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EFFECT OF ANTHRACYCLINES ON PHOSPHOLIPASE A_2 ACTIVITY AND PROSTAGLANDIN E_2 PRODUCTION IN RAT GASTRIC MUCOSA

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Abstract—The purpose of this study was to investigate in rats the effects of three anthracyclines, pirarubicin, doxorubicin and epirubicin on gastric prostaglandin E_2 (PGE_2) metabolism and phospholipase A_2 (PLA_2, EC 3.1.1.4) activity. The level of the membrane precursor, arachidonic acid, and the stability of the membrane were investigated by analysis of the composition of fatty acids. Enzymatic activities involved in the turnover of membrane phospholipids such as lysophospholipase (LPase, EC 3.1.1.5) and acyl-CoA lysophosphatidylcholine: acyltransferase (ACLAT, EC 2.3.1.23), and in the detoxification of lipid hydroperoxides, selenium-dependent glutathione-peroxidase (GSH-PX, EC 1.11.1.9) were measured after injection of the drugs for 4 consecutive days. Pirarubicin does not give rise to any changes in these activities but doxorubicin and epirubicin decreased PGE_2 production and the activities of PLA_2, LPase and ACLAT. GSH-PX activity was not changed by any of the drugs. The decrease in PLA_2 activity does not seem to be related to variations in membrane lipid composition because the total phospholipids content was unchanged. The P/S (polyunsaturated/saturated) ratio increased in the doxorubicin group and decreased in the epirubicin group, and the unsaturation index was moderately modified. Arachidonic acid was increased only in the doxorubicin group. *In vitro*, PLA_2 activity was not inhibited by the three drugs in the micromolar range. A marked inhibition was observed at 2.5 mM for pirarubicin and at 1.0 mM for doxorubicin and epirubicin. The Lineweaver–Burk representation showed that these inhibitions were of an uncompetitive type. Pirarubicin may therefore be considered to be an anthracycline without marked side-effects on gastric mucosa. However, the *in vitro* inhibition of PLA_2 activity by anthracyclines does not fully explain the *in vitro* decrease in PLA_2 specific activity observed after doxorubicin and epirubicin treatment, and in this context membrane structure modifications unconnected with the lipid composition can not be excluded. *In vivo* these phenomena may affect PGE_2 synthesis, whose level was lower in the doxorubicin and epirubicin groups than in control group.

Anticancer drug therapy using anthracyclines [1] is often impeded by their secondary side-effects. One of the most deleterious and widely studied side-effects is their cardiotoxicity [2, 3], but gastrointestinal disorders have also been reported. In most cases nausea and vomiting are observed. In anticancer drug-treated rodents, macro and micro examination of mucosa revealed damage when the drugs were given intraperitoneally [4–6] or intravenously [7, 8]. A decrease in the synthesis of gastric phospholipids has been described [9], and this may contribute to ulcer formation, as the integrity of the gastric membrane is of crucial importance in this pathology. The mechanisms leading to these alterations are poorly understood since few investigations have been devoted to this aspect. It has recently been described in both minipigs [10] and humans [11] that administration of a mixture of antimitotic drugs leads to a significant decline in gastric potential 2 hr after drug infusion, indicating that the gastric mucosal function is altered.

In the stomach, the biochemical mechanisms leading to the gastric lesions are known to be closely linked to arachidonic acid metabolism, which provides the bioactive derivatives such as prostaglandins. In the stomach, these compounds mostly of the E-series (PGE_2†), are known to exert a cytoprotective function, thus preventing the development of gastric ulcers [12, 13]. An illustration of this effect is provided by non steroidal anti-inflammatory drugs (such as aspirin) that inhibit the cyclo-oxygenase pathway [14] and steroidal anti-inflammatory drugs (such as glucocorticoids) that inhibit phospholipase A_2 (PLA_2) [15], both drugs being able to induce visible ulceration of the gastric mucosa.

PLA_2, by releasing arachidonic acid from membrane phospholipids, is the key enzyme in initiating the production of prostaglandins [16]. This activity has been characterized in the gastric glandular part of the stomach [17–19], mainly in the pepsinogen secreting cells [20] and this activity was localized on the apical part of the plasma membrane [21] of rat gastric mucosa. A PLA_2 belonging to the pancreatic type (type I) has been purified from a soluble extract of whole rat gastric homogenate [22, 23].

However, to our knowledge, there is no

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† Abbreviations: PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; LPase, lysophospholipase; ACLAT, acyl-CoA lysophosphatidylcholine: acyltransferase; GSH-PX, selenium-dependent glutathione-peroxidase; Cont, control; Pir, pirarubicin; Dox, doxorubicin; Epi, epirubicin; POPC, 1-palmitoyl-2[1-^{14}C]oleoylphospatidylcholine; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; FAME, fatty acid methyl esters.

information on the *in vivo* effect of anthracyclines on the gastric PGE_2 metabolism as well as on the activity of gastric PLA_2. Therefore, the purpose of this study was to investigate the effects of acute administration of various anthracyclines on gastric PGE_2 metabolism on the level of its precursor in the membrane (i.e. arachidonic acid) and on PLA_2 activity on samples prepared from gastric mucosa. Since membrane stability plays a fundamental role in ulceration, we also investigated the effects of the drugs on the fatty acid composition and on the activities of the enzymes involved in the elimination of cytotoxic lysocompounds and membrane hydroperoxides [24] both generated by PLA_2. The enzymes studied were lysophospholipase (LPase) and acylCoA lysophosphatidylcholine: acyltransferase (ACLAT) which catalyse the elimination of the lysophospholipids and the resynthesis of the parent phospholipids, respectively, and the selenium-dependent glutathione peroxidase (GSH-PX) that reduces the released hydroperoxides in the cytosol to prevent any further decomposition into alkoxyl radicals. The anthracyclines [doxorubicin (Dox), epirubicin (Epi) and pirarubicin (Pir)] have been chosen on the basis of their clinical safety profiles and on their differential secondary side-effect, Epi and Pir being recognized to be less toxic than the parent compound Dox. Results showed that the drugs, Dox and Epi, decreased PLA_2 specific activity and consecutively the *in vitro* PGE_2 production in gastric mucosa. These results cannot be explained entirely by uncompetitive inhibition of PLA_2 activity by these antitumor drugs.

MATERIALS AND METHODS

Animals, experimental procedures and sample treatments. Male Wistar rats (IFFA-CREDO, Paris, France) weighing between 270 and 280 g were fed a standard diet. They were divided into four groups of eight rats each and housed in pairs. Drugs were intraperitoneally injected, for four consecutive days, alternatively on the left and right side. The Dox group received 4 mg/day/kg (6.89 μmol) of Dox (gift from Carlo-Erba Farmitalia, Rueil-Malmaison, France). The Epi group received 4 mg/day/kg (6.89 μmol) of Epi (gift from Carlo-Erba Farmitalia, Rueil-Malmaison, France). The Pir group received 4 mg/day/kg (6.37 μmol) of Pir (Roger Bellon, Neuilly/Seine, France). The control group (Cont) received a solution of 0.15 M NaCl. In all groups, the volume injected was 0.55 mL. After the last injection, rats were fasted overnight. They were killed by rapid decapitation the next morning (fifth day). No mortality due to drugs was observed during the course of the experiment. Homogenates of gastric mucosa were prepared as described in [25] and stored at −60°.

Protein assay. Proteins were measured by the method of Lowry *et al.* [26] using bovine serum albumin as standard.

PLA_2 and lysophospholipid-metabolizing enzyme assays. Gastric PLA_2 (EC 3.1.1.4) was assayed as previously described [19]. The incubation mixture contained 0.2 mM 1-palmitoyl-2[1-^{14}C]oleoylphosphatidylcholine (NEN, Paris, France) and 50 mM sodium cacodylate at pH 7.0 in a final volume of 0.2 mL. Phosphatidylcholine was dispersed in liposome form by sonication for 5 min under nitrogen. In the optimal assay conditions, the reaction was started by adding 0.1 mg protein homogenate then 5 mM calcium chloride. The mixtures were incubated at 37° for 20 min. The [1-^{14}C]oleic acid was isolated by Dole extraction. One milliliter of the heptane phase was passed over a small silica gel column and the [1-^{14}C]oleic acid in the eluate was counted by liquid scintillation spectrometry (Beckman LS 9000) in 10 mL of Readysolve MP (Beckman) scintillation fluid. The results were corrected for a control incubated without protein. LPase (EC 3.1.1.5) and ACLAT (EC 2.3.1.23) were assayed with [1-^{14}C]palmitoyl-lysophosphatidylcholine (NEN, Paris, France) as described previously [27, 28]. The method described by Levander *et al.* [29] to assay the selenium-dependent GSH-PX (EC 1.11.1.9) was used to determine its activity in gastric mucosa. The reaction was initiated by adding *t*-butylhydroperoxide.

In vitro *production of PGE_2.* PGE_2 was produced by incubating mucosa homogenates (5 mg protein) at 37° for 10 min. The pH of the homogenates was then adjusted to 3.5 by adding 3% formic acid and prostaglandins were extracted with ethyl acetate according to the method of Lokesh *et al.* [30]. *Ex vivo* produced PGE_2 was quantified by radioimmunoassay (RIA), using a [^{125}I] PGE_2 RIA kit (NEK-020) purchased from NEN. Appropriate blanks for non-specific binding, and tubes for total binding determinations were included. Assays were done in duplicate and the measurements were on the linear portion of the standard curve.

Lipid analysis. Lipids were extracted from the gastric mucosa homogenate by the method of Folch *et al.* [31]. A fraction of this extract was used for determination of lipid phosphorus [32]. Another part of this extract was used to analyse fatty acid composition. Their fatty acid methyl esters (FAME) were prepared according to a rapid and convenient method [33]. FAME recovered in hexane were stored under nitrogen at −20° pending gas liquid chromatography analysis. Separation of FAME was performed as described [34], using a gas liquid chromatograph (Girdel 3000, Paris, France) equipped with a peak integrator (Delsi, Enica 10, Suresne, France) and a 50 m capillary column (Spirawax FS, 1493, Spiral, Dijon, France).

Statistical analysis. Results presented in the tables are means ± SD. As we compared the effect of each drug with the control and not with another drug, statistical significance of mean differences was investigated by the Student's *t*-test.

In vitro *study of gastric PLA_2 activity.* Rat gastric PLA_2 used in this study has been purified in our laboratory [35] (manuscript in preparation). The final enzyme fraction was 700-fold purified and gave a single protein band on SDS–polyacrylamide gel electrophoresis. For the *in vitro* inhibition studies the final fraction was dialysed against water, to avoid interaction between drugs and activity co-factors. After dialysis the specific activity was 35.4×10^{-3} μmol/min/mg in normal assay conditions.

Table 1. Fatty acid composition of total lipids in gastric mucosa of rat treated with anthracyclines

	Control	Pir	Dox	Epi
20:4 n − 6	8.55 ± 1.65	7.80 ± 3.00	11.94* ± 2.22	9.10 ± 0.24
MUFA	24.71	26.34	24.22	22.29
PUFA	33.10	34.26	36.11	28.23
SFA	42.30	39.54	39.23	49.51
P/S	0.78	0.86	0.92	0.57
UI	113.14	114.91	124.60	100.84

Fatty acids in weight per cent. P/S, polyunsaturated/saturated ratio. UI, unsaturation index obtained by ΣNx where, for a given fatty acid, N is the number of double bonds and x is the weight percentage.

* Significantly different ($P < 0.05$) from control group (Student's t-test), N = 8. Values are means ± SD.

Table 2. PGE_2 production and activities of enzymes of phospholipid metabolism and of detoxification of lipid hydroperoxides in gastric mucosa of rat treated with anthracyclines

	Controls	Pir	Dox	Epi
PGE_2	83.27 ± 25.77	99.09 ± 49.43	44.29* ± 19.53	55.52* ± 28.64
PLA_2	633 ± 70	674 ± 49	417* ± 217	533* ± 41
LPase	53 ± 12	33 ± 12	8* ± 2	13* ± 3
ACLAT	304 ± 63	338 ± 107	147* ± 26	163* ± 34
GSH-PX	454 ± 81	548 ± 136	494 ± 31	513 ± 93
P/A	2.08	1.99	2.83	3.26

PGE_2 production in ng/g mucosa. PLA_2 specific activity in nmol/hr/mg. ACLAT specific activity in nmol/hr/mg. LPase specific activity in nmol/min/mg. GSH-PX specific activity in nmol/min/mg. P/A: PLA_2/ACLAT.

* Significantly different ($P < 0.05$) from control group (Student's t-test) N = 6. Values are means ± SD.

The activity of purified enzyme was assayed after an incubation time of 20 min at 4°, with different quantities of each drug or water for control. Each incubation and assay contained 1.3 mU of PLA_2 (38 μg), 5 mM $CaCl_2$ and 50 mM sodium cacodylate at pH 7.0. The reaction was started by adding 1-palmitoyl-2[1-^{14}C]oleoylphosphatidylcholine (POPC) substrate in cacodylate buffer. Consecutively, for the Lineweaver–Burk representation, the drug concentrations were selected in order to have a residual PLA_2 activity between 30 and 50%. Final drug concentrations in the assay were: Pir 2.5, Dox 1.0 and Epi 1.0 mM.

RESULTS

Over the 5 days experimental period, gastric mucosa weights were not significantly different among the four groups of rats and no visible evidence of gastric ulceration was observed. The protein content and total phospholipids remained unchanged among the four groups, the mean values were 132.6 mg/g and 4.2 mg/g mucosa, respectively.

Total lipid fatty acid composition of rat gastric mucosa

The fatty acid composition of gastric mucosa is shown in Table 1 and demonstrated a significant increase in arachidonic acid (+39%) in the Dox group. In the Pir and Epi groups the variations were not significant (−9% and +6%, respectively). The percentage of monounsaturated fatty acid (MUFA) is unchanged among the four groups. Compared with the control group, the polyunsaturated fatty acid (PUFA) content was slightly increased in the Dox group (+9%) and markedly decreased in the Epi group (−15%). This was essentially due to the variation of N-6 PUFA percentage (linoleic, linolenic and arachidonic acid). The saturated fatty acid (SFA) content was markedly increased in the Epi group (+17%) (essentially, palmitic and stearic acids). These differences were reflected by the polyunsaturated/saturated (P/S) ratio and the unsaturation index which were increased in the Dox group (+18 and +10%, respectively) and decreased in the Epi group (−27 and −11%, respectively).

Selenium-dependent GSH-PX activity

GSH-PX activity was assayed to estimate the ability of gastric mucosa to neutralize the hydroperoxides of PUFA under the various treatments. As shown in Table 2, GSH-PX activity was not modified by drugs.

PGE_2 in vitro production and phospholipid-metabolizing enzyme activities

In comparison with the control group (Table 2)

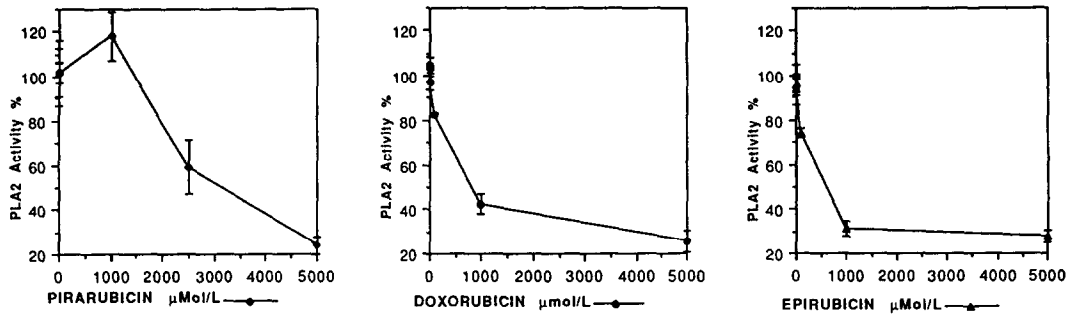

Fig. 1. *In vitro* study of gastric PLA_2 activity as function of the antitumor drug concentration. Incubation mixtures contained 1.3 mU of PLA_2, antitumoral drug [Pir (-◆-), Dox (-●-), Epi (-▲-)] or water for control. Each representation is the result of four experiments for Pir and two experiments for Dox and Epi.

the *in vitro* production of PGE_2 remained constant in the Pir group whereas the value decreased significantly in the Dox group (−47%) and in the Epi group (−33%). Gastric PLA_2 specific activity was unchanged in the Pir group but was significantly lower in the Dox group (−35%) and in the Epi group (−13%) compared with the control group. The same behavior is observed with gastric LPase and ACLAT specific activities. Compared with the control group, these activities were unchanged in the Pir group and were significantly lower in the Dox and Epi groups: LPase −85 and −75%, respectively, ACLAT: −51 and −46%, respectively. The activities of PLA_2 relative to ACLAT could represent the theoretical net turnover of arachidonate and the status of free fatty acids available for prostaglandin synthesis.

In vitro *study of gastric PLA_2 activity in the presence of the different drugs*

The activity of purified enzyme was assayed after incubation for 20 min at 4°, with different quantities of each drug. The results are presented in Fig. 1. The enzymatic activity was affected slightly in the micromolar range of Dox and Epi and decreased markedly from 1 mM concentration. To determine the type of inhibition the activity of PLA_2 was studied with drugs at different substrate concentrations. The drug concentrations were selected to have a residual PLA_2 activity between 30 and 50%. For each drug the Lineweaver–Burk representation of PLA_2 activity (Fig. 2) showed a parallel behavior when compared to the native enzyme activity. The V_{max} and apparent K_m values of PLA_2 activity, on POPC, were 52.6×10^{-3} µmol/min and 0.268 mM, respectively. These two values are similarly decreased by a factor of 3.00, 2.74 and 7.15 in the presence of Pir, Dox and Epi, respectively. These results allowed classification of this phenomenon to be of the uncompetitive inhibition type.

DISCUSSION

Cellular dysfunction has been reported after anthracycline administration and these adverse effects have been related to ultrastructural lesions. Many studies suggested that membrane lipid peroxidation may be at the basis of the development of this cellular dysfunction [36, 37]. In gastric mucosa, recent results suggest that active oxygen species and lipid peroxidation may play a role in the formation of gastric mucosal damage induced by stress [38].

The specific activity of PLA_2 in the gastric mucosa is one of the highest compared to those measured in other organs [39]. This may be related to the high prostaglandin turnover in the gastric mucosa. This is illustrated by the results of Takano *et al.* [40] who showed a concomitant decrease in PLA_2 activity and prostaglandin levels in stomachs of rats stressed by water immersion. Furthermore, there was a clear reciprocal correlation between the PLA_2 activity and the ulcer index. These authors concluded that a decrease in the PLA_2 activity resulting in decreases in the tissue level of prostaglandins played a key role in the genesis of gastric ulcers in these rats.

In many cases of antitumor drug therapy, gastric lesions are observed [4–8]. However, the *in vivo* effect of anthracyclines on the gastric PGE_2 metabolism and PLA_2 activity is unknown. The aim of this work was to study these secondary side-effects. After acute administration of three anthracyclines (Pir, Dox, and Epi) the *in vitro* PGE_2 production and the PLA_2 activity of gastric mucosa were measured. The level of the prostaglandin membrane precursor, arachidonic acid, and the stability of membrane PUFA were investigated by the analysis of the composition of fatty acids. In the same way the other enzymatic activities involved in the turnover of membrane phospholipids (LPase and ACLAT) and in the detoxification of lipid hydroperoxides (GSH-PX) were examined.

Under our experimental conditions, no visible evidence of gastric ulceration was observed after acute administration of antitumor drugs and saline buffer. The contents of proteins remained unchanged among the four groups of animals. In all of the parameters studied the Pir group does not show any change. Among the three groups studied, Dox causes the most marked modifications. The *in vitro* PGE_2 production and specific activity of PLA_2 were significantly decreased in the Dox and Epi groups.

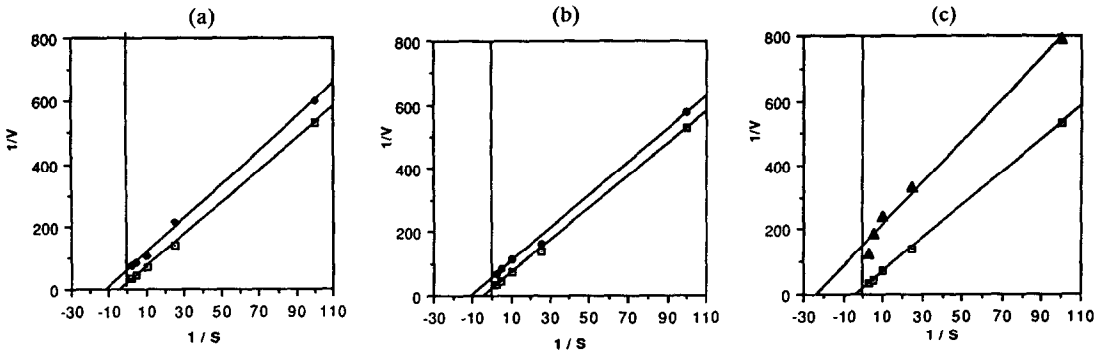

Fig. 2. Lineweaver–Burk representation of PLA_2 activity as a function of substrate concentration in the presence of antitumor drug. V, PLA_2 activity in μmol/mg/min. S, POPC concentration in mM. Incubation mixtures and assay conditions are the same as in Fig. 1, with a fixed antitumoral concentration for each case: (a) Pir 2.5 mM (–◆–); (b) Dox 1.0 mM (–●–); (c) Epi 1.0 mM (–▲–); (a, b and c), PLA_2 activity without drug (–⊡–).

The lower PLA_2 specific activity may be due to a direct inhibitory effect by the drugs or physical changes in the lipid micro-environment surrounding the enzyme (such as lipid composition) allowing the protein to be less active. When considering our results, the decrease in PLA_2 activity can not be explained by variations of membrane lipid composition because the total phospholipid content was unchanged in all groups, and concerning the fatty acid composition the polyunsaturated/saturated (P/S) ratio increased in the Dox group and decreased in the Epi group and the unsaturation index is moderately modified. However many drugs alter structure without altering lipid composition and in this respect the anthracycline Dox has previously been shown to influence membrane fluidity [41] and these modulations can result in variations in membrane enzyme activities. The decrease in *in vitro* PGE_2 production can not be related to the arachidonic acid level because this fatty acid was significantly increased in the Dox group only.

As previously described [42] in heart and liver, these contrasting changes in the membrane fatty acid composition in the gastric mucosa after anthracyclin administration strongly suggest that the process of elongation and desaturation is somewhat modified by/or in response to the drug.

The theoretical net turnover of arachidonate available for prostaglandin synthesis is not altered in drug treated groups and the decrease in production of PGE_2 is not similar to the decrease of PLA_2 activity after Dox and Epi. Prostaglandin synthesis is mediated by cyclo-oxygenase activity and this membrane enzyme activity can be inhibited if arachidonic acid is in a state that is inaccessible to cyclo-oxygenase. This situation arises when there is less PLA_2 stimulation [43], or when the concentration of hydroperoxide needed for initiation of cyclo-oxygenase is reduced [44]. The hydroperoxide detoxification process seems not to be affected since GSH-PX was maintained at the same level in all treated groups.

Like PLA_2 activity, the LPase and ACLAT specific activities are significantly decreased in the Dox and the Epi groups. To understand the decreased PLA_2 specific activity in the Dox and Epi groups, the *in vitro* study of purified gastric PLA_2 activity in the presence of each antitumor drug was carried out. Our attention was focused on PLA_2 activity because this enzyme represents the rate-limiting step of PGE_2 synthesis. The PLA_2 activity was not inhibited by the three drugs in the micromolar range. A marked inhibition was observed only at 2.5 mM for Pir and at 1.0 mM for Dox and Epi. These results are not fully compatible with the drug concentrations used *in vivo*. The *in vitro* study confirms that in our experimental conditions Pir may be considered as an antitumor drug without drastic secondary side-effects on gastric mucosa.

The Lineweaver–Burk representations show that these inhibitions are of an uncompetitive type. The inhibitor binds only to the enzyme–substrate complex and not to the free enzyme, indeed the inhibitor decreases V_{max} because a fraction of the enzyme–substrate complex is always diverted by the inhibitor to the inactive enzyme–substrate–inhibitor complex. Since the inhibitor decreases the concentration of the enzyme–substrate complex, it also decreases the apparent K_m [45].

The inhibitory effects of anthracyclines have been reported for some plasma membrane and mitochondrial membrane enzymatic activities (for a review see Ref. 46). The lipid–enzyme interactions are of prime importance for the mitochondrial cytochrome C oxidase activity. The mechanism of its inhibition by anthracyclines was shown to result from the complexation of the enzyme phospholipid environment rather than from a direct drug–enzyme interaction [47]. In the same way, an *in vitro* study [48] shows an inhibition by Dox of hydrolysis by porcine pancreatic PLA_2 of the phosphatidylcholine analogs. The authors suggested that this inhibition results from a drug-lipid interaction. Gastric PLA_2 acts at nearly the same rate on the two major gastric membrane phospholipids, namely phosphatidylcholine and phosphatidylethanolamine [21].

The *in vitro* results on the inhibition of PLA_2 activity by anthracyclines do not fully explain the *in*

vivo decrease of PLA_2 specific activity observed after our treatment conditions with Dox and Epi. In this context membrane structure modifications unconnected with the lipid composition can not be excluded. We may infer that LPase and ACLAT which are membrane enzymes like PLA_2 may be decreased in the same way, but GSH-PX being a cytosolic enzyme is not affected by the drugs. *In vivo* changes of PLA_2 activity may modify the availability or accessibility of arachidonic acid and affect the PGE_2 synthesis pathway, whose level was lower in the Dox and Epi groups than in control group. In addition, long term treatment with antitumoral drugs may disrupt the cytoprotective function of PGE_2 and thus contribute to ulcer formation.

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