA₂α was present as both diffuse and structured pools throughout the cytoplasm and nucleus of subconfluent HUVECs (supplemental Fig. 2B), similar to previous observations with endothelial cells and fibroblasts (16, 32, 33). To study the relocation of cPLA₂α in response to cytosolic Ca²⁺ elevation, we used the Ca²⁺ ionophore, A23187. This agent is ideal for studying confluence-dependent changes in cPLA₂α relocation, since A23187 raises cytosolic Ca²⁺ to similar levels in both subconfluent and confluent endothelial cells (supplemental Fig. 2C). Signaling mediated by other agonists that elevate cytosolic Ca²⁺ varies with endothelial cell density (34, 35). In subconfluent HUVECs, upon elevation of cytosolic Ca²⁺, cPLA₂α relocated to the nuclear periphery and less disperse cytoplasmic structures (see supplemental Fig. 2). Relocation occurred rapidly (<1 min) and cPLA₂α immunoreactivity co-distributed extensively with calreticulin and ERGIC-53 (Fig. 1A and supplemental Fig. 2, D–E), consistent with translocation to the endoplasmic reticulum (ER) and ER-Golgi intermediate compartments (ERGIC) (36–38). Quantitation of this co-localization revealed a 2.5-fold increase in overlap between cPLA₂α and calreticulin and a 2.3-fold increase in overlap between cPLA₂α and ERGIC-53 upon A23187 treatment (Fig. 1A). A23187-induced relocation of cPLA₂α to the ER and ERGIC promoted its interaction with mem-

brane substrate, resulting in a 12-fold increase in AA release from subconfluent cells (Fig. 1B).

cPLA₂α Is Coupled to COX-1 and -2 in Subconfluent Endothelial Cells—AA may be converted into prostaglandin H₃ by the action of the COX enzymes. Targeting of cPLA₂α to the specific intracellular membranes in which COX enzymes are located can lead to the coupling of these enzymes to facilitate efficient conversion of AA into prostaglandins (2, 11). Functional coupling does not require the direct interaction of AA synthesizing and metabolizing enzymes but relies on both enzymes being in close apposition. Consequently, AA released by cPLA₂α is statistically more likely to encounter the required downstream enzyme than if synthesized at a distant site. We predicted that recruitment of cPLA₂α to the ER of subconfluent

FIGURE 1. cPLA₂α targets to the ER/ERGIC and functionally couples with the COX enzymes in nonconfluent endothelial cells. A, subconfluent HUVECs were directly fixed or stimulated with 5 μM A23187 for 1 min prior to fixation. cPLA₂α and either calreticulin or ERGIC-53 were detected by immunofluorescence microscopy. Quantification of cPLA₂α co-distribution with calreticulin and ERGIC-53 was performed, and the percentage of co-localization was calculated using the IMARIS computer package as described under “Experimental Procedures” (n = 13, ±S.E.). Subconfluent cells loaded with 1 μCi/ml [³H]AA were stimulated with 5 μM A23187 for 15 min, and released AA was determined by scintillation counting. Results are expressed as a percentage of the total [³H]AA incorporated (n = 3, ±S.E.), *p < 0.001 versus unstimulated cells. Subconfluent HUVECs were directly fixed or stimulated with 5 μM A23187 for 1 min prior to fixation. cPLA₂α and either COX-1 or -2 were detected by immunofluorescence microscopy, and the co-distribution of cPLA₂α with COX-1 and -2 was quantified. Percentage co-localization was calculated using the IMARIS computer package as described under “Experimental Procedures” (n = 15, ±S.E.). D, generation of PGE₂ by subconfluent HUVECs in response to A23187 stimulation. Cells were treated with combinations of A23187 and or BEL for 30 min prior to stimulation with A23187 for 15 min in the presence or absence of 10 μM AA. PGE₂ generation was quantified using a high sensitivity ELISA (n = 3, ±S.E.), *p < 0.001 versus unstimulated cells, **p < 0.001 versus A23187-stimulated cells. All results are representative of three separate experiments.

TABLE 1. Percent overlap between cPLA₂α and calreticulin and ERGIC-53 upon A23187 treatment

| Treatment         | % overlap | p value |
|-------------------|-----------|---------|
| Unstimulated      | 0%        |         |
| A23187            | 25%       | <0.001  |

Percentage co-localization was calculated using the IMARIS computer package as described under “Experimental Procedures” (n = 3, ±S.E.).
HUVECs would promote functional coupling, since both COX-1 and -2 are located at the ER of endothelial cells (39).

Subconfluent cells were stimulated to elevate intracellular Ca\(^{2+}\), and the co-distribution of cPLA\(_2\)α with COX-1 and -2 was assessed (Fig. 1C and supplemental Fig. 2, F and G). Quantitation revealed a 3.2-fold increase in overlap between cPLA\(_2\)α and COX-1 and a 2.3-fold increase between cPLA\(_2\)α and COX-2 upon cytosolic Ca\(^{2+}\) elevation (Fig. 1C). We then assessed the ability of subconfluent HUVECs to generate PGE\(_2\), a major downstream product of both COX-1 and -2 activity (Fig. 1D). PGE\(_2\) plays a key role in a variety of key vascular processes, such as angiogenesis and the regulation of vascular tone (40, 41). Prior to A23187 treatment, PGE\(_2\) generation was minimal (0.35 pg/1000 cells) but rose 24-fold upon cytosolic Ca\(^{2+}\) elevation. Pretreatment of HUVECs with BEL, an inhibitor of iPLA\(_2\) activity (42), had no effect on Ca\(^{2+}\)-induced PGE\(_2\) generation. This suggests that iPLA\(_2\)-mediated AA release is not involved in PGE\(_2\) production in endothelial cells. Inhibition of cPLA\(_2\)α with AACOCF\(_3\) (43) inhibited PGE\(_2\) generation by 88% (Fig. 2D). Thus, Ca\(^{2+}\)-induced PGE\(_2\) generation was

**FIGURE 2.** Sequestration of cPLA\(_2\)α at the Golgi apparatus inhibits membrane recruitment. A, the localization of cPLA\(_2\)α in subconfluent (sub-confl.) and confluent (confl.) HUVECs was determined by immunofluorescence microscopy. B, HUVECs were transfected with either recombinant GFP alone or recombinant cPLA\(_2\)α tagged with GFP to the N terminus (GFP-cPLA\(_2\)α) or C terminus (cPLA\(_2\)α-GFP). After 48 h, transfected HUVECs were directly fixed, and both GFP and TGN46 were detected by immunofluorescence microscopy. C and D, confluent HUVECs were directly fixed or stimulated with 5 \(\mu\)M A23187 for 1 min prior to fixation. cPLA\(_2\)α and either calreticulin (C) or ERGIC-53 (D) were detected by immunofluorescence microscopy. E, quantification of cPLA\(_2\)α co-distribution with calreticulin and ERGIC-53. Percentage co-localization was calculated using the IMARIS computer package as described under “Experimental Procedures” (n = 13, ±S.E.), *p < 0.001 versus subconfluent cells. All results are representative of three separate experiments. All images represent 0.2-\(\mu\)m sections through cell nuclei. *, EC nuclei. Scale bar, 25 \(\mu\)m.
almost entirely dependent on cPLA2α activity. The maximal capacity of the cells to produce PGE2 was assessed by incubating cells with exogenous AA. Exogenous AA reversed the inhibitory effect of AACOCF3 but did not elevate PGE2 generation any higher than that liberated by cytosolic Ca2+ elevation. Thus, specific targeting of cPLA2α to the ER/ERGIC and co-localization with the COX enzymes appears to result in maximal conversion of AA into PGE2. This demonstrates coupling between these enzymes in endothelial cells and indicates that release of AA by cPLA2α is the rate-limiting step in PGE2 production in endothelial cells.

**Sequestration of cPLA2α at the Golgi Apparatus of Quiescent Endothelial Cells Blocks Its Translocation to Other Membranes**—By immunofluorescence microscopy, in confluent endothelial cells, cPLA2α was seen to become associated with a reticular juxtanuclear region (Fig. 2A) corresponding to the Golgi apparatus. Similar results were obtained with recombinant GFP-tagged cPLA2α (N-terminal and C-terminal linked constructs; Fig. 2B) and with a separate antibody targeted to the C terminus of cPLA2α (supplemental Fig. 3). In other cell types, association of cPLA2α with the Golgi apparatus promotes AA release (44, 45), whereas in confluent endothelial cells, cPLA2α activity is inhibited (14–16). Furthermore, interaction with the Golgi blocked targeting of cPLA2α to the ER and ERGIC upon Ca2+ elevation (Fig. 2, C–E). In confluent HUVECs, the co-distribution of cPLA2α with calreticulin (Fig. 2C) and ERGIC-53 (Fig. 2D) positive structures was not enhanced upon cytosolic Ca2+ elevation. Quantitation revealed that overlap between cPLA2α and calreticulin was only 4% that of subconfluent cells upon cytosolic Ca2+ elevation (Fig. 2E). Overlap between cPLA2α and ERGIC-53 was also reduced by 47% relative to A23187-treated subconfluent cells (Fig. 2E). The reduction in overlap with ERGIC-53 was not to the extent seen with calreticulin but is probably due to background overlap with ERGIC-53-positive vesicles that have fused with the Golgi apparatus. Most importantly, when quantified, overlap of cPLA2α with the ER and ERGIC was not enhanced upon cytosolic Ca2+ elevation (Fig. 2E). Thus, association with the Golgi apparatus sequesters
cPLA₂α away from its intracellular substrate, accounting for the reduced AA release seen at endothelial cell confluence (15, 16).

Sequestration of cPLA₂α at the Golgi Apparatus Inhibits Its Functional Coupling with the COX Enzymes—Confluent endothelial cells generate lower levels of prostaglandins than subconfluent cells (14, 15). We predicted that the sequestration of cPLA₂α at confluence would be important to the control of prostaglandin generation. In confluent HUVECs, no significant overlap between cPLA₂α and COX-1 or -2 was observed prior to A23187 stimulation (Fig. 3, A and B). Furthermore, sequestration at the Golgi apparatus blocks the Ca²⁺-induced co-localization of cPLA₂α with COX-1 and -2 (Fig. 3, A and B). Quantitation revealed that overlap of cPLA₂α with COX-1 and -2 was only 6 and 22% that of subconfluent cells upon cytosolic Ca²⁺ elevation (Fig. 3C). As a result, in confluent HUVECs, Ca²⁺-induced PGE₂ generation was inhibited by 95.6% relative to subconfluent cells (Fig. 3D). Furthermore, cytosolic Ca²⁺ elevation did not induce any greater PGE₂ generation than unstimulated controls (Fig. 3D). The low levels of PGE₂ generated from confluent HUVECs were not due to iPLA₂α or cPLA₂α-mediated AA release, since preincubation with BEL and AACOCF₃ had no effect (Fig. 3D). These results were not due to variations in endogenous COX activity, since both subconfluent and confluent HUVECs generate similar levels of PGE₂ when supplied with exogenous AA (Fig. 3E). Thus, sequestration of cPLA₂α at the Golgi apparatus of endothelial cells represents a novel mechanism for the regulation of prostaglandin generation by blocking its targeting to the specific intracellular membranes at which COX-1 and -2 reside.

The Interaction of cPLA₂α with the Golgi Apparatus is Ca²⁺-independent—Treatment of subconfluent HUVECs with A23187 does not enhance the co-distribution of cPLA₂α with the Golgi-resident protein mannosidase II (46) (Fig. 4A, ManII). This is despite other reports documenting the Ca²⁺-independent, since treatment of confluent HUVECs with mannosidase II was not influenced by intracellular Ca²⁺ elevation (Fig. 4B). Thus, in confluent HUVECs, cPLA₂α is immobilized at the Golgi apparatus and is insensitive to Ca²⁺ elevation. The interaction of cPLA₂α with the Golgi is entirely Ca²⁺-independent, since treatment of confluent monolayers with the intracellular Ca²⁺ chelator, 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid acetoxyethyl ester, did not affect the Golgi association of cPLA₂α (Fig. 4C). Furthermore, the Golgi association of cPLA₂α was...
not affected by incubation with the extracellular Ca\(^{2+}\) chelator, EGTA (Fig. 4C), which is known to decrease intracellular Ca\(^{2+}\) levels (7).

Since this interaction has no requirement for Ca\(^{2+}\), it cannot be mediated by the Ca\(^{2+}\)-dependent lipid binding property of the CalB domain of cPLA\(_2\). Thus, we hypothesized that association with the Golgi apparatus may occur via a novel mechanism.

**cPLA\(_2\) Interacts with Annexin A1 at the Golgi Apparatus of Confluent Endothelial Cells**—There is previous evidence to suggest that cPLA\(_2\) activity can be modulated by its interaction with a number of binding partners. The CalB domain of cPLA\(_2\) binds the head domain of vimentin in far Western and co-immunoprecipitation experiments (48). In addition, overexpression of the head domain of vimentin inhibits AA and prostanoid production in rat fibroblasts (48). However, when confluent HUVECs were co-stained for cPLA\(_2\) and vimentin, no evidence for a co-association was apparent at the Golgi apparatus (Fig. 5A). From yeast two-hybrid and immunoprecipitation experiments, the catalytic domain of cPLA\(_2\) has also been found to bind p11, a member of the S100 family of calcium-binding proteins (49). In vitro, p11 inhibits cPLA\(_2\), and in vivo knockdown of p11 elevates AA release in human bronchial epithelial cell lines. However, from immunofluorescence co-staining experiments, no evidence for an association of p11 with cPLA\(_2\) was found at the Golgi apparatus of confluent HUVECs (Fig. 5A).

The phospholipid-binding protein, annexin A1, is known to interact with the CalB domain of cPLA\(_2\) in vitro (20, 21). In addition, annexin A1 has been implicated in the regulation of cellular cPLA\(_2\) activity in a number of studies (17, 19). We therefore compared the locations of cPLA\(_2\) and annexin A1 in confluent endothelial cells (Fig. 5A). Results showed that a pool of annexin A1 was located at the Golgi apparatus of confluent endothelial cells and that this pool extensively co-distributed with cPLA\(_2\) and the Golgi marker protein, TGN46 (Fig. 5, A and B). In subconfluent endothelial cells, annexin A1 is not enriched at the Golgi apparatus to the same extent as confluent cells (Fig. 5B). Cellular levels of annexin A1 protein do not vary between confluent and subconfluent endothelial cells (supplemental Fig. 4); therefore, an enrichment of annexin A1 at the Golgi apparatus of confluent HUVECs must be a consequence of the cell density-dependent redistribution of annexin A1. Evidence for a physical association between annexin A1 and cPLA\(_2\) was further obtained upon co-immunoprecipitation of annexin A1 with cPLA\(_2\) from confluent HUVEC lysates (Fig. 5C). To confirm the specificity of the interaction, immunoprecipitations were also performed after preincubation of anti-cPLA\(_2\) antibodies with antigenic peptide. Under these conditions, neither cPLA\(_2\) nor annexin A1 were immunoprecipitated (Fig. 5C). To further assess the confluence dependence of the association between annexin A1 and cPLA\(_2\), immunoprecipitations of annexin A1 were performed from confluent and subconfluent HUVEC lysates and immunoblotted for the presence of cPLA\(_2\). Consistent with the immunofluorescence data, only annexin A1 from confluent endothelial cells was associated with cPLA\(_2\) (Fig. 5D).
Interaction of cPLA2α with Annexin A1 Inhibits Prostaglandin Synthesis in Confluent Endothelial Cells—To test the hypothesis that interaction with annexin A1 at the Golgi apparatus is responsible for inhibition of cPLA2α activity in confluent endothelial cells, the siRNA knockdown of annexin A1 was performed. In agreement with the hypothesis, knockdown of endogenous annexin A1 resulted in a significant increase in AA-dependent PGE2 generation relative to HUVECs transfected with nontargeting siRNA (Fig. 6, A and B, mock). However, the knockdown of endogenous annexin A1 was not sufficient to elevate PGE2 generation from HUVECs stimulated in the presence of free AA (Fig. 6C). Thus, the increased ability of siRNA-treated HUVECs to generate PGE2 was a consequence of an increased ability to release free AA and not due to an effect on downstream aspects of PGE2 generation. Furthermore, this effect was not a consequence of increased cPLA2α, COX-1, or COX-2 expression, since knockdown of endogenous annexin A1 had no effect on their protein levels (Fig. 6A). To determine whether the interaction of cPLA2α with annexin A1 is responsible for sequestering cPLA2α at the Golgi apparatus of confluent HUVECs, the subcellular localization of cPLA2α after annexin A1 knockdown was investigated (Fig. 6D). Upon knockdown, endogenous annexin A1 cPLA2α becomes distributed throughout the cell in a manner similar to subconfluent cells. Furthermore, in annexin A1 siRNA-treated cells, cPLA2α does not appear to co-distribute as extensively with the Golgi marker protein, TGN46, relative to control siRNA-treated HUVECs (Fig. 6D). Quantification of this overlap confirmed that significantly less cPLA2α co-distributed with the Golgi apparatus of annexin A1 siRNA-treated cells versus control cells (Fig. 6E). Thus, the association of cPLA2α with annexin A1 at the Golgi apparatus represents a novel mechanism for the control of endothelial cell AA release and prostaglandin production.

DISCUSSION

Here we have defined a novel mechanism for the regulation of prostaglandin biosynthesis in endothelial cells. Generation of prostaglandins by the endothelium regulates vascular homeostasis. For example, PGE2 acts as a potent vasodilator/vasoconstrictor and proangiogenic stimuli (40, 41). Here we find that targeting of cPLA2α to the ER/ERGIC is required for its functional coupling to the COX enzymes and maximal PGE2 generation. Furthermore, sequestration of cPLA2α at the Golgi apparatus is sufficient to abolish Ca2+-induced prostaglandin

**FIGURE 6.** Inhibition of cPLA2α activity at confluence occurs upon interaction with annexin A1. A, confluent HUVECs were transfected with either no siRNA (control), nontargeted control siRNA (mock), or annexin A1-targeted siRNA (siRNA). 48 h after transfection, cells were lysed, and annexin A1 levels were analyzed by Western blotting. Membranes were reprobed for cPLA2α, COX1, and COX2 to show equal protein loading. Relative amounts of annexin A1, cPLA2α, and COX1 were determined using densitometry software and then plotted (n = 3, ±S.E.). B, transfected cells were incubated with 5 μM A23187 for 15 min, and the ability of cells to generate PGE2 was then assessed using an ELISA (n = 3, ±S.E.). C, transfected cells were incubated with 5 μM A23187 and 10 μM arachidonic acid for 15 min, and the ability of cells to generate PGE2 was then assessed using an ELISA (n = 3, ±S.E.). D, confluent transfected cells were directly fixed, and then cPLA2α, annexin A1, and TGN46 were detected by confocal immunofluorescence microscopy. E, quantification of the co-distribution of cPLA2α with TGN46 upon the siRNA-mediated knockdown of annexin A1 performed using LSM510Meta software (Zeiss). Results represent the ratio of cPLA2α/TGN46 fluorescence intensity at TGN46-positive Golgi structures (n = 40, ±S.E.), *, p < 0.05 versus mock.
FIGURE 7. A model for the regulation of cPLA₂α activity in endothelial cells. In subconfluent cells, cPLA₂α is free to associate with the ER and ERGIC upon cytosolic Ca²⁺ elevation. The resulting spatiotemporal colocalization with the COX enzymes then facilitates the conversion of AA into prostaglandins. Upon cell confluence, cPLA₂α becomes associated with the Golgi apparatus and interacts with the cPLA₂α-inhibitory protein, annexin A1. Consequently, the blocking of membrane targeting and inhibition of cPLA₂α activity abolishes cPLA₂α-mediated signaling.

generation in confluent endothelial cells (Fig. 7). This represents a novel molecular switch for the control of endothelial prostaglandin generation and accounts for earlier observations that confluent endothelial cells generate lower levels of prostaglandins compared with nonconfluent endothelial cells (14, 15).

In this study, we describe the Ca²⁺-induced association of cPLA₂α with the ER/ERGIC of nonconfluent HUVECs. Previously, we demonstrated that intracellular Ca²⁺ elevation targets cPLA₂α to membranes distinct from ER/Golgi apparatus of EA.hy.926 cells (31). The EA.hy.926 cell line is a continuous hybrid line formed from the fusion of primary HUVECs and the human lung carcinoma cell line, A549. Consequently, any difference in the localization of cPLA₂α in EA.hy.926 cells versus their HUVEC donor line must represent characteristics associated with the A549 fusion partner. These differences emphasize the highly cell type-specific nature of the subcellular localization of cPLA₂α and highlight the potential deficiencies of hybrid cell lines as model cell systems. Sequestration at the Golgi apparatus and inactivation of cPLA₂α appears to be unique to endothelial cells and is not displayed by any other cell type tested (HeLa, Madin-Darby canine kidney, A549, saphenous vein smooth muscle, and EA.hy.926 cells; data not shown). Furthermore, this mechanism appears to be common to all endothelial cells, since we find that Golgi-localized cPLA₂α is seen in confluent cell cultures of HUVECs, human dermal microvascular endothelial cells, and HCAEC (not shown). Golgi-localized cPLA₂α is also seen in endothelial cells in isolated rat mesenteric arteries (data not shown).

It has been suggested previously that confluence-dependent fluctuations in endothelial cell PGE₂ generation are a consequence of differential COX activity (50). However, the previous study was conducted on serum-starved, unstimulated endothelial cells in the absence of intracellular Ca²⁺ elevation. In their model system, AA must be derived from cPLA₂α-independent sources, and the resultant PGE₂ production is minimal (<1.4 pg/1000 cells). In addition, we find that total COX activity remains constant independent of endothelial cell density, consistent with previous observations (15). Thus, regulation of prostaglandin generation from endothelial cells is critically dependent on the ability of cPLA₂α to be targeted to specific intracellular membranes.

Here we establish that inhibition of cPLA₂α at the Golgi apparatus of confluent endothelial cells occurs upon interaction with annexin A1. A number of cPLA₂α-binding proteins that negatively regulate cPLA₂α activity have been previously identified and include the intermediate filament protein vimentin (48, 51), the calcium-binding protein p11/S100A10 (49), and the phospholipid–binding protein annexin A1 (20, 21). Using co-immunoprecipitation assays, we found no evidence for binding of cPLA₂α to vimentin or p11/S100A10 in confluent cells (data not shown); however, cPLA₂α was seen to specifically associate with annexin A1 only at the Golgi apparatus of confluent unstimulated endothelial cells. This interaction was responsible for inhibition of cPLA₂α activity, since siRNA-mediated knockdown of annexin A1 released cPLA₂α from the Golgi and significantly elevated AA-dependent PGE₂ production. Thus, interaction of annexin A1 with cPLA₂α at the Golgi apparatus represents a novel regulatory mechanism for the control of endothelial cell AA release and prostaglandin production. This is consistent with previous studies documenting the importance of annexin A1-mediated inhibition of cPLA₂α to the anti-inflammatory action of glucocorticoids (17–19).

In this study, we also describe the cell density-dependent relocation of annexin A1 to the Golgi apparatus of confluent endothelial cells. Consequently, the accumulation of annexin A1 at the Golgi apparatus inactivates cPLA₂α upon its interaction with annexin A1 and the sequestration of cPLA₂α away from its intracellular substrates. When endothelial cells reach confluence, they characteristically undergo the contact inhibition of cellular proliferation and exit the cell cycle to form quiescent monolayers. The molecular mechanisms regulating the contact inhibition are not clearly defined but are known to involve the inhibition of growth factor receptor signaling, activation of protein phosphatases, and inactivation of Rac upon the formation of cell-cell contacts (34, 52, 53). Similar mechanisms may promote the confluence-dependent redistribution of annexin A1 to the Golgi apparatus and subsequent inactivation of cPLA₂α. However, the distinct mechanisms involved in the redistribution of annexin A1 remain to be elucidated.

In subconfluent endothelial cells, cPLA₂α is distributed throughout the cytoplasm and nucleus of resting cells and
remains largely inactive. Upon Ca\textsuperscript{2+} elevation, cPLA\textsubscript{2}\textalpha{} can then interact with intracellular membrane substrates via its Ca\textsuperscript{2+}-dependent lipid-binding domain (C2 domain). In confluent cells, the Ca\textsuperscript{2+}-dependent ability of cPLA\textsubscript{2}\textalpha{} to interact with membranes is blocked upon its sequestration at the Golgi apparatus. Sequestration of signaling proteins away from their endogenous substrates is an emerging concept in the regulation of a variety of enzymes. The Golgi apparatus is known to possess an “exoskeleton” of structural proteins, which includes the molecular scaffold Sef. Interaction with Sef sequesters MEK/ERK at the Golgi apparatus and excludes it from nuclear targets (54). Similarly, annexin A1 is also known to form membrane scaffolds (55). However, isolation of endothelial cell Golgi membranes using differential centrifugation, sucrose density gradients, or iodixanol (OptiPrep) gradients does not produce annexin A1- or cPLA\textsubscript{2}\textalpha{}-enriched Golgi fractions when isolated from confluent cells lysed in the absence of high Ca\textsuperscript{2+} concentrations (>1 mM) (supplemental Figs. 5 and 6, A and B). It is only upon the addition of Ca\textsuperscript{2+} and the absence of chelators that cPLA\textsubscript{2}\textalpha{} and annexin A1 become associated with membrane-containing fractions. Thus, in confluent endothelial cells, cPLA\textsubscript{2}\textalpha{} may not directly interact with components of the Golgi membrane but may interact with an associated structure or scaffold that is disrupted upon mechanical lysis and subcellular fractionation. The Golgi-localized pool of annexin A1 may represent such a labile scaffold to which cPLA\textsubscript{2}\textalpha{} is immobilized in confluent endothelial cells. An analogous situation has been reported for phospholipase D, which is blocked from accessing its phospholipid substrate by binding to the cytoskeletal, spectrin-related protein, fodrin (56). The next challenge will be to elucidate the molecular details of annexin A1-mediated inhibition of cPLA\textsubscript{2}\textalpha{} in the endothelial cell.

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