

Clinical Concentrations of Doxorubicin Inhibit Activity of Myocardial Membrane-associated, Calcium-independent Phospholipase A₂¹

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ABSTRACT

Use of the anticancer antibiotic doxorubicin continues to be limited by its cumulative dose-related cardiotoxicity. Our study reports inhibition of myocardial intracellular calcium-independent phospholipase A₂ (iPLA₂) activity by clinically relevant concentrations of the drug. The effect was first shown *in vitro* using suspensions of freshly isolated rat and rabbit cardiomyocytes. Addition of 0.1–10 μM doxorubicin to these cells led to a concentration- and time-dependent inhibition of total iPLA₂, as measured using (16:0, [³H]18:1) plasmenylcholine and phosphatidylcholine substrates in the presence or absence of calcium. Subcellular fractionation into cytosolic and membrane fraction revealed that the drug selectively inhibits membrane-associated iPLA₂ activity, without altering activity of the cytosolic enzyme. Doxorubicin treatment of cells prelabeled with [³H]arachidonic acid led to a depression of baseline arachidonic acid release levels, corroborating iPLA₂ inhibition. Reducing agents blocked PLA₂ inhibition in cardiomyocyte suspensions, suggesting that the doxorubicin effect is mediated by oxidation of susceptible cysteines. *In vivo* experiments, in which adult rats were *i.v.* injected with a bolus dose of 4 mg/kg doxorubicin, confirmed *in vitro* findings, revealing a 2-fold decrease in membrane-associated Ca²⁺-independent iPLA₂ activity in the heart tissue of treated animals. The observed phenomenon has important implications for myocyte signaling cascades and membrane remodeling.

INTRODUCTION

DOX³ and related anthracycline antibiotics are among the most powerful anticancer drugs used in clinical medicine (1, 2). Unfortunately, anthracycline therapy is associated with an acute as well as cumulative dose-related cardiomyopathy (3, 4). A variety of mechanisms have been suggested to explain the cardiotoxicity of anthracyclines, with many of the proposed pathways implying increased formation of oxygen free radicals (3–8). The oxyradical hypothesis of DOX cardiotoxicity was also supported by our studies, which revealed that cardiomyocytes with a diminished level of superoxide dismutase are more susceptible to DOX exposure (7) and that an increase in intracellular oxidation after exposure to high concentrations of DOX can be visualized directly in isolated cardiac cells (8). Despite numerous supporting pieces of evidence, however, the oxyradical hypothesis as well as other presumptive mechanisms of DOX cardiotoxicity continues to be a matter of controversy, mainly because most of the data came from studying subcellular fractions, often in combination with extremely high concentrations of DOX not encountered in clinical practice (3, 4). Furthermore, none of the therapeutic venues to alleviate the cardiotoxicity of the drug, suggested by the above mechanisms, have been particularly successful (2, 3). Therefore, the search

for the exact pathways leading to anthracycline-induced cardiomyopathy continues.

PLA₂ represents a diverse family of enzymes that hydrolyze the fatty acyl group from the *sn*-2 position of glycerophospholipids, releasing lysophospholipids and free fatty acids (9–12). A number of PLA₂s display enhanced catalytic activity in response to cell membrane lipid peroxidation (10, 11). The conformational changes imposed by oxidized phospholipids are consistent with reported increases in PLA₂ activity at membrane sites having packing defects and appear to be most prominent for membranes containing phospholipid hydroperoxides (10).

On the basis of the above observations (10–12), the known ability of DOX to promote lipid peroxidation (4, 5, 13), and incidental reports of activation of PLA₂ by anthracyclines *in vitro* (14, 15), one would expect to observe an increase in PLA₂ activity upon treatment with these drugs. However, our study in isolated cardiomyocytes and intact heart tissue revealed the opposite effect. Specifically, we have shown for the first time that high nanomolar concentrations of DOX inhibit intracellular myocardial PLA₂ activity, because of the rapid inactivation of the membrane-associated, calcium-independent form of the enzyme. This novel observation has implications for cardiomyocyte signaling cascades and membrane remodeling and suggests novel pathways for anthracycline cardiotoxicity.

MATERIALS AND METHODS

Materials. Collagenase (type II) was purchased from Worthington Biochemical (Lakewood, NJ). [³H]JAA and [³H]oleic acid were purchased from NEN (Boston, MA). BEL was a gift from Hoffmann-LaRoche (Nutley, NJ). Doxorubicin, MEM, gentamicin, albumin, HEPES, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Rat and Rabbit Ventricular Cardiomyocytes. Cells were obtained from adult Sprague Dawley male rats (200–300 g) and New Zealand rabbits of either sex (2–3 kg) using retrograde Langendorff perfusion with collagenase (8, 16). A yield of 5–7 × 10⁶ myocytes/rat heart and 14–20 × 10⁶ myocytes/rabbit heart was obtained routinely. Myocyte viability was evaluated by the microscopic determination of the number of rod-shaped cells and the number of the myocytes that excluded trypan blue (7).

Short-term Treatment of Myocytes with DOX. Myocytes in suspensions (0.25 × 10⁶ cell/ml of Tyrode, supplemented with 10 mM HEPES, pH 7.3) were incubated at room temperature with designated DOX concentrations. GSH and DTT were prepared fresh and added to the myocytes 30 min prior DOX. For measurement of PLA₂ activity and Western blots, cells were transferred to ice-cold PLA₂ assay buffer containing 250 mM sucrose, 10 mM KCl, 10 mM imidazole, 5 mM EDTA 5, 2 mM DTT, and 10% glycerol (pH 7.8).

Long-term Treatment of Myocytes with DOX. Cardiomyocytes were plated onto laminin-covered glass coverslips and placed in MEM supplemented with 5 mM HEPES, 10 μg/ml gentamicin, 0.1 μg/ml streptomycin, and 0.1 unit/ml penicillin for 4–5 h. Cells were then preincubated with or without 10 μM BEL for 30 min, and 10 μM DOX was added thereafter. This schedule was repeated every 12 h for a total of four treatments. Corresponding controls were run in parallel. Within 48 h, the total average viability of each slip was estimated by LDH assay and morphology.

Preparation of Cytosolic and Membrane Fractions. Myocytes suspended in ice-cold PLA₂ assay buffer were sonicated six times for 10 s, and the sonicate was centrifuged at 14,000 × *g* for 20 min to remove cell debris, nuclei, and mitochondria. The resultant supernatant fraction was centrifuged at

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³ The abbreviations used are: DOX, doxorubicin; PLA₂, phospholipase A₂; iPLA₂, calcium-independent PLA₂; BEL, bromoenol lactone; GSH, glutathione; LDH, lactate dehydrogenase; AA, arachidonic acid release; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; ROS, reactive oxygen species; GPX, cytosolic glutathione peroxidase; HPGPX, hydroperoxide glutathione peroxidase.

100,000 × *g* for 1 h to separate the membrane fraction (pellet) from the cytosolic fraction (supernatant).

In Vivo Experiments with DOX. Male Sprague Dawley rats (300–400 g) were injected via tail vein with 0.75–1 ml volume of either saline or DOX (single dose of 4 mg/kg). Four h after the hearts were removed, trimmed of connective tissue, perfused with saline, homogenized in ice-cold PLA₂ assay buffer, sonicated, and assayed for enzyme activity as described above.

Western Blot Analysis of PLA₂. Proteins (2–40 μg) from each sample were mixed with an equal volume of SDS sample buffer, boiled at 95°C for 5 min, and subjected to the SDS-PAGE and Western blot procedure as described previously (17). The nitrocellulose membranes were incubated with primary antibodies against iPLA₂, washed, and treated with horseradish peroxidase-linked secondary antibody. The regions of antibody binding were detected with the enhanced chemiluminescence method (SuperSignal kit; Pierce). Immunoblots were quantified using densitometric analysis (Multi-Analyst; Bio-Rad).

Assay of PLA₂ Activity. PLA₂ activity was quantified by incubating enzyme (200 μg of total protein, 8 μg of membrane protein, or 200 μg of cytosolic protein) with 100 μM (16:0, [³H]18:1) plasmeylcholine or phosphatidylcholine (18) in assay buffer containing 100 mM Tris, 10% glycerol (pH 7.0), with either 4 mM EGTA or 1 mM CaCl₂ at 37°C for 5 min in a total volume of 200 μl. Reactions were terminated by the addition of 100 μl of butanol. Released radiolabeled fatty acid was isolated by TLC on silica G plates, followed by development in petroleum ether-diethyl ether-acetic acid (70:30:1 v/v/v), and quantification by liquid scintillation spectrometry. The reaction conditions selected resulted in linear reaction velocities with respect to both time and total protein concentration for each substrate examined. Protein content was determined by the Lowry method.

AA Release. Rabbit myocytes were incubated overnight with [³H]AA (3 μCi/ml cells). Myocytes were washed three times with Tyrode solution containing 3.6% BSA to remove unincorporated [³H]AA and incubated at 37°C for the duration of DOX exposure. The percentage of released [³H]AA was quantified by rapidly centrifuging the myocyte suspension and measuring the amount of radioactivity in the supernatant and in the cell pellet after lysis with 10% SDS.

Statistics. Statistical comparison of values was performed by Student's *t* test. All results are expressed as mean ± SE. Statistical significance was considered to be *P* < 0.05.

RESULTS

Myocardial PLA₂ Activity. PLA₂ activity was measured both in total homogenate and subcellular fractions from rat hearts and isolated

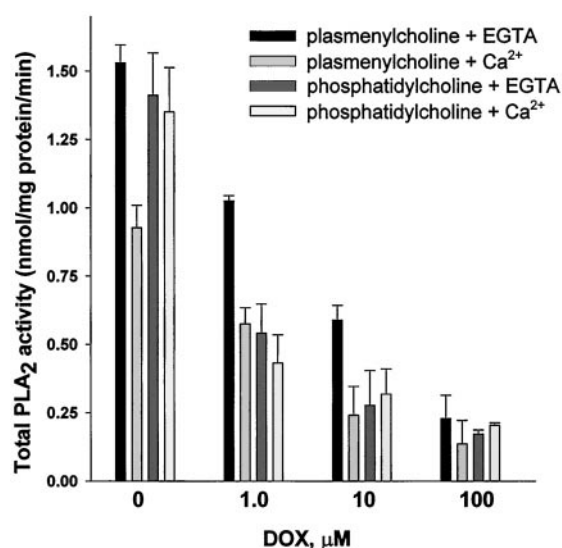


Fig. 1. Total PLA₂ activity in control and DOX-treated rat cardiomyocytes. The cells were incubated with designated concentrations of DOX for 30 min. PLA₂ activity of whole-cell sonicates was measured using two phospholipid substrates in the presence of 4 mM EGTA or 1 mM Ca²⁺. Values are means for individual values derived from three separate preparations; bars, SE.

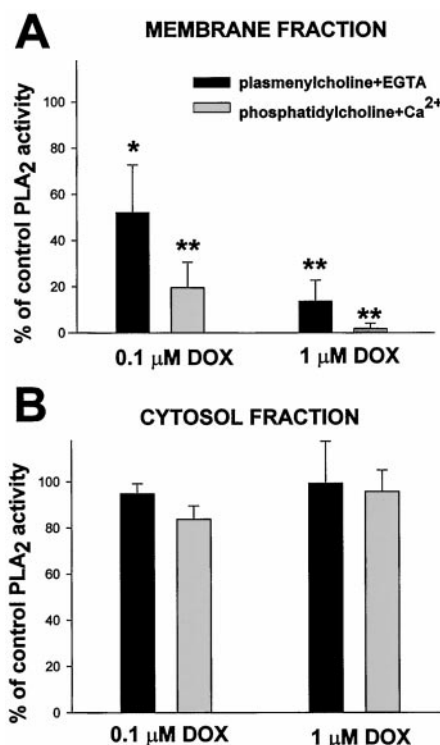


Fig. 2. PLA₂ activity in membrane (A) and cytosolic fractions (B) of control and DOX-treated rat cardiomyocytes. Activity was measured using (16:0, [³H]18:1) plasmeylcholine in the presence of 4 mM EGTA or (16:0, [³H]18:1) phosphatidylcholine in the presence of 1 mM Ca²⁺. Values are means (*n* = 3); bars, SE. *, *P* < 0.05; **, *P* < 0.01 versus corresponding controls.

rat and rabbit ventricular cardiomyocytes. PLA₂ assays were conducted using both plasmeylcholine and phosphatidylcholine substrates radiolabeled with oleic acid at the *sn*-2 position. The presence of Ca²⁺ in the assay buffer did not increase enzyme activity, indicating that the PLA₂ activity was largely Ca²⁺ independent (iPLA₂, Fig. 1). The majority of myocardial PLA₂ activity was represented by membrane-associated enzyme, similar to our earlier reports (16–18). The membrane-associated iPLA₂ activity was BEL sensitive and was not affected by sonication or submicellar concentration of detergents, as reported by others (19) and in accordance with our previous studies (16–18). Membrane-associated iPLA₂ activity, equivalent to the rat and rabbit enzyme measured in our study, is present in human myocardium (20), indicating that observed effects are most likely to occur in the course of anthracycline therapy in humans.

DOX Effect on Cardiomyocyte PLA₂ Activity. Addition of 0.1–10 μM DOX to suspensions of isolated rat cardiomyocytes led to a concentration-dependent inhibition of total iPLA₂ activity under all assay conditions studied (Fig. 1). Subcellular fractionation into cytosolic and membrane fractions revealed that the drug selectively inhibits membrane-associated PLA₂ activity (Fig. 2). The membrane PLA₂ inhibition was DOX concentration and incubation time dependent and was observed using both rat and rabbit cardiomyocytes (Fig. 3). No change in cytosolic PLA₂ activity was detected after either 10 or 30 min DOX treatment or when concentrations of the drug were increased to 10 μM (Fig. 2 and data not shown).

DOX Effect on AA Release. We examined the effect of DOX on AA release using isolated rabbit cardiomyocytes (Fig. 4). DOX treatment inhibited the release of radioactive label, thus corroborating decreased enzyme activity (PLA₂ cleaves AA from the *sn*-2 position of membrane phospholipids, releasing AA into the medium). The inhibition of AA release occurred minutes after drug addition and persisted for the duration of DOX treatment (data not shown).

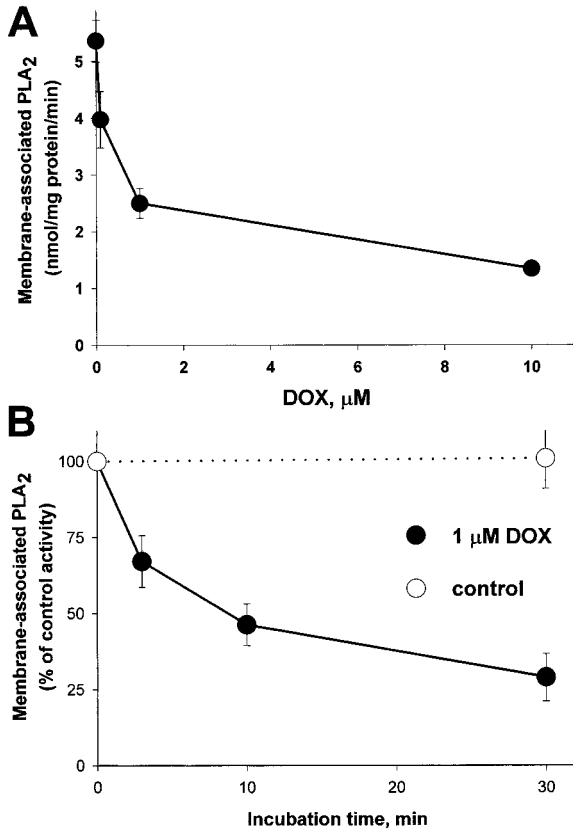


Fig. 3. DOX inhibition of membrane-associated PLA₂. *A*, the concentration course obtained using rabbit myocytes, 30 min incubation. *B*, time course obtained using isolated rat myocytes, 1 μM DOX. Activity was measured using (16:0, [³H]18:1) plasmenylcholine in the presence of 4 mM EGTA. Values are means (*n* = 3–6); bars, SE. *, *P* < 0.05; **, *P* < 0.01 compared with controls.

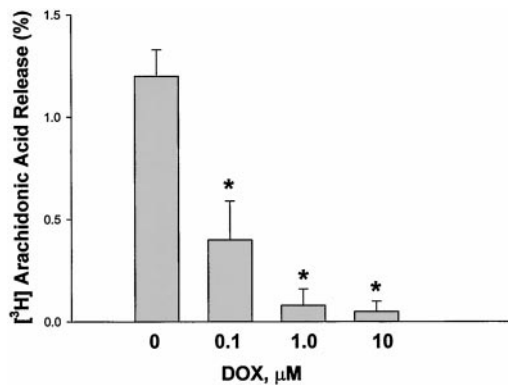


Fig. 4. Effect of DOX on [³H]AA release from rabbit ventricular myocytes. Myocytes were incubated with designated DOX concentrations for 30 min. Basal [³H]AA release levels were constant in the absence of DOX (data not shown). Values represent means of six determinations from two separate preparations; bars, SE.

In Vivo Effects of DOX on Heart PLA₂ Activity. Plasma concentrations of DOX during bolus administration can reach as high as 10 μM, followed by exponential decline with 24–30 h half-time (5). Therefore, the concentrations we used in *in vitro* experiments with myocytes (0.1–1 μM DOX) are expected to be present in a patient’s plasma for at least 2 h. However, toxic effects of DOX can be substantially less *in vivo* because of the presence of albumin, glutathione, and other antioxidants in the plasma. Therefore, it was important to confirm that myocardial PLA₂ inhibition occurs during *i.v.* drug treatment. A bolus dose of 4 mg/kg DOX was administered to the rats according to the protocols, which led to plasma drug concentra-

tions similar to those found in clinical settings (21, 22). After 4 h, the hearts of the control and treated animals were assessed for PLA₂ activity, as described in “Materials and Methods.” A 2-fold decrease in membrane-associated iPLA₂ activity was highly significant (*P* < 0.005) and was measured with both with plasmenylcholine and phosphatidylcholine substrates (Fig. 5).

Western Blots of Cytosolic and Membrane PLA₂. In an effort to understand the mechanism of DOX effect, we assessed the amount of iPLA₂ protein in both cytosol and membrane fractions after cardiomyocyte treatment with DOX. The densities of the iPLA₂ bands in membrane and cytosol samples were not affected by DOX treatment, and no additional bands were detected (Fig. 6 and data not shown), suggesting that exposure to DOX did not alter the amount of the iPLA₂ protein in either fraction or result in cleavage of iPLA₂ protein. Immunoblot analysis of purified iPLA₂ protein (Genetics Institute) was used as a positive control and demonstrated the presence of iPLA₂ at the same molecular mass (~85 kDa).

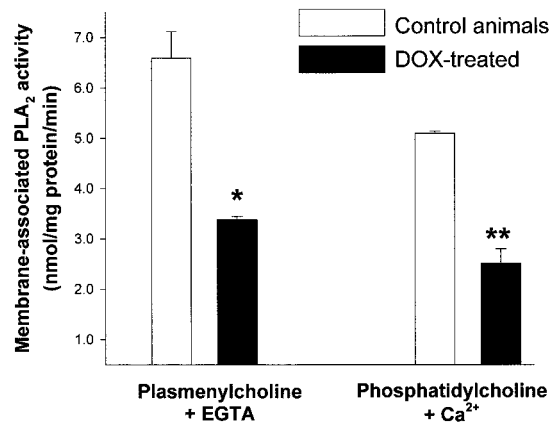


Fig. 5. Membrane-associated PLA₂ activity in hearts from control and DOX-treated rats. Animals were given bolus injection of either saline or DOX (4 mg/kg weight), and myocardial enzyme activity was assessed 4 h later. PLA₂ activity was measured using 100 μM plasmenylcholine in the presence of 4 mM EGTA or phosphatidylcholine in the presence of 1 mM CaCl₂. Values are means from three animals in each group; bars, SE. *, *P* < 0.05; **, *P* < 0.01 versus control animals.

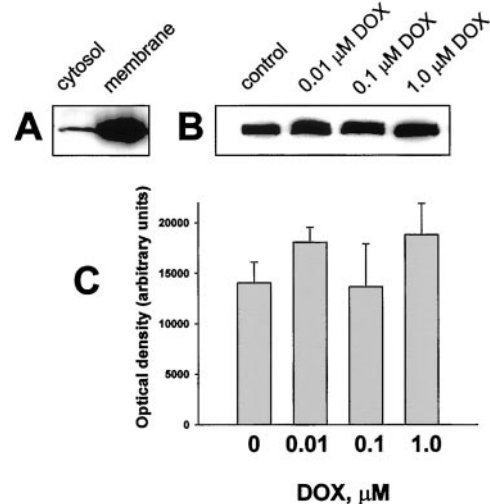


Fig. 6. Immunoblot analysis of PLA₂. *A*, relative intensity of the cytosolic and membrane-associated PLA₂ staining in rat cardiomyocytes (both lanes were loaded with identical amount of protein). *B*, representative experiment (of three), which shows iPLA₂ staining in membranes of cells treated with increasing concentrations of DOX for 30 min. *C*, average density of the bands for the experiment shown in *B*. Density values are means (*n* = 3); bars, SE.

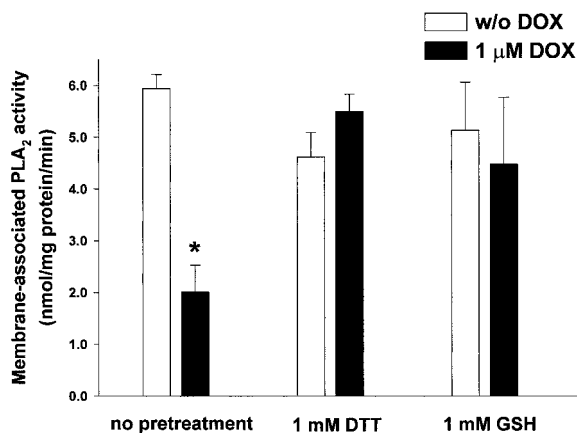


Fig. 7. Effect of pretreatment with thiol-reducing agents on DOX-induced PLA₂ inhibition. Cardiomyocytes were pretreated with either 1 mM DTT or 1 mM glutathione for 30 min, followed by addition of 1 μM DOX for another 30 min. Activity was measured using (16:0, [³H]18:1) plasmenylcholine in the presence of 4 mM EGTA. Values represent means ($n = 3$); bars, SE. *, $P < 0.05$ compared with corresponding sample without DOX.

DTT and GSH Alleviate DOX-mediated PLA₂ Inhibition. Several studies have shown an existence of essential cysteines in a variety of lipases, including Cys-331 of cytosolic Ca²⁺-dependent PLA₂ (23, 24). Moreover, DTNB, an agent that covalently modifies the SH group, has been shown to directly inhibit myocardial membrane-associated PLA₂ activity (19). We therefore hypothesized that DOX effects are mediated by oxidation of essential SH groups and tested the ability of two reducing agents to alleviate DOX effect. Specifically, the cells were pretreated with either 1 mM GSH or DTT, and 1 μM DOX was added thereafter. Reducing agents prevented DOX-induced PLA₂ inhibition, strongly suggesting that the effect of the drug is mediated by oxidation of cysteines (Fig. 7). Importantly, the presence of 2 mM DTT in PLA₂ assay buffer did not restore the activity of PLA₂ in DOX-treated samples.

Potential of DOX Toxicity by iPLA₂ Inhibitor BEL. To establish a causal relationship between the observed inhibition of PLA₂ activity and the deleterious effects of DOX, we designed an experiment that tested the ability of a specific iPLA₂ inhibitor, BEL (25), to alter DOX toxicity. Specifically, isolated cardiomyocytes were subcultured in a serum-free medium that allows cells to retain their rod-shaped phenotype for several days (26). Ten μM DOX was administered every 12 h, and cell viability was assessed after 48 h of treatment using both rod-shaped cell morphology and LDH release (total LDH content of the cells was determined after cell disruption with saponin). If 10 μM BEL was added 30 min before DOX treatment, it substantially potentiated the toxic effect of the drug. BEL by itself did not adversely affect myocyte viability within 48 h (Fig. 8).

DISCUSSION

We report a marked inhibition of the intracellular, membrane-associated iPLA₂ by clinically relevant DOX concentrations. DOX administration caused a concentration- and time-dependent inactivation of this protein in cardiac tissue both *in vitro* and *in vivo* (Figs. 1–5). Three main types of PLA₂ have been described in mammalian tissues: secretory, cytosolic Ca²⁺-dependent, and the iPLA₂. Several independent studies have shown that the majority of PLA₂ activity in myocardium tissue and in ventricular myocytes is constituted by a membrane-associated iPLA₂ (16–19, 27). The most recent report cloned and confirmed the importance of this enzyme in human myocardium (20).

For the most part, the diverse functions of these enzymes can be

divided into two major categories, “housekeeping” and “signaling.” Housekeeping functions of PLA₂ include phospholipid turnover, membrane remodeling, and removal of phospholipid peroxides. Under pathological situations, altered PLA₂ activity may result in the loss/gain of essential membrane glycerophospholipids and oxidized lipids, leading to changes in membrane fluidity, permeability, and ion homeostasis (9, 27). The second category includes the essential role of PLA₂ in cell signaling (9, 12). The products of PLA₂-catalyzed hydrolysis of membrane phospholipids can be converted to eicosanoids, platelet-activating factor, and lysophosphatidic acid or act as second messengers themselves. Hundreds of studies report the activation of PLA₂, release of AA and other PLA₂ products during inflammation, oxidative stress, and other pathophysiological events.

What is the mechanism of iPLA₂ inhibition by DOX? At least three possibilities can be readily suggested: changes in phospholipid environment, loss of PLA₂ protein, or modification of the enzyme itself. The interaction of anthracyclines with lipids has been the subject of numerous studies (13, 28). At neutral pH, DOX is partially protonated with a net positive charge and is suggested to electrostatically bind to acidic head groups of cardiolipin and other acidic phospholipids (28). Many earlier studies have shown marked effects of DOX on membrane fluidity and cholesterol clustering (13). On the other hand, the catalytic activity of PLA₂ has been shown to be extremely sensitive to the topology of the bilayer, the membrane fluidity, surface charge, and saturation index (10, 11). Therefore, one can suggest that observed inactivation of PLA₂ by DOX may be attributable to the alteration in membrane structure and its physical properties. However, the PLA₂ assay procedure involves repetitive sonication and several washing steps that should minimize the presence of the drug in the membrane fraction. Moreover, the addition of different amounts of labeled substrate does not alter the degree of PLA₂ inhibition by DOX (data not

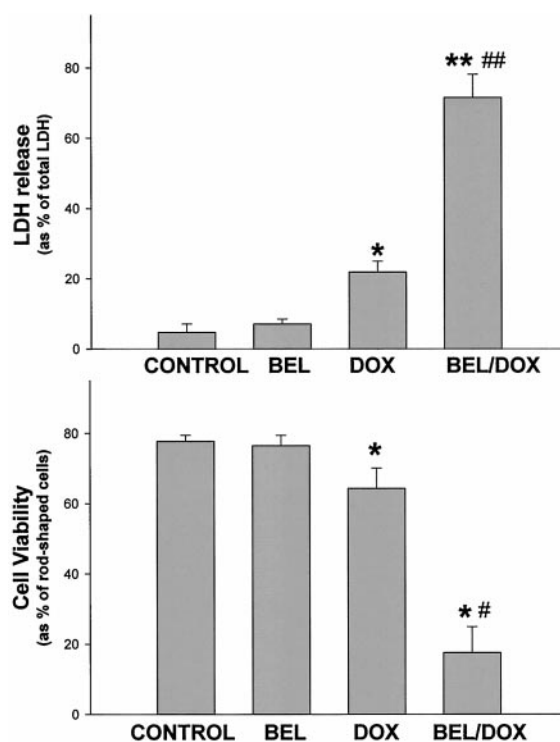


Fig. 8. BEL effect on DOX toxicity. Myocytes were pretreated with 10 μM BEL for 30 min before each DOX application. An application of 10 μM DOX was made every 12 h, and the viability of myocytes was assessed 48 h later. Values represent means of average values from four separate preparations; bars, SE. Each preparation consisted of duplicate coverslips for the control and BEL samples and quadruplicate coverslips for the DOX and DOX/BEL treatments. *, $P < 0.05$; **, $P < 0.01$ (versus control samples); #, $P < 0.05$; ##, $P < 0.01$ (versus DOX samples).

shown). These data argue against the substrate-based mechanism of DOX inhibition.

The second possibility may be a loss of membrane iPLA₂. We hypothesized that the drug treatment may result in a loss of membrane iPLA₂ protein, by either causing its translocation to the cytosol (albeit cytosolic activity did not increase, one can conceive translocation of inactive form of the enzyme) or by cleavage into a nonfunctional protein. However, the density of the iPLA₂ bands in membrane and cytosol samples from DOX-treated cells were identical to the bands from untreated cells (Fig. 6), and no additional bands of lower molecular weight appeared, indicating that DOX treatment did not affect the amount of the actual membrane PLA₂ protein.

The third possibility is a covalent modification of the iPLA₂ protein. The experiments with reducing agents suggest strongly that oxidation of essential thiol residues is responsible for the observed inhibition (Fig. 7). Indeed, an earlier study has shown that 1 mM DTNB, a compound that covalently modifies thiol groups, inhibits myocardial membrane PLA₂ (19). Interestingly, the cytosolic iPLA₂ activity was not affected by DOX (Fig. 2). More data are needed to determine what makes membrane-associated iPLA₂ more sensitive than the cytosolic enzyme—peculiarity of the enzyme structure that can make cysteine residues less accessible, or the fact that DOX exhibits high affinity toward acidic phospholipids and its proximity to iPLA₂ within the lipid bilayer may increase the likelihood of the encounter of DOX-generated ROS and iPLA₂ cysteines. The distinct sensitivity of PLA₂s to DTNB in different cell fractions was also observed in human myocardium (19), highlighting differences in the chemical moieties of organelle-specific iPLA₂s.

Several earlier studies reported the ability of DOX to promote oxidation of protein thiols in a variety of proteins (4, 29, 30). However, most of the effects were observed *in vitro* by incubating heart microsomes with high micromolar concentrations of the drug or/and in the presence of metal salts. In contrast, the DOX effect on myocardial PLA₂ was observed *in vivo* using 1000-fold lower concentration of the drug. At these DOX concentrations, most studies (5), including ours (8), failed to detect any changes in baseline ROS formation, and oxidation of PLA₂ thiols indicates the distinctive sensitivity of this enzyme to the drug.

There are several ways to incorporate previous findings about DOX effect on cardiac tissue with observed inactivation of iPLA₂. Chronic inhibition of PLA₂ activity may impact membrane lipid composition and physical properties, which in turn causes alterations in the function of integral membrane proteins, such as ion channels or receptors. Moreover, reaction products of PLA₂ are important second messengers, and alterations in their response can markedly affect mitochondrial or sarcoplasmic reticulum function. These broad possibilities aside, we suggest a series of steps through which DOX-induced inhibition of PLA₂ can lead to increase in lipid peroxides and thus directly couple free radical hypothesis of DOX cardiotoxicity to our new data (Fig. 9). DOX has been shown to produce ROS through either enzyme-mediated cycling of semiquinone radical or drug complexation with iron (5, 30), and it was suggested that DOX-induced increase in superoxide anions leads to membrane peroxidation. It is, however, doubtful that ROS produced by clinically relevant drug concentrations are capable of significantly changing the amount of membrane peroxides, especially if the cell antioxidant defense system is intact. Nevertheless, several studies have found enhanced lipid peroxidation in myocardium of DOX-treated animals and patients who had anthracycline therapy (3, 5, 21, 22). This apparent paradox may be resolved if one assumes that DOX-induced PLA₂ inhibition severely compromises cell capacity to restore oxidized phospholipids (Fig. 9). Previous studies have established that phospholipid hydroperoxides are not susceptible to direct reduction by GPX (31).

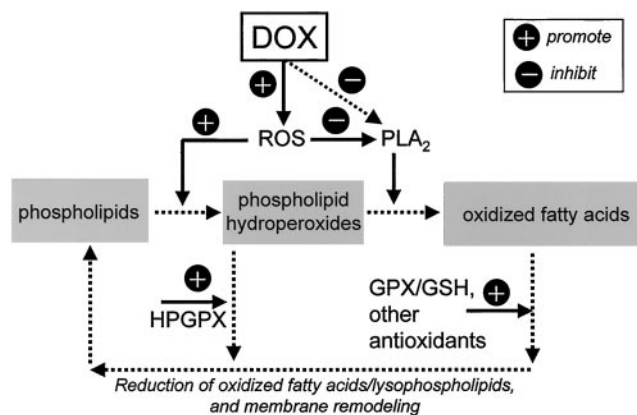


Fig. 9. Proposed mechanism of DOX cardiotoxicity and its relationship with oxyradical hypothesis.

Instead, the oxidized *sn*-2 fatty acyl groups must first be hydrolyzed by PLA₂, and GPXs then act on liberated fatty acid hydroperoxides. Therefore, we hypothesize that the ability of cells to deal with DOX-enhanced lipid peroxidation is severely compromised by DOX-induced PLA₂ inhibition, with ensuing deleterious effects. Another selenium-dependent, enzyme-phospholipid HPGPX have been shown to reduce phospholipid hydroperoxides *in situ* without the necessity of prior hydrolysis by PLA₂ (32). However, the activity of membrane-bound HPGPX in cardiac muscle is 100 times lower than the activity of soluble GPX (33). Interestingly, a significant increase in membrane-associated HPGPX activity (with no changes in cytosolic GPX levels) was found in the heart tissue of rats treated with DOX (22), suggesting some adaptive mechanisms to deal with increased phospholipid peroxides when PLA₂ activity is diminished.

The proposed sequence of events is further supported by our experiments with BEL, a selective inhibitor for Ca²⁺-independent PLA₂s (Fig. 8). DOX by itself has little effect on cell viability under the conditions used, and BEL-treated samples were not different from controls. However, pretreatment with BEL significantly potentiated DOX toxicity. We thus hypothesize that for the samples treated with DOX only, partial inhibition of PLA₂ and/or restoration of membrane enzyme activity between DOX applications can alleviate DOX-induced changes in lipid content. Moreover, the cytosolic PLA₂ is not inhibited by DOX and may assist in hydrolyzing peroxidized phospholipids. Treatment with BEL irreversibly inactivates both membrane-bound and cytosolic iPLA₂ isoforms, making DOX-induced peroxidation much more toxic [in cardiomyocytes, the activities of other PLA₂s, *e.g.*, secretory PLA₂ and Ca²⁺-dependent PLA₂ are insignificant as compared with iPLA₂ (20, 27)]. On the basis of the same reasoning, we also suggest that under normal, nonstressed conditions, cells can compensate baseline levels of lipid peroxidation even if most of the iPLA₂ activity is inhibited by BEL. However, during DOX exposure, as the concentration of phospholipid peroxides increases, the inhibition of the detoxification pathway leads to cell death. Thus, the proposed mechanism explains, at least in part, how ROS, undetectable at low DOX levels, may lead to measurable membrane peroxidation. It also suggests that an increase in tissue antioxidant capacity is capable of alleviating DOX toxicity because of: (a) relief of PLA₂ inactivation by restoring active cysteines; (b) return to baseline levels of ROS; and (c) detoxification of accumulated phospholipid peroxides.

In conclusion, this study shows the marked inhibition of myocardial membrane-associated calcium-independent PLA₂ by a clinically relevant concentration of DOX. The effect was first observed *in vitro* using isolated rat and rabbit ventricular myocytes and was then

confirmed *in vivo* in myocardium of rats injected with the drug. This novel observation has significant implications for the elucidation of the mechanisms underlying DOX cardiotoxicity and pharmacological interventions aimed at its prevention.

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