Anthracycline-induced phospholipase A$_2$ inhibition

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Abstract The purpose of this essay is to overview our findings that membrane-associated calcium-independent phospholipase A$_2$ is markedly inhibited by low, clinically relevant concentrations of anthracyclines. Our studies suggest that due to the essential role of this enzyme in membrane homeostasis, its inhibition can be one of the early culprits leading to anthracycline-induced cardiac dysfunction. The clinical importance and potential pharmaceutical use of this new phenomenon await further studies.

Keywords Anthracyclines · Phospholipase A$_2$ · Phospholipids · Cardiomyocytes · Cardiomyopathy

Introduction

Anthracyclines are powerful anticancer antibiotics, the therapeutic efficiency of which is abridged by their prominent cardiotoxicity. Numerous studies have ascribed anthracycline cardiotoxicity to specific cellular pathways, including an increase in free radicals formation, interference of calcium dynamics, adverse effects on RNA and protein synthesis and other putative mechanisms. Unfortunately, many of these studies have been conducted using exceedingly high concentrations of the drugs, making their

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relevance to clinical toxicity questionable. Moreover, therapeutic interventions, based on the above mechanisms, have had limited success. Therefore there is an urgent need to explore alternative hypotheses, which can explain cardiovascular toxicity of these drugs at clinically relevant concentrations. The purpose of this essay is to overview our findings that membrane-associated calcium-independent phospholipase A$_2$ (iPLA$_2$), is markedly inhibited by low, clinically relevant concentrations of anthracyclines [1–4]. For the purpose of brevity we will refer to this newly observed phenomenon as AIPI (Anthracycline-Induced Phospholipase A2 Inhibition). As a result of our studies we suggest that due to the essential role of iPLA$_2$ in membrane homeostasis, AIPI can be one of the early culprits leading to anthracycline-induced cardiac dysfunction.

Doxorubicin and oxidative stress

We start by briefly mentioning our earlier work, which was done to confirm the oxidative theory of anthracycline cardiotoxicity. Specifically we have shown that doxorubicin is toxic to cardiomyocytes with low levels of CuZn superoxide dismutase and this effect can be correlated with the rate of superoxide anion production in the myocyte cytosolic fractions [5]. To visualize the involvement of free radicals in doxorubicin-mediated myocyte injury we used confocal laser scanning microscopy and the oxidant-sensitive fluorescent dye, 2′,7′-dichlorofluorescin. Exposure to doxorubicin produced a rapid and concentration-dependent increase in the fluorescence that appeared to occur in close proximity to the mitochondria [6, 7]. We have also shown that pretreatment of the cells with dexrazoxane inhibited doxorubicin-induced increase in oxidant-sensitive fluorescence. Although these experiments have confirmed the

ability of doxorubicin to generate reactive oxygen species in intact cells, they have also suggested that at clinically relevant concentrations of the drug (<5 μM), their levels are negligibly low and thus will not directly harm the cells. We then hypothesized that reactive oxygen species generated by the drug do not directly damage tissue via oxidative stress, but act as second messengers triggering downstream events. In search of possible culprits we turned our attention to the enzymes that may be affected by low-level oxidative stress. One of the potential targets was myocardial phospholipase A_2.

Anthracycline-induced phospholipase A_2 inhibition

To our surprise, the measurements of total PLA_2 activity in homogenates of control and doxorubicin-treated myocytes have revealed that the enzyme was markedly inhibited [1]. Importantly, the inhibition occurred at low, clinically relevant drug concentrations (as low as 0.1 μM). Our subsequent studies have shown that the effect is both concentration- and time-dependent and that the drug selectively inhibits membrane-associated PLA_2 activity, without altering activity of the cytosolic enzyme (Fig. 1).
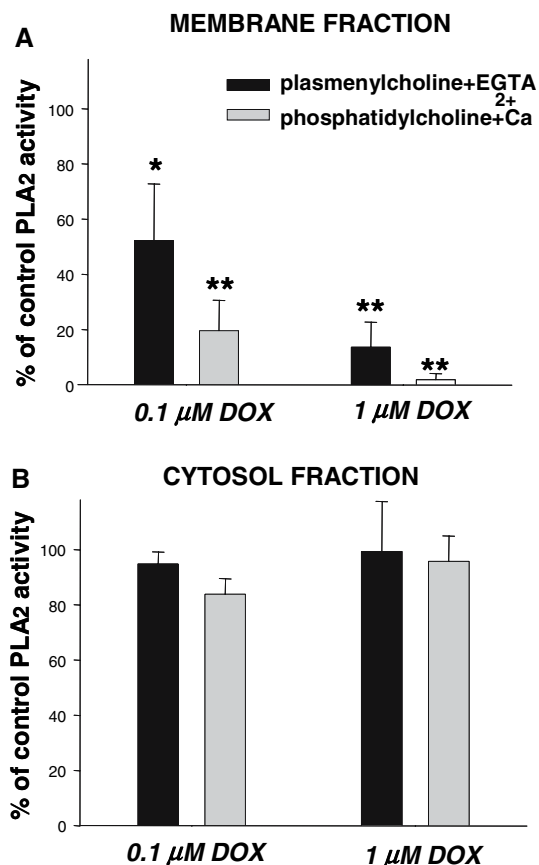

Fig. 1 PLA_2 activity in (**A**) membrane and (**B**) cytosolic fractions of control and doxorubicin-treated rat cardiomyocytes. [1]

The functional impact of iPLA2 inhibition was confirmed by treating cells pre-labeled with [H^3]-arachidonic acid. Decrease in the baseline values of arachidonic acid (the latter being the product of iPLA2 activity) occurred minutes after doxorubicin's addition and persisted for the duration of the treatment (Fig. 2). Finally in vivo experiments, in which adult rats were intravenously injected with 4 mg/kg doxorubicin, confirmed our in vitro findings, revealing a two-fold decrease in membrane-associated Ca^{2+}-independent $iPLA_2$ activity in the heart tissue of drug-treated animals (Fig. 3.).

To explore the mechanism of $iPLA_2$ inhibition we have tested whether it can be caused by the loss of membrane $iPLA_2$ protein to the cytosol (although cytosolic activity did not increase, one can conceive translocation of the inactive form of the enzyme). However, the density of the $iPLA_2$ bands on SDS-PAGE for the membrane and cytosol samples from doxorubicin-treated cells were identical to the bands from untreated cells and no additional bands of lower molecular weight appeared, indicating that doxorubicin treatment did not affect the amount of the actual membrane $iPLA_2$ protein. The second possibility was a covalent modification of the $iPLA_2$. Reducing agents blocked $iPLA_2$ inhibition in cardiomyocyte suspensions, suggesting that the doxorubicin effect is mediated by oxidation of susceptible cysteines [1]. Notably, while tert-butyl hydroperoxide (tBOOH) and H_2O_2 also inhibited iPLA2 activity, it required exceedingly high concentration of the oxidants (Fig. 4) and the effect was not specific to the membrane form of the enzyme [2].

Effect of different analogues

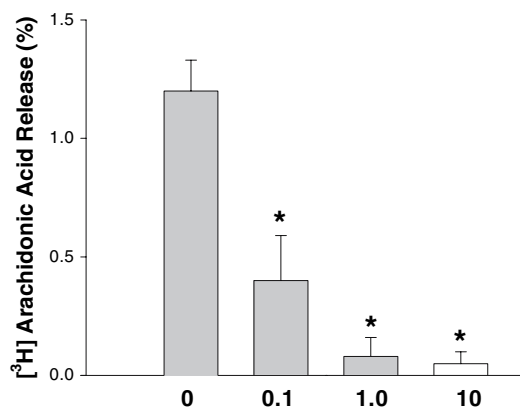We then compared effects of four anthracycline analogues widely used in clinic: doxorubicin, daunorubicin,

Fig. 2 Effect of DOX on [^{3}H]arachidonic acid release. Myocytes were incubated with designated doxorubicin concentrations for 30 min. Basal [^{3}H] arachidonic acid release levels remained constant in the absence of the drug. [1]
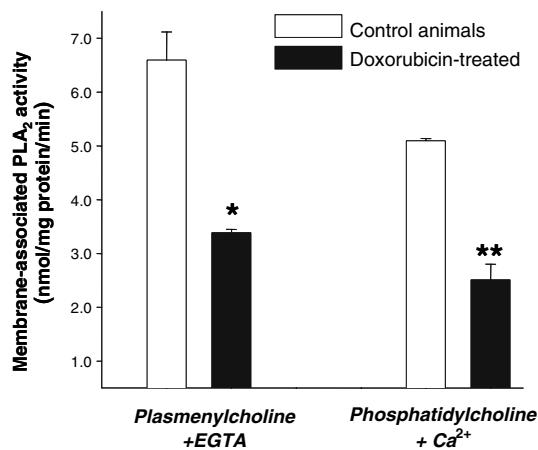
Fig. 3 Membrane-associated iPLA$_2$ activity in hearts from control and DOX-treated rats. Animals were given bolus injection of either saline or doxorubicin (4 mg/kg weight) and myocardial enzyme activity was assessed 4 h later [1]

idarubicin, and epirubicin [3]. We argued that if a correlation is observed between the analogue's effect on iPLA$_2$ activity and clinical indices of cardiotoxicity, it would serve as an important sign that this phenomenon is responsible for the deleterious effects of anthracyclines. For all examined analogues iPLA$_2$ inhibition was concentration- and time-dependent, preceded detectable changes in cell viability and was specific to the membrane-associated enzyme. The degree of inhibition by equimolar concentrations of epirubicin and idarubicin was less than that of doxorubicin or daunorubicin. Overall the ''ranking order'' of analogue's ability to inhibit iPLA$_2$ (doxorubicin $\approx$ daunorubicin > epirubicin > idarubicin) was in agreement with clinical reports and studies in chronic animal models. Interestingly the pecking order of the acute drug's toxicity was quite different (idarubicin > daunorubicin > epirubicin > doxorubicin). The latter sequence was derived from our data on cell necrosis observed after 24 h exposure to 20 μM of each analogue [4]. Such a discrepancy between in vitro and in vivo cardiotoxicity has been noted before, and is evident from a literature survey on comparative studies of analogues' cardiac effects [4]. Notably, the differences in analogues' pharmacokinetics and cardiac accumulation do not necessarily explain the discrepancies between in vivo and in vitro data [8, 9].

Chronic administration of anthracyclines and its effect on phospholipid content

Our next step was to examine whether iPLA$_2$ activity is altered during chronic administration of anthracyclines. These experiments employed a rat model of anthracycline cardiomyopathy, which involves weekly injections of
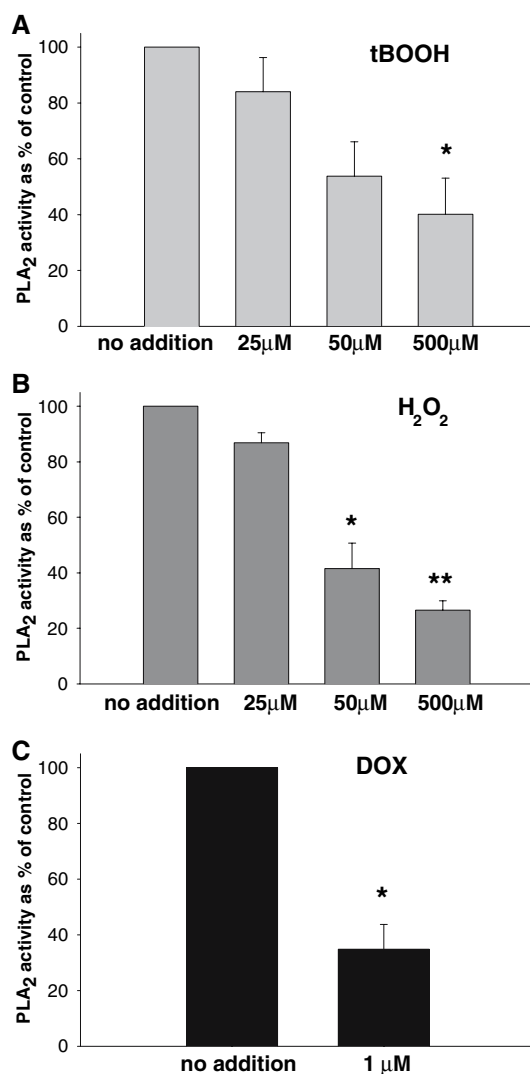

Fig. 4 Activity of iPLA$_2$ in membrane fraction of myocytes pre-treated with (**A**) tert-butyl peroxide, (**B**) hydrogen peroxide and (**C**) doxorubicin. Note the difference is effective oxidant concentrations [2]

doxorubicin [10]. This protocol results in a weight loss, increased heart/body ratio, structural lesions, decreased ejection fraction and eventually leads to the development of congestive heart failure [11]. We employed low, sub-toxic cumulative doses of the drug [8] mg/kg) at which no prominent cardiac lesions were observed (they starts to appear at >15 mg/kg in rat [10]). The goal was to observe changes in iPLA$_2$ activity and membrane composition *before* the development of cardiomyopathic lesions. Indeed, we saw a marked decrease in myocardial iPLA$_2$ activity in animals chronically treated with doxorubicin [4]. Interestingly, a hypothesis that chronically decreased myocardial membrane-associated iPLA$_2$ activity can be detrimental to the heart function was supported by recent data that myocardial infarction decreases membrane iPLA$_2$ protein and

activity [12]. Although reasons for such a decrease remain to be established, one may speculate that myocardial iPLA$_2$ loss is detrimental for both anthracycline-treated and post-infarcted subjects.

Our second goal while conducting chronic animal studies was to observe possible changes in phospholipid content. But why should one expect changes in phospholipid composition in the hearts of doxorubicin-treated animals? The reasons are as follows: The membrane phospholipids are in a constant dynamic state of deacetylation/acetylation with different incorporation rates for individual fatty acids [13]. The iPLA$_2$ has shown substrate preference for plasmalogens and arachidonylated phospholipids [14, 15]. A certain degree of substrate specificity was also observed for AIPI itself [1, 2]. Together this implies that chronic inhibition of the enzyme is highly likely to alter phospholipid composition of both sarcolemma and sarcoplasmic reticulum. To test this assumption we have quantified the phospholipid content of control rats and animals chronically treated with doxorubicin. After HPLC separation of phospholipids into individual classes, the choline (CGP) and ethanolamine glycerophospholipids (EGP) were separated by reverse-phase HPLC, and individual molecular species were collected and quantified using the microphosphate assay. Doxorubicin treatment resulted in significant alterations in the distribution of fatty acyl moieties esterified to the sn-2 position of CGP while EGP-esterified fatty acids remained unaffected. The effect included a selective loss of 20:4 and 18:1 fatty acids in diacyl CGPs and increase in esterified 20:4 and 18:2 fatty acids in choline plasmalogens. The elevation in the amount of arachidonate and linoleate (20:4 and 18:2) esterified to the sn-2 position of plasmalogens is consistent with the notion that iPLA2 displays selectivity for plasmalogens, therefore enzyme inhibition may affect de-esterification of these species. More studies are required to understand the specificity of these changes as well as why mainly choline glycerophospholipids are affected. Notably the above described changes have been seen at low cumulative dose of 8 mg/kg at which very few structural lesions or functional changes occur. Therefore we conclude that doxorubicin-induced phospholipid changes appear to be both specific and early signs, which precede morphological alterations.

Effect on cell survival

Finally we sought direct evidence that a reduction in iPLA2 influenced myocyte survival/function [16]. Cardiomyocytes were pretreated with the iPLA2 inhibitor bromoenol lactone (BEL) followed by additions of low micromolar concentration of tert-butyl hydroperoxide (tBOOH). While either the oxidant or the iPLA2 inhibitor itself had little effects on cells, acting together they significantly decreased cell viability (Fig. 5). The effects of doxorubicin were essentially identical to the effect of the iPLA2 inhibitor, i.e., drug-treated myocytes become more susceptible to the low-level oxidative stress. These data suggest the following explanation. Under normal, non-stressed conditions cells survived even if most of the iPLA2 activity was inhibited. In the samples treated with oxidant only, the remaining iPLA2 and/or the restoration of membrane enzyme activity between the applications alleviated induced lipid peroxidation, preserving the cells. However, during the combined oxidant/inhibitor treatment, inhibition of the detoxification pathway under conditions of stress proved to be lethal.

Linkage to other mechanisms of anthracycline cardiotoxicity

All together our data lead us to suggest that AIPI (Anthracycline-Induced iPLA2 Inhibition) can be linked to chronic anthracycline cardiotoxicity as illustrated in Fig. 6.
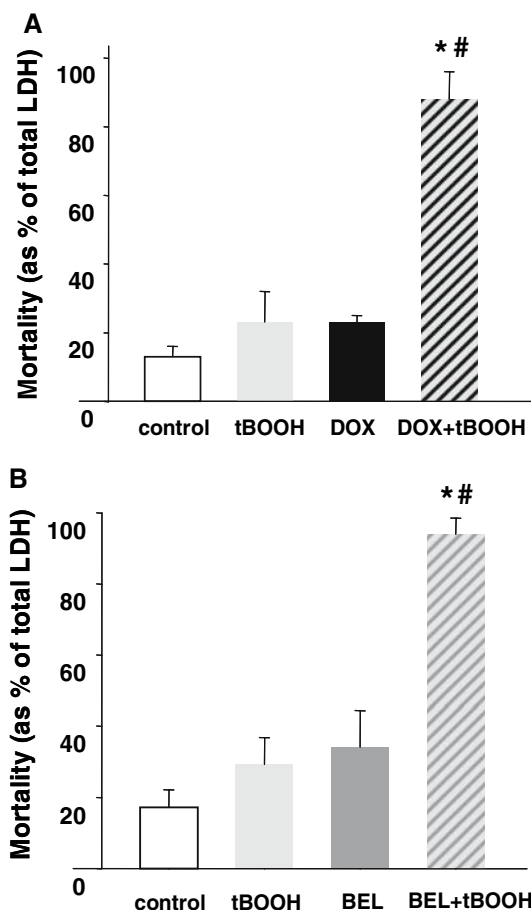

Fig. 5 Potentiation of tert-butyl peroxide toxicity by doxorubicin and iPLA$_2$ inhibitor BEL [3]
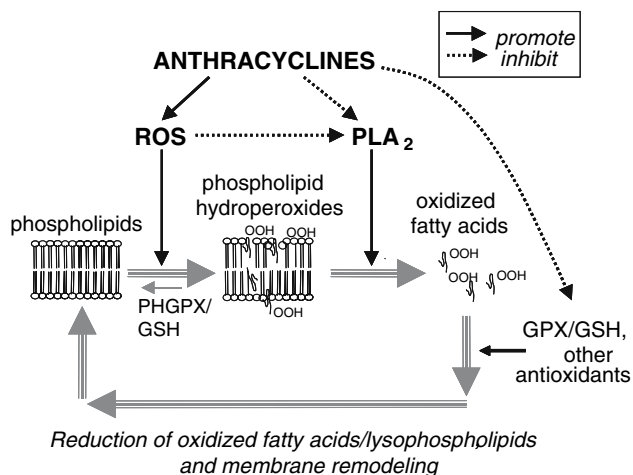
Fig. 6 Proposed role of AIPI (Anthracycline-induced iPLA$_2$ inhibition) in phospholipid turnover and repair cycle. ROS- reactive oxygen species, GPX – glutathione peroxidase, PHGPX – phospholipid hydroperoxide glutathione peroxidase, GSH – glutathione

Phospholipid hydroperoxides are not susceptible to direct reduction by cytosolic glutathione peroxidase. Instead, the oxidized sn-2 fatty acyl groups must first be hydrolyzed by PLA$_2$, and then glutathione peroxidase can act on liberated fatty acid hydroperoxides. Therefore, the ability of cells to deal with anthracycline-enhanced lipid peroxidation is severely compromised by AIPI. It also explains how anthracyclines can lead to measurable lipid peroxidation [17], while no significant increases in free radical formation have been detected at clinically relevant doxorubicin concentrations [6, 18]. Another selenium-dependent enzyme - membrane-bound phospholipid hydroperoxide glutathione peroxidase (PHGPX) has been shown to reduce phospholipid hydroperoxides *in situ* without the necessity of prior hydrolysis by PLA$_2$ [19]. However, the activity of PHGPX in cardiac muscle is a hundred times lower than the activity of soluble glutathione peroxidase [20]. Interestingly, a significant increase in membrane-associated PHGPX activity (with no changes in the cytosolic enzyme) was found in the heart tissue of rats treated with doxorubicin [21], suggesting some adaptive mechanisms to deal with increased phospholipid peroxides when PLA$_2$ activity is diminished.

Another point where oxidative stress is likely to be involved is iPLA$_2$ inhibition itself via oxidation of cysteines [1]. Thus, the fact that an increase in tissue antioxidant capacity is capable of alleviating anthracycline toxicity may be due: (i) relief of iPLA$_2$ inactivation by restoring active cysteines; and (ii) detoxification of accumulated phospholipid peroxides. Therefore, the AIPI-based mechanism allows one to narrow down the previous oxyradical hypothesis to more specific pathways.

The calcium hypothesis, which implicates abnormal Ca$^{++}_{in}$ cycling, with ryanodine receptors as the major cul-

prit, can also be linked to AIPI. Several independent laboratories have shown that chronic doxorubicin treatment causes an impairment of calcium-induced-calcium-release [22, 23]. The latter requires a coordinated effort from L-type calcium channels, ryanodine receptors, SERCA and other membrane proteins [24]. Thus, it is intriguing to suggest that AIPI-induced changes in the phospholipid environment adversely affect the finely-tuned system of calcium-induced-calcium-release with a resulting decrease in contractility and cardiac function.

Cardiac sarcolemmal cis-unsaturated fatty acid sensitive phospholipase D (cis-UFA phospholipase D) is modulated by iPLA$_2$ activity via intramembrane release of cis-UFA [12, 25]. As phospholipase D-derived phosphatidic acid influences intracellular Ca^{2+} concentration and contractile performance [26], changes in iPLA$_2$ activity may contribute to abnormal function of the failing heart. Thus, the AIPI might contribute to the defective Ca^{2+} handling and contractile performance of the failing heart via decreased activity of cis-UFA phospholipase D.

We have recently observed an abundance of iPLA2 protein within the intercalated disc areas of cardiac myocytes (Fig. 7). This finding suggests that these sites possess high metabolic activity of iPLA2, which may be important for the maintenance of complex protein-phospholipid structures at the cellular interface. Since ruptured intercalated disks have been observed in electron micrographs from subjects with anthracycline cardiomyopathy, it is tempting to suggest a link between AIPI, intercalated disk stability, titin attachment and/or cardiac remodeling.

Interestingly, the AIPI also allows one to explain the ''paradoxically'' decreased circulating levels of conjugated dienes and hydroperoxides shown to occur after intravenous administration of doxorubicin to cancer patients [27]. This effect is likely to be a direct manifestation of AIPI, which decreases the release of conjugated dienes and hydroperoxides from oxidized cardiac membranes. Therefore, the above mentioned study may provide the first indirect evidence that AIPI occurs in humans.
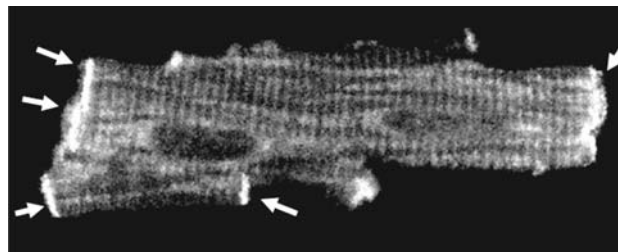

Fig. 7 Immunostaining of adult rat cardiac myocyte for iPLA$_2$ (polyclonal antibody from Cayman Chemicals). The cross-striated pattern suggests presence of iPLA$_2$ protein in the T-tubules. The arrows point to the abundance of the protein in the intercalated disks

Conclusion

In summary, we have shown that low micromolar concentrations of anthracyclines inhibit iPLA2, a major housekeeping enzyme. A series of follow-up studies led us to suggest that this phenomenon may serve as one of the initial steps in a series of events leading to anthracycline cardiomyopathy. While intriguing, this hypothesis awaits further experimental confirmation. Its' clinical importance as well as potential pharmaceutical application also remain to be explored.

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