Involvement of Calcium-independent Phospholipase A2 in Hydrogen Peroxide-induced Accumulation of Free Fatty Acids in Human U937 Cells

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Phospholipase A2 (PLA2) is a key regulatory step in the production of prostaglandins, because it catalyzes the release of arachidonic acid (AA) from the sn-2 position of phospholipids, making the free fatty acid accessible to prostaglandin synthases. At present, 14 different PLA2 groups have been identified (1, 2). These include ten groups of enzymes utilizing a catalytic histidine, which show millimolar requirements for Ca2+ and are collectively referred to as the secreted PLA2s (Groups I, II, III, V, IX, X, XI, XII, XIII, and XIV) (1, 2), and two groups of intracellular, high molecular mass enzymes, which utilize a catalytic serine (Groups IV and VI). Group IV PLA2 has also been known as cytosolic PLA2 (cPLA2), and Group VI PLA2, or iPLA2, is Ca2+-independent (1, 2).

Among these PLA2s, Groups II, V, and IV have repeatedly been shown to be responsible for AA release and prostaglandin generation in different systems (3–5). In phagocytic cells, Group VI PLA2 has been primarily implicated in basal fatty acid reacylation reactions by controlling the cellular level of lysophosphatidylcholine acceptors (6). In other cell types, notably heart and pancreatic islets, the enzyme has also been implicated in receptor-mediated AA release, based on the effects of a bromoenol lactone suicide inhibitor (BEL) (6).

Recent work has shown that reactive oxygen intermediates enhance AA release and prostaglandin production in different cell systems, but the molecular mechanism responsible for these effects has not been clarified. Activation of an intracellular PLA2 has been pointed out as the most likely mechanism for AA mobilization in vascular smooth muscle cells, stromal cells, and striatal neurons exposed to H2O2 (7–11). In other systems however, diminished AA incorporation into phospholipids, not PLA2 activation, has been suggested to be the event responsible for free AA accumulation (12, 13). In an attempt to reconcile these conflicting results, we sought to investigate the ability of H2O2 to induce AA mobilization from human monocytic U937 cells and the molecular mechanism involved in this process. U937 cells contain both cPLA2 and iPLA2 and have been shown to release AA and produce prostaglandins in response to a variety of receptor-mediated and soluble agonists in a cPLA2-regulated manner (14, 15). Utilizing a variety of approaches, we show here that H2O2 induces AA mobilization in U937 cells by a Ca2+-independent mechanism that involves not cPLA2, but rather iPLA2. Importantly, however, the results indicate that the iPLA2-mediated AA release does not reflect a true activation of the enzyme (i.e. a stable increase in the specific activity of the enzyme) but rather an increased accessibility of the iPLA2 toward its substrate. These results underscore the key role of iPLA2 in modulating basal fatty acid deacylation reactions.

**EXPERIMENTAL PROCEDURES**

Materials—[5,6,8,9,11,12,14,15-3H]AA (100 Ci/mmol) was from Amersham Biosciences. BEL and methyl arachidonyl fluorophosphonate (MAFP) were from Cayman (Ann Arbor, MI). The specific cPLA2, inhibitor pyrrophenone was generously provided by Dr. K. Seno

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The abbreviations used are: PLA2, phospholipase A2; AA, arachidonic acid; cPLA2, cytosolic phospholipase A2; iPLA2, Ca2+-independent phospholipase A2; BEL, bromoenol lactone; MAFP, methyl arachidonyl fluorophosphonate; ConA, concanavalin A.
Cell Culture—U937 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and gentamicin (24 μg/ml). The cells were incubated at 37 °C in a humidified atmosphere of CO2/O2 (1:19) at a cell density of 0.5–1 × 106 cells/ml in 12-well plastic culture dishes (Costar). Cell differentiation was induced by treating the cells with 35 ng/ml PMA for 24 h (19, 20).

AA Release Experiments—The cells were labeled with 0.5 μCi/ml [3H]AA for 18 h. After this period, the cells were washed and placed in serum-free medium for 1 h before the addition of the appropriate stimulus in the presence of 0.5 mg/ml bovine serum albumin. The supernatants were removed, cleared of cells by centrifugation, and assayed for radioactivity by liquid scintillation counting.

For analysis of [3H]AA metabolites released into the supernatant, the stimulations were conducted in the absence of albumin. The supernatant was acidified to pH 3.5 with 5 M formic acid and extracted twice with 3 ml of ethyl acetate. The ethyl acetate was dried under a stream of nitrogen, and the residue was dissolved in a few drops of chloroform/methanol (2:1, v/v) and chromatographed on Silicagel G-60 plates. Unlabeled prostaglandin standards were used as carriers. The solvent system used was chloroform/methanol/acetic acid/water (90:3:1:0.8, by volume) (21).

Treatment of the Cells with Antisense Oligonucleotides—The antisense oligonucleotides utilized in these studies were derived from prior publications reporting their effects (22–24). The iPLA2 antisense sequence corresponded to nucleotides 59–78 in the murine group VI iPLA2 sequence, which is conserved in human group VI iPLA2 (25, 26). 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/μl, respectively. Oligonucleotide treatment and culture conditions were not toxic for the cells as assessed by the trypan blue dye exclusion assay and by quantitating adherent cell protein.

Preparation of Substrates for the iPLA2 Assay—[3H]AA-labeled choline glycerophospholipids and ethanolamine glycerophospholipids were isolated from cellular lipids of U937 cells incubated for 24 h with the exogenous [3H]-labeled fatty acid (0.5 μCi/ml). Labeled phospholipids were purified by thin-layer chromatography and tested for purity as described previously (15). Labeled U937 cell membranes were prepared by adding 0.5 μCi/ml [3H]AA to the U937 cell cultures for 18 h. Total cellular membranes were prepared by sucrose centrifugation exactly as described by Diez et al. (28).

Lipid Peroxide Determination—The amount of lipid peroxides in membranes was quantified by the thiobarbituric acid-reactive substance assay (29). The samples were mixed with 1 ml of 0.67% thiobarbituric acid and 0.5 ml of 20% trichloroacetic acid, and the mixtures were incubated in a boiling water bath for 20 min. After cooling the tubes on ice, the reaction mixture was centrifuged at 3000 × g for 10 min, and absorbance of the supernatant was read at 532 nm. The concentration of thiobarbituric acid-reactive substances, which is directly proportional to the amount of lipid peroxides in the samples, was calculated using tetraethoxy propane as a reference standard.

RESULTS

AA Mobilization in H2O2-treated U937 Cells—We began the current study by determining whether H2O2 was capable of causing the extracellular release of AA from U937 cells. To this end, the cells, labeled with 0.5 μCi of [3H]AA, were exposed to different concentrations of H2O2 for various periods of time. As shown in Fig. 1, H2O2 did induce a concentration- and time-dependent release of [3H]AA from the cells (Fig. 1). Maximal effects of H2O2 on AA release were observed at a concentration of 500 μM (Fig. 1A). Such a concentration was therefore used in all subsequent experiments. Fig. 1B shows that, after a lag of about 5–15 min, H2O2-induced AA release proceeded linearly for the following hour, proceeding at a slower rate thereafter. That the kinetics of AA release in response to H2O2 does not show saturation within 1 h of treatment is in stark contrast with the kinetics of AA release in response to the receptor-directed agonist ConA (20), which is also shown in Fig. 1B for comparison.

The composition of the 3H-released material was analyzed by thin-layer chromatography, and the results are shown in Table I. Treatment of the cells with H2O2 significantly increased prostaglandin production, most notably of prostaglandin E2 and D2, but unmetabolized free AA was the most abundant labeled compound released into the medium.

PLA2 Inhibition Studies—To address the involvement of the different PLA2 forms in H2O2-induced AA release, we first utilized MAPF, a dual cPLA2/iPLA2 inhibitor (30). As shown in Fig. 2A, MAPF significantly blocked the response to H2O2. To distinguish whether the inhibition of MAPF on AA release was because of either cPLA2 or iPLA2, we conducted studies with BEI, a compound that manifests a marked selectivity for inhibition of iPLA2 versus cPLA2 in vitro (6). Fig. 2A also shows that BEI, at concentrations that are known to block cellular iPLA2, exerted a significant inhibitory effect on the H2O2-induced AA mobilization. As a control for these experiments,
TABLE I  
Prostaglandin production by U937 cells exposed to H2O2 
U937 cells, prelabeled with [3H]AA, were untreated or treated with 500 1/4M H2O2 for 60 min. Supernatants were taken off, and the different metabolites were separated by thin-layer chromatography as described under “Experimental Procedures.” The data are expressed in dpm × 10−3. PGF2α, prostaglandin E2, PGD2, prostaglandin D2, 6-keto-PGF1α, 6-ketoprostaglandin F1α, TXB2, thromboxane B2.

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>Control</th>
<th>H2O2</th>
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<tbody>
<tr>
<td>PGE2</td>
<td>2.7 ± 0.4</td>
<td>7.1 ± 0.2</td>
</tr>
<tr>
<td>PGD2</td>
<td>1.9 ± 0.3</td>
<td>6.7 ± 0.2</td>
</tr>
<tr>
<td>6-Keto-PGF1α</td>
<td>0.9 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>TXB2</td>
<td>0.5 ± 0.1</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>AA</td>
<td>8.7 ± 0.4</td>
<td>20.7 ± 0.4</td>
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Fig. 2. Effect of MAFP and BEL on [3H]AA release from U937 cells. The cells were treated with the indicated concentrations of MAFP (open circles) or BEL (closed circles) for 30 min before the addition of 500 μM H2O2 (open symbols) or 100 μg/ml ConA (open symbols), and the incubations proceeded for 60 min. Results are given as a percentage of the response obtained in the absence of inhibitors.

Fig. 3. Effect of pyrrophenone on [3H]AA release from U937 cells. The cells were treated with the indicated amounts of pyrrophenone for 30 min before the addition of 500 μM H2O2 (closed symbols) or 100 μg/ml ConA (open symbols), and the incubations proceeded for 60 min. Results are given as a percentage of the response obtained in the absence of inhibitors.

man counterpart of the murine one that we and others have successfully employed elsewhere (22–24). Using this antisense, an ~70% decrease of both the immunoreactive iPLA2 protein (Fig. 4A) and of cellular iPLA2 activity (Fig. 4B) was achieved, in agreement with previous estimates (22, 23). The antisense to iPLA2 had no effect on the expression of cPLA2α (Fig. 4A). Under these conditions, a significant decrease in the AA release response of H2O2-treated cells was observed (Fig. 4C), which provides additional evidence for the involvement of the iPLA2 in this process. Control antisense experiments utilizing ConA as a trigger for AA release revealed the expected lack of effect of the iPLA2 antisense (Fig. 4C).

Characterization of the H2O2 Effect on iPLA2—Collectively, the above data suggest the involvement of iPLA2 in the AA mobilization response induced by H2O2 in U937 cells. Because iPLA2 is a Ca2+-independent enzyme, one might expect the H2O2-induced AA mobilization process to be Ca2+-independent, as well. To evaluate this possibility, the following approaches were undertaken. In the first place, the cells were exposed to H2O2 in the absence of Ca2+ in the incubation medium, and the effect on AA mobilization was studied. Fig. 5 shows that this strategy did not modify the H2O2 response. As a control, the effect of Ca2+ deprivation on the ConA response was also studied, and the response was strongly blunted (Fig. 5). In the next series of experiments, the cells were depleted of their intracellular Ca2+ by treating them with 40 μM quin2/AM plus 1 mM EGTA in a Ca2+-free medium. This procedure buffers and clamps the intracellular calcium concentration at very low levels (about 10−8 M) (32). Under these conditions, the AA response to H2O2 remained unchanged, whereas the ConA response was abolished (Fig. 5). Collectively, these results indicate that AA mobilization in response to H2O2 does not require Ca2+, which is consistent with the participation of an iPLA2.

Unlike cPLA2α, iPLA2 does not show any apparent substrate specificity (6). Thus, if iPLA2 is involved in fatty acid release in the H2O2-treated cells, one might expect to observe the release of not only AA but also of other fatty acids. To address this possibility, experiments were conducted where the cells were labeled with [3H]oleic acid prior to exposure to H2O2. H2O2 induced a low but measurable release of oleic acid. When the cells were exposed to ConA instead, release of oleic acid was not observed (Fig. 6). Altogether, the results are consistent with the finding that ConA signals through the AA-specific cPLA2α but not through the iPLA2. H2O2, in contrast, appears to cata-
lyze fatty acid mobilization through the fatty acid-nonspecific iPLA₂.

Studies on the Regulation of iPLA₂ Activity—If the H₂O₂ effect on the iPLA₂ is truly an activating one, an increase in the specific activity of the enzyme is to be expected. Homogenates of U937 cells, either untreated or treated with H₂O₂, were prepared, and assays were conducted to assess iPLA₂ activity utilizing a vesicle substrate assay. Under these conditions we failed to detect any change in the iPLA₂-specific activity of homogenates from H₂O₂-treated cells versus untreated cells. Conversely, definite increases in the Ca²⁺/H₁₁₀₀₁-dependent activity of the homogenates could be detected if the cells were previously treated with ConA (Fig. 7). These changes, which most likely correspond to increases in cPLA₂ activity (14, 15), suggest that our inability to detect changes in the iPLA₂ specific activity may not be because of technical issues. Experiments in which iPLA₂ activity was measured utilizing the mixed micelle assay described by Dennis and co-workers (27) also failed to reveal any change in the iPLA₂ activity of the homogenates (not shown).

As a third approach, we utilized the mammalian membrane assay system described by Diez and co-workers (28). In this system, purified [³H]AA-labeled mammalian membranes are used as a substrate. Utilizing this assay, again no differences in the iPLA₂ activity of untreated cells versus H₂O₂-treated cells could be demonstrated. Importantly however, when iPLA₂ activity of homogenates from either untreated cells or H₂O₂-treated cells was assayed toward H₂O₂-treated membranes, a low but significant increase in the iPLA₂ activity could be...
PLA2 activity was expressed as the percentage of hydrolysis of the intrinsic activity of the iPLA2 does not change after exposure of membranes from H2O2-treated cells. Moreover, the membranes from H2O2-treated cells showed significantly elevated levels of lipid peroxides, as quantified by measuring thiobarbituric acid-reactive substances (73 ± 12 pmol/mg protein in H2O2-treated membranes versus 31 ± 9 pmol/mg protein in membranes from untreated cells; mean ± S.E., n = 4).

**DISCUSSION**

Phagocytic cells produce reactive oxygen intermediates such as superoxide anion and hydrogen peroxide in response to a variety of agonists (33). Although the production of these oxygen metabolites plays an important role in cellular signaling and host defense, their uncontrolled production constitutes a serious pathophysiological factor for a wide variety of vascular-based disorders (35). Oxidative damage is often associated with AA mobilization from cells from the vascular system, such as endothelial cells, smooth muscle cells, platelets, and phagocytes. Thus, interactions between reactive oxygen intermediates and AA metabolites are of particular importance.

In this study, H2O2 was used to investigate mechanisms of AA mobilization in phagocytic cells under an oxidative stress, and the data suggest that oxidant-induced fatty acid mobilization from U937 phagocytes does not depend on cPLA2 activity, which is based on several lines of evidence, such as the use of chemical inhibitors and antisense oligonucleotide techniques. Examination of the time course of AA mobilization in response to H2O2 revealed that, after a short lag, the response proceeded linearly with time, showing signs of saturation only after 2 h of exposure to the oxidant. Such a kinetics strongly contrasts with the response of the cells to ConA, a well known receptor agonist of U937 cells, which shows the typical saturation kinetics that is expected from a highly regulated cellular response such as AA release. In keeping with the above, when assayed in a cell-free system, cellular iPLA2 activity did not change. Of note, assays were conducted under three different experimental conditions, namely a vesicle assay, a mixed micelle assay, and a natural membrane assay. Because the results were the same regardless of the assay system utilized, it appears likely that the intrinsic activity of the iPLA2 does not change after exposure of the cells to H2O2. This conclusion argues against the possibility of a stable activation of the iPLA2 as the mechanism for H2O2-mediated AA release in U937 cells.

Interestingly, when membranes from H2O2-treated cells were used in the assay, the iPLA2 activity measured was found to be significantly higher than that found in membranes from otherwise unstimulated cells. Therefore, treating the cells with H2O2 results in facilitated iPLA2 attack on membrane phospholipids. We have found that membranes from H2O2-treated cells contain significantly higher amounts of lipid peroxides than membranes from untreated cells. Thus the data suggest that lipid hydrolysis by iPLA2 occurs more readily in H2O2-treated cells because of changes in the physical state of membrane substrates, which may result, at least in part, from lipid peroxide accumulation. How this facilitated catalysis occurs is presently unknown, but a number of factors that alter membrane lipid packing are well documented to increase fatty acid release both in vitro and in vivo (34).

Taken together, these results suggest a model for fatty acid mobilization in H2O2-treated cells whereby the oxidant induces lipid oxidation, which results in accumulation of lipid peroxides at the membrane. These lipid peroxides destabilize the membrane and render it susceptible to attack by the iPLA2, which then starts releasing increased amounts of fatty acids. An important aspect of the above model is that this fatty acid release occurs in the absence of cPLA2 activation, which underscores the apparent lack of a regulated signaling component in the process. Still, a mechanism such as the one proposed here may be of importance under certain pathophysiological settings (i.e. oxidative stress), where increased iPLA2 activity may account for a significant phospholipid hydrolysis before cellular homeostasis is re-established. In turn, these results highlight the key role of iPLA2 in modulating basal fatty acid deacylation reactions.

Whether iPLA2 is also involved in regulated phospholipid hydrolysis in phagocytic cells is unknown at present. However, the fact that multiple splice variants of iPLA2 exist in some cells and that other iPLA2S distinct from the classical group VI enzyme have recently been described (6) suggest the possibility that iPLA2 may be subject to complex regulatory mechanisms that differ among cell types. Two recent reports utilizing cells overexpressing group VI iPLA2 have shown the enzyme to be responsive to Ca2+ ionophore in HEK293 cells (35) and to glucose plus cAMP-elevating agents in INS-1 insulinoma cells (36), thus suggesting that the enzyme is capable of playing some signaling roles in cells. Whether, in addition to its housekeeping role in U937 cells and phagocytic cells in general, the group VI iPLA2 also plays a signaling role is currently under study.

Analysis of the AA metabolites produced after exposure to the cells to H2O2 revealed a significant production of prostaglandins, particularly the pro-inflammatory prostaglandins E2 and D2. This suggests that an immediate biological consequence of H2O2-induced AA release is to generate mediators that propagate and/or amplify the oxidative injury. Interestingly, a major portion of the material released after H2O2 exposure remained as free unmetabolized AA, which raises the possibility that its metabolism to eicosanoid mediators might not be its only biological fate. H2O2 is known to induce apoptosis in a number of cells including phagocytes (37, 38), and there is evidence that unesterified AA within cells can signal apoptosis (39, 40). Moreover, treating U937 cells with BEL has been shown to retard Fas- and tumor necrosis factor α-mediated apoptosis (41, 42). Taking all these findings together, it is tempting to speculate that the AA liberated by iPLA2 in H2O2-treated cells may play a role in oxidant-induced apoptosis in these cells. Studies are currently in progress to investigate this attractive possibility.

**Fig. 8. Time course of PLA2 activity using a natural membrane as substrate.** Untreated (open circles) and H2O2-treated (closed circles) [3H]AA-labeled membranes were incubated with U937 cell homogenates (as a source of enzyme). Reactions were stopped at different time points, and free [3H]AA was isolated by thin-layer chromatography. PLA2 activity was expressed as the percentage of hydrolysis of the labeled membrane substrate.
REFERENCES