Clinical Concentrations of Doxorubicin Inhibit Activity of Myocardial Membrane-associated, Calcium-independent Phospholipase A₂ ¹

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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 [18:1] plasmalogen 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 [H]arachidonic acid led to a depression of baseline arachidonic acid release levels, corroborating iPLA₂ inhibition. Reducing agents blocked PLA₂ activity in cardiomyocyte suspensions, suggesting that the doxorubicin effect is mediated by oxidation of susceptible cysteines. In vivo experiments, in which adult rats were 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

DOX ³ and related anthracyclines 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 formation of oxygen free radicals (3–8). The oxyradical hypothesis of anthracycline cardiotoxicity is based on the fact that cardiomyocytes with a diminished level of superoxide dismutase are more susceptible to DOX exposure (7) and that an increase in ROS, reactive oxygen species; GPX, cytosolic glutathione peroxidase; HPGPX, hydroperoxide dehydrogenase; AA, arachidonic acid release; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; ROS, reactive oxygen species; GPX, cytosolic glutathione peroxidase; HPGPX, hydroperoxide dehydrogenase 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 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), [1H]AA 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 Langendorf perfusion with collagenase (8, 16). A yield of 5–7 X 10⁶ myocytes/rat heart and 14–20 X 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 X 10⁶/ml of Tyrode, supplemented with 10 mM HEPES, pH 7.3) were incubated at room temperature with designated DOX concentrations. Gh 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, 2 mM DTT, and 1% 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 PLA₂ 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 X g for 20 min to remove cell debris, nuclei, and mitochondria. The resultant supernatant fraction was centrifuged at 10,000 X g for 30 min to remove nuclei and mitochondria, and the resultant supernatant fraction was used for the assay.
100,000 × g for 1 h to separate the membrane fraction (pellet) from the cytosolic fraction (supernantant).

**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-Analyyst; 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, [1H]18:1) plasmenylcholine 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/mg 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 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 rat and rabbit ventricular cardiomyocytes. PLA2 assays were conducted using both plasmenylcholine 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).

**DOX Effect on Cardiomyocyte PLA2 Activity.** PLA2 activity was measured both in the presence of 4 mM EGTA or 1 mM CaCl2 at 37°C. The presence of Ca2+ in the assay buffer did not increase enzyme activity, indicating that the PLA2 activity was largely Ca2+ independent (iPLA2).

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CLINICAL CONCENTRATIONS OF DOXORUBICIN INHIBIT iPLA₂

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-

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.
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 Ca\(^{2+}\)-dependent, and the iPLA2. Several independent studies have shown that the majority of PLA2 activity in heart tissues: secretory, cytosolic Ca\(^{2+}\)-dependent, and the iPLA2. Several independent studies have shown that the majority of PLA2 activity in heart

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 Ca\(^{2+}\)-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 \(\mu\)M 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.

Potentiation 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 \(\mu\)M 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 \(\mu\)M 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).

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 \(\mu\)M DOX for another 30 min. Activity was measured using (1Y0, [\(^{3}H\)]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.

Fig. 8. BEL effect on DOX toxicity. Myocytes were pretreated with 10 \(\mu\)M BEL for 30 min before each DOX application. An application of 10 \(\mu\)M 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 cysteic 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 iPLA2.

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 cause 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, difficult to prove 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 anthracyclene 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).

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, 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 Ca++-independent PLA2 (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 Ca++-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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