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Changes in Phospholipid Content and Myocardial Calcium-Independent Phospholipase A_2 Activity during Chronic Anthracycline Administration

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ABSTRACT

Despite numerous investigations, the causes underlying anthracycline cardiomyopathy are yet to be established. We have recently reported that acute treatment with anthracyclines inhibits membrane-associated calcium-independent phospholipase A_2 ($iPLA_2$) activity both in vitro and in vivo. This study presents data that $iPLA_2$ activity is also suppressed during *chronic* drug administration. Adult Sprague-Dawley rats were given weekly 1 mg/kg i.v. injections of doxorubicin for a total of 8 weeks. One week after the last injection, the animals were sacrificed, and heart tissue was assessed for phospholipid content and $iPLA_2$ activity. Membrane-associated $iPLA_2$ activity in the myocardium of doxorubicin-treated animals was 40% lower than that in control hearts. In addition, doxorubicin treatment resulted in significant alterations in the distribution of fatty acyl moieties esterified to the *sn*-2 position of choline glycerophospholipids. The ethanolamine species remained unaffected. Elevation in the amount of arachidonate and linoleate esterified to the sn-2 position of choline plasmalogens was consistent with the hypothesis that $iPLA_2$ displays selectivity for plasmalogen phospholipids; therefore, enzyme inhibition may affect hydrolysis of these phospholipid subclasses. Notably, the changes in phospholipid content occurred at a low cumulative dose of 8 mg/kg at which appearance of structural lesions was minimal. Therefore, these alterations seem to be both specific and early signs of cardiomyocyte pathology. The results support our hypothesis that myocardial $iPLA_2$ inhibition may be one of the steps that leads to the functional and structural changes associated with chronic anthracycline treatment.

Therapeutic efficacy of anthracyclines remains limited by their cardiotoxicity. After a cumulative dose of these potent anticancer drugs exceeds critical levels (400 mg/m^2 for doxorubicin in humans; Swain et al., 2003), the patient's risk of developing irreversible, dilated cardiomypathy progressively increases (Gewirtz, 1999). Many investigators have attempted to understand the specific cellular pathways that lead to structural and functional changes associated with anthracycline cardiotoxicity. These putative mechanisms include an increase in the formation of free radicals (Doroshow, 1983), toxicity of drug metabolites (Gambliel et al., 2002), changes in calcium dynamics (Pessah et al., 1992; Boucek et al., 1997), adverse effects on RNA synthesis (Jeyaseelan et al., 1997), apoptosis (Kotamraju et al., 2000), and more recent hypotheses such as titin proteolysis (Lim et al., 2004). Another important pathway is related to the ability of doxorubicin to extract iron from ferritin (Winterbourn et al., 1991). The doxorubicin-iron complex, in the presence of intracellular thiols such as reduced glutathione or cysteine may generate free radicals by a cyclic mechanism, leading to the continuous formation of superoxide and hydroxyl radicals. Additional arguments for iron involvement come from studies with the chelator ICRF-187 (dexrazoxane), which has been shown to attenuate chronic cardiotoxicity of anthracyclines (Imondi et al., 1996; Herman et al., 1997). Despite many leads, however, the therapeutic interventions, based on the earlier reported mechanisms, have had limited success (Dorr, 1996). Such limited success can be explained, at least in part, by the fact that many putative mechanisms relied on studies that were conducted using exceedingly high concentrations of the drugs, and/or were performed in in vitro preparations only. Therefore, we are still in search of the cellular and molecular mechanisms that can explain how low, clinically relevant doses of these drugs can cause progressive deterioration of myocardial function in vivo.

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ABBREVIATIONS: $iPLA_2$, calcium-independent phospholipase A_2; AIPI, anthracycline-induced phospholipase A_2 inhibition; PLA_2, phospholipase A_2; HPLC, high-performance liquid chromatography; CGP, choline glycerophospholipid; EGP, ethanolamine glycerophospholipid; PVDF, polyvinylidene difluoride; *cis*-UFA PLD, *cis*-unsaturated fatty acid-sensitive form of phospholipase D.

Studies by our group have been focused on a newly reported phenomenon that membrane-associated calcium-independent phospholipase A_2 (iPLA$_2$) is inhibited by low, clinically relevant concentrations of doxorubicin (McHowat et al., 2001b). Moreover, we have recently shown a correlation between reported clinical cardiotoxicity of four anthracycline analogs, namely, doxorubicin, daunorubicin, idarubicin, and epirubicin, and their effect on iPLA$_2$ activity (Swift et al., 2003). Together, these data suggest an intriguing possibility that anthracycline-induced iPLA$_2$ inhibition (AIPI) can be linked to anthracycline cardiotoxicity (McHowat et al., 2001c). To obtain further support for this new hypothesis, we have expanded our earlier observations (McHowat et al., 2001b), which documented occurrence of AIPI in isolated cardiomyocytes and in rats injected with a single dose of the drug (4 mg/kg doxorubicin, tail i.v. injection) to a more clinically relevant scenario. Specifically, this study presents evidence that a *repetitive*, *weekly* administration of doxorubicin leads to AIPI in the myocardium of treated rats. Notably, we have employed low subtoxic accumulative dose of the drug (8 mg/kg weight) to reveal whether AIPI and associate phospholipid changes occur *before* apparent myocardial lesions and functional impairment of the heart muscle.

Materials and Methods

Animal Model and Sample Preparation. Adult Sprague-Dawley rats (200–250 g initial weight) were maintained at the Texas Tech University Health Sciences Center (Lubbock, TX) animal care facilities. Animals were housed in plastic cages at 22°C on a 12-h light/dark cycle and were given laboratory chow and tap water ad libitum. Doxorubicin was administered weekly as 1-mg/kg dose via tail vein injection. The treatment lasted 8 weeks. Controls received an equivalent volume of saline. Body weight was measured at weekly intervals after the injection. Six days after the last injection, animals were anesthetized with pentobarbital, and their hearts were rapidly removed. The hearts were trimmed from surrounding tissue, weighed, and flushed with cold saline using retrograde Langendorff perfusion. The small piece of ventricular tissue close to the apex was removed and transferred to a fixative for electron microscopy analysis. The left ventricle was then split into two parts. The first piece was immediately frozen in liquid nitrogen for membrane phospholipid assessment. The second half was processed for iPLA$_2$ activity determination.

Measurement of PLA$_2$ Activity. Ventricular tissue from the control and doxorubicin-treated animals were homogenized at 4°C in homogenization buffer containing 250 mmol/l sucrose, 10 mmol/l KCl, 10 mmol/l imidazole, 5 mmol/l EDTA, and 2 mmol/l dithiothreitol with 10% glycerol, pH 7.8. The homogenate was centrifuged at 14,000*g* for 10 min to remove unbroken cells, nuclei, and mitochondria. The resultant supernatant fraction was centrifuged at 100,000*g* for 60 min to separate the membrane fraction (pellet) from the cytosolic fraction (supernatant). The membrane fraction was washed twice with buffer to remove residual cytosol and resuspended in ice-cold homogenization buffer. PLA$_2$ activity was assessed by incubating the enzyme (8 μg of membrane protein, 200 μg of cytosolic protein) with 100 μM (16:0, [^{3}H]18:1) plasmenylcholine substrate. Incubations were performed in assay buffer containing 10 mM Tris, 10% glycerol, pH 7.0, and 4 mM EGTA at 37°C for 5 min in a total volume of 200 μl. Reactions were terminated by the addition of 100 μl of butanol and then vortexed and centrifuged at 2000*g* for 5 min. Released radiolabeled fatty acid was isolated by application of 25 μl of the butanol phase to channeled Silica Gel G plates and development in petroleum ether/diethyl ether/acetic acid [70:30:1 (v/v/v)] and was subsequently quantified by liquid scintillation spectrometry.

Electron Microscopy Analysis of Myocardial Morphology. The tissue samples taken from the left ventricles were cut into 1-mm^3 blocks and stored in 2% paraformaldehyde and 2.5% glutaraldehyde fixative overnight at 4°C. The samples were postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series with 100% propylene oxide as a transitional solvent, and embedded in LX-112 resin (Ladd Research Industries, Burlington, VT). Ultrathin sections were obtained with an ultramicrotome (LKB Instruments, Gaithersburg, MD) and were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Philips, Eindhoven, The Netherlands). From each animal, two blocks of ventricular tissue were used. Each block was cut into sections, and four images from different sections were taken. Total number of analyzed images was $4 \times 2 \times 4 = 32$ for the control and 32 for the doxorubicin-treated group.

Extraction, Separation, and Quantification of Individual Choline and Ethanolamine Glycerophospholipid Molecular Species. Cellular phospholipids were extracted using chloroform and methanol by the method of Bligh and Dyer (1959) at 0–4°C. Phospholipids were separated into different classes by injecting them onto an Ultrasphere-Si (5-μm silica), 4.6 × 250-mm HPLC column (Beckman Coulter, Fullerton, CA) using gradient elution with hexane/isopropanol/water. Individual choline glycerophospholipid (CGP) and ethanolamine glycerophospholipid (EGP) molecular species were isolated by reverse-phase HPLC with the use of an Ultrasphere ODS (5 μm; C-18) column, 4.6 × 250 mm (Beckman Coulter). Individual molecular species were separated by means of gradient elution with acetonitrile/methanol/water with 20 mM choline chloride (McHowat et al., 1996). The molecular identity of individual molecular species was established by GLC characterization (McHowat et al., 1996). Quantification of individual phospholipid molecular species was achieved by determination of lipid phosphorus in reverse-phase HPLC column effluents. For lipid phosphorus determination, column effluents were taken to dryness under N_2 and electrically heated at 150°C for 2 h with 400 μl of perchloric acid. The samples were allowed to cool to room temperature, and excess perchloric acid was neutralized by addition of 1 ml of 4.5 N KOH. The samples were centrifuged at 2000*g* for 10 min to sediment the $KClO_4$ precipitate, and 600 μl of the supernatant was removed for assay of lipid phosphorus (Itaya and Ui, 1966).

Immunoblot Analysis of iPLA$_2$. Membrane fractions were prepared as described above for iPLA$_2$ activity determination. Samples were mixed with an equal volume of SDS sample buffer and heated at 95°C for 5 min before loading onto a 10% polyacrylamide gel. Protein was separated by SDS-polyacrylamide gel electrophoresis at 200 V for 45 min and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Richmond, CA) with 45V overnight at 4°C. Adsorptive PVDF sites were blocked with Tris buffer solution containing 0.1% (v/v) Tween 20 and 5% (w/v) nonfat milk. The blocked PVDF membrane was incubated with antibodies to iPLA$_2$ (1:2000 dilution; Cayman Chemical, Ann Arbor, MI), washed with Tris buffer solution containing 0.1% (v/v) Tween 20, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:50,000 dilution). Regions of antibody binding were detected by enhanced chemiluminescence (Super Signal Ultra; Pierce Chemical, Rockford, IL) after exposure to preflashed film (Hyperfilm; Amersham Biosciences Inc., Piscataway, NJ). Optical density of the bands was quantified using Visage 2000 densitometer (Bio Image, Ann Arbor, MI).

Statistics. Statistical comparison of values was performed by Student's *t* test. All results are expressed as means ± S.E.M. Statistical significance was considered to be $p < 0.05$.

Results

Body and Heart Weight and Overall Mortality/Morbidity. Changes in animal heart and body weight are shown in Fig. 1A. Body and heart weights were found to be slightly

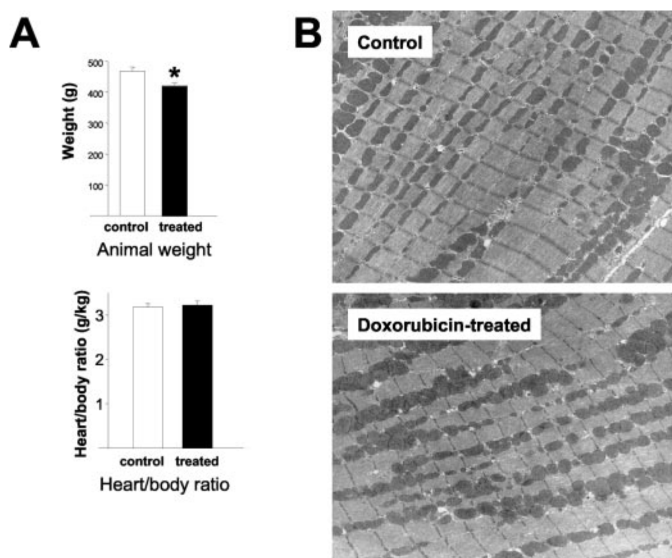

Fig. 1. Evidence of minimal adverse effects on the heart at the end of 8 mg/kg cumulative doxorubicin dose in Sprague-Dawley rats (weekly i.v. injections). A, weight and heart/body ratio for control (n = 4) and doxorubicin-treated animals (n = 4). B, intact myocardial infrastructure in control and doxorubicin-treated animals.

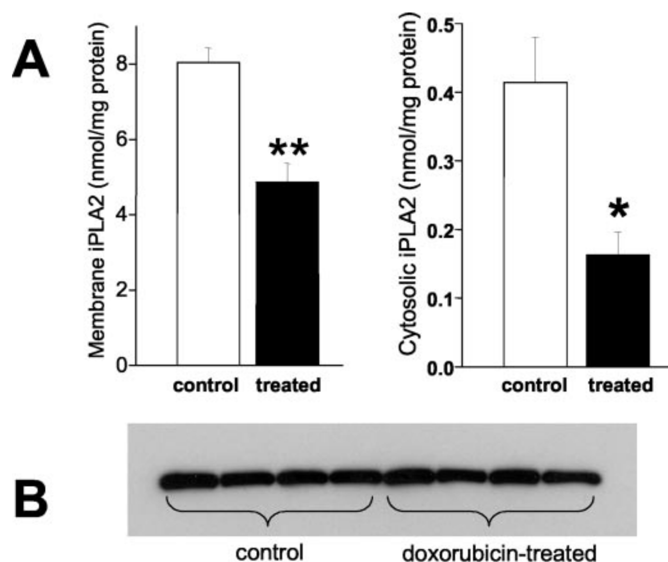

Fig. 2. A, inhibition of myocardial membrane-associated and cytosolic $iPLA_2$ in chronically treated animals (n = 4 in each group; *, $p < 0.05$; **, $p < 0.005$). The majority of $iPLA_2$ activity was associated with the membrane fraction (note difference in the y-axes). B, immunoblot analysis of $iPLA_2$ in the membrane fraction of the control and doxorubicin-treated animals (each lane was loaded with equal amount of total protein).

lower in doxorubicin-treated animals than in controls, whereas the heart/body weight ratio remained the same. At the end of the protocol, doxorubicin-treated animals seemed less active compared with the controls; however, no mortality or morbidity was observed.

Assessment of Myocardial Ultrastructure. Electron microscopy images were evaluated for possible cytoplasmic vacuolization, myofibrillar loss, dilatation of sarcoplasmic reticulum and t-tubules, mitochondrial swelling, intra- and extracellular edema, dissociation of intercellular junctions, and nuclear chromatin appearance. Thorough analysis of electron microscopy images did not reveal any gross alterations in the hearts of doxorubicin-treated animals (Fig. 1B). The blind assessment of samples for the frequency and severity of cardiac lesions using a semiquantative 0 to 3 scale similar to Billingham (Billingham, 1991; Zhang et al., 1996) also did not reveal significant differences between the doxorubicin-treated and the control group. These data are consistent with previous studies that report the absence of myocardial lesions when a subtoxic cumulative dose is used (Herman et al., 1985).

$iPLA_2$ Activity in the Hearts of Control and Doxorubicin-Treated Animals. Cytosolic and membrane-associated $iPLA_2$ activity was measured using (16:0, [^{3}H] 18:1) plasmenylcholine substrate in the absence of calcium (4 mM EGTA). Eight-week treatment of Sprague-Dawley rats (1 mg/kg weekly i.v. dose) led to 40% loss of enzyme activity in the membrane fraction (Fig. 2A). Cytosolic $iPLA_2$ activity was also markedly decreased (Fig. 2A).

Presence of $iPLA_2$ Protein in the Hearts of Control and Doxorubicin-Treated Animals. Immunoblot analysis was used to determine whether the level of expression of the $iPLA_2$ protein was altered by doxorubicin treatment. The optical density of the $iPLA_2$ bands in membrane samples from myocardium of doxorubicin-treated animals was not different from the controls (2.7 ± 0.3 versus 2.8 ± 0.2; Fig. 2B). Immunoblot analysis of purified $iPLA_2$ protein (Genetics Institute, Cambridge, MA) demonstrated the presence of $iPLA_2$ at the same molecular mass (~85 kDa, positive control; data not shown).

Doxorubicin-Induced Changes in Myocardial Phospholipid Composition. After HPLC separation of phospholipids into individual classes, the CGP and EGP were separated by reverse-phase HPLC, and individual molecular species were collected and quantified using the microphosphate assay. As shown in Fig. 3 and Table 1, doxorubicin treatment resulted in highly significant alterations in the distribution of fatty acyl moieties esterified to the sn-2 position of CGP, whereas EGP species remained unaffected. When comparing the esterified sn-2 fatty acid composition of CGP, the selective loss of 20:4 and 18:1 fatty acids in diacyl CGP and increase in esterified 20:4 and 18:2 fatty acids in choline plasmalogens was clearly evident (Table 1). An 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 $iPLA_2$ displays selectivity for plasmalogens; therefore, long-term inhibition of this enzyme may affect hydrolysis rates of these phospholipid molecular species. The increase in the amount of plasmalogens corresponded to the decrease in arachidonic (20:4) and oleic (18:1) fatty acids esterified to diacylglycerophospholipids and may represent compensatory changes.

Discussion

Our earlier studies in isolated cardiac myocytes have revealed that clinically relevant doxorubicin concentrations inhibit myocardial $iPLA_2$ activity (McHowat et al., 2001b; Swift et al., 2003). The effect was specific to the membrane-associated enzyme and was ascribed to $iPLA_2$. Importantly, we have also confirmed occurrence of this phenomenon in vivo (McHowat et al., 2001b). Specifically, we have shown that activity of the myocardial membrane-associated $iPLA_2$ is di-

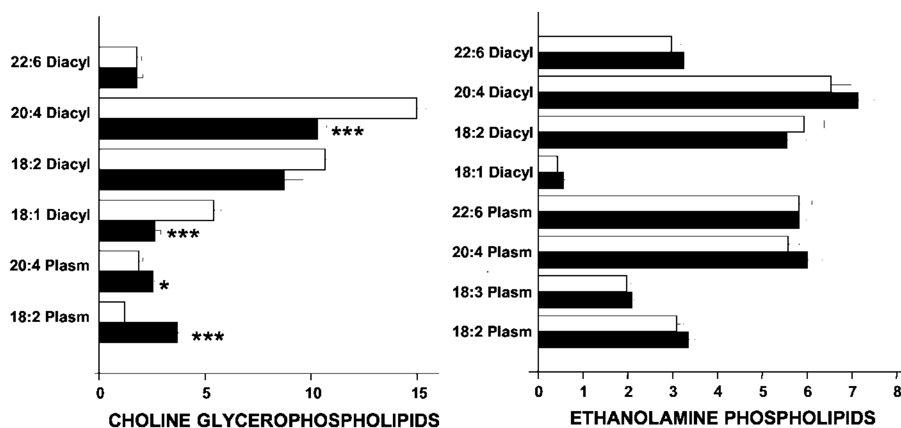

Fig. 3. Doxorubicin-induced alterations in the composition of unsaturated aliphatic chains esterified to choline and ethanolamine phospholipids in rat myocardium (nanomoles of PO_4 per milligram of protein). Plasm, plasmalogen; Diacyl, diacylphospholipids; (18:1) oleic acid; (18:2) linoleic acid; (20:4) arachidonic acid; (22:6) docosahexaenoic acid. *, $p < 0.05$; ***, $p < 0.001$ for comparison of values in control ($n = 4$) and doxorubicin-treated ($n = 4$) animals.

TABLE 1

Quantification of phospholipid molecular species in myocardium from control and doxorubicin-treated animals

Individual phospholipid molecular species were isolated by reverse-phase HPLC and identified as described under *Materials and Methods*. The composition of the fatty acids is represented by shorthand notation (x:y), where x represents the number of carbon atoms and y the number of double bonds in the aliphatic chain. Phospholipids in bold are significantly different between control and DOX-treated subjects. Values are in nanomoles of PO_4 per milligram of protein ($n = 4$ in each group).

Composition	Control	Doxorubicin-Treated	p
Choline glycerophospholipids			
18:2, 22:6 Ptd Cho	0.40 ± 0.08	0.27 ± 0.03	NS
18:2, 20:4 Ptd Cho	1.00 ± 0.22	1.13 ± 0.16	NS
18:2, 18:2 Ptd Cho	1.53 ± 0.13	2.01 ± 0.17	NS
16:0, 22:6 Ptd Cho	1.40 ± 0.16	1.53 ± 0.21	NS
16:0, 20:4 Ptd Cho	5.65 ± 0.15	5.98 ± 0.18	NS
16:0, 18:2 Ptd Cho	4.76 ± 0.24	3.93 ± 0.34	NS
16:0, 20:4 Plas Cho	1.88 ± 0.17	2.51 ± 0.10	0.05
16:0, 18:2 Plas Cho	1.20 ± 0.16	3.71 ± 0.37	0.001
16:0, 18:1 Ptd Cho	5.41 ± 0.34	2.64 ± 0.28	0.001
18:0, 20:4 Ptd Cho	8.34 ± 0.44	3.20 ± 0.40	0.0005
18:0, 18:2 Ptd Cho	4.38 ± 0.61	2.81 ± 0.85	NS
Ethanolamine glycerophospholipids			
18:2, 22:6 Ptd Eth	0.21 ± 0.03	0.24 ± 0.03	NS
18:2, 20:4 Ptd Eth	0.64 ± 0.06	0.58 ± 0.04	NS
18:2, 18:2 Ptd Eth	0.38 ± 0.06	0.33 ± 0.04	NS
16:0, 22:6 Ptd Eth	0.67 ± 0.25	0.63 ± 0.08	NS
16:0, 20:4 Ptd Eth	0.52 ± 0.10	0.58 ± 0.07	NS
16:0, 22:6 Plas Eth	2.34 ± 0.09	2.45 ± 0.16	NS
16:0, 20:4 Plas Eth	1.72 ± 0.15	1.88 ± 0.11	NS
18:1, 22:6 Plas Eth	1.72 ± 0.19	1.55 ± 0.08	NS
18:1, 20:4 Plas Eth	2.49 ± 0.12	2.51 ± 0.11	NS
16:0, 18:2 Plas Eth	2.70 ± 0.14	2.92 ± 0.15	NS
16:0, 18:1 Ptd Eth	0.43 ± 0.01	0.55 ± 0.05	NS
18:0, 22:6 Ptd Eth	2.10 ± 0.13	2.38 ± 0.05	NS
18:0, 20:4 Ptd Eth	5.39 ± 0.42	5.98 ± 0.44	NS
18:0, 18:2 Ptd Eth	5.56 ± 0.39	5.23 ± 0.43	NS
18:0, 22:6 Plas Eth	1.78 ± 0.09	1.82 ± 0.09	NS
18:0, 20:4 Plas Eth	1.38 ± 0.15	1.63 ± 0.14	NS
18:0, 18:3 Plas Eth	1.97 ± 0.11	2.09 ± 0.04	NS
18:0, 18:2 Plas Eth	0.40 ± 0.02	0.44 ± 0.03	NS

Ptd Cho, phosphatidylcholine; NS, not significant; Plas Cho, plasmenylcholine; Ptd Eth, phosphatidylethanolamine; Plas Eth, plasmenylethanolamine.

minished after acute doxorubicin administration (as assessed 4 h after single 4 mg/kg i.v. dose). Testing of other anthracyclines has confirmed that they also decrease $iPLA_2$ activity (Swift et al., 2003). Moreover, data revealed a correlation between analog's inhibitory effects and reported clinical toxicity. Intrigued by these findings, we have decided to examine whether myocardial $iPLA_2$ activity is altered by chronic anthracycline administration. Our second goal while conducting chronic animal studies was to observe possible changes in phospholipid content.

In the past, different animal protocols have been used to mimic anthracyclines cardiomyopathy. One of the most common protocols employs adult rats and weekly i.v. tail injection of doxorubicin (Czarnecki, 1984; Herman et al., 1985). After cumulative dose exceeds 15 mg/kg (Czarnecki, 1984), the significantly increased heart-to-body ratio, structural lesions, and functional changes such as decreased ejection fraction become evident. The protocol eventually leads to the development of congestive heart failure and animal death (Della Torre et al., 1996). In our experiments, we used similar administration schedule, but used low, subtoxic doses of the drug that resulted in a cumulative dose of 8 mg/kg.

The reason for choosing the subtoxic cumulative dose was to avoid comparing healthy and severely damaged myocardium, because the latter is likely to exhibit multiple nonspecific changes (Robison and Giri, 1986; Robison and Giri, 1987). We hypothesized that if doxorubicin-induced changes in myocardial $iPLA_2$ activity and/or membrane composition play a role in the *development* of myocardial lesions, they should *precede* gross ultrastructural or functional impairments induced by the drug. Indeed, our data revealed marked decreases in both membrane and cytosolic $iPLA_2$ activity (Fig. 2A), whereas structural changes in myocardial structure were negligible. Although the enzyme activity was diminished, the $iPLA_2$ protein levels remained unaffected (Fig. 2B). This was similar to our earlier studies in which we examined the effects of acute doxorubicin treatment (McHowat et al., 2001b). Although oxidation of essential cysteines is a suspected cause of $iPLA_2$ inhibition (McHowat et al., 2001b), more studies will be required to determine the molecular mechanism by which the enzyme activity is decreased in chronically doxorubicin-treated animals.

Notably, an earlier study (Robison and Giri, 1987) did not reveal changes in cardiac PLA_2 activity in doxorubicin-treated animals, although it used a much higher cumulative dose of the drug. The difference between our results and these earlier studies can be explained mainly by the fact that the measurements of PLA_2 activity have been significantly refined in the past 20 years. We have recently illustrated the importance of the reaction conditions for PLA_2 activity measurements, including the amount of protein used, the time and temperature of incubation, and the concentration of substrate (McHowat et al., 2001a). Notably, the $iPLA_2$ activity in our study was about 8 nmol/mg of protein/min, whereas cited study reports 0.5 nmol/mg of protein/min. Given the extremely low activity measurements they saw, it is not sur-

prising that difference between the control and doxorubicin-treated animals was not detected.

But why would one expect that altered $iPLA_2$ activity can affect phospholipid composition? First, it is important to note that in the heart, membrane $iPLA_2$ activity is the highest among other PLA_2 isoforms (McHowat and Creer, 2001). In addition to its role as a cell signaling enzyme, $iPLA_2$ is believed to be a "housekeeping" enzyme involved in membrane phospholipid turnover and repair (Six and Dennis, 2000). The membrane phospholipids are in a constant dynamic state of deacylation/reacylation with different incorporation rates for individual fatty acids (Sevanian, 1988). Myocardial $iPLA_2$ has shown to have substrate preference for arachidonylated plasmalogen phoshopholipids (McHowat and Creer, 2001). A certain degree of substrate specificity was also observed for AIPI itself (McHowat et al., 2001b,c). Together, these findings imply that chronic inhibition of the enzyme is likely to alter phospholipid composition of sarcolemma and/or sarcoplasmic reticulum.

Interestingly, the notion that chronically decreased myocardial membrane-associated $iPLA_2$ activity can be detrimental to the heart function is supported by another independent line of evidence. Specifically, a decrease in membrane $iPLA_2$ protein and activity was observed in a rat model of heart failure induced by myocardial infarction (McHowat et al., 2001d). Although reasons for such decrease remain to be established, one may speculate that myocardial $iPLA_2$ loss is detrimental for both anthracycline-treated and postmyocardial infarction subjects.

Based on our results and data by others, we suggest that AIPI can be linked to chronic anthracycline cardiotoxicity as illustrated in Fig. 4. The suggested scheme allows one to incorporate other tentative pathways, including the most commonly accepted oxyradical hypothesis. As we argued in our previous studies (McHowat et al., 2001c; Swift et al., 2003), AIPI augments oxidative damage caused by anthracycline treatment. Specifically, as the repair cycle is interrupted, oxidized phospholipids are likely to accumulate in cell membranes (Fig. 4A). Thus, AIPI-based mechanism helps to explain how anthracyclines can lead to a measurable lipid peroxidation (Thayer, 1984), whereas no significant increases in free radical formation have been detected at clinically relevant doxorubicin concentrations (Malisza et al., 1996; Sarvazyan, 1996). Another point where oxidative stress is likely to be involved is $iPLA_2$ inhibition itself via oxidation of cysteines (McHowat et al., 2001b). Thus, the fact that an increase in tissue antioxidant capacity is capable of alleviating anthracycline toxicity may be due to 1) relief of $iPLA_2$ inactivation by restoring active cysteines, and 2) detoxification of accumulated phospholipid peroxides. Therefore, the AIPI-based mechanism allows one to narrow oxyradical hypothesis to more specific pathways.

The calcium hypothesis, which implicates abnormal Ca^{2+} cycling, with ryanodine receptors as a major culprit, can also be linked to AIPI (Fig. 4B). Several independent laboratories have shown that chronic doxorubicin treatment causes an impairment of calcium-induced calcium release (Pessah et al., 1992; Boucek et al., 1999). This requires the coordinated effort from L-type calcium channel, ryanodine receptor, calcium pump, and other membrane proteins (Bers, 2002). Thus, it is intriguing to suggest that AIPI-induced changes in phospholipid environment adversely affect the finely tuned system of calcium-induced calcium release with a resulting decrease in contractility and cardiac function.

Another interesting possibility is the involvement of phospholipase D. It has been reported that a cardiac sarcolemmal *cis*-unsaturated fatty acid-sensitive form of this enzyme (*cis*-UFA PLD) can be modulated by $iPLA_2$ activity via intramembrane release of unsaturated fatty acids (Liu et al., 1998; McHowat et al., 2001d). The *cis*-UFA PLD-derived phosphatidic acid, in turn, influences intracellular Ca^{2+} concentration and contractile performance (Xu et al., 1996). Therefore, changes in $iPLA_2$ activity via AIPI might contribute to the defective Ca^{2+} handling and contractile performance of the failing heart due to *cis*-UFA PLD-mediated pathway (Fig. 4B).

Finally, AIPI 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 (Minotti et al., 1996). 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 study by Minotti et al. (1996) is consistent with the notion that AIPI occurs in humans.

On a cautious note, we want to add that although the pathways depicted in Fig. 4B are supported by indirect evidence, it remains speculative to suggest that the AIPI and

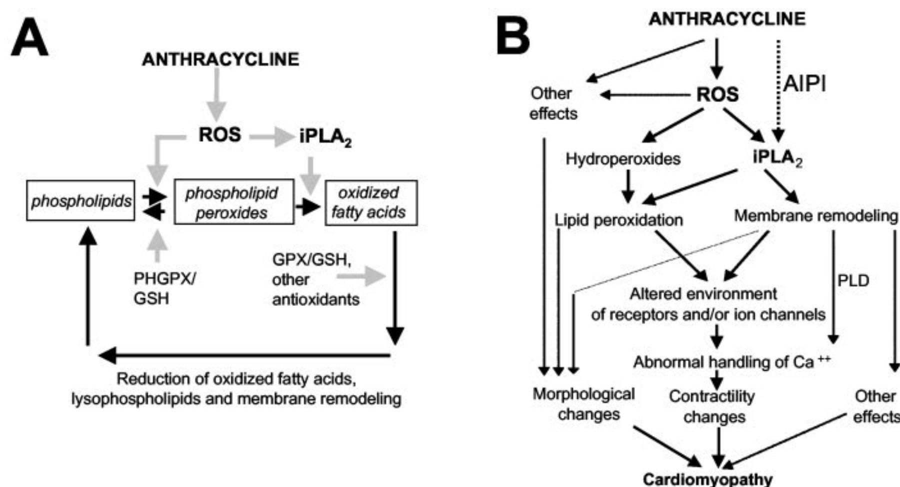

Fig. 4. Proposed pathways that may link AIPI to cardiomyopathy and heart failure. A, impairment of membrane phospholipid repair cycle. B, relationship between AIPI and other known anthracycline targets. ROS, reactive oxygen species; PHGPX, phospholipid glutathione peroxidase. See text for details.

the observed changes in lipid composition serve as a precursor to anthracycline cardiotoxicity. Further studies are needed to confirm this link.

A careful reader can also point to a small change in animal body and heart weight at the end of the anthracycline protocol. This effect is consistent with the loss of appetite associated with anthracycline therapy. It has been shown, however, that the alterations in cardiac function observed in doxorubicin-treated rats are not due to a reduction in the food intake (Canepari et al., 1994). Similarly, we believe that weight loss was not a cause of the specific changes observed in our study. Notably, the alterations in measured endpoints were marked and ranged from 40 to 200%, whereas body weight difference was barely detectable ($<10\%$; $p = 0.03$) and heart/body weight ratio remained the same. Therefore, we believe that marked changes in iPLA$_2$ activity and phospholipid composition of cardiac muscle occur primarily due to the doxorubicin treatment and not to a small decrease in body or heart weight. To completely eliminate such an alternative, however, one might use a pair-feeding approach to control for animal weight loss.

In summary, we have shown for the first time that chronic treatment of adult rats with subtoxic doxorubicin concentrations diminishes activity of both cytosolic and membrane-associated myocardial iPLA$_2$ and leads to changes in membrane phospholipid content. These data support our hypothesis that doxorubicin-induced iPLA$_2$ inhibition can be an initial step in the series of events leading to anthracycline cardiomyopathy.

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