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Group V Phospholipase A_2 Induces Leukotriene Biosynthesis in Human Neutrophils through the Activation of Group IVA Phospholipase A_2*

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Young Jun Kim‡, Kwang Pyo Kim‡, Sang Kyou Han‡§, Nilda M. Munoz¶, Xiangdong Zhu¶, Hiroyuki Sano¶, Alan R. Leff¶, and Wonhwa Cho‡‖

From the ‡Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois 60607 and the ¶Department of Medicine, University of Chicago, Chicago, Illinois 60307

We reported previously that exogenously added human group V phospholipase A_2 (hVPLA$_2$) could elicit leukotriene B_4 (LTB$_4$) biosynthesis in human neutrophils (Han, S. K., Kim, K. P., Koduri, R., Bittova, L., Munoz, N. M., Leff, A. R., Wilton, D. C., Gelb, M. H., and Cho, W. (1999) *J. Biol. Chem.* 274, 11881–11888). To determine the mechanism of the hVPLA$_2$-induced LTB$_4$ biosynthesis in neutrophils, we thoroughly examined the effects of hVPLA$_2$ and their lipid products on the activity of group IVA cytosolic PLA_2 (cPLA$_2$) and LTB$_4$ biosynthesis under different conditions. As low as 1 nM exogenous hVPLA$_2$ was able to induce the release of arachidonic acid (AA) and LTB$_4$. Typically, AA and LTB$_4$ were released in two phases, which were synchronized with a rise in intracellular calcium concentration ($[Ca^{2+}]_i$) near the perinuclear region and cPLA$_2$ phosphorylation. A cellular PLA_2 assay showed that hVPLA$_2$ acted primarily on the outer plasma membrane, liberating fatty acids and lysophosphatidylcholine (lyso-PC), whereas cPLA$_2$ acted on the perinuclear membrane. Lyso-PC and polyunsaturated fatty acids including AA activated cPLA$_2$ and 5-lipoxygenase by increasing $[Ca^{2+}]_i$ and inducing cPLA$_2$ phosphorylation, which then led to LTB$_4$ biosynthesis. The delayed phase was triggered by the binding of secreted LTB$_4$ to the cell surface LTB$_4$ receptor, which resulted in a rise in $[Ca^{2+}]_i$ and cPLA$_2$ phosphorylation through the activation of mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2. These results indicate that a main role of exogenous hVPLA$_2$ in neutrophil activation and LTB$_4$ biosynthesis is to activate cPLA$_2$ and 5-lipoxygenase primarily by liberating from the outer plasma membrane lyso-PC that induces $[Ca^{2+}]_i$ increase and cPLA$_2$ phosphorylation and that hVPLA$_2$-induced LTB$_4$ production is augmented by the positive feedback activation of cPLA$_2$ by LTB$_4$.

Phospholipase A_2 (PLA_2)[1] catalyzes the release from the *sn*-2 position of certain membrane phospholipids of arachidonic acid (AA) that can be transformed into potent inflammatory lipid mediators, including prostaglandins, leukotrienes, and thromboxanes. Multiple forms of mammalian PLA_2 have been identified from mammalian tissues, which include several forms of secretory PLA_2 (sPLA$_2$) (1), group IVA Ca^{2+}-dependent cytosolic PLA_2 (cPLA$_2$) (2), and group VI Ca^{2+}-independent PLA_2 (iPLA$_2$) (3). Recent studies have indicated that sPLA$_2$s work in concert with cPLA$_2$ to induce eicosanoid formation in different cells (4–6). Neutrophils are inflammatory cells that release AA and 5-lipoxygenase products, most notably leukotriene B_4 (LTB$_4$), upon activation by various agonists, including a bacterial peptide, formyl-Met-Leu-Phe (fMLP). It was reported that human neutrophils contain several forms of endogenous PLA$_2$s, including cPLA$_2$, iPLA$_2$, and group V and group X sPLA$_2$s (7). However, roles of these PLA$_2$s in inflammatory actions of neutrophils, biosynthesis and release of LTB$_4$ in particular, have not been elucidated fully. Based on the effects of exogenously added sPLA$_2$, AA, and 5-lipoxygenase (5-LO) products on cPLA$_2$ activity, it was postulated earlier that stimulus-induced AA release or exocytosis of sPLA$_2$ activates cPLA$_2$ by initiating the formation of LTB$_4$ which leads to the phosphorylation of cPLA$_2$ (8). Similarly, both sPLA$_2$ and cPLA$_2$ were shown to be involved in the fMLP-stimulated AA release from human neutrophils (9). Recently, however, it was reported that fMLP-induced secretion of group V sPLA$_2$ from human neutrophils did not lead to LTB$_4$ biosynthesis (7). Instead, cPLA$_2$ was reported to be entirely responsible for the fMLP-stimulated LTB$_4$ release from human neutrophils (7). Independently, we showed that exogenously added human group V PLA_2 (hVPLA$_2$) could induce AA and LTB$_4$ release from unprimed human neutrophils half as effectively as fMLP (10)

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§ Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

‖ Established investigator of American Heart Association. To whom correspondence should be addressed: Dept. of Chemistry (M/C 111), University of Illinois at Chicago, 845 West Taylor St., Chicago, IL 60607-7061. Tel.: 312-996-4883; Fax: 312-996-2183; E-mail: wcho@uic.edu.

[1] The abbreviation used are: PLA_2, phospholipase A_2; AA, arachidonic acid; $AACOCF_3$, arachidonyltrifluoromethyl ketone; BSA, bovine serum albumin; cPLA$_2$, group IVA cytosolic PLA_2; DiIC12, 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; ERK, extracellular signal-regulated kinase; fMLP, formyl-Met-Leu-Phe; FRET, fluorescence resonance energy transfer; HBSS, Hanks' balanced salt solution; hIIaPLA$_2$, human group IIa PLA_2; hVPLA$_2$, human group V PLA_2; HPLC, high performance liquid chromatography; iPLA$_2$, group VI Ca^{2+}-independent PLA_2; 5-LO, 5-lipoxygenase; LTB$_4$, leukotriene B_4; LTB$_4$DMA, LTB$_4$ dimethyl amide; lyso-PC, 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine; MAP kinase, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; OA, oleic acid; PED6, *N*-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt; PGB_2, prostaglandin B_2; SAPC, 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; sPLA$_2$, secretory PLA_2.

and that hVPLA$_2$ bound to cell surface heparan sulfate proteoglycans was eventually internalized and degraded (11). To understand better the interplay between sPLA$_2$ and cPLA$_2$ in leukotriene biosynthesis in neutrophils, we thoroughly examined the effects of sPLA$_2$s and their lipid products on cPLA$_2$ activity and leukotriene biosynthesis under different conditions. Results not only account for the discrepancy in previous reports but also provide new insights into the mechanism by which sPLA$_2$ and cPLA$_2$ work in concert to achieve effective and controlled leukotriene biosynthesis in neutrophils.

EXPERIMENTAL PROCEDURES

Materials—Arachidonyltrifluoromethyl ketone ($AACOCF_3$) was purchased from Biomol (Plymouth Meeting, PA). Surfactin was a generous gift from Dr. C. H. Lee of CheilJedang Co. (Incheon, Korea). The p38 mitogen-activated (MAP) kinase inhibitor SB203580 was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-cPLA$_2$ monoclonal antibody was generously provided by Dr. James Clark of the Genetics Institute (Cambridge, MA). [^{3}H]AA and ^{14}C-labeled oleic acid (OA) were purchased from American Radiochemical Co. (St. Louis, MO). 1-Stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphatidylcholine ([^{14}C]SAPC), goat anti-rabbit immunoglobin conjugated with horseradish peroxidase, goat anti-mouse immunoglobin conjugated with horseradish peroxidase, enhanced chemiluminescence and molecular weight markers were purchased from Amersham Biosciences. Anti-Activ™-extracellular signal-regulated kinase (ERK)1/2 antibody, Anti-Active™-p38 MAP kinase antibody, and a mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) inhibitor, U0126, were purchased from Promega Co. LTB$_4$, LTB$_4$ dimethyl amide (LTB$_4$DMA) and iPLA$_2$ inhibitor (*E*)-6-(bromoethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (bromoenol lactone) were from Cayman Chemical Co. (Ann Arbor, MI). *N*-((6-(2,4-dinitrophenyl)amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (PED6), Fluo-4 AM and 1,1′-didodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiIC12) were purchased from Molecular probes (Eugene, OR). 1-Palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine (lyso-PC) was purchased from Avanti Polar Lipids (Alabaster, AL). AA and ionomycin were purchased from Sigma.

Expression and Purification of sPLA$_2$—Recombinant human group IIa PLA$_2$ (hIIaPLA$_2$) was prepared as described (12). Recombinant hVPLA$_2$ and mutants were expressed in *Escherichia coli*, refolded, and purified as described previously (10, 13). The purity of enzymes assessed by SDS-PAGE was consistently higher than 90%.

Isolation of Human Neutrophils—Human neutrophils were prepared from heparinized venous blood collected from healthy medication-free donors by dextran sedimentation, followed by isolymph centrifugation and removal of remaining red blood cells by hypotonic lysis (14). The resultant cell population consisted of >95% neutrophils with >98% viability as judged by trypan blue exclusion.

Fatty Acid and LTB$_4$ Release from Human Neutrophils—Dual radiolabeling of neutrophils was achieved by incubating 10^7 cells with [^{3}H]AA and [^{14}C]OA (0.1 μCi/ml each) in calcium-free Hanks' balanced salt solution (HBSS) for 3 h. [^{3}H]AA and [^{14}C]OA that had not been incorporated into cellular lipids were removed by washing the cells three times with HBSS containing 0.2% bovine serum albumin (BSA). Radiolabeled cells (10^6) were resuspended in 160 μl of HBSS containing 1.2 mM $CaCl_2$ and 0.2% BSA, preincubated with a selected inhibitor for 20 min at 37 °C if necessary, and then were stimulated with hVPLA$_2$. The reaction was quenched by centrifugation, and the radioactivity in the cell pellet and the medium was measured separately by a two-channel liquid scintillation counter. LTB$_4$ levels were determined using a LTB$_4$ enzyme immunoassay kit from Cayman and then corrected for background signals from control cells that were not treated with hVPLA$_2$.

Measurement of cPLA$_2$ Activity—Neutrophils (2×10^6 cells) were stimulated with varying concentrations of hVPLA$_2$. The reaction was quenched by adding 1 ml of ice-cold water, and the reaction mixture was centrifuged. The pellet was resuspended in 70 μl of lysis buffer (20 mM Tris-HCl, pH 8.0, containing 2.5 mM EDTA, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 2 mM VO_4, 50 mM NaF, and 5 μg/ml pepstatin) and sonicated briefly. The resulting cell lysate was pretreated with 10 μl of dithiothreitol (final concentration 10 mM) on ice for 5 min to inactivate sPLA$_2$, and 10 μl of 1 mM $CaCl_2$ (final concentration 0.1 mM) was then added to each sample. The cPLA$_2$ substrate solution was prepared by drying a chloroform solution of [^{14}C]SAPC under a stream of N_2 and suspending the film in 100 μl of 10% aqueous ethanol by vortexing. The reaction was initiated by adding a 10 μl-portion of the substrate solution (final concentration 9 μM) to each cell lysate. The reaction was carried out for 30 min at 37 °C and was quenched by adding 560 μl of Dole's reagent (heptane, 2-propanol, 1 N H_2SO_4, 400:390:10 (v/v)), followed by 110 μl of H_2O, vortexed for 20 s, and then centrifuged at 13,000 × *g*. The 180-μl upper layer was transferred to 800 μl of hexane containing 25 mg of silica gel. The 800-μl samples was then mixed with 2 ml of scintillation fluids, and the radioactivity was counted in a liquid scintillation counter.

Immunoblotting—Aliquots of neutrophils (2×10^6 cells/sample) were centrifuged at 13,000 × *g* for 20 s, and cell pellets were lysed by the addition of the ice-cold lysis buffer (20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 40 mM NaCl, 30 mM $Na_4P_2O_7$, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml chymostatin, 2 mM diisopropylfluorophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na_3VO_4, 50 mM NaF, and 5 μg/ml pepstatin, 1% Nonidet P-40, and 0.5% deoxycholic acid). After 20 min on ice, the samples were centrifuged (13,000 × *g*, 10 min) to remove nuclear and cellular debris. Supernatants were mixed with the gel loading buffer (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% SDS, 0.005% bromphenol blue), and boiled for 5 min. Equivalent amounts of protein (~50 μg) were loaded onto SDS-polyacrylamide gels (10% of cPLA$_2$ and 12% for p38 and ERK1/2 MAP kinases). Electrotransfer of protein from the gel to the polyvinylidene difluoride membrane was achieved using a semidry system (400 mA, 60 min). The membrane was blocked with 1% BSA for 60 min, then incubated with primary antibodies (anti-cPLA$_2$, Anti-Active™-ERK1/2, or Anti-Active™-p38 antibody).

Measurement of [Ca^{2+}]$_i$—Measurement of intracellular calcium concentration ([Ca^{2+}]$_i$) was performed with a Zeiss LSM 510 laser scanning confocal microscope using Fluo-4 AM as the indicator. Neutrophils (10^7 cells/ml) were incubated in HBSS containing 1.2 mM Ca^{2+}, 1% BSA, and 2 μM Fluo-4 for 30 min at 37 °C. Labeled cells were seeded into each of eight wells on a sterile Nunc Lak-TeKII™ chambered cover glass filled with 400 μl of HBSS containing 1.2 mM Ca^{2+}, and incubated at 37 °C with 5% CO_2 for 10 min. After washing once with HBSS containing 1.2 mM Ca^{2+}, 10 nM hVPLA$_2$ (or 3 μM lyso-PC, 3 μM AA, 0.3 μM LTB$_4$) was added, and the fluorescence intensity of Fluo-4 was monitored with a 488 nm argon/krypton laser and a 530 nm linepass filter. A 63× (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. [Ca^{2+}]$_i$ was calibrated as described previously using the reported calcium dissociation constant value (*i.e.* 345 nM) of Fluo-4 (15).

Confocal Microscopy Imaging of PLA$_2$ Activity—Neutrophils (10^6 cells/ml) were seeded into each of eight wells on a sterile Nunc Lak-TeKII™ chambered cover glass filled with 400 μl of HBSS and incubated at 37 °C with 5% CO_2 for 10 min. After the cells were washed once with HBSS, they were overlaid with 10 μl of PED6 vesicle solution (0.75 mM POPS/cholesterol/POPG/PED6/DiIC12 (107:31:20:1:1 in a mol ratio) mixed vesicles in HBSS) and incubated for 50 min at 37 °C with 5% CO_2. After rinsing the labeled cells six times with HBSS containing 1.2 mM Ca^{2+}, 10 nM hVPLA$_2$ was added to cells. Imaging was done with a Zeiss LSM 510 laser scanning confocal microscope with the detector gain adjusted to eliminate the background autofluorescence. The fluorescence resonance energy transfer (FRET) from the BODIPY™ group of the hydrolyzed PED6 to DiIC12 was monitored with a 488 nm argon/krypton laser and a 585 nm linepass filter. A 63× (1.2 numerical aperture) water immersion objective was used for all experiments.

Leukotriene Analysis by HPLC-Electrospray Ionization-Mass Spectrometry—The analysis of leukotriene composition secreted to the growth medium was performed as reported previously (16, 17). After neutrophils (10^8 cells) were incubated with 1 or 10 nM hVPLA$_2$, they were treated with 2 volumes of ice-cold methanol containing 5 ng of internal standard, prostaglandin B$_2$ (PGB$_2$). The samples were diluted with water to a final methanol concentration lower than 20%, and extraction was quickly carried out using a Supelclean LC-18 solid phase cartridge (Supelco, Bellafonte, PA). The retained material was eluted using 90% aqueous methanol. After evaporation of solvent, the dried extract was solubilized in 100 μl of solvent A (methanol/acetonitrile/water/acetic acid, 10:10:80:0.02, v/v/v/v, pH 5.5, adjusted with ammonium hydroxide), injected into a C18 reverse phase HPLC column (5 × 150 mm; Waters, Milford, MA) that was interfaced directly with the electrospray source of a triple quadrupole mass spectrometer (Micromass Qurattro II). The column was eluted with a linear gradient of solvent B (acetonitrile/methanol, 65:35) from 40 to 100% at a flow rate of 100 μl/min over 30 min. Each leukotriene peak was analyzed by mass spectrometry measuring the ion abundance for the following collision-induced transformation at the corresponding retention times: LTB$_4$ and

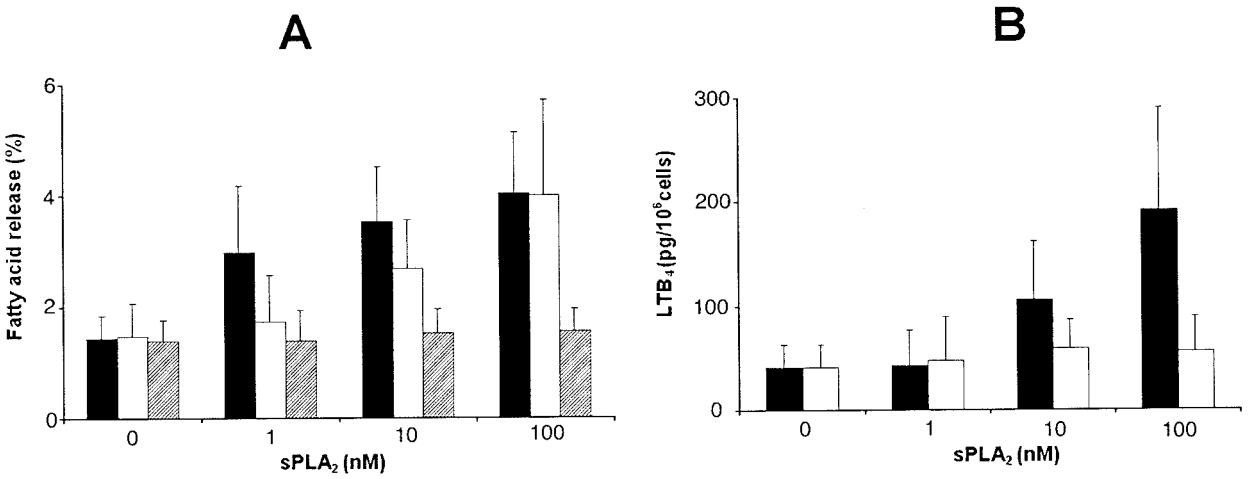

FIG. 1. **Dependence of fatty acid and LTB$_4$ release on hVPLA$_2$ concentration.** *A*, human neutrophils were labeled for 3 h with [^{3}H]AA and [^{14}C]OA and incubated for 20 min at 37 °C with different concentrations (1–100 nM) of hVPLA$_2$ or hIIaPLA$_2$ in HBSS containing 1.2 mM Ca^{2+} and 0.2% BSA. *Black* and *white bars* indicate [^{3}H]AA and [^{14}C]OA release, respectively, by hVPLA$_2$. *Gray bars* indicate [^{3}H]AA release by hIIaPLA$_2$. *B*, human neutrophils were incubated for 30 min at 37 °C with different concentrations (1–100 nM) of hIIaPLA$_2$ or hVPLA$_2$ in HBSS containing 1.2 mM Ca^{2+}. The supernatants were collected, and the LTB$_4$ level was assayed as described under "Experimental Procedures." *Black* and *white bars* indicate LTB$_4$ secretion by hVPLA$_2$ and hIIaPLA$_2$, respectively. Data represent the mean ± S.E. from triplicate measurements.

6-*trans*-LTB$_4$ isomers (*m/z* 335 → 195), 20-hydroxy-LTB$_4$ (*m/z* 351 → 195), 20-carboxy-LTB$_4$ (*m/z* 365 → 195). Quantitation of individual peaks was carried out using their mass ion abundance relative to that of PGB$_2$.

RESULTS

hVPLA$_2$-induced Fatty Acid and LTB$_4$ Release from Neutrophils—We showed previously that exogenously added hVPLA$_2$ triggers AA release and LTB$_4$ secretion from unprimed human neutrophils (10). To understand better the mechanism of hVPLA$_2$-induced LTB$_4$ biosynthesis, we carefully examined the time course of the fatty acid and LTB$_4$ release from neutrophils in the presence of varying concentrations of exogenously added hVPLA$_2$. First, we measured the hVPLA$_2$ concentration dependence of fatty acid release from neutrophils double labeled with [^{3}H]AA and [^{14}C]OA. Because of the high AA specificity of cPLA$_2$ and lack of fatty acid selectivity of sPLA$_2$s, the [^{3}H]AA release in this system would reflect both sPLA$_2$ and cPLA$_2$ activities, whereas the [^{14}C]OA release would largely represent sPLA$_2$ activity. As illustrated in Fig. 1*A*, the [^{3}H]AA release was detected at a lower hVPLA$_2$ concentration than was [^{14}C]OA release. For instance, at 1 nM hVPLA$_2$ the [^{3}H]AA release was twice larger than the control, whereas the [^{14}C]OA release was essentially the same as the control. At 100 nM hVPLA$_2$, however, both [^{3}H]AA and [^{14}C]OA releases were about three times higher than the control. This suggests that at lower concentrations of exogenously added hVPLA$_2$, cPLA$_2$ is mainly responsible for the AA release, whereas at higher concentrations (*i.e.* ≥10 nM) hVPLA$_2$ also contributes to the overall AA release. Although it is difficult to monitor the secretion of endogenous hVPLA$_2$ in response to the exogenously added hVPLA$_2$, the contribution of the former to the AA release should be insignificant under our experimental conditions. This is because at lower concentrations of exogenously added hVPLA$_2$, cPLA$_2$ plays a predominant role in overall AA release, whereas at higher concentrations (*i.e.* >10 nM) of exogenously added hVPLA$_2$, the amount of secreted endogenous hVPLA$_2$ (~1.5 nM) (7) should be much smaller than that of exogenous hVPLA$_2$. As reported previously (10, 11), hIIaPLA$_2$ up to 100 nM had little effect on fatty acid release under the same conditions, underscoring the unique ability of hVPLA$_2$ to release fatty acid from unprimed neutrophils. We then measured the LTB$_4$ release from neutrophils as a function of hVPLA$_2$ concentration (Fig. 1*B*). Unlike the AA release that occurred with as low as 1 nM exogenously added hVPLA$_2$, the LTB$_4$ release was not detectable at 1 nM hVPLA$_2$. However, the LTB$_4$ release was clearly seen with ≥10 nM hVPLA$_2$. As reported earlier (10, 11), 100 nM hVPLA$_2$ was about half as effective as 1 μM fMLP + 5 μg/ml cytochalasin B in inducing the LTB$_4$ release. As a negative control, hIIaPLA$_2$ up to 100 nM was shown to have little effect on LTB$_4$ release. Together, these data suggest that the release of AA and LTB$_4$ biosynthesis in human neutrophils have disparate dependence on hVPLA$_2$ concentration acting on their cell surfaces.

We then monitored the kinetics of fatty acid release from dual labeled neutrophils by 10 nM hVPLA$_2$. As shown in Fig. 2*A*, the [^{14}C]OA release showed simple saturation kinetics, whereas the [^{3}H]AA release exhibited more complex two-phase kinetics under the same conditions, suggesting that at least two distinct pathways are involved in the latter case. The early phase of [^{3}H]AA release reached a plateau in 10–20 min, as was the case with the [^{14}C]OA release; however, the delayed phase of [^{3}H]AA release followed after ~20 min and extended for about 1 h. When neutrophils were incubated with a cPLA$_2$ inhibitor, AACOCF$_3$ (25 μM) (18) or surfactin (10 μM) (19), prior to the addition of hVPLA$_2$, the delayed phase AA release was abrogated, whereas the early phase AA release was modestly (about 30%) reduced. On the other hand, OA release remained essentially unchanged after treatment with cPLA$_2$ inhibitors. An iPLA$_2$ inhibitor, bromoenol lactone (10 μM) had little effect on the time course of fatty acid release (data not shown). In conjunction with the data shown in Fig. 1, these data suggest that in the presence of 10 nM exogenous hVPLA$_2$, both hVPLA$_2$ and cPLA$_2$ are involved in the early phase of [^{3}H]AA release, whereas cPLA$_2$ is primarily responsible for the delayed phase of [^{3}H]AA release. The involvement of hVPLA$_2$ only in the early phase of AA release is also consistent with our previous finding that the exogenously added hVPLA$_2$ is internalized and degraded in neutrophils within the first 10 min under similar experimental conditions (11).

We also monitored the time course of LTB$_4$ release by 1–100 nM exogenous hVPLA$_2$ under the same experimental conditions (Fig. 2*B*). With 10 nM hVPLA$_2$, the LTB$_4$ release reached a saturation in 5–10 min, consistent with the early phase AA release curve, and started to decline until it increased again at 15–20 min, which is approximately synchronized with the delayed phase AA release. The delayed phase LTB$_4$ release was

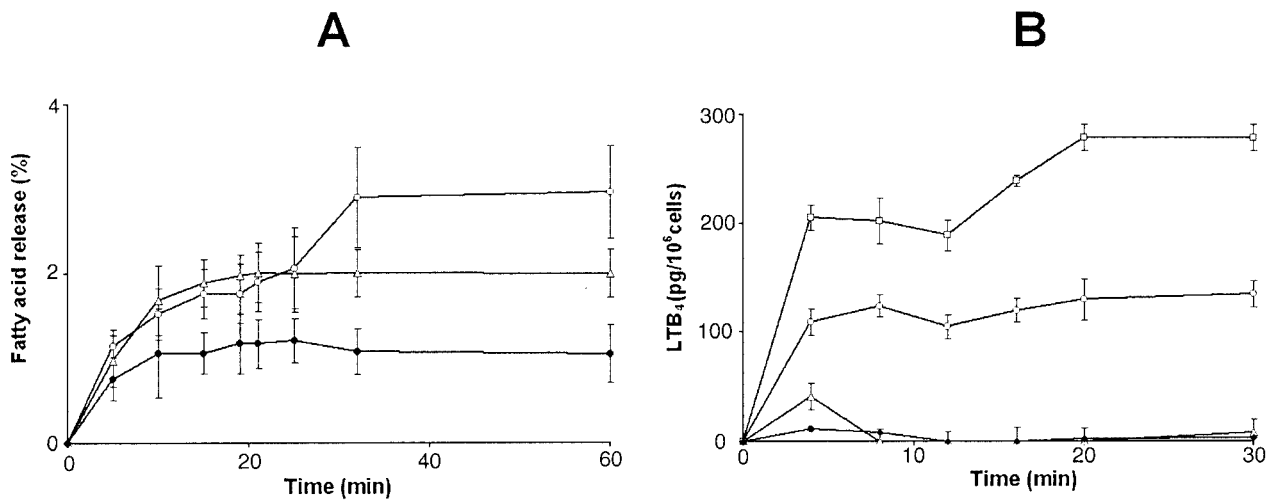

FIG. 2. **Time courses of hVPLA$_2$-induced fatty acid and LTB$_4$ release from neutrophils.** *A*, dual radiolabeled neutrophils were incubated with 10 nM hVPLA$_2$, and the time courses of [^{3}H]AA (○) and [^{14}C]OA (△) releases were monitored. Also, the [^{3}H]AA release was monitored for neutrophils preincubated for 30 min with 25 μM AACOCF$_3$ before the addition of 10 nM hVPLA$_2$ (●). *B*, time courses of LTB$_4$ secretion were measured in the presence of 1 nM (△), 10 nM (○), and 100 nM (□) hVPLA$_2$. Also, the LTB$_4$ release from neutrophils preincubated for 30 min with 25 μM AACOCF$_3$ before the addition of 10 nM hVPLA$_2$ was measured (●). Data represent the mean ± S.E. from triplicate measurements.

much more pronounced with 100 nM hVPLA$_2$. Importantly, treatment of neutrophils with AACOCF$_3$ abrogated the LTB$_4$ release, indicating that cPLA$_2$ is mainly responsible for LTB$_4$ biosynthesis under these conditions. With 1 nM hVPLA$_2$, the LTB$_4$ release rapidly reached a maximal point at 5 min and then decreased to a basal level after 10 min, which is consistent with the lack of LTB$_4$ release at 10 min shown in Fig. 1*B*. These data indicate, however, that as low as 1 nM exogenous hVPLA$_2$ can stimulate the LTB$_4$ biosynthesis.

In neutrophils AA is transformed into several different 5-LO products including LTB$_4$ (16). Also, LTB$_4$ is known to be degraded and inactivated by microsomal ω-oxidation and peroxisomal β-oxidation in myeloid cells (16). To understand better the fate of AA liberated in neutrophils, we analyzed the composition of lipid products that neutrophils released to the medium when they were challenged with 1 and 10 nM exogenous hVPLA$_2$, respectively. The chromatogram in Fig. 3*A* shows that at 1 nM hVPLA$_2$ two main leukotriene products are 20-carboxy-LTB$_4$ and 20-hydroxy-LTB$_4$, which are produced as a consequence of LTB$_4$ oxidation (16). These data thus confirm that even 1 nM exogenous hVPLA$_2$ can induce the biosynthesis of a considerable amount of LTB$_4$. It appears, however, that LTB$_4$ is degraded relatively rapidly to oxidation products that do not cross-react with the LTB$_4$ antibody used in the commercial LTB$_4$ detection kit, hence there is no LTB$_4$ signal with 1 nM hVPLA$_2$ in Fig. 1*B*. With 10 nM hVPLA$_2$, LTB$_4$ was clearly seen along with other leukotrienes. As was the case with 1 nM hVPLA$_2$, 20-carboxy-LTB$_4$ was the most abundant component.

hVPLA$_2$-induced Activation of cPLA$_2$ in Neutrophils—Accumulating evidence has indicated that cPLA$_2$ plays a pivotal role in the receptor-mediated mobilization of AA and eicosanoid biosynthesis in neutrophils (7, 9, 20). Furthermore, several reports have indicated that exogenously added sPLA$_2$s activate cPLA$_2$ in neutrophils (8) and other mammalian cells (21, 22). Also, the occurrence of the delayed phase AA release in our studies implies that cPLA$_2$ is activated during or after the early phase of AA release. We therefore measured the effect of hVPLA$_2$ on cPLA$_2$ activities in neutrophils. It has been established that cPLA$_2$ can be activated by a rise in $[Ca^{2+}]_i$ (23) and the phosphorylation of Ser residues, most notably Ser^{505} (24). In neutrophils, it was shown previously that exogenously added pancreatic sPLA$_2$ phosphorylated and activated cPLA$_2$ through the formation of 5-LO products, including LTB$_4$ (8). To elucidate the mechanism by which hVPLA$_2$ activates cPLA$_2$, we monitored the time-dependent changes in cPLA$_2$ activity and the phosphorylation by enzyme assay and electrophoretic mobility assay, respectively, upon incubating neutrophils with 10 nM hVPLA$_2$. First, we measured the time course of cPLA$_2$ activity from neutrophil lysates. To eliminate residual sPLA$_2$ activities in the cell lysates, the lysates were incubated with 10 mM dithiothreitol before the addition of a cPLA$_2$ substrate, [^{14}C]SAPC. As shown in Fig. 4A, the cPLA$_2$ activity of neutrophils was enhanced by exogenously added hVPLA$_2$, but the time course of activation was rather complex. The cPLA$_2$ activity increased about 2.3-fold in first 5 min but then started to decrease until it rose again at ~10 min and reached a plateau in 20 min. As was the case with AA and LTB$_4$ release, it thus appears that cPLA$_2$ activation also occurs in two phases. Interestingly, preincubation of neutrophils with a LTB$_4$ receptor antagonist, LTB$_4$DMA (0.3 μM) abrogated the delayed phase activation of cPLA$_2$, suggesting that it is mediated through the binding of LTB$_4$ to its cell surface receptor. Because the cPLA$_2$ assay of the lysates was done in the presence of a saturating concentration of calcium for cPLA$_2$ (0.1 mM), the activity enhancement should reflect mainly the protein phosphorylation. Indeed, Fig. 4*B* shows that the extent of cPLA$_2$ phosphorylation is synchronized with the change in cPLA$_2$ activity shown in Fig. 4*A*.

We then measured the effect of exogenously added hVPLA$_2$ on $[Ca^{2+}]_i$. We monitored the fluctuation of $[Ca^{2+}]_i$ with a fluorescence indicator, Fluo-4. Although UV-excitable Ca^{2+} indicators, such as Indo-1 and Fura-2, allow more accurate $[Ca^{2+}]_i$ measurement by a ratiometric analysis, we used Fluo-4 in our studies because the UV irradiation severely damages human neutrophils. We monitored the fluorescence intensity changes of Fluo-4 in the perinuclear region by confocal microscopy. As shown in Fig. 5, the addition of 10 nM hVPLA$_2$ evoked an immediate increase in $[Ca^{2+}]_i$ (to ~500 nM) in the perinuclear region. Interestingly, a second $[Ca^{2+}]_i$ spike was seen in the perinuclear region, which was about 50% of the first one in magnitude. The timing of the second spike varied between 10 and 15 min among different cells. As seen with the progress curve of cPLA$_2$ activation (see Fig. 4*A*), the second $[Ca^{2+}]_i$ peak was completely abrogated when the cells were pretreated with a LTB$_4$ receptor antagonist, LTB$_4$DMA. In conjunction with cPLA$_2$ phosphorylation data, these data suggest that the addi-

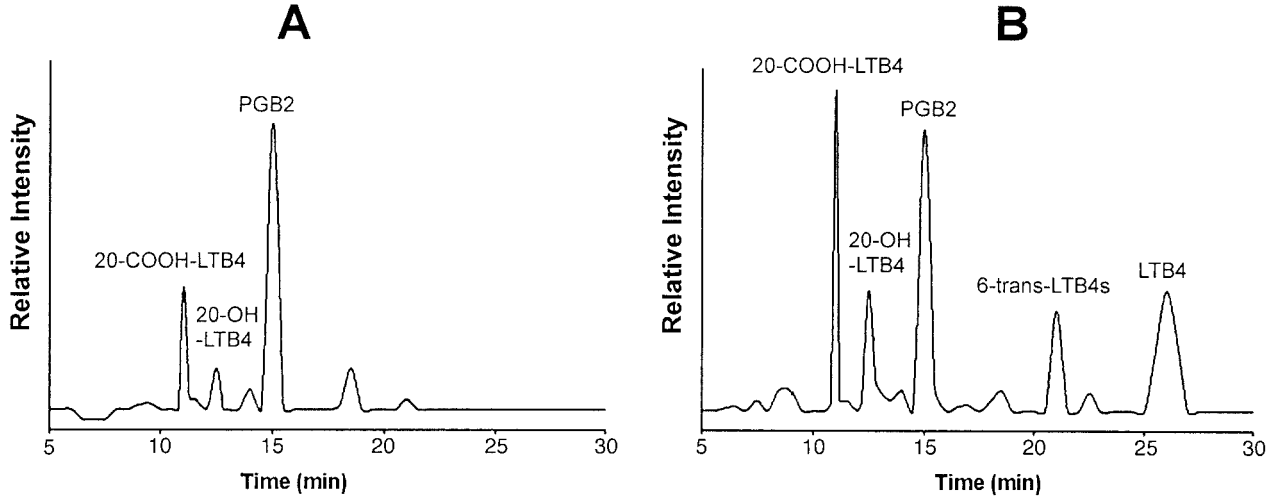

FIG. 3. **HPLC-MS analysis of LTB$_4$ derivatives secreted from human neutrophils.** Representative chromatograms are shown for neutrophils treated with 1 nM (*A*) and 10 nM (*B*) hVPLA$_2$. Extracts of growth medium dissolved in 10:10:80:0.02 methanol/acetonitrile/water/acetic acid (pH 5.5) with the internal standard PGB$_2$ were injected into a C18 reverse phase HPLC column (5 × 150 mm) that was directly interfaced with the electrospray source of a triple quadrupole mass spectrometer. The column was eluted using a linear gradient of acetonitrile/methanol (65:35) from 40 to 100%. Quantitation of individual peaks was carried out using their mass ion abundance relative to that of PGB$_2$.

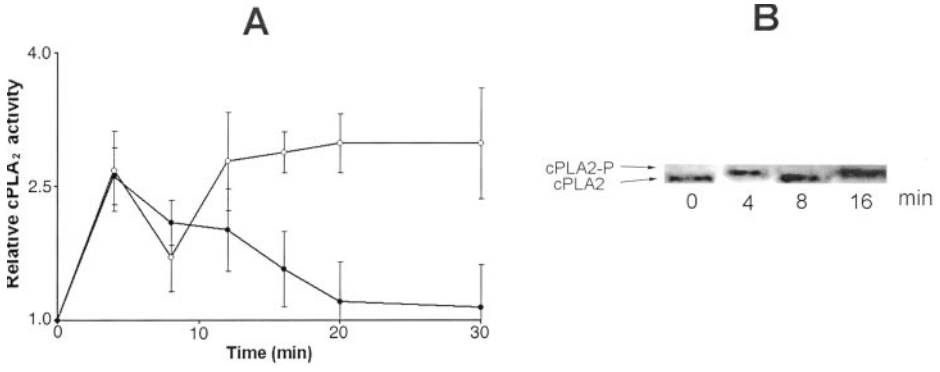

FIG. 4. **Time-dependent activation of neutrophil cPLA$_2$ by exogenous hVPLA$_2$.** *A*, neutrophils (2 × 10^6 cells) suspended in 200 μl of HBSS containing 1.2 mM $CaCl_2$ were incubated with 10 nM hVPLA$_2$ in the absence (●) or presence (○) of 0.3 μM LTB$_4$DMA for a given period and were pelleted and lysed. The cPLA$_2$ activity of the cell lysate was measured using [^{14}C]SAPC as substrate in the presence of 10 mM dithiothreitol and 0.1 mM $CaCl_2$. Data represent the mean ± S.E. from triplicate measurements. *B*, immunoblotting of the cell lysates using an anti-cPLA$_2$ antibody after incubation with 10 nM hVPLA$_2$ for different periods.

tion of hVPLA$_2$ activates cPLA$_2$ by increasing the $[Ca^{2+}]_i$ and inducing cPLA$_2$ phosphorylation in both the early and the delayed phases and that the delayed phase activation is mediated through the binding of LTB$_4$ to its cell surface receptor.

Sites of hVPLA$_2$ and cPLA$_2$ Actions in Neutrophils—To determine the exact site of actions for hVPLA$_2$ and cPLA$_2$ in neutrophils, we performed a cellular PLA$_2$ activity assay using a fluorescent phospholipid, PED6. We recently reported the use of PED6 in the real time activity assay for hVPLA$_2$ internalized into human embryonic kidney 293 cells (25). Because cPLA$_2$ has much lower specific activity than sPLA$_2$ for this phospholipid (25, 26), the cellular cPLA$_2$ activity would yield only a low fluorescence signal from PED6 hydrolysis. To improve the sensitivity of assay for cPLA$_2$, we double labeled neutrophil membranes with DiIC12 and PED6. DiIC12 is a nonhydrolyzable fluorescent lipid that shows a greatly enhanced fluorescent intensity at 585 nM by FRET from hydrolyzed PED6. Indeed, the *in vitro* FRET assay using POPS/cholesterol/POPG/PED6/DiIC12 (107:31:20:1:1) vesicles yielded a three times larger fluorescence change than the assay using POPS/cholesterol/POPG/PED6 (107:31:20:1) vesicles in the presence of the same concentration of cPLA$_2$ (10 nM) (data not shown). When neutrophils double labeled with DiIC12 and PED6 were incubated with 10 nM exogenous hVPLA$_2$, prominent signals appeared at the plasma membrane and the perinuclear region after 5 min (Fig. 6). Because of the high laser power necessary for visualization of the signal change, real time monitoring was not attempted in this case because it would lead to serious photobleaching. Most importantly, the perinuclear signal was abrogated when the labeled neutrophils were pretreated with 25 μM $AACOCF_3$ before the addition of hVPLA$_2$. No change was observed, however, when the cells were treated with 10 μM bromoenol lactone. This clearly indicates that the perinuclear signal is the result of cPLA$_2$ activity and that hVPLA$_2$ primarily acts on the plasma membrane in neutrophils. Taken together, these results indicate that hVPLA$_2$-induced activation of cPLA$_2$ by calcium increase and phosphorylation results in the lipolytic action of cPLA$_2$ in the perinuclear region.

Effects of hVPLA$_2$ Products and LTB$_4$ on cPLA$_2$ Activity—To determine the mechanism by which hVPLA$_2$ activates cPLA$_2$, we first measured the effect of exogenously added lipid products of hVPLA$_2$, fatty acids (AA and OA) and lysophospholipids (lyso-PC) on the [^{3}H]AA and LTB$_4$ release from neutrophils. Lyso-PC was selected as a representative lysophospholipid because the main phospholipid component of the outer plasma membrane of mammalian cells is phosphatidylcholine. As illustrated in Fig. 7*A*, 3 μM lyso-PC had the same potency as 10 nM hVPLA$_2$ in eliciting [^{3}H]AA release from labeled neutrophils. Even 1 μM lyso-PC was able to induce significant [^{3}H]AA release (data not shown). Although less potent than lyso-PC, AA was also able to induce [^{3}H]AA release. In this case, 10 μM exogenous AA was as effective as 10 nM hVPLA$_2$. In contrast, OA up to 30 μM showed negligible effects on [^{3}H]AA release. Lyso-PC and AA showed an additive effect when used in combination. A similar trend was seen with the LTB$_4$ release. Lyso-PC (3 μM) was nearly twice as effective as 10 nM hVPLA$_2$ in net LTB$_4$ release activity (see Fig. 7*B*), whereas the same concentration of AA was about 30% active. OA up to 30 μM

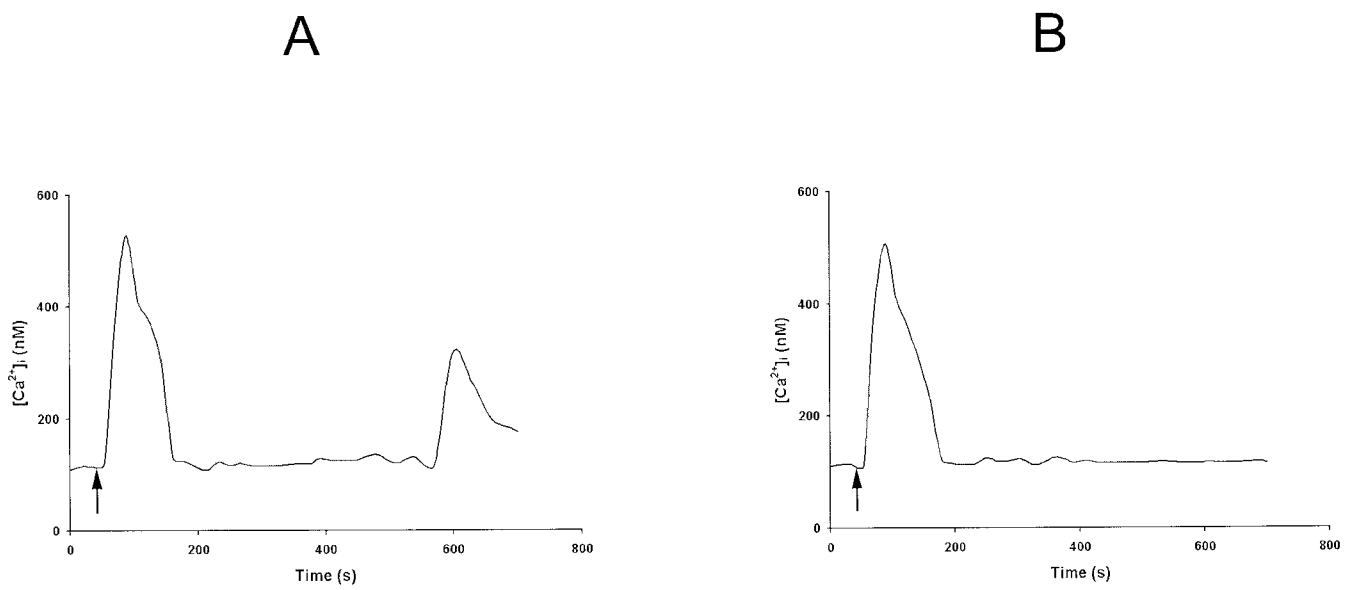

FIG. 5. **Time lapse changes in $[Ca^{2+}]_i$ of human neutrophils caused by exogenous hVPLA$_2$.** The fluorescence intensity of Fluo-4 near the perinuclear region of neutrophils was monitored upon adding 10 nM hVPLA$_2$ (*arrows*), and $[Ca^{2+}]_i$ values were calculated from the intensity values using a calibration curve (see "Experimental Procedures"). $[Ca^{2+}]_i$ changes are shown for a single representative cell in the absence (*A*) and presence (*B*) of 0.3 μM LTB$_4$DMA (total number of cells = 12).

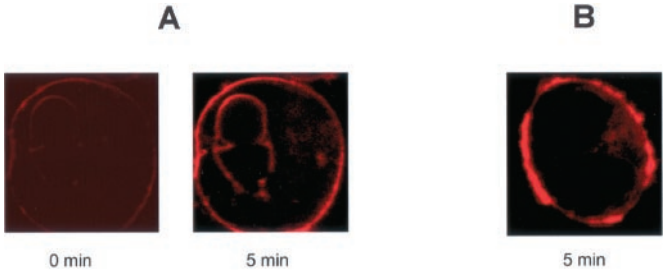

FIG. 6. **Confocal microscopic imaging of PLA$_2$ activities in human neutrophils.** *A*, imaging was performed after adding 10 nM hVPLA$_2$ to human neutrophils that were incubated with POPS/cholesterol/POPG/PED6/DiIC12 (107:31:20:1:1 in mol ratio) for 50 min at 37 °C. *B*, neutrophils were treated with 25 μM AACOCF$_3$ before the addition of hVPLA$_2$.

failed to induce LTB$_4$ release. Given that AA constitutes only a small fraction (~5%) of fatty acids incorporated into the phospholipids in the outer plasma membrane, these data indicate that the cellular effect of hVPLA$_2$ is mediated largely through the formation of lyso-PC.

Interestingly, the exogenous addition of 0.3 μM LTB$_4$ enhanced the [^{3}H]AA release as much as 10 nM hVPLA$_2$ (Fig. 7*A*). Furthermore, preincubation of labeled neutrophils with 0.3 μM LTB$_4$DMA significantly reduced the positive effects of hVPLA$_2$, lyso-PC, and AA on [^{3}H]AA release, suggesting that a large part of their effects is mediated through the activation of LTB$_4$ receptors on the neutrophil surfaces. To investigate this aspect further, we measured the time course of cPLA$_2$ activation by lyso-PC in the presence and absence of LTB$_4$DMA. As described above, the cPLA$_2$ activity assay of the lysates was performed in the presence of 0.1 mM calcium and 10 mM dithiothreitol, and the activity enhancement should mainly reflect cPLA$_2$ phosphorylation. Fig. 8 shows that lyso-PC increased the cPLA$_2$ activity (and phosphorylation) in two phases, which is reminiscent of two-phase cPLA$_2$ activation and phosphorylation by exogenous hVPLA$_2$. Again, the delayed phase activation was abrogated by preincubation of neutrophils with LTB$_4$DMA. This indicates that lyso-PC induces the phosphorylation of cPLA$_2$ in both phases of cPLA$_2$ activation and that the delayed phase phosphorylation takes place via LTB$_4$ formation and its receptor binding.

We also measured the effects of lyso-PC, fatty acids, and LTB$_4$ on the change of $[Ca^{2+}]_i$ in the perinuclear region. It has been reported that AA (27) and LTB$_4$ (28) can increase $[Ca^{2+}]_i$ in human neutrophils. We also observed that 3 μM AA or 0.3 μM LTB$_4$ rapidly enhanced $[Ca^{2+}]_i$ in the perinuclear region to 400–600 nM. Similarly, 3 μM lyso-PC spontaneously raised $[Ca^{2+}]_i$ to 500 nM in the perinuclear region as shown in Fig. 9. These effects on $[Ca^{2+}]_i$ are reminiscent of the effect of hVPLA$_2$ illustrated in Fig. 5. Finally, OA up to 10 μM had no effect on $[Ca^{2+}]_i$ (data not shown).

MAP Kinases Involved in cPLA$_2$ Phosphorylation—It has been reported that cPLA$_2$ is phosphorylated and activated by different kinases in mammalian cells (29–35). In neutrophils, cPLA$_2$ was shown to be phosphorylated by p38 MAP kinase, ERK1/2 MAP kinase, or both, depending on how neutrophils are activated (20, 36). To determine how these MAP kinases are involved in the hVPLA$_2$-induced cPLA$_2$ activation and LTB$_4$ biosynthesis in neutrophils, we first measured the effect of hVPLA$_2$ on p38 and ERK1/2 MAP kinase activation. Phosphorylation of these MAP kinases is commonly used as an indicator of their activation. As shown in Fig. 10*A*, hVPLA$_2$ caused a time-dependent phosphorylation of p38 and ERK1/2 MAP kinases. The phosphorylation of ERK1/2 exhibited a biphasic pattern, peaking at 5 and 20 min, respectively, but the delayed phase phosphorylation was more pronounced. In contrast, p38 phosphorylation peaked at 10 min and declined thereafter. This suggests that both p38 and ERK1/2 MAP kinases are involved in the early phase cPLA$_2$ phosphorylation, whereas ERK1/2 plays a predominant role in the delayed phase cPLA$_2$ phosphorylation. To test this notion, we measured the effects on the time course of cPLA$_2$ activation of specific inhibitors of two MAP kinase pathways: SB203580, which specifically inhibits p38 MAP kinase (37), and U0126, which specifically inhibits MEK, which is an upstream kinase of ERK1/2 (38). As shown in Fig. 10*B*, 30 μM SB203580 significantly inhibited the early phase cPLA$_2$ activation with a lesser effect on the delayed phase. 10 μM U0126, however, had a much more pronounced effect on the delayed phase cPLA$_2$ activation while also showing a significant effect on the early phase. Together,

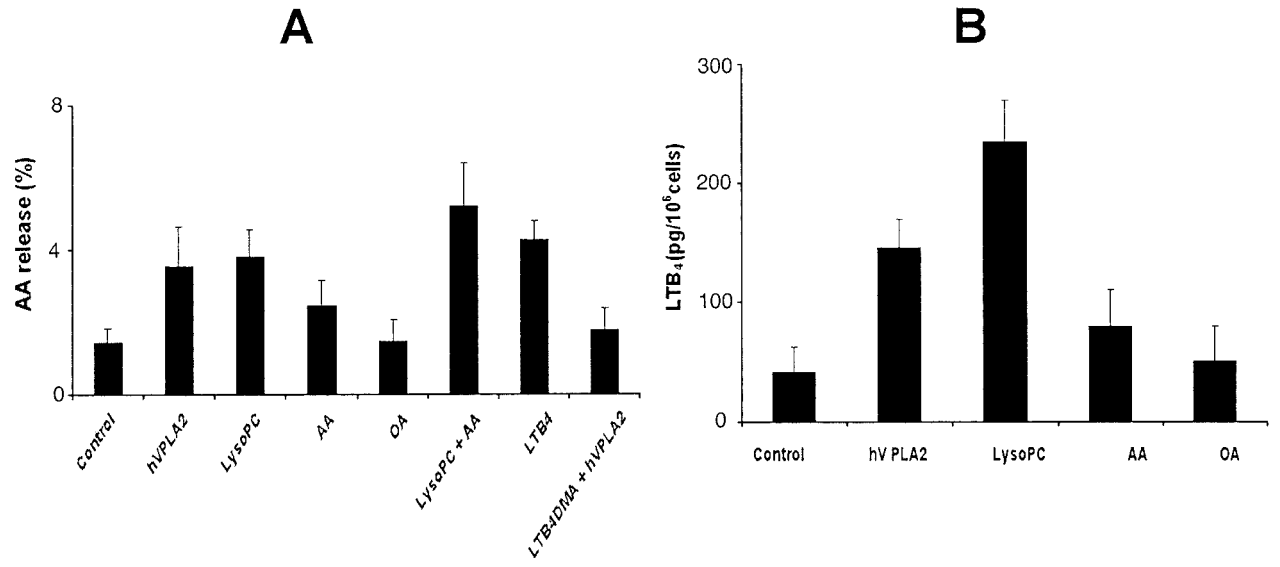

FIG. 7. **Effects of hVPLA$_2$ hydrolysis products and LTB$_4$ on neutrophil activation.** AA (*A*) and LTB$_4$ (*B*) release was measured after neutrophils were treated with each agonist (10 nM hVPLA$_2$, 3 μM AA, 3 μM OA, 3 μM lyso-PC, 0.3 μM LTB$_4$, or 0.3 μM LTB$_4$DMA) for 20 min. Data represent the mean ± S.E. from triplicate measurements.

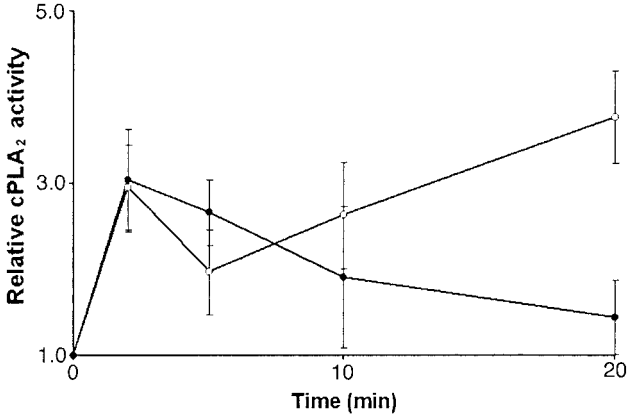

FIG. 8. **Time-dependent activation of neutrophil cPLA$_2$ by exogenous lyso-PC.** The effect of 3 μM lyso-PC was measured in the absence (○) and presence (●) of 0.3 μM LTB$_4$DMA. Experimental conditions were the same as described for Fig. 4. Data represent the mean ± S.E. from triplicate measurements.

these results indicate that both ERK1/2 and p38 MAP kinases are involved in the early phase of the hVPLA$_2$-induced cPLA$_2$ activation in neutrophils, whereas ERK1/2 is involved primarily in the delayed phase.

DISCUSSION

Neutrophils that play a key role in defense against microbial infection release AA, LTB$_4$, and other 5-LO products in response to various stimuli, including bacterial peptides. Recent studies on fMLP-induced activation of human neutrophils have indicated that both sPLA$_2$ and cPLA$_2$ are involved in AA release (9), whereas cPLA$_2$ is responsible primarily for LTB$_4$ release (7). We showed previously that exogenously added hVPLA$_2$ could also elicit the release of AA and LTB$_4$ from unprimed human neutrophils almost as effectively as fMLP (10). This neutrophil activation involves the direct binding of hVPLA$_2$ to the outer plasma membrane and the hydrolysis of phosphatidylcholine (10) and is terminated by the internalization and degradation of cell surface-bound hVPLA$_2$ in a heparan sulfate proteoglycan-dependent manner (11). The present study shows that exogenous hVPLA$_2$ as low as 1 nM is able to induce AA and LTB$_4$ release from unprimed human neutrophils. Furthermore, the study reveals that the hVPLA$_2$-induced formation of AA and leukotrienes in human neutrophils is a complex and dynamic process that involves cPLA$_2$ activation by $[Ca^{2+}]_i$ increase and phosphorylation. The most salient feature of hVPLA$_2$-induced neutrophil activation is the two-phase kinetics. All phenomena associated with neutrophil activation, AA and LTB$_4$ release, $[Ca^{2+}]_i$ increase, and cPLA$_2$ phosphorylation, follow similar two-phase kinetic patterns.

The time course of hVPLA$_2$-induced OA release as well as the effect of cPLA$_2$ inhibition on the time course of hVPLA$_2$-induced AA release indicate that both hVPLA$_2$ and cPLA$_2$ contribute to the early phase AA release, whereas cPLA$_2$ is responsible primarily for the delayed phase. Also, the primary sites of action for hVPLA$_2$ and cPLA$_2$ are the outer plasma membrane and the perinuclear region, respectively (see Fig. 6). Given that the AA composition of neutrophil plasma membrane is less than 5% (39) and that cell surface-bound hVPLA$_2$ is readily internalized and degraded (11), a relatively high concentration of exogenous hVPLA$_2$ would be necessary to liberate a significant amount of AA from the outer plasma membrane. Indeed, direct AA production by hVPLA$_2$ becomes significant only when its concentration reaches 10 nM (see Fig. 1*A*). Importantly, the abrogation of LTB$_4$ release in the presence of cPLA$_2$ inhibitors points to the predominant role of cPLA$_2$ in LTB$_4$ biosynthesis under our experimental conditions. This in turn indicates that the AA liberated from the outer plasma membrane of neutrophils by direct lipolytic action of hVPLA$_2$ in the early phase is not conducive to LTB$_4$ biosynthesis by 5-LO. Thus, it would seem that the primary role of this hVPLA$_2$-produced AA is to activate cPLA$_2$. In fact, AA and other polyunsaturated fatty acids have been shown to activate cPLA$_2$ in neutrophils (40). In this regard, it is noteworthy that lyso-PC is about three times more potent than AA in inducing LTB$_4$ biosynthesis in neutrophils. Also, lyso-PC should be produced in a much larger amount than AA and polyunsaturated fatty acids from the outer plasma membrane of neutrophils because of the abundance of phosphatidylcholine. This and other results presented herein support the notion that hVPLA$_2$-induced activation of neutrophils is largely mediated by lyso-PC. Lyso-PC species containing a saturated acyl chain in the *sn*-1 position, including the palmitoyl derivative employed in this study, have been shown to activate a cell surface G protein-coupled receptor (41) and thereby regulate a broad range of cell processes, including increases in cAMP (42) and $[Ca^{2+}]_i$ (41) and the activation of

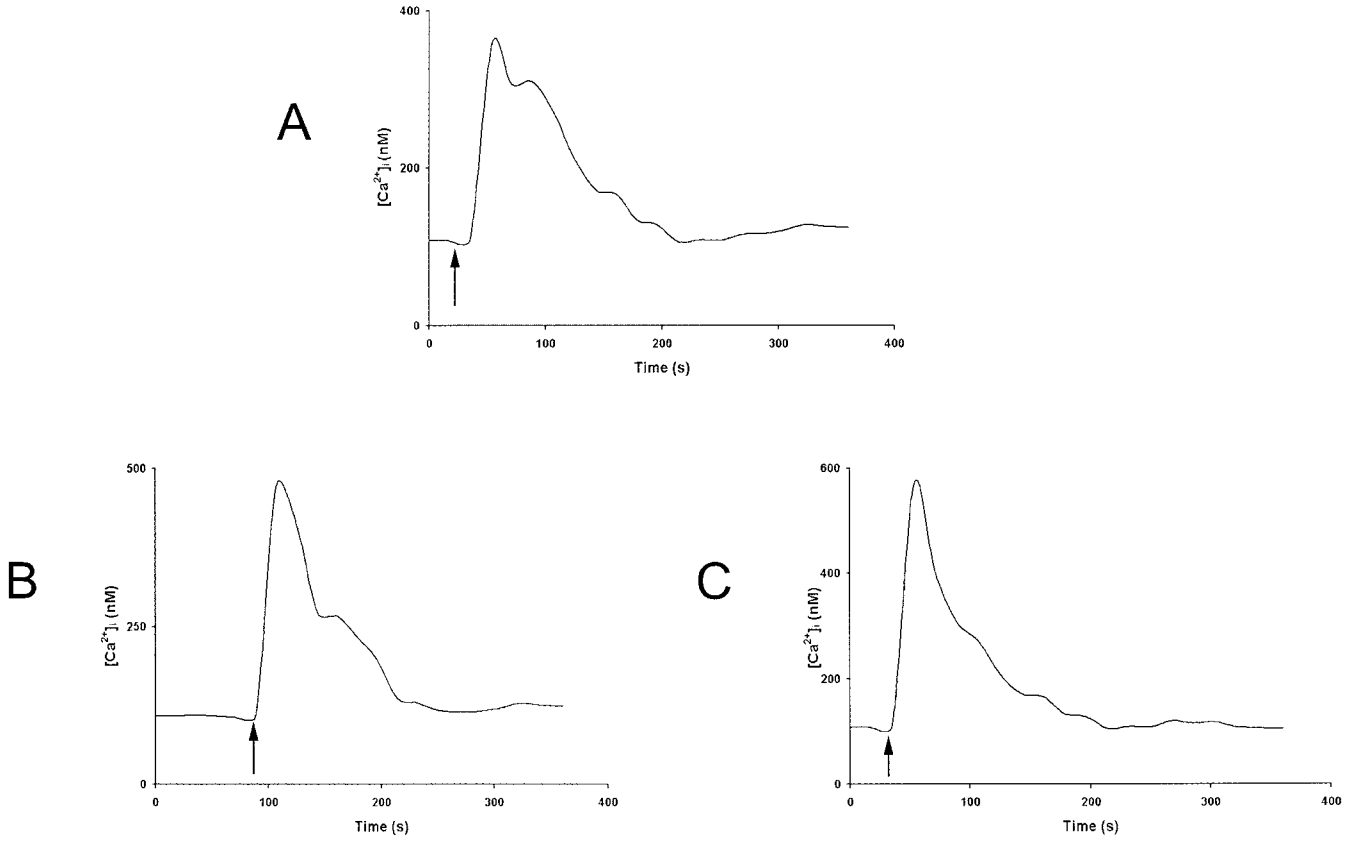

FIG. 9. **Time lapse changes in $[Ca^{2+}]_i$ of human neutrophils caused by AA (*A*), lyso-PC (*B*), and LTB$_4$ (*C*).** Experimental conditions were the same as described for Fig. 5.

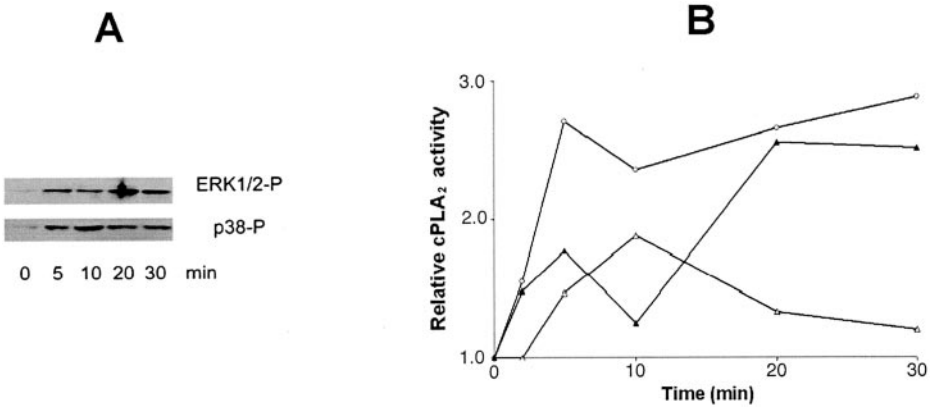

FIG. 10. **MAP kinase activation in hVPLA$_2$-induced neutrophil activation.** *A*, time lapse phosphorylation of ERK1/2 and p38 MAP kinases in human neutrophils treated with 10 nM hVPLA$_2$. *B*, effect of the p38 inhibitor SB203580 and MEK inhibitor U0126 on neutrophil cPLA$_2$ activation. Untreated neutrophils (○) and neutrophils pretreated with 30 μM SB203580 (▲) and 10 μM U0126 (△), respectively, were incubated with 10 nM hVPLA$_2$ and the cPLA$_2$ activity measured. Experimental conditions were the same as described for Fig. 4. Data represent an average of duplicate measurements.

MAP kinase (41) and protein kinase C (43). In particular, 100 μM lyso-PC was shown to induce AA release and increase $[Ca^{2+}]_i$ in rat heart myoblastic H9c2 cells (43). In our study, a few micromolar lyso-PC effectively simulated all activities of hVPLA$_2$ on human neutrophils, including AA and LTB$_4$ release, a rise in $[Ca^{2+}]_i$, and cPLA$_2$ phosphorylation. In particular, lyso-PC activates cPLA$_2$ by inducing both $[Ca^{2+}]_i$ increase and cPLA$_2$ phosphorylation in both early and delayed phases. A rise in Ca^{2+} by lyso-PC would also activate 5-LO by inducing its translocation to the nuclear envelope (44), thereby promoting LTB$_4$ synthesis.

It has been shown that LTB$_4$ can activate neutrophils by an autocrine, positive feedback mechanism (49). Neutrophils contain a cell surface G protein-coupled LTB$_4$ receptor (50), and the binding of LTB$_4$ to the receptor leads to various cells activation, including a rise in $[Ca^{2+}]_i$ and the MAP kinase activation (50). It was shown previously that the agonist-induced biosynthesis of LTB$_4$ in neutrophils leads to cPLA$_2$ phosphorylation (8). Our results clearly show that the biosynthesis of LTB$_4$ and its binding to the cell surface receptor play a pivotal role in the delayed phase of hVPLA$_2$-induced cPLA$_2$ activation by causing both a rise in $[Ca^{2+}]_i$ and cPLA$_2$ phosphorylation. Because blocking the LTB$_4$ receptor with LTB$_4$DMA abrogates the $[Ca^{2+}]_i$ increase and the cPLA$_2$ phosphorylation only in the delayed phase, it is unlikely that LTB$_4$ is involved in the early phase cPLA$_2$ activation that is mediated primarily by lyso-PC (and polyunsaturated fatty acids). Our results also indicate that a certain threshold concentration of LTB$_4$ is required for its positive feedback effect because of the relatively rapid oxidative degradation of LTB$_4$ in neutrophils. In the case of neutrophil activation by exogenous hVPLA$_2$, this threshold concentration of LTB$_4$ is achieved by ~10 nM hVPLA$_2$. The threshold LTB$_4$ concentration was not determined directly in this study because of difficulties involved in distinguishing between exogenous and endogenous LTB$_4$.

In neutrophils, cPLA$_2$ is phosphorylated by p38 MAP kinase,

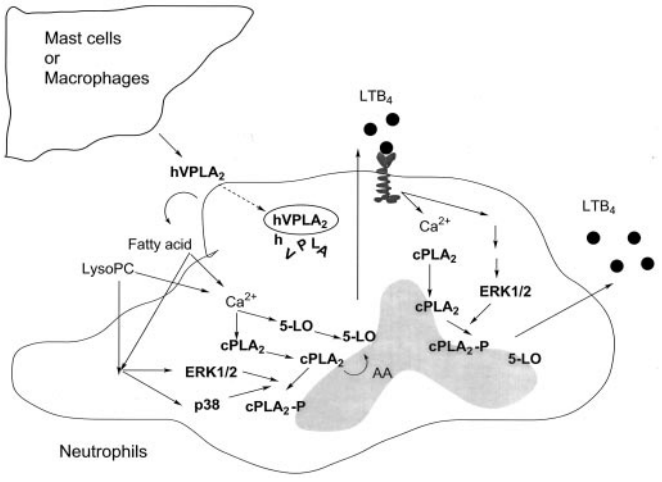

FIG. 11. **A proposed mechanism of hVPLA$_2$-induced LTB$_4$ biosynthesis in human neutrophils.** *Arrows* indicate the flow of signaling pathways, and the *broken arrow* indicates the internalization. The secreted hVPLA$_2$ acts directly on the outer cell membranes of neutrophils to release fatty acids (including AA) and lyso-PC, both of which can induce the immediate membrane translocation of 5-LO and cPLA$_2$ by increasing $[Ca^{2+}]_i$. They also activate cPLA$_2$ via phosphorylation. At the same time, activated 5-LO produces and releases LTB$_4$, which binds the cell surface LTB$_4$ receptor in an autocrine manner and triggers a MAP kinase cascade to phosphorylate and activate cPLA$_2$ in the delayed phase. This delayed phase phosphorylation of cPLA$_2$ will then lead to amplified and prolonged production of AA, LTB$_4$, and other eicosanoids.

ERK1/2, or both, depending on the nature of agonists (20). Although the identification of the network of protein kinases involved in hVPLA$_2$-induced cPLA$_2$ phosphorylation and the site of cPLA$_2$ phosphorylation are beyond the scope of this investigation, our results indicate that both ERK1/2 and p38 MAP kinases are involved in the early phase cPLA$_2$ activation, whereas ERK1/2 is involved primarily in the delayed phase. The direct role of ERK1/2 in cPLA$_2$ phosphorylation in neutrophils has been well documented. In particular, LTB$_4$ was shown to activate ERK1/2 (45) but not p38 MAP kinase (46). This is consistent with our finding that ERK1/2 is involved mainly in the delayed phase cPLA$_2$ phosphorylation that is mediated by the binding of LTB$_4$ to its receptor. It has been shown that lyso-PC (43) and AA (and other polyunsaturated fatty aids) (47) can activate protein kinase C. Furthermore, the phorbol ester-induced activation of protein kinase C in neutrophils was shown to phosphorylate cPLA$_2$ via ERK1/2 activation (20). Thus, it appears that at least one signaling pathway to cPLA$_2$ phosphorylation in the early phase involves the protein kinase C activation that leads to ERK1/2 activation. A previous study reported that AA stimulated p38 phosphorylation in neutrophils (48), Thus, AA and polyunsaturated fatty acids released by hVPLA$_2$ might be responsible for the p38 phosphorylation in the early phase of neutrophil activation by hVPLA$_2$. It is not clear, however, whether the activated p38 is directly or indirectly involved in cPLA$_2$ phosphorylation. Further studies are necessary to sort out the effects of different protein kinases in the activation of cPLA$_2$ in neutrophils.

On the basis of our present and previous studies, we propose a mechanism by which hVPLA$_2$ induces the LTB$_4$ biosynthesis in human neutrophils as shown in Fig. 11. In this model, hVPLA$_2$ directly acts on the outer cell membranes of neutrophils to release fatty acids (including AA) and lysophospholipids, most likely lyso-PC. Both polyunsaturated fatty acids (including AA) and lyso-PC induce the immediate membrane translocation of 5-LO and cPLA$_2$ with transient Ca^{2+} influx. Also, they activate cPLA$_2$ via phosphorylation, which leads to the liberation of AA at the perinuclear region. cPLA$_2$ activated by hVPLA$_2$ products then returns to the resting state as cells internalize hVPLA$_2$ via heparan sulfate proteoglycan binding and degrade them to avoid extensive lipolytic damage of the outer plasma membrane. In the meantime, activated 5-LO produces LTB$_4$, which binds the cell surface LTB$_4$ receptor in an autocrine manner and triggers a MAP kinase cascade to rephosphorylate and reactivate cPLA$_2$ in the delayed phase. This delayed phase phosphorylation of cPLA$_2$ will then lead to amplified and prolonged production of AA, LTB$_4$, and other eicosanoids.

It should be noted that this model focuses mainly on the action of exogenous hVPLA$_2$ on neutrophils but not on the role of endogenous hVPLA$_2$ in neutrophil activation. Based on the lack of LTB$_4$ release from human neutrophils stimulated with fMLP and cytochalasin B, it was postulated that the endogenous hVPLA$_2$ in neutrophils is not involved in LTB$_4$ biosynthesis (7). In this report, the concentration of hVPLA$_2$ released from neutrophils by fMLP and cytochalasin B was estimated to be in the low nanomolar range (7). Our study shows that even this concentration of hVPLA$_2$ can induce the formation of a significant amount of LTB$_4$ but cannot trigger the receptor-mediated positive feedback effect because of rapid oxidative degradation. However, the amount of hVPLA$_2$ in human neutrophils seems to vary to a large extent depending on the allergic state of donors (52),[2] suggesting that higher concentrations of endogenous hVPLA$_2$ could be secreted by activated neutrophils. Furthermore, the sPLA$_2$ concentration in serum and inflammatory exudates was reported be much higher (51). In particular, mast cells and macrophages release a significant amount of group V PLA$_2$ in response to different stimuli.[2] It is therefore likely that exogenous hVPLA$_2$ is able to trigger LTB$_4$ biosynthesis in neutrophils, either alone or in combination with other stimuli, under pathophysiological conditions. Undoubtedly, further studies are necessary to address this important question.

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