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Studies on a Mechanism by Which Cytosolic Phospholipase A$_2$ Regulates the Expression and Function of Type IIA Secretory Phospholipase A$_2$

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Although it has been proposed that arachidonate release by several secretory phospholipase A$_2$ (sPLA$_2$) isozymes is modulated by cytosolic PLA$_2$ (cPLA$_2$), the cellular component(s) that intermediates between these two signaling PLA$_2$s remains unknown. Here we provide evidence that 12- or 15-lipoxygenase (12/15-LOX), which lies downstream of cPLA$_2$, plays a pivotal role in cytokine-induced gene expression and function of sPLA$_2$-IIA. The sPLA$_2$-IIA expression and associated PGE$_2$ generation induced by cytokines in rat fibroblastic 3Y1 cells were markedly attenuated by antioxidants that possess 12/15-LOX inhibitory activity. 3Y1 cells expressed 12/15-LOX endogenously, and forcible overexpression of 12/15-LOX in these cells greatly enhanced cytokine-induced expression of sPLA$_2$-IIA, with a concomitant increase in delayed PG generation. Moreover, studies using 293 cells stably transfected with sPLA$_2$-IIA revealed that stimulus-dependent hydrolysis of membrane phospholipids by sPLA$_2$-IIA was enhanced by overexpression of 12/15-LOX. These results indicate that the product(s) generated by the cPLA$_2$-12/15-LOX pathway following cell activation may play two roles: enhancement of sPLA$_2$-IIA gene expression and membrane sensitization that leads to accelerated sPLA$_2$-IIA-mediated hydrolysis. The Journal of Immunology, 2000, 165: 4024–4031.

The phospholipase A$_2$ (PLA$_2$) family represents a diverse group of enzymes that hydrolyze the sn-2 fatty acids from membrane glycerophospholipids and play a role in a wide range of physiological functions. Individual members of this family are classified into several subfamilies according to their structure, localization, and biochemical properties (1). Current evidence suggests that cytosolic PLA$_2$ (cPLA$_2$; type IV) and two of the secretory PLA$_2$ (sPLA$_2$) isozymes (types IIA and V) are the signaling PLA$_2$s, which are functionally coupled with the cyclo-oxygenase (COX) pathway for stimulus-initiated production of bioactive PGs (2–4). These sPLA$_2$s can supply arachidonic acid (AA) to both COX-1 and -2 and predominantly to COX-2 in the immediate and delayed PG biosynthetic responses, respectively. The functional segregation of the two COX isozymes may be accounted for at least in part by their differing substrate concentration requirements (4) and distinct coupling with terminal PG synthases (5, 6).

Secretory PLA$_2$-IIA is a widely distributed sPLA$_2$ isozyme whose expression level increases dramatically during inflammation (7). In some cells, sPLA$_2$-V, an isozyme closely related to sPLA$_2$-IIA, appears to substitute for sPLA$_2$-IIA (3, 4, 8, 9). The delayed biosynthesis of PG, which is elicited by proinflammatory stimuli such as IL-1, TNF, and LPS, is accompanied by the continuous supply of AA over long periods of culture spanning several hours, during which de novo induction of these two sPLA$_2$s often occurs, depending on cell type (10–13). Current evidence has suggested that these inducible sPLA$_2$s are crucial for optimal COX-2-dependent PG production (3–5, 12–14). Moreover, these sPLA$_2$s often up-regulate COX-2 expression in several cell types (15–17). The AA-releasing action of these sPLA$_2$s is influenced by the cell activation state, in that only agonist-stimulated cellular membranes become sensitive to them (7, 18). Perturbation of plasma membrane asymmetry by phospholipid scrambling, which carries anionic phospholipids from the inner to the outer leaflet of the plasma membrane, appears to contribute at least in part to sensitization of cells toward the action of sPLA$_2$ (17). More intriguingly, endogenously expressed sPLA$_2$-IIA binds preferentially to glypican, a glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan, and accumulates in caveolae and perinuclear sites in cytokine-stimulated cells (19). This particular compartmentalization may allow relatively small amounts of the heparin-binding sPLA$_2$s to be efficiently coupled with perinuclear COX-2.

Several lines of evidence suggest that cPLA$_2$ is required for sPLA$_2$-IIA or -V to act properly (3, 9, 13, 16, 20). Supporting this idea are observations that sPLA$_2$-dependent AA release was blocked by cPLA$_2$ inhibitors and restored by supplementation of exogenous AA in fibroblasts, mast cells, and macrophages (3, 9, 13), and that cotransfection of cPLA$_2$ and sPLA$_2$-IIA augments

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Abbreviations used in this paper: PLA$_2$, phospholipase A$_2$; sPLA$_2$, secretory PLA$_2$; cPLA$_2$, cytosolic PLA$_2$; AA, arachidonic acid; COX, cyclo-oxygenase; CDC, cyto-namyl-3,4-dihydroxy-cyanocinnamate; ETYA, 5,8,11,14-eicosatetraynoic acid; LOX, lipoxygenase; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; NDGA, nordihydroguaiaretic acid; HEK, human embryonic kidney; LysPC, lysophosphatidylycholine; AADCF$_2$, arachidonyl trifluoromethyl ketone; t-BuOOH, tert-butylhydroperoxide; BHT, butylated hydroxytoluene; PPAR, peroxisome proliferator-activated receptor.
AA release in a synergistic manner in human embryonic kidney (HEK) 293 cells (3, 20). In addition, we (12) and others (13, 16) have obtained complementary results suggesting that cPLA₂ is required for the induction of sPLA₂-IIA and sPLA₂-V expression in fibroblasts and macrophages, respectively, at the transcriptional level. However, the factor(s) that intermediates between cPLA₂ and sPLA₂ has remained unclear.

In the present study we show that 12/15-lipoxygenase (LOX), a dual specificity LOX isozyme that produces 12- and 15-hydroperoxyeicosatetraenoic acids (HPETEs) from AA substrate and directly oxidizes membrane lipids (21–23), lies downstream of cPLA₂, contributing to the stimulation of sPLA₂-IIA gene transcription. Moreover, 12/15-LOX-mediated lipid oxidation facilitates sPLA₂-IIA-mediated membrane hydrolysis, leading to AA release. Our results have revealed unexplored functional cross-talk between the constitutive cPLA₂-12/15-LOX and the inducible sPLA₂-IIA-COX-2 pathways.

Materials and Methods

Materials

Mouse and human IL-1β and human TNF-α were purchased from Genzyme (Cambridge, MA). Rabbit antisera to mouse COX-1 was provided by W. L. Smith (Michigan State University, East Lansing, MI), and mouse COX-2 cDNA and the COX-2 inhibitor NS-399 were provided by J. Trzasko (Merck, Kenilworth, NJ). Rabbit antisera to mouse COX-2, human 12/15-LOX cDNA, AA, 12-s-HPETE, and the PGE₂ enzyme immunnoassay kit were purchased from Cayman Chemical (Ann Arbor, MI). The LOX inhibitors, including nordihydroguaiaretic acid (NDGA; general LOX inhibitor), AA-861 (5-LOX inhibitor), cinnamonyl-3,4-dihydroxy-α-cyclohexanecinnamate (CDC; 12-LOX inhibitor) and 5,8,11,14-eicosatetraynoic acid (ETYA; 15-LOX inhibitor), were purchased from Biomol (Plymouth Meeting, PA). The cDNAs for rat sPLA₂-IIA (24) and porcine leukocyte-type 12-LOX (12/15-LOX) (25) and rabbit polyclonal Ab against rat sPLA₂-IIA (26) were prepared as described previously. The cPLA₂ inhibitor arachidonyl trifluoromethyl ketone (AAOCOF₃) was purchased from Calbiochem (La Jolla, CA). Aspirin, n-propyl gallate, butylated hydroxytoluene (BHT), 1-oleoyl-lysophosphatidylcholine (LysoPC), and tert-butyldihydroperoxide (t-BuOOH) were purchased from Sigma (St. Louis, MO). Lipofectamine Plus reagent, Opti-MEM medium, and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA). Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were purchased from BD PharMingen (San Diego, CA). nordihydroguaiaretic acid (NDGA; general LOX inhibitor), AA-861 (5-LOX inhibitor), cinnamonyl-3,4-dihydroxy-α-cyclohexanecinnamate (CDC; 12-LOX inhibitor) and 5,8,11,14-eicosatetraynoic acid (ETYA; 15-LOX inhibitor), were purchased from Biomol (Plymouth Meeting, PA). The cDNAs for rat sPLA₂-IIA (24) and porcine leukocyte-type 12-LOX (12/15-LOX) (25) and rabbit polyclonal Ab against rat sPLA₂-IIA (26) were prepared as described previously. The cPLA₂ inhibitor arachidonyl trifluoromethyl ketone (AAOCOF₃) was purchased from Calbiochem (La Jolla, CA). Aspirin, n-propyl gallate, butylated hydroxytoluene (BHT), 1-oleoyl-lysophosphatidylcholine (LysoPC), and tert-butyldihydroperoxide (t-BuOOH) were purchased from Sigma (St. Louis, MO). Lipofectamine Plus reagent, Opti-MEM medium, and TRIzol reagent were purchased from Life Technologies (Gaithersburg, MD). FITC-conjugated goat anti-rabbit IgG Ab was purchased from Zymed (South San Francisco, CA). 32P)dCTP (NEN Life Science Products) by random priming was obtained from New England Nuclear (Boston, MA).

Activation of 3Y1 cells

Rat fibroblastic 3Y1 cells were a gift from Dr. Y. Uehara (National Institute of Infectious Disease, Tokyo, Japan). The cells were maintained in culture medium composed of DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) FCS, penicillin/streptomycin (100 U/ml and 100 µg/ml, respectively; Flow Laboratories, Rockville, MD), and 2 mM glutamine (Life Technologies) at 37°C in a CO₂ incubator flushed with 5% CO₂ in humidified air. The media of 3Y1 cells that had attained 60–80% confluence in six-well plates (Iwaki Glass, Tokyo, Japan) were replaced with 2 ml of DMEM supplemented with 2% FCS. After culture for 24 h, 1 ng/ml mouse IL-1β and 100 U/ml human TNF-α were added to the cultures to assess the delayed PGE₂ biosynthetic response. The PGE₂ released into the culture medium was determined by enzyme immunnoassay. For the RNA blot analysis, TRIzol was directly added to the cell monolayer.

Activation of 293 cells

Culture of HEK 293 cells (Human Science Research Resources Bank, Osaka, Japan) and establishment of stable transfectants expressing mouse sPLA₂-IIA, rat sPLA₂-V, and mouse cPLA₂ were detailed in our previous papers (3, 4). The cells (5 × 10⁵ cells in 1 ml of culture medium) were seeded into 24-well plates. To assess AA release, 0.1 µCi/ml of [3H]AA (Amersham, Arlington Heights, IL) was added to the cells on day 3 when they had nearly reached confluence, and culture was continued for another day. After three washes with fresh medium, 250 µl of RPMI 1640 with or without A23187 or human IL-1β was added to each well, and the amount of free [3H]AA released into the supernatant during culture (30 min with A23187 and 4 h with IL-1β) was measured. The percent release of AA was calculated using the formula [(S–P) × 100, where S and P are the radioactivities measured in equal portions of the supernatant and cell pellet, respectively.

RNA blotting

All procedures were performed as described previously (14). Briefly, equal amounts (5 µg) of total RNA, purified using TRizol reagent, were applied to each lane of 1% (w/v) formaldehyde–agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore, Bedford, MA). The resulting blots were then sequentially probed with sPLA₂-IIA, COX-2, and GAPDH (Clontech, Palo Alto, CA) cDNA probes that had been labeled with [32P]dCTP (NEN Life Science Products) by random priming (Takara Biomedical, Otsu, Japan). All hybridizations were conducted at 42°C overnight in a solution comprising 50% (v/v) formamide, 0.75 M NaCl, 75 mM sodium citrate, 0.1% (w/v) SDS, 1 mM EDTA, 10 mM sodium phosphate (pH 6.8), 5 × Denhardt’s solution (Nacalai Tesque, Kyoto, Japan), 10% (w/v) dextran sulfate (Sigma), and 100 µg/ml salmon sperm DNA (Sigma).

RT-PCR

Synthesis of cDNA was performed using 1 µg of total RNA and AMV reverse transcriptase, according to the manufacturer’s instructions supplied with the RNA PCR kit (AMV, version 2.1, Takara Biomedical). Subsequent amplifications of the partial cDNA encoding LOX isozymes were performed using 1 µl of the reverse transcribed mixture as a template with specific oligonucleotide primers (Greiner Japan, Tokyo, Japan) as follows: rat 5-LOX: sense, 5'-CTT CCT ACA CTG TCA CCG TAG-3'; and antisense, 5'-GTC CAC TCC CTT TTC ACT ATC-3'; rat 12/15-LOX: sense, 5'-CCA GTA GAA CCA ATC CAG CGT-3'; and antisense, 5'-TTG ATG CTT TTC CTG GAC-3'. The expected sizes of the PCR products for rat 5-LOX and rat 12/15-LOX were 523 and 563 bp, respectively. The PCR mixtures were subjected to 30 cycles of amplification by
denaturation (30 s at 94°C), annealing (30 s at 60°C), and elongation (1 min at 72°C). The PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide. The gels were further subjected to Southern blot hybridization using specific LOX probes as required for the experiments.

Transfection of 12/15-LOX cDNA
Porcine 12/15-LOX cDNA was inserted into a mammalian expression vector pcDNA3.1 at the XhoI site and transfected into rat fibroblastic 3Y1 cells and HEK 293 cells using Lipofectamine Plus reagent according to the manufacturer’s instructions. Briefly, 1 μg of 12/15-LOX cDNA was mixed with 6 μl of Lipofectamine Plus reagent in 200 μl of Opti-MEM medium, incubated for 30 min at room temperature, and then added to cells that had attained 70% confluence in six-well dishes (Iwaki Glass) containing 1 ml of Opti-MEM. After incubation for 3 h, the medium was replaced with 2 ml of fresh culture medium. After overnight culture, the medium was replaced again with 2 ml of fresh medium. To obtain stable transfectants, the cells were harvested 3 days after transfection and were cloned by limiting dilution in 96-well plates in culture medium supplemented with 800 μg/ml hygromycin (Invitrogen) to establish stable transformants expressing both sPLA₂-IIA and 12/15-LOX.

Measurement of 12/15-LOX activity
The lysate was incubated with 0.1 μCi of [14C]AA for 10 min at 24°C in 100 μl of PBS in the presence or the absence of inhibitors. The reaction was terminated by addition of 0.3 ml of diethyl ether/methanol/1 M citric acid (30/4/1, v/v). The extracts were separated by TLC with a solvent system of diethyl ether/petroleum ether/acetic acid (85/15/0.1, v/v) at 20°C (25). Distribution of radioactivities on the TLC plates was detected by BAS2000 imaging analyzer (Fujix, Tokyo, Japan). The positions of [14C]AA, 12-HETE, and 15-HETE were determined by comparing their Rf values with those of authentic standards (Cayman Chemical).

Results and Discussion
12/15-LOX plays a role in inducing sPLA₂-IIA expression
When 3Y1 cells were treated with IL-1β and TNF-α, delayed PGE₂ generation occurred over 48 h, which was regulated by functional coupling of two inducible enzymes, sPLA₂-IIA and COX-2 (12). AACOCF₃, a cPLA₂ inhibitor, attenuated the expression of sPLA₂-IIA without affecting that of COX-2 (Fig. 1A), accompanied by reduction of PGE₂ generation (12). Addition of AA and LysoPC modestly, but significantly, restored this AACOCF₃ suppression of sPLA₂-IIA induction (Fig. 1A), suggesting that cPLA₂-derived AA, LysoPC, or their metabolites play some role in the induction of sPLA₂-IIA. Cytokine-induced sPLA₂-IIA expression was also markedly reduced by NDGA (Fig. 1B), which suppresses several LOX isozymes (27), but not by COX inhibitors such as

![Figure 2](image_url)

**FIGURE 2.** Detection of LOX isozymes in 3Y1 cells. Expression of 5-LOX and 12/15-LOX in 3Y1 cells and rat peritoneal macrophages was assessed by RT-PCR. Lanes 1 and 3, 5-LOX; lanes 2 and 4, 12/15-LOX. A representative result of two independent experiments is shown.

![Figure 3](image_url)

**FIGURE 3.** Establishment of 12/15-LOX-overexpressing 3Y1 cells. A, 12/15-LOX activity (upper panel) and expression of its transcripts (bottom panel) in 12/15-LOX-overexpressing cells (right lane) and control cells (left lane) were assessed. B, The effects of antioxidants (20 μM NDGA, 50 μM BHT, or 50 μM n-propyl gallate) on 12/15-LOX activity in the 12/15-LOX-overexpressing cells were assessed. Representative results of three independent experiments are shown.
aspirin and NS-398 (12). As NDGA is an antioxidant, the effects of other general antioxidants (n-propyl gallate and BHT) on sPLA2-IIA expression were next examined. As shown in Fig. 1C, n-propyl gallate, but not BHT, markedly suppressed cytokine induction of sPLA2-IIA (as described below, this suppressive effect was correlated with potency to inhibit 12/15-LOX). Consistent with these observations, PGE2 generation 24 h after stimulation with IL-1β/TNF-α was reduced by >80% by either NDGA or n-propyl gallate.

To clarify which LOX isozymes were expressed in 3Y1 cells, RT-PCR was conducted using specific primers for 5-LOX and 12/15-LOX. After 30 cycles of amplification, under which conditions these two LOX cDNAs were amplified from the control cells (rat peritoneal macrophages), a signal for 12/15-LOX, but not for 5-LOX, was obtained from 3Y1 cells (Fig. 2).

To determine whether 12/15-LOX indeed plays a role in the induction of sPLA2-IIA, 12/15-LOX cDNA was subcloned into the mammalian expression vector pcDNA3.1 and was transfected into 3Y1 cells to establish drug-resistant transfectants stably overexpressing 12/15-LOX. Functional expression of 12/15-LOX in the transfectants was confirmed by its activity (Fig. 3A, top), which was assessed by the conversion of [14C]AA to [14C]12-HETE using cell lysates, and its mRNA expression, which was assessed by RNA blotting (Fig. 3A, bottom). The expression level of 12/15-LOX in the transfectants was estimated to be ~20 times higher than that in control cells. NDGA and n-propyl gallate each markedly decreased 12/15-LOX activity, whereas BHT showed little effect (Fig. 3B). Thus, the antioxidants that decreased sPLA2-IIA expression (Fig. 1B) had the capacity to inhibit 12/15-LOX activity (Fig. 3B).

When the 12/15-LOX-overexpressing cells were cultured for 24 h with IL-1β and TNF-α alone or in combination, the induction of sPLA2-IIA mRNA was markedly increased relative to that of replicate mock-transfected cells, and these two cytokines acted in synergy (Fig. 4A). On the basis of quantification of sPLA2-IIA expression, it increased ~16- and 120-fold in IL-1β/TNF-α-stimulated control and 12/15-LOX-transfected cells, respectively, over that in unstimulated control cells. Low, but significant, sPLA2-IIA expression was already detectable in 12/15-LOX-overexpressing, but not in control, cells even without cytokine stimulation. In contrast, cytokine-induced expression of COX-2 mRNA was increased only minimally (Fig. 4A), and constitutive expression of cPLA2 and COX-1 was unchanged (data not shown), following overexpression of 12/15-LOX. IL-1β/TNF-α-stimulated PGE2 generation reached ~1.8 ± 0.3 (mean ± SE; n = 3; p < 0.05 vs unstimulated control cells) and 17.5 ± 2.8 (n = 3; p < 0.05 vs unstimulated 12/15-LOX-transfected cells and IL-1β/TNF-α-stimulated control cells) ng/ml PGE2 in mock- and 12/15-LOX-transfected cells, respectively. Thus, increased sPLA2-IIA expression in IL-1β/TNF-α-stimulated 12/15-LOX-transfected cells appeared to be roughly correlated with increased PGE2 biosynthesis (Fig. 4B). Furthermore, 12/15-LOX-enhanced sPLA2-IIA expression (Fig. 4C) and PGE2 generation (Fig. 4D) were suppressed dramatically by AACOCF3 or NDGA almost in parallel, whereas the expression without IL-1β, TNF-α, or their combination, as assessed by RNA blotting, B. PGE2 generation by cells stimulated for 24 h with IL-1β/TNF-α was measured. C and D. Effects of AACOCF3 (10 μM) and NDGA (20 μM) on sPLA2-IIA and COX-2 protein expression, as assessed by immunoblotting (C), and PGE2 generation (D) 24 h after stimulation with IL-1β/TNF-α. Representative results of three independent experiments are shown. In B and D, the results are expressed as the fold increase in PGE2 relative to that in unstimulated control cells.
of COX-2 was unaffected by these inhibitors (Fig. 4C). The addition of 10 μM AACOCF₃ also reduced the production of 12-HETE in 12/15-LOX-transfected cells by 80% (data not shown), suggesting a role of endogenous cPLA₂ in supplying AA to 12/15-LOX.

Immunocytostaining using an anti-sPLA₂-IIA Ab revealed that sPLA₂-IIA protein, which resided in the dot-like compartments and the perinuclear region, was significantly increased after cytokine stimulation in the mock-transfected cells (Fig. 5, A and B). A previous study has shown by colocalization that these compartments are caveolae vesicles (19). This particular sPLA₂-IIA signal was markedly elevated in the cells overexpressing 12/15-LOX, in which the sPLA₂-IIA signal was already detectable under unstimulated conditions (Fig. 5C) and was increased markedly after cytokine stimulation (Fig. 5D). Colocalization of sPLA₂-IIA, an AA donor, and COX-2, an AA acceptor, in the same perinuclear area may facilitate their efficient coupling during the delayed PGE₂-biosynthetic response.

These results together with our previous finding that cPLA₂ is activated immediately after IL-1β/TNF-α stimulation of 3Y1 cells (12) and the present observation that cPLA₂ and 12/15-LOX are functionally linked (also see Fig. 7D) suggest that the cPLA₂-12/15-LOX pathway precedes the sPLA₂-IIA/COX-2 pathway, contributing to up-regulation of cytokine-induced sPLA₂-IIA expression and accompanying PGE₂ biosynthesis. This regulatory mechanism is not peculiar in 3Y1 cells, because IL-1β induction of sPLA₂-IIA gene expression in rat aortic smooth muscle cells is also NDGA sensitive (28). As the ligands for the nuclear receptor peroxisome proliferator-activated receptor (PPARγ) are fatty acids and their oxidative derivatives, including 12/15-LOX metabolites (29), and the sPLA₂-IIA gene promoter region contains a motif.
showing homology with the PPAR-binding sites (positions −160 to −133) (28), we conducted EMSA to address the involvement of this putative PPAR binding site in 12/15-LOX-mediated hyperinduction of sPLA2-IIA. However, activation of PPARγ in cytokine-stimulated 3Y1 cells was unchanged regardless of 12/15-LOX overexpression (data not shown). Moreover, activation of NF-κB, which is crucial for sPLA2-IIA induction by cytokines (30), was also unaffected by 12/15-LOX overexpression (data not shown). Thus, activation of these transcription factors alone appears to be insufficient to fully explain the 12/15-LOX-mediated induction of sPLA2-IIA. Whether some other regulatory elements are responsible for 12/15-LOX induction of sPLA2-IIA is now under investigation.

12/15-LOX plays a role in sensitizing cellular membranes to sPLA2

In view of the fact that AA release by sPLA2s from activated cells often occurs even without accompanying de novo induction of its expression, it has been suggested that membrane rearrangement during cell activation also represents a crucial event leading to efficient sPLA2-mediated membrane phospholipid hydrolysis (7, 18). Among the models proposed to date (3, 9, 13, 16, 17–20, 31–34), several studies have shown that treatment of cells with agents provoking the oxidative response makes the membranes susceptible to sPLA2-IIA (34–36).

To verify that membrane oxidation indeed induces such an alteration, we exploited HEK293 transfectants that stably express sPLA2-IIA, 12/15-LOX, or both were prelabeled with [3H]AA, washed, and then stimulated with the indicated concentrations of A23187 for 30 min to assess [3H]AA release. Expression of sPLA2-IIA and 12/15-LOX, as assessed by RNA blotting, is shown in the top panel. D. Functional coupling between PLA2 and 12/15-LOX. 293 cells transfected with 12/15-LOX alone or in combination with either cPLA2 or sPLA2-IIA were prelabeled with [14C]AA, stimulated for 4 h with 1 ng/ml IL-1β, and subjected to TLC analysis to assess the production of [14C]12/15-HETE. Production of [14C]12/15-HETE was undetectable when these cells were cultured without stimulus. Representative results of three independent experiments are shown.
to a similar extent (data not shown). Our previous study has shown that these 12/15-LOX inhibitors suppress sPLA2-IIA induction in 3Y1 cells (12). Thus, these results suggest that endogenous 12/15-LOX may participate in IL-1β-induced activation of sPLA2-IIA-mediated membrane hydrolysis. In support of this idea, supplementation of the sPLA2-IIA-expressing, but not parental, cells with 12-HPETE, a primary product of 12/15-LOX, resulted in a significant increase in AA release even without stimulation (Fig. 7B). Moreover, the cells cotransfected with sPLA2-IIA and 12/15-LOX released more AA than the cells expressing sPLA2-IIA alone following A23187 stimulation, even though the sPLA2-IIA expression levels in both clones were comparable, and AA release by the clone expressing 12/15-LOX alone did not increase significantly (Fig. 7C). More importantly, the clones coexpressing sPLA2-IIA and 12/15-LOX than in replicate cells expressing 12/15-LOX alone (Fig. 7D). These results suggest that 12/15-HPETE or some other oxidative lipid metabolite produced by 12/15-LOX during cell activation may cause membrane perturbation, leading to accelerated AA release by sPLA2s.

The finding that AA released by either cPLA2 or sPLA2-IIA is efficiently converted by 12/15-LOX to 12/15-HETE (Fig. 7D) implies the presence of an autocatalytic activation loop in the sPLA2-IIA-dependent response. Thus, cPLA2 activation immediately after cell activation leads to the production of 12/15-LOX metabolites, which, in turn, trigger sPLA2-IIA-mediated AA release. The AA thus released is further oxidized by 12/15-LOX, thereby amplifying the lipid oxidation-directed membrane rearrangement process and eventually leading to sustained activation of sPLA2-IIA. As the cellular actions of sPLA2-V are very similar (3, 4, 12, 13, 15–17), if not identical (38), to those of sPLA2-IIA, we speculate that cellular functions of the group II subfamily of sPLA2s may be generally influenced by 12/15-LOX.

Conclusion: on the mechanisms by which signaling sPLA2s are activated

Although several studies have suggested that cPLA2, a well-recognized initiator of AA metabolism, precedes the prolonged PG biosynthetic response mediated by the two related heparin-binding signaling sPLA2s, sPLA2-IIA and sPLA2-V (3, 9, 13, 16, 20), the mechanisms by which cPLA2 modulates the functions of sPLA2s have remained unclear. In search of a regulatory molecule that links cPLA2 and sPLA2, we have found that 12/15-LOX, a LOX isozyme that oxygenates free AA as well as esterified polysaturated fatty acids in the cellular membranes (21–23), may play a pivotal role in the regulation of signaling sPLA2s. Although overexpression experiments indicate only the possibility that a 12/15-LOX-sPLA2 pathway could operate in parental cells, they are the best recourse open to us in view of the nature of LOX inhibitors. 12/15-LOX regulation of sPLA2 occurs in two ways: 1) it up-regulates the induction of sPLA2-IIA expression; and 2) it accelerates sPLA2-IIA-mediated membrane phospholipid hydrolysis, probably through oxidizing and thereby sensitizing the cellular membranes. This scenario may also occur in immunologically relevant cells such as macrophages, in which 12/15-LOX is expressed (21–23), and sPLA2 expression and function require prior activation of cPLA2 (8, 13). Thus, our present results have revealed a functional array of enzymes in separate arms of the AA cascade, the LOX and COX pathways, and substantiated a long-held hypothesis that membrane perturbation following cell activation is a prerequisite for sPLA2s to exert their proper actions on cells. Moreover, our results provide new insight into the biological importance of LOX-directed lipid oxidation signaling in regulating the expression and function of particular lipid-metabolizing enzymes and imply that the intracellular redox state affects the AA metabolic activity of cells. Thus, 12/15-LOX inhibitors would be a potential target for the development of therapeutic and prophylactic drugs for diseases or tissue disorders in which sPLA2s are significantly involved.

Finally, our current studies have led to the identification of several factors that affect the cellular actions of sPLA2-IIA. Glypican delivers endogenous heparin-binding sPLA2s into caveolar signalosomes of activated cells, a process crucial for their efficient functional coupling with COX in the PG biosynthetic response (19). Alteration of plasma membrane asymmetry by phospholipid scramblase enhances AA release by signaling sPLA2s (17). The M-type sPLA2 receptor appears to mediate some biological actions of sPLA2-IIA, at least in some animal species (39). Here we have shown that the cPLA2-12/15-LOX pathway is a key regulatory step for sPLA2 functions. Given that sensitivity to sPLA2s differs according to cell type, it is likely that cells showing limited expression of either of these modifying factors may be refractory to the actions of sPLA2s even in the presence of the appropriate stimuli.

References


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