Internalized Group V Secretory Phospholipase A$_2$ Acts on the Perinuclear Membranes.

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Running Title: sPLA$_2$ internalization

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SUMMARY

Mammalian secretory phospholipases A₂ (sPLA₂) have been implicated in cellular eicosanoid biosynthesis but the mechanism of their cellular action remains unknown. To elucidate the spatiotemporal dynamics of sPLA₂ mobilization and determine the site of its lipolytic action, we performed time-lapse confocal microscopic imaging of fluorescently labeled sPLA₂ acting on human embryonic kidney (HEK) 293 cells the membranes of which are labeled with a fluorogenic phospholipid, N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-hexadecanoyl-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-sn-glycero-3-phosphoethanolamine. The Western blotting analysis of HEK293 cells treated with exogenous sPLA₂s showed that not only the affinity for heparan sulfate proteoglycan but also other factors, such as sPLA₂ hydrolysis products or cytokines, are necessary for the internalization of sPLA₂ into HEK293 cells. Live cell imaging showed that the hydrolysis of fluorogenic phospholipids incorporated into HEK293 cell membranes was synchronized with the spatiotemporal dynamics of sPLA₂ internalization, detectable initially at the plasma membrane and then at the perinuclear region. Lastly, immunocytostaining showed that human group V sPLA₂ induced the translocation of 5-lipoxygenase to the nuclear envelope at which they were colocalized. Together, these studies provide the first experimental evidence that the internalized sPLA₂ acts on the nuclear envelope to provide arachidonate for other enzymes involved in the eicosanoid biosynthesis.
INTRODUCTION

Phospholipases A$_2$ (PLA$_2$) catalyze the hydrolysis of membrane phospholipids, the products of which can be transformed into potent inflammatory lipid mediators, platelet activating factor and eicosanoids that include prostaglandins, thromboxanes, leukotrienes and lipoxins. Multiple forms of secretory PLA$_2$s (sPLA$_2$) and intracellular PLA$_2$s have been found in mammalian tissues (1). Recent cell studies have indicated that some sPLA$_2$ isoforms work in concert with group IV cytosolic PLA$_2$ (cPLA$_2$) to induce immediate and delayed eicosanoid formation (2-4). At present, the identity of pro-inflammatory sPLA$_2$, the spatiotemporal dynamics of sPLA$_2$ mobilization, and the signaling mechanism that links sPLA$_2$, cPLA$_2$, and other enzymes involved in eicosanoid biosynthesis are not fully understood. It has been reported that the heparan sulfate proteoglycan (HSPG)-mediated internalization of sPLA$_2$ is an important step in sPLA$_2$ actions on mammalian cells (3-6); however, functional consequences of sPLA$_2$ internalization remain controversial. In agonist-induced human embryonic kidney 293 (HEK293) cells transfected with various sPLA$_2$s, the sPLA$_2$ internalization resulted in arachidonic acid (AA) release and prostaglandin synthesis (3-6), whereas in human neutrophils (7) and mast cells (8) the sPLA$_2$ internalization led to protein degradation. This study was undertaken to clarify the effect of sPLA$_2$ internalization on the cellular eicosanoid biosynthesis and determine the location of sPLA$_2$ lipolytic actions. Results described herein provide the first experimental evidence that the internalized sPLA$_2$ liberates fatty acids from the phospholipids in the nuclear envelope at which other eicosanoid-producing enzymes are localized during the cellular eicosanoid biosynthesis.
EXPERIMENTAL PROCEDURES

Materials – 1,1'-didodecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiIC12), N-((6-(2,4-dinitrophenyl)amino)hexanoyl)-1-hexadecanoyl-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-sn-glycero-3-phosphoethanolamine triethylammonium salt (PED6) and Texas Red® C₂ maleimide were purchased from Molecular Probes, Inc. (Eugene, OR). Cholesterol, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS) were from Avanti Polar Lipids, Inc. (Alabaster, AL) and used without further purification. Phospholipid concentrations were determined by phosphate analysis (9). Dubbleco's Modified Eagles Medium (DMEM) and inactivated fetal bovine serum (FBS) were from Gibco (Grand Island, NY). HEK293 cells and zeocin were from Invitrogen (San Diego, CA). Fatty acid-free bovine serum albumin (BSA) was from Bayer Inc. (Kankakee, IL). Arachidonyl trifluoromethyl ketone was from Calbiochem (San Diego, CA). Recombinant human group V PLA₂ (hVPLA₂) (10), its mutants (7,11), and human group IIa PLA₂ (hIIaPLA₂) (12) were expressed and purified as described previously.

Western Blotting Analysis of hVPLA₂-treated HEK293 Cells — HEK293 cells were treated with 100 nM of hVPLA₂-W79A, W79A/W31A, W79A/R100E/K101E, and hIIaPLA₂ for the indicated period, and the incubation was quenched by adding a solution of ice-cold 0.6 M NaCl in DMEM. After washing with the same solution, the pellet was collected by scrapping and centrifugation, then lysed in 70 µl of lysis buffer (20 mM Tris-HCl, 30 mM Na₄P₂O₇, 50 mM NaF, 40 mM NaCl, 5 mM EDTA, pH 7.4) containing 1% Nonidet P-40, 10 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na₃VO₄, and 0.5% deoxycholic
acid. After 10 min on ice, the cell lysates were centrifuged at 12,000g for 3 min to remove the cell debris. The supernatants were then mixed with 14 µl of gel loading buffer (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% sodium dodecyl sulfate, 0.005% bromphenol blue), and the mixtures were boiled for 5 min. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing condition using 16% acrylamide gels. The electrotransfer of proteins from the gels to polyvinylidene fluoride membrane was achieved using a semidry system (400 mA, 120 min). The membrane was blocked with 2% BSA for 60 min, then incubated with 1 µg/ml of either the anti-hVPLA2 monoclonal antibody 3G1 (14) or a commercial hIIaPLA2 antibody (Upstate Biotechnology) diluted in Tris-buffered saline plus 0.05% Tween 20 (TBS-T) overnight. The membranes were washed three times for 20 min with TBS-T. Goat anti-mouse IgG conjugated with horseradish peroxidase was diluted 3000-fold in TBS-T and incubated with polyvinylidene fluoride membrane for 60 min. The membrane was washed three times with TBS-T and assayed with an ECL chemiluminescence system (Amersham Pharmacia Biotech).

Arachidonic Acid Release — Radiolabeling of human neutrophils cells with [3H]-AA was performed as described previously (7). Radiolabeling of HEK293 cells was achieved by incubating the cells (10^6) with 0.05 µCi/ml [3H]-AA for 4 h at 37 °C. Unincorporated [3H]-AA was removed by washing the cells three times with DMEM containing 0.2% BSA. The reaction was quenched by adding 3 ml of ice-cold DMEM and the cell and the medium were separated by centrifugation, then the radioactivity of pellet and supernatant, respectively, was measured by liquid scintillation.
Texas Red Labeling of W79C hVPLA2 — To create a single free cysteine for chemical labeling, the W79C mutation was performed as described previously (11). W79C was expressed, refolded and purified according to the protocol used for hVPLA2 (10). Purified W79C (0.5 mg) was dissolved in 1 ml 25 mM Tris-HCl, pH 7.5, containing 0.5 M guanidinium chloride and treated with 10-fold molar excess of Texas Red® C₂ maleimide for 2 h at room temperature. The labeled protein was fractionally precipitated with 50% ammonium sulfate on ice, collected by centrifugation at 50,000 g and at 4 °C for 15 min, and resuspended in 1 ml of 25 mM Tris-HCl buffer, pH 7.5, containing 0.2 M guanidinium chloride. The labeled protein was purified using a Hitrap™ Heparin column (Amersham-Pharmacia) that was attached to a Äkta FPLC system (Amersham-Pharmacia) and equilibrated in the same buffer. Labeled protein was eluted with the linear gradient of NaCl to 0.5 M in the same buffer. The fractions corresponding to a major protein peak were dialyzed against 25 mM Tris-HCl, pH 8.0, for 24 h at 4 °C and then stored at −20 °C.

Confocal Microscopy Imaging of hVPLA2 Internalization and Activity — The labeling of cell membranes by PED6 was performed as described previously (13) with some modifications. A mixture of POPS/cholesterol/POPG/PED6 (107:31:20:1 in molar ratio, 300 nmoles total) in chloroform was dried under N₂ and resuspended in ethanol (10 µl), followed by the addition of DMEM (10 µl). The solution was dried again under N₂ until the volume was reduced to ~7 µl to ensure that most of ethanol was evaporated. Additional 10 µl of DMEM was added to the mixture and vesicles were prepared by sonication of the mixture on ice (20 min). HEK293 cells (3-5 x 10⁴ cells) were seeded into each of eight wells on a sterile Nunc Lak-TeK II™ chambered
cover glass filled with the DMEM supplemented with 10% FBS and 250 μg/ml of zeocin™, and incubated at 37 °C with 5% CO₂ for 48 hours. The vesicle solution (10 μl) was then added to each of eight wells and incubated with HEK293 cells for 25-50 min at 37 °C. HEK293 cells were rinsed with phosphate-buffered saline (PBS) five times, resuspended in 300 μl of DMEM media, and 150 nM (or higher) of sPLA₂ and 2 mM CaCl₂ (final concentration) were added. Imaging was done with a Zeiss LSM510 laser scanning confocal microscope with the detector gain adjusted to eliminate the background autofluorescence. The signal from the Texas Red® attached to W79C was observed directly upon excitation with a 568-nm Argon/Krypton laser and a 650-nm linepass filter whereas the BODIPY® signal from the hydrolyzed PED6 was visualized with a 488-nm Argon/Krypton laser and a 530-nm bandpass filter. A 63x (1.2 numerical aperture) water immersion objective was used for all experiments. Images were analyzed using the analysis tools provided in the Zeiss biophysical software package. Using these tools, regions of interest in the cytosol and the membranes were defined, and the average fluorescence intensity in a square (1 mm x 1 mm) was obtained as a function of time.

Confocal Microscopy Imaging of Intracellular Vesicle Formation — To label HEK293 cell membranes with DiIC12, each of 1 μl dye solution in ethanol (2 mg/ml) was added rapidly to 400 μl of HEK293 cells that were cultured for 48 hours in DMEM supplemented with 10 % FBS and 250 μg/ml of zeocin™ in each of eight wells on a sterile Nunc Lak-TeK II™ chambered cover glass. Unbound dye was removed by washing four times with PBS. Washed HEK293 cells were imaged in 400 μl of DMEM media without phenol red. The preparation was placed on the stage of a Zeiss Pascal laser scanning confocal microscope fitted with a 570-nm linepass filter and a
543-nm He/Ne laser. 150 nM of sPLA₂ and 2 mM CaCl₂ (final concentration) were added and the imaging was performed as described above.

**Immunocytostaining** — HEK293 cells were plated onto a sterile cover glass and incubated at 37 °C with 5% CO₂. The stable HEK293 cell line expressing 5-lipoxygenase (5-LO) was generated by transfecting the cells with pcDNA3.1-human 5-LO plasmid using lipofectamine (Life Technologies, Inc), followed by selection of clones in the presence of geneticin (800 µg/ml) for 3-4 weeks. The cells were treated with 150 nM (final concentration) of hVPLA₂ in DMEM for 5, 10, and 30 min in a 37 °C, 5% CO₂, humidified incubator. At the given time, cells were washed twice with cold PBS, and then were fixed at room temperature with 3.6% paraformaldehyde in PBS for 10 min. After fixation, the cells were washed six times with PBS and placed in a blocking solution (10% normal goat serum and 100 µM goat IgG in PBS) at room temperature for 3 hours. The cells were then permeabilized with PBS containing 0.1% Triton X-100 and 2% BSA for 1 h at room temperature, washed four times with PBS, and incubated with the monoclonal antibodies raised against hVPLA₂ (2 µg/ml) (14) and human 5-LO polyclonal antibody (Santa Cruz Biotechnology, CA) (500-fold diluted), respectively, in the presence of 2% BSA. After 2-hour incubation at room temperature, the antibodies were removed, and cells were washed six times with PBS. Secondary antibodies, Alexa⁴₈₈ goat anti-goat antibody and Alexa⁵₆₈ goat anti-mouse antibody (Molecular Probes) diluted in PBS containing 2% BSA were applied for 1 h at room temperature. After washing six times with PBS, the slide was mounted with Fluoromount-G (Southern Biotech Associates, AL). Imaging was done with a Zeiss LSM510 laser scanning confocal microscope.
In vitro Assay of sPLA$_2$ with PED6 Vesicles — The sPLA$_2$-catalyzed hydrolysis of PED6 in the mixed vesicles of POPS/cholesterol/POPG/PED-6 (107:31:20:1) was carried out at 37° C in 2 ml of 10 mM Tris-HCl, pH 7.4, containing 0.16 M KCl, 0.01 mM EDTA, 2.5 mM Ca$^{2+}$. The progress of hydrolysis was monitored as an increase in fluorescence emission at 520 nm using a Hitachi F4500 fluorescence spectrometer with the excitation wavelength set at 488 nm. Spectral bandwidth was set at 10 nm for both excitation and emission. Values of specific activity were determined from the initial rates of hydrolysis.

RESULTS

Requirements for sPLA$_2$ Internalization — The internalization of sPLA$_2$ to mammalian cells has been observed when the cells were treated with exogenously added sPLA$_2$ (7,8) or the cells expressing sPLA$_2$s were stimulated with agonists, such as interleukin-1 (IL-1) (3-6). To rigorously and systematically determine the requirements for sPLA$_2$ internalization, we selected the exogenous addition method that allows the use of the protein chemically labeled with a fluorescent probe for real-time monitoring. The chemical labeling was preferred to the genetic incorporation of a green fluorescence protein tag because the latter significantly altered either enzymatic activity or HSPG affinity (data not shown). First, we measured the internalization of hVPLA$_2$ (W79A), its mutants (W79A/W31A and W79A/K100E/R101E), and hIIaPLA$_2$ to unstimulated HEK293 cells by Western blotting analysis of cell extracts after sPLA$_2$ treatment. For hVPLA$_2$, the W79A mutant is used in place of wild type because it is fully active and gives a higher yield of refolding than wild type (11). We previously showed that hVPLA$_2$ (and W79A) has much higher activity on mammalian cells than hIIaPLA$_2$ because of its ability to effectively
bind and hydrolyze PC (11). We also showed that W79A/W31A of hVPLA2 has ~50 times lower activity on PC membranes than W79A (11) and that W79A/R100E/K101E has full PC activity but has much reduced affinity for HSPG (7). As shown in Fig. 1, only hVPLA2-W79A showed a significant degree of internalization at 20 min. Even after 60 min, W79A/R100E/K101E and hIIaPLA2 did not show detectable internalization (data not shown). W79A/W31A was internalized at a greatly reduced rate: a faint band appeared at 20 min, which only after 60 min became comparable to that of wild type measured at 20 min (Fig. 1C). Interestingly, when HEK293 cells were treated with W79A/W31A in the presence of N. n. naja PLA2 that was shown to have high PC activity (15), W79A/W31A was internalized as well as wild type (Fig. 1D). The dark 14 kDa band was not due to internalized N. n. naja PLA2 because our hVPLA2 monoclonal antibodies do not cross-react with N. n. naja PLA2 (14) and because N. n. naja PLA2 owing to its extremely low HSPG affinity was not internalized under our experimental conditions. Thus, both HSPG affinity and the ability to hydrolyze the outer plasma membrane (i.e., PC membranes) are required for a sPLA2 to enter unstimulated HEK293 cells. In contrast, W79A, W79A/W31A and hIIaPLA2 (Fig. 1F), but not W79A/R100E/K101E, were internalized when HEK293 cells were primed with IL-1β. This indicates that HSPG affinity is both necessary and sufficient for the internalization of sPLA2 into IL-1β-primed HEK293 cells. Lastly, the internalized sPLA2 remained intact after several hours in HEK293 cells, which is in sharp contrast to the rapid degradation of internalized sPLA2 in neutrophils (7). Taken together, these results indicate that although HSPG affinity is a critical factor for the internalization of sPLA2 into HEK293 cells, other factors, such as PLA2 hydrolysis products and cytokines, are also necessary for the internalization.
Activity of hVPLA₂ to Release AA from HEK293 Cells — Although several reports have suggested that sPLA₂s might act intracellularly, whether they are intracellularly localized (16) or re-internalized after secretion (3-6), no direct experimental evidence for the notion has been documented. To determine the correlation between the internalization of sPLA₂ and its intracellular lipolytic activities, we treated [³H]-AA-labeled human neutrophils and HEK293 cells with W79A and W79A/R100E/K101E and measured the time courses of AA release. As we reported previously (7), the liberation of AA from human neutrophils by W79A reached a plateau after ~15 min whereas the AA release by non-internalizing W79A/R100E/K101E continued to proceed even after 1 h (Fig 2A). The saturation of the AA release by W79A is due to its internalization into neutrophils and subsequent degradation (7). Interestingly, the AA release from HEK293 cells by W79A and W79A/R100E/K101E showed similar biphasic patterns and the slower second phases lasted for more than an hour (Fig 2B). In view of the different fate of the internalized hVPLA₂ in neutrophils and HEK293 cells (i.e., degradation versus retention), these data imply that the second phase of W79A-induced AA release from HEK293 cells is due to the action of internalized enzyme on intracellular membranes.

Dual Monitoring of sPLA₂ Internalization and Phospholipid Hydrolysis — To corroborate the notion that the internalized sPLA₂ is active on intracellular membranes and to determine the intracellular location of sPLA₂ lipolytic action, we labeled HEK293 cells with a fluorogenic phospholipid, PED6, which has been used for in vivo PLA₂ assays (17,18). In this lipid, the fluorescent BODIPY® moiety in the sn-2 position is quenched by the dinitrophenyl group in the head group, which is relieved when the PLA₂-catalyzed hydrolysis releases the BODIPY®-labeled fatty acid. As summarized in Table I, all sPLA₂s used in these studies showed relatively
high activity on PED6 in the in vitro vesicle assay; however, cPLA₂ had less than 0.1% of hVPLA₂-W79A activity. Since the BODIPY® fluorescence in PED6 is not completely quenched, the cellular distribution of intact PDE6 can be monitored if cells were illuminated with a higher laser power. As shown in Fig. 3A, PDE6 was primarily localized in the plasma membrane within first 20 min of incubation but more evenly distributed among various cellular membranes after 25 to 50 min of incubation under our experimental conditions.

We first treated PED6-labeled (unstimulated) HEK293 cells with hIIPLA₂, hVPLA₂-W79A and mutants. As shown in Fig. 3B, the addition of W79A to the cells resulted in the appearance of BODIPY® fluorescence, first at the plasma membrane and then intracellularly with a clear annular pattern around the nucleus. The time-lapse relative fluorescence intensity profiles of the region of interest clearly show that the signal at the plasma membrane peaks at ~2 min and the signal at the nuclear envelope reaches the plateau at ~4 min (Fig. 3C). The cytoplasmic signal that is much weaker than nuclear envelope signal initially (i.e., <4 min) continued to rise until up to 6 min. Thus, this relatively diffuse cytoplasmic signal seems to reflect the diffusion of the short-chain BODIPY fatty acid from the nuclear membranes. Consistent with our Western blotting data, hIIPLA₂ did not induce any appreciable fluorescence signal (Fig. 4A). Also, W79A/R100E/K101E showed the fluorescence signal mainly at the plasma membrane (Fig. 4B). A weaker intracellular fluorescence signal seen with the mutant seems to be due to the small amount of internalized protein, since the mutation would not completely block the HSPG binding and internalization. This also reflects the higher sensitivity of fluorescence imaging in comparison with the Western blotting. To preclude the possibility that the intracellular lipid signals, cytoplasmic signal in particular, are due to the intracellular uptake of BODIPY® fatty acid released from the outer plasma membrane, we performed the control experiments in which the
PED6 is primarily labeled in the plasma membrane of HEK293 cells (Fig. 5A). Under this condition, both W79A and W79A/R100E/K101E yielded the fluorescence signals almost exclusively at the plasma membranes and in the medium (Fig. 5B). As shown in the relative fluorescence intensity profile, the intracellular signal was negligible (Fig. 5C), corroborating the notion that the intracellular BODIPY® fluorescence signal seen with HEK293 cells whose membranes are evenly labeled with PED6 derives from the intracellular membrane hydrolysis. It is also unlikely that the intracellular signal comes from either cPLA₂ or group VI calcium-independent PLA₂ because HEK293 cells contain extremely low level of these intracellular PLA₂s (3) and because the pre-treatment of cells with 10 µM arachidonyl trifluoromethyl ketone had no effect on lipid hydrolysis. We then incubated hIIPLA₂ and hVPLA₂-W79A with IL-1β-primed, PED6-labeled HEK293 cells. W79A acts on IL-1β-primed HEK293 cells as effectively as unstimulated cells, producing BODIPY® fluorescence at the plasma membrane and the perinuclear region (data not shown). In case of hIIPLA₂, the fluorescent signal at the plasma membrane was not detectable, as expected from its low PC activity, but the intracellular signal was clearly visible (see Fig. 4C). Interestingly, hIlaPLA₂ did not produce a distinct annular fluorescent signal at the nuclear envelope but instead yielded a diffuse cytoplasmic signal, implying that it might have a different site of lipolytic action. More importantly, these data, in conjunction with our Western blotting data, indicate that HSPG-binding sPLA₂s, including hIIPLA₂, are internalized into the agonist-primed HEK293 cells and act on intracellular membranes.

We then treated the PDE-labeled HEK293 cells with the Texas Red®-labeled hVPLA₂ to simultaneously monitor the enzyme internalization and the lipid hydrolysis by two-channel detection. Our labeling strategy is to attach a Texas Red® fluorophore to a single free cystein
residue introduced by mutation. For hVPLA₂, the W79C mutation was selected based on our previous observation that the W79A mutant is fully active and gives a higher yield of refolding than wild type (11). The labeling of hVPLA₂-W79C by Texas-Red C2 maleimide and the purification yielded the pure modified protein that is chromatographically distinct from the unlabeled W79C. Both W79C and Texas Red®-labeled W79C were as active as wild type hVPLA₂ toward PED6 by a vesicle activity assay (see Table I). When Texas Red®-labeled W79C was added to unstimulated PED6-labeled HEK293 cells, the labeled hVPLA₂-W79C appeared intracellularly after 90 seconds and then was predominantly localized in the perinuclear region (Fig. 6). The release of BODIPY® fatty acid was synchronized and colocalized with the hVPLA₂ internalization, appearing initially at the plasma membrane and then moving toward the perinuclear region.

Lastly, we measured the change in membrane structure during sPLA₂ internalization using non-hydrolyzable membrane probe, DiIC12. As shown in Fig. 7, the addition of exogenous hVPLA₂ to unstimulated HEK293 cells led to the formation of vesicles near the inner plasma membrane. The budding of lipid vesicles was not a spontaneous cellular process because hIIaPLA₂ did not induce vesicle formation under the same conditions. Incubation of HEK293 cells with AA or 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (each at 2 µM) also induced the vesicle formation. Thus, it appears that products of hVPLA₂ hydrolysis at the outer plasma membrane promote protein internalization via vesicle formation.

Co-localization of Internalized sPLA₂ with 5-LO — To better define the localization of internalized sPLA₂ and understand its interactions with other key proteins involved in eicosanoid biosynthesis, we treated HEK293 cells stably expressing 5-LO with hVPLA₂ and measured their
relative cellular location by double immunocytostaining. It has been shown that 5-LO is present in the cytoplasm and the nucleus in resting cells and translocates to the nuclear envelope upon cell activation (19). As shown in Fig. 8, 5-LO is evenly distributed in the cytoplasm and the nucleus before hVPLA₂ was added to the cells. Within 5 min of hVPLA₂ addition, most 5-LO molecules migrated to the nuclear envelope, as indicated by a bright annular signal around the nucleus. hVPLA₂ also translocated to the nuclear envelope, albeit at a slower rate. After 10 min, hVPLA₂ and 5-LO are co-localized exclusively at the nuclear envelope. In combination of the above data, these results suggest that sPLA₂ has a dual function in cellular eicosanoid biosynthesis; the production of AA at the nuclear envelope and the translocation of other eicosanoid–synthesizing enzymes, including 5-LO, to the nuclear envelope.

**DISCUSSION**

Although much has been reported on the expression and secretion of sPLA₂ isoforms under different inflammatory conditions, less is known about the spatiotemporal dynamics of sPLA₂ mobilization. In particular, the mechanism and the functional consequences of sPLA₂ internalization remain unclear. The present investigation provides a new insight into these important questions. We selected HEK293 cells for these studies because much of sPLA₂ internalization studies have been performed with these cells transfected with various sPLA₂ isoforms (3,5,6). Our Western blotting analysis show that at least for two sPLA₂ isoforms used in these studies, hVPLA₂ and hIIaPLA₂, the HSPG affinity is an essential but not sufficient factor for the internalization of sPLA₂ into HEK293 cells: other factors, such as PLA₂ hydrolysis products and cytokines, are also necessary for the internalization. This finding also accounts for
the reported discrepancy between the measurements on unstimulated human neutrophils and those on agonist (e.g., IL-1β)-stimulated HEK293 cells. In the former case, only hVPLA2 that has both PC activity and HSPG affinity was internalized (7), whereas in the latter case all HSPG-binding sPLA2s were internalized (3,5,6). Since the mechanism of sPLA2 internalization is unknown at present, it is difficult to figure out the exact role of the auxiliary factors in sPLA2 internalization. The imaging of HEK293 cells using a non-hydrolyzable membrane probe, DiIC12 shows that PLA2 hydrolysis products promote the formation of large intracellular vesicles. It is unclear, however, whether they facilitate the endocytosis by changing the physical state of plasma membrane or by a receptor-mediated mechanism. Undoubtedly, further studies are needed to address this question.

Regardless of the mechanism of internalization, our measurements with tritiated AA-labeled HEK293 cells suggest that the internalized hVPLA2 is active on intracellular membranes. Furthermore, the live cell imaging using sPLA2s, including Texas Red®-labeled hVPLA2, and PED6-labeled HEK293 cells demonstrates the time-dependent appearance of the fluorescent fatty acid molecules inside the cells. Our control experiments with HEK293 cells the plasma membrane of which is selectively labeled with PED6 preclude the possibility that the intracellular BODIPY® fatty acid signal arises from the internalization of hydrolyzed fatty acid from the outer plasma membrane either by fatty acid transfer proteins or via sPLA2-induced vesicle formation. The time-lapse relative fluorescence intensity plot indicates that the nuclear envelope is the primary site of action for the internalized hVPLA2 (see Fig. 3C). The cytoplasmic fluorescence signal could derive from either the diffusion of short-chain BODIPY® fatty acid from the perinuclear membranes or the hydrolysis of PED6 incorporated in sPLA2-containing internalized vesicles. The fluorescence intensity profiles in which the cytoplasmic signal
definitely lags behind the nuclear envelope signal (see Fig. 3C) strongly supports the former mechanism. In the latter case, one would expect that the cytoplasmic signal precede the signal at the nuclear envelope. Both the live cell imaging of Texas Red®-labeled hVPLA₂ and immunocytostaining of hVPLA₂ and 5-LO show that the internalized hVPLA₂ is co-localized with 5-LO at the nuclear envelope. The translocation of 5-LO to the nuclear envelope is induced by the rise in intracellular Ca²⁺ concentration (19), which has been shown to be induced by PLA₂ products, lysophosphatidylcholine and fatty acids, at the outer plasma membrane². Together, these results indicate that the nuclear envelope is the main site of action at least for internalized hVPLA₂. This notion is also consistent with the findings that the nuclear envelope is relatively rich in PC (20) and hVPLA₂ has high activity on PC membranes (10,11). Since disulfide-rich sPLA₂s are labile in the reducing environment of cytoplasm and require millimolar Ca²⁺ for full activity, it is not clear how internalized hVPLA₂ can directly act on the nuclear envelope. Presumably, the local Ca²⁺ concentration in the cytoplasm near the nuclear envelope is high enough to allow sPLA₂ catalysis², albeit sub-optimally, and the sPLA₂ bound to the perinuclear membrane surface is not readily reduced and denatured by cellular glutathione.

Given the distinct phospholipid head group specificities of sPLA₂s and the different lipid composition of various intracellular membranes (21,22), it is possible that other sPLA₂s might act on different intracellular membranes. For instance, hIIaPLA₂ that has low PC activity did not produce a clear fluorescent signal at the nuclear envelope (see Fig. 4C); instead, it yielded a diffuse cytoplasmic signal. Further studies are necessary to accurately determine the intracellular site of action for other sPLA₂ isoforms and how these enzymes are delivered to their target membrane sites. Our cPLA₂ inhibition study in HEK293 cells indicates that the contribution of cPLA₂ to the production of fatty acid signal at the nuclear envelope is negligible under our
experimental conditions. This notion is further corroborated by the finding that neither cPLA$_2$ inhibition nor cPLA$_2$ overexpression by transfection had any appreciable effect on both BODIPY$^*$ fatty acid release and AA release$^3$. It should be noted, however, that HEK293 cells contain a very low level of endogenous cPLA$_2$ activity. When sPLA$_2$ acts, whether in an autocrine or paracrine manner, on other mammalian cells that show significant cPLA$_2$ activity, sPLA$_2$ might work in concert with cPLA$_2$ as reported previously (23-25).

In sum, our data provide the first experimental evidence that the internalized sPLA$_2$ acts on the nuclear envelope where other key enzymes in the eicosanoid biosynthesis, including cPLA$_2$, cyclooxygenase and 5-LO, are localized during eicosanoid biosynthesis. The new experimental approach used in these studies will serve as a useful tool for further studies on the mechanisms by which different sPLA$_2$ isoforms are internalized and delivered to a target membrane, and act intracellularly in various mammalian cells under different physiological conditions.
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FOOTNOTES

1 The abbreviation used are: AA, arachidonic acid; BSA, bovine serum albumin; cPLA₂, group VI cytosolic PLA₂; DilC₁₂, 1,1'‑didodecyl‑3,3,3',3'‑tetramethylinodocarbocyanine perchlorate; DMEM, Dublico's modified eagles medium; FBS, fetal bovine serum; HEK, human embryonic kidney; hIIaPLA₂, human group IIa PLA₂; HSPG, heparan sulfate proteoglycan; hVPLA₂, human group V PLA₂; 5-LO, 5‑lipoxigenase; PBS, phosphate-buffered saline; PC, phosphatidyl choline; PED₆, N‑((6‑(2,4‑dinitrophenyl)amino)hexanoyl)‑1‑hexadecanoyl‑2‑(4,4‑difluoro‑5,7‑dimethyl‑4‑bora‑3a,4a‑diaza‑s‑indacene‑3‑pentanoyl)‑sn‑glycero‑3‑phosphoethanolamine triethylammonium salt; PLA₂, phospholipase A₂; POPG, 1‑palmitoyl‑2‑oleoyl‑sn‑glycero‑3‑phosphoglycerol; POPS, 1‑palmitoyl‑2‑oleoyl‑sn‑glycero‑3‑phosphoserine; sPLA₂, secretory PLA₂.

2 Kim, Y. J. and Cho, W., unpublished observation.

3 Kim, K. P. and Cho, W., manuscript in preparation.
FIGURE LEGENDS

Fig. 1. Internalization of sPLA₂s into HEK293 cells detected by Western blotting analysis. HEK293 cells in DMEM were incubated PLA₂ for 20 min at 37 °C with (A) 100 nM of hVPLA₂-W79A, (B) W79A/R100E/K101E, (C) W79A/W31A, (D) 100 nM of W79A/W31A + 100 nM N. a. atra PLA₂. For W79A/W31A, the incubation was extended to 60 min. Also, 100 nM of hIIaPLA₂ was incubated for 20 min with untreated HEK293 cells (E) and HEK293 cells pretreated with 1 ng/ml IL-1β for 12 hours (F), respectively. W79A and W79A/W31A behaved similarly to hIIaPLA₂ when incubated with IL-1β-treated HEK293 cells. After washing with DMEM containing 0.6 M NaCl, the pellet was collected by scrapping and centrifugation, lysed, and subjected to SDS-electrophoresis on 16% polyacrylamide gels. Essentially the same electropherograms were obtained from triplicate experiments.

Fig. 2. Time courses of AA release by W79A hVPLA₂ and W79A/R100E/K101E. 100 nM of W79A (open circle) and W79A/R100E/K101E (closed circle) were incubated with AA-labeled neutrophils (A) and HEK293 cells (B) at 37 °C for a given period. Each data point represents an average of triplicate measurements.

Fig. 3. Confocal microscopic Imaging of W79A hVPLA₂ and relative fluorescence intensity profiles. A. Time-dependent distribution of PED6 prior to sPLA₂ addition visualized with a higher laser power. B. 150 nM of hVPLA₂-W79A was added with 2 mM CaCl₂ to HEK293 cells that were incubated with PED6 containing vesicles for 25-50 min at 37 °C and the
images were taken continuously. C. The relative fluorescence intensities were determined in the
defined regions of interest in various parts of the cell, including the nuclear envelope (square
symbols), cytoplasm (circles), and plasma membrane (triangles).

**Fig. 4.** Confocal microscopic imaging of hIIaPLA₂ and W79A/R100E/K101E hVPLA₂
activities. 250 nM of hIIaPLA₂ (A) and 150 nM of hVPLA₂-W79A/R100E/K101E (B) were
added with 2 mM CaCl₂ to HEK293 cells that were incubated with PED6 containing vesicles for
25-50 min at 37 °C. 700 nM of hIIaPLA₂ (C) was added to HEK293 cells pretreated with 1
ng/ml IL-1β for 12 hours. A higher concentration of hIIaPLA₂ was required because of its lower
activity for PED6 and, presumably, the lower efficiency of IL-1β-induced internalization.

**Fig. 5.** Confocal microscopic imaging of W79A hVPLA₂ activity on plasma membrane-
labeled HEK293 cells and relative fluorescence intensity profiles. Experimental conditions
are the same as described for Fig. 3 except that PED6 is primarily labeled in the plasma
membrane after 15-min incubation. A. Distribution of PED6 prior to sPLA₂ addition visualized
with a higher laser power. B. Time-dependent distribution of hydrolyzed BODIPY fatty acid. C.
Relative fluorescence intensity profiles were determined at the nuclear envelope (squares),
cytoplasm (circles), and plasma membrane (triangles).

**Fig. 6.** Dual imaging of Texas Red-labeled hVPLA₂ internalization and its lipolytic
activity. 150 nM of Texas Red®-labeled hVPLA₂ (W79C) was added to HEK293 cells as
described under "Experimental Procedures," and the dual images were taken at 0.8 min and 7 min
with a Zeiss LSM 510 confocal microscope. A, Images of Texas Red®-labeled hVPLA₂ (W79C);
B, Images of released BODIPY® fatty acid from PED6; C, Merged images of A and B. Real-time movies of hVPLA₂ internalization and PED6 hydrolysis are included in the “Supplemental Materials.”

**Fig. 7. Confocal microscopic imaging of intracellular vesicle formation.** After HEK293 cells were labeled with DiIC12 and washed four times with PBS, 150 nM of hVPLA₂-W79A (A) or hIIaPLA₂ (B) was added with 2 mM CaCl₂ to the medium and the images were taken with a Zeiss Pascal confocal microscope. Arrows indicate the formation of intracellular vesicles. A real-time movie of vesicle formation is included in the “Supplemental Materials.”

**Fig. 8. Colocalization of hVPLA₂ and 5-LO.** HEK293 cells stably expressing 5-LO were treated with 150 nM of hVPLA₂-W79 and cells were fixed immediately after PLA₂ addition and after 30 min. The permeabilized cells were then stained with hVPLA₂ monoclonal antibodies and human 5-LO polyclonal antibody, respectively, and imaged with a Zeiss LSM 510 confocal microscope.
TABLE I

*Relative activities of PLA₂ on PED6 substrate*. Each activity value was determined as an average of triplicate measurements.

| Enzyme                                    | Relative activity |
|-------------------------------------------|-------------------|
| hVPLA₂ W79A                               | 1.00<sup>b</sup>  |
| hVPLA₂ W79A/W31A                          | 0.25              |
| hVPLA₂ W79A/R100E/K101E                   | 0.70              |
| hVPLA₂ W79C                               | 0.95              |
| Labeled hVPLA₂ W79C                       | 0.90              |
| hIIaPLA₂                                  | 0.54              |
| cPLA₂                                     | 0.0008            |

<sup>a</sup>The vesicle composition was POPS/cholesterol/POPG/PED6 = 107:31:20:1 in mole ratio.

<sup>b</sup>The absolute specific activity value for W79A was 20 ± 2 µmol/min/mg.
Fig 2
Fig 3

A

10 min

30 min

B

0 min

2 min

10 min

C

Relative Fluorescence Intensity

Time, sec

0 100 200 300 400 500
Fig 4
Fig 5
Fig 7
Fig. 8
Internalized group V secretory phospholipase A2 acts on the perinuclear membranes
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Wonhwa Cho

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