

# Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells

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**Abstract** Arachidonic acid (AA) participates in a reacylation/deacylation cycle of membrane phospholipids, the so-called Lands cycle, that serves to keep the concentration of this free fatty acid in cells at a very low level. To manipulate the intracellular AA level in U937 phagocytes, we have used several pharmacological strategies to interfere with the Lands cycle. We used inhibitors of the AA reacylation pathway, namely thimerosal and triacsin C, which block the conversion of AA into arachidonoyl-CoA, and a CoA-independent transacylase inhibitor that blocks the movement of AA within phospholipids. In addition, we used cells overexpressing group VIA phospholipase A<sub>2</sub>, an enzyme with key roles in controlling basal fatty acid deacylation reactions in phagocytic cells. All of these different strategies resulted in the expected increase of cellular free AA but also in the induction of cell death by apoptosis. Moreover, when used in combination with any of the aforementioned drugs, AA itself was able to induce apoptosis at doses as low as 10 μM. Blocking cyclooxygenase or lipoxygenases had no effect on the induction of apoptosis by AA. Collectively, these results indicate that free AA levels within the cells may provide an important cellular signal for the onset of apoptosis and that perturbations of the mechanisms controlling AA reacylation, and hence free AA availability, may decisively affect cell survival.—Pérez, R., X. Matabosch, A. Llebaria, M. A. Balboa, and J. Balsinde. **Blockade of arachidonic acid incorporation into phospholipids induces apoptosis in U937 promonocytic cells.** *J. Lipid Res.* 2006. 47: 484–491.

**Supplementary key words** calcium-independent phospholipase A<sub>2</sub> • deacylation • reacylation • Lands cycle

The availability of free, unesterified arachidonic acid (AA) is known to be one of the limiting factors for the production of prostaglandins and leukotrienes by activated inflammatory cells. AA is the intermediate in a deacylation/reacylation cycle of membrane phospholipids, the so-

called Lands cycle, in which the fatty acid is hydrolyzed from phospholipids by phospholipase A<sub>2</sub> (PLA<sub>2</sub>) and reincorporated by the concerted action of fatty acyl-CoA synthetase and lysophospholipid acyltransferase (1–3). In unstimulated cells, the reacylation pathway dominates over the phospholipolytic step; hence, AA levels are kept very low. However, given this dominance of AA reacylation over AA deacylation in cells of phagocytic origin, the accumulation of free fatty acid may also occur if the reacylation is inhibited (2). When cellular stimulation occurs, agonist-activated PLA<sub>2</sub> shifts the cycle toward the accumulation of free AA, which is then available for eicosanoid synthesis. Nevertheless, AA reacylation is still very significant in activated cells, as manifested by the fact that only a minor portion of the AA released by PLA<sub>2</sub> is converted into oxygenated metabolites, the remainder being rapidly incorporated back into phospholipids (2).

AA incorporation into phospholipids is critically dependent on the availability of certain lysophospholipid acceptors, particularly lysophosphatidylcholine [1-acyl-2-lyso-*sn*-glycero-3-phosphocholine (lysoPC)] and, to a lesser degree, 2-lysophosphatidylinositol. In phagocytes, maintenance of the steady-state levels of lysoPC involves the continuing action of group VIA calcium-independent phospholipase A<sub>2</sub> (iPLA<sub>2</sub>-VIA) on cellular phospholipids (3–5). Thus, inhibition of iPLA<sub>2</sub> by different approaches results in a decrease of the steady-state level of lysoPC and, hence, of AA incorporation into phospholipids (3, 6, 7). Conversely, cellular overexpression of iPLA<sub>2</sub> results in increased lysoPC levels and the increased capacity of the cells to incorporate AA into phospholipids (8).

Abbreviations: AA, arachidonic acid; DAPI, 4',6-diamidino-2-phenylindole; IHP, diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)hepatine phosphonate; iPLA<sub>2</sub>-VIA, group VIA calcium-independent phospholipase A<sub>2</sub>; lysoPC, lysophosphatidylcholine (1-acyl-2-lyso-*sn*-glycero-3-phosphocholine); PARP, poly(ADP-ribose) polymerase; PC, choline glycerophospholipid; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PS, phosphatidylserine.

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Once the AA is initially incorporated into choline glycerophospholipids (PCs), it is slowly transferred to certain lysophospholipids, particularly 1-alkenyl-2-lyso-*sn*-glycero-3-phosphoethanolamine, in a reaction catalyzed by CoA-independent transacylase. The CoA-independent transacylase-driven AA phospholipid-remodeling reactions appear to be of the utmost importance for the cell to place the AA in the appropriate phospholipid pools (2, 9).

Recently, several lines of evidence have suggested that the control of AA levels may be important in signaling apoptotic cell death. For example, inhibition of cytosolic PLA<sub>2</sub>α-mediated AA release has been shown to prevent tumor necrosis factor-α-mediated apoptosis in colonocytes (10). On the other hand, overexpression of either long-chain fatty acyl-CoA synthetase or cyclooxygenase-2, which scavenge free AA by converting it into the CoA thioester derivative or prostaglandin, respectively, protects cells from apoptosis (11).

To address the link between the generation of free AA and the induction of apoptosis in U937 promonocytes, we have used several strategies to increase the cellular level of this fatty acid. These include the overexpression of iPLA<sub>2</sub>-VIA as well as the inhibition of the enzymatic mechanisms controlling AA reacylation and remodeling at several steps, including arachidonoyl-CoA synthetase and CoA-independent transacylase. The results described in this work demonstrate that increases of unesterified AA in U937 cells attributable to a blockade of reacylation result in apoptotic cell death.

## EXPERIMENTAL PROCEDURES

### Reagents

[5,6,8,9,11,12,14,15-<sup>3</sup>H]AA (200 Ci/mmol) was purchased from Amersham Ibérica (Madrid, Spain). pcDNA3.1 vector containing the mouse iPLA<sub>2</sub>-VIA gene was kindly provided by Dr. Suzanne Jackowski (St. Jude Children's Research Hospital, Memphis, TN). Pyrrolidine-1 was synthesized in our laboratory exactly as described elsewhere (12). Triacsin C was from BioMol (Plymouth Meeting, PA). 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 U/ml), and streptomycin (100 µg/ml). For experiments, the cells were incubated at 37°C in a humidified atmosphere of CO<sub>2</sub>/air (1:19) at a cell density of 0.5–1 × 10<sup>6</sup> cells/ml in 12-well plastic culture dishes (Costar). In some experiments, cells were maintained for at least 7 days in serum-free medium to obtain AA-depleted cells (13, 14).

### Measurement of [<sup>3</sup>H]AA incorporation into phospholipids

U937 cells were placed in serum-free medium for 60 min before exposure to exogenous [<sup>3</sup>H]AA (1 µM, 0.5 µCi/ml). 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, v/v). For separation of

phospholipid classes, a mobile phase consisting of chloroform-methanol-acetic acid-water (25:20:3:0.3, v/v) was used. This system allows a good resolution between major phospholipids, including phosphatidylinositol and phosphatidylserine.

### Measurement of [<sup>3</sup>H]AA phospholipid remodeling

For these experiments, the U937 cells were pulse-labeled with [<sup>3</sup>H]AA (1 µM, 0.5 µCi/ml) for 30 min at 37°C. The cells were then washed four times with medium containing 1 mg/ml bovine serum albumin to remove the nonincorporated label. Afterward, the cells were placed in serum-free medium and incubated at 37°C for the indicated periods of time. The lipids were extracted and separated as described above.

### Measurement of apoptosis

Apoptosis was analyzed using three methods: chromatin condensation, poly(ADP-ribose) polymerase (PARP) cleavage, and externalization of phosphatidylserine (PS). Chromatin condensation was analyzed by nuclear staining with the DNA binding fluorophore 4',6-diamidino-2-phenylindole (DAPI). Briefly, cells were fixed with 4% paraformaldehyde in PBS, stained for 30 min with DAPI (1 µg/ml), and mounted in an antifading medium. Fluorescence was analyzed with a Nikon TE-2000U inverted microscope with an ultraviolet light filter. PARP hydrolysis was detected by immunoblot analysis. Cells were lysed in an ice-cold lysis buffer, and 50 µg of cellular protein from each sample was separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Dilution of both primary and secondary antibodies was made in PBS containing 5% defatted dry milk and 0.1% Tween 20. After 1 h of incubation with anti-PARP antibody (Santa Cruz Technologies) at 1:1,000 dilution, blots were washed four times and the secondary peroxidase-conjugated antibody was added for another 1 h. Immunoblots were developed using the Amersham ECL system. PS externalization was determined by labeling with the Annexin-V FITC Apoptosis Detection Kit (Pharmingen), which recognizes PS exposure on the outer leaflet of the plasma membrane, according to the manufacturer's instructions. 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. Paired Student's *t*-tests were used to compare treated samples with controls. *P* values are given in the figure legends.

## RESULTS

### Effect of AA on apoptosis in U937 cells

In previous studies, we have reported that H<sub>2</sub>O<sub>2</sub> rapidly and efficiently induces apoptosis in U937 phagocytes (8) and promotes the liberation of unmetabolized free AA (15). Recent studies have suggested that AA or any of its oxidative products may be involved in the induction of apoptosis in certain cell types (11, 14). To begin testing this hypothesis in our system, we initially cultured the U937 cells in medium containing different amounts of exogenous AA (0–20 µM, 24 h incubation). Treatment of the cells with H<sub>2</sub>O<sub>2</sub> resulted in the expected increased exposure of PS on the cell surface (8); however, the extent of H<sub>2</sub>O<sub>2</sub>-induced PS externalization was not affected by the amount

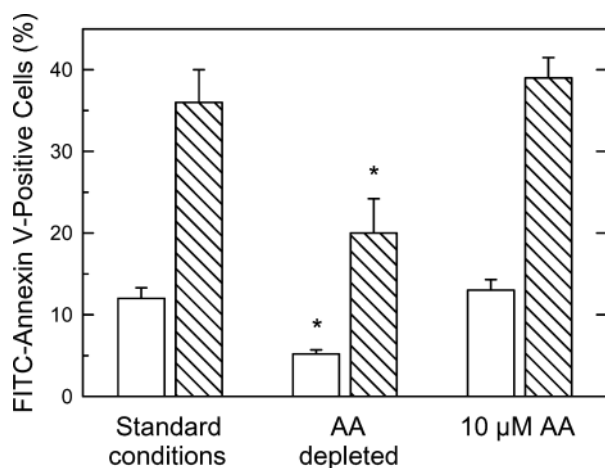
of AA initially present in the medium. H<sub>2</sub>O<sub>2</sub>-induced chromatin condensation, as analyzed by DAPI staining, was also not affected by the presence of AA (data not shown).

In the next set of experiments, we prepared cells depleted of AA by incubating them in serum-free, essential fatty acid-deficient medium for at least 7 days. This procedure results in cells containing far less AA in phospholipids than cells normally grown in serum (13, 14). **Figure 1** shows that AA-depleted cells were significantly less sensitive to H<sub>2</sub>O<sub>2</sub>-induced apoptosis than cells maintained under standard culture conditions or cells supplemented with 10 μM AA. Apoptosis induced by H<sub>2</sub>O<sub>2</sub> was also lower in the AA-depleted cells (Fig. 1).

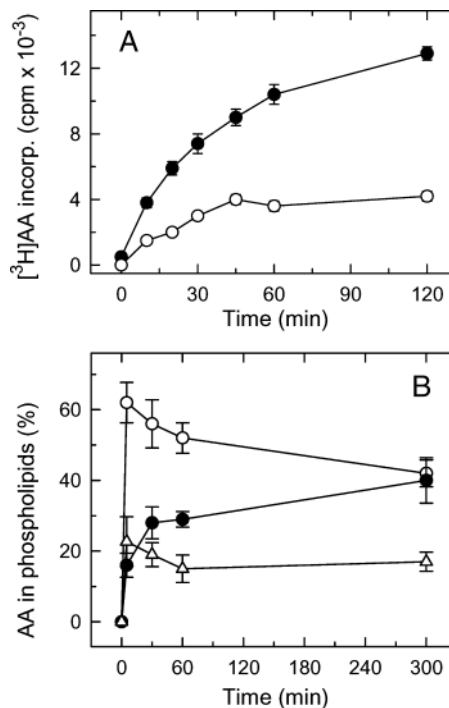
#### Inhibitors of AA esterification induce apoptosis in U937 cells

The data shown in Fig. 1 suggested that the presence of cellular AA is critical for the full induction of apoptosis. However, this observation appears to contrast with the results indicating that supplementation with exogenous AA does not significantly influence the induction of apoptosis.

Cultured cell lines of monocytic origin are known to incorporate and remodel AA into phospholipids at rates manyfold greater than their primary counterparts (i.e., blood leukocytes) (6, 16–19). In this regard, **Fig. 2** shows that U937 cells did indeed incorporate AA and remodeled it through phospholipids at a high rate. Approximately 35–40% of the total AA added to the medium was found to be esterified into phospholipids at 30 min, whereas neutral lipids incorporated lesser amounts (Fig. 2A). At early incubation times, PC incorporated the majority of AA. At longer times, the AA content in PC decreased gradually, paralleling the increased incorporation of AA in ethanolamine glycerophospholipid. Overall, these changes are



**Fig. 1.** Apoptosis of arachidonic acid (AA) depleted and non-depleted U937 cells. Cells were maintained in serum-free medium (AA depleted) or in serum-containing medium (standard conditions) supplemented with or without 10 μM AA (see abscissa). Cells were incubated for 20 h in the presence (hatched bars) or absence (open bars) of 500 μM H<sub>2</sub>O<sub>2</sub>, and apoptosis was measured by the FITC-annexin V binding assay as described in Experimental Procedures. Data are shown as means ± SEM of three independent experiments carried out in duplicate. \* *P* < 0.05.



**Fig. 2.** AA incorporation and remodeling within phospholipids in U937 cells. A: Cells were exposed to 1 μM AA (0.5 μCi/ml) for the times indicated. Afterward, AA incorporated into phospholipids (closed circles) or triacylglycerol (open circles) was determined. B: Cells, labeled with [<sup>3</sup>H]AA, were washed and then incubated without label for different periods of time. Radioactivity in choline phospholipids (open circles), ethanolamine phospholipids (closed circles), and phosphatidylinositol (open triangles) was determined. Values are given as percentages of total radioactivity present in phospholipids. Data are shown as means ± SEM of three independent experiments carried out in duplicate.

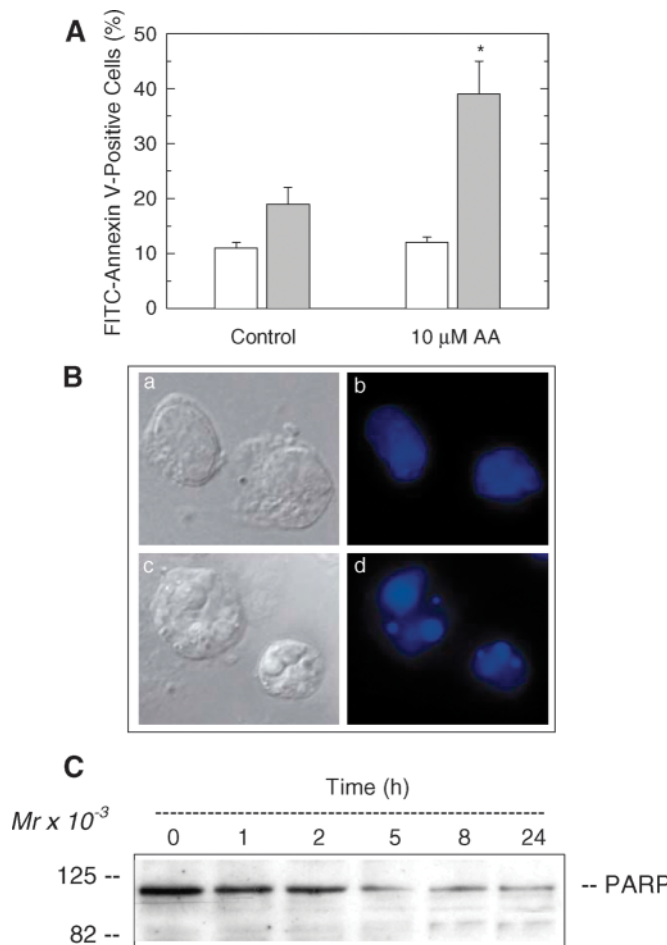
consistent with data in other systems (6, 16, 20) and reflect the remodeling action of CoA-independent transacylase on cellular phospholipids.

The high rate of AA incorporation and remodeling in U937 cells may help reconcile the results reported in Fig. 1. It is conceivable that the lack of a significant effect on apoptosis in cells exposed to exogenous AA is attributable to an exceedingly high AA reacylation rate that prevents the accumulation of unesterified AA within the cell, and hence the onset of apoptosis. If this hypothesis is correct, circumstances that prevent AA reacylation should increase the extent of apoptosis in U937 cells.

To verify this hypothesis, we first used thimerosal, an organometallic compound that is known to prevent fatty acid incorporation into phospholipids by blocking the enzyme long-chain fatty acid-CoA synthetase (21). Initial experiments demonstrated that thimerosal at concentrations up to 3 μM effectively impeded the incorporation of AA (10 μM) into phospholipids (3.61 ± 0.42 nmol of AA incorporated per million cells in the absence of thimerosal versus 0.86 ± 0.10 nmol of AA incorporated per million cells in the presence of 3 μM thimerosal; means ± SEM, *n* = 4). Higher thimerosal concentrations led to substantial cell death by necrosis, as judged by the trypan blue

exclusion assay. **Figure 3A** shows that 3  $\mu\text{M}$  thimerosal had a slight impact on its own on PS externalization but, importantly, also substantially increased PS externalization in U937 cells incubated with 10  $\mu\text{M}$  exogenous AA, which, on its own, was ineffective.

To ensure that the increase of PS externalization observed in Fig. 3A was indeed linked to apoptotic cell death, other indices of apoptosis, such as chromatin condensation and the appearance of fragments of the caspase substrate PARP, were measured. Chromatin condensation was analyzed by nuclear staining with the DNA binding fluorophore DAPI. Figure 3B shows that U937 cells treated with AA plus thimerosal for 24 h demonstrated clear signs of chromatin condensation, indicating apoptosis. Cleavage



**Fig. 3.** Effect of thimerosal on U937 cell apoptosis. **A:** Cells were incubated without (open bars) or with 3  $\mu\text{M}$  thimerosal (shaded bars) for 20 h in the absence (control) or presence of 10  $\mu\text{M}$  AA, as indicated on the abscissa. Apoptosis was measured by the FITC-annexin V binding assay as described in Experimental Procedures. Data are shown as means  $\pm$  SEM of three independent experiments carried out in duplicate. \*  $P < 0.01$ . **B:** Cells were untreated (a, b) or treated with thimerosal plus AA (c, d) for 20 h, stained with 4',6-diamidino-2-phenylindole, and examined by fluorescence microscopy. Panels a and c are Nomarski images, and panels b and d show the fluorescence of the same cells. **C:** Cells were treated with thimerosal plus AA for the indicated times and analyzed for poly(ADP-ribose) polymerase (PARP) expression by immunoblotting. Mr, relative molecular mass.

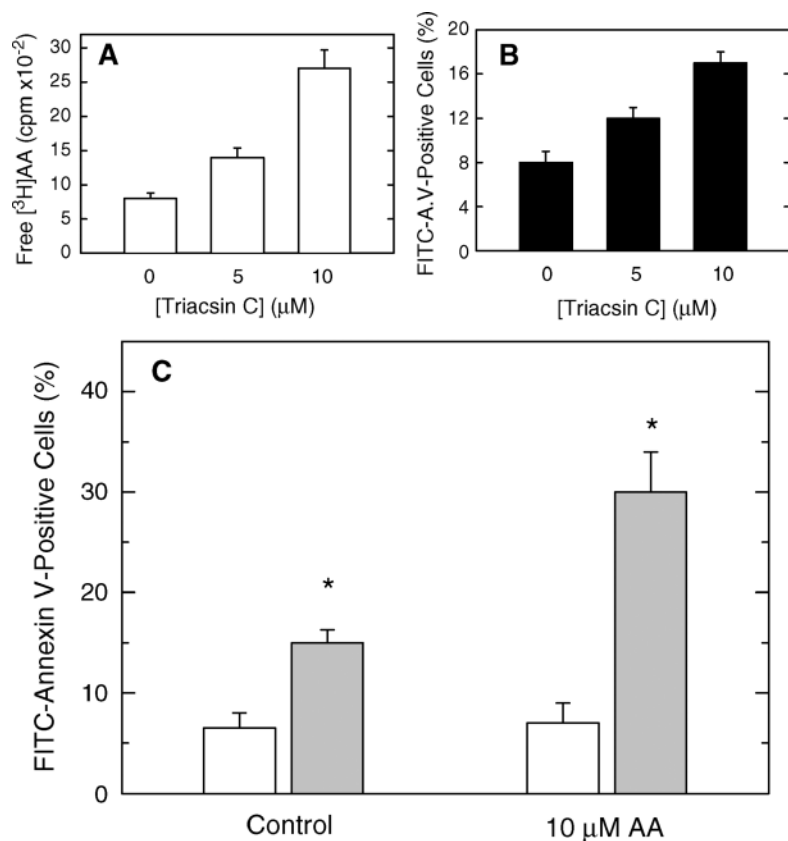
of PARP was evaluated by immunoblot analysis. Untreated cells expressed the intact PARP 112 kDa polypeptide (Fig. 3C), which was found to rapidly disappear in a time-dependent manner after exposure of the cells to AA plus thimerosal. Control experiments using cells treated with thimerosal alone or AA alone did not show any nuclear morphology changes or PARP cleavage, confirming that it is the impairment of AA esterification that is associated with apoptosis.

We next evaluated whether triacsin C, a well-established inhibitor of long-chain fatty acid-CoA synthetase (22), exerted a similar effect as thimerosal on the induction of apoptosis. **Figure 4** reveals that triacsin C inhibited AA incorporation into phospholipids and caused a concentration-dependent increase of PS externalization. Cotreatment of exogenous AA with triacsin C caused apoptotic cell death to a much greater extent (Fig. 4). Collectively, the experiments depicted in Figs. 3 and 4 suggest that conditions that lead to increased levels of unesterified AA result in apoptosis. Conversely, the data shown in Fig. 1 indicate that AA depletion blunts apoptosis.

In the experiments described above, unesterified AA levels were manipulated by inhibiting the first step of the AA incorporation route, the formation of the arachidonyl-CoA that is needed for direct incorporation into phospholipid. In the next series of experiments, we used the alternative strategy of blocking the AA phospholipid-remodeling step by inhibiting the activity of the CoA-independent transacylase. To this end, we used the selective CoA-independent transacylase inhibitor diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)heptane phosphonate (IHP) (23). Long-term incubation with IHP resulted in significant alterations of the distribution of AA among cellular lipids. The data shown in **Fig. 5A** demonstrate that IHP reduced the level of AA in phospholipids and concomitantly increased the levels of this fatty acid in triacylglycerol. Given the blockade of AA phospholipid remodeling by IHP, the shift of AA from phospholipid to triacylglycerol was to be expected and probably arises from the direct acylation of diacylglycerol via de novo synthesis (14, 24, 25). Importantly, the intracellular (cell-associated) level of unesterified AA was also noticeably increased after treatment with IHP (Fig. 5A). **Figure 5B** shows that incubation of the U937 cells with IHP resulted in increased death by apoptosis, as judged by the FITC-annexin V binding assay, and that cotreatment with exogenous AA augmented the extent of apoptosis. These results provide further evidence that conditions that lead to the accumulation of unesterified AA induce apoptosis in U937 cells.

#### Overexpression of calcium-independent PLA<sub>2</sub> augments apoptosis in U937 cells

In addition to blocking either AA incorporation or AA phospholipid remodeling, we used a third strategy to manipulate the levels of unesterified AA in cells: the use of cells transfected with the iPLA<sub>2</sub>-VIA gene. Small amounts of AA and other fatty acids are constantly being generated under resting conditions, and in certain cell types such as



**Fig. 4.** Effect of triacsin C on U937 cell apoptosis. A: Cells, labeled with [<sup>3</sup>H]AA, were incubated with the indicated concentrations of triacsin C for 20 h. Afterward, extracellular free [<sup>3</sup>H]AA was measured as described in Experimental Procedures. B: Cells were exposed to the indicated concentrations of triacsin C for 20 h. Afterward, cell death was measured by the FITC-annexin V binding assay. C: Cells were incubated without (open bars) or with 10 μM triacsin C (shaded bars) for 20 h in the absence (control) or presence of 10 μM AA, as indicated on the abscissa. Apoptosis was measured by the FITC-annexin V binding assay. Data are shown as means ± SEM of three independent experiments carried out in duplicate. \* *P* < 0.01.

phagocytic cells, this step appears to be mediated mostly by iPLA<sub>2</sub>-VIA (5–7). In accordance with this, we have previously shown that U937 cells stably overexpressing iPLA<sub>2</sub>-VIA exhibit increased levels of free AA (8). Thus, the iPLA<sub>2</sub>-VIA-overexpressing U937 cell may constitute a useful model to verify the hypothesis that unesterified AA acts as a signal for the induction of apoptosis. In these experiments, apoptosis was monitored by annexin V staining of the cell surface. Under basal conditions, the extent of apoptosis in the iPLA<sub>2</sub>-VIA-overexpressing cells was slightly higher than in control cells transfected with an empty vector. However, this difference failed to reach statistical significance (14 ± 2% of iPLA<sub>2</sub>-VIA-overexpressing cells underwent apoptosis after a 24 h incubation under standard incubation conditions, whereas 11 ± 2% of control cells transfected with an empty vector underwent apoptosis under the same conditions; means ± SEM, *n* = 7). Interestingly, when treated with H<sub>2</sub>O<sub>2</sub> for 24 h, the iPLA<sub>2</sub>-VIA-overexpressing cells underwent apoptosis to a significantly greater extent than control cells transfected with an empty vector (79 ± 2% of iPLA<sub>2</sub>-VIA-overexpressing cells vs. 38 ± 2% of control cells; means ± SEM, *n* = 6; *P* < 0.01). We have previously shown that incubation of U937 cells with H<sub>2</sub>O<sub>2</sub> results in the liberation of relatively high amounts of unmetabolized free AA (8, 15).

#### Effect of cyclooxygenase and lipoxygenase inhibitors on U937 cell apoptosis

In the next series of experiments, we sought to investigate whether the proapoptotic effect associated with

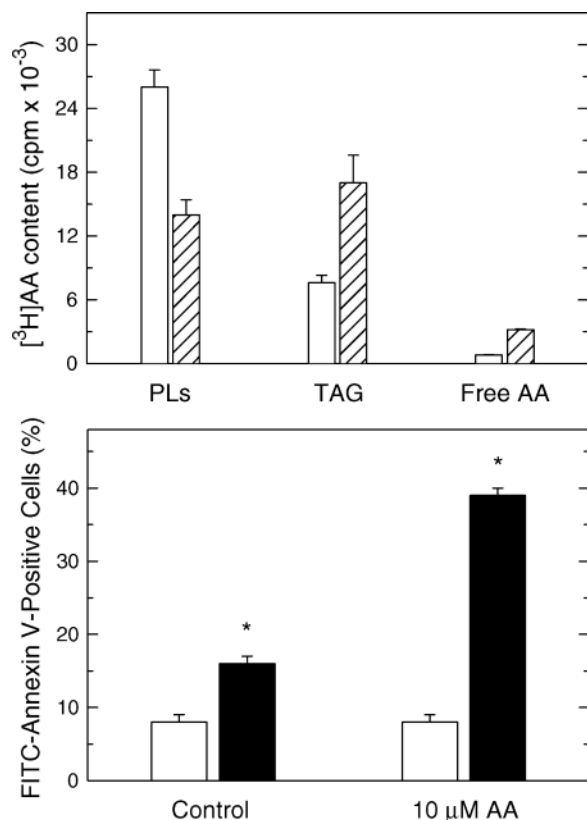
increased levels of free AA was attributable to the fatty acid itself or to oxygenated metabolites of AA generated by the cyclooxygenase or lipoxygenase pathways. The cells were incubated with the cyclooxygenase inhibitors indomethacin, aspirin, and NS-398 or the general lipoxygenase inhibitors baicalein and ebselen in the presence or absence of exogenous AA and thimerosal. Neither of these inhibitors of AA metabolism affected apoptosis induced by exogenous AA plus thimerosal (**Fig. 6**). These findings suggest that it is AA itself, and not an oxygenated metabolite, that is responsible for the proapoptotic effect.

As another control for the effect of free AA, we conducted experiments using exogenous palmitic acid. The cells were incubated with exogenous free palmitic acid (0–20 μM) in the absence or presence of 3 μM thimerosal, 10 μM triacsin C, or 25 μM IHP. None of these treatments led to U937 cell apoptosis, as judged by the annexin V binding assay.

## DISCUSSION

Using different approaches to increase the level of unesterified AA in U937 cells, we show in this study that this free fatty acid is an inducer of apoptosis.

Small amounts of free AA and other fatty acids are continuously generated during the deacylation/reacylation cycle of membrane phospholipids that occurs under resting conditions. The deacylation step involves the participation of a PLA<sub>2</sub>, and in phagocytes, it is believed



**Fig. 5.** Effect of diethyl 7-(3,4,5-triphenyl-2-oxo-2,3-dihydroimidazol-1-yl)hepatine phosphonate (IHP) on U937 cell apoptosis. A: Cells, labeled with [<sup>3</sup>H]AA, were incubated without (open bars) or with 25 μM IHP (hatched bars) for 20 h. Afterward, [<sup>3</sup>H]AA content in phospholipids (PLs), triacylglycerol (TAG), or as a free fatty acid form (free AA) was quantified. B: Cells were incubated without (open bars) or with 25 μM IHP (closed bars) for 20 h in the absence (control) or presence of 10 μM AA, as indicated on the abscissa. Apoptosis was measured by the FITC-annexin V binding assay. Data are shown as means ± SEM of three independent experiments carried out in duplicate. \* *P* < 0.01.

that a major contributor to this basal activity is iPLA<sub>2</sub>-VIA (5–7). We have recently shown that stably transfecting the U937 cells with a plasmid containing iPLA<sub>2</sub>-VIA results in notable alterations in AA metabolism. Pertinent to the results of this study, iPLA<sub>2</sub>-VIA-transfected cells show increased basal levels of unesterified AA and increased AA release responses to an oxidative insult (8).

We found that U937 cells transfected with iPLA<sub>2</sub>-VIA displayed higher apoptosis rates than control untransfected cells when exposed to H<sub>2</sub>O<sub>2</sub>. Under these conditions, the amount of unesterified AA is greatly increased, and the cell population undergoing apoptosis is doubled.

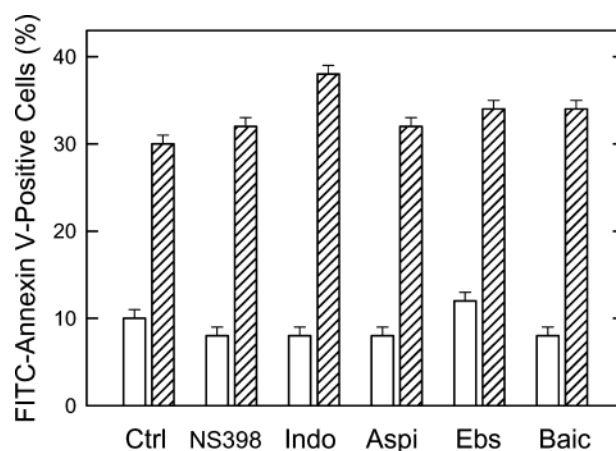
We also show in this work that, in common with most phagocytic cells, U937 cells readily and efficiently incorporate AA into cellular phospholipids. The existence of such an efficient mechanism for keeping free AA at low levels is the likely reason why resting U937 cells transfected with iPLA<sub>2</sub>-VIA do not show significant apoptosis unless treated with H<sub>2</sub>O<sub>2</sub>. In the presence of H<sub>2</sub>O<sub>2</sub>, free AA

accumulates because the oxidant activates the PLA<sub>2</sub>-mediated deacylation step, which then dominates over the fatty acid reacylation step (8, 15).

The existence of a highly active AA reacylation pathway in U937 cells may also explain why exogenous AA even at micromolar concentrations does not promote apoptosis on its own. It follows from all of these observations that impeding AA reacylation by pharmacological means would result in increased free AA and, hence, in apoptotic cell death. We have demonstrated this to be the case using two distinct, chemically unrelated inhibitors of arachidonyl-CoA synthetase, thimerosal and triacsin C. At nontoxic doses, as judged by the trypan blue exclusion assay, both compounds significantly impair AA incorporation into phospholipids and sensitize the cells to apoptotic cell death on exposure to exogenous AA.

As a third independent approach to manipulate unesterified AA levels in the cells, we used the CoA-independent transacylase inhibitor IHP. By blocking AA phospholipid remodeling (i.e., the direct transfer of AA from 1-acyl-linked choline phospholipids to 1-ether-linked ethanolamine phospholipids), AA accumulates in choline phospholipids, which are a preferred substrate for iPLA<sub>2</sub>-VIA (7, 20, 26). This should result in an increased basal hydrolysis of AA-containing phospholipids, and hence of free AA, as shown in this work. In addition, blockade of the CoA-independent transacylase pathway may also decrease the cellular levels of lysoPC, thus reducing AA reacylation, as discussed elsewhere (14).

Analogous to the effects of thimerosal and triacsin C indicated above, IHP promotes cell death when given to the cells along with exogenous AA. That IHP only moderately induces apoptosis when added without the supply



**Fig. 6.** Effect of cyclooxygenase or lipoxygenase inhibition on AA-induced U937 cell apoptosis. Cells were incubated with 5 μM NS-398, 25 μM indomethacin (Indo), 25 μM aspirin (Aspi), 10 μM ebselen (Ebs), 10 μM baicalein (Baic), or no additions [control (Ctrl)] in the absence (open bars) or presence (hatched bars) of 3 μM thimerosal plus 10 μM AA to induce apoptosis. After 20 h, apoptosis was measured by the FITC-annexin V binding assay. Data are shown as means ± SEM of three independent experiments carried out in duplicate.



of exogenous AA can be explained by the participation of alternative metabolic pathways to attenuate the level of unesterified fatty acid under these conditions. In accord with this, increased acylation of AA into the triacylglycerol fraction is detected in cells treated with IHP compared with untreated cells. This indicates that a significant portion of the free AA produced after CoA-independent transacylase inhibition is diverted to the low-affinity, high-capacity de novo route for triacylglycerol biosynthesis, a route that is seldom used under normal conditions by cultured cell lines of the phagocytic lineage (2, 24, 26).

The concentrations of AA that were able to induce apoptosis when combined with drugs inhibiting fatty acid incorporation and remodeling are in the low micromolar range. These concentrations are likely to be reached locally in vivo during an inflammatory process and therefore may be of pathophysiological relevance. It is worth noting that palmitic acid fails to elicit an apoptotic response either alone or in combination with thimerosal, triacsin C, or IHP. This indicates that the proapoptotic effect of AA described in this study is not a detergent effect. Using well-established cyclooxygenase and lipoxigenase inhibitors, we have also demonstrated that the AA effects are attributable to the fatty acid itself and not to an oxidized metabolite. These studies, along with results by others (11, 14), raise the question of how increasing cellular free AA levels puts into motion the machinery that leads to programmed cell death. Although much work is clearly needed to define the molecular steps involved in AA-induced apoptosis, it is interesting that AA at low micromolar concentrations is known to affect a number of intracellular effectors and signaling pathways, including the generation of apoptosis-inducing ceramides (27–30). Overall, our work strongly suggests that the enzymatic mechanisms regulating free AA availability in cells must be regarded as potential candidates for the design of drugs with antitumoral potential (31, 32). ■

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## REFERENCES

- Lands, W. E. M. 2000. Stories about acyl chains. *Biochim. Biophys. Acta.* **1483**: 1–14.
- Chilton, F. H., A. H. Fonteh, M. Surette, M. Triggiani, and J. D. Winkler. 1996. Control of arachidonate levels within inflammatory cells. *Biochim. Biophys. Acta.* **1299**: 1–15.
- Balsinde, J., and E. A. Dennis. 1997. Function and inhibition of intracellular calcium-independent phospholipase A<sub>2</sub>. *J. Biol. Chem.* **272**: 16069–16072.
- Winstead, M. V., J. Balsinde, and E. A. Dennis. 2000. Calcium-independent phospholipase A<sub>2</sub>: structure and function. *Biochim. Biophys. Acta.* **1488**: 28–39.
- Balsinde, J., and M. A. Balboa. 2005. Cellular regulation and proposed biological functions of group VIA calcium-independent phospholipase A<sub>2</sub> in activated cells. *Cell. Signal.* **17**: 1052–1062.
- Balsinde, J., S. E. Barbour, I. D. Bianco, and E. A. Dennis. 1994. Arachidonic acid mobilization in P388D<sub>1</sub> macrophages is controlled by two distinct Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> enzymes. *Proc. Natl. Acad. Sci. USA.* **91**: 11060–11064.
- Balsinde, J., M. A. Balboa, and E. A. Dennis. 1997. Antisense inhibition of group VI Ca<sup>2+</sup>-independent phospholipase A<sub>2</sub> blocks phospholipid fatty acid remodeling in murine P388D<sub>1</sub> macrophages. *J. Biol. Chem.* **272**: 29317–29321.
- Pérez, R., R. Melero, M. A. Balboa, and J. Balsinde. 2004. Role of group VIA calcium-independent phospholipase A<sub>2</sub> in arachidonic acid release, phospholipid fatty acid incorporation, and apoptosis in U937 cells responding to hydrogen peroxide. *J. Biol. Chem.* **279**: 40385–40391.
- Yamashita, A., T. Sugiura, and K. Waku. 1997. Acyltransferases and transacylases involved in fatty acid remodeling of phospholipids and metabolism of bioactive lipids in mammalian cells. *J. Biochem. (Tokyo).* **122**: 1–16.
- Dong, M., K. Guda, P. R. Nambiar, A. Rezaie, G. S. Belinsky, S. Lambeau, C. Giardina, and D. W. Rosenberg. 2003. Inverse association between phospholipase A<sub>2</sub> and COX-2 expression during mouse colon tumorigenesis. *Carcinogenesis.* **24**: 307–315.
- Cao, Y., A. T. Pearman, G. A. Zimmerman, T. M. McIntyre, and S. M. Prescott. 2000. Intracellular unesterified arachidonic acid signals apoptosis. *Proc. Natl. Acad. Sci. USA.* **97**: 11280–11285.
- Seno, K., T. Okuno, K. Nishi, Y. Murakami, F. Watanabe, T. Matsuura, M. Wada, Y. Fujii, M. Yamada, T. Ogawa, et al. 2000. Pyrrrolidine inhibitors of human cytosolic phospholipase A<sub>2</sub>. *J. Med. Chem.* **43**: 1041–1044.
- Blank, M. L., Z. L. Smith, and F. Snyder. 1992. Contributing factors in the trafficking of [<sup>3</sup>H]arachidonate between phospholipids. *Biochim. Biophys. Acta.* **1124**: 262–272.
- Surette, M. E., A. N. Fonteh, C. Bernatchez, and F. H. Chilton. 1999. Perturbations in the control of cellular arachidonic acid levels block cell growth and induce apoptosis in HL-60 cells. *Carcinogenesis.* **20**: 757–763.
- Balboa, M. A., and J. Balsinde. 2002. Involvement of calcium-independent phospholipase A<sub>2</sub> in hydrogen peroxide-induced accumulation of free fatty acids in human U937 cells. *J. Biol. Chem.* **277**: 40384–40389.
- Balsinde, J., B. Fernández, and J. A. Solís-Herruzo. 1994. Increased incorporation of arachidonic acid into phospholipids in zymosan-stimulated mouse peritoneal macrophages. *Eur. J. Biochem.* **221**: 1013–1018.
- Surette, M. E., and F. H. Chilton. 1998. The distribution and metabolism of arachidonate-containing phospholipids in cellular nuclei. *Biochem. J.* **330**: 915–921.
- Joly, F., M. Breton, C. Wolf, E. Nimio, and O. Colard. 1992. Heterogeneity of arachidonate and paf-acether precursor pools in mast cells. *Biochim. Biophys. Acta.* **1125**: 305–312.
- Boillard, E., and M. E. Surette. 2001. Anti-CD3 and concanavalin A-induced human T cell proliferation is associated with an increased rate of arachidonate-phospholipid remodeling. Lack of involvement of group IV and group VI phospholipase A<sub>2</sub> in remodeling and increased susceptibility of proliferating T cells to CoA-independent transacylase inhibitor-induced apoptosis. *J. Biol. Chem.* **276**: 17568–17575.
- Balsinde, J. 2002. Roles of various phospholipases A<sub>2</sub> in providing lysophospholipid acceptors for fatty acid phospholipid incorporation and remodelling. *Biochem. J.* **364**: 695–702.
- Kaever, V., U. Firla, and K. Resch. 1988. Sulfhydryl reagents as model substances for eicosanoid research. *Eicosanoids.* **1**: 49–57.
- Hartman, E. J., S. Omura, and M. Laposata. 1989. Triacsin C: a differential inhibitor of arachidonoyl-CoA synthetase and nonspecific long chain acyl-CoA synthetase. *Prostaglandins.* **37**: 655–671.
- Chilton, F. H., A. N. Fonteh, C. M. Sung, D. M. Hinkley, T. J. Torphy, R. J. Mayer, L. A. Marshall, J. D. Heravi, and J. D. Winkler. 1995. Inhibitors of CoA-independent transacylase block the movement of arachidonate into 1-ether-linked phospholipids of human neutrophils. *Biochemistry.* **34**: 5403–5410.
- Balsinde, J., and E. A. Dennis. 1996. The incorporation of arachidonic acid into triacylglycerol in P388D<sub>1</sub> macrophage-like cells. *Eur. J. Biochem.* **235**: 480–485.
- Blank, M. L., Z. L. Smith, and F. Snyder. 1993. Arachidonate-containing triacylglycerols: biosynthesis and a lipolytic mechanism for the release and transfer of arachidonate to phospholipids in HL-60 cells. *Biochim. Biophys. Acta.* **1170**: 275–282.
- Balsinde, J., I. D. Bianco, E. J. Ackermann, K. Conde-Frieboes, and

- E. A. Dennis. 1995. Inhibition of calcium-independent phospholipase A<sub>2</sub> prevents arachidonic acid incorporation and phospholipid remodeling in P388D<sub>1</sub> macrophages. *Proc. Natl. Acad. Sci. USA*. **92**: 8527–8531.
27. Chan, T. A., P. J. Morin, B. Vogelstein, and K. W. Winzler. 1998. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proc. Natl. Acad. Sci. USA*. **95**: 681–686.
28. Jayadev, S., C. M. Linares, and Y. A. Hannun. 1994. Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha. *J. Biol. Chem.* **269**: 5757–5763.
29. Jayadev, S., H. L. Hayter, N. Andrieu, C. J. Gamard, B. Liu, R. Balu, M. Hayakawa, F. Ito, and Y. A. Hannun. 1997. Phospholipase A<sub>2</sub> is necessary for tumor necrosis factor alpha-induced ceramide generation in L929 cells. *J. Biol. Chem.* **272**: 17196–17203.
30. Cai, Z., A. Bettaieb, N. E. Mahdani, L. G. Legres, R. Stancou, J. Masliah, and S. J. Chouaib. 1997. Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF7 cells to tumor necrosis factor-alpha-mediated cytotoxicity. *J. Biol. Chem.* **272**: 6918–6926.
31. Monjaneb, A. M., C. E. Clay, K. P. High, and F. H. Chilton. 2002. Antineoplastic properties of arachidonic acid and its metabolites. *Prostaglandins Leukot. Essent. Fatty Acids*. **66**: 5–12.
32. Das, U. N. 1999. Essential fatty acids, lipid peroxidation and apoptosis. *Prostaglandins Leukot. Essent. Fatty Acids*. **61**: 157–163.