Role of Group VIA Calcium-independent Phospholipase A₂ in Arachidonic Acid Release, Phospholipid Fatty Acid Incorporation, and Apoptosis in U937 Cells Responding to Hydrogen Peroxide*

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The abbreviations used are: PLA$_2$, phospholipase A$_2$; cPLA$_2$, cytosolic PLA$_2$; iPLA$_2$, Ca$^{2+}$-independent PLA$_2$; sPLA$_2$, secreted PLA$_2$; AA, arachidonic acid; BEL, bromoenol lactone; MAFP, methyl arachidonyl fluorophosphonate; lysoPC, lysophosphatidylcholine.
ABSTRACT

Group VIA calcium-independent phospholipase A₂ (iPLA₂) has been shown to play a major role in regulating basal phospholipid deacylation reactions in certain cell types. More recently, roles for this enzyme have also been suggested in the destruction of membrane phospholipid during apoptosis, and after oxidant injury. Proposed iPLA₂ roles have rested heavily on the use of bromoenol lactone as a selective iPLA₂ inhibitor, but this compound actually inhibits other enzymes and lipid pathways unrelated to PLA₂, which makes it difficult to define the contribution of iPLA₂ to specific functions. In previous work we pioneered the use of antisense technology to decrease cellular iPLA₂ activity as an alternative approach to study iPLA₂ functions. In the present study we have followed the opposite strategy and have prepared U937 cells that exhibit enhanced iPLA₂ activity by stably expressing a plasmid containing iPLA₂ cDNA. Compared with control cells, the iPLA₂-overexpressing U937 cells show elevated responses to hydrogen peroxide with regard to both arachidonic acid mobilization and incorporation of the fatty acid into phospholipids, thus providing additional evidence for the key role that iPLA₂ plays in these events. Long-term exposure of the cells to hydrogen peroxide results in cell death by apoptosis, and this process is accelerated in the iPLA₂-overexpressing cells. Increased phospholipid hydrolysis and fatty acid release also occur in these cells. Unexpectedly however, abrogation of U937 cell iPLA₂ activity by either methyl arachidonyl fluorophosphonate or an antisense oligonucleotide does not delay or decrease the extent of apoptosis induced by hydrogen peroxide. These results indicate that although iPLA₂-mediated phospholipid hydrolysis occurs during apoptosis, iPLA₂ may actually be dispensable for the apoptotic process to occur. Thus, beyond a mere destructive role, iPLA₂ may be playing other roles during apoptosis.
Phospholipase A₂s (PLA₂) constitute a diverse group of enzymes whose common feature is to hydrolyze the fatty acid at the sn-2 position of phospholipids. Several mammalian intracellular and extracellular PLA₂s have been characterized in recent years and classified into fourteen group types on the basis of sequence data [1, 2]. Attending to their biochemical characteristics, the PLA₂s are generally grouped into three major subfamilies, namely secreted PLA₂s (sPLA₂), cytosolic Ca²⁺-dependent PLA₂ (cPLA₂), and cytosolic Ca²⁺-independent PLA₂ (iPLA₂) [1-6]. sPLA₂s are extracellular, low molecular mass enzymes that require millimolar Ca²⁺ concentrations for activity. cPLA₂, specifically the α isoform, is an intracellular enzyme that plays a pivotal role in receptor-coupled arachidonic acid (AA) release and prostaglandin production. Whereas cPLA₂α has a striking selectivity for AA-containing phospholipids, sPLA₂s do not exhibit acyl chain specificity.

iPLA₂s are Ca²⁺-independent cytosolic enzymes whose functional role(s) in cells has recently gained interest [7, 8]. Among the iPLA₂s, the better studied is the one classified as Group VIA. This is an 85-kDa enzyme that shows no fatty acid selectivity and is potently and irreversibly inhibited by bromoenol lactone (BEL) [7, 8]. Evidence suggests that Group VIA iPLA₂ is directly involved in maintaining the homeostatic levels of lysophosphatidylcholine (lysoPC) in resting cells [9]. Since lysoPC is the main acceptor of free AA for its incorporation into phospholipid pools [10], Group VIA iPLA₂ has been implicated in phospholipid fatty acyl chain deacylation/reacylation reactions (i.e. the Lands cycle). In these reactions, a phospholipid containing a saturated fatty acid in the sn-2 position is cleaved by Group VIA iPLA₂, and the resulting lysophospholipid acceptor is acylated by CoA-dependent acyl transferases with a polyunsaturated fatty acid such as AA. This proposal, based on the demonstration that inhibition of cellular iPLA₂ by BEL or a
specific antisense oligonucleotide blocks AA incorporation, was originally described in P388D1 macrophages [11, 12] and later confirmed by others in a number of mammalian cells [13-15].

It seems likely however, that neither Group VIA iPLA2 is the only enzyme involved in maintaining homeostatic lysophospholipid levels [16] nor is that the only cellular function of Group VIA iPLA2 in cells. Recent evidence has implicated this enzyme in the destruction of membrane phospholipid subsequent to the cells entering apoptosis, resulting in the liberation of various free fatty acids to the extracellular medium [17-19]. Another instance wherein iPLA2-mediated phospholipolysis occurs in a seemingly receptor-uncontrolled manner is during oxidative stress [20, 21]. Finally, cells from schizophrenic patients have been reported to exhibit a constitutively elevated phospholipid fatty acid turnover, which appears to be mediated by an iPLA2-like activity [22].

To further expand our investigations on cellular functions of Group VIA iPLA2 in human U937 cells [9, 16, 20, 23, 24], we have prepared stably transfected cells that overexpress Group VIA iPLA2. Utilizing this strategy, we have re-assessed the role of iPLA2 in oxidant-induced AA release and incorporation into phospholipids, and extended our studies to the role of iPLA2 in oxidant-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials.** [5,6,8,9,11,12,14,15-3H]AA (200 Ci/mmol) and [3H]choline chloride (80 Ci/mmol) were purchased from Amersham Ibérica (Madrid, Spain). BEL and the
Group VIA iPLA₂ antibody were from Cayman (Ann Arbor, MI). pcDNA3.1 vector containing the mouse Group VIA iPLA₂ gene was kindly provided by Dr. Suzanne Jackowski (St. Jude Children’s Research Hospital, Memphis, TN) [25]. All other reagents were from Sigma (St. Louis, MO).

**Cell culture.** U937 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, penicillin (100 units/ml) and streptomycin 100 µg/ml. For experiments, the cells were incubated at 37°C in a humidified atmosphere of CO₂/O₂ (1:19) at a cell density of 0.5-1 x 10⁶ cells/ml in 12-well plastic culture dishes (Costar). Differentiation was achieved by treating the cells with 35 ng/ml PMA for 24 h.

**Production of transfectants stably expressing Group VIA iPLA₂.** The plasmid containing Group VIA iPLA₂ (approx. 2 µg per 10⁶ cells) was transfected by electroporation at 270 V (975 µF) using a Gene Pulser II electroporator (Bio-Rad). To select for the transfected cells, they were incubated in medium containing 1 mg/ml geneticin. To obtain the transfectants stably expressing Group VIA iPLA₂, the transfected cells were cloned by limiting dilution in medium containing 300 µg/ml geneticin. After two weeks, wells containing a single colony were chosen for further expansion and the iPLA₂ expression was analyzed by immunoblot and measurement of iPLA₂ activity. The clones were always grown in medium containing 300 µg/ml geneticin.

**Immunoblot analyses.** Cells were lysed in an ice-cold lysis buffer, and 15 µg of cellular protein from each sample were separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Dilution of both primary and secondary antibodies was made in phosphate-buffered saline containing 0.5% defatted dry milk and 0.1% Tween 20. After 1 h incubation with primary antibody at 1:1000, blots were
washed 3 times and an anti-rabbit secondary peroxidase-conjugated antibody was added for another h. Immunoblots were developed using the Amersham ECL system.

*iPLA₂ assay* – Briefly, 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-[³H]palmitoyl-glycero-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. The reactions were quenched by adding 3.75 volumes of chloroform/methanol (1:2). After lipid extraction, free [³H]palmitic acid was separated by thin-layer chromatography.

*Measurement of lysoPC.* Cells labeled with 0.5 µCi/ml [³H]choline for 2 days were used. After the incubations, lipids were extracted with ice-cold n-butanol and separated by thin-layer chromatography with chloroform/methanol/acetic acid/water (50:40:6:0.6) as a solvent system. Spots corresponding to lysoPC were scraped into scintillation vials and the amount of radioactivity was estimated by liquid scintillation counting.

*Measurement of [³H]AA release, and of [³H]AA incorporation into phospholipids.* For the AA release experiments, the cells were labeled with 0.5 µCi/ml [³H]AA for 18 h. Under these conditions, equilibrium labeling of AA pools with [³H]AA is reached. After this period, the cells were washed and placed in serum-free medium for 1 h before the addition of the appropriate stimulus in the presence of 0.5 mg/ml bovine serum albumin. The supernatants were removed, cleared of cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

[³H]AA release under these equilibrium conditions represents a balance between what is liberated directly from phospholipids minus what is re-incorporated
back into phospholipids by the action of acyltransferases. \(^{3}H\)AA incorporation into phospholipids cannot be measured simultaneously with \(^{3}H\)AA release, because the former could not be distinguished from the endogenous phospholipid-bound \(^{3}H\)AA that has not been mobilized by PLA\(_2\). To circumvent this problem, AA incorporation experiments were conducted in parallel under the same experimental conditions as those employed above for \(^{3}H\)AA release, but unlabeled cells were used instead and exogenous \(^{3}H\)AA was added together with the stimulus. Briefly, the cells were placed in serum-free medium for 1 h before exposure to exogenous \(^{3}H\)AA (10 µM, 0.5 µCi/ml) in the presence or absence of the indicated stimuli. At the indicated times, supernatants were removed and the cell monolayers were scraped twice with 0.1% Triton X-100. Total lipids were extracted and were separated by thin-layer chromatography with n-hexane/diethyl ether/acetic acid (70:30:1 by volume). Spots corresponding to phospholipid were scraped and their radioactive content was determined by scintillation counting.

**Antisense oligonucleotide treatments.** The iPLA\(_2\) antisense oligonucleotide utilized in this study has been described in previous studies from our laboratory [12, 20, 23, 24]. The iPLA\(_2\) antisense sequence corresponds to nucleotides 59-78 in the murine group VIA iPLA\(_2\) sequence, which is conserved in human group VIA iPLA\(_2\) [26, 27]. The antisense or sense oligonucleotides were mixed with lipofectamine, and complexes were allowed to form at room temperature for 10-15 min. The complexes were then added to the cells, and the incubations were allowed to proceed under standard cell culture conditions. The final concentrations of oligonucleotide and lipofectamine were 1 µM and 10 µg/ml, respectively. Oligonucleotide treatment and culture conditions were not toxic for the cells as assessed by the Trypan blue dye exclusion assay and by quantitating adherent cell protein.
Conditions for the use of a human cPLA$_2$ antisense oligonucleotide were taken from Tomassini and Cantoni [28]. The oligonucleotides used were: antisense: 5’-TAC AGT AAA TAT CTA GGA ATG-3’; and sense (random sequence of the antisense bases): 5’-CCT ACT GAG GGT ACG GTA CAT-3’). The oligonucleotides were phosphorothioate-modified (MWG Biotech, Ebersberg, Germany). The U937 cells were washed twice with serum-free medium and seeded at a cell density of 10$^6$ per ml in serum-free medium for 6 h in the absence of presence of the oligonucleotides (10 µM). A final concentration of 5% fetal bovine serum was added and the cells were cultured for an additional 48 h, and finally used for experiments.

Measurement of apoptosis. Apoptosis was analyzed by labeling with the Annexin-V FITC Apoptosis Detection Kit (Pharmingen), that recognizes phosphatidylserine exposure on outer leaflet of the plasma membrane. The cells were analyzed by flow cytometry using a Coulter-Epics XL-MCL cytofluorimeter.

Data presentation. Assays were carried out in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Unless otherwise indicated, the data presented are from representative experiments.

RESULTS

Characterization of U937 cells overexpressing Group VI iPLA$_2$ - Transfection of U937 cells with a plasmid containing mouse Group VIA iPLA$_2$ followed by selection of geneticin-resistant clones resulted in the isolation of a stably transfected clone expressing a 3-4-fold increase in iPLA$_2$ activity as compared to control cells
transfected with an empty plasmid (Fig. 1A). The stably transfectants also showed increased levels of an 85 kDa protein that was recognized by the anti-iPLA2 antibody from Cayman (Fig. 1A, inset). In addition, the stably transfected cells also exhibited a 2-3 increase in the steady-state level of cellular lysoPC, as measured in cells labeled with [3H]choline (Fig. 1B). Importantly, increased iPLA2 expression was not a permanent phenotype of the transfected cells, as it did not persist on serial passages in culture. Appreciable losses of both iPLA2 activity and immunoreactive protein were detected at approx. 20 cell passages. Interestingly, we have consistently failed to obtain expression --either transient or stable--, of an iPLA2 dominant negative mutant in which the catalytic Ser465 had been replaced by Ala. These data appear to suggest that large increases or decreases in the intracellular iPLA2 activity content are injurious for the U937 cells, which in turn highlight the importance that this class of enzymes may have in the regulation of homeostatic phospholipid metabolism.

When exposed to H2O2, U937 macrophage-like cells have been shown to liberate fatty acids, including AA, in a process that appears to depend on iPLA2 [20]. Fig. 2 shows that the iPLA2-overexpressing cells liberated more AA than did control cells when exposed to H2O2. Basal release (i.e, that determined in the absence of H2O2) was also increased in the iPLA2-overexpressing cells. As a control for these experiments, the effect of concanavalin A on AA release from these cells was also investigated. Concanavalin A is known to signal to AA release in U937 cells by directly activating the cPLA2, and the iPLA2 is not involved [20]. In accordance with these previous findings, iPLA2 overexpression did not result in an increased AA release response of the cells to concanavalin A (data not shown). Thus, iPLA2 overexpression does not impact on cellular functions that do not depend on iPLA2. To further strengthen the lack of involvement in H2O2-induced AA mobilization,
experiments were conducted in which cPLA$_2$ expression was knocked out by antisense technology (Fig. 3). Only partial inhibition of cPLA$_2$ could be achieved (38 ± 5% inhibition as assessed by immunoblot, Fig. 3 inset), which was not unexpected, given the high levels of cPLA$_2$ that the U937 cells are known to express [29, 30]. Despite such a low inhibition, we were still able to detect significant inhibition of the Con A-induced AA release (Fig. 3). Importantly, parallel measurement of the H$_2$O$_2$-induced AA mobilization showed no detectable effect (Fig. 3). These data, along with our previous data showing no inhibition of the H$_2$O$_2$-induced AA release by the cPLA$_2$-specific inhibitor pyrrophenone [20], strengthen the lack of a role for cPLA$_2$ in oxidant-induced AA mobilization in U937 cells.

Role of iPLA$_2$ in AA incorporation into U937 cell phospholipids exposed to H$_2$O$_2$ - Rather than reflecting enzyme activation per se, iPLA$_2$-mediated fatty acid release in response to H$_2$O$_2$ is thought to occur because of a facilitated interaction of the enzyme with its substrate, secondary to H$_2$O$_2$-induced membrane oxidation [20]. However, previous studies by Sporn et al. [31] utilizing alveolar macrophages have suggested that a major route by which H$_2$O$_2$ induces AA mobilization in these cells is by impairing fatty acid esterification into phospholipid. Similar results have been reported by Cane et al. [32] in vascular smooth muscle cells. Since AA mobilization in response to stimuli represents a balance between what is released from phospholipids by phospholipases minus what is reincorporated back into phospholipids by acyltransferases, we explored whether in the U937 cells, H$_2$O$_2$ induced AA release also involves inhibition of fatty acid incorporation into phospholipids.

In the first series of experiments, we assayed H$_2$O$_2$-induced AA release in the presence of thimerosal, a well known inhibitor of fatty acyl-CoA synthetases and
hence, of fatty acid incorporation into phospholipids [33, 34]. Figure 4 shows that thimerosal did not affect AA release on its own, but markedly augmented [3H]AA release in response to H2O2. This clearly suggests that the H2O2 effect on AA release did not involve an inhibitory effect on AA acylation into phospholipids.

We directly assayed the effect of H2O2 on AA incorporation into phospholipids in the experiments depicted in Fig. 5. Treating the cells with H2O2 not only did not inhibit exogenous AA incorporation but actually enhanced it. Given our previous data showing that H2O2 increases iPLA2 activity in the U937 cells [20], this unexpected finding correlates well with the proposed role of iPLA2 as a major provider of the lysoPC acceptors utilized in the initial incorporation of AA into cellular phospholipids. Thus, the simplest explanation for the enhancing effect of H2O2 reported in Fig. 5 is that H2O2, by increasing iPLA2 activity, acts to elevate the intracellular pool of lysoPC and this increases AA incorporation.

Further proof to the above view was obtained when AA incorporation was studied in the iPLA2 stably transfectants which, as indicated above, present higher amounts of lysoPC to serve as the acyl acceptors. The iPLA2 overexpressing cells incorporated more exogenous AA into phospholipids than did control cells transfected with an empty vector (Fig. 6). Moreover, when the AA incorporation experiments were conducted in the presence of H2O2, additional increases were observed again in both the untrasfected and the iPLA2-overexpressing cells (Fig. 6). All these increases were significantly blunted by BEL (~40% inhibition), confirming the involvement of iPLA2 in the response (Fig. 7). It should be noted however, that the fact that BEL does not completely blunt AA incorporation indicates that there are other pathways in addition to iPLA2 that may significantly contribute to overall AA incorporation into cellular phospholipids [16]. Interestingly, pyrrophnone, a specific
cPLA2 inhibitor, failed to exert any significant effect on the response (not shown). Collectively, findings in Figs. 5-7 do support an important role for iPLA2 in AA incorporation into U937 cell phospholipids.

**Role of iPLA2 in H2O2-induced apoptosis** - H2O2 is known to induce apoptosis in a number of cells [35, 36], and there is evidence that unesterified fatty acids such as AA inside the cells can signal apoptosis [37]. Since iPLA2 is responsible for liberating fatty acids in response to H2O2, the possibility arises that iPLA2 may be a key signaler of H2O2-induced apoptosis. Figure 8 shows that almost 40% of the U937 cells exposed to 500 µM H2O2 underwent apoptosis at 20 h, as measured by the annexin-V-FITC assay. Importantly, the fraction of cells undergoing apoptosis was doubled if iPLA2 overexpressing cells were used (Fig. 8). As a control for these experiments we used BEL, which is known to induce apoptosis in U937 cells in an iPLA2-independent manner [24]. More than 90% of the cell population underwent apoptosis in the presence of BEL and this happened in both control and iPLA2-overexpressing U937 cells (Fig. 8). Fig. 9A shows that in [3H]AA-labeled cells, the iPLA2 transfectants exhibited larger losses of cellular phospholipid content than did control cells exposed to H2O2. These phospholipid losses correlated with higher levels of [3H]AA in the incubation media, as compared to parental cells (Fig. 8B). Thin-layer chromatographic analyses of the radioactivity accumulating in the extracellular medium revealed that more than 80% of it corresponded to free fatty acid. Collectively, data in Figs. 8 and 9 suggest that iPLA2 participates in H2O2-induced apoptosis of U937 cells, and promotes destruction of membrane phospholipid.

The effect of iPLA2 inhibition on H2O2 apoptosis was assayed next. The cells were incubated with MAFP, a dual cPLA2/iPLA2 inhibitor that, unlike BEL, does not
induce apoptosis of the U937 cells on its own [24]. Preliminary experiments carried out with the specific cPLA₂ inhibitor pyrrophenone had shown no effect of this compound on H₂O₂-induced apoptosis, which rules out a role for cPLA₂ in the process, in agreement with previous reports [17, 18]. Unexpectedly, incubating the cells for 6, 8 or 20 h with 10-25 µM MAFP ---concentrations which we have previously shown to completely inhibit cellular iPLA₂ activity [24]-- also failed to decrease the extent of H₂O₂-induced apoptosis, as measured by the annexin V-FITC surface binding assay (Fig. 10A). In agreement with these data, U937 cells made deficient in iPLA₂ by antisense treatment exhibited no decreased apoptosis in response to H₂O₂ (Fig. 10B). These results suggest that, although iPLA₂-mediated phospholipid breakdown does occur during H₂O₂-induced apoptosis, the apoptotic process itself still can occur in the absence of iPLA₂.

**DISCUSSION**

H₂O₂ is an oxidant generated in large quantities by phagocyte cells by the action of superoxide dismutase on superoxide anion. Excessive accumulation of H₂O₂ is known to cause lipid peroxidation, which may compromise cellular function and, ultimately leading to cytotoxicity.

H₂O₂ is widely used as an oxidant stressor for the study of oxidation-induced signaling events in different cell models. In previous work, we have described the molecular mechanism for fatty acid mobilization during H₂O₂-induced oxidative
damage in U937 cells and found an unexpected role for Group VIA iPLA\textsubscript{2} as a main participant in the process [20]. Probably by increasing the amount of lipid peroxides at the membrane, the oxidant was found to increase the accessibility/susceptibility of iPLA\textsubscript{2} towards its substrate, which resulted in increased fatty acid release [20]. From a biochemical viewpoint this iPLA\textsubscript{2} role is striking since, under true activation conditions --i.e. those involving the activation of receptor-dependent or -independent intracellular signaling cascades-- there is general recognition that cPLA\textsubscript{2α}, not iPLA\textsubscript{2}, is an absolute requirement for AA mobilization in phagocyte cells. While inhibition of cPLA\textsubscript{2α} strongly blunts receptor-induced AA mobilization in phagocytes, inhibition of iPLA\textsubscript{2} by BEL does not generally affect the response [20, 38-42].

iPLA\textsubscript{2} involvement in oxidant-induced phospholipid hydrolysis has also been recognized to occur in uterine stromal cells [21] and, more recently, in astrocytes as well [43]. Interestingly however, in cells such as alveolar macrophages [31] and vascular smooth muscle cells [32], oxidant-induced AA mobilization was found to result from impairment of fatty acid incorporation into phospholipids. Given the key role that iPLA\textsubscript{2} appears to play in AA deacylation/reacylation reactions in immunoinflammatory cells [7, 8], it was of interest to assess the effect of H\textsubscript{2}O\textsubscript{2} on the AA incorporation mechanisms of U937 macrophage-like cells. Initial studies were carried out with thimerosal, an organometallic compound that blocks AA reacylation but spares deacylation via PLA\textsubscript{2} [33, 34]. This compound markedly enhanced the H\textsubscript{2}O\textsubscript{2}-induced AA mobilization, a finding that is consistent with the H\textsubscript{2}O\textsubscript{2} effect being on the phospholipolytic step and not on the reacylation step. Thus, if an inhibitory effect of H\textsubscript{2}O\textsubscript{2} on AA reacylation was the cause of the AA release, one would expect an additive or less-than-additive effect of thimerosal on the H\textsubscript{2}O\textsubscript{2} response, as demonstrated in the studies by Sporn et al. [31] and Cane et al. [32]. On the contrary,
if a stimulatory effect of H$_2$O$_2$ on the PLA$_2$-mediated deacylation step was the cause of the AA release, then the effects of H$_2$O$_2$ plus thimerosal should be supra-additive or synergistic. This is exactly what we observed in the experiments described in this report.

Analyses of the effect of H$_2$O$_2$ on the AA incorporation capacity of the cells indicate that the oxidant not only does not block AA esterification into phospholipids, but actually enhances it. Thus, despite the oxidant increasing fatty acid esterification into phospholipids, the net result is an increase in fatty acid mobilization, which indicates that the effect of H$_2$O$_2$ on the iPLA$_2$ is stronger and thus prevails.

Taking into account that H$_2$O$_2$ does not affect either positively or negatively the activities of the AA reacylating enzymes arachidonoyl-CoA synthetase or the arachidonoyl-CoA acyltransferase [31], a plausible explanation for the phenomena herein described is that accelerated hydrolysis of phospholipids by iPLA$_2$ in the presence of H$_2$O$_2$ leads to accumulation of intracellular lysophospholipid acceptors, which in turn triggers feed-back increase of AA incorporation into phospholipids. Thus, the biochemical significance of the AA incorporation data in the H$_2$O$_2$-treated cells is that not all of the AA released from phospholipids by iPLA$_2$ will be available for further metabolism. Rather, a significant portion of free AA will be incorporated back into phospholipids, limiting in this way the amount of free AA available for further metabolic reactions.

Control of the intracellular level of lysophospholipid acceptors utilized for incorporation of AA into phospholipids is one of the earliest proposed roles for iPLA$_2$ in phagocyte cells. This role was demonstrated by studies in which iPLA$_2$ activity was reduced in cells by either pharmacological or antisense inhibition approaches [7, 8, 11, 12]. In the current study we have employed a third different approach, which is
the opposite of the other two previously employed. We have prepared cells that stably overexpress iPLA2 to confirm previous functional roles proposed for the enzyme, and to study new ones. As would be expected from the model discussed above, the iPLA2 overexpressing cells exhibit a significantly higher capacity to incorporate AA into phospholipids than control cells. Also, the iPLA2 overexpressing cells mobilize larger quantities of free AA in response to H2O2. Thus, these results provide new evidence for the key role of iPLA2 in mediating phospholipid hydrolysis during oxidative stress by H2O2. In turn, the results provide additional evidence for the key role of iPLA2 in regulating the intracellular levels of lysoPC to be used for fatty acid incorporation via the Lands cycle.

Studies on whether lysoPC levels limit the initial rate of AA incorporation into phospholipids in cells not of the phagocytic lineage have also recently been carried out. In pancreatic islet cells, the steady-state levels of lysoPC appear to be so high that even after acute inhibition of endogenous iPLA2, the cells still retain at least 80% of their initial lysoPC content [44]. Thus the initial rate of AA incorporation into islet phospholipids is not altered, suggesting that in these cells the iPLA2 is not required for the initial AA incorporation into phospholipids. Nevertheless, it should be noted that islet iPLA2 is estimated to provide at least 20% of the very high lysoPC levels that these cells contain [44], which suggests that the enzyme still possesses significant *housekeeping* activity with regard to the maintenance of endogenous lysoPC levels.

Transient overexpression of iPLA2 into COS cells has been found to significantly increase lysoPC levels without a concomitant increase in the incorporation of exogenous AA into phospholipids, suggesting that under these settings, lysoPC levels do not limit the initial rate of AA incorporation into
phospholipids [25]. Since the results obtained in COS cells were performed in transiently transfected cells, it is possible that acute alterations in phospholipid metabolism induced by transient overexpression of iPLA$_2$ may not trigger normal physiological responses, as discussed elsewhere [45]. It is also possible that COS cells have a very limited capacity to incorporate AA into membrane phospholipids, and that the steady-state level of lysoPC in untrasfected cells is already high enough to account for a normal rate of incorporation of AA into the phospholipids of these cells. Thus, the excess amount of lysophospholipid produced by the iPLA$_2$ overexpressing cells would not be needed for AA incorporation.

Our results in phagocytic cells, together with those in COS [25] and pancreatic islet cells [44, 46], appear to suggest that the mechanisms for lysophospholipid generation and the PLA$_2$s involved in phospholipid fatty acyl chain remodeling may be cell-type specific. However, the results are also compatible with the hypothesis that a certain threshold level of intracellular lysophospholipid is necessary to support AA incorporation into phospholipids. In cells with a limited capacity of AA incorporation or in those with an exceedingly high steady-state lysophospholipid level, increasing and/or partially decreasing the intracellular level of lysoPC --by either iPLA$_2$ overexpression [25, 46] or pharmacological inhibition [44], respectively-- may have little or no effect on the initial rate of AA incorporation. Conversely, in cells specialized in AA metabolism such as phagocytes, decreasing [11, 12] or increasing (this work) the intracellular level of lysoPC may lead to significant changes in the initial rate of AA incorporation. Thus lysoPC-dependent regulation of AA incorporation into phospholipids may be strikingly characteristic of some cell types but not of others, and other factor(s) in addition to lysophospholipid availability may limit AA incorporation in certain cell types.
Beyond the *housekeeping* role of iPLA$_2$ in phospholipid fatty acid reacylation/deacylation reactions and in non-specific fatty acid release during oxidant injury discussed above, a role for iPLA$_2$ in apoptosis has been suggested by the finding that apoptosis induction by either anti-Fas antibody or tumor necrosis factor plus cycloheximide in U937 cells is associated with iPLA$_2$-mediated hydrolysis of membrane phospholipids [17, 18]. We have observed in this work that the extent of H$_2$O$_2$-induced apoptosis in U937 cells is higher if iPLA$_2$-overexpressing cells are used. Increased destruction of membrane phospholipid and concomitant release of fatty acid to the supernatant were also observed under these conditions, confirming that iPLA$_2$-mediated phospholipid hydrolysis does occur during apoptosis.

Importantly however, treating the cells with an iPLA$_2$ antisense oligonucleotide, or with MAFP under conditions that result in total inhibition of cellular iPLA$_2$, does not prevent the H$_2$O$_2$-induced apoptosis. This suggests that iPLA$_2$ activity is, in the strict sense, not necessary for the apoptosis to take place. In support of this view, BEL induces apoptosis in a variety of cells via a caspase-3-mediated pathway that necessarily proceeds in the absence of functional iPLA$_2$ activity [24; see also Fig. 8], and Atsumi *et al.* [18] have also noted that MAFP treatment of U937 cells does not prevent apoptosis in response to both anti-Fas antibody or tumor necrosis factor plus cycloheximide, although in this case, the drug was found to partially decrease apoptosis at early time periods. More recently, Lauber *et al.* [19] have reported that apoptosis of caspase-3-transfected MCF7 cells exposed to UV is not prevented by arachidonyl trifluoromethyl ketone. Although the latter result was taken by the authors to rule out a role for cPLA$_2$ in apoptosis [19], arachidonyl trifluoromethyl ketone is also known to strongly inhibit iPLA$_2$ [47, 48], which makes it likely that UV-induced apoptosis of caspase-3-transfected MCF7 cells
is also independent of iPLA₂ [19].

Collectively, all of the aforementioned examples suggest that, even though iPLA₂ may participate in the early phase of apoptosis under certain conditions, the enzyme may actually be dispensable for the apoptotic process to fully develop. It is therefore conceivable that, beyond a mere destructive role, iPLA₂-mediated phospholipid hydrolysis during oxidant injury may serve to provide those accessory signals —e.g. eat me, or attraction signals— that are triggered along with the destructive process itself [49, 50]. Thus, products of iPLA₂ hydrolysis of cellular phospholipids during apoptosis, namely free fatty acids or perhaps lysophospholipids such as lysoPC [19, 51], might be involved in providing these signals. Experiments are currently under way to test this possibility during oxidant-induced apoptosis.

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FIGURE CAPTIONS

Figure 1. Overexpression of iPLA₂ in U937 cells. A) The stably transfected cells (referred to as iPLA₂ in the abscissa) were prepared as described under “Experimental Procedures”, and the levels of iPLA₂ activity and immunoreactive protein were compared to those of parental cells transfected with an empty vector (pcDNA3.1). B) The cells were labeled with [³H]choline, and the level of radioactivity in lysoPC in the stably transfected cells (iPLA₂) and control cells transfected with an empty vector (pcDNA3.1) was measured as described under “Experimental Procedures”.

Figure 2. Effect of H₂O₂ on AA release from iPLA₂-overexpressing (iPLA₂) and control (pcDNA3.1) cells. AA release was measured at 1 h in cells exposed (closed bars) or not (open bars) to 500 µM H₂O₂.

Figure 3. Effect of a cPLA₂ antisense oligonucleotide on stimulus-induced AA release. The cells were treated with a sense oligonucleotide (gray bars), antisense oligonucleotide (black bars) or neither (open bars) as described under Experimental Procedures. Afterwards the cells were exposed to 500 H₂O₂, 100 µg/ml Con A or neither (Control) for 1 h, and AA release was determined. Inset shows the cPLA₂ protein content of control (C), sense-treated (S) or antisense-treated (A) cells, as measured by immunoblot.

Figure 4. Effect of thimerosal on H₂O₂-induced AA release. The cells were either untreated (open symbols) or treated (closed symbols) with 500 μM H₂O₂ for 1 h in the
presence of the indicated concentrations of thimerosal.

Figure 5. Effect of H$_2$O$_2$ on AA incorporation into U937 cell phospholipids. The cells were treated with exogenous [$^3$H]AA for the indicated amounts of time in the absence (open symbols) or presence (closed symbols) of 500 µM H$_2$O$_2$, and AA incorporation into cellular phospholipids was measured as described under “Experimental Procedures”.

Figure 6. Effect of H$_2$O$_2$ on AA incorporation into phospholipids in iPLA$_2$-overexpressing (iPLA$_2$) and control (pcDNA3.1) cells. [$^3$H]AA incorporation was measured at 1 h in cells exposed (closed bars) or not (open bars) to 500 µM H$_2$O$_2$, as described under “Experimental Procedures”.

Figure 7. Effect of BEL on the incorporation of AA into phospholipids in iPLA$_2$-overexpressing (iPLA$_2$) and control (pcDNA3.1) cells. [$^3$H]AA incorporation was measured at 1 h in cells treated (cross-hatched bars) or not (open bars) with 25 µM BEL and in the absence (Ctrl) or presence of 500 µM H$_2$O$_2$.

Figure 8. Annexin V-FITC labeling of iPLA$_2$-overexpressing (iPLA$_2$) and control (pcDNA3.1) cells. The cells were treated with 500 µM H$_2$O$_2$ (upper panels) or 25 µM BEL (lower panels) for 20 h in serum-free media, and stained with annexin V-FITC as described under “Experimental Procedures”. Labeling obtained after H$_2$O$_2$ or BEL treatment (open traces) is compared with that of cells treated with vehicle alone (gray-filled traces).
Figure 9. Changes of \(^{3}\)H-radioactivity in phospholipids (A) and extracellular fatty acid release (B) in \([^{3}\)H\]AA-labeled U937 cells exposed to \(\text{H}_2\text{O}_2\). The iPLA\(_2\)-overexpressing (cross-hatched bars) or control (open bars) cells were exposed to \(\text{H}_2\text{O}_2\) for the indicated amounts of time. Afterward, radioactivity in phospholipids and in the extracellular medium was quantified as described under “Experimental Procedures”.

Figure 10. Role of iPLA\(_2\) in \(\text{H}_2\text{O}_2\)-induced apoptosis. A, The cells were treated without (open bars) or with (hatched bars) 25 µM MAFP, and then incubated with 500 µM \(\text{H}_2\text{O}_2\) for the indicated times. After the incubations, the cells were stained with annexin V-FITC as described under “Experimental Procedures” and the number of apoptotic cells was determined by cytometry. Apoptosis in cells not treated with \(\text{H}_2\text{O}_2\) did not exceed 17% at any time. B, The cells were treated with iPLA\(_2\) sense oligonucleotide (gray bars), iPLA\(_2\) antisense oligonucleotide (black bars) or neither (open bars) as described under Experimental Procedures. Afterwards the cells were incubated without (Control) or with 500 \(\text{H}_2\text{O}_2\) for 20 h, and the number of apoptotic cells was determined by cytometry after staining with annexin V-FITC. Inset shows the iPLA\(_2\) protein content of control (C), sense-treated (S) or antisense-treated (A) cells, as measured by immunoblot.
Figure 1

A

![Graph showing iPLA2 Activity (% hydrolysis)]

B

![Graph showing LysoPC (cpm x 10^-3)]
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10

Control                    H$_2$O$_2$

FITC-Annexin V-Positive Cells (%)

0                   10                   20                   30                   40                   50

6 h               8 h               20 h

A

FITC-Annexin V-Positive Cells (%)

0                   10                   20                   30                   40                   50

C   S   A

Control                    H$_2$O$_2$

B

Figure 10