Amplification Mechanisms of Inflammation: Paracrine Stimulation of Arachidonic Acid Mobilization by Secreted Phospholipase A_2 Is Regulated by Cytosolic Phospholipase A_2-Derived Hydroperoxyeicosatetraenoic Acid[1]

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In macrophages and other major immunoinflammatory cells, two phospholipase A_2 (PLA_2) enzymes act in concert to mobilize arachidonic acid (AA) for immediate PG synthesis, namely group IV cytosolic phospholipase A_2 ($cPLA_2$) and a secreted phospholipase A_2 ($sPLA_2$). In this study, the molecular mechanism underlying cross-talk between the two PLA_2s during paracrine signaling has been investigated. U937 macrophage-like cells respond to Con A by releasing AA in a $cPLA_2$-dependent manner, and addition of exogenous group V $sPLA_2$ to the activated cells increases the release. This $sPLA_2$ effect is abolished if the cells are pretreated with $cPLA_2$ inhibitors, but is restored by adding exogenous free AA. Inhibitors of cyclooxygenase and 5-lipoxygenase have no effect on the response to $sPLA_2$. In contrast, ebselen strongly blocks it. Reconstitution experiments conducted in pyrrophenone-treated cells to abolish $cPLA_2$ activity reveal that 12- and 15-hydroperoxyeicosatetraenoic acid (HPETE) are able to restore the $sPLA_2$ response to levels found in cells displaying normal $cPLA_2$ activity. Moreover, 12- and 15-HPETE are able to enhance $sPLA_2$ activity in vitro, using a natural membrane assay. Neither of these effects is mimicked by 12- or 15-hydroxyeicosatetraenoic acid, indicating that the hydroperoxy group of HPETE is responsible for its biological activity. Collectively, these results establish a role for 12/15-HPETE as an endogenous activator of $sPLA_2$-mediated phospholipolysis during paracrine stimulation of macrophages and identify the mechanism that connects $sPLA_2$ with $cPLA_2$ for a full AA mobilization response. ***The Journal of Immunology,*** **2003, 171: 989–994.**

The phospholipase A_2 (PLA_2)[3] superfamily represents a heterogeneous group of enzymes with key roles in inflammation whose common feature is to hydrolyze the fatty acid present at the sn-2 position of phospholipids (1, 2). Multiple forms of PLA_2 have been described in mammalian tissues, including several forms of secreted PLA_2 ($sPLA_2$) (3), group IV Ca^{2+}-dependent cytosolic PLA_2 ($cPLA_2$) (4), and group VI Ca^{2+}-independent PLA_2 (5). Of those, $cPLA_2$ and two of the $sPLA_2$ enzymes, namely groups IIA and V, have repeatedly been shown to be responsible for generating free arachidonic acid (AA) for PG synthesis (6, 7).

An interesting feature of the AA release process is that cross-talk appears to exist between the PLA_2 involved. During stimulation of the cells with agents that promote an immediate AA mobilization response, $cPLA_2$ activation precedes and appears to be required for the subsequent action of $sPLA_2$ (either group IIA or V) (8–12). The first evidence in support of this view was provided by studies in macrophages showing that the $sPLA_2$-dependent release of AA was blocked by $cPLA_2$ inhibitors, and restored by elevating the intracellular levels of free AA by exogenous addition of the fatty acid, which mimics $cPLA_2$ activation (8, 9). These results were later confirmed in other cell types (10, 11), and also by transfection studies showing a synergistic $sPLA_2$-dependent AA release in cells overexpressing $cPLA_2$ (12). Because $sPLA_2$ activity is particularly sensitive to the physical state of the membrane, different events that alter membrane dynamics, such as ceramide generation (13), membrane oxidation (14), and loss of membrane asymmetry (15, 16), have been proposed as possible mechanisms involved in facilitating $sPLA_2$ hydrolysis of the agonist-stimulated cellular membranes. However, the factor that intermediates between $cPLA_2$ and $sPLA_2$ has not been identified.

To understand better the interplay between $cPLA_2$ and $sPLA_2$ in AA mobilization in macrophages, we have examined the effects of exogenous group V PLA_2 on AA mobilization from activated human U937 macrophage-like cells. These settings mimic an instance of paracrine amplification of the inflammatory response in that exogenous $sPLA_2$ being released to the inflammatory foci acts on neighboring cells to increase the response.

Activated U937 cells exhibit an immediate AA release response when exposed to a variety of receptor-mediated and soluble agonists. This AA release is sensitive to inhibitors of $cPLA_2$, but not to inhibitors of other PLA_2s, implying that only $cPLA_2$ is responsible for the release (17–19). Results described in this work identify 12/15-hydroperoxyeicosatetraenoic acid (12/15-HPETE) as the $cPLA_2$-downstream product that enables exogenous group V $sPLA_2$ to properly act on the membranes of activated U937 macrophages.

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[3] Abbreviations used in this paper: PLA_2, phospholipase A_2; AA, arachidonic acid; $cPLA_2$, cytosolic PLA_2; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; $iPLA_2$, calcium-independent PLA_2; $sPLA_2$, secreted PLA_2.

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Materials and Methods

Materials

The [5, 6, 8, 9, 11, 12, 14, 15-^{3}H]AA (100 Ci/mmol) was from Amersham (Arlington Heights, IL). The 12(*S*)-HPETE, 15(*S*)-HPETE, 12-hydroxyeicosatetraenoic acid (12-HETE), and 15-HETE were purchased from Cayman (Ann Arbor, MI). Lipoxygenase inhibitors were from BioMol (Plymouth Meeting, PA). DNA polymerase was from BioTools (Madrid, Spain). Primers for PCR were from MWG-Biotech AG (Ebersberg, Germany). Recombinant rat group V sPLA$_2$ was generously provided by A. Aarsman (Utrecht University, Utrecht, The Netherlands) (20). The specific cPLA$_2\alpha$ inhibitor pyrrophenone was generously provided by K. Seno (Shionogi, Osaka, Japan) (21). All other reagents were from Sigma-Aldrich (St. Louis, MO).

Cell culture

U937 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were incubated at 37°C in a humidified atmosphere of CO_2/O_2 (1:19) at a cell density of 0.5–1 $\times$ 10^6 cells/ml in 12-well plastic culture dishes (Costar, Cambridge, MA). Cell differentiation was induced by treating the cells with 35 ng/ml PMA for 24 h (22, 23).

AA release experiments

The cells were labeled with 0.5 μCi/ml [^{3}H]AA for 18 h. After this period, the cells were washed and placed in serum-free medium for 1 h before the addition of 100 μg/ml Con A in the presence of 0.5 mg/ml BSA. When free AA, HPETEs, or HETEs were added to the cells, they were dissolved in ethanol. Appropriate controls were conducted to exclude an effect of the solvent. The supernatants were removed, cleared of cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

Enzyme assays

For the measurement of cellular iPLA$_2$, aliquots of U937 cell homogenates were incubated for 2 h at 37°C in 100 mM HEPES (pH 7.5) containing 5 mM EDTA and 100 μM labeled phospholipid substrate (1-palmitoyl-2-[^{3}H]palmitoylglycero-3-phosphocholine, sp. act. 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO) in a final volume of 150 μl. The phospholipid substrate was used in the form of sonicated vesicles in buffer. For group V sPLA$_2$ enzyme activity assay, the mammalian membrane substrate assay described by Diez et al. (24) was used.

RT-PCR

cDNA from U937 cells was produced using the kit Cells-to-cDNA (Ambion, Austin, TX), following the manufacturer's instructions. The cDNA was then amplified by PCR using the following primers: 15-LOX (15-LOX-1), upstream primer (5′-GAGTTGACTTTGAGGTTTCGC-3′), downstream primer (5′-GCCCGTCTGTCTTATAGTGG-3′) (25); 15-LOX-2, upstream primer (5′-TGCCTCTCGCCATCCAGCT-3′), downstream primer (5′-TGTTCCCCTGGGATTTAGATGGA-3′) (26); and 12-LOX, upstream primer (5′-CGTAAGGATGATCTACCTCC-3′), downstream primer (5′-TTGGGGTTGGAGAGCTGGGG) (27). The expected sizes for PCR products using these primers were: 952, 1065, and 519 bp, respectively. PCR conditions were: 30–35 cycles, denaturation at 94°C for 1–2 min; annealing at 58°C for 75 min for 15-LOX, 60°C for 1 min for 15-LOX-2, and 63°C for 1 min for 12-LOX, and extension at 72°C for 2 min. An additional extension at 72°C for 10 min was performed at the end of the cycles. The amplified DNA was analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide.

Separation of AA metabolites

For these experiments, the cells were labeled with 5 μCi [^{3}H]AA for 18 h and the stimulations were conducted in the absence of albumin. The supernatant was acidified to pH 3.5 with 5 M formic acid, and extracted twice with 3 ml of isopropanol-diethyl ether (1:1.5). The organic phase was dried under a stream of nitrogen, and the residue was dissolved in a few drops of chloroform-methanol (2:1, v/v) and analyzed by reverse-phase HPLC. Separation of lipoxygenase metabolites was performed on a 4.6 $\times$ 250-mm ODS reverse-phase column (Beckman, Palo Alto, CA), using an isocratic mobile phase of methanol-water-acetic acid (70:30:1) at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected, and radioactivity content was measured by liquid scintillation counting. Retention times of the different products were identified by coelution with authentic standards (Cayman).

Data presentation

Assays were conducted in triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments, and are shown as means $\pm$ SD.

Results

Exogenous group V sPLA$_2$ effects on AA release from U937 cells

When PMA-differentiated U937 cells are stimulated by Con A, immediate AA release occurs by a mechanism that is entirely attributable to cPLA$_2$ activation, with no involvement of the one other PLA$_2$ that these cells express, namely iPLA$_2$ (18, 19). This conclusion is based on the complete inhibition of the AA release response by the specific cPLA$_2$ inhibitor pyrrophenone at concentrations higher than 0.5 μM (19), and the lack of any detectable effect of the iPLA$_2$ inhibitor bromoenol lactone even at concentrations as high as 25 μM (18). U937 cells have been reported not to exhibit measurable sPLA$_2$ activity (17, 28). In accordance with this, we have not detected expression of groups IB, IIA, IIC, IID, IIE, IIF, III, V, and X in U937 cells by RT-PCR (M. A. Balboa and J. Balsinde, unpublished data). Fig. 1*A* shows that pyrrophenone concentrations equal to those leading to complete inhibition of Con A-induced AA release had no effect on the iPLA$_2$ activity of U937 cell homogenates, as measured in an in vitro assay. Even at concentrations of 10 μM, pyrrophenone exerted little effect on cellular iPLA$_2$ activity (Fig. 1*B*). At the same concentrations, pyrrophenone also failed to minimally affect the activity of pure group V sPLA$_2$ (Fig. 1*B*).

Addition of exogenous group V sPLA$_2$ to the Con A-activated U937 cells resulted in a dramatic increase in the amount of [^{3}H]AA mobilized (Fig. 2). Such an elevated response was inhibited by the specific cPLA$_2$ inhibitor pyrrophenone (Fig. 2), suggesting that in U937 macrophages, cPLA$_2$ activation modulates

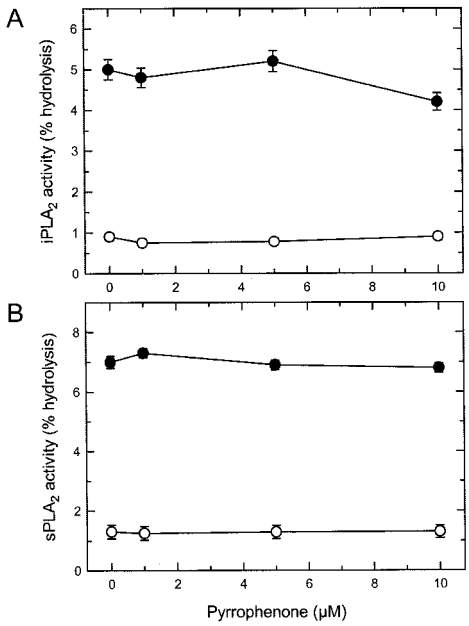

FIGURE 1. Effect of pyrrophenone on PLA$_2$ activities. *A*, Homogenates from U937 cells (●) were assayed for calcium-independent PLA$_2$ activity in the presence of the indicated concentrations of pyrrophenone. ○, Denote control incubations in the absence of homogenate. *B*, Recombinant group V sPLA$_2$ was assayed for activity using a natural membrane as substrate. [^{3}H]AA-labeled membranes were incubated without (○) or with (●) 15 nM group V sPLA$_2$ for 1 h in the presence of the indicated concentrations of pyrrophenone.

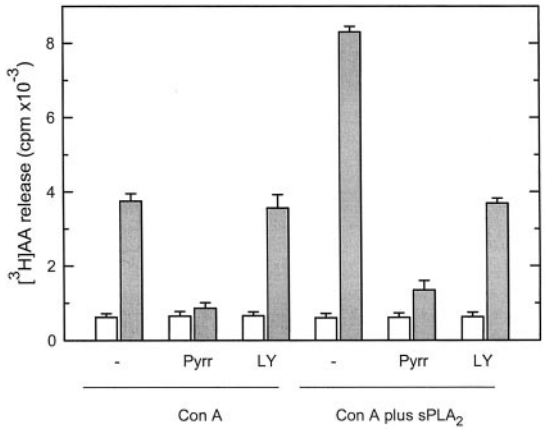

FIGURE 2. Exogenous $sPLA_2$-induced [^{3}H]AA release from U937 macrophage-like cells. The cells were untreated (□) or treated with 100 μg/ml Con A or 100 μg/ml Con A plus 15 nM group V $sPLA_2$, as indicated (▣), for 15 min in the absence or presence of the indicated inhibitors. Pyrrophenone (Pyrr) was used at 1 μM. LY311727 (LY) was used at 25 μM.

$sPLA_2$. The $sPLA_2$-dependent AA release was blocked by the specific inhibitor LY311727, indicating that the hydrolytic activity of the enzyme is required for the response to take place (Fig. 2).

Role of 12/15-HPETE in facilitating $sPLA_2$ activity

The above data indicate that in common with other cell types (8–12), activation of $cPLA_2$ in U937 cells appears to facilitate the action of $sPLA_2$ on cellular membranes. To further stress this notion, [^{3}H]oleic acid release experiments were conducted. $cPLA_2$ releases little, if anything, of this fatty acid, whereas $sPLA_2$ does it readily (29, 30). Thus, determination of [^{3}H]oleic acid release allows one to separate the contribution of $sPLA_2$ to phospholipid hydrolysis from the one of $cPLA_2$ and, in turn, provides a straightforward tool to study the effect of $cPLA_2$ inhibition on $sPLA_2$ activation. Fig. 3 shows that U937 cells exposed to exogenous group V $sPLA_2$ released modest quantities of [^{3}H]oleic acid. Such a release was completely blocked by LY311727, thus confirming that it was actually due to $sPLA_2$ (not shown). When the cells were activated by Con A and then exposed to exogenous $sPLA_2$, a marked potentiation of the response occurred. Con A-activated cells released no oleic acid in the absence of exogenous $sPLA_2$, in agreement with previous data (19). Importantly, the enhanced response was blocked by treating the cells with the $cPLA_2$ inhibitor pyrrophenone (Fig. 3). The inhibitory effect of pyrrophenone could be reversed by exposing the cells to 1 μM exogenous AA for 2 min before $sPLA_2$ addition. At the concentration used, exogenous AA did not exert any effect on its own (Fig. 3). Addition of lysophosphatidylcholine to the $cPLA_2$-activity-deficient cells did not restore the $sPLA_2$ effect (Fig. 3). These results suggest that $cPLA_2$-derived AA, or an oxygenated metabolite, plays a role in mediating the action of $sPLA_2$ on cellular membranes.

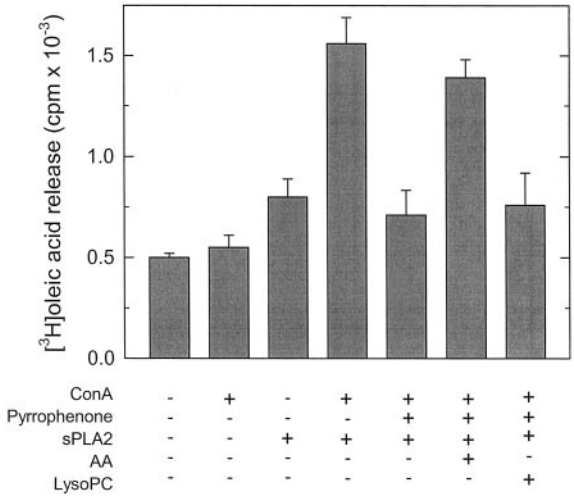

FIGURE 3. Arachidonic acid restores the enhancing effect of exogenous $sPLA_2$ on [^{3}H]oleic acid mobilization in activated U937 macrophage-like cells. The cells were preincubated with 1 μM pyrrophenone where indicated. Subsequently, the cells were stimulated by Con A (100 μg/ml) in the absence or presence of 15 nM exogenous group V $sPLA_2$ and in the absence or presence of 1 μM AA or 10 μM lyso-phosphatidylcholine (LysoPC), as indicated.

Fig. 4*A* shows that the contribution of exogenous $sPLA_2$ to total [^{3}H]AA mobilization in cells exposed to both Con A and exogenous $sPLA_2$ was markedly reduced by the unspecific 15-lipoxygenase inhibitor ebselen (31), but not by inhibitors of cyclooxygenase (aspirin), 5-lipoxygenase-activating protein (MK-886) (32), or platelet-type 12-lipoxygenase (baicalein) (33). Moreover, inhibition by ebselen could be overcome by addition of 15-HPETE, the immediate product of 15-lipoxygenase action on free AA. The 15-HETE was considerably less effective than 15-HPETE in restoring the $sPLA_2$ effect, and 12-HPETE also restored it completely (Fig. 4*B*). These results suggest that the hydroperoxy group of 15-HPETE (and of 12-HPETE) is responsible for the enhancing effect on $sPLA_2$.

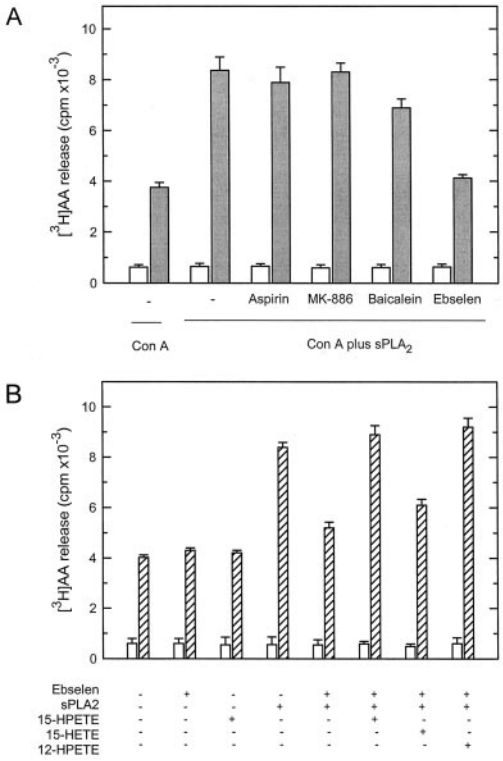

FIGURE 4. Effect of inhibitors of AA metabolism on [^{3}H]AA release in activated U937 macrophages. *A*, The cells were untreated (□) or treated with 100 μg/ml Con A or 100 μg/ml Con A plus 15 nM group V $sPLA_2$, as indicated (▣) , for 15 min in the absence or presence of the indicated inhibitors. All of the inhibitors were used at 25 μM. *B*, The cells were incubated with 25 μM ebselen where indicated. Subsequently, the cells were treated with (▨) or without (□) 100 μg/ml Con A in the presence or absence of 15 nM $sPLA_2$, 0.5 μM 15-HPETE, 0.5 μM 15-HETE, or 0.5 μM 12-HPETE, as indicated.

The 15-HPETE also enabled $sPLA_2$ action on cellular membranes in cells deficient in $cPLA_2$ activity, and 12-HPETE reproduced the effect (Fig. 5). Collectively, the results of Figs. 4 and 5 suggest that 12/15-HPETE is/are the factor(s) that, lying downstream of $cPLA_2$, enables $sPLA_2$ to properly act on cellular membranes. The 15-HPETE and 12-HPETE are both produced by reticulocyte-type 15-lipoxygenase in human myeloid cells, with 15-HPETE being the major product (34). However, because the relative proportion of products synthesized varies among species, this enzyme is also frequently called 12/15-lipoxygenase, and hereinafter it will be referred as such. This enzyme is also called 15-lipoxygenase-1 to distinguish it from the more recently described 15-lipoxygenase-2 (26). RT-PCR analysis of RNA from PMA-differentiated U937 cells revealed that these cells did express 12/15-lipoxygenase (Fig. 6*A*). PCR product specificity was confirmed by DNA sequence analysis. No expression of 12-lipoxygenase (platelet type) or 15-lipoxygenase-2 could be demonstrated (data not shown).

Production of 15-HPETE by activated U937 cells

Reverse-phase HPLC determinations were conducted to verify whether activated U937 macrophage-like cells produced 12/15-lipoxygenase metabolites in a $cPLA_2$-dependent manner. Stimulation of the [^{3}H]AA-labeled U937 cells with Con A resulted in a significant production of 15-[^{3}H]HPETE (Fig. 6*B*). Low levels of 12-[^{3}H]HPETE were also detected (Fig. 6*B*). Unstimulated [^{3}H]AA-labeled U937 cells did not produce significant amounts of these products. Importantly, when the experiments were conducted in the presence of pyrrophenone to block $cPLA_2$ activity, a strong inhibition of 12/15-[^{3}H]HPETE production was detected (93 $\pm$ 2% inhibition, mean $\pm$ SD, $n = 3$). Thus, activated cells produce 12/15-lipoxygenase products downstream of $cPLA_2$ activation.

The 12/15-HPETE enhance $sPLA_2$ activity

The 12/15-lipoxygenase is known to catalyze endogenous membrane oxidation, which may have profound effects on cellular physiology (34). Given that $sPLA_2$ are particularly sensitive to physical changes of the membranes (35), it is likely that the hydroperoxy metabolites produced by 12/15-lipoxygenase may act to influence $sPLA_2$ activity by altering membrane structure. To investigate this possibility, $sPLA_2$ activity measurements were conducted using the natural membrane assay described by Diez et al. (24). In this system, purified [^{3}H]AA-labeled membranes are used as substrate. Addition of 15-HPETE to the assay mix resulted in a marked increase in $sPLA_2$ activity (Fig. 7). Such an increase was not observed if 15-HETE was added instead. The 12-HPETE exerted the same stimulatory effect as 15-HPETE. As a positive control, H_2O_2-oxidized membranes were used (19), and marked increases in $sPLA_2$ activity were observed as well (data not shown). Thus, membrane peroxidation sensitizes membranes to $sPLA_2$ attack.

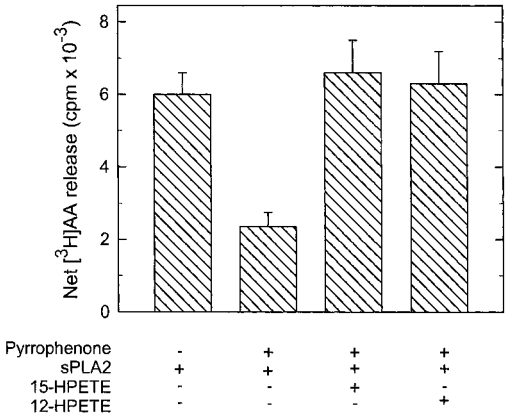

FIGURE 5. The 12- and 15-HPETE restore the enhancing effect of exogenous $sPLA_2$ on [^{3}H]AA mobilization in activated U937 macrophages. [^{3}H]AA-labeled Con A-activated cells were preincubated with 1 μM pyrrophenone for 15 min where indicated. Subsequently, 0.5 μM 15-HPETE or 12-HPETE was added 3 min before adding 15 nM group V $sPLA_2$, as indicated. To highlight the contribution of $sPLA_2$ to the [^{3}H]AA release, the responses in the absence of $sPLA_2$ were subtracted from those in the presence of $sPLA_2$ at each condition.

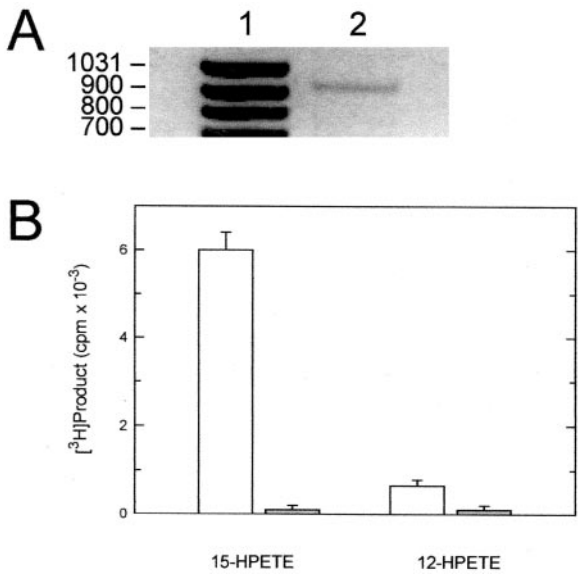

FIGURE 6. Involvement of 12/15-lipoxygenase in AA mobilization in U937 cells. *A*, RT-PCR analysis of 12/15-lipoxygenase in U937 cells. cDNA from U937 cells was obtained and amplified by PCR using the primers set described in *Materials and Methods*. *Lane 1*, Corresponds to a 1000-bp ladder; *lane 2*, corresponds to the analysis of the product (952 bp) obtained by PCR from U937 cDNA. *B*, The 12- and 15-HPETE production by activated U937 macrophages. The [^{3}H]AA-labeled cells were treated with 100 μg/ml Con A for 15 min in the presence (gray bars) or absence (open bars) of 1 μM pyrrophenone, as indicated. The 12/15-HPETE production was determined by reverse-phase HPLC, as described in *Materials and Methods*.

Discussion

Major immunoinflammatory cells such as macrophages and mast cells mobilize AA for PG production in two temporally distinct

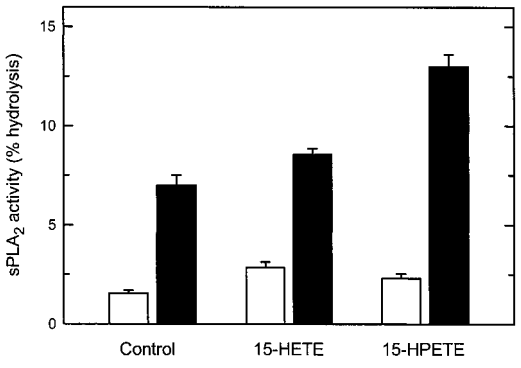

FIGURE 7. The 12/15-HPETE enhance $sPLA_2$ activity using a natural membrane as substrate. [^{3}H]AA-labeled membranes were incubated with (■) or without (□) 15 nM group V $sPLA_2$ for 1 h in the presence of 0.5 μM 12/15-HETE, 0.5 μM 12/15-HPETE, or neither, as indicated.

phases, namely the immediate and delayed pathways (6, 10, 35–37). The immediate pathway, which is typically triggered by Ca^{2+}-mobilizing agonists, goes on for short periods of time (up to 1 h) and occurs at the expense of pre-existing effectors. The delayed pathway, spanning several hours, is strikingly dependent on protein synthesis. Both pathways appear to involve two PLA_2 effectors, namely $cPLA_2$ and $sPLA_2$, although the mechanisms dramatically differ. In general terms, $cPLA_2$ appears to act as the initiator and key regulator of the response, while $sPLA_2$ amplifies the $cPLA_2$-generated signal. The $sPLA_2$ may act both as an autocrine and paracrine effector (i.e., on the same cells that secreted it, or on neighboring cells), ensuring in this manner an efficient amplification of the response (6, 10, 30, 35–37).

In this work, we have studied the interactions between $cPLA_2$ and exogenous $sPLA_2$ during the immediate AA release response triggered by Con A on U937 macrophage-like cells. AA release in this cellular system depends on $cPLA_2$ activation, as judged by complete inhibition of the response by the highly selective $cPLA_2$ inhibitor pyrrophenone (19) (Fig. 1). Although no $sPLA_2$ activity has been demonstrated to occur in U937 cells (28), this study shows that $sPLA_2$ can still participate in the immediate response of the U937 cells if applied exogenously. This strategy is pathophysiologically sound in that it mimics a paracrine mechanism for amplification of the inflammatory response. In turn, the use of exogenous enzyme provides a straightforward means to study the influence of $cPLA_2$ activation on the action of $sPLA_2$.

Group V $sPLA_2$ is produced by human and murine macrophages and mast cells, and has been repeatedly shown to play key roles in proinflammatory AA signaling (38) and, importantly, to be capable of activating cells in the vicinity of those that secreted it (25). Unlike the group IIA enzyme, group V $sPLA_2$ can act on the outer membrane of otherwise unstimulated cells (24, 25). However, the effect is more prominent on agonist-activated cells (39), reflecting the need for some kind of membrane rearrangement for group V $sPLA_2$ to fully express its hydrolytic activity (35).

Our previous studies in murine macrophages demonstrated that the elevated activity that group V $sPLA_2$ displays toward agonist-activated cells can be greatly diminished if cellular $cPLA_2$ activity is blocked, indicating the existence of cross-talk between the two signaling PLA_2s (8, 9). Importantly, the inhibitory effect of $cPLA_2$ can be overcome by exogenous AA, suggesting that a $cPLA_2$-derived AA metabolite intermediates between $cPLA_2$ and $sPLA_2$ (8, 9). In this study, we provide direct evidence that such a metabolite is 12/15-HPETE, the immediate product of 12/15-lipoxygenase action on free AA. Thus, cell treatment with ebselen blocks the enhanced action of group V $sPLA_2$ on activated U937 cells, and reconstitution experiments show that overcoming 12/15-lipoxygenase inhibition by exogenous supply of 12/15-HPETE fully restores the action of $sPLA_2$ on the activated cells. Thus, these results support a model whereby agonist activation of $cPLA_2$ results in the immediate generation of free AA, which will be used by 12/15-lipoxygenase to produce 12/15-HPETE. Subsequently, 12/15-HPETE serves a signaling role by enabling full activation of group V $sPLA_2$ and thus allowing for a further amplification of the AA mobilization response.

The $sPLA_2$-activating effect of 12/15-HPETE was not mimicked by 12/15-HETE, indicating that the hydroperoxy group of 12/15-HPETE is responsible for its biological activity. In turn, this clearly suggests a role for 12/15-HPETE-mediated oxidization of membrane phospholipids as the mechanism for membrane sensitization leading to enhanced group V $sPLA_2$ activity. Fully supporting this view, we have found significantly higher $sPLA_2$ activity in vitro when the membrane substrate was pretreated with 12/15-HPETE.

It has been recognized that a prominent biological action of 12/15-lipoxygenase metabolites on cells is to induce lipid peroxidation reactions, to initiate a series of structural membrane changes (34). For this kind of peroxidation reaction, 12/15-lipoxygenase appears to typically act on esterified substrate, not necessarily on the free fatty acid (34). The current results establish, however, that $cPLA_2$ generation of free AA is required, and hence, that the involvement of 12/15-lipoxygenase in $sPLA_2$ activation takes place through the production of free 12/15-HPETE. This confers on the system greater versatility in that the peroxidizing effect of 12/15-HPETE can be exerted at places far away from its site of synthesis. This is important because 12/15-lipoxygenase is an intracellular enzyme, while exogenous $sPLA_2$ acts primarily on the outer surface of the cells (24, 25, 38). Because 12/15-HPETE can readily be taken up and esterified by the cells (40), it could also be envisioned that this metabolite may exit the cells to amplify the inflammatory response.

Although the results of this study have established a cascade of events for full AA mobilization involving the sequential participation of $cPLA_2$, 12/15-lipoxygenase, and $sPLA_2$, elegant studies by Cho and coworkers (30) have demonstrated that in otherwise unstimulated cells, exogenous group V $sPLA_2$ action leads to activation of $cPLA_2$ and the immediate metabolism of free AA by lipoxygenase pathways. It is tempting to speculate that in analogy with the results of our study, part of the hydroperoxy fatty acids produced under these settings (25) may act to enhance $sPLA_2$ attack on cellular membranes and in this manner amplify the immediate response. In contrast, overexpression studies by Kuwata et al. (41) have suggested a role for 12/15-lipoxygenase in regulating the expression of group IIA $sPLA_2$ during the delayed phase of AA mobilization of 3Y1 fibroblastic cells. Although the mechanisms implicating 12/15-lipoxygenase in the immediate (this study) and delayed (41) AA mobilization pathways obviously differ, it is nonetheless striking that the same effectors appear to be used to elicit these two separate responses.

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