Group V Phospholipase A2 Induces Leukotriene Biosynthesis in Human Neutrophils through the Activation of Group IVA Phospholipase A2*

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We reported previously that exogenously added human group V phospholipase A2 (hVPLA2) could elicit leukotriene B4 (LTB4) 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 hVPLA2-induced LTB4 biosynthesis in neutrophils, we thoroughly examined the effects of hVPLA2 and their lipid products on the activity of group IVA cytosolic PLA2 (cPLA2) and LTB4 biosynthesis under different conditions. As low as 1 nM exogenous hVPLA2 was able to induce the release of arachidonic acid (AA) and LTB4. Typically, AA and LTB4 were released in two phases, which were synchronized with a rise in intracellular calcium concentration ([Ca2+]i) near the perinuclear region and cPLA2 phosphorylation. A cellular PLA2 assay showed that hVPLA2 acted primarily on the outer plasma membrane, liberating fatty acids and lysophosphatidylcholine (lyso-PC), whereas cPLA2 acted on the perinuclear membrane. Lyso-PC and polyunsaturated fatty acids including AA activated cPLA2 and 5-lipoxigenase by increasing [Ca2+]i, and inducing cPLA2 phosphorylation, which then led to LTB4 biosynthesis. The delayed phase was triggered by the binding of secreted LTB4 to the cell surface LTB4 receptor, which resulted in a rise in [Ca2+]i and cPLA2 phosphorylation through the activation of mitogen-activated protein kinase, extracellular signal-regulated kinase 1/2. These results indicate that a main role of exogenous hVPLA2 in neutrophil activation and LTB4 biosynthesis is to activate cPLA2 and 5-lipoxygenase primarily by liberating from the outer plasma membrane lyso-PC that induces [Ca2+]i increase and cPLA2 phosphorylation and that hVPLA2-induced LTB4 production is augmented by the positive feedback activation of cPLA2 by LTB4.

Phospholipase A2 (PLA2) 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 PLA2 have been identified from mammalian tissues, which include several forms of secretory PLA2 (sPLA2) (1), group IVA Ca2+-dependent cytosolic PLA2 (cPLA2) (2), and group VI Ca2+-independent PLA2 (iPLA2) (3). Recent studies have indicated that sPLA2s work in concert with cPLA2 to induce eicosanoid formation in different cells (4–6). Neutrophils are inflammatory cells that release AA and 5-lipoxygenase products, most notably leukotriene B4 (LTB4), 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 PLA2s, including cPLA2, iPLA2, and group V and group X sPLA2s (7). However, roles of these PLA2s in inflammatory actions of neutrophils, biosynthesis and release of LTB4, in particular, have not been elucidated fully. Based on the effects of exogenously added sPLA2, AA, and 5-lipoxygenase (5-LO) products on cPLA2 activity, it was postulated earlier that stimulus-induced AA release or exocytosis of sPLA2 activates cPLA2 by initiating the formation of LTB4 which leads to the phosphorylation of cPLA2 (8). Similarly, both sPLA2 and cPLA2 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 sPLA2 from human neutrophils did not lead to LTB4 biosynthesis (7). Instead, cPLA2 was reported to be entirely responsible for the fMLP-stimulated LTB4 release from human neutrophils (7). Independently, we showed that exogenously added human group V PLA2 (hVPLA2) could induce AA and LTB4 release from unprimed human neutrophils half as effectively as fMLP (10).

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and that hVPLA2 bound to cell surface heparan sulfate proteoglycans was eventually internalized and degraded (11). To understand better the interplay between sPLA2 and cPLA2 in glycans was eventually internalized and degraded (11). To understand better the interplay between sPLA2 and cPLA2 in leukotriene biosynthesis in neutrophils, we thoroughly examined the effects of sPLA2 and their lipid products on cPLA2 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 sPLA2 and cPLA2 work in concert to achieve effective and controlled leukotriene biosynthesis in neutrophils.

**EXPERIMENTAL PROCEDURES**

**Materials**—Arachidonoyltriuroyl methyl ketone (AACOCF3) 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-cPLA2 monoclonal antibody was generously provided by Dr. James Clark of the Genetics Institute (Cambridge, MA). [3H]AA and [14C]-labeled oleic acid (OA) were purchased from American Radiochemical Co. (St. Louis, MO). 1-Stearoyl-2-[14C]arachidonoyl-sn-glycero-3-phosphatidylcholine ([14C]SAPC), goat anti-rabbit immunoglobulin conjugated with horseradish peroxidase, goat anti-mouse immunoglobulin conjugated with horseradish peroxidase, and ionomycin were purchased fromSigma. 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (lyso-PC) was purchased from Avanti Polar Lipids (Alabaster, AL). AA (Eugene, OR). 1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (PED6), Fluo-4 AM and 1,1'deconoyleurono-5,7-dimethyl-4-bora-3a,4a-diaza-2H-pyran-2-one (bromoenol lactone) were from Cayman Chemical Co. (Ann Arbor, MI).

**Immunoblotting**—Aliquots of neutrophils (2 × 10^6 cells/sample) were centrifuged at 13,000 × g for 20 s, and cell pellet was re-suspended in the addition of the cold-ice lysis buffer (20 mM Tris-HCl, pH 8.0, containing 5 mM EDTA, 40 mM NaCl, 30 mM Na2PO4, 10 μM leu-leu-leu, 5 μg/ml aprotinin, 5 μg/ml chymostatin, 2 mM disopropylfluorophosphate, 1 mM phenylmethylsulfonyl fluoride, 2 mM Na3VO4, 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 at 13,000 × g, 10 min) to remove nuclear and cellular debris. Supernatants were mixed with the gel loading buffer (0.125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% SDS, 0.05% bromphenol blue), and boiled for 5 min. Equivalent amounts of protein (∼50 μg) were loaded onto SDS-polyacylamide gels (10% of SAPC, and 12% for p38 and ERK1/2 MAP kinases). Electrophoretic transfer of protein from the gel to the polyvinylidene difluoride membrane was performed at 100 V for 1 h. The membrane was blocked with 1% BSA for 30 min, then incubated with primary antibodies (anti-cPLA2, Anti-Active'-ERK1/2, or Anti-Active™-p38 antibody).

**Measurement of (Ca2+)^2—**Measurement of intracellular calcium concentration ([Ca2+]c) was performed with a Zeiss LSM 510 laser scanning confocal microscopy using Fluo-4 AM as the indicator. Neutrophils (10^6 cells/ml) were incubated in HBSS containing 1.2 mM Ca2+, 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 Lek-ToK™ chambered cover glass filled with 400 μl of HBSS containing 1.2 mM Ca2+, and incubated at 37°C with 5% CO2 for 10 min. After washing once with HBSS containing 1.2 mM Ca2+, 10 μM hVPLA2 for 3 μM lyso-PC, 3 μM AA, 0.3 μM LTβ 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 with the Zeiss biological software package. [Ca2+]c, was calibrated as described previously using the reported calcium dissociation constant value (i.e. 345 nM) of Fluo-4 (15).

**Confocal Microscopy Imaging of PLA2 Activity—**Neutrophils (10^6 cells/ml) were seeded into each of eight wells on a sterile Nunc Lek-ToK™ chambered cover glass filled with 400 μl of HBSS and incubated at 37°C with 5% CO2 for 10 min. After the cells were washed once with HBSS, they were overlaid with 10 μM of PED6 vesicle solution (0.75 mM POPS/cholesterol/POPG/PE/DiC12 (107:31:20:1:1 in a mol ratio) mixed vesicles in HBSS) and incubated for 50 min at 37°C with 5% CO2. After rinsing the labeled cells six times with HBSS containing 1.2 mM Ca2+, 10 μM hVPLA2, and 0.3 μM LTβ was added. Each leukotriene peak was analyzed by mass spectrometry measuring the ion abundance for the following collision-induced transformation at the corresponding retention times: LTβ and
RESULTS

hVPLA2-induced Fatty Acid and LTB4 Release from Neutrophils—We showed previously that exogenously added hVPLA2 triggers AA release and LTβ secretion from unprimed human neutrophils (10). To understand better the mechanism of hVPLA2-induced LTβ biosynthesis, we carefully examined the time course of the fatty acid and LTβ release from neutrophils in the presence of varying concentrations of exogenously added hVPLA2. First, we measured the hVPLA2 concentration dependence of fatty acid release from neutrophils double labeled with [3H]AA and [14C]OA. Because of the high AA specificity of cPLA2 and lack of fatty acid selectivity of sPLA2s, the [3H]AA release in this system would reflect both sPLA2 and cPLA2 activities, whereas the [14C]OA release would largely represent sPLA2 activity. As illustrated in Fig. 1A, the [3H]AA release was detected at a lower hVPLA2 concentration than was [14C]OA release. For instance, at 1 nM hVPLA2, the [3H]AA release was twice higher than the control, whereas the [14C]OA release was essentially the same as the control. At 100 nM hVPLA2, however, both [3H]AA and [14C]OA releases were about three times higher than the control. This suggests that at lower concentrations of exogenously added hVPLA2, cPLA2 is mainly responsible for the AA release, whereas at higher concentrations (i.e. >10 nM) of exogenously added hVPLA2, the amount of secreted endogenous hVPLA2 (~1.5 nM) (7) should be much smaller than that of exogenous hVPLA2. As reported previously (10, 11), hIaPLA2 up to 100 nM had little effect on fatty acid release under the same conditions, underscoring the unique ability of hVPLA2 to release fatty acid from unprimed neutrophils. We then measured the LTβ4 release from neutrophils as a function of hVPLA2 concentration (Fig. 1B). Unlike the AA release that occurred with so low as 1 nM exogenously added hVPLA2, the LTβ4 release was not detectable at 1 nM hVPLA2. However, the LTβ4 release was clearly seen with ≥10 nM hVPLA2. As reported earlier (10, 11), 100 nM hVPLA2 was about half as effective as 1 μM fMLP + 5 μg/ml cytochalasin B in inducing the LTβ release. As a negative control, hIaPLA2 up to 100 nM was shown to have little effect on LTβ4 release. Together, these data suggest that the release of AA and LTβ biosynthesis in human neutrophils have disparate dependence on hVPLA2 concentration acting on their cell surfaces.

We then monitored the kinetics of fatty acid release from dual labeled neutrophils by 10 nM hVPLA2. As shown in Fig. 2A, the [14C]OA release showed simple saturation kinetics, whereas the [3H]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 [3H]AA release reached a plateau in 10–20 min, as was the case with the [14C]OA release; however, the delayed phase of [3H]AA release followed after ~20 min and extended for about 1 h. When neutrophils were incubated with a cPLA2 inhibitor, AACOCF3 (25 μM) (18) or surfactin (10 μM) (19), prior to the addition of hVPLA2, 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 cPLA2 inhibitors. An iPLA2 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 hVPLA2, both hVPLA2 and cPLA2 are involved in the early phase of [3H]AA release, whereas cPLA2 is primarily responsible for the delayed phase of [3H]AA release. The involvement of hVPLA2 only in the early phase of AA release is also consistent with our previous finding that the exogenously added hVPLA2 is internalized and degraded in neutrophils within the first 10 min under similar experimental conditions (11).

We also monitored the time course of LTβ4 release by 1–100 nM exogenous hVPLA2 under the same experimental conditions (Fig. 2B). With 10 nM hVPLA2, the LTβ4 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 LTβ4 release was
nM hVPLA_2. Importantly, treatment of neutrophils with AAOCCF_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. 1B. 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. 3A 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 as shown in Fig. 1B. 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_2s 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+}] (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 inciting 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, [1^4]C]OA. 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_4DMA (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. 4B shows that the extent of cPLA_2 phosphorylation is synchronized with the change in cPLA_2 activity shown in Fig. 4A.

We then measured the effect of exogenously added hVPLA_2 on [Ca^{2+}]. We monitored the fluctuation of [Ca^{2+}] with a fluorescence indicator, Fluo-4. Although UV-excitable Ca^{2+} indicators, such as Indo-1 and Fura-2, allow more accurate 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+}] (to ~500 nM) in the perinuclear region. Interestingly, a second [Ca^{2+}] peak 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. 4A), the second [Ca^{2+}] peak was completely abrogated when the cells were pretreated with a LTB_4 receptor antagonist, LTB_4DMA. In conjunction with cPLA_2 phosphorylation data, these data suggest that the addi-
tion of hVPLA2 activates cPLA2 by increasing the [Ca$^{2+}$], and inducing cPLA2 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 hVPLA2 and cPLA2 Actions in Neutrophils**—To determine the exact site of actions for hVPLA2 and cPLA2 in neutrophils, we performed a cellular PLA2 activity assay using a fluorescent phospholipid, PED6. We recently reported the use of PED6 in the real time activity assay for hVPLA2 internalized into human embryonic kidney 293 cells (25). Because cPLA2 has much lower specific activity than sPLA2 for this phospholipid (25, 26), the cellular cPLA2 activity would yield only a low fluorescence signal from PED6 hydrolysis. To improve the sensitivity of assay for cPLA2, we double labeled neutrophil membranes with DiIC12 and PED6. DiIC12 is a nonhydrolyzable fluorescent lipid that shows a greatly enhanced fluorescent signal when bleached. Most importantly, the perinuclear signal was abrogated when the labeled neutrophils were pretreated with 25 mM AACOCF$_3$ before the addition of hVPLA2. No change was observed, however, when the cells were treated with 10 mM bromo-phenol lactone. This clearly indicates that the perinuclear signal is the result of cPLA2 activity and that hVPLA2 primarily acts on the plasma membrane in neutrophils. Taken together, these results indicate that hVPLA2-induced activation of cPLA2 by calcium increase and phosphorylation results in the lipolytic action of cPLA2 in the perinuclear region.

**Effects of hVPLA2 Products and LTB$_4$ on cPLA2 Activity**—To determine the mechanism by which hVPLA2 activates cPLA2, we first measured the effect of exogenously added lipid products of hVPLA2, fatty acids (AA and OA) and lysophospholipids (lyso-PC) on the [3H]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. 7A, 3 mM lyso-PC had the same potency as 10 nM hVPLA2 in eliciting [3H]AA release (data not shown). Although less potent than lyso-PC, AA was also able to induce [3H]AA release. In this case, 10 mM exogenous AA was as effective as 10 nM hVPLA2. In contrast, OA up to 30 mM showed negligible effects on [3H]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 mM) was nearly twice as effective as 10 nM hVPLA2 in net LTB$_4$ release activity (see Fig. 7B), whereas the same concentration of AA was about 30% active. OA up to 30 mM...
human neutrophils. A using a calibration curve (see "Experimental Procedures"). cPLA2 activity assay of the lysates was performed in the presence (B) of 0.3 mM LTB4DMA (total number of cells ~12).

This indicates that lyso-PC induces the phosphorylation of cPLA2 in both phases of cPLA2 activation and that the delayed phase phosphorylation takes place via LTB4 formation and its receptor binding.

We also measured the effects of lyso-PC, fatty acids, and LTB4 on the change of [Ca2+]i in the perinuclear region. It has been reported that AA (27) and LTB4 (28) can increase [Ca2+]i in human neutrophils. We also observed that 3 μM AA or 0.3 μM LTB4 rapidly enhanced [Ca2+]i in the perinuclear region to 400–600 nM. Similarly, 3 μM lyso-PC spontaneously raised [Ca2+]i to 500 nM in the perinuclear region as shown in Fig. 9. These effects on [Ca2+]i, are reminiscent of the effect of hVPLA2 illustrated in Fig. 5. Finally, OA up to 10 μM had no effect on [Ca2+]i (data not shown).

**MAP Kinases Involved in cPLA2 Phosphorylation**—It has been reported that cPLA2 is phosphorylated and activated by different kinases in mammalian cells (29–35). In neutrophils, cPLA2 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 hVPLA2-induced cPLA2 activation and LTB4 biosynthesis in neutrophils, we first measured the effect of hVPLA2 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. 10A, hVPLA2 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 cPLA2 phosphorylation, whereas ERK1/2 plays a predominant role in the delayed phase cPLA2 phosphorylation. To test this notion, we measured the effects on the time course of cPLA2 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. 10B, 30 μM SB203580 significantly inhibited the early phase cPLA2 activation with a lesser effect on the delayed phase, 10 μM U0126, however, had a much more pronounced effect on the delayed phase cPLA2 activation while also showing a significant effect on the early phase. Together,
these results indicate that both ERK1/2 and p38 MAP kinases are involved in the early phase of the hVPLA2-induced cPLA2 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, LTB4, 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 sPLA2 and cPLA2 are involved in AA release (9), whereas cPLA2 is responsible primarily for LTB4 release (7). We showed previously that exogenously added hVPLA2 could also elicit the release of AA and LTB4 from unprimed human neutrophils almost as effectively as fMLP (10). This neutrophil activation involves the direct binding of hVPLA2 to the outer plasma membrane and the hydrolysis of phosphatidylcholine (10) and is terminated by the internalization and degradation of cell surface-bound hVPLA2 in a heparan sulfate proteoglycan-dependent manner (11). The present study shows that exogenous hVPLA2 as low as 1 nM is able to induce AA and LTB4 release from unprimed human neutrophils. Furthermore, the study reveals that the hVPLA2-induced formation of AA and leukotrienes in human neutrophils is a complex and dynamic process that involves cPLA2 activation by [Ca2+]i increase and phosphorylation. The most salient feature of hVPLA2-induced neutrophil activation is the two-phase kinetics. All phenomena associated with neutrophil activation, AA and LTB4 release, [Ca2+]i increase, and cPLA2 phosphorylation, follow similar two-phase kinetic patterns.

The time course of hVPLA2-induced OA release as well as the effect of cPLA2 inhibition on the time course of hVPLA2-induced AA release indicate that both hVPLA2 and cPLA2 contribute to the early phase AA release, whereas cPLA2 is responsible primarily for the delayed phase. Also, the primary sites of action for hVPLA2 and cPLA2 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 hVPLA2 is readily internalized and degraded (11), a relatively high concentration of exogenous hVPLA2 would be necessary to liberate a significant amount of AA from the outer plasma membrane. Indeed, direct AA production by hVPLA2 becomes significant only when its concentration reaches 10 nM (see Fig. 1A). Importantly, the abrogation of LTB4 release in the presence of cPLA2 inhibitors points to the predominant role of cPLA2 in LTB4 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 hVPLA2 in the early phase is not conducive to LTB4 biosynthesis by 5-LO. Thus, it would seem that the primary role of this hVPLA2-produced AA is to activate cPLA2. In fact, AA and other polyunsaturated fatty acids have been shown to activate cPLA2 in neutrophils (40). In this regard, it is noteworthy that lyso-PC is about three times more potent than AA in inducing LTB4 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 hVPLA2-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 [Ca2+]i (41) and the activation of...
MAP kinase (41) and protein kinase C (43). In particular, 100 μM lyso-PC was shown to induce AA release and increase 

\[ \text{Ca}^{2+} \] in rat heart myoblastic H9c2 cells (43). In our study, a few micromolar lyso-PC effectively simulated all activities of hVPLA₂ on human neutrophils, including AA and LTB₄ release, a rise in 

\[ \text{Ca}^{2+} \], and cPLA₂ phosphorylation. In particular, lyso-PC activates cPLA₂ by inducing both 

\[ \text{Ca}^{2+} \] increase and cPLA₂ phosphorylation in both early and delayed phases. A rise in 

\[ \text{Ca}^{2+} \] by lyso-PC would also activate 5-LO by inducing its translocation to the nuclear envelope (44), thereby promoting LTB₄ synthesis.

It has been shown that LTB₄ can activate neutrophils by an autocrine, positive feedback mechanism (49). Neutrophils contain a cell surface G protein-coupled LTB₄ receptor (50), and the binding of LTB₄ to the receptor leads to various cell activation, including a rise in 

\[ \text{Ca}^{2+} \], and the MAP kinase activation (50). It was shown previously that the agonist-induced biosynthesis of LTB₄ in neutrophils leads to cPLA₂ phosphorylation (8). Our results clearly show that the biosynthesis of LTB₄ and its binding to the cell surface receptor play a pivotal role in the delayed phase of hVPLA₂-induced cPLA₂ activation by causing both a rise in 

\[ \text{Ca}^{2+} \], and cPLA₂ phosphorylation. Because blocking the LTB₄ receptor with LTB₄DMA abrogates the 

\[ \text{Ca}^{2+} \] increase and the cPLA₂ phosphorylation only in the delayed phase, it is unlikely that LTB₄ is involved in the early phase cPLA₂ activation that is mediated primarily by lyso-PC (and polyunsaturated fatty acids). Our results also indicate that a certain threshold concentration of LTB₄ is required for its positive feedback effect because of the relatively rapid oxidative degradation of LTB₄ in neutrophils. In the case of neutrophil activation by exogenous hVPLA₂, this threshold concentration of LTB₄ is achieved by ~10 nM hVPLA₂. The threshold LTB₄ concentration was not determined directly in this study because of difficulties involved in distinguishing between exogenous and endogenous LTB₄.

In neutrophils, cPLA₂ is phosphorylated by p38 MAP kinase,
ERK1/2, or both, depending on the nature of agonists (20). Although the identification of the network of protein kinases involved in hVPLA2-induced cPLA2 phosphorylation and the site of cPLA2 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 cPLA2 activation, whereas ERK1/2 is involved primarily in the delayed phase. The direct role of ERK1/2 in cPLA2 phosphorylation in neutrophils has been well documented. In particular, LTB4 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 cPLA2 phosphorylation that is mediated by the binding of LTB4 to its receptor. It has been shown that lyso-PC (43) and AA (and other polyunsaturated fatty acids) (47) can activate protein kinase C. Furthermore, the phorboI ester-induced activation of protein kinase C in neutrophils was shown to phosphorylate cPLA2 via ERK1/2 activation (20). Thus, it appears that at least one signaling pathway to cPLA2 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 hVPLA2 might be responsible for the p38 phosphorylation in the early phase of neutrophil activation by hVPLA2. It is not clear, however, whether the activated p38 is directly or indirectly involved in cPLA2 phosphorylation. Further studies are necessary to sort out the effects of different protein kinases in the activation of cPLA2 in neutrophils.

On the basis of our present and previous studies, we propose a mechanism by which hVPLA2 induces the LTB4 biosynthesis in human neutrophils as shown in Fig. 11. In this model, hVPLA2 directly acts on the outer cell membranes of neutrophils to release fatty acids (including AA) and lyso phospholipids, most likely lyso-PC. Both polyunsaturated fatty acids (including AA) and lyso-PC induce the immediate membrane translocation of 5-LO and cPLA2 with transient Ca2+ influx. Also, they activate cPLA2 via phosphorylation, which leads to the liberation of AA at the perinuclear region. cPLA2 activated by hVPLA2 products then returns to the resting state as cells internalize hVPLA2 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 LTB4, which binds to the cell surface LTB4 receptor in an autocrine manner and triggers a MAP kinase cascade to rephosphorylate and reactivate cPLA2 in the delayed phase. This delayed phase phosphorylation of cPLA2 will then lead to amplified and prolonged production of AA, LTB4, and other eicosanoids.

It should be noted that this model focuses mainly on the action of exogenous hVPLA2 on neutrophils but not on the role of endogenous hVPLA2 in neutrophil activation. Based on the lack of LT6, release from human neutrophils stimulated with fMLP and cytochalasin B, it was postulated that the endogenous hVPLA2 in neutrophils is not involved in LT6 biosynthesis (7). In this report, the concentration of hVPLA2 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 hVPLA2 can induce the formation of a significant amount of LT6 but cannot trigger the receptor-mediated positive feedback effect because of rapid oxidative degradation. However, the amount of hVPLA2 in human neutrophils seems to vary to a large extent depending on the allergic state of donors (52), suggesting that higher concentrations of endogenous hVPLA2 could be secreted by activated neutrophils. Furthermore, the sPLA2 concentration in serum and inflammatory exudates was reported to be much higher (51). In particular, mast cells and macrophages release a significant amount of group V PLA2 in response to different stimuli. It is therefore likely that exogenous hVPLA2 is able to trigger LT6 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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