Involvement of Calcium-independent Phospholipase A\(_2\) in Hydrogen Peroxide-induced Accumulation of Free Fatty Acids in Human U937 Cells* 

Received for publication, June 20, 2002, and in revised form, July 29, 2002

Published, JBC Papers in Press, August 13, 2002, DOI 10.1074/jbc.M206155200

Maria A. Balboa‡ and Jesus Balsinde§

From the Institute of Molecular Biology and Genetics, University of Valladolid School of Medicine, E-47005 Valladolid, Spain

Previous studies have demonstrated that U937 cells are able to mobilize arachidonic acid (AA) and synthesize prostaglandins in response to receptor-directed and soluble stimuli by a mechanism that involves the activation of Group IV cytosolic phospholipase A\(_2\)\(_\alpha\). In this paper we show that these cells also mobilize AA in response to an oxidative stress induced by H\(_2\)O\(_2\) through a mechanism that appears not to be mediated by cytosolic phospholipase A\(_2\)\(_\alpha\) but by the calcium-independent Group VI phospholipase A\(_2\) (iPLA\(_2\)). This is supported by the following lines of evidence: (i) the response is essentially calcium-independent, (ii) it is inhibited by bromoeno lactone, and (iii) it is inhibited by an iPLA\(_2\) antisense oligonucleotide. Enzyme assays conducted under a variety of conditions reveal that the specific activity of the iPLA\(_2\) does not change as a result of H\(_2\)O\(_2\) exposure, which argues against the activation of a specific signaling cascade ending in the iPLA\(_2\). Rather, the oxidant acts to perturb membrane homeostasis in a way that the enzyme susceptibility/accessibility to its substrate increases, and this results in altered fatty acid release. In support of this view, not only AA, but also other fatty acids, were found to be liberated in an iPLA\(_2\)-dependent manner in the H\(_2\)O\(_2\)-treated cells. Collectively, these studies underscore the importance of the iPLA\(_2\) in modulating homeostatic fatty acid deacylation reactions and document a potentially important route under pathophysiological conditions for increasing free fatty acid levels during oxidative stress.

Phospholipase A\(_2\) (PLA\(_2\))\(^1\) constitutes 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 PLA\(_2\) groups have been identified (1, 2). These include ten groups of enzymes utilizing a catalytic histidine, which show millimolar requirements for Ca\(^{2+}\) and are collectively referred to as the secreted PLA\(_2\)s (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 IVA PLA\(_2\), also known as cytosolic PLA\(_2\)\(_\alpha\) (cPLA\(_2\)\(_\alpha\)), is a highly regulated, Ca\(^{2+}\)-dependent enzyme (1, 2), whereas Group VI PLA\(_2\), or iPLA\(_2\), is Ca\(^{2+}\)-independent (1, 2).

Among these PLA\(_2\)s, Groups II, V, and IV have repeatedly been shown to be responsive for AA release and prostaglandin generation in different systems (3–5). In phagocytic cells, Group VI PLA\(_2\) 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 bromoeno 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 PLA\(_2\) has been pointed out as the most likely mechanism for AA mobilization in vascular smooth muscle cells, stromal cells, and striatal neurons exposed to H\(_2\)O\(_2\) (7–11). In other systems however, diminished AA incorporation into phospholipids, not PLA\(_2\) 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 H\(_2\)O\(_2\) to induce AA mobilization from human monocytic U937 cells and the molecular mechanism involved in this process. U937 cells contain both cPLA\(_2\)\(_\alpha\) and iPLA\(_2\) and have been shown to release AA and produce prostaglandins in response to a variety of receptor-mediated and soluble agonists in a cPLA\(_2\)\(_\alpha\)-regulated manner (14, 15). Utilizing a variety of approaches, we show here that H\(_2\)O\(_2\)-induces AA mobilization in U937 cells by a Ca\(^{2+}\)-independent mechanism that involves not cPLA\(_2\)\(_\alpha\), but rather iPLA\(_2\). Importantly, however, the results indicate that the iPLA\(_2\)-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 iPLA\(_2\) toward its substrate. These results underscore the key role of iPLA\(_2\) in modulating basal fatty acid deacylation reactions.

**EXPERIMENTAL PROCEDURES**

Materials—[5,6,8,9,11,12,14,15-\(^3\)H]AA (100 Ci/mmol) was from American Bioscience. BEL and methyl arachidonyl fluorophosphonate (MAFP) were from Cayman (Ann Arbor, MI). The specific cPLA\(_2\)\(_\alpha\) inhibitor pyrrophenone was generously provided by Dr. K. Seno.
**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, preceding 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 1. 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 MAFF, a dual cPLA₂/iPLA₂ inhibitor (30). As shown in Fig. 2A, MAFF significantly blocked the response to H₂O₂. To distinguish whether the inhibition of MAFF on AA release was because of either cPLA₂α or iPLA₂, we conducted studies with BEL, a compound that manifests a marked selectivity for inhibition of iPLA₂ versus cPLA₂α in vitro (6). Fig. 2A also shows that BEL, 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

<table>
<thead>
<tr>
<th>Eicosanoid</th>
<th>Control</th>
<th>H2O2</th>
</tr>
</thead>
<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>
</tr>
</tbody>
</table>

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 or 100 µg/ml ConA, 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 or 100 µg/ml ConA, and the incubations proceeded for 60 min. Results are given as a percentage of the response obtained in the absence of inhibitors.

Fig. 4. Characterization of the H2O2 Effect on iPLA2—Collectively, the above data suggest the involvement of cPLA2 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 (Control) 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

Fig. 4. iPLA₂ antisense oligonucleotide inhibits iPLA₂ protein expression and activity and AA release in H₂O₂-treated U937 cells. The cells were either untreated (Control) or treated with sense or antisense oligonucleotides. A, total cytoplasmic protein was evaluated by immunoblot for iPLA₂ (top) or cPLA₂ (bottom). B, effect on cellular iPLA₂ activity. C, effect on the AA mobilization response triggered by 500 μM H₂O₂ (closed bars), 100 μg/ml ConA (open bars), or neither (gray bars).

Fig. 5. Effect of Ca²⁺ on [³H]AA release from U937 cells. The cells were treated with 500 μM H₂O₂, 100 μg/ml ConA, or neither (Control) as indicated for 60 min in medium with 1.3 mM CaCl₂ (open bars), Ca²⁺-free medium with 1 mM EGTA (closed bars), or Ca²⁺-free medium with 1 mM EGTA plus 40 mM quin2/AM (hatched bars). Afterward, supernatants were assayed for [³H]AA release.

Fig. 6. Oleic acid release from U937 cells. The cells, labeled with [³H]oleic acid, were treated with 500 μM H₂O₂, 100 μg/ml ConA, or neither (Control) as indicated, for 60 min. Afterward, supernatants were assayed for [³H]oleic acid release.

Fig. 7. PLA₂ activity of homogenates from U937 cells. Homogenates from untreated cells (Control) or from cells treated with either 500 μM H₂O₂ or 100 μg/ml ConA were prepared, and PLA₂ activity was measured in the absence (open bars) or presence (closed bars) of 1 mM CaCl₂ in the assay mix.
Fatty Acid Release by Hydrogen Peroxide

**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.

measured (Fig. 8). Thus, it must be the physical state of the substrate and not the intrinsic activity of the enzyme that changes after H₂O₂ exposure. 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 but rather on an iPLA₂-like activity. This is based on several lines of evidence, such as the use of chemical inhibitors and of 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 play some role 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 prosta-landins, 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 receptor-mediated apoptosis (41, 42). Tackling 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.
REFERENCES

8. Birbes, H., Gothie
Involvement of Calcium-independent Phospholipase A₂ in Hydrogen Peroxide-induced Accumulation of Free Fatty Acids in Human U937 Cells
Mari?a A. Balboa and Jesús Balsinde

doi: 10.1074/jbc.M206155200 originally published online August 13, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M206155200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 21 of which can be accessed free at
http://www.jbc.org/content/277/43/40384.full.html#ref-list-1