Involvement of Group VIA Calcium-Independent Phospholipase A_2 in Macrophage Engulfment of Hydrogen Peroxide-Treated U937 Cells[1]

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Hydrogen peroxide-induced apoptosis of U937 cells results in substantial hydrolysis of membrane phospholipids by calcium-independent group VIA phospholipase A_2 (iPLA$_2$-VIA). However, abrogation of cellular iPLA$_2$-VIA neither delays nor decreases apoptosis, suggesting that, beyond a mere destructive role, iPLA$_2$-VIA may serve other specific roles. In this study, we report that phagocytosis of apoptosing U937 cells by macrophages is blunted if the cells are depleted of iPLA$_2$-VIA by treatment with an inhibitor or an antisense oligonucleotide, and it is augmented by overexpression of iPLA$_2$-VIA in the dying cells. Thus, the magnitude of macrophage phagocytosis correlates with the level of iPLA$_2$-VIA activity of the dying cells. Eliminating by antisense oligonucleotide technology of cytosolic group IVA phospholipase A_2 does not attenuate phagocytosis of U937 dying cells by macrophages. Incubation of U937 cells with different fatty acids has no effect on either the extent of hydrogen peroxide-induced apoptosis or the degree of phagocytosis of the dying cells by macrophages. However, preincubation of the macrophages with lysophosphatidylcholine before exposing them to the dying cells blocks phagocytosis of the latter. These results indicate that formation of lysophosphatidylcholine by iPLA$_2$-VIA in hydrogen peroxide-treated U937 cells to induce apoptosis directly contributes to their efficient clearance by macrophages. *The Journal of Immunology,* **2006, 176: 2555–2561.**

Apoptosis is a type of cell death that does not involve an inflammatory response and occurs in a tightly controlled manner. In contrast with cell death by necrosis, apoptosis involves the activation of catabolic mediators and enzymes before cytolysis. Many cellular changes that take place during apoptosis play important roles in the safe and efficient recognition and clearance of the dying cells by neighboring phagocytes. One of these changes is the scrambling of plasma membrane phospholipids, which results in loss of membrane asymmetry and the exposure of anionic phospholipids such as phosphatidylserine (PS)[3] on the cell surface (1, 2). This event constitutes one of the best-described "eat-me" signals that allow professional phagocytes to identify an apoptotic cell as such and proceed to its engulfment. PS is recognized by a recently cloned receptor on the phagocyte surface whose blockade abrogates the phagocytosis of the apoptotic cell (1–4).

Apoptosis induced by different agents also is associated with the hydrolysis of membrane phospholipids by calcium-independent group VIA phospholipase A_2 (iPLA$_2$-VIA) (5–9). In addition to merely serving a destructive function, iPLA$_2$-VIA-catalyzed phospholipid hydrolysis gives rise to the formation of lysophosphatidylcholine (lysoPC) in the apoptotic cell. A portion of this lysoPC is secreted to the extracellular milieu, where it is thought to function as an attraction signal for the phagocytes to find the apoptotic cell (7).

The involvement of iPLA$_2$-VIA in apoptosis was first demonstrated by Atsumi et al. (5, 6) in U937 promonocytes treated with Fas ligand or TNF-α plus cycloheximide. We have shown recently that apoptosis of U937 cells in response to hydrogen peroxide also is associated with increased iPLA$_2$-VIA activity (9). In this system, overexpression of iPLA$_2$-VIA increases membrane phospholipid hydrolysis and augments the rate of apoptosis (9). However, importantly, inhibition of endogenous iPLA$_2$-VIA activity by either methylarachidonyl fluorophosphonate (MAFP) or an antisense oligonucleotide does not prevent apoptosis, suggesting that, although iPLA$_2$-VIA does participate in membrane phospholipid destruction during apoptosis, it is not absolutely required for the apoptotic process to fully develop (9). Collectively, these results suggest that, beyond a merely destructive role, iPLA$_2$-VIA may be centrally involved in providing accessory signals that are triggered along with the destructive process itself.

In the present study, we provide evidence that iPLA$_2$-VIA-mediated membrane phospholipid hydrolysis in apoptotic cells plays a role in enabling macrophages to engulf the dying cells. We show that the iPLA$_2$-VIA metabolite responsible for this effect is lysoPC. Thus, iPLA$_2$-VIA-induced lysoPC within the dying cell may function as a direct "eat me" signal that helps macrophages recognize an engulf the apoptotic cell.

Materials and Methods

Reagents

Human U937 cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. P388D$_1$ macrophages stably expressing the enhanced GFP (EGFP) were provided by Dr. Y. Shirai (Biosignal Research Center, Kobe University, Kobe, Japan). Pyrrophenone was provided by Dr. T. Ono (Shionogi, Osaka, Japan). MAFP (methylacetate solution), free fatty acids, and the anti-iPLA$_2$-VIA were from Cayman Chemical. Anti-cPLA$_2\alpha$ Ab was from BD. All other reagents were obtained from either Sigma-Aldrich or BIOMOL.

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[3] Abbreviations used in this paper: PS, phosphatidylserine; PL, phospholipase; iPLA$_2$-VIA, calcium-independent group VIA PLA$_2$; cPLA$_{2\alpha}$, cytosolic group IVA PLA$_2$; MAFP, methylarachidonyl flurophosphonate; lysoPC, lysophosphatidylcholine (1-acyl-2-lyso-*sn*-glycero-3-phosphocholine); EGFP, enhanced GFP.

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Cell culture

Murine P388D$_1$ macrophages and human U937 promonocytic cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Phagocytosis assay

Phagocytosis of apoptotic cells by macrophages was determined by flow cytometry. Murine P388D$_1$ macrophages stably transfected with EGFP were used as effector cells. One million P388D$_1$ cells were seeded in 24-well culture plates in RPMI 1640 medium containing 10% serum. As a cell target, apoptotic U937 cells were used. Apoptotic cell death was induced in these cells by treating them with 500 μM H_2O_2 for 20 h (9). Afterward, the U937 cells were stained with 500 μM propidium iodide for 20 min. In some experiments, the apoptotic cells were labeled with Cy3-annexin V for 5 min. After washing the cells twice with PBS, target cells were added to the P388D$_1$ macrophage monolayers in a final volume of 1.5 ml of RPMI 1640 medium without serum. The plates were centrifuged at 300 $\times$ *g* for 3 min so as to bring the target cells into direct contact with the macrophages, thus avoiding the necessity for "find me" signals. The ratio of target cells to macrophages was 3:1. The phagocytosis reaction was allowed to proceed for 2 h in a humidified CO_2 incubator at 37°C, after which the macrophages were trypsinized by incubating them with a solution 0.25% trypsin/EDTA (Sigma-Aldrich) for 5 min. This additional step helps to detach undigested U937 cells from the macrophages. The macrophages were then scraped from the wells by using a cell scraper, washed twice with PBS, and analyzed by flow cytometry using a Coulter Epics XL-MCL cytofluorimeter. Green fluorescence from the EGFP was analyzed in FL1 (505–545 nm), while red fluorescence from propidium iodide or annexin V-Cy3 was analyzed in FL2 (555–600 nm). To quantify phagocytosis, red fluorescence was analyzed only in the cell populations exhibiting significant green fluorescence (EGFP-positive cells, i.e., macrophages). The EGFP-containing macrophages are positive for red fluorescence only if they have ingested the propidium iodide-labeled U937 cells. Phagocytosis was confirmed by confocal microscopy using a laser scanning system (Radiance 2100; Bio-Rad) coupled to a Nikon inverted microscope with a thermostatized chamber. The objective was a $\times$60, 1.4 numerical aperture, oil immersion. Green fluorescence was monitored at 488-nm argon excitation using a combination of HQ500 long-bandpass and a HQ560 short-bandpass blocking filters. Red fluorescence was monitored sequentially at 543-nm HeNe excitation using a HQ590/570 long-bandpass blocking filter.

Measurement of apoptosis

Apoptosis was analyzed by labeling with the Annexin-V Apoptosis Detection Kit (BD Pharmingen) that recognizes PS exposure on the outer leaflet of the plasma membrane. The cells were analyzed by flow cytometry using an Epics XL-MCL cytofluorimeter.

Antisense oligonucleotide treatments

The iPLA$_2$-VIA antisense oligonucleotide used in this study has been described in previous studies from our laboratory (9–11). 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 measuring adherent cell protein.

The human cPLA$_2\alpha$ antisense oligonucleotide used in this work was originally described by Tomassini and Cantoni (12). The oligonucleotides used were as follows: (antisense) 5′-GTA AGG ATC TAT AAA TGA CAT-3′; and (sense; random sequence of the antisense bases), 5′-GAT GAT CAG ATA TAC GAT AAT-3′). The oligonucleotides were phosphorothioate-modified (MWG Biotech). Nucleotides were introduced into the cell by the Nucleofector method (Amaxa Biosystems), following the manufacturer's instructions (program V-001). The cells were used 48 h after nucleofection.

Production of transfectants stably expressing iPLA$_2$-VIA

pcDNA3.1 vector containing group VIA iPLA$_2$ (provided by Dr. S. Jackowski, St. Jude Children's Research Hospital, Memphis, TN) ($\sim$2 μg per 10^6 cells) was transfected by electroporation at 270 V (975 μF) using a Gene Pulser II electroporator (Bio-Rad). To select for the transfected cells, they were incubated in medium containing 1 mg/ml geneticin. To obtain the transfectants stably expressing iPLA$_2$-VIA, the transfected cells were cloned by limiting dilution in medium containing 300 μg/ml geneticin. After 2 wk, wells containing a single colony were chosen for further expansion, and the iPLA$_2$-VIA expression was analyzed by immunoblot and measurement of iPLA$_2$ activity (9). The clones were always grown in medium containing 300 μg/ml geneticin.

Data presentation

Assays were conducted in duplicate or triplicate. Each set of experiments was repeated at least three times with similar results. Paired Student's *t* test was used to compare treated samples with controls. Values of *p* are given in the figure legends.

Results

We have demonstrated previously that iPLA$_2$-VIA-mediated phospholipid hydrolysis occurs during oxidative stress-induced apoptosis (9, 10). However, iPLA$_2$-VIA appears to be dispensable for the apoptotic process to occur, because inhibition of enzyme activity does not prevent apoptosis (9). These data suggested that, beyond a mere destructive role, iPLA$_2$-VIA may be playing other roles during apoptosis.

We set up a flow cytometry assay of phagocytosis of apoptotic cells in which U937 cells exposed to H_2O_2 to induce apoptosis were coincubated with P388D$_1$ macrophages, and a centrifugation was conducted to bring target and effector cells together. This procedure ensured that the extent of phagocytosis was not limited by the generation of attraction signals by the apoptotic cells, because macrophages were already put into direct contact with the dying cells. The apoptotic cells were stained with propidium iodide and/or Cy3-annexin V and exposed to P388D$_1$ macrophages stably expressing EGFP. The number of macrophage cells positive for both red and green colors is determined by flow cytometry, and this provides a direct estimation of phagocytosis of apoptotic cells by the macrophages (Fig. 1). For most experiments described below, propidium iodide-labeled cells were used, which indicates cells in late apoptosis. However, we also used cells in early apoptosis (Cy3 annexin V-positive, propidium iodide-negative; treatment with H_2O_2 for periods of time up to 12 h) and obtained essentially the same results. Thus, the results described below apply to both H_2O_2-induced early and late apoptosis

As a first approach to study whether iPLA$_2$-VIA was responsible for inducing changes that lead to recognition of the apoptotic cells by the macrophages, we used the pharmacological inhibitors MAFP (a dual cPLA$_2\alpha$-iPLA$_2$ inhibitor) (13) and pyrrophenone (a selective cPLA$_2\alpha$ inhibitor) (14). The iPLA$_2$-selective inhibitor bromoenol lactone cannot be used for these studies, because long-term incubation with this compound induces apoptosis per se (15). Apoptosis was induced by treating the U937 cells with H_2O_2 for 24 h in the absence or presence of the aforementioned inhibitors. In previous studies, we characterized the H_2O_2-induced U937 cell apoptosis by using the annexin V binding assay (9). Here, we confirmed that H_2O_2 does render the U937 cells apoptotic, as judged by chromatin condensation, measured by 4′,6′-diamidino-2-phenylindole staining (Fig. 2). We have shown previously that neither MAFP nor pyrrophenone modifies the H_2O_2-induced apoptosis of U937 cells (9). After the different treatments, the cells were labeled with propidium iodide and coincubated with the EGFP-labeled P388D$_1$ macrophages. Results in Fig. 3*A* show that phagocytosis of apoptotic U937 cells was markedly reduced if the cells were treated with MAFP but not with pyrrophenone. In vitro activity assays confirmed that, at the concentrations used, MAFP and pyrrophenone completely abrogated iPLA$_2$ and cPLA$_2\alpha$ activities, respectively (data not shown). These results suggest that inhibition of iPLA$_2$-VIA but not of cPLA$_2\alpha$- impairs phagocytosis of apoptotic cells.

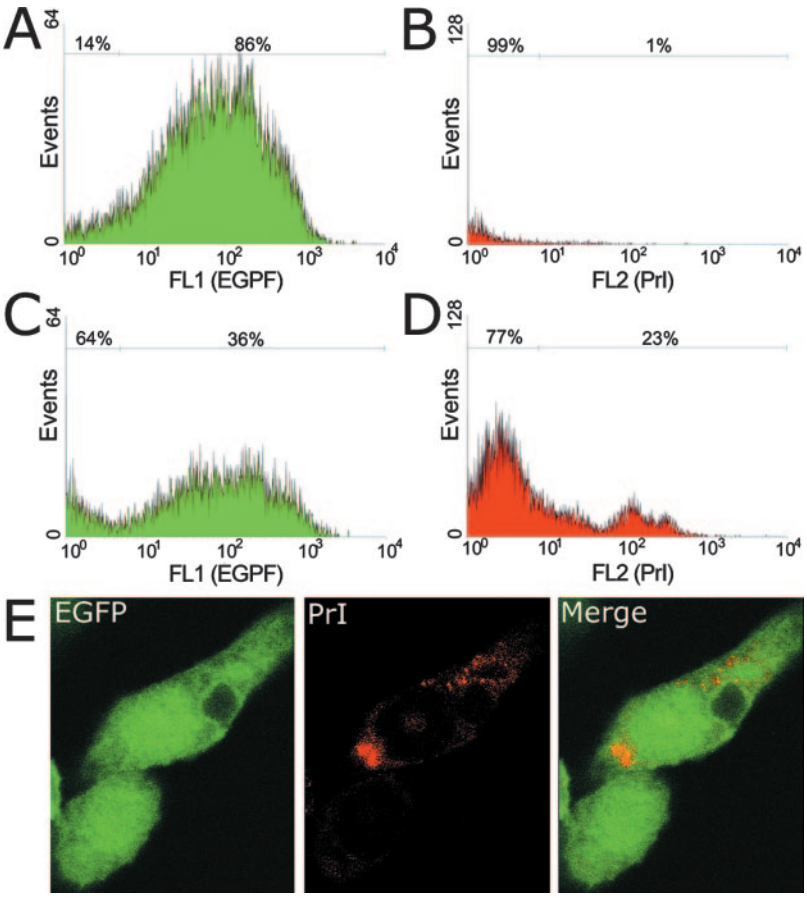

FIGURE 1. Phagocytosis of apoptotic U937 cells by $P388D_1$ macrophages. EGFP-transfected $P388D_1$ macrophages were analyzed by flow cytometry for green (*A* and *C*) and red fluorescence (*B* and *D*) before (*A* and *B*) and after (*C* and *D*) coincubating them with propidium iodide (PrI)-loaded apoptotic U937 cells for 2 h. Apoptosis was induced in these cells by treating them with 500 μM H_2O_2 for 20 h. Red fluorescence was analyzed only in the population of cells with intense green fluorescence (macrophages; 86% of the cells in *A* and 36% of the cells in *C*). Phagocytosis also was studied by confocal microscopy (*E*).

To test the above suggestion more rigorously, the effect of an iPLA-$_2$-VIA antisense oligonucleotide on phagocytosis of apoptotic cells was evaluated. The antisense oligonucleotide used is the human counterpart of the murine one that we and others have successfully used elsewhere (16–18). Using this antisense in the U937 cells, we typically detect a 70–75% decrease of both immunoreactive $iPLA_2$-VIA protein and cellular $iPLA_2$ activity (Fig. 3*B* and Refs. 9–11). Phagocytosis of the $iPLA_2$-VIA-deficient cells by $P388D_1$ macrophages was considerably lower (Fig. 3*B*), thus providing again strong evidence for the involvement of $iPLA_2$-VIA in this process. Because cells made deficient in $iPLA_2$-VIA by this antisense procedure undergo apoptosis at levels similar to those exhibiting normal $iPLA_2$-VIA levels (Ref. 9 and data not shown), the percent apoptotic cells in the experiments depicted in Fig. 3 is the same under all conditions.

In the next set of experiments, U937 cells stably overexpressing $iPLA_2$-VIA (9) were used. $iPLA_2$-VIA-overexpressing cells rendered apoptotic by treatment with H_2O_2 were found to be phagocytosed by $P388D_1$ macrophages to a greater extent than apoptotic cells expressing normal $iPLA_2$-VIA levels (Fig. 3*C*). Collectively, the data in Fig. 3 do support the involvement of $iPLA_2$-VIA of apoptosing U937 cells in enabling phagocytosis by the macrophages.

To study whether the role of $iPLA_2$-VIA in H_2O_2-treated cells is generalizable to other cell types, we conducted studies with the lymphocyte-like cell line Jurkat. Although in these cells H_2O_2 did induce apoptosis, as judged by the annexin V binding assay, phagocytosis of apoptotic Jurkat cells by macrophages was not altered by inhibiting endogenous $iPLA_2$ activity with 10 μM. In keeping with this finding, and in stark contrast with U937 cells (10), Jurkat cells did not readily release fatty acids into the incubation medium in response to H_2O_2. These data suggest that the role of $iPLA_2$ during apoptosis appears to be peculiar to cells in which $iPLA_2$-VIA can be potently activated by H_2O_2 (i.e., U937 cells but not Jurkat cells). In this regard, it is interesting to note that Jurkat cells appear to contain 2- to 3-fold less $iPLA_2$ activity than U937 cells (R. Pérez, M. A. Balboa, and J. Balsinde, unpublished data).

In the next series of experiments, we used TNF-α, a well known inducer of apoptosis in the various cell types, including U937 cells (5, 6). We confirmed that U937 cells exposed to TNF-α for 24 h underwent apoptosis, as judged by the annexin V binding assay, and were readily engulfed by the $P388D_1$ macrophages when assayed according to our phagocytosis protocol described above. However, phagocytosis of TNF-α-treated cells was not inhibited if the cells were first treated with 10 μM MAFP to block cellular $iPLA_2$. These results indicate that the role of $iPLA_2$-VIA during H_2O_2-induced apoptosis may not be generalizable to other apoptotic stimuli or cell systems.

In the H_2O_2-treated U937 cells, the data show a correlation between the levels of cellular $iPLA_2$-VIA activity within the apoptotic cell and the extent of phagocytosis. Thus, it could be that a metabolite generated by $iPLA_2$-VIA in the apoptotic cell is responsible for facilitated phagocytosis by the macrophage.

$iPLA_2$-VIA-mediated fatty acid release occurs abundantly during H_2O_2-induced apoptosis of U937 cells (9, 10). Thus, we investigated whether $iPLA_2$-VIA-mediated fatty acid deacylation reactions during apoptosis could be involved in providing signals that facilitate phagocytosis. In the first series of experiments, we prepared cells enriched in different fatty acids by culturing them for 3 days in medium containing 10 μM corresponding fatty acid. The fatty acids tested were arachidonic acid, eicosapentaenoic

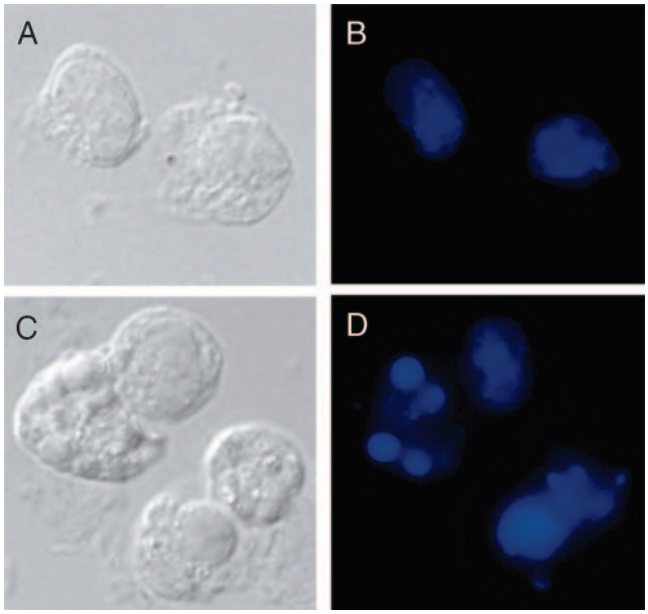

FIGURE 2. Effect of H_2O_2 on nuclear morphology of U937 cells. The cells were treated without (*A* and *B*) or with (*C* and *D*) 500 μM H_2O_2 for 20 h, stained with 4′,6′-diamidino-2-phenylindole, and examined by fluorescence microscopy. *A* and *C* are the Nomarski images, and *B* and *D* show the fluorescence of the same cells.

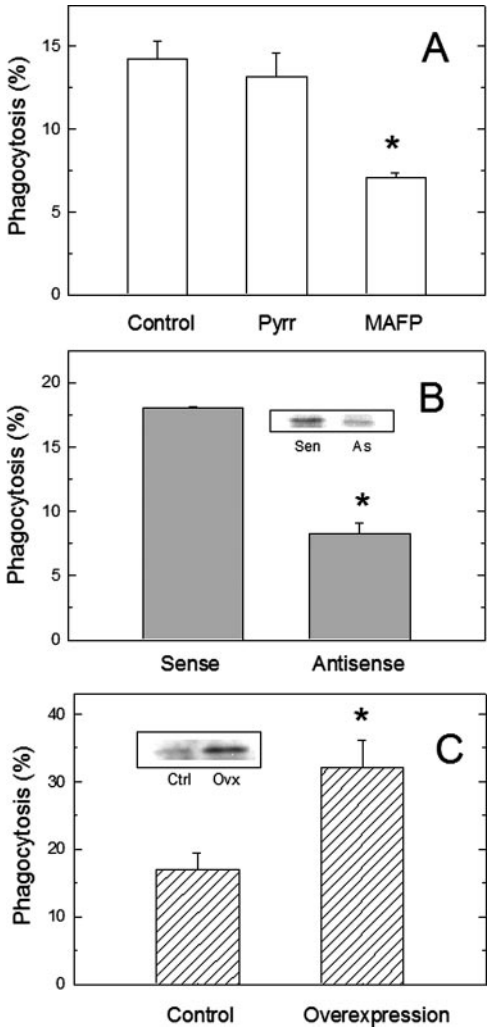

FIGURE 3. iPLA$_2$-VIA modulates phagocytosis of apoptotic cells by macrophages. *A*, U937 cells were rendered apoptotic by incubating them with 500 μM H_2O_2 for 20 h in the absence (Control) or presence of the cPLA$_2\alpha$ inhibitor pyrrophenone (Pyrr; 2 μM) or the dual cPLA$_2\alpha$-iPLA$_2$-VIA inhibitor MAFP (10 μM), as indicated. *B*, U937 cells were treated with an antisense oligonucleotide specific for iPLA$_2$-VIA or a control sense oligonucleotide, as indicated. iPLA$_2$ protein level after the treatment with the oligonucleotides was analyzed by immunoblot (see *inset*). *C*, U937 cells stably overexpressing iPLA$_2$-VIA (referred to in the abscissa as Overexpression, Ovx) were prepared, and the level of iPLA$_2$ immunoreactive protein was analyzed by immunoblot (see *inset*). Apoptosis was induced by incubating the U937 cells with 500 μM H_2O_2 for 20 h. The apoptotic cells were washed and coincubated with the EGFP-transfected macrophages for 2 h. Phagocytosis was determined by flow cytometry, and it is presented as a percent of the number of green cells exhibiting red fluorescence with respect to the total number of green cells. Results are given as means ± SEM of three independent determinations (*, $p < 0.01$).

acid, docosahexaenoic acid, and palmitic acid. If a free fatty acid was responsible for favoring macrophage phagocytosis of dying cells, one would expect these fatty acid-enriched cells to be ingested by macrophages to a greater extent than cells incubated under standard conditions (i.e., no fatty acid supply). Apoptosis of the fatty acid-enriched cells in response to H_2O_2 was no different from that observed in cells cultured under normal incubation conditions. Similarly, phagocytosis of the fatty acid-enriched apoptotic cells was not changed (data not shown). Next, we added different fatty acids at the same time the apoptotic cells were coincubated with the macrophages. The fatty acids tested were arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and palmitic acid, all at 10 μM. Phagocytosis was again not changed whether or not any fatty acid was present during the 2-h incubation period (data not shown). Collectively, these data suggest that fatty acids and/or phospholipid fatty acid composition do not affect phagocytosis of apoptotic U937 cells.

LysoPC is the other major by-product of iPLA$_2$-VIA-mediated hydrolysis of cellular phospholipids (19–21). We have shown previously that U937 cells made deficient in iPLA$_2$-VIA by treatment with the antisense oligonucleotide exhibit decreased lysoPC levels, compared with untreated cells (11), and, conversely, iPLA$_2$-VIA-overexpressing cells exhibit larger lysoPC levels than untreated cells (9). To study the possibility that lysoPC on the surface of apoptotic cells facilitates engulfment by the macrophages, we exposed the H_2O_2-treated U937 cells to P388D$_1$ macrophages that had been preincubated with different concentrations of lysoPC for 30 min. Thus, if engulfment by macrophages of apoptotic cells occurs at least in part by recognition of lysoPC on the surface of the dying cells, blockade of the putative lysoPC receptors on the macrophage surface by soluble lysoPC should inhibit phagocytosis. Fig. 4 shows that phagocytosis of apoptotic cells by the macrophages was inhibited by lysoPC in a concentration-dependent manner. Importantly, if lysoPC was added after the macrophages had been exposed to the apoptotic cells, no significant inhibition of phagocytosis was observed. For these experiments, lysoPC was added in the absence of serum or albumin in the incubation medium. The experiments also were repeated using resident peritoneal macrophages from C57BL/6 mice (University of Valladolid Animal House, Valladolid, Spain), and the results obtained were the same as those obtained with P388D$_1$ macrophage-like cells and shown in Fig. 4.

The concentrations of lysoPC used in these experiments (<5 μM) were not stimulatory for the macrophages, as judged by Ca^{2+} mobilization and p42/p44MAPK phosphorylation measurements. In addition, 5 μM lysoPC did not induce apoptosis or necrosis of P388D$_1$ macrophages, or enhanced cell death of the targets, as

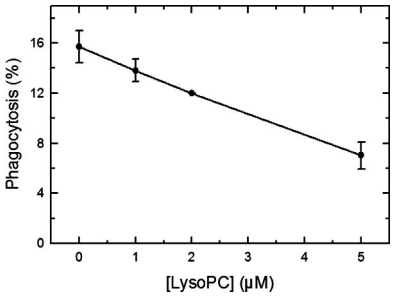

FIGURE 4. LysoPC blocks phagocytosis of apoptotic cells by macrophages. U937 cell apoptosis was induced by incubating the cells with 500 μM H_2O_2 for 20 h. The EGFP-transfected macrophages were exposed to the indicated amounts of lysoPC for 30–60 min before coincubating them with the dying cells for 2 h. Phagocytosis was determined by flow cytometry, and it is presented as a percent of the number of green cells exhibiting red fluorescence with respect to the total number of green cells. Results are given as means ± SEM of three independent determinations.

judged by the annexin V binding assay or the trypan blue exclusion assay (results not shown). Concentrations of lysoPC >5 μM could not be used, because they activated the macrophages per se. This is likely due to the changes in the physical state of the phospholipid at higher concentrations, because lysoPC's critical micellar concentration under these conditions is ~7 μM (22). As a control for these experiments, the effect of other lysophospholipids, namely lysophosphatidylethanolamine and lysophosphatidic acid, on macrophage phagocytosis of apoptotic cells also was assayed. Neither of these lysophospholipids, used at 5 μM, exerted any effect, thus reinforcing the notion that lysoPC is specifically involved in the recognition of apoptotic cells by the macrophages.

As another control, we studied the effect of lysoPC on macrophage phagocytosis of yeast-derived zymosan. Alexa Fluor 594-labeled zymosan (Molecular Probes) was used at a concentration of 5 particles per 10^6 macrophages. The extent of zymosan phagocytosis by lysoPC-treated macrophages was no different from the one observed with macrophages not exposed to the lysophospholipids. Thus, the inhibitory effect of exogenous lysoPC appears to be specific for the engulfment of apoptotic cells.

Although the molecular composition of the fatty acid at the sn-1 position of cellular lysoPC can be regulated by multiple CoA-dependent and -independent transacylation reactions, formation of lysoPC in cells can be conducted only by A-type PLs. Having established previously that $iPLA_2$-VIA does regulate lysoPC levels in U937 cells (9, 11), we wanted to investigate whether other PLA_2 forms present in the U937 cells also catalyzed lysoPC formation during apoptosis. Using RT-PCR, we investigated the expression of different PLA_2 forms in the U937 cells. PLA_2s sought for were those of groups IB, IIA, IID, IIE, IIF, III, IVA, IVB, IVC, V, VIB, and X. Of those, only cytosolic group IVA PLA_2 ($cPLA_2\alpha$) was

Table I. *Oligonucleotide primers used for amplification of PLA_2 groups from U937 cell cDNA*[a]

PLA_2 Group	Product Sequences	Product (bp)	Detection
IB PLA_2		341	
Sense	5′-TCCTTGTGCTAGCTGTGCTG-3′		
Antisense	5′-TGAAGGCCTCACACTCTTTG-3′		
IIA PLA_2		443	
Sense	5′-CTTACCATGAAGACCCTCCTACTGTTGGCA-3′		
Antisense	5′-GAGGGGACTCAGCAACGAGGGGTGCT-3′		
IID PLA_2		390	
Sense	5′-ATGGAACTTGCACTGCTGTGTG-3′		
Antisense	5′-CAGTCGCTTCTGGTAGGTGTCC-3′		
IIE PLA_2		369	
Sense	5′-ATGAAATCTCCCCACGTGCTGG-3′		
Antisense	5′-TGTAGGTGCCCAGGTTGCGGCG-3′		
IIF PLA_2		474	
Sense	5′-ATGAAGAAGTTCTTCACCGTG-3′		
Antisense	5′-ACTGCAGGTGACCTCCTGAGG-3′		
III PLA_2		500	
Sense	5′-TGCCTACAGAATCAGCACGA-3′		
Antisense	5′-TTGAGCAGCTGGAACTCGAT-3′		
IVA PLA_2		450	Yes
Sense	5′-GAGTTTTGGGCGTTTCTGGT-3′		
Antisense	5′-ACGGCAGGTTAAATGTGAGC-3′		
IVB PLA_2		420	
Sense	5′-GAGCGAGTTACGAGAATTCC-3′		
Antisense	5′-CACATGGGATTCCAGATCAG-3′		
IVC PLA_2		287	
Sense	5′-TACTCTCTGACCGACTTCTG-3′		
Antisense	5′-GTGAGTTCTGACCAGTCTTC-3′		
VPLA_2		359	
Sense	5′-CAAGGAGGCTTGCTGGACCTAA-3′		
Antisense	5′-CAGAGGATGTTGGGAAAGT-3′		
VIA PLA_2		2420/2256	Yes
Sense	5′-ATGCAGTTCTTTGGACGCCTG-3′		
Antisense	5′-TGGGTGAGAGCAGCAGCT-3′		
VIB PLA_2		476	
Sense	5′-AAAGCTTTTGTGTTCAGAAAC-3′		
Antisense	5′-CCCTTCCAACTGCAGCTGATC-3′		
X PLA_2		327	
Sense	5′-GATCCTGGAACTGGCAGGAA-3′		
Antisense	5′-TCAGTCACACTTGGGCGAGT-3′		

[a] Expression of the different PLA_2 groups was identified by RT-PCR using the primers indicated above.

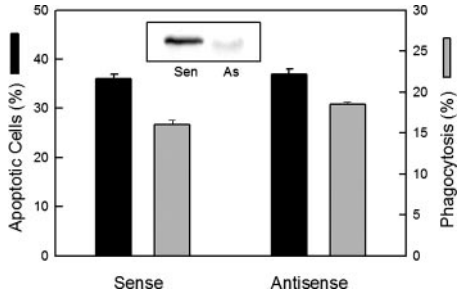

FIGURE 5. Antisense inhibition of cPLA$_2\alpha$ does not affect U937 cell apoptosis or phagocytosis of the apoptotic cells by macrophages. U937 cells were treated with an antisense oligonucleotide specific for cPLA$_2\alpha$ or a control random oligonucleotide, as indicated. cPLA$_2\alpha$ protein level after the treatment with the oligonucleotides was analyzed by immunoblot (see *inset*). U937 cell apoptosis was induced by incubating the cells with 500 μM H_2O_2 for 20 h. U937 cell apoptosis (■) was analyzed by the annexin V-binding assay. Apoptotic U937 cells were washed and coincubated with the macrophages for 2 h. Phagocytosis (▨) was determined by flow cytometry, and it is presented as a percentage of the number of green cells exhibiting red fluorescence with respect to the total number of green cells (EGFP-transfected macrophages). Results are given as means ± SEM of three independent determinations (differences are statistically not significant).

detected in the U937 cells (Table I). To study the role of this enzyme in lysoPC production during apoptosis, we inhibited its expression by antisense oligonucleotide technology. Fig. 5 shows that, 48 h after transfection, immunoreactive cPLA$_2\alpha$ was barely detectable, but robust expression was observed in homogenates treated with a random oligonucleotide control. The apoptotic response of the cPLA$_2\alpha$-depleted cells to H_2O_2 was identical with those of cells expressing normal cPLA$_2\alpha$ levels (Fig. 5). These data are consistent with the lack of effect of pyrrophenone (Fig. 3*A*) and, together, demonstrate that cPLA$_2\alpha$ is not required for this type of apoptosis. Importantly, phagocytosis of the cPLA$_2\alpha$-deficient apoptotic cells by the macrophages was also not impaired, indicating that, unlike iPLA$_2$-VIA, cPLA$_2\alpha$ is not involved in providing "eat me" signals (Fig. 5).

Discussion

Efficient clearance of apoptotic cells, a critical process for tissue homeostasis, is conducted by phagocytic cells. This process is initiated by the display of "eat me" signals on the surface of the dying cells. Thus, "eat me" signals represent markers for phagocytes to identify and engulf dying cells. Different types of "eat me" signals have been identified, including new molecules that appear on the surface (e.g., PS or annexin I) (1–4, 23), surface molecules that undergo modifications (e.g., ICAM-3 or CD31) (24, 25), and indirect signals generated by interaction of serum components with the apoptotic cell surface (26).

In the present study, we have found that a functionally active iPLA$_2$-VIA enzyme within the H_2O_2-treated, apoptosing cells is required for them to be efficiently recognized and engulfed by macrophages. By either blunting or increasing the levels of iPLA$_2$-VIA in the apoptotic cells, we have found a correlation between iPLA$_2$-VIA activity and the extent of phagocytosis of the dying cells by the macrophages, suggesting that a metabolic product of iPLA$_2$-VIA is involved in the process. We have identified this metabolite as lysoPC. Thus, the present studies identify lysoPC as a direct "eat me" signal for phagocytes. Importantly, it is the lysoPC that is generated specifically by iPLA$_2$-VIA that functions as an "eat me" signal, because abrogation of the one other PLA$_2$ present in the cells, cPLA$_2\alpha$, has no discernible effect. The latter result would be consistent with previous studies suggesting that cPLA$_2\alpha$ is inactivated during U937 cell apoptosis induced by Fas ligand or TNF-α (5, 6, 27).

However, the role of lysoPC as an "eat me" signal for apoptosis may be limited to certain conditions involving sustained iPLA$_2$-VIA activity. The lysoPC role could not be appreciated with Jurkat cells or with U937 cells when TNF-α was used as an inducer of apoptosis. In both of these cases, iPLA$_2$-VIA-mediated phospholipid hydrolysis does not occur at the high levels observed in H_2O_2-treated U937 cells (9, 10). Thus, it appears that the lysoPC role as an "eat me" signal may be significant only when iPLA$_2$-VIA substantially participates in phospholipid degradation during apoptosis.

Of considerable interest to our present results, elegant studies by Kim et al. (26) have demonstrated that apoptotic cells generate a surface epitope recognized by natural IgM Abs that results in complement activation and phagocytosis. The epitope recognized was the phosphoryl choline moiety of lysoPC (26). Interestingly, enhanced IgM binding could be suppressed by the unspecific iPLA$_2$ inhibitor bromoenol lactone, making it likely that also in this system, lysoPC was being generated by an iPLA$_2$, although the molecular identity of the actual iPLA$_2$ form involved was not determined (26).

In H_2O_2-treated, apoptosing U937 cells, different molecular approaches, together with the fact that U937 appear not to express the group VIB iPLA$_2$ isoform, have clearly indicated the specific involvement of the iPLA$_2$-VIA isoform in lysoPC production. Also, our phagocytosis studies were conducted in the total absence of serum, thus ruling out the participation of any serum component in the recognition of apoptotic cells by the macrophages. Moreover, phagocytosis of dying cells by macrophages could be inhibited by preincubating the macrophages with lysoPC, clearly suggesting that macrophages can recognize lysoPC directly. The ability of lysoPC to act as a direct (this study) or indirect (26) "eat me" signal may depend on cell type or apoptotic stimuli used, but it appears likely that the two lysoPC functions may coexist during phagocytosis of apoptotic cells.

It is interesting to speculate with the mechanism by which lysoPC produced by iPLA$_2$-VIA attack on membrane phospholipids gets exposed to the cell surface and how the lipid is recognized by phagocytes. As discussed elsewhere (28), it appears likely that phospholipid scrambling may represent the main mechanism for lysoPC exposure to occur. Phospholipid scrambling is an early event in apoptosis and results in the outer exposure of the negatively charged aminophospholipid PS. PS exposure constitutes a primary "eat me" signal for phagocytes, and these cells possess receptors for PS that mediate engulfment of the dying cell (1–4), Although recent studies have questioned the view that the PS receptor primarily functions in apoptotic cell clearance (29), it is possible that macrophages possess receptors that specifically recognize lysoPC on the surface of the dying cell. This lysoPC receptor may either mediate engulfment of the dying cell or act indirectly to promote binding and recruit other signaling receptors involved in the engulfment. A G-protein-coupled receptor present in macrophages and other cells, G2A, was reported to bind lysoPC with high affinity (30). Although more recent results have failed to confirm that lysoPC directly binds to G2A (31), contrasted evidence exists that expression of G2A in Jurkat T cells confers the ability to migrate in response to lysoPC (32, 33) Thus, G2A may effect certain cellular responses to lysoPC by an indirect mechanism in which lysoPC modifies another receptor or process that in turn regulates the G2A receptor.

To conclude, our studies highlight a key role for iPLA$_2$-VIA and its by-product lysoPC in mediating the recognition and subsequent engulfment of H_2O_2-treated, apoptosing cells by macrophages. It

is noteworthy that the control of the steady-state level of lysoPC is one the earliest biological functions discovered for iPLA$_2$-VIA in cells (for reviews, see Refs. 19–21). In resting cells, iPLA$_2$-VIA activity is quite significant, as judged by the measurable decrease of lysoPC levels after acute inhibition of the enzyme (19–21). Importantly, iPLA$_2$-VIA is cleaved by caspase-3 during apoptosis, yet the specific activity of the enzyme remains unchanged (5, 6). This suggests that certain apoptotic signals may render the membrane more susceptible to iPLA$_2$-VIA (10), or that a putative iPLA$_2$-VIA-regulatory protein may be modified in vivo, leading to increased phospholipid hydrolysis and the accumulation of lysoPC. Studies are currently in progress to elucidate the mechanisms of regulation of iPLA$_2$-VIA activity during apoptosis induced by H_2O_2.

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Disclosures

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