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Antisense Inhibition of Group VI Ca\(^{2+}\)-independent Phospholipase A\(_2\) Blocks Phospholipid Fatty Acid Remodeling in Murine P388D\(_1\) Macrophages*  

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A major issue in lipid signaling relates to the role of particular phospholipase A\(_2\) isoforms in mediating receptor-triggered responses. This has been difficult to study because of the lack of isoform-specific inhibitors. Based on the use of the Group VI Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)) inhibitor bromoenol lactone (BEL), we previously suggested a role for the iPLA\(_2\) in mediating phospholipid fatty acid turnover (Balsinde, J., Bianco, I. D., Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92: 8527–8531). We have now further evaluated the role of the iPLA\(_2\) in phospholipid remodeling by using antisense RNA technology. We show herein that inhibition of iPLA\(_2\) expression by a specific antisense oligonucleotide decreases both the steady-state levels of lysophosphatidylcholine and the capacity of the cell to incorporate arachidonic acid into membrane phospholipids. These effects correlate with a decrease in both iPLA\(_2\) activity and protein in the antisense-treated cells. Collectively these data provide further evidence that the iPLA\(_2\) plays a major role in regulating phospholipid fatty acyl turnover in P388D\(_1\) macrophages. In stark contrast, experiments with activated cells confirmed that the iPLA\(_2\) does not play a significant role in receptor-coupled arachidonate mobilization in these cells, as manifested by the lack of an effect of the iPLA\(_2\) antisense oligonucleotide on PAF-stimulated arachidonate release.

The phospholipase A\(_2\) (PLA\(_2\))\(^{1}\) superfamily of enzymes includes a heterogeneous collection of proteins with diverse roles in cell function (1). The PLA\(_{2}\)s catalyze the hydrolysis of the sn-2 fatty acyl moiety of phospholipids, generating a free fatty acid and a 2-lysophospholipid, both of which may serve significant biological roles. The latter reaction is particularly relevant when the free fatty acid generated is arachidonic acid (AA), as this is the common precursor of the biologically active prostaglandins, leukotrienes, thromboxane, and lipoxins (2).

Based on sequence data, nine different PLA\(_2\) Groups have been identified to date (1). However, based on biochemical properties and structural features, the PLA\(_2\) superfamily can be subdivided into three main types, i.e. the Ca\(^{2+}\)-dependent secretory enzymes (sPLA\(_2\)), the Ca\(^{2+}\)-dependent cytosolic enzymes (cPLA\(_2\)) and the Ca\(^{2+}\)-independent cytosolic enzymes (iPLA\(_2\)).

It is difficult to demonstrate specificity of function for a single PLA\(_2\) isoform in vivo because most of the PLA\(_2\) inhibitors currently available are not isoform-specific. However, bromoenol lactone (BEL) had been regarded as a specific iPLA\(_2\) inhibitor since it manifests greater than 1000-fold selectivity for the iPLA\(_2\) versus the sPLA\(_2\) and cPLA\(_2\) (3, 4). We recently found that BEL inhibits AA esterification into P388D\(_1\) cell phospholipids in a dose-dependent and saturable manner, with the decrease being directly related to inhibition of both cellular iPLA\(_2\) activity and steady-state lysophospholipid levels (5). These data, along with the finding that the process takes place in a Ca\(^{2+}\)-independent manner, led us to implicate the iPLA\(_2\) as the enzyme providing the lysophospholipid acceptors employed in the reaction (5, 6). Recently however, BEL has been found to inhibit another cellular phospholipase, the Mg\(^{2+}\)-dependent phosphatidate phosphohydrolase, with similar potency to that shown for the iPLA\(_2\) (7).

The nucleotide sequence of the macrophage iPLA\(_2\) has recently been elucidated (8). This has now allowed us to achieve the specific inhibition of the iPLA\(_2\) by using antisense RNA technology. In this manner, the inherent lack of specificity associated with the use of chemical inhibitors such as BEL is circumvented. We have previously taken advantage of this technique to unravel the very important role that sPLA\(_2\) plays in AA metabolism in P388D\(_1\) cells (9–11). We report herein our results on the antisense inhibition of the macrophage iPLA\(_2\), which provide further evidence for the involvement of this enzyme in regulating fatty acid remodeling reactions among membrane phospholipids.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse P388D\(_1\), macrophage-like cells were obtained from the American Type Culture Collection (Rockville, MD). Iscove's modified Dulbecco's medium (endotoxin <0.05 ng/ml) was from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum was from Hyclone Labs. (Logan, UT). Nonessential amino acids were from Irvine Scientific (Santa Ana, CA). [5,6,8,9,11,12,14,15-\(^{3}H\)]Arachidonic acid (specific activity 100 Ci/mmol) and [methyl-\(^{3}H\)]choline chloride (specific activity 79 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Bacterial lipopolysaccharide (LPS) and platelet-activating factor (PAF) were from Sigma. Group VI iPLA\(_2\) antisemur was generously provided by Dr. S. Jones (Genetics Institute, Cambridge, MA) (8, 12).

Oligonucleotide Treatment and Cell Culture Conditions—A 20-base-
long antisense corresponding to nucleotides 59–75 in the murine Group VI iPLA₂ sequence (8) was utilized (ASGVI-18; 5'-CUC CUU CAC CCG GAA UGG GU). As a control, the sense complement of ASGVI-18 was used (SGV-18; 5'-ACC CAU UCC GGG UGA AGG AG). Both ASGVI-18 and SGVI-18 contained phosphorothioate linkages to limit degradation. The antisense reaction procedures were adapted from that described by Lecat et al. (13) for inhibition of the cPLA₂. Briefly, 2.5 × 10⁶ cells were cultured in the presence of different oligonucleotide concentrations in Iscove’s modified Dulbecco’s medium for 4 h at 37 °C in a humidified atmosphere at 90% air and 10% CO₂. A final concentration of 10% fetal bovine serum was then added, and the cells were kept in culture for an additional 48-h time period. The culture medium was supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids. Oligonucleotide treatment and culture conditions were not toxic for the cells as assessed by the trypan blue dye exclusion assay and by quantitation of adherent cell protein.

Measurement of [³H]AA Incorporation into Cellular Phospholipids—After the treatment described above, the cells were placed in phosphate-buffered saline containing 1 mM EDTA for 60 min, washed, and then exposed to exogenous [³H]AA (5 nM, 0.5 μCi/ml). After 10 min, supernatants were removed, and the cell monolayers were gently washed with buffer containing 5 mg/ml albumin to remove the labeled AA that had not been incorporated into cellular lipids. The cell monolayers were scraped twice with 0.5% Triton X-100, and total lipids were extracted according to Bligh and Dyer (14). Phospholipids were separated from the rest of cellular lipids by thin-layer chromatography with n-hexane/ethyl ether/acetic acid (70:30:1). In this system, phospholipids remain at the origin of the plate. Radioactivity content in phospholipids was quantitated by liquid scintillation counting. When BEL was used (25 μM), it was added to the cells 30 min before addition of [³H]AA.

Measurement of Lysophospholipid Levels—The cells were labeled with 0.5 μg/ml [³H]choline or 0.5 μg/ml [³H]ethanolamine for 2 days. When oligonucleotides were used, they were added to the cells at the same time as the radioactive compounds. Cellular uptake of the radioactive compounds was not affected by the oligonucleotides. Afterward, the cell monolayers were scraped in 0.5 ml of 0.5% Triton X-100. For separation of lyso-PC and lyso-PE, the lipids were extracted with ice-cold n-butyl alcohol and separated by thin-layer chromatography with chloroform/methanol/acetic acid/water (50:40:0.6) as a solvent system. Spots corresponding to lyso-PC or lyso-PE were scraped into scintillation vials, and the amount of radioactivity was estimated by liquid scintillation counting.

Immunoblot Analysis of iPLA₂—The cells were washed twice with serum-free medium and homogenized by 25 strokes in a Dounce homogenizer in a buffer consisting of 20 mM Tris-HCl, 2 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μM leupeptin, 20 μM aprotinin, 0.1% 2-mercaptoethanol, pH 7.5. The homogenates were centrifuged at 500 × g for 5 min at 4 °C to separate nuclei. Samples (50 μg) were separated by SDS-PAGE (10% acrylamide gel) and transferred to Immobilon-P (Millipore). Nonspecific binding was blocked by incubating the membranes with a buffer consisting of 5% nonfat milk, 10 mM Tris-HCl, pH 5.0, 1 mM EDTA, 150 mM NaCl, and 0.1% Triton X-100 for 60 min. Membranes were then incubated with anti-iPLA₂ antiserum at a 1:200 dilution for 30 min and then treated with horseradish peroxidase-conjugated protein A (Amersham Life Science, Inc.). Bands were detected by enhanced chemiluminescence (ECL, Amersham Life Science, Inc.).

iPLA₂ Assay—Homogenates from P388D₁ cells, were prepared by sonication as described previously (5). iPLA₂ activity in the homogenates was assayed in a buffer consisting of 100 mM Hepes, 400 μM Triton X-100, 100 mM phospholipid substrate (1-palmitoyl-2-[¹⁴C]palmitoyl-sn-glycerol-3-phosphocholine; 300,000 cpm), 5 mM EDTA, and the indicated amounts of ATP in a final volume of 0.2 ml (pH 7.5) (15, 16). The mixture was incubated at 40 °C for 1 h with shaking. The reaction was stopped by adding 0.75 ml of chloroform/methanol (1:2). The products were extracted according to Bligh and Dyer (14) and separated by thin-layer chromatography with n-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase.

³H]AA Release Measurement—The standard procedure for activating the cells with LPS and PAF has been previously described (4). Briefly, the cells (10⁶ cells/ml), labeled for 20 h with 0.5 μCi/ml of [³H]AA, were placed in serum-free medium for 1 h before the addition of LPS (200 ng/ml) for 1 h. Subsequently, 10 μl of a 10 mg/ml albumin solution was added per well (final concentration of albumin in each well was 0.1 mg/ml), and after 5 min, PAF (100 nM) was added. After a 15-min incubation period, supernatants were removed, cleared of detached cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

**RESULTS**

**Effect of an iPLA₂ Antisense Oligonucleotide on AA Incorporation into Phospholipids**—The iPLA₂ of P388D₁ cells, now designated as a Group VI PLA₂ (1), is an 80–85 kDa protein that shows no specificity for the fatty acid present at the sn-2 position of the phospholipid (16). This enzyme appears to be implicated in regulating basal phospholipid remodeling in P388D₁ cells (4, 5). Fig. 1A shows that treatment of the P388D₁ cells with an iPLA₂ antisense oligonucleotide (referred to as ASGVI-18) led to a marked decrease in their capacity to incorporate exogenous [³H]AA into membrane phospholipids. This inhibition was not due to loss of cell viability, as judged by trypan blue exclusion and by quantitation of adherent cell protein. Cell viability was further assessed by monitoring [³H]thymidine incorporation, which was the same in control or antisense-treated cells (4, 5).

![FIG. 1. iPLA₂ antisense oligonucleotides inhibit iPLA₂ protein expression and activity and AA incorporation into phospholipids in P388D₁ macrophages](http://www.jbc.org/)

A. [³H]AA incorporation into phospholipids (%)

B. iPLA₂ activity (% hydrolysis)

C. iPLA₂ protein (arbitrary units)

Lane 1, control (C); lane 2, SGVI-18 (S); lane 3, ASGVI-18 (A).

Downloaded from http://www.jbc.org/ at Biomedical Library, UCSD on December 24, 2020
Antisense Inhibition of Macrophage iPLA₂

Fig. 2. AA incorporation into phospholipids is inhibited by ASGVI-18 in a concentration- and time-dependent manner. A, the extent of AA incorporation into phospholipids was determined in cells that had been treated with the indicated amounts of ASGVI-18 (●), or SGVI-18 (○) for 40 h. B, AA incorporation was measured at the indicated times in cells treated with ASGVI-18 (●), SGVI-18 (○), or neither (×).

The dose dependence and time course of the effect of the iPLA₂ antisense oligonucleotide were investigated, and the results are shown in Fig. 2. Concentrations of ASGVI-18 below 1 μM exerted little effect; whereas, maximal effects were observed at an oligonucleotide concentration of 10 μM (Fig. 2A). Oligonucleotide concentrations higher than 10 μM induced excessive detachment of the cells from the plastic wells and, therefore, could not be used. Consistent with an antisense-type inhibition, ASGVI-18 decreases cellular AA esterification at all times measured (Fig. 2B).

The above experiments determined incorporation of AA at short times in cells that were already deficient in iPLA₂. Thus, it was important to determine whether iPLA₂ depletion changes the endogenous AA pools. To that end, experiments were conducted wherein the radioactive AA was added at the time the cells were exposed to the oligonucleotides. As AA incorporation into phospholipids in P388D₁ takes place very rapidly (10), it will be completed long before the cellular iPLA₂ levels begin to drop as a result of the antisense treatment. Thus, the relative distribution of AA among phospholipids in iPLA₂-depleted cells can be determined and compared with that in control untreated cells. As shown in Table I, the relative distribution of AA among phospholipids did not change after ASGVI-18 treatment, indicating that ASGVI-18 treatment does not change the endogenous AA pools.

Fig. 3 shows that ASGVI-18 treatment of P388D₁ cells resulted in a 50–55% decrease of the steady-state levels of lysophosphatidylethanolamine (lyso-PE), which corresponds well with the decrease in AA incorporation into phospholipids shown in Fig. 1A. Lyso-PC levels in SGVI-18-transfected cells were the same as those found in control untreated cells (Fig. 3). Within experimental error, no effect of ASGVI-18 on lyso-PE levels could be detected (data not shown).

Effect of BEL on iPLA₂ Antisense-treated Cells—Collectively, the above data suggest that selective inhibition of iPLA₂ expression by antisense RNA technology reduces AA incorporation due to a decrease in the amount of cellular lyso-PC acceptors available for the esterification reaction. These data are consistent with our previous results using BEL to inhibit the iPLA₂ (5). However, it is now known that BEL also inhibits the Mg²⁺-dependent phosphatidate phosphohydrolase, which is another key enzyme in phospholipid metabolism (7, 17). To verify the specificity of BEL in our previous studies (5), it seemed important to study the effects of BEL on AA esterification in antisense-treated cells. The results from these experiments are summarized in Fig. 4. In agreement with our previous data (8), AA incorporation into phospholipids was blocked up to 60% by BEL in control and SGVI-18-treated cells. In ASGVI-18-treated cells, which already showed a 60% decrease in AA incorporation, BEL was ineffective in further increasing this inhibition (Fig. 4). We have previously found that BEL has no effect on the relative distribution of AA among the different phospholipids of P388D₁ cells (5).

Effect of iPLA₂ Antisense on Receptor-coupled AA Release—The above data suggest that the Group VI iPLA₂ may function as a 'housekeeping' enzyme involved in the regulation of basal deacylation/acylation reactions among phospholipids. To further assess its role in cellular function, we sought to assess its
role during receptor activation conditions. The effect of ASGVI-18 on LPS/PAF-stimulated AA release from P388D1 cells is shown in Fig. 5. Stimulation of murine P388D1 macrophages with nanomolar amounts of the inflammatory mediator PAF results in negligible cellular responses unless the cells are first treated with LPS. LPS acts just as a primer, i.e. it does not stimulate the P388D1 cells by itself but enables the cells to optimally respond to PAF (23). The sense and antisense oligonucleotides both slightly decreased the AA release response, which was detected both in resting and PAF-activated cells. However, the ratio of stimulated versus unstimulated release remained the same under all conditions (Fig. 5). This result indicates that the iPLA2 does not play a significant role in mediating agonist-induced AA mobilization in these cells.

DISCUSSION

It is now well established that the availability of free AA limits the synthesis of the biologically active eicosanoids (2). As AA is mainly found esterified at the sn-2 position of cellular phospholipids, PLA2 has emerged as a key enzyme responsible for controlling the levels of free fatty acid (18). However, the amount of free AA available for eicosanoid synthesis represents a balance between what is being liberated by the activated PLA2s, minus what is reincorporated back into phospholipids by the highly active acyltransferases (19). Thus, free fatty AA levels are also efficiently controlled by the AA reacylation pathway (19).

Even under resting conditions, the capacity of certain cells such as macrophages to incorporate AA into phospholipids via reacylation reactions is exceedingly high (6, 20, 21). Thus these cells should possess a basal PLA2 activity high enough to account for their high AA reacylation capacity. Interestingly, AA incorporation into phospholipids in macrophages is Ca2+-independent, i.e. it takes place normally at free Ca2+ concentrations lower than 10 nM (5). As under these conditions neither the cPLA2 nor the sPLA2 must be active, these results suggest that the enzyme providing lysophospholipid acceptors for the AA reacylation pathway is an iPLA2. Further evidence for such a role was obtained by the use of BEL (5). Using this inhibitor, we found a direct correlation between endogenous iPLA2 activity, steady-state lysophospholipid levels, and AA incorporation capacity of the cells (5). However, BEL, being selective for the iPLA2, over the other PLA2s (3, 4), is not devoid of other effects. For example, we have recently found that BEL also potently blocks the Mg2+-dependent phosphatidate phosphohydrolase, a key enzyme in cellular phospholipid metabolism (7).

The inherent problems associated with the use of chemical inhibitors can be potentially circumvented by inhibiting expression of the iPLA2 with antisense RNA oligonucleotides. Using this strategy, we have successfully achieved the specific inhibition of the macrophage Group VI PLA2 and confirmed our previous findings with BEL. Thus, the iPLA2 antisense oligonucleotide ASGVI-18 reduces the cellular iPLA2 activity by 75–80% (Fig. 1B) and iPLA2 protein by at least 50–60% (Fig. 1C), which results in a decrease of the capacities of the cells to incorporate AA into phospholipids (Figs. 1A and 2) as well as the steady-state lyso-PC levels (Fig. 3). These latter effects correspond precisely, and are specific, as parallel experiments using the sense control SGVI-18 did not reproduce any of the effects induced by ASGVI-18. Specificity of ASGVI-18 is also stressed by the fact that sPLA2 antisense oligonucleotides do not affect macrophage AA esterification (10). These results provide definitive evidence for the key role that the iPLA2 plays in regulating basal phospholipid remodeling reactions in macrophages.

Strikingly, ASGVI-18 treatment was found to significantly decrease the steady state of lyso-PC levels but not of lyso-PE. It is likely that our inability to detect any effect of ASGVI-18 on lyso-PE levels merely reflects some sort of experimental limitations. However, it could indicate as well that the Group VI PLA2 preferentially attacks PC over PE in vivo even though the enzyme has been found to lack headgroup specificity in vitro (12, 16). It is possible that PC pools within the cells are more accessible to iPLA2 attack than the PE pools. Interestingly, in regards to AA metabolism, it is lyso-PC, not lyso-PE, that is the major acceptor for esterification of free AA (19). Over time, the AA accumulates into PE as a consequence of direct transfer from PC by CoA-independent transacylation, not by direct acylation of lyso-PE with AA (19). Therefore, selective inhibition of lyso-PC but not of lyso-PE would result in decreased labeling of phospholipids with AA, and the ratio AA-PC to AA-PE remaining unchanged. This is exactly what was found in the experiments determining the effect of ASGVI-18 treatment on AA distribution among phospholipids (Table I). Thus, the current data provide additional support to the notion that the different phospholipid classes and subclasses serve different roles for AA incorporation and redistribution within different pools (19).

Under our experimental conditions, we were not able to achieve complete inhibition of the iPLA2, but did achieve a 60% or 80% disappearance at best, as judged by protein content or activity, respectively. This reduces AA incorporation into phospholipids by about 60% in antisense-treated cells. It could thus be argued that achieving 100% inhibition of Group VI iPLA2
expression would result in almost complete ablation of the capacity of the cell to incorporate AA into phospholipids. However, the fact that the decrease in iPLA₂ protein detected by Western blot does not correspond with the decrease in cellular iPLA₂ activity suggests the possibility that, in addition to the iPLA₂, the antibody used may be recognizing another 85 kDa protein in P388D₁ macrophages. If this were the case, then the remainder of the 85-kDa protein band that is not blocked by ASGVI-18 could correspond to an antigenically related protein. Moreover, it is also possible that the 20% of iPLA₂ activity that is not eliminated by ASGVI-18, corresponds to another iPLA₂ distinct from the Group VI enzyme. The possibility that the P388D₁ cells contain more than one iPLA₂ form has previously been considered (16). Finally, BEL, either alone (5) or together with the iPLA₂ antisense (Fig. 4) fails to completely inhibit the response. Saturation of inhibition by BEL is reached at about 60–70% (5). Altogether, these facts seem to suggest that the cell possesses other mechanisms for regulating AA esterification besides the one provided by the Group VI iPLA₂. It should be remarked here that the current experiments were done in the absence of Ca²⁺. Therefore, if a second PLA₂ distinct from the Group VI enzyme was involved in regulating AA esterification, it would have to be another Ca²⁺-independent isoform.

As discussed elsewhere (5, 6), the contribution of the de novo biosynthetic pathway to AA incorporation into phospholipids is minimal in P388D₁ macrophages. This contrasts with results obtained with peritoneal macrophages (21) and neutrophils (22), wherein the de novo pathway has been demonstrated to provide a minor but significant route for the generation of highly polyunsaturated phospholipid species such as 1,2-di-arachidonoyl-sn-glycerol-3-phosphocholine. However the phospholipids of peritoneal macrophages and neutrophils are already very enriched in AA (21, 22). Such a circumstance likely explains why a significant portion of the free AA is shunted to the low-affinity biosynthetic route in these cells. In contrast, P388D₁ cells are very scarce in endogenous AA (23). Hence, most of the AA incorporation in these cells occurs through the high-affinity remodeling pathway (5, 6). Nonetheless, recent data have highlighted the fact that the sn-1 position of phospholipids is also extensively remodeled in macrophages (21), which means that a phospholipase A₁ could also eventually participate in regulating AA esterification via fatty acid remodeling. Such a possibility, as well as the possible involvement of the recently described CoA-dependent transacylase reaction (24, 25), is currently under investigation in our laboratory.

In summary, in the present study we have utilized antisense RNA technology to obtain independent conclusive confirmation that the macrophage Group VI iPLA₂ does play an important role in modulating phospholipid fatty acid turnover by providing the 2-lysophospholipid acceptors required for the reaction. In addition, our results demonstrate that the Group VI iPLA₂ does not appear to play a significant role in the stimulation of AA release mediated via the PAF surface receptor.

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