Inhibition of Membrane-associated Calcium-independent Phospholipase A₂ as a Potential Culprit of Anthracycline Cardiotoxicity

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ABSTRACT

Administration of anthracyclines, a family of highly effective anticancer drugs, is associated with a cumulative dose-related cardiomyopathy, the etiology of which remains poorly understood. We have discovered that administration of the anthracyclines leads to a marked inhibition of membrane-associated calcium-independent phospholipase A₂ (iPLA₂) both in vitro and in vivo. To elucidate the clinical relevance of this effect and to correlate it with known cardiotoxicity of the individual anthracyclines, we have compared four anthracycline analogues: doxorubicin, daunorubicin, idarubicin, and epirubicin for their ability to inhibit iPLA₂. Isolated adult rat cardiomyocytes were treated with each analogue at concentrations of 0.1–100 μM, and PLA₂ activity was assessed in cytosolic and membrane fractions using (16:0, [3H]18:1) plasmenylcholine in the absence of calcium. For all of the examined analogues, iPLA₂ inhibition was concentration and time dependent, preceded detectable changes in cell viability, and was specific to the membrane-associated enzyme. The degree of iPLA₂ inhibition by equimolar concentrations of epirubicin and idarubicin was significantly less than that of doxorubicin or daunorubicin, which correlates with the reported in vivo cardiotoxicity of these drugs. Because membrane iPLA₂ represents the majority of myocardial PLA₂ activity, its inhibition by anthracyclines would critically impair the ability of cardiomyocytes to repair oxidized phospholipids. Indeed, anthracycline-pretreated myocytes become more susceptible to the low-level oxidative stress imposed by repetitive additions of tert-butyl peroxide. The results suggest that iPLA₂ inhibition may be the initial step in a chain of events leading to chronic cardiotoxicity of the anthracyclines.

INTRODUCTION

Anthracyclines are powerful anticancer antibiotics, the therapeutic efficiency of which is abridged by the prominent cardiotoxicity of the drugs (1–3). Numerous studies have ascribed the cardiotoxicity of these drugs to specific cellular pathways, including an increase in the formation of free radicals, interference of calcium dynamics, adverse effects on RNA synthesis, and other putative mechanisms (4, 5). Unfortunately, most of these studies have been conducted using exceedingly high concentrations of the drugs, making their relevance to clinical toxicity questionable (1). Moreover, therapeutic interventions, based on the above mechanisms, have had limited success (5). Therefore, our recent findings that membrane-bound iPLA₂ (6) is markedly inhibited by low, clinically relevant concentrations of DOX both in vitro and in vivo (6), presents an intriguing possibility that this new phenomenon may underlie cardiotoxicity of anthracyclines. To obtain further support for this hypothesis, we have compared the inhibitory effects of four widely used anthracycline analogues, namely, DOX, DNR, IDA, and EPI. We argued that if a correlation is observed between the analogue’s effect on iPLA₂ activity and clinical indices of cardiotoxicity, it would serve as important evidence that inhibition of this essential myocardial enzyme can be responsible for the deleterious effects of anthracyclines.

MATERIALS AND METHODS

Materials. Collagenase (type II) was purchased from Worthington Biochemical. [1H]Arachidonic acid and [3H]oleic acid were purchased from NEN. Clinical grade EPI, IDA (Pharmacia and Upjohn, respectively), DOX, and DNR (both from Bedford Laboratories) were obtained from the Southwest Cancer Center. BEL was a gift from Hoffmann-LaRoche Inc., Nutley, NJ. Gentamicin, MEM (Joklik’s modified minimum essential medium), BSA, HEPES, t-BOOH, and other reagents were purchased from Sigma.

Preparation of Rat Ventricular Cardiomyocytes. Two-month-old Sprague Dawley rats (200–300 g) were injected i.p. with 500 units/kg sodium heparin. After 20–25 min, the rats were anesthetized i.p. with sodium pentobarbital (45 mg/kg), and the excised hearts were perfused for 10 min with MEM supplemented with 1.25 mM CaCl₂. This was then followed by a 5-min perfusion with a nominally calcium-free MEM, supplemented with 20 mM creatine and 60 mM taurine, and 6–10 min of perfusion with the same medium containing 0.5–1 mg/ml of type II collagenase and 0.1% BSA. The ventricles were then minced and vigorously shaken in the same medium containing 2% BSA. After two washes in collagenase-free medium, the CaCl₂ concentration in the medium was gradually increased to 1.25 mM. With this method, a yield of 5–7 × 10⁶ calcium-tolerant cells per heart was routinely obtained.

Short-Term Treatment of Cells with Anthracyclines. Fifteen-mÌl tubes containing myocytes in suspension (0.25 × 10⁶ cell/ml Tyrode, supplemented with 10 mM HEPES (pH 7.3)) were gently shaken (0.5 Hz) at room temperature with designated anthracycline concentrations. At the end of the incubation period, cells were washed twice with analogue-free Tyrode and processed for iPLA₂ activity.

Long-Term Treatment of Cells with Anthracyclines. To access cardiotoxicity of the analogues during longer periods, cardiomyocytes were cultured in serum-free Tyrode solution, conditions which allow retention of the cell’s rod shape for an extended period (7). Specifically, freshly isolated cells were plated onto 12-mm laminin-covered glass coverslips and were preincubated for 4–5 h in MEM supplemented with 5 mM HEPES, 10 μg/ml gentamicin, 0.1 μg/ml streptomycin, and 0.1 units/ml penicillin. Each slip was then placed in a separate well of a 24-well plate with 1 ml of MEM, followed by the addition of DOX, DNR, IDA, EPI, t-BOOH, or BEL to achieve the indicated concentrations. At the end of the incubation period, the total average viability of each slip was evaluated by LDH assay and visual assessment of rod-shaped morphology.

Preparation of Cytosolic and Membrane Fractions. Myocytes were suspended in ice-cold buffer containing: 250 mM sucrose, 10 mM KCl, 10 mM imidazole, 4 mM EDTA, and 2 mM DTT (pH 7.8; PLA₂ assay buffer). To separate membrane and cytosolic fractions, cell sonicates were centrifuged at 14,000 × g for 20 min to remove nuclei and mitochondria, and the supernatant fraction was centrifuged at 100,000 × g for 1 h. The membrane fraction (pellet) was separated from the cytosolic fraction (supernatant) and was resuspended in PLA₂ assay buffer.

Assay of PLA₂ Activity. PLA₂ activity was quantified by incubating the enzyme (8 μg of membrane protein or 200 μg of cytosolic protein) with 100 μM (16:0, [3H]18:1) plasmenylcholine in assay buffer containing 100 mM Tris, 10% glycerol (pH 7.0), 4 mM EGTA at 37°C for 5 min in a total volume of 200 μl. Synthesis of radiolabeled phospholipid substrate (1-0-hexadec-1'-eny1-2-[3H]oleoyl-sn-glycero-3-phosphocholine) has been described in detail previ-
The reaction was initiated by adding the substrate as a concentrated stock solution in ethanol (5-µl total volume), which was injected into a total volume of 200-µl aqueous buffer to achieve a final substrate concentration of 100 µM. Reactions were terminated by the addition of 100 µl of butanol. Released radiolabeled fatty acid was isolated by thin-layer chromatography 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 (9). Protein content was determined by the Lowry method.

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

**RESULTS**

**Inhibition of iPLA₂ Activity by DNR.** Suspensions of freshly isolated myocytes were incubated with 0.1, 1.0, and 10 µM DNR for either 10 or 30 min and were washed, sonicated, and assessed for iPLA₂ activity in both the membrane and cytosolic fractions. The enzyme activity was measured using (16:0, [³H]18:1) plasmenylcholine substrate in the absence of calcium (4 mM EGTA). The membrane iPLA₂ activity was about 20 times higher than of the cytosolic enzyme (4906 ± 738 versus 206 ± 28 pmol/mg protein/min). A half-hour incubation of myocytes with micromolar concentrations of the drug resulted in a concentration-dependent decrease in iPLA₂ activity associated with the membrane fraction (Fig. 1B). The degree of enzyme inhibition by higher concentrations of DNR (up to 100 µM) was only slightly greater than the one produced by 1 µM. The effect was rapid (Fig. 1C), with a majority of the enzyme activity affected during first 10 min of incubation. Cytosolic iPLA₂ activity was not affected by the DNR treatment (Fig. 1D). For all of the experimental conditions presented in Fig. 1, the viability of the cells remained similar to that of the control samples and did not decrease below 90% of initial viability values.

**Inhibition of iPLA₂ Activity by IDA and EPI.** Similar sets of experiments were conducted using two other widely used anthracyclines, IDA and EPI (Fig. 2 shows data for IDA only). Analogously to the DOX (6) and DNR data (Fig. 1), only the membrane-associated

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**Fig. 1. DNR effect on iPLA₂ activity.** A, chemical structure of the analogue. B, changes in membrane iPLA₂ activity after 10 and 30 min incubation with 1 µM DNR. Average from four experiments with each assay run in duplicate. Enzyme activity was measured using (16:0,[³H]18:1) plasmenylcholine in the presence of 4 mM EGTA. *, significant difference at *P* < 0.05 (as compared with pretreatment values). No changes in control samples were observed after 30 min of myocytes incubation in drug-free medium. C, effect of 30-min incubation with increasing DNR concentrations on membrane-associated iPLA₂ activity. ***, significant difference at *P* < 0.005, as compared with controls. Average from 10 experiments, with each assay run in duplicate. iPLA₂ activity was measured using (16:0,[³H]18:1) plasmenylcholine in the presence of 4 mM EGTA. D, effect of 30-min incubation with increasing DNR concentrations on cytosolic iPLA₂ activity. Average from 10 experiments, with each assay run in duplicate. iPLA₂ activity was measured using (16:0,[³H]18:1) plasmenylcholine in the presence of 4 mM EGTA.
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**Fig. 2.** IDA effect on iPLA2 activity. A, chemical structure of the analogue. B, changes in membrane iPLA2 activity after 10- and 30-min incubations with 100 µM IDA. Average from four experiments, with each assay run in duplicate. Enzyme activity was measured using (16:0,[3H]18:1) plasmenylcholine in the presence of 4 mM EGTA. *p<0.05 (as compared with pretreatment values). No changes in control samples were observed after 30 min of myocytes incubation in drug-free medium. C, effect of 30-min incubation with increasing IDA concentrations on membrane-associated iPLA2 activity. *p<0.05, as compared with controls. Average from seven experiments, with each assay run in duplicate. Enzyme activity was measured using (16:0,[3H]18:1) plasmenylcholine in the presence of 4 mM EGTA. D, effect of 30-min incubation with increasing IDA concentrations on cytosolic iPLA2 activity. Average from seven experiments, with each assay run in duplicate. iPLA2 activity was measured using (16:0,[3H]18:1) plasmenylcholine in the presence of 4 mM EGTA.

Form of the enzyme was affected. The concentrations required to reach statistically significant inhibition of membrane iPLA2, however, were considerably higher for both IDA and EPI. No effect on cytosolic iPLA2 activity was observed even at the highest concentrations of the drugs (Fig. 2D and data not shown).

**Comparative Experiments with Four Analogues.** To minimize factors that can compromise direct comparison between different analogues (e.g., inherent differences between animals, variability between PLA2 assays, quality of myocyte preparation, and so forth) we conducted a set of experiments in which all four of the anthracyclines were tested simultaneously using the cardiomyocyte preparation from the same rat. These experiments used a low, clinically relevant concentration of the analogues (1 µM) and confirmed that the inhibitory effect on iPLA2 activity exhibited by DNR is similar to that with DOX, whereas EPI and IDA are less effective at inhibiting the enzyme (Fig. 3). Therefore, in respect to their ability to affect membrane-associated iPLA2, the anthracyclines can be ranked in the following order: DOX > DNR > EPI > IDA. To compare these results with previous studies on cardiotoxicity of these analogues, we have compiled data from previously published in vitro and in vivo studies (Table 1). The data revealed that the ranking of analogues based on their ability to inhibit iPLA2 correlates well with clinical cardiotoxicity of these drugs. It has also been suggested that no direct relationship exists between the ability of individual anthracyclines to inhibit iPLA2 and acute in vitro toxicity of the drug. The next series of experiments tested this assumption directly.

*Fig. 3.** Comparative effect of analogues on iPLA2 activity. Cardiomyocytes from the same preparation were incubated for 30 min with each analogue (1 µM). Enzyme activity was assayed using (16:0,[3H]18:1) plasmenylcholine in the presence of 4 mM EGTA. Experiment was repeated using four different animals.
Acute Toxicity of Anthracyclines at Concentrations Exceeding the Clinical Range. Treatment of myocytes with clinically relevant anthracycline concentrations (1–5 \(\mu M\) for up to 60 h) did not lead to any detectable cell damage (data not shown). Indices of cell necrosis (both morphological and biochemical) become apparent only when cells were exposed to 20–\(\mu M\) concentrations of the analogues for at least 24 h (Fig. 4). The descending order of acute toxicity of the drugs (IDA > DNR > DOX > EPI) in these experiments was in agreement with in vitro data obtained by others (10) but has little correlation with clinical studies (Table 1). Notably, the acute toxicity of the drugs had no correlation with the ability of a particular analogue to inhibit membrane iPLA₂ (compare Figs. 3 and 4).

Anthracyclines and Myocyte Susceptibility to Low-Level Oxidative Stress. Acute cardiomyocyte damage (observed at anthracycline concentrations exceeding clinical range by 10–100-fold) appears to have little relevance to the chronic deleterious effects of these drugs on a patient’s heart (1, 2). In contrast, we believe that decreased iPLA₂ activity can be clinically relevant and can lead to a slow deterioration of the cardiac function. Specifically, we hypothesize that because of the essential role of iPLA₂ enzyme in the detoxification of lipid peroxides (6), anthracycline treatment renders cardiomyocytes more susceptible to an oxidative stress. The next series of experiments tested this assumption directly. The cells were pretreated with DOX for 30 min and then were exposed to a low, subtoxic dose of t-BOOH (0.1–5 \(\mu M\)). The treatment was repeated every 12 h for a total of 48 h. Although selected concentrations of either t-BOOH or anthracycline alone were not toxic to the cells during the 48-h protocol, the combined anthracycline/t-BOOH treatment proved lethal (Fig. 5A). To obtain further evidence that such an effect can be a consequence of iPLA₂ inhibition, we conducted a similar set of experiments substituting DOX with iPLA₂ inhibitor BEL. BEL by itself did not affect myocyte viability, but it markedly augmented susceptibility to t-BOOH-imposed oxidative stress (Fig. 5B).

### DISCUSSION

The most dangerous adverse effect that limits therapeutic potency of anthracyclines is their chronic cardiotoxicity. The latter is characterized by progressive left-ventricular dysfunction and congestive heart failure, the risk of which increases precipitously after cumulative doses exceed critical values established for each of its analogues (11, 12). Clinical practice reveals considerable variation in the individual susceptibility to the cardiotoxic effects of these drugs (2). Unfortunately, no specific biochemical, molecular, or genetic markers are currently available to predict an individual patient’s susceptibility so that the clinician could tailor anthracycline chemotherapy to minimize its cardiac side-effects. Absence of such markers, as well as the failure of most protocols based on existent hypotheses of anthracycline cardiotoxicity to prevent cardiac dysfunction in clinical settings (5), is largely attributable to an inadequacy of in vitro models of anthracycline cardiotoxicity. Specifically, although some pathways have been confirmed on a whole animal level (13–16), the majority of adverse effects exhibited by DOX and its analogues have been studied using sarcolemmal vesicles, mitochondria, isolated cardiomyocytes, or heart slices (reviewed in Refs. 1 and 4) and Table 1). Because of the short-term viability of these preparations, exceedingly high concentrations of the drugs (usually 10–800 \(\mu M\) versus peak plasma values of 1–5 \(\mu M\)) must be used to detect significant changes. The reported effects, therefore, reflect the acute cell injury rather than the chronic toxicity of the drug. The only in vitro model that allows somewhat questionable (19).

The discrepancy between in vitro data and the results of clinical trials has been noted before (1, 12) and is evident when one briefly...
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affect myocyte viability (25). Conversely, we suggest that AIPI may be responsible for chronic toxicity of these drugs via an impairment of oxidized phospholipids recycling and the slow, accumulative changes in membrane composition and the redox status of the cell. One must take into account that (a) oxidized sn-2 fatty acyl groups must first be hydrolyzed by PLA2 to be repaired by cytosolic glutathione peroxidase [followed by reacylation of the phospholipids by CoA-dependent acyltransferase, and CoA-independent transacylase (26)]; and (b) that membrane iPLA2 accounts for the majority of PLA2 activity in the heart (27). Therefore, as suggested in Fig. 6, in cardiac muscle, the anthracyclines may not only augment free radical formation but also disable the repair process.

This hypothesis was further supported by our experiments in which anthracycline-treated cells were exposed to low, subtoxic concentrations of t-BOOH (Fig. 5). The data has revealed that combined DOX/t-BOOH treatment is substantially more toxic to the cells, presumably because of the impaired ability on the part of the myocyte to repair oxidized phospholipids (Fig. 5A). One may argue, however, that a DOX-mediated increase in free radicals simply adds to t-BOOH-induced oxidative stress. However, substitution of DOX with the iPLA2 inhibitor BEL had the equivalent effect [in cardiomyocytes, the activities of other PLA2s, e.g., secretory sPLA2 and Ca2+-dependent cPLA2, are insignificant as compared with those of iPLA2 (27)].

The suggested pathway through which AIPI may lead to anthracycline cardiotoxicity is depicted in Fig. 6. It allows one to reconcile several apparently contradictory results while incorporating existing free-radical hypotheses of anthracycline cardiotoxicity. First, it helps

Fig. 4. Acute toxicity of high anthracycline concentrations. Cardiomyocytes in short-term primary cultures were incubated with each analogue (20 µM), and indices of cell viability were assessed at 24 h and 60 h. The data are the means from triplicate experiments for the control and analogue-treated samples. *, P < 0.05; **, P < 0.005 (versus control samples). A, mortality as determined by the release of LDH into the medium after 24-h incubation. Insert (gray), shows corresponding measurements of cell viability using rod-shaped morphology. B, mortality as determined by the release of LDH into the medium after 60-h incubation. Insert (gray) shows corresponding measurements of cell viability using rod-shaped morphology.

surveys comparative studies on the cardiotoxicity of the analogues (Table 1). Specifically, IDA has been shown to be more potent in stimulating superoxide formation by mitochondrial NADH-dehydrogenase (20), increasing the rate of semiquinone formation (21), or damaging both adult and neonatal cardiac cells (22, 23). At the same time clinical trials report that cardiac side effects of IDA are much less pronounced than those of DOX, especially at equimolar concentrations (12, 24). Similar discordance is evident for EPI treatment (Table 1). Notably, differences in the pharmacokinetics of the analogues and cardiac accumulation do not explain the discrepancies between in vivo and in vitro data (1, 12).

Therefore, the correlation between the AIPI observed in this study (Figs. 1–3) and clinical cardiotoxicity of anthracyclines (Table 1) is particularly intriguing. Notably, acute injury to the cells caused by the application of 20 µM DOX, DNR, EPI, or IDA (Fig. 4) was in accordance with both the partition coefficient and cellular intake of the drug (22). Yet, the loss of viability did not correlate with AIPI (Figs. 3 and 4). Thus, we believe that AIPI is not a major cause of acute cell necrosis elicited by high anthracycline concentrations. In fact, our previous studies have shown that a similar degree of iPLA2 inhibition attained by the exposure to iPLA2 inhibitor BEL, did not

Fig. 5. Potentiation of t-BOOH toxicity by DOX and BEL. A, cardiomyocytes in short-term primary cultures were pretreated with 10 µM DOX for 30 min before each t-BOOH application. t-BOOH additions were made every 12 h, and the viability of the cells was assessed at the end of the 48-h protocol using a LDH assay. Values represent mean of four separate preparations.

B, cardiomyocytes in short-term primary cultures were pretreated with 10 µM BEL for 30 min before each t-BOOH application. t-BOOH additions were made every 12 h, and the viability of the cells was assessed at the end of the 48-h protocol using LDH assay. Values represent mean of four separate preparations.
to explain how anthracyclines can lead to a measurable lipid peroxidation (28), whereas no significant increases in free-radical formation have been detected at a low micromolar range (21, 29). Secondly, AlPI could explain decreased circulating levels of conjugated dienes and hydroperoxides shown to occur after i.v. administration of DOX to cancer patients (30). The effect (originally ascribed to “paradoxical” inhibition of cardiac lipid peroxidation) is likely to be a direct manifestation of phospholipase inhibition, which decreases the release of conjugated dienes and hydroperoxides from oxidized cardiac membranes. The mechanism shown in Fig. 6 also adds a new aspect to the crucial role of glutathione peroxidases in prevention of anthracycline cardiotoxicity (31). Notably, in the cardiac muscle, the activity of membrane-bound phospholipid glutathione peroxidase (which does not require PLA2 to reduce oxidized fatty acids (32)) is 100 times lower than the activity of cytosolic glutathione peroxidase (26, 33).

Furthermore, several studies have reported a loss of glutathione peroxidase activity in the hearts of anthracycline-treated animals (34–36), suggesting further weakening of the repair cycle in cardiac tissue.

The ability of anthracyclines to inhibit PLA2 allows one to suggest that at least some of the drugs’ anticancer effects may come through the inhibition of this enzyme and/or that PLA2 inhibitors might have anticancer properties. This intriguing possibility requires further investigation and was not addressed in the present study.

In summary, using isolated rat cardiomyocytes, we have shown for the first time that (a) DNR, EPI, and IDA inhibit PLA2 activity in a time- and concentration-dependent manner; (b) these anthracyclines affect activity of the membrane-associated enzyme, whereas no detectable changes occur in the cytosolic fraction; (c) the degree of PLA2 inhibition by a particular analogue is not associated with the drugs’ intracellular accumulation or acute in vitro toxicity, but correlates with reported in vivo cardiotoxicity of these drugs; and (d) pretreatment with anthracyclines renders cardiomyocytes more susceptible to oxidative stress.

Overall, the data strongly support our original hypothesis that anthracycline-induced PLA2 inhibition is involved in the chronic cardiotoxicity of these drugs. 

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