Polyunsaturated fatty acids potentiate interleukin-1-stimulated arachidonic acid release by cells overexpressing type IIA secretory phospholipase A_2

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Received 16 April 1999; received in revised form 11 May 1999

Abstract By analyzing human embryonic kidney 293 cell transfectants stably overexpressing various types of phospholipase A_2 (PLA_2), we have shown that polyunsaturated fatty acids (PUFAs) preferentially activate type IIA secretory PLA_2 ($sPLA_2$-IIA)-mediated arachidonic acid (AA) release from interleukin-1 (IL-1)-stimulated cells. When 293 cells prelabeled with [^{3}H]AA were incubated with exogenous PUFAs in the presence of IL-1 and serum, there was a significant increase in [^{3}H]AA release (in the order AA > linoleic acid > oleic acid), which was augmented markedly by $sPLA_2$-IIA and modestly by type IV cytosolic PLA_2 ($cPLA_2$), but only minimally by type VI Ca^{2+}-independent PLA_2, overexpression. Transfection of $cPLA_2$ into $sPLA_2$-IIA-expressing cells produced a synergistic increase in IL-1-dependent [^{3}H]AA release and subsequent prostaglandin production. Our results support the proposal that prior production of AA by $cPLA_2$ in cytokine-stimulated cells destabilizes the cellular membranes, thereby rendering them more susceptible to subsequent hydrolysis by $sPLA_2$-IIA.

Key words: Phospholipase A_2; Arachidonic acid; Prostaglandin

1. Introduction

The phospholipase A_2 (PLA_2) family comprises a growing number of lipolytic enzymes that play pivotal roles in phospholipid remodeling, host defense, digestion, and signal transduction via the production of lipid mediators such as arachidonic acid (AA)-derived eicosanoids, lysophosphatidic acid and platelet-activating factor. More than 10 PLA_2 isozymes are found in mammalian cells, and these can be subdivided into several classes based upon their primary structures, enzymatic properties, subcellular distributions, and functions [1]. Cytosolic Ca^{2+}-dependent PLA_2 (type IV $cPLA_2$), which undergoes translocation from the cytosol to the perinuclear membrane in response to submicromolar increases in cytoplasmic Ca^{2+} levels and is activated by mitogen-activated protein kinase-directed phosphorylation, is thought to be essential for lipid mediator generation in activated cells [2]. Cytosolic Ca^{2+}-independent PLA_2 (type VI $iPLA_2$) forms a multimeric complex and is implicated mainly in the phospholipid remodeling pathway [3]. The secretory PLA_2 ($sPLA_2$) group includes five isozymes (IB, IIA, IIC, V, and X), all of which require millimolar Ca^{2+} for enzymatic activity in vitro [4]. The expression of $sPLA_2$-IIA and attendant delayed prostaglandin (PG) generation are often strongly induced in several types of cell after proinflammatory stimuli such as interleukin-1 (IL-1) [5,6]. Our recent analysis using transfectants overexpressing each PLA_2 confirmed that $cPLA_2$ and the two heparin-binding $sPLA_2$ isozymes, $sPLA_2$-IIA and $sPLA_2$-V, are 'signaling' PLA_2s that contribute to PG biosynthesis via either of the cyclooxygenase (COX) isozymes, COX-1 or COX-2, depending upon the phase of cell activation, while $iPLA_2$ is involved in phospholipid remodeling rather than PG generation [7–9]. $sPLA_2$-IIA and $sPLA_2$-V, which are functionally redundant, are capable of augmenting IL-1-stimulated AA metabolism via the transcellular route, a property not shared by intracellular $cPLA_2$ [9]. However, the way in which AA metabolism is regulated by particular $sPLA_2$ isoforms remains obscure.

The observation that AA release by $sPLA_2$-IIA generally occurs in agonist-stimulated, but not quiescent, cells suggests that membrane perturbation during cell activation is a crucial event leading to $sPLA_2$-mediated membrane phospholipid hydrolysis [10]. Several previous studies have shown that polyunsaturated fatty acids (PUFAs) or their oxidative products contribute to enhanced $sPLA_2$-IIA-mediated membrane hydrolysis [11–13]. However, results obtained from in vitro studies often vary according to the assay methods employed, and may not always reflect events occurring in vivo. In addition, the utilization of relatively non-specific PLA_2 inhibitors in these previous studies may limit generalizations based on interpretation of their data.

To verify whether PUFAs do indeed affect the functions of PLA_2s in mammalian cells, we utilized HEK293 cell transfectants overexpressing each $cPLA_2$ isozyme [8,9]. Here we provide unequivocal evidence that PUFAs, AA in particular, activate AA release mediated by $sPLA_2$-IIA in preference to $cPLA_2$ and $iPLA_2$ in IL-1-stimulated HEK293 transfectants. Our observations appear to be in line with the hypothesis proposed by Balsinde et al. [14,15] that prior activation of $cPLA_2$ is required for the adequate functioning of $sPLA_2$ in activated cells.

2. Materials and methods

2.1. Materials

AA, PGE_2, 5-hydroxyeicosatetraenoic acid (HETE), 12-HETE, and methyl arachidonylfluorophosphate (MAFP) were purchased from

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Abbreviations: PLA_2, phospholipase A_2; $sPLA_2$-IIA, type IIA secretory PLA_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; COX, cyclooxygenase; AA, arachidonic acid; PUFA, polyunsaturated fatty acid; PG, prostaglandin; IL-1, interleukin-1; FCS, fetal calf serum; HETE, hydroxyeicosatetraenoic acid; MAFP, methyl arachidonylfluorophosphate

0014-5793/99/$20.00
PII: S0014-5793(99)00702-4

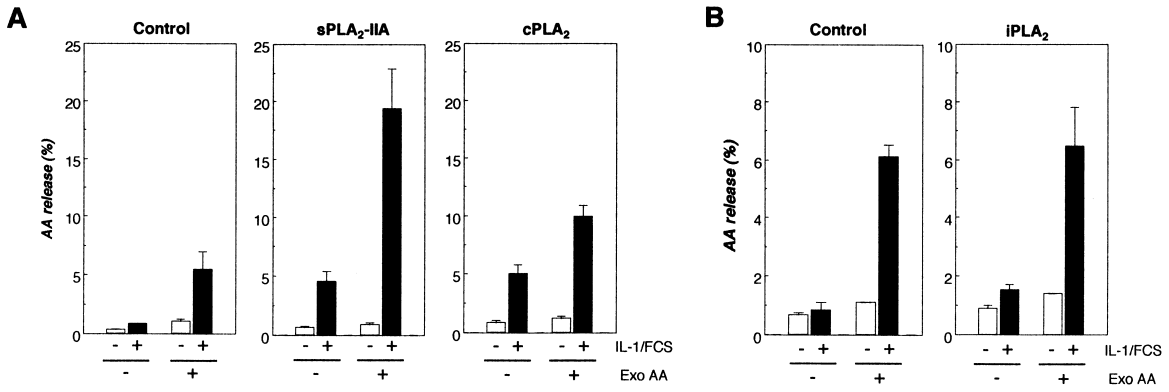

Fig. 1. Effects of exogenous AA on endogenous AA release by HEK293 transfectants. Control cells, transfectants expressing $sPLA_2$-IIA or $cPLA_2$ (A), and transfectants expressing $iPLA_2$ (B) were prelabeled with [^{3}H]AA, and then cultured for 4 h with (solid bars) or without (open bars) IL-1/FCS stimulation in the presence or absence of 50 μM AA. Values represent means ± S.E.M. ($n = 3$).

Cayman Chemical. Stearic acid, oleic acid and linoleic acid were obtained from Sigma. Human IL-1β was purchased from Genzyme.

2.2. Cell culture

HEK293 cells were obtained from Health Science Research Resources Bank and maintained in RPMI 1640 medium (Nissui Pharmaceutical) supplemented with 10% fetal calf serum (FCS). Transformants of 293 cells that stably expressed $cPLA_2$, $sPLA_2$-IIA and its mutant G30S, and $iPLA_2$ were established as described previously [8,9]. For the co-transfection experiments, a 293 transformant expressing $sPLA_2$-IIA was subjected to a second transfection with $cPLA_2$, which had previously been subcloned into pCDNA3.1/Zeo(+) (Invitrogen), using Lipofectamine Plus reagent (Life Technologies). The cells were then seeded into 96-well plates and cloned by culture in the presence of 50 μg/ml zeocin (Invitrogen) in order to establish stable transformants overexpressing both PLA_2s. The expression of each enzyme was confirmed by RNA blotting.

2.3. RNA blotting

Approximately equal amounts (~10 μg) of the total RNAs obtained from the transfected cells were applied to individual lanes on 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with their respective cDNA probes, which had been labeled with [^{32}P]dCTP (Amersham) by random priming (Takara Shuzo). All hybridizations were carried out as described previously [7,8].

2.4. Cell activation

Cells (5×10^4 in 1 ml of culture medium) were seeded into individual wells on 24-well plates. In order to assess AA release, 0.1 μCi/ml [^{3}H]AA (Amersham) was added to the cells in each well 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 1 ng/ml IL-1β and 10% FCS was added to each well and the [^{3}H]AA released into the supernatant during culture was measured. The percentage release of [^{3}H]AA was calculated using the formula $[S/(S+P)] \times 100$, where S and P are the radioactivities measured in equal portions of the supernatant and cell pellet, respectively. This procedure is described in detail in our previous reports [8,9].

3. Results

3.1. Exogenous PUFAs preferentially activate $sPLA_2$-IIA-mediated AA release

In the present study, we used HEK293 cell stable transfectants that expressed $sPLA_2$-IIA, $cPLA_2$ and $iPLA_2$, and confirmed their expression by Northern and/or Western blotting [8,9]. Parental 293 cells expressed only a trace of $cPLA_2$, and $sPLA_2$ and $iPLA_2$ were undetectable (see Fig. 4A) [8,9]. When 293 transfectants expressing $sPLA_2$-IIA or $cPLA_2$ were prelabeled with [^{3}H]AA and cultured for 4 h with IL-1 in the culture medium, there was a marked increase in [^{3}H]AA release, reaching 4–5%, from both transfectants, whereas release from replicate parental cells increased only minimally (Fig. 1A). These results confirmed our previous findings that both $sPLA_2$-IIA and $cPLA_2$ are capable of mediating IL-1/FCS-induced delayed AA metabolism [7–9]. We found that when AA was added exogenously to the control cells, IL-1/FCS-dependent, but not basal, release of endogenous [^{3}H]AA was significantly augmented (Fig. 1A). Furthermore, IL-1/FCS-dependent [^{3}H]AA release by $sPLA_2$-IIA-expressing cells was dramatically increased by exogenous AA, reaching nearly 20% release. The catalytically inactive $sPLA_2$-IIA mutant G30S [8] did not elicit enhanced [^{3}H]AA release by exogenous AA (data not shown), implying that the enzymatic activity of $sPLA_2$-IIA is essential for this event. A significant increase in IL-1/FCS-dependent [^{3}H]AA release caused by exogenous AA was also observed in $cPLA_2$-transfected cells, although this increment was smaller than that in $sPLA_2$-IIA-expressing cells

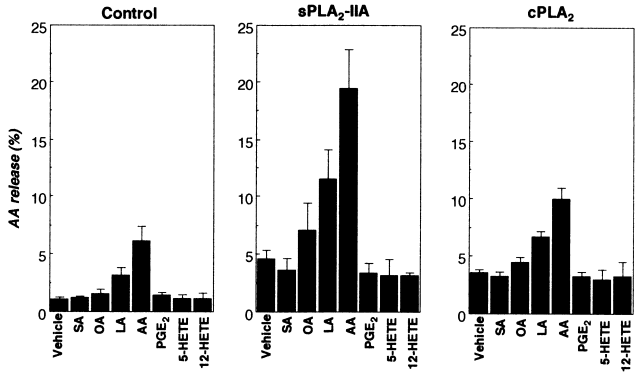

Fig. 2. Effects of various fatty acids and AA metabolites on endogenous AA release by HEK293 transfectants. Control cells and transfectants expressing $sPLA_2$-IIA or $cPLA_2$, prelabeled with [^{3}H]AA, were incubated for 4 h with IL-1/FCS in the presence of 50 μM AA, linoleic acid (LA), oleic acid (OA) or stearic acid (SA), or with 500 ng/ml PGE_2, 5-HETE or 12-HETE. Values represent means ± S.E.M. ($n = 3$).

(Fig. 1A). Basal and IL-1/FCS-mediated [^{3}H]AA release approximately doubled after iPLA$_2$ transfection, as reported previously [8,9], but exogenous AA had no appreciable effect on [^{3}H]AA release by iPLA$_2$-expressing cells (Fig. 1B).

Assessments of fatty acid specificity showed that AA was the most potent enhancer of IL-1/FCS-stimulated [^{3}H]AA release, followed in order by linoleic acid and oleic acid, whereas stearic acid was virtually without effect (Fig. 2). sPLA$_2$-IIA augmented the exogenous PUFA-dependent incremental release of [^{3}H]AA more efficiently than cPLA$_2$ under experimental conditions in which both PLA$_2$s produced comparable [^{3}H]AA release in response to IL-1/FCS in the absence of PUFAs. Enhanced [^{3}H]AA release from parental 293 cells was evident at concentrations of exogenous AA more than 5 μM, and sPLA$_2$-IIA augmented release several-fold when compared with control cells at all AA concentrations examined (Fig. 3A). In sPLA$_2$-IIA transfectants, IL-1/FCS-stimulated [^{3}H]AA release increased to reach a plateau by 4–8 h, whereas it continued to increase throughout the culture period if exogenous AA was added further (Fig. 3B).

PGE$_2$, a major AA metabolite of the COX pathway, produced no appreciable effect on IL-1/FCS-induced [^{3}H]AA release, whether or not sPLA$_2$-IIA and cPLA$_2$ were introduced (Fig. 2). Moreover, the increase in [^{3}H]AA release caused by exogenous AA in control cells and sPLA$_2$-IIA-expressing cells was not appreciably suppressed by several COX inhibitors (data not shown), implying that prostanoids are not involved in this process. 5-HETE and 12-HETE, by-products of the lipoxygenase pathway, also failed to augment sPLA$_2$-IIA- and cPLA$_2$-mediated AA release (Fig. 2). Although we observed that nordihydroguaiaretic acid, a lipoxygenase-inhibiting antioxidant [16], significantly suppressed IL-1/FCS-induced [^{3}H]AA release in sPLA$_2$-IIA-expressing cells whether or not exogenous AA was added (Kuwata et al., unpublished observation), it remains unclear at present whether certain lipoxygenase metabolites (other than 5- and 12-HETEs) or other PUFA oxidative products participate in this pathway.

3.2. Coexpression of cPLA$_2$ and sPLA$_2$-IIA coordinately enhances AA release

The observation that exogenous PUFAs potently stimulate sPLA$_2$-IIA-mediated [^{3}H]AA release prompted us to ask whether the production of endogenous AA by cPLA$_2$ would affect the function of sPLA$_2$-IIA. To address this issue, we employed 293 transfectants coexpressing cPLA$_2$ and sPLA$_2$-IIA, and compared their AA-releasing and PGE$_2$-producing abilities with those of transfectants expressing each PLA$_2$ alone. The expression of each PLA$_2$ was verified by RNA blotting (Fig. 4A). IL-1-dependent release of [^{3}H]AA (Fig. 4B) and PGE$_2$ (Fig. 4C) by cells expressing cPLA$_2$ or sPLA$_2$-IIA alone was increased modestly relative to release by control cells, as we have recently reported [8,9]. Most importantly, coexpression of cPLA$_2$ and sPLA$_2$-IIA synergistically augmented [^{3}H]AA release (Fig. 4B) and PGE$_2$ production (Fig. 4C) in IL-1-stimulated cells. [^{3}H]AA release preceded PGE$_2$ generation, probably because the latter requires de novo induction of COX-2, which occurs after 1–4 h of IL-1 stimulation [8].

Taking these results together, it seems reasonable to consider that the AA produced endogenously by the action of cPLA$_2$ sensitized the cells, leading to increased sPLA$_2$-IIA-dependent AA release.

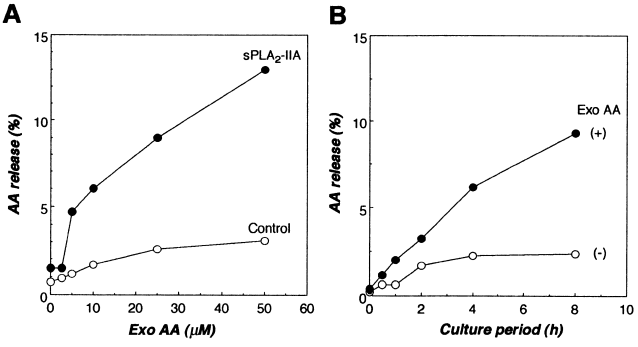

Fig. 3. Analyses of the effects of exogenous AA on sPLA$_2$-IIA-mediated AA release. A: AA dose response. sPLA$_2$-expressing (solid circles) and control (open circles) 293 cells, prelabeled with [^{3}H]AA, were incubated for 4 h with IL-1/FCS in the presence of the indicated concentrations of AA. B: Time course. sPLA$_2$-IIA-expressing 293 cells were cultured for the indicated periods with IL-1/FCS in the presence (solid circles) or absence (open circles) of 50 μM AA. A representative result of two reproducible experiments is shown.

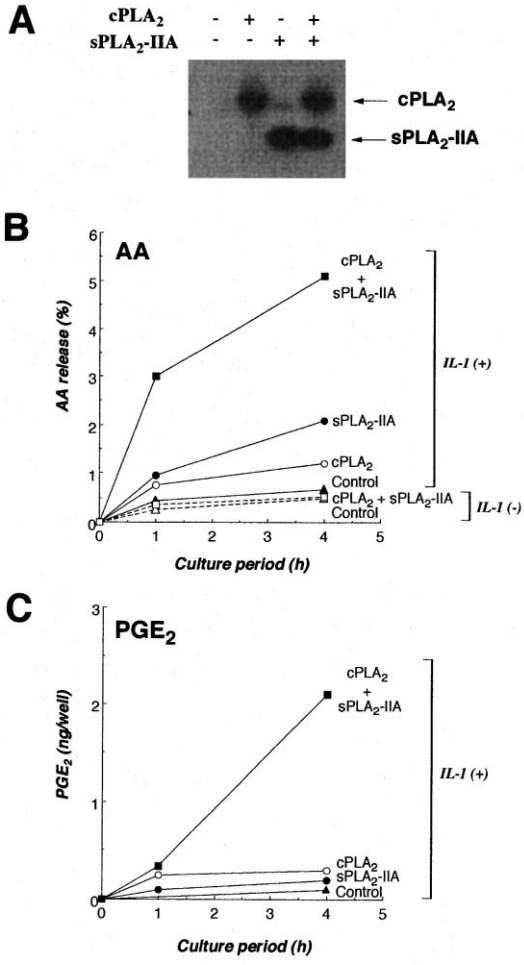

Fig. 4. Effects of cPLA$_2$ and sPLA$_2$-IIA coexpression on AA and PGE2 release. A: Expression of cPLA$_2$ and sPLA$_2$-IIA in HEK293 transfectants as assessed by RNA blotting. [^{3}H]AA release (B) and PGE2 generation (C) in each transfectant after culture for the indicated periods with or without IL-1. A representative result of three independent experiments is shown.

4. Discussion

P388The observation that AA release by $sPLA_2$-IIA generally occurs only in agonist-stimulated cells [10] prompted us to hypothesize that membrane rearrangement during cell activation is a crucial event leading to $sPLA_2$-mediated membrane phospholipid hydrolysis. In this model, the outer leaflet of the plasma membrane of quiescent cells, rich in PC and sphingomyelin, is fairly resistant to the enzymatic action of $sPLA_2$-IIA, and cell activation perturbs the plasma membrane, loosening the otherwise tightly packed membrane bilayer to be susceptible to this enzyme. Several possible explanations have been proposed to account for this phenomenon, among which the roles of PUFAs in eliciting changes in membrane structures have been demonstrated by several workers [11–15]. Balsinde et al. [14,15] have provided evidence that prior activation of $cPLA_2$ is necessary for $sPLA_2$ (in this case, the type V isozyme, a close homolog of $sPLA_2$-IIA) to exert its function in $P388D_1$ macrophage-like cells, and that supplementing these cells with exogenous AA restores the requirement of $cPLA_2$ for $sPLA_2$ activation. Robinson et al. [13] have reported that $sPLA_2$ and $cPLA_2$, but not $iPLA_2$, are activated by PUFAs in human neutrophils. Moreover, we have recently demonstrated that $sPLA_2$-IIA-dependent eicosanoid generation is sensitive to several $cPLA_2$ inhibitors [6,8].

In the present study, we have shown that $sPLA_2$-IIA-mediated AA release from intact cells is markedly potentiated by exogenous PUFAs, among which AA elicits the most potent effect. In contrast, AA release by $cPLA_2$ was enhanced only modestly, and release by $iPLA_2$ was unaffected, by exogenous PUFAs, indicating that the augmentative effect of PUFAs on endogenous AA release by intact cells is somewhat selective for $sPLA_2$-IIA. Of note, this PUFA effect was observed only in cells stimulated with IL-1/FCS. A likely explanation for this is that IL-1/FCS may initiate the signaling transduction pathway leading to $sPLA_2$-IIA-mediated membrane hydrolysis in particular cellular compartments, and PUFAs or their metabolites may accelerate this hydrolytic process by perturbing membrane bilayers which are attacked by $sPLA_2$-IIA. Importantly, the striking synergism between $cPLA_2$ and $sPLA_2$-IIA observed in cotransfectants supports the theory of a positive-feedback model, in which agonist-induced release of AA by $cPLA_2$ promotes further stimulation of $sPLA_2$-mediated membrane hydrolysis and subsequent production of PGE_2 via COX-2. Certain oxidative PUFA metabolites might be involved in this membrane sensitization process, since some oxidants have been shown to increase $sPLA_2$-IIA-mediated phospholipid hydrolysis in vesicles and intact cells [12,17]. This possibility is now under investigation.

A smaller but still significant increase in $[^3H]$AA release by PUFAs occurred in $cPLA_2$-expressing cells. This suggests that $cPLA_2$-mediated membrane hydrolysis is also facilitated by membrane perturbation caused by PUFAs. Alternatively, the ability of PUFAs to mobilize intracellular Ca^{2+} [18,19] and activate several protein kinases [20] might lead to the post-translational activation of $cPLA_2$. Given that $sPLA_2$ activates $cPLA_2$ in some cell types [21,22], a bidirectional interaction between $sPLA_2$ and $cPLA_2$ may contribute to the synergistic augmentation of the eicosanoid-biosynthetic response seen in the cotransfectants.

Acknowledgements: This work is supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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