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Role of Group VIA Calcium-independent Phospholipase A_2 in Arachidonic Acid Release, Phospholipid Fatty Acid Incorporation, and Apoptosis in U937 Cells Responding to Hydrogen Peroxide*

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Group VIA calcium-independent phospholipase A_2 ($iPLA_2$) 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 $iPLA_2$ roles have rested heavily on the use of bromoenol lactone as an $iPLA_2$-specific inhibitor, but this compound actually inhibits other enzymes and lipid pathways unrelated to PLA_2, which makes it difficult to define the contribution of $iPLA_2$ to specific functions. In previous work, we pioneered the use of antisense technology to decrease cellular $iPLA_2$ activity as an alternative approach to study $iPLA_2$ functions. In the present study, we followed the opposite strategy and prepared U937 cells that exhibited enhanced $iPLA_2$ activity by stably expressing a plasmid containing $iPLA_2$ cDNA. Compared with control cells, the $iPLA_2$-overexpressing U937 cells showed 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 $iPLA_2$ plays in these events. Long-term exposure of the cells to hydrogen peroxide resulted in cell death by apoptosis, and this process was accelerated in the $iPLA_2$-overexpressing cells. Increased phospholipid hydrolysis and fatty acid release also occurred in these cells. Unexpectedly, however, abrogation of U937 cell $iPLA_2$ activity by either methyl arachidonyl fluorophosphonate or an antisense oligonucleotide did not delay or decrease the extent of apoptosis induced by hydrogen peroxide. These results indicate that, although $iPLA_2$-mediated phospholipid hydrolysis occurs during apoptosis, $iPLA_2$ may actually be dispensable for the apoptotic process to occur. Thus, beyond a mere destructive role, $iPLA_2$ may play other roles during apoptosis.

Phospholipases A_2 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 phospholipase A_2 (PLA_2)[1] enzymes have been characterized in recent years and classified into 14 group types on the basis of sequence data (1, 2). According to their biochemical characteristics, the PLA_2 enzymes are generally grouped into three major subfamilies, *viz.* secreted PLA_2 ($sPLA_2$) enzymes, cytosolic Ca^{2+}-dependent PLA_2 ($cPLA_2$), and cytosolic Ca^{2+}-independent PLA_2 ($iPLA_2$) (1–6). $sPLA_2$ enzymes are extracellular low molecular mass enzymes that require millimolar Ca^{2+} concentrations for activity. $cPLA_2$, specifically the α isoform, is an intracellular enzyme that plays a pivotal role in receptor-coupled arachidonic acid (AA) release and prostaglandin production. Whereas $cPLA_2\alpha$ has a striking selectivity for AA-containing phospholipids, $sPLA_2$ enzymes do not exhibit acyl chain specificity.

$iPLA_2$ enzymes are Ca^{2+}-independent cytosolic enzymes whose functional role(s) in cells has recently gained interest (7, 8). Among the $iPLA_2$ enzymes, 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 $iPLA_2$ is directly involved in maintaining the homeostatic levels of lysophosphatidylcholine (lyso-PC) in resting cells (9). Since lyso-PC is the main acceptor of free AA for its incorporation into phospholipid pools (10), Group VIA $iPLA_2$ 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 at the *sn*-2 position is cleaved by Group VIA $iPLA_2$, and the resulting lysophospholipid acceptor is acylated by CoA-dependent acyltransferases with a polyunsaturated fatty acid such as AA. This proposal, based on the demonstration that inhibition of cellular $iPLA_2$ by BEL or a specific antisense oligonucleotide blocks AA incorporation, was originally described in $P388D_1$ macrophages (11, 12) and was later confirmed by others in a number of mammalian cells (13–15).

It seems likely, however, that Group VIA $iPLA_2$ is not the only enzyme involved in maintaining homeostatic lysophospholipid levels (16) and that this is not the only cellular function of Group VIA $iPLA_2$ in cells. Recent evidence has implicated this enzyme in the destruction of membrane phospholipid subsequent to cells entering apoptosis, resulting in the liberation of various free fatty acids to the extracellular medium (17–19). Another instance wherein $iPLA_2$-mediated phospholipolysis occurs in a seemingly receptor-uncontrolled manner is during oxidative stress (20, 21). Finally, cells from schizophrenic pa-

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $sPLA_2$, secreted PLA_2; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; AA, arachidonic acid; BEL, bromoenol lactone; lyso-PC, lysophosphatidylcholine; FITC, fluorescein isothiocyanate; MAFP, methyl arachidonyl fluorophosphonate.

tients have been reported to exhibit a constitutively elevated phospholipid fatty acid turnover, which appears to be mediated by an iPLA$_2$-like activity (22).

To further expand our investigations on cellular functions of Group VIA iPLA$_2$ in human U937 cells (9, 16, 20, 23, 24), we prepared stably transfected cells overexpressing Group VIA iPLA$_2$. Utilizing this strategy, we reassessed the role of iPLA$_2$ in oxidant-induced AA release and incorporation into phospholipids and extended our studies to the role of iPLA$_2$ in oxidant-induced apoptosis.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol) and [^{3}H]choline chloride (80 Ci/mmol) were purchased from Amersham Biosciences. BEL and the anti-Group VIA iPLA$_2$ antibody were from Cayman Chemical Co., Inc. (Ann Arbor, MI). The pcDNA3.1 vector containing the mouse Group VIA iPLA$_2$ gene was kindly provided by Dr. Suzanne Jackowski (St. Jude Children's Research Hospital, Memphis, TN) (25). All other reagents were from Sigma.

Cell Culture—U937 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. For experiments, 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 Corp.). Differentiation was achieved by treating the cells with 35 ng/ml phorbol 12-myristate 13-acetate for 24 h.

Production of Transfectants Stably Expressing Group VIA iPLA$_2$—The plasmid containing Group VIA iPLA$_2$ (~2 μg/10^6 cells) was transfected by electroporation at 270 V (975 microfarads) 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 transfectants stably expressing Group VIA iPLA$_2$, the transfected cells were cloned by limiting dilution in medium containing 300 μg/ml Geneticin. After 2 weeks, wells containing a single colony were chosen for further expansion, and iPLA$_2$ expression was analyzed by immunoblotting and measurement of iPLA$_2$ activity. The clones were always grown in medium containing 300 μg/ml Geneticin.

Immunoblot Analyses—Cells were lysed in 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 performed in phosphate-buffered saline containing 0.5% defatted dry milk and 0.1% Tween 20. After a 1-h incubation with primary antibody at 1:1000, blots were washed three times, and a peroxidase-conjugated anti-rabbit secondary antibody was added for another hour. Immunoblots were developed using the Amersham Biosciences ECL system.

iPLA$_2$ 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-[^{3}H]palmitoylglycero-3-phosphocholine, specific activity of 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 [^{3}H]palmitic acid was separated by thin-layer chromatography.

Measurement of Lyso-PC—Cells labeled with 0.5 μCi/ml [^{3}H]choline for 2 days were used. After the incubations, lipids were extracted with ice-cold 1-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 lyso-PC were scraped into scintillation vials, and the amount of radioactivity was estimated by liquid scintillation counting.

Measurement of [^{3}H]AA Release and of [^{3}H]AA Incorporation into Phospholipids—For the AA release experiments, the cells were labeled with 0.5 μCi/ml [^{3}H]AA for 18 h. Under these conditions, equilibrium labeling of AA pools with [^{3}H]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.

[^{3}H]AA release under these equilibrium conditions represents a balance between what is liberated directly from phospholipids minus what is reincorporated 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 cannot 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 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 cellular protein.

The conditions for using a human cPLA$_2$ antisense oligonucleotide were as described by Tommasini and Cantoni (28). The oligonucleotides used were 5′-TAC AGT AAA TAT CTA GGA ATG-3′ (antisense) and 5′-CCT ACT GAG GGT ACG GTA CAT-3′ (sense; random sequence of the antisense bases). 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 cells/ml in serum-free medium for 6 h in the absence or 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-fluorescein isothiocyanate (FITC) apoptosis detection kit (Pharmingen), which recognizes phosphatidylserine exposure on the outer leaflet of the plasma membrane. The cells were analyzed by flow cytometry using a Coulter Epics XL-MCL cytofluorometer.

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

RESULTS

Characterization of U937 Cells Overexpressing Group VIA 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 3–4-fold increased levels of iPLA$_2$ activity compared with control cells transfected with an empty plasmid (Fig. 1*A*). The stable transfectants also showed increased levels of an 85-kDa protein that was recognized by the anti-iPLA$_2$ antibody from Cayman Chemical Co., Inc. (Fig. 1*A*, *inset*). In addition, the stably transfected cells also exhibited a 2–3-fold increase in the steady-state level of cellular lyso-PC as measured in cells labeled with [^{3}H]choline (Fig. 1*B*). Importantly, increased iPLA$_2$ expression was not a permanent phenotype of the transfected cells, as it did not persist upon serial passages in culture. Appreciable losses of both iPLA$_2$ activity and immunoreactive protein were detected at ~20 cell passages. Interestingly, we consistently failed to obtain expression (either transient or stable) of an iPLA$_2$ dominant-negative mutant in which the catalytic Ser^{465} had been replaced by Ala. These data appear to suggest that large increases or decreases in the intracellular iPLA$_2$ activity content are injurious to U937 cells, highlighting the importance that this class of enzymes may have in the regulation of homeostatic phospholipid metabolism.

When exposed to H_2O_2, U937 macrophage-like cells have been shown to liberate fatty acids, including AA, in a process

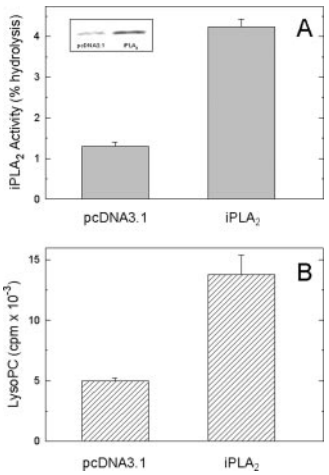

FIG. 1. **Overexpression of iPLA$_2$ in U937 cells.** *A*, stably transfected cells (iPLA$_2$) were prepared as described under "Experimental Procedures," and the levels of iPLA$_2$ activity and immunoreactive protein were compared with those in parental cells transfected with an empty vector (pcDNA3.1). *B*, the cells were labeled with [^{3}H]choline, and the level of radioactivity in lyso-PC in the stably transfected cells (iPLA$_2$) and control cells transfected with an empty vector (pcDNA3.1) was measured as described under "Experimental Procedures."

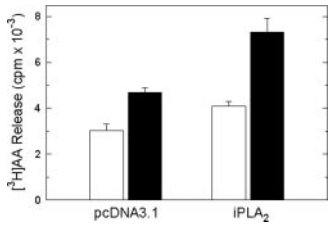

FIG. 2. **Effect of H$_2$O$_2$ on AA release from iPLA$_2$-overexpressing and control (pcDNA3.1) cells.** AA release was measured at 1 h in cells exposed (*closed bars*) or not (*open bars*) to 500 μM H$_2$O$_2$.

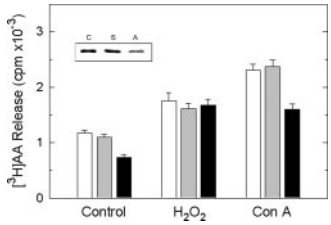

FIG. 3. **Effect of a cPLA$_2$ antisense oligonucleotide on stimulus-induced AA release.** The cells were treated with a sense oligonucleotide (*shaded bars*), antisense oligonucleotide (*closed bars*), or neither (*open bars*) as described under "Experimental Procedures." Afterward, the cells were exposed to 500 H$_2$O$_2$, 100 μg/ml concanavalin A (*Con A*), or neither (*Control*) for 1 h, and AA release was determined. The *inset* shows the cPLA$_2$ protein content of control (*C*), sense oligonucleotide-treated (*S*), or antisense oligonucleotide-treated (*A*) cells as measured by immunoblotting.

that appears to depend on iPLA$_2$ (20). Fig. 2 shows that the iPLA$_2$-overexpressing cells liberated more AA than did control cells when exposed to H$_2$O$_2$. Basal release (*i.e.* that determined in the absence of H$_2$O$_2$) was also increased in the iPLA$_2$-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 cPLA$_2$, and iPLA$_2$ is not involved (20). In accordance with these previous findings, iPLA$_2$ overexpression did not result in an increased AA release response of the cells to concanavalin A (data not shown). Thus, iPLA$_2$ overexpression does not impact on cellular functions that do not depend on iPLA$_2$. To further support the lack of involvement of cPLA$_2$ in H$_2$O$_2$-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 $\pm$ 5% inhibition as assessed by immunoblotting) (Fig. 3, *inset*), which was not unexpected, given the high levels of cPLA$_2$ that U937 cells are known to express (29, 30). Despite such a low inhibition, we were still able to detect significant inhibition of the concanavalin 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), support 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 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). Fig. 4 shows that thimerosal did not affect AA release on its own, but markedly augmented [^{3}H]AA release in response to H$_2$O$_2$. This clearly suggests that the H$_2$O$_2$ effect on AA release did not involve an inhibitory effect on AA acylation into phospholipids.

We directly assayed the effect of H$_2$O$_2$ on AA incorporation into phospholipids in the experiments depicted in Fig. 5. Treating the cells with H$_2$O$_2$ did not inhibit exogenous AA incorporation, but actually enhanced it. Given our previous data showing that H$_2$O$_2$ increases iPLA$_2$ activity in U937 cells (20), this unexpected finding correlates well with the proposed role of iPLA$_2$ as a major provider of the lyso-PC acceptors utilized in the initial incorporation of AA into cellular phospholipids. Thus, the simplest explanation for the enhancing effect of H$_2$O$_2$ reported in Fig. 5 is that H$_2$O$_2$, by increasing iPLA$_2$ activity, acts to elevate the intracellular pool of lyso-PC and that this increases AA incorporation.

Further proof for the above view was obtained when AA incorporation was studied in the iPLA$_2$ stable transfectants, which, as indicated above, presented higher amounts of lyso-PC to serve as the acyl acceptors. The iPLA$_2$-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 H$_2$O$_2$, additional increases were observed again in both the untransfected and the iPLA$_2$-overexpressing cells (Fig. 6). All these increases were significantly blunted by BEL (~40% inhibition), confirming the involvement of iPLA$_2$ in the response (Fig. 7). It should be noted, however, that the fact that BEL did not completely blunt AA incorporation indicates that there are other pathways in addition to iPLA$_2$ that may significantly contribute to overall AA incorporation into cellular phospholipids (16). Interestingly, pyrrophe-

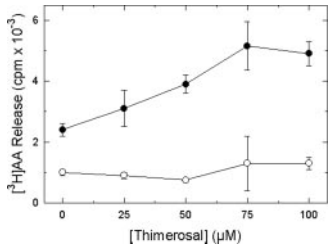

FIG. 4. **Effect of thimerosal on H_2O_2-induced AA release.** The cells were either left untreated (*open symbols*) or treated with 500 μM H_2O_2 for 1 h (*closed symbols*) in the presence of the indicated concentrations of thimerosal.

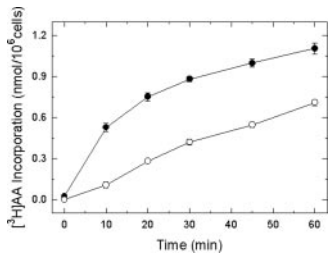

FIG. 5. **Effect of H_2O_2 on AA incorporation into U937 cell phospholipids.** The cells were treated with exogenous [^{3}H]AA for the indicated amounts of time in the absence (*open symbols*) or presence (*closed symbols*) of 500 μM H_2O_2, and AA incorporation into cellular phospholipids was measured as described under "Experimental Procedures."

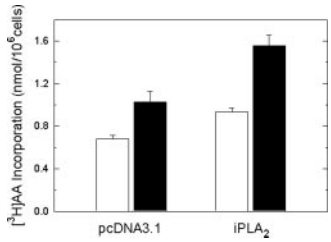

FIG. 6. **Effect of H_2O_2 on AA incorporation into phospholipids in iPLA$_2$-overexpressing and control (pcDNA3.1) cells.** [^{3}H]AA incorporation was measured at 1 h in cells exposed (*closed bars*) or not (*open bars*) to 500 μM H_2O_2 as described under "Experimental Procedures."

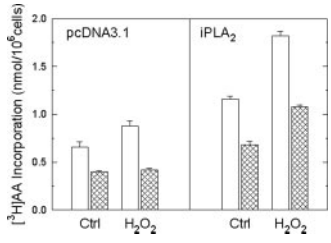

FIG. 7. **Effect of BEL on the incorporation of AA into phospholipids in iPLA$_2$-overexpressing and control (pcDNA3.1) cells.** [^{3}H]AA incorporation was measured at 1 h in cells treated (*hatched bars*) or not (*open bars*) with 25 μM BEL in the absence (control (*Ctrl*)) or presence of 500 μM H_2O_2.

none, a cPLA$_2$-specific inhibitor, failed to exert any significant effect on the response (data not shown). Collectively, the findings in Figs. 5–7 do support an important role for iPLA$_2$ in AA incorporation into U937 cell phospholipids.

Role of iPLA$_2$ in H_2O_2-induced Apoptosis—H_2O_2 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 iPLA$_2$ is responsible for liberating fatty acids in response to H_2O_2, the possibility arises that iPLA$_2$ may be a key signaler of H_2O_2-induced apoptosis. Fig. 8 shows that almost 40% of the U937 cells exposed to 500 μM H_2O_2 underwent apoptosis at 20 h as measured by the

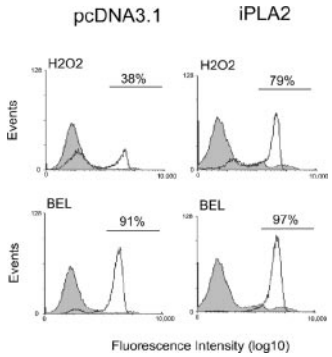

FIG. 8. **Annexin V-FITC labeling of iPLA$_2$-overexpressing and control (pcDNA3.1) cells.** The cells were treated with 500 μM H_2O_2 (*upper panels*) or 25 μM BEL (*lower panels*) for 20 h in serum-free medium and stained with annexin V-FITC as described under "Experimental Procedures." Labeling obtained after H_2O_2 or BEL treatment (*open traces*) is compared with that in cells treated with vehicle alone (*shaded traces*).

annexin V-FITC assay. Importantly, the fraction of cells undergoing apoptosis was doubled if iPLA$_2$-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 iPLA$_2$-independent manner (24). More than 90% of the cell population underwent apoptosis in the presence of BEL, and this happened in both control and iPLA$_2$-overexpressing U937 cells (Fig. 8). Fig. 9*A* shows that, in [^{3}H]AA-labeled cells, the iPLA$_2$ transfectants exhibited larger losses of cellular phospholipid content than did control cells exposed to H_2O_2. These phospholipid losses correlated with higher levels of [^{3}H]AA in the incubation medium compared with the parental cells (Fig. 9*B*). Thin-layer chromatographic analyses of the radioactivity accumulating in the extracellular medium revealed that >80% of it corresponded to free fatty acid. Collectively, the data in Figs. 8 and 9 suggest that iPLA$_2$ participates in H_2O_2-induced apoptosis of U937 cells and promotes destruction of membrane phospholipid.

The effect of iPLA$_2$ inhibition on H_2O_2 apoptosis was assayed next. The cells were incubated with methyl arachidonyl fluorophosphonate (MAFP), a dual cPLA$_2$/iPLA$_2$ inhibitor that, unlike BEL, does not induce apoptosis of U937 cells on its own (24). Preliminary experiments carried out with the cPLA$_2$-specific inhibitor pyrrophenone had shown no effect of this compound on H_2O_2-induced apoptosis, which rules out a role for cPLA$_2$ 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 that we have previously shown to completely inhibit cellular iPLA$_2$ activity (24)) also failed to decrease the extent of H_2O_2-induced apoptosis as measured by the annexin V-FITC surface binding assay (Fig. 10*A*). In agreement with these data, U937 cells made deficient in iPLA$_2$ by antisense treatment exhibited no decreased apoptosis in response to H_2O_2 (Fig. 10*B*). These results suggest that, although iPLA$_2$-mediated phospholipid breakdown does occur during H_2O_2-induced apoptosis, the apoptotic process itself still can occur in the absence of iPLA$_2$.

DISCUSSION

H_2O_2 is an oxidant generated in large quantities by phagocyte cells by the action of superoxide dismutase on superoxide anion. Excessive accumulation of H_2O_2 is known to cause lipid peroxidation, which may compromise cellular function and ultimately lead to cytotoxicity.

H_2O_2 is widely used as an oxidant stressor for the study of oxidation-induced signaling events in different cell models. In previous work, we described the molecular mechanism for fatty

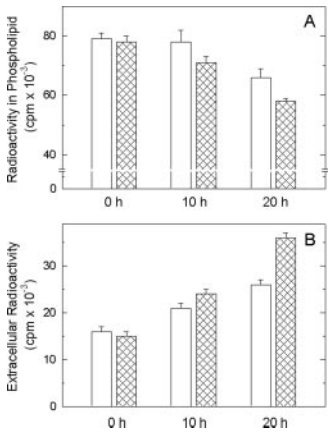

FIG. 9. **Changes in ^{3}H radioactivity in phospholipids (*A*) and extracellular fatty acid release (*B*) in [^{3}H]AA-labeled U937 cells exposed to H_2O_2.** The iPLA$_2$-overexpressing (*cross-hatched bars*) and control (*open bars*) cells were exposed to H_2O_2 for the indicated amounts of time. Afterward, radioactivity in phospholipids and in the extracellular medium was quantified as described under "Experimental Procedures."

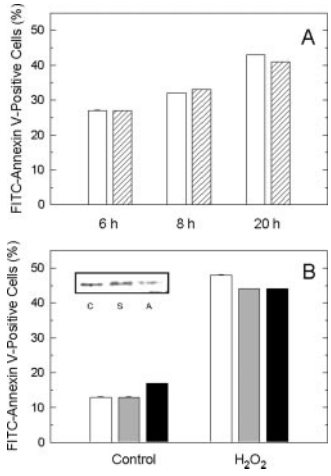

FIG. 10. **Role of iPLA$_2$ in H_2O_2-induced apoptosis.** *A*, the cells were treated without (*open bars*) or with (*hatched bars*) 25 μM MAFP and then incubated with 500 μM H_2O_2 for the indicated times. After the incubations, the cells were stained with annexin V-FITC as described under "Experimental Procedures," and the number of apoptotic cells was determined by cytometry. Apoptosis in cells not treated with H_2O_2 did not exceed 17% at any time. *B*, the cells were treated with the iPLA$_2$ sense oligonucleotide (*shaded bars*), the iPLA$_2$ antisense oligonucleotide (*closed bars*), or neither (*open bars*) as described under "Experimental Procedures." Afterward, the cells were incubated without (*Control*) or with 500 H_2O_2 for 20 h, and the number of apoptotic cells was determined by cytometry after staining with annexin V-FITC. The *inset* shows the iPLA$_2$ protein content of control (*C*), sense oligonucleotide-treated (*S*), or antisense oligonucleotide-treated (*A*) cells as measured by immunoblotting.

acid mobilization during H_2O_2-induced oxidative damage in U937 cells and found an unexpected role for Group VIA iPLA$_2$ 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$_2$ toward its substrate, resulting in increased fatty acid release (20). From a biochemical viewpoint, this iPLA$_2$ 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$_2\alpha$, not iPLA$_2$, is an absolute requirement for AA mobilization in phagocyte cells. Whereas inhibition of cPLA$_2\alpha$ strongly blunts receptor-induced AA mobilization in phagocytes, inhibition of iPLA$_2$ by BEL does not generally affect the response (20, 38–42).

iPLA$_2$ 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$_2$ appears to play in AA deacylation/reacylation reactions in immunoinflammatory cells (7, 8), it was of interest to assess the effect of H_2O_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$_2$ (33, 34). This compound markedly enhanced the H_2O_2-induced AA mobilization, a finding that is consistent with the H_2O_2 effect being on the phospholipolytic step and not on the reacylation step. Thus, if an inhibitory effect of H_2O_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_2O_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_2O_2 on the PLA$_2$-mediated deacylation step was the cause of the AA release, then the effects of H_2O_2 plus thimerosal should be supra-additive or synergistic. This is exactly what we observed in the experiments described in this study.

Analyses of the effect of H_2O_2 on the AA incorporation capacity of the cells indicated that the oxidant did not block AA esterification into phospholipids, but actually enhanced it. Thus, despite the oxidant increasing fatty acid esterification into phospholipids, the net result was an increase in fatty acid mobilization, indicating that the effect of H_2O_2 on iPLA$_2$ is stronger and thus prevails.

Taking into account that H_2O_2 does not affect either positively or negatively the activities of the AA-reacylating enzymes arachidonoyl-CoA synthetase and arachidonoyl-CoA acyltransferase (31), a plausible explanation for the phenomena described herein is that accelerated hydrolysis of phospholipids by iPLA$_2$ in the presence of H_2O_2 leads to accumulation of intracellular lysophospholipid acceptors, which in turn triggers feedback increases in AA incorporation into phospholipids. Thus, the biochemical significance of the AA incorporation data in the H_2O_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 a pharmacological or antisense inhibition approach (7, 8, 11, 12). In this study, we employed a third approach, which is the opposite of the two employed previously. We prepared cells stably overexpressing iPLA$_2$ to confirm previous functional roles proposed for the enzyme and to study new ones. As would be expected from the model discussed above, the iPLA$_2$-overexpressing cells exhibited a significantly higher capacity to incorporate AA into phospholipids than did control cells. Also, the iPLA$_2$-overexpressing cells mobilized larger quantities of free AA in response to H_2O_2. Thus, these results provide new evidence for the key role of iPLA$_2$ in mediating phospholipid hydrolysis during oxidative stress by H_2O_2. In turn, the results provide additional evidence for the key role of iPLA$_2$ in regulating the intracellular levels of

lyso-PC to be used for fatty acid incorporation via the Lands cycle.

Studies on whether lyso-PC 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 lyso-PC appear to be so high that, even after acute inhibition of endogenous iPLA$_2$, the cells still retain at least 80% of their initial lyso-PC content (44). Thus, the initial rate of AA incorporation into islet phospholipids is not altered, suggesting that, in these cells, iPLA$_2$ is not required for the initial AA incorporation into phospholipids. Nevertheless, it should be noted that islet iPLA$_2$ is estimated to provide at least 20% of the very high lyso-PC levels that these cells contain (44), which suggests that the enzyme still possesses significant *housekeeping* activity with regard to the maintenance of endogenous lyso-PC levels.

Transient overexpression of iPLA$_2$ into COS cells has been found to significantly increase lyso-PC levels without a concomitant increase in the incorporation of exogenous AA into phospholipids, suggesting that, under these settings, lyso-PC 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 lyso-PC in untransfected 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 phagocyte cells, together with those in COS cells (25) and pancreatic islet cells (44, 46), appear to suggest that the mechanisms for lysophospholipid generation and the PLA$_2$ enzymes 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 lyso-PC (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 lyso-PC may lead to significant changes in the initial rate of AA incorporation. Thus, lyso-PC-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 nonspecific 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_2O_2-induced apoptosis in U937 cells was higher if iPLA$_2$-overexpressing cells were 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 resulting in total inhibition of cellular iPLA$_2$ did not prevent H_2O_2-induced apoptosis. This suggests that iPLA$_2$ activity is, in the strict sense, not necessary for 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 (Ref. 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 and 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) reported that apoptosis of caspase-3-transfected MCF7 cells exposed to UV light 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 light-induced apoptosis of caspase-3-transfected MCF7 cells is also independent of iPLA$_2$ (19).

Collectively, all of the aforementioned examples suggest that, even though iPLA$_2$ 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, iPLA$_2$-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 iPLA$_2$ hydrolysis of cellular phospholipids during apoptosis, *viz.* free fatty acids or perhaps lysophospholipids such as lyso-PC (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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