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DOXORUBICIN INDUCED ALTERATIONS IN LIPID METABOLISM OF CULTURED MYOCARDIAL CELLS

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Abstract—Doxorubicin (DX) was found to inhibit the incorporation of [1-^{14}C]linoleic acid and [1(3)-^{3}H]glycerol into the major membrane phosphoglycerides, phosphatidylcholine and phosphatidylethanolamine of cultured myocardial cells in a dose-dependent manner (0.16–16 μM). It is suggested that DX affects *de novo* biosynthesis of these lipids. In contrast, DX-treatment of the cells stimulated incorporation of [1-^{14}C]linoleic acid into triacylglycerol. The effects of DX on lipid metabolism were only demonstrable 20–24 hr after a 1 hr exposure of the cells to the drug indicating that DX exerts little or no direct effect on the enzymes participating in lipid synthesis and that the alterations in lipid metabolism induced by DX probably are secondary to inhibition of protein synthesis and progressive cell injury. Extensive peroxidative decomposition of membrane lipids appeared not to take place in the DX-treated cells as judged from fatty acid analysis of total membrane phosphoglyceride.

The anthracycline antibiotic doxorubicin (adriamycin) (DX)‡ has a significant antitumor activity against a wide range of human malignant neoplasms [1]. Besides the common side effects of cancer chemotherapeutics the clinical usefulness of DX is limited by a cumulative dose-related cardiomyopathy, that manifests as congestive heart failure [2, 3]. The cardiotoxicity associated with the use of DX has long been recognized and much effort has been devoted to unravel the pathogenetic mechanisms underlying DX-induced cardiotoxicity. However, the mechanism of action of DX has not been clarified.

DX interacts with nucleic acids in DNA with subsequent inhibition of DNA, RNA and protein synthesis in its target cells [4–6]. This effect has been implicated as the primary event leading to DX-toxicity. Evidence that interaction of DX with cell membranes could contribute to the development of DX-induced cardiotoxicity originates from studies in which DX has been demonstrated (1) to bind to membrane lipids [7–10], (2) to induce peroxidative decomposition of membrane lipids in the presence of trace concentrations of iron salts [11–13], (3) to alter membrane fluidity [14–16], (4) to have a cytotoxic effect apparently without entering the cell [17], and (5) to inhibit membrane-bound enzymes [18–20]. Membrane-bound enzymes are involved in the synthesis and degradation of membrane lipids [21, 22]. The present study investigates the possibility that DX affects the lipid metabolism of myocardial cells.

‡ Abbreviations: DX, doxorubicin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; Sph, sphingomyelin; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin; TG, triacylglycerol; DG, diacylglycerol; FFA, free fatty acid. 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 20:4, arachidonic acid; 22:5, docosapentaenoic acid; 22:6, docosahexaenoic acid.

MATERIALS AND METHODS

Materials. Hank's calcium- and magnesium-free balanced salt solution and culture medium Medium 199 were obtained from Gibco Bio Cult. Crude collagenase was from Boehringer (Mannheim, F.R.G.), [1-^{14}C]linoleic acid (58 Ci/mole) and [1(3)-^{3}H]glycerol (2500 Ci/mole) from Amersham International (Amersham, U.K.). Crystalline bovine serum albumin and linoleic acid were from Sigma Chemical Co. (St. Louis, U.S.A.). Organic solvents of analytical grade were distilled before use. Doxorubicin was kindly supplied by Farmitalia Carlo Erba (Milan, Italy).

Cell cultures. Myocardial cells were prepared from hearts of neonatal Wistar rats (0–2 days old) by step-wise enzyme digestion according to Harary and Farley [23] with minor modifications. Heart ventricles were isolated and the tissue cut into small pieces and washed twice with Hank's salt solution containing 10 mM Hepes (pH 7.3), penicillin (75 U/ml) and streptomycin (75 μg/ml). The tissue was then placed in Hank's salt solution containing 0.05% collagenase and gently agitated for five cycles of 15 min each. The harvest of the first cycle was discarded to avoid mesenchymal cells and debris. The following harvests containing myocardial cells were combined and centrifuged at 1000 rpm for 5 min at 4°. After resuspension in Hank's solution another centrifugation was made.

The cell pellets were resuspended in culture medium and transferred to a Petri dish (Nunclon, Nunc, Denmark) for cell cultures for 5 min. The culture medium was Medium 199 with Hank's salts,

25 mM Hepes (pH 7.3), 10% heat-inactivated horse serum, penicillin (75 U/ml) and streptomycin (75 μg/ml). Advantage was taken from the faster rate of attachment to the dish of fibroblasts and endothelial cells than of muscle cells. The cells remaining in suspension were removed, counted in a haemacytometer after trypan blue staining and plated in multi-dishes (Nunclon, Nunc, Denmark) with 0.26 mill cells/cm^2 and 0.5 mill cells/ml culture medium. The cultures were kept in a water-saturated (90%) incubator at 37° (Hotpack, model 351820, Philadelphia, PA, USA). All dissections and preparation procedures were carried out in a laminar-flow hood at 37°.

Doxorubicin treatment. All experiments were carried out with cells cultured for a 68-hr period. The purity of these cultures with regard to muscle cells was about 80% as evaluated by PAS-staining [24].

Treatment of cells with DX (0.16, 1.6 or 16 μM) was carried out in culture medium for 1 hr at 37°. The DX-containing medium was then removed and the cells washed twice with DX-free culture medium. The cells were hereafter reincubated in DX-free medium. Untreated controls from each cell culture were run parallel in a similar way.

Labelling of cell lipids. Labelling of lipids in control and DX-treated cells was carried out by incubation of the cells at 37° in culture medium containing either 0.25 μCi [1-^{14}C]linoleic acid (58 Ci/mole) (added as the serum albumin complex) or 12.5 μCi [1(3)-^{3}H]glycerol (2500 Ci/mole) per ml culture medium. The radioactive medium was aspirated at the end of the incubation period and the cells were washed rapidly three times with 0.9% NaCl solution (4°). Thereafter, the cells were detached from the multi-dishes by adding 500 μl 0.5% sodium deoxycholate in water and transferred to a lipid extraction mixture [25] composed of 2.5 ml methanol and 1 ml chloroform containing 50 μg 2,6-di-*tert*-butyl-*p*-cresol as an anti-oxidant and 200 μg total phospholipid extracted from pig heart as a cold carrier. The extracts were stored at −20° for less than 3 days before further processing.

Cells for chase experiments were treated with 16 μM DX for 1 hr, reincubated in DX-free culture medium for 20 hr and prelabelled with [1-^{14}C]linoleic acid for a 4-hr period as described above. The labelled cells were washed twice with culture medium and chased with non-labelled linoleic acid (6 μM) in culture medium for 4, 7.5 or 10 hr. Cell lipids were extracted as above.

Lipid analysis. The chloroform phases obtained by adding 1 ml chloroform and 2.5 ml water to the lipid extraction mixtures were removed and evaporated to dryness under nitrogen. The lipid residue was redissolved in 300 μl chloroform and 100 μl aliquots were subjected to TLC on 0.25 mm silica gel plates (Merck, Darmstadt, FRG.). Phosphoglycerides were separated by means of two-dimensional TLC with the solvents chloroform/methanol/conc. NH_3 (74:36:5, v/v/v) and chloroform/methanol/acetic acid (46:15:6, v/v/v). TG, DG and FFA were separated using the solvent diethylether/petrolether/acetic acid (70:35:0.5, v/v/v).

Lipids were visualized by I_2-vapour and scraped off the plates into 5 ml scintillation mixture prepared by mixing 5.5 g PPO, 0.1 g dimethyl-POPOP, 333 ml Triton X-100 and 667 ml xylol. Radioactivity was determined in a Nuclear Chicago scintillation counter.

Cell lipids for quantitation of total lipid phosphorus and fatty acid analysis were extracted and separated by TLC as described above but without addition of carrier lipid. Lipid phosphorus was determined by the method of Bartlett [26]. Fatty acid methyl esters were prepared using the method of Mason and Waller [27] and separated by GLC using a 2 m × 3 mm column containing 10% SP 2330 on Chromosorb W,AW, 100/120 mesh (Supelco). Samples were injected at 150° and the temperature was increased at a rate of 3°/min up to 220°. Separation of fatty acid methyl esters by TLC was carried out using $AgNO_3$–silica gel plates prepared as described [28] and with the solvent *n*-hexane/diethyl ether (40:60, v/v).

Preparation of linoleic acid–albumin complex. Complexation of linoleic acid to bovine serum albumin was carried out as follows: linoleic acid in benzene (0.5 mg/ml) was evaporated to dryness under nitrogen and fatty acid free bovine serum albumin in water (4.5 mg/ml) was added to give a molar ratio of linoleic acid to albumin of 5:1. The mixture was incubated at 37° for 4 hr and stored over night at 4° before use.

Other methods. Fatty acid free serum albumin was prepared by treatment of crystalline bovine serum albumin with charcoal [29].

Cell protein was determined on cells dissolved in 500 μl 0.5 M NaOH using the method of Lowry *et al.* [30]. Serum albumin was used as a standard.

Statistical analysis was performed by Student's *t*-test.

RESULTS

The time course for incorporation of the lipid precursors [1-^{14}C]linoleic acid and [1(3)-^{3}H]glycerol into the major membrane phosphoglycerides phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL), and into triacylglycerol (TG) of cultured myocardial cells is shown in Fig. 1. The rate of incorporation of radioactivity into PC, PE and TG decreased with time during the 4-hr labelling period. Incorporation of [^{14}C]linoleic acid into CL took place with an initial lag period of 1–1.5 hr. More than 90% of the radioactivity associated with the lipids after incubation with either [^{14}C]linoleic acid or [^{3}H]glycerol was contained in the esterified linoleic acid or the glycerol moity of the cell lipids respectively (data not shown). A 4-hr pulse period was chosen in all subsequent experiments examining the effects of DX in order to obtain adequate incorporation of radioactivity especially into CL.

Figure 2 shows the radioactivity incorporated into lipids of myocardial cells pretreated with DX (16 μM for 1 hr) 20 hr before the incubation with either [^{14}C]linoleic acid or [^{3}H]glycerol, and of untreated control cells. A significantly reduced incorporation of both [^{14}C]linoleic acid and [^{3}H]glycerol into PC and PE was demonstrated in the DX-treated cells ($P < 0.001$

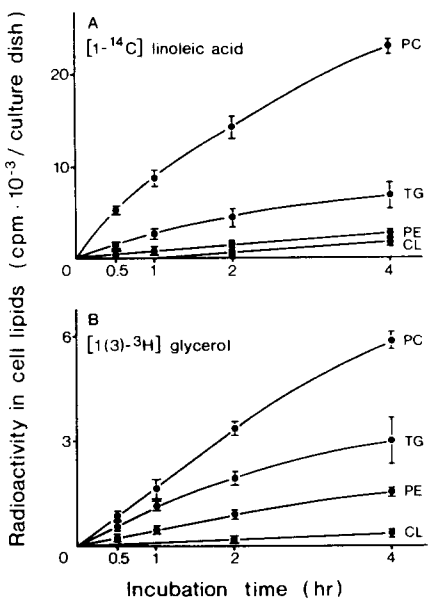

Fig. 1. Time course for the incorporation of [1-^{14}C]linoleic acid (A) and [1(3)-^{3}H]glycerol (B) into glycerolipids of myocardial cell cultures. Experimental conditions as described under Materials and Methods. Results are expressed as mean and S.D. of four determinations from two independent experiments.

Table 1. Analysis of lipid phosphorus and major fatty acids in total phosphoglyceride of control and doxorubicin-treated myocardial cell cultures

	Control	DX-treated
Total lipid phosphorus	(nmoles/mg cell protein)	
	140.4 ± 23.0	125.7 ± 16.3
Fatty acids	(% of total)	
14:0	3.5 ± 1.0	3.9 ± 0.3
16:0	18.2 ± 3.3	17.8 ± 3.2
18:0	27.4 ± 1.5	28.2 ± 1.0
18:1	18.5 ± 0.5	17.0 ± 0.4
18:2	7.7 ± 0.4	6.7 ± 2.5
20:4	17.7 ± 3.0	17.9 ± 2.5
22:5	0.9 ± 0.2	0.8 ± 0.2
22:6	1.3 ± 0.3	1.2 ± 0.2

Lipid analysis was carried out 24 hr after treatment of the cultures with 16 μM doxorubicin for 1 hr. Experimental conditions and procedures for lipid analysis as described under Materials and Methods. Data are mean ± S.D. from 4–5 separate experiments.

and $P < 0.02$, respectively). A significant reduction of radioactivity in CL was demonstrated in DX-treated cells after incubation with [^{14}C]linoleic acid ($P < 0.05$), but not after incubation with [^{3}H]glycerol. In contrast, incorporation of [^{14}C]linoleic acid into TG was significantly enhanced ($P < 0.05$) in DX-treated cells.

A dose-dependent effect of DX on the incorporation of radioactivity into cell lipid was observed when the cells were pretreated for 1 hr with 0.16, 1.6 or 16 μM of DX 20 hr before incubation with either [^{14}C]linoleic acid or [^{3}H]glycerol. The data for PE are presented in Fig. 3.

Figure 4 shows the results from experiments in which the myocardial cells were incubated with [^{14}C]linoleic acid immediately following exposure to DX (16 μM for 1 hr) and 24 or 48 hr hereafter. The effects of DX on incorporation of [^{14}C]linoleic acid into PC, PE, CL and TG developed with time during the reincubation of the DX-treated cells in drug-free medium. Immediately after the DX-treatment there was no significant effect of DX on the incorporation of [^{14}C]linoleic acid into PC, PE and CL whereas

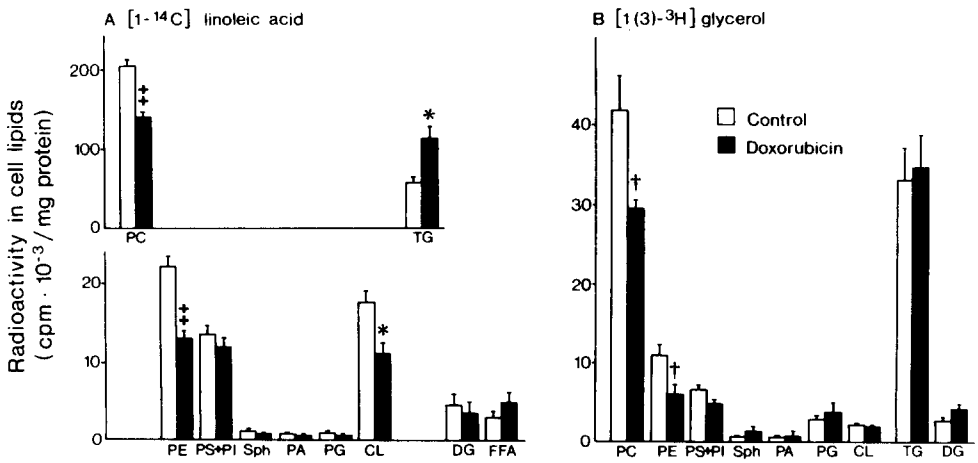

Fig. 2. Incorporation of [1-^{14}C]linoleic acid (A) and [1(3)-^{3}H]glycerol (B) into lipids in control (□) and doxorubicin-treated (■) myocardial cell cultures. Cultures treated with 16 μM doxorubicin for 1 hr and untreated control cultures were labelled for a 4-hr period (20 hr after the doxorubicin treatment) as described under Materials and Methods. Data are presented as mean and S.E.M. from two to eight separate experiments each performed in duplicate. Statistically significant difference from the control: *$P < 0.05$ ($N = 8$), †$P < 0.02$ ($N = 8$), ‡$P < 0.001$ ($N = 8$).

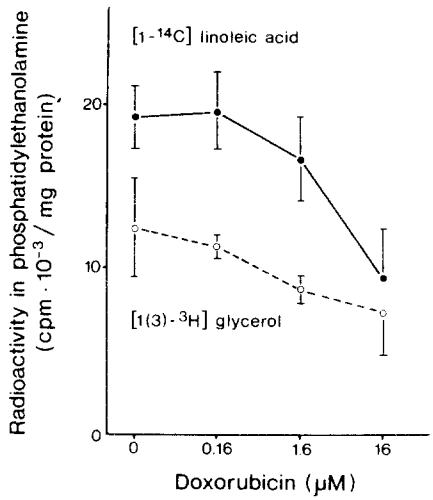

Fig. 3. Doxorubicin dose–response curves for incorporation of [1-^{14}C]linoleic acid (●——●) and [1(3)-^{3}H]glycerol (○- - -○) into phosphatidylethanolamine of myocardial cell cultures. Experimental conditions as described under Materials and Methods. Data are presented as the mean and S.E.M. from two to five separate experiments each performed in duplicate.

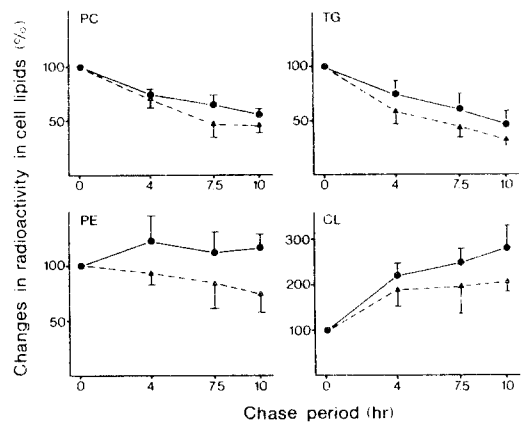

Fig. 5. Changes in [1-^{14}C]linoleic acid labelled lipids of myocardial cell cultures during chase incubation. Conditions as described under Materials and Methods. Controls ●——●, doxorubicin-treated △——△. Results are presented as mean and S. D. (% of initial) from five separate experiments each performed in duplicate.

incorporation of [^{14}C]linoleic acid into TG appeared to be significantly reduced ($P < 0.01$).

Pulse-chase experiments were carried out in which cells treated with 16 μM DX for 1 hr and untreated control cells were labelled with [^{14}C]linoleic acid 20 hr after DX-treatment and then chased in medium containing unlabelled linoleic acid. The results are shown in Fig. 5. Linoleic acid in PC and TG showed a relative high rate of turnover (half-time about 10 hr) whereas the labelling of PE did not change significantly during the 10 hr chase-incubation. Incorporation of [^{14}C]linoleic acid into CL continued during the chase-incubation although the cells contained little labelled free fatty acid (cf. Fig. 2). Turnover of linoleic acid in the lipids was not significantly enhanced in DX-treated cells.

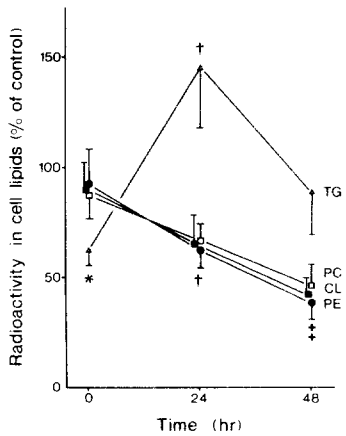

Fig. 4. Incorporation of [1-^{14}C]linoleic acid into lipids of myocardial cell cultures at different times after doxorubicin treatment. Cultures treated with 16 μM doxorubicin for 1 hr were labelled for a 4 hr period immediately after doxorubicin-treatment (0 hr) and 24 hr or 48 hr hereafter. [^{14}C]Linoleic acid incorporated into lipid per mg cell protein is presented as a percentage of control cells. TG (△); PC (□); CL (■); PE (●). Data are mean and S.D. from three to five separate experiments each performed in duplicate. Statistically significant difference from the control: *$P < 0.01$ (TG, N = 3); †$P < 0.05$ (TG, N = 5), $P < 0.001$ (PC and PE, N = 5), $P < 0.05$ (CL, N = 5); ‡$P < 0.02$ (PC and PE, N = 3), $P < 0.05$ (CL, N = 3).

Quantitation of lipid phosphorus and analysis of fatty acids in phosphoglyceride of DX-treated and control cells was carried out. As can be seen from Table 1, no significant change in the ratio of total lipid phosphorus to total cell protein or in the fatty acid profile of the total phosphoglyceride could be demonstrated 24 hr after a 1 hr exposure to DX (16 μM) where phosphoglyceride synthesis was reduced to 60–70% of the control cells.

DISCUSSION

DX-induced morphological changes in membranes of sarcoplasmic reticulum and of mitochondria [31–35], together with an increase in osmophilic lipid containing vacuoles [5, 32, 36], in myocardial cells, has been described in electron microscopic studies, suggesting DX-induced alterations in lipid metabolism. In the present study it is demonstrated that exposure of cultured myocardial cells to DX causes inhibition of incorporation of both [^{14}C]linoleic acid and [^{3}H]glycerol into the major membrane phosphoglycerides PC and PE. Incorporation of both lipid precursors was inhibited by DX to about the same extent (Fig. 2) and inhibition was characterized by similar dose–response curves (Fig. 3). Furthermore, no significant enhancement in the rate of linoleic acid turnover in lipids of DX-treated cells could be demonstrated (Fig. 5). We therefore suggest that DX-treatment of the myocardial cells gives rise to inhibition of *de novo* biosynthesis of PC and PE.

CL is localized exclusively in the inner mitochondrial membranes [37] and is synthesized by mito-

chondrial enzymes [38]. Linoleic acid is a major fatty acid in CL [37, 39]. The pulse-chase experiments indicate that linoleic acid incorporated into CL to a great extent is provided through the turnover of other lipids in the myocardial cells. It is not possible to decide from the present data whether the reduced rate of labelling of CL in DX-treated cells by [^{14}C] linoleic acid is due to a lowering of the specific activity of these linoleic acid pools or to inhibition of linoleic acid incorporation into CL. No significant effect of DX on incorporation of [^{3}H]glycerol into CL could be demonstrated.

Accurate lipid analysis on cells cultured for more than 24 hr after exposure to 16 μM DX could not be carried out as the cells tended to detach from the culture-dishes. Considering that the half-time for phosphoglyceride-turnover probably is in the order of 2–5 days [39, 40] it could not be expected that significant alterations in overall lipid composition of the DX-treated cells (with only a partial inhibition of lipid synthesis) were obtainable during this short culture-period (Table 1). Extensive peroxidative decomposition of membrane lipids in the DX-treated cells did not take place as judged from the lipid-analysis data.

The finding that DX-induced inhibition of incorporation of lipid precursors into phosphoglycerides of the cells developed with a 20–40 hr delay indicate that the drug has little or no direct effect on the activity of the enzymes participating in membrane phosphoglyceride biosynthesis in myocardial cells. We suggest that the alterations in lipid metabolism observed in the cultured cells after exposure to DX are secondary to a general cytotoxic effect of the drug and to progressive impairment of cell functions. The myocardial cells showed clear morphological changes at the light microscope level and the beating frequence was reduced 24 hr after DX-treatment (16 μM for 1 hr), where phosphoglyceride synthesis was inhibited to 60–70% of the controls. The cells tended to detach from the culture dishes 48–72 hr after treatment. DX probably has multiple effects on the cells including: inhibition of protein synthesis [5], impairment of mitochondrial functions [41–43], depletion of ATP [44, 45] and enhancement of free radical damage to DNA, membrane lipids and enzyme activities [46]. Work is in progress to characterize the effect of DX on mitochondrial functions and energy production in the cells. Preliminary results (S. Christensen, K. Wassermann and E. Steiness, unpublished) indicate that DX causes a time-dependent inhibition of $^{14}CO_2$ production from cells incubated either with [^{14}C]lactate or [^{14}C]glutamate.

The data in Fig. 4 clearly indicate that DX-treatment of the myocardial cells redirects incorporation of [^{14}C]linoleic acid from the membrane phosphoglycerides and into TG. This effect of DX could be the direct consequence of the inhibition of phosphoglyceride synthesis, but may also reflect a decreased mitochondrial function. It is well known that inhibition of mitochondrially fatty acid oxidation in myocardial cells can give rise to accumulation of TG [47].

The present study does not address the question as to whether the DX-induced alterations in lipid metabolism are caused by a specific effect of DX in myocardial cells. However, it is well established that the functions of cell membranes and associated enzyme activities are influenced by the composition of the membrane lipid [48–50], and a disturbance of lipid metabolism in myocardial cells by DX may therefore contribute to the series of events which leads to irreversible cell injury. The effects of DX on membrane phosphoglyceride synthesis described may be of importance for the development of the special cardiotoxicity of the drug.

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