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HEART AND LIVER MEMBRANE PHOSPHOLIPID HOMEOSTASIS DURING ACUTE ADMINISTRATION OF VARIOUS ANTITUMORAL DRUGS TO THE RAT

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Abstract—The purpose of this study was to investigate in the rat heart and liver the effects of an acute administration of three anthracyclines, doxorubicin, epirubicin and pirarubicin, and an anthracenedione, mitoxantrone, on the membrane peroxidative status, which was estimated by the composition of polyunsaturated fatty acids (PUFA), and on the activities of the enzymes involved in membrane repair processes and lipid hydroperoxide detoxification. Rats were injected for four consecutive days with the drugs or saline (control) and killed 24 hr after the last injection. All the drugs induced an increase in plasma thiobarbituric reactive substances and α-tocopherol concentrations, both expressed per milligram of plasma lipids. Plasma vitamin A was decreased by about a factor of two by all the drugs. The fatty acid profile in the heart lipids showed that the polyunsaturated species (20:4 n-6, 22:6 n-3) remained at the same or even higher levels after anthracycline treatment. This can be explained by the fact that the activities of the enzymes involved in either the recycling of membrane phospholipids, such as phospholipases A1 and A2 (EC 3.1.1.4 and EC 3.1.1.32), lysophospholipases (EC 3.1.1.5) and acylCoA:lysophosphatidylcholine acyltransferases (EC 2.3.1.23), or hydroperoxide detoxification, such as selenium-dependent glutathione peroxidase (GSH-PX, EC 1.11.1.9) and glutathione *S*-transferases (GSH-T, EC 2.1.5.18), were maintained at the same level of activity after the antitumoral treatment. In liver, membrane phospholipid levels of PUFA were maintained as well as the activities of phospholipid-metabolizing enzymes. GSH-PX activity was not affected whereas that of GSH-T was slightly lowered by the drugs. These results suggest that during acute antitumoral-induced lipid peroxidation of membranes, the multi-enzymatic complex of the immediate processes of repair and detoxification is fully operational, allowing the membrane to rapidly recover its functional status. The results are discussed in the context of the equivocal relationships between antitumoral-induced lipid peroxidation and cardiac disturbances.

Anticancer drug therapy using anthracyclines is often impeded by their secondary side-effects, one of the most deleterious being their cardiotoxicity. Systolic and diastolic dysfunctions have been reported after anthracycline administration [1, 2]. These adverse effects have been related to ultrastructural lesions observed in the sarcoplasmic reticulum, sarcolemma and mitochondria [3, 4]. The mechanisms leading to these alterations are not fully understood. Since early studies in the late 1970s [4, 5] suggested that membrane lipid peroxidation may be at the origin of the development of cardiac dysfunction, a multitude of works has been devoted to validate this hypothesis. Unfortunately, no firm conclusion can be drawn because many arguments for and against this hypothesis exist (for a review see Ref 6). It is obvious that membrane lipid peroxidation can be deleterious for cell function, particularly in the heart [7, 8], but whether the cardiotoxicity of anthracyclines is due solely to free radical-induced membrane peroxidation still remains an open question. In the *in vivo* studies, the equivocal results obtained may arise in part from the various experimental protocols used, such as the duration of treatment, the accumulated dose, the method of drug administration and the animal species. The drug-induced extent of tissue lipid peroxidation has been investigated mostly in tissue homgenates by the classical thiobarbituric acid reactive substances (TBARS§) assay, a method that is criticized because of its lack of specificity [9–11]. Moreover, measurement of TBARS in whole tissue homogenate is not an indication of the real peroxidative status of membranes, because cellular lipid peroxides that accumulate mainly in intracytoplasmic structures [12] may reflect an early peroxidative stress.

Whether membrane lipid peroxidation is deleterious for cell functions is likely to be dependent on the efficiency of the repair process of peroxidized membrane phospholipids. The repair process includes a variety of factors (enzymatic and

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§ Abbreviations: TBARS, thiobarbituric acid reactive substances; GSH-PX, selenium-dependent glutathione peroxidase; GSH-T, glutathione *S*-transferases; DOX, doxorubicin; EPI, epirubicin; PIR, pirarubicin; MIT, mitoxantrone; TG, triglycerides; TC, total cholesterol; UC, unesterified cholesterol; CE, cholesteryl ester; PUFA, polyunsaturated fatty acids; ACLAT, acyl-CoA:lysophosphatidylcholine acyltransferase.

non-enzymatic) acting sequentially to allow the membrane to rapidly recover its initial composition. The enzymes that play a fundamental role in this emergency process are, according to the cycle described by Lands [13], phospholipases A1 and A2, lysophospholipases, and acylCoA:lysophospholipid acyltransferases (ACLATs). It may be alleged that any inadequate adjustment of these activities to peroxidative stress perturbs the stability of the membrane. In this sequential event, phospholipase A2 should play an antioxidant role since its activity was demonstrated to be higher on phospholipid-hydroperoxides than on intact phospholipids [14, 15]. This is because the structural alterations induced by lipid peroxidation are, as compared with intact structures, recognized more easily by phospholipase A2 [14]. Phospholipase A2 works in concert with the selenium-dependent glutathione peroxidase (GSH-PX) [15] since the released hydroperoxide is then reduced in the cytosol by this enzyme to prevent any further decomposition into alkoxyl radicals. A glutathione peroxidase acting directly on phospholipid hydroperoxides is another means of detoxification [16], and in this case also, the phospholipase A2 has to cleave the reduced hydroperoxide of phospholipid. In both cases, as the lysophospholipid issued from phospholipase A2 action is a cytotoxic detergent compound its reacylation into a new phospholipid by acyltransferase or its elimination by a lysophospholipase is of crucial importance for proper membrane integrity and function.

To the best of our knowledge, almost no information is available concerning the effect of anthracyclines on these phospholipid-metabolizing enzyme activities. Ogawa *et al.* [17] reported that heart mitochondria isolated from doxorubicin (DOX)-treated rats induced *in vitro* a 65% increase in the release of linoleic acid from phospholipids. *In vitro*, Adriamycin® was shown to interact with pyrene-labeled phospholipids, thus altering the kinetic properties of pancreatic phospholipase A2 [18].

The purpose of this study was to investigate the effects of an acute administration of various anthracyclines and an antitumoral anthracenedione on the enzyme activities involved in membrane phospholipid recycling in rat heart and liver. These compounds have been chosen on the basis of their clinical safety profiles. DOX is the mother compound of the anthracycline class, and newer anthracyclines, such as epirubicin (EPI) and pirarubicin (PIR), are clinically accepted as being far less cardiotoxic. Mitoxantrone (MIT) has a mechanism of action on DNA targets very close to that of anthracyclines and its cardiotoxicity is comparable to that of EPI or PIR. Evaluation of membrane peroxidative status was performed by fatty acid analysis.

Results have shown that administration of antitumoral drugs for 4 consecutive days did not alter the peroxidative status of membrane phospholipids in the heart and liver, although a higher level of circulating peroxides was observed. These results are explained by the fact that the membrane repair enzymatic process was not inactivated by the drugs, allowing the membrane to recover its integrity.

MATERIALS AND METHODS

Animals, experimental procedures and sample treatments. Male Wistar rats (IFFA-CREDO, Paris, France) weighing between 270 and 280 g were fed standard chow. They were divided into five groups of eight rats each and housed in pairs. Drugs were injected intraperitoneally, for four consecutive days, on the left and right sides alternately. The DOX group received 4 mg/day/kg of DOX (Adriablastine®, gift from Carlo-Erba Farmitalia, Rueil-Malmaison, France). The EPI group received 4 mg/day/kg of EPI (Farmorubicine®, gift from Carlo-Erba Farmitalia). The PIR group received 4 mg/day/kg (Theprubicine®, Roger Bellon, Neuilly/Seine, France) and the MIT group received the anthracenedione MIT (Novantrone®, gift from Lederle, Rungis, France) at a dose of 0.81 mg/day/kg. This lower dose was chosen because of the higher therapeutical activity of this drug. The control group 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 (5th day). No mortality due to drugs was observed during the course of the experiment.

Blood was collected in heparinized tubes and centrifuged immediately at 2500 rpm for 30 min at 4° to obtain plasma. The heart and liver were excised immediately. Biopsies for ultramicroscopic examination were taken randomly from three hearts and three livers and treated as described previously [19]. Briefly, pieces of tissues were fixed for 2 hr in 2% glutaraldehyde. After several washes, samples were treated for 1 hr with 1% OsO_4. After graded dehydration, they were embedded in Epon 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate. Organs were thoroughly rinsed at 4° in a sucrose 0.25 M, Tris–HCl 20 mM pH 7.4 buffer, minced and divided into two parts: one for a direct lipid extraction and the other for homogenization in the same buffer. These two procedures have been described previously [19]. Lipid extracts that contained 0.05% butylated hydroxytoluene as an antioxidant were stored at −80° under nitrogen pending fatty acid analysis and lipid phosphorus determination. Homogenates and plasma were stored at −80° pending protein and enzyme activity determinations.

Plasma parameters and tissue lipid analyses. Some plasma parameters were determined with a Technicon SMAC II (Tarrytown, NY, U.S.A.), an apparatus routinely used for clinical assay on plasma. The parameters assayed with SMAC were: triglycerides (TG), total cholesterol (TC), unesterified cholesterol (UC), proteins, albumin, glucose, creatinine and alkaline phosphatase (EC 3.1.3.1). Plasma α-tocopherol and vitamin A were separated and assayed by HPLC on a RP8 LiChrosorb (7 μ, 250 × 4 mm) column (Merck, Darmstadt, Germany) as described by De Leenher *et al.* [20]. Detection was done at 295 nm. Fatty acid methyl esters of total lipids isolated from the heart and liver were prepared and separated as described before [21]. Lipid phosphorus was assayed as already published [22]. Lipid peroxides were measured by quantification of

the TBARS according to the method described by Dousset *et al.* [23] and modified as published recently [24]. Particularly, it was found that 0.025% butylated hydroxytoluene afforded protection of lipids during the heating process at 95°. Consequently, this antioxidant was added at this final concentration in all the assays. Fluorescence was measured (λ_{ex} 532 nm and λ_{em} 553 nm) using a Jobin-Yvon JY3D spectrofluorimeter.

Protein and enzyme assays. Protein was assayed according to Lowry *et al.* [25] using bovine serum albumin as the standard. Alkaline phospholipase A1 and A2 (EC 3.1.1.4 and EC 3.1.1.32) activity was assayed at pH 8.4 at a final calcium concentration of 5 mM for 20 min using di[1-^{14}C]oleoylphosphatidylcholine (Du Pont NEN, Paris, France) with a specific radioactivity of 50 Bq/nmol as described previously [26]. Lysophospholipase (EC 3.1.1.5) was assayed at pH 8.0 for 20 min [26] using 1-[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphocholine (NEN) with a specific radioactivity of 20 Bq/nmol. ACLAT (EC 2.3.1.23) activity was determined for 15 min at pH 7.4 according to a method already published [27]. The substrate was identical to that used for the lysophospholipase assay and cold oleoyl-CoA (Sigma, La Verpillière, France) served as the donor. The method described by Levander *et al.* [28] to assay the selenium-dependent glutathione peroxidase (GSH-PX, EC 1.11.1.9) was used to determine its activity in the heart and liver. The reaction was initiated by adding *t*-butylhydroperoxide and developed in the presence of glutathione, glutathione reductase and NADPH Type X (all from Sigma). Consumption of NADPH was automatically recorded at 340 nm for 4–5 min with a DU-40 spectrophotometer (Beckman, Paris, France). Glutathione *S*-transferase (GSH-T, EC 2.1.5.18) activity was assayed according to Habig *et al.* [29] using 1-chloro-2,4-dinitrobenzene as substrate. All the enzyme assays were done at least in duplicate with respect to the linear range of protein concentration and time dependence, and a blank without proteins was included systematically in all the assays.

Chemicals and statistical analysis. Fatty acid methyl ester standards (99% pure) were from Interchim (Paris, France). Standards of α-tocopherol and vitamin A, and enzyme substrates were obtained from Sigma at the highest purity. As we compared the effect of each drug with the control and not with another drug, statistical significance of mean differences was investigated using the Student's *t*-test, first at $P < 0.05$ and then at $P < 0.01$.

RESULTS

Plasma parameters (Table 1)

Concentrations of TG tended to be lower in the drug-treated rats. The decrease was found to be significant on the case of EPI treatment only (−24%). TC concentrations were unchanged. However, significant changes in the proportions of cholesteryl ester (CE) and UC were induced by all the drugs. In general, the UC concentration was increased, particularly with PIR and EPI treatment where the increases attained a factor of 3.4 and 3.6, respectively. Conversely, the CE concentration was decreased, but with DOX treatment the decrease was not found to be significant. Total protein and albumin concentrations were lowered by drug treatment, the most pronounced effect being on albumin in DOX-treated rats (−22%). Since the creatinine concentration was not augmented by drug treatment, the decrease in protein and albumin concentrations can be attributed to drug-induced hepatic dysfunction but not to renal dysfunction. Since α-tocopherol is carried by lipoproteins, the α-tocopherol concentration was expressed per milligram of TG + TC (Table 1). The concentration was higher in all treated groups, the highest increase being with PIR (+40%). This may be explained by the fact that TG tended to be lower and also by the fact that the absolute concentration of α-tocopherol (μmol/L) tended to be higher in the drug-treated rats (data not shown). On the other hand, the vitamin A concentration was drastically and significantly decreased by more than 50% after anthracycline administration and by 35% after MIT administration. Since TBARS, like α-tocopherol, are transported by lipoproteins, they were expressed per milligram of TG + TC. They were increased significantly by all the drugs tested, the highest increases being after EPI and MIT administration (around 80%).

Ultrastructure of the heart

Electron microscopic appreciation of the degree of myocardial alteration caused by the various drugs was carried out according to the grading system proposed by Billingham *et al.* [30]. The alterations (data not shown) concerned at least one heart of the three examined and ranged between grade 0 (normal) and 1 (some cells with moderate alterations). In myocytes, both the myofibrillar system and nucleus content were preserved. Only the sarcoplasmic reticulum and mitochondria showed modification in their appearance. Some dilatation of the sarcoplasmic reticulum appeared after PIR, MIT and EPI treatments, being most evident after the latter. Mitochondrial alterations (swelling, disorganization of the crests and myelinization) seemed more abundant after MIT treatment. The endothelial cells appeared to be unaffected and exhibited an intense pinocytic activity. The unusual presence of collagen bundles was however occasionally noticed after PIR and DOX treatment.

Lipid composition and lipid peroxidation status in rat heart

As Table 2 shows, lipid phosphorus was not altered by the various drug treatments. The effect of antitumoral drugs on the peroxidative status of the heart was investigated. As mentioned above, the sole measure of TBARS in tissue may give an erroneous estimation of the peroxidative status of the membrane lipids since lipid peroxides are normally released into the cytosol. Accordingly, the fatty acid composition of heart lipids was determined to give an index of the stability of the membrane polyunsaturated fatty acids (PUFA). In the heart, total fatty acids are, at 90%, esterified on phospholipids [21, 31, 32]. Moreover, highly polyunsaturated fatty acids such as 20:4 n-6 and 22:6 n-3

Table 1. Plasma parameters of rats treated with saline or antitumoral drugs

	Control	PIR	DOX	EPI	MIT
TG (mmol/L)	0.56 ± 0.17	0.46 ± 0.18	0.45 ± 0.15	0.43 ± 0.10*	0.45 ± 0.14
TC (mmol/L)	1.82 ± 0.33	1.91 ± 0.24	1.91 ± 0.38	1.88 ± 0.33	1.57 ± 0.24
UC (mmol/L)	0.23 ± 0.08	0.79 ± 0.07*	0.54 ± 0.12*	0.83 ± 0.08*	0.40 ± 0.08*
CE (mmol/L)	1.58 ± 0.25	1.12 ± 0.20*	1.37 ± 0.27	1.05 ± 0.25*	1.17 ± 0.15*
Proteins (g/L)	62.2 ± 2.87	55.4 ± 3.74*	56.4 ± 3.85*	56.6 ± 2.07*	54.5 ± 2.80*
Albumin (μmol/L)	551 ± 15.6	450 ± 25.0*	432 ± 24.3*	447 ± 13.9*	469 ± 28.4*
Glucose (mmol/L)	6.85 ± 1.29	8.03 ± 0.87*	9.20 ± 1.0*	7.87 ± 0.59*	6.67 ± 0.52
Creatinine (μmol/L)	53.5 ± 3.07	44.5 ± 4.34*	41.1 ± 5.33*	43.7 ± 3.10*	49.7 ± 3.45*
Alkaline phosphatase (U/L)	257 ± 74.9	135 ± 32.1*	140 ± 23.7*	130 ± 29.9*	235 ± 41.3
α-Tocopherol (mg/g TC + TG)	2.78 ± 0.27	3.91 ± 0.28*	3.20 ± 0.24*	3.59 ± 0.23*	3.40 ± 0.31*
Vitamin A (mg/L)	0.56 ± 0.04	0.23 ± 0.03*	0.24 ± 0.05*	0.23 ± 0.03*	0.35 ± 0.04*
TBARS (μmol/mg TC + TG)	0.60 ± 0.18	0.92 ± 0.28*	0.89 ± 0.31*	1.09 ± 0.31*	1.06 ± 0.18*

Assays are described in Materials and Methods, CE was calculated as difference between total and unesterified cholesterol.

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

Table 2. Total phospholipid content and fatty acid composition of total lipids in the hearts of rats treated with saline or antitumoral drugs

	Control	PIR	DOX	EPI	MIT
			Fatty acids (weight %)		
16:0	14.1 ± 2.88	13.2 ± 0.61	12.9 ± 1.60	11.6 ± 0.22*	14.1 ± 3.42
16:1	0.81 ± 0.39	0.71 ± 0.28	0.59 ± 0.27	0.86 ± 0.49	0.86 ± 0.39
18:0	22.7 ± 4.32	22.0 ± 0.86	22.0 ± 0.80	25.1 ± 0.27	21.8 ± 0.87
18:1 n-9	3.97 ± 0.85	3.32 ± 0.65	3.20 ± 0.99	1.70 ± 0.22*	3.01 ± 0.30*
18:1 n-7	3.90 ± 0.33	3.44 ± 0.22*	3.53 ± 0.31*	2.57 ± 0.16*	3.66 ± 0.33
18:2 n-6	22.5 ± 3.23	16.5 ± 1.51*	19.4 ± 2.00*	17.6 ± 0.47*	23.8 ± 1.32
18:3 n-3	0.12 ± 0.06	0.12 ± 0.03	0.10 ± 0.03	0.05 ± 0.02*	0.10 ± 0.02
20:4 n-6	18.2 ± 2.32	21.5 ± 1.18*	21.1 ± 1.57*	21.6 ± 0.41*	18.3 ± 0.07
22:3	0.46 ± 0.08	0.46 ± 0.05	0.46 ± 0.05	0.47 ± 0.07	0.47 ± 0.06
22:4 n-6	0.71 ± 0.12	0.76 ± 1.00	0.71 ± 0.04	0.73 ± 0.05	0.67 ± 0.07
22:5 n-3	1.31 ± 0.11	1.62 ± 0.18*	1.43 ± 0.10*	1.48 ± 0.13*	1.85 ± 0.10*
22:6 n-3	11.0 ± 2.35	15.9 ± 1.09*	14.2 ± 1.86*	15.9 ± 0.24*	11.0 ± 1.15
Others	0.42	0.46	0.42	0.28	0.32
Total n-6	41.4	38.8	41.2	39.9	42.8
Total n-3	12.4	17.7	15.7	18.2	12.9
UI	204	237	229	236	210
			Total phospholipids (mg/g wet tissue)		
	27.4 ± 4.67	25.6 ± 3.79	28.8 ± 1.01	25.8 ± 1.25	28.6 ± 0.77

* Significantly different at $P < 0.01$ (Student's t-test, $N = 8$) from control. Values are means, or means ± SD.

Others, mainly long chain monounsaturated and polyunsaturated fatty acids. 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.

are quite exclusively esterified in phospholipids [33, 34].

Fatty acid composition of the heart total lipids is shown in Table 2. In general, most of the modifications induced by anthracyclines concerned the PUFA, except in the EPI group in which changes also concerned saturated and monounsaturated fatty acids. In treated groups, the levels of PUFA with more than two double-bonds were never lower than in the control group. In the case of 20:4 n-6 and 22:6 n-3, levels were actually increased by 16% and 36%, respectively. It is worth noting the contrasting changes in the levels of 20:4 n-6 and 18:2 n-6 induced by anthracyclines, the latter being significantly lowered (−20%) whereas 20:4 n-6 was increased by 17%. The sum of n-6 PUFA was not changed whereas that of n-3 PUFA was increased. MIT administration induced modifications in the n-9 monounsaturated species only. Except in the group treated with MIT, the unsaturation index was slightly increased in the drug-treated groups. GSH-PX and selenium-independent GSH-T activities were assayed to estimate the ability of heart to neutralize the hydroperoxides of PUFA under the various

Table 3. Activities of enzymes involved in phospholipid metabolism and detoxification of lipid hydroperoxides in the hearts of rats treated with saline or antitumoral drugs

	Phospholipase A	Lysophospholipase (nmol/hr/mg)	ACLAT	GSH-PX (nmol/min/mg)	GSH-T (μmol/min/mg)
Control	1.42 ± 0.37	10.16 ± 1.24	174 ± 14	312 ± 18	16.9 ± 2.42
DOX	1.43 ± 0.37	9.82 ± 1.27	199 ± 25*	355 ± 47*	17.5 ± 2.07
EPI	1.72 ± 0.34	9.74 ± 1.48	166 ± 21	307 ± 65	23.2 ± 3.53*
MIT	1.56 ± 0.37	10.13 ± 0.72	191 ± 16*	336 ± 41	17.0 ± 2.19
PIR	1.30 ± 0.38	11.63 ± 2.21	193 ± 15*	367 ± 50*	19.9 ± 3.94

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

treatments. As shown in Table 3, GSH-PX activity was either unchanged or slightly stimulated by drug. DOX and PIR significantly induced a slight stimulation of 14% and 17%, respectively. GSH-T activity was not significantly altered by drug except EPI which induced 37% stimulation (Table 3).

Phospholipid-metabolizing enzyme activities in the heart

The results are shown in Table 3. Phospholipase A and lysophospholipase activities were not significantly altered by any of the antitumoral drugs administrated. Modest but significantly higher activities were observed with ACLAT in the case of DOX (+14%), MIT (+10%) and PIR (+11%).

Ultrastructure of the liver

In the liver, only EPI and DOX induced lesions, which, in both cases, were in foci neighboring entirely normal parenchyma (data not shown). After DOX treatment there was, in the foci, an invasion of parenchyma by collagen bundles. Hepatocytes were filled with numerous lipid droplets and the mitochondria were less abundant and showed shorter crests. Endoplasmic reticulum appeared as dilated tubules and cisternae. Bile canaliculi were absent between the cells, and the membranes facing the Disse space possessed some digitations. After EPI treatment, collagen and lipid droplets in the foci were less marked. The endoplasmic reticulum was dilated and autophagic vacuoles were numerous. However, the bile canaliculi were maintained, but apical microvilli were lost.

Lipid composition and lipid peroxidation status in the liver

As shown in Table 4, liver lipid phosphorus was not significantly altered by antitumoral treatment. The same parameters of membrane peroxidative status as those for the heart were studied. The fatty acid composition is given in Table 4 and shows a quite different effect of the drugs when compared with that of the heart. In the liver, the modifications were mainly to the saturated and monosaturated species: stearic acid was increased by 20% and oleic and palmitoleic acids were lowered by 30% and 50%, respectively. Palmitoleic acid was not affected by MIT treatment. PUFA were not affected by the drugs indicating that their levels were maintained in liver membranes since about 85% of total 20:4 n-6 and 90% of total 22:6 n-3 are esterified in liver phospholipids [35]. The unsaturation index did not change under antitumoral treatment. The activity of GSH-PX was not significantly altered except in the group treated with EPI where the decrease attained 25%. GSH-T activities were slightly but significantly lowered (18–20%) by DOX, MIT and PIR, and the decrease attained 26% with EPI.

Phospholipid-metabolizing enzyme activities in the liver

Phospholipase A and lysophospholipase activities were not affected by the various antitumoral drugs. The ACLAT activity remained quite stable except in the PIR group where it was increased by 24% (Table 5).

DISCUSSION

Although the hypothesis of lipid peroxidation has probably been the most extensively investigated to understand the cardiotoxicity of anthracyclines, a satisfactory explanation has not been provided because contradictory reports are numerous. That anthracyclines behave *in vivo* and *in vitro* as a free radical generator system leading to membrane injury appears uncontestable [4–6, 36–38], but that the anthracycline-induced peroxidation of membranes is itself sufficient to account for the pathophysiological effects is still debatable [6]. Indeed, it should be pointed out that most of the studies establishing a relationship between lipid peroxidation and membrane dysfunction have been performed with isolated subcellular fractions or artificial membranes. Such models do not allow the membranes to be efficiently restored, in contrast to the *in vivo* situation in which free radical-damaged membranes should be rapidly repaired through the deacylation–reacylation process. So, any *in vitro* functional alteration to membranes due to antitumoral-induced lipid peroxidation is of physiological relevance if one can demonstrate that the repair process has also been injured. This was the reason which motivated us to determine whether the enzymes involved in the deacylation–reacylation process could overcome the antitumoral-induced peroxidative stress affecting cardiac membranes. We consequently focused our work on the effect of acute administration of antitumoral drugs on the enzyme activities involved in the turnover of membrane phospholipids and those

Table 4. Total phospholipid content and fatty acid composition of total lipids in the livers of rats treated with saline or antitumoral drugs

	Control	PIR	DOX	EPI	MIT
			Fatty acids (weight %)		
16:0	19.7 ± 1.40	17.9 ± 1.19*	19.3 ± 1.42	20.1 ± 1.16	17.3 ± 1.40*
16:1	1.10 ± 0.60	0.53 ± 0.37*	0.59 ± 0.31*	0.42 ± 0.40*	1.11 ± 0.70
18:0	20.5 ± 1.17	24.9 ± 0.58*	23.6 ± 2.34*	24.6 ± 1.26*	24.4 ± 1.19*
18:1	7.07 ± 1.02	4.69 ± 0.31*	5.24 ± 1.35*	4.36 ± 0.80*	5.48 ± 0.72*
18:2 n-6	17.0 ± 1.00	16.5 ± 1.42	17.6 ± 1.70	17.4 ± 1.41	17.1 ± 0.84
18:3 n-3	0.23 ± 0.06	0.14 ± 0.02*	0.15 ± 0.06*	0.11 ± 0.04*	0.18 ± 0.08
20:4 n-6	26.8 ± 0.94	28.3 ± 2.20	26.2 ± 1.95	25.4 ± 1.45*	26.8 ± 1.34
22:4 n-6	0.32 ± 0.05	0.34 ± 0.02	0.36 ± 0.04	0.31 ± 0.03	0.31 ± 0.03
22:5 n-3	0.58 ± 0.09	0.53 ± 0.06	0.57 ± 0.10	0.54 ± 0.06	0.58 ± 0.07
22:6 n-3	5.98 ± 1.12	5.54 ± 0.27	5.90 ± 0.60	6.31 ± 0.66	6.22 ± 0.71
Others	0.68	0.70	0.52	0.37	0.41
Total n-6	44.1	45.1	44.2	43.1	44.2
Total n-3	6.21	5.68	6.05	6.42	6.40
UI	189	187	184	181	188
			Total phospholipids (mg/g wet tissue)		
	30.1 ± 1.44	28.7 ± 3.55	29.8 ± 3.68	31.3 ± 1.2	30.6 ± 2.13

* Significantly different at $P < 0.01$ (Student's t-test, $N = 8$) from control.
Others, essentially long chain monounsaturated and polyunsaturated fatty acids. UI, as in Table 2. Values are means, or means ± SD.

Table 5. Activities of enzymes involved in phospholipid metabolism and detoxification of lipid hydroperoxides in the livers of rats treated with saline or antitumoral drugs

	Phospholipase A	Lysophospholipase (nmol/hr/mg)	ACLAT	GSH-PX (nmol/min/mg)	GSH-T (μmol/min/mg)
Control	12.7 ± 1.7	25.5 ± 2.7	470 ± 45	842 ± 71	441 ± 44
DOX	10.5 ± 2.4	24.7 ± 2.7	514 ± 52	823 ± 81	348 ± 35*
EPI	9.90 ± 0.8	22.8 ± 2.8	547 ± 60	647 ± 77*	326 ± 37*
MIT	13.5 ± 2.7	23.2 ± 2.9	492 ± 60	794 ± 177	364 ± 38*
PIR	10.3 ± 1.5	24.6 ± 2.0	583 ± 70*	856 ± 73	371 ± 30*

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

involved in the detoxifiation of lipid hydroperoxides. The stability of membrane PUFA was investigated by the analysis of the composition of phospholipid fatty acids.

Under our experimental conditions, the acute administration of antitumoral drugs induced modest alterations in the ultrastructure of the heart which are in good agreement with those described by Doroshow [39]. In the liver, structural alterations were restricted to some foci of hepatic parenchyma in the case of EPI and DOX treatments only. These structural alterations seen in the liver are consistent with the fact that its function was somewhat modified as judged by the decrease in protein synthesis, and the alteration in circulating lipid metabolism. However, since plasma alkaline phosphatase was not increased, hepatic injury remained within acceptable limits. Taken as a whole, these observations allowed us to assume that the doses administrated were moderately subtoxic.

All the antitumoral drugs tested resulted in a higher level of circulating peroxides, even MIT which should not generate free radicals [40], although contrasting results have been reported [41]. This higher level of peroxides cannot be explained by a depletion of tocopherol, since its plasma concentration was paradoxically increased. A possible explanation is that the peroxides measured in the plasma did not arise from the plasma itself but originated in the tissues. Thayer [42] demonstrated that in rats chronically treated with DOX the tissue levels of TBARS, including the heart, was not enhanced by drug treatment. However, as higher levels of lipid peroxides were detected in the plasma, the author suggested that these peroxides were cleaved from membranes, released into the plasma and transported by lipoproteins. Our present observations support and extend this hypothesis for the following reasons. Firstly, the fatty acid composition of the heart and liver shows that the levels of highly peroxidizable PUFA, mainly 20:4 n-6 and 22:6 n-3, as well as the unsaturation index were not decreased in the membranes after antitumoral treatment; they were

even slightly increased in the heart. Secondly, it had been demonstrated *in vitro* with heart membranes that radical-induced peroxidation may cause the formation of lipid–protein adducts, which renders the lipids inextractable with lipid solvents [43]. In this study [43], the levels of 20:4 n-6 and 22:6 n-3 in the extracted lipids were significantly reduced. In our study, the recoveries of phospholipids in the lipid extracts were not different between control and drug-treated groups, confirming that the physiochemical state of the membranes was conserved. Thirdly, enzymes of membrane phospholipid metabolism were not inactivated by drug since our results show that both phospholipase A and ACLAT of the heart and liver were not inactivated by any of the drugs administrated. Moreover, the efficiency with which phospholipase A cleaves peroxidized phospholipids does not apparently require activation of the enzyme since peroxidized phospholipids are preferred substrates [15]. Accordingly, Ogawa *et al.* [17] described a 65% increase in the liberation of linoleic acid from an *in vitro* mixture of exogenous dilinoleoyl phosphatidylcholine and endogenous phospholipids (rich in linoleic acid) of the heart mitochondria of rats receiving doxorubicin for 4 consecutive days. Since, in this study [17], the pool of endogenous phospholipids of the mitochondria is important, it is likely that these authors measured a susceptibility of endogenous phospholipids to hydrolysis rather than a true phospholipase A activation.

The slight stimulation of heart ACLAT observed with some drugs may indicate that, following acute administration, there is an early activation of the repair process. In addition, the slight stimulation of or at least the lack of change in the activity of GSH-PX and GSH-T in the heart confirmed that, in this organ, the multi-enymatic complex represented by phospholipase A, ACLAT and glutathione peroxidases was fully functional in eliminating lipid hydroperoxides and rebuilding the membrane. Also, the direct elimination of lysophospholipids was maintained as judged by the stability of lysophospholipase activity. However, comparing the specific activity of lysophospholipase with ACLAT, the contribution of the latter in eliminating lysocompounds is far more important.

In the liver, the hydroperoxide detoxification process seems to be somewhat affected. However, the modest decrease in GSH-T activity induced by all the drugs probably did not affect this process since GSH-PX activity was maintained at the same level.

Two specific points deserve comment. First, the contrasting changes in linoleic and arachidonic acids in the heart after anthracycline administration strongly suggest that the process of elongation and desaturation is somewhat stimulated by/or in response to the drug. The same process may operate for n-3 PUFA since 22:6 n-3 was significantly increased in the drug-treated groups. It has been reported that long-chain PUFA inhibit Δ6 desaturation [44]. So, during a peroxidative stress an enhanced turnover of membrane PUFA may result in a partial removal of this inhibitory effect. The second point is the drastic diminution in circulating vitamin A, independent of the nature of the drug administrated. Besides the problems of growth and xerophthalmia that are related to vitamin A deficiency, an inverse relationship between plasma vitamin A and the risk of cancer has been proposed [45]. Vitamin A deficiency, if it is confirmed during long-term chemotherapy, should be considered as an additional deleterious side-effect during anticancer therapy. This is illustrated by the beneficial effect of a combination of vitamins A and E on myocardial damage in rabbits treated with doxorubicin [46].

In the present study, we provide evidence that under our experimental conditions, acute administration of anthracyclines and MIT at mild subtoxic doses does not affect the efficiency of the heart and liver to restore their membranes when injured by drug-induced free radicals. Our results may contribute to clarify the paradoxical reports on the relationships between drug-induced lipid peroxidation and cardiac dysfunctions [6]. These dysfunctions could appear as soon as the emergency repair process, when overwhelmed by peroxidative stress, is not able to restore injured membranes, leading to partial or total inactivation of membrane-bound proteins.

To conclude, the measurement of the efficiency of the repair process during chemotherapy, especially during long-term administration, appears to be an additional useful parameter to evaluate objectively the effects of antitumoral drugs.

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