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Enzyme Induction by Daunorubicin in Neonatal Heart Cells in Culture

Dimitrios Galaris and Jan Rydström[1)]

Department of Biochemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm, Sweden

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The effect of the antineoplastic agent daunorubicin on beating heart cells from neonatal rats was investigated with respect to cell damage and induction of enzymes possibly involved in drug metabolism. Of the enzymes assayed DT-diaphorase and glutathione-S-transferase showed a two-to-four fold increase in activity: higher concentrations of daunorubicin inactivated glutathione-S-transferase. Daunorubicin toxicity increased in the presence of dicoumarol, a specific inhibitor of DT-diaphorase. These results indicate that both DT-diaphorase and glutathione-S-transferase may be involved in the metabolism of daunorubicin.

The anthracycline antibiotics are highly efficient antineoplastic agents, but their use is limited due to serious cardiotoxic side effects [1]. Although several different mechanisms for the anthracycline-mediated cardiotoxicity have been proposed [2-7] the most discussed one is that of semiquinone free radical formation by enzymatic single-electron reduction of anthracyclines [8]. These semiquinones can then undergo rapid autooxidation with the formation of superoxide radicals ($O_2^{\cdot-}$), which may initiate lipid peroxidation resulting in membrane damage and other types of injuries [9]. In the case of the heart, these types of injuries are magnified because of the limited capacity of the heart to detoxify reactive oxygen species [10].

It has been reported previously that DT-diaphorase constitutes a cellular device controlling the level of quinones by a direct reduction to relatively stable hydroquinones [11]. In several tissues a competing reac-

1) To whom correspondence should be addressed.

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tion in the removal of quinones is the one-electron reduction by e.g. NADPH-cytochrome c reductase, producing the $O_2^{\cdot-}$-generating semiquinones, which eventually also will be converted into hydroquinones (cf. ref. 12); all forms of quinones may be conjugated with e.g. glutathione. Under in vivo-like conditions it appears that quinone reduction in rat liver is predominantly catalyzed by the DT-diaphorase pathway [11].

In the present investigation the levels of quinone-metabolizing enzymes have been measured in cultured neonatal heart cells as model system. It is shown that both DT-diaphorase and glutathione-S-transferase are markedly induced by daunorubicin in a manner which suggests that they may regulate the cardiotoxicity of daunorubicin.

2. Materials and Methods

The method of isolating heart cells from neonatal rats and the subsequent establishment of primary cultures of these cells has been described previously [13]. Daunorubicin was included at the concentrations indicated in the minimum essential medium (MEM) prior to the daily medium exchange. 3-Methylcholanthrene, Sudan III (1-(p-phenylazo-phenylazo)-2-naphthol) and dicoumarol were added as DSMO solutions. When the treatment was completed the medium was discarded, the cells were washed twice with 1 ml of buffer containing 20 mM Tris-Cl (pH 7.2) and 0.25 M sucrose and finally scraped from the petri dish bottom, and collected in 1 ml of the same buffert. The cells were subsequently sonicated 2 x 30 sec in a batch sonicator, with cooling in ice between the sonications; homogenates were then used for assaying enzyme activities. Lactate dehydrogenase activity of cellhomogenates was assayed spectrophotometrically at 340 nm in the presence of 200 μM NADH and 0.4 mM pyruvate in a buffer containing 20 mM Tris-Cl (pH 7.3). DT-diaphorase was measured using cytochrome c as acceptor [14]. Glutathione-S-transferase was assayed with CDNB (chlorodinitrobenzene) as acceptor [15]. The activity of NADPH-cytochrome c reductase was measured by following the reduction of cytochrome c [16]. Reduction of 3-acetylpyridine adenine nucleotide by NADPH was used as a measure of nicotinamide nucleotide transhydrogenase activity [17]. Glutathione reductase was assayed as the oxidation of 200 μM NADPH in the presence of 2 mM glutathione disulfide [18]. Protein was determined by the method of Lowry et al [19]. 3-methylcholanthrene and other biochemicals were obtained from Sigma Chem. Co. (St. Louis, Mo., USA). Daunorubicin was a gift from the Department of Radiology, Huddinge University Hospital, (Huddinge, Sweden).

3. Results

As may be seen in Table 1, the specific activities of DT-diaphorase and glutathione-S-transferase in cultured neonatal rat heart cells were increased about 2-4-fold following treatment with 0.35 μM daunorubicin for

Table I

Specific activities of enzymes in daunorubicin treated heart cells in culture

The cells were treated with 0.35 μM daunorubicin as described in Methods. Treatment lasted for two days.

Activity	specific activity[1] (nmoles · min^{-1} · mg $prot^{-1}$)					
	control			treated		
DT-diaphorase	183 ± 25	(6)		655 ± 95	(6)	
Glutathione-S-transferase	82 ± 21	(7)		175 ± 40	(7)	
NADPH-cytochrome c red	26 ± 4	(7)		22 ± 1	(7)	
Glucose-6P-dehydrogenase	24 ± 3	(3)		22 ± 5	(4)	
Transhydrogenase	40 ± 10	(3)		33 ± 6	(3)	
Glutathione reductase	3.0 ± 0.6	(4)		4.5 ± 0.8	(4)	
Lactate dehydrogenase	1510 ± 160	(7)		1110 ± 180	(8)	

[1] number within parenthesis denote number of experiments

two days. It has previously been shown that neonatal heart cells stop beating after treatment for 2 days with daunorubicin at concentrations between 0.17 ml and 1.7 μM [20]. NADPH-cytochrome c reductase, glucose-6--phosphate dehydrogenase and nicotinamide nucleotide transhydrogenase were essentially unaffected whereas glutathione reductase and lactate dehydrogenase were slightly increased and decreased, respectively, by this treatment. Titration experiments indicated that maximal induction of DT-diaphorase (Fig. 1) and glutathione-S-transferase (Fig. 2) was obtained at a concentra-

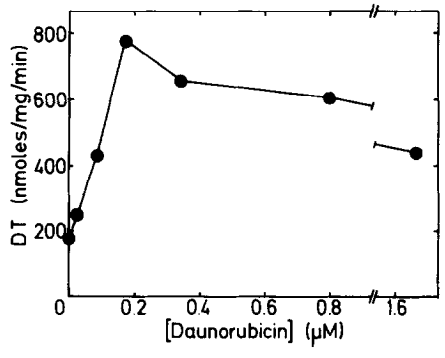

Fig. 1. Induction of DT-diaphorase (DT) by daunorubicin

The treatment by different concentrations of daunorubicin lasted for two days. Every point is the mean of three different experiments.

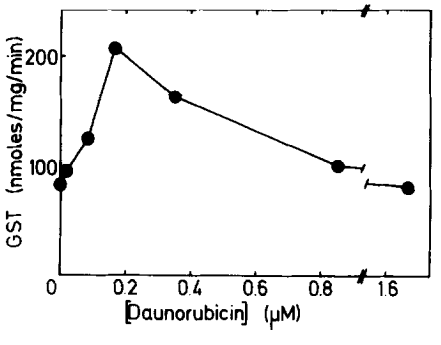

Fig. 2. Induction of glutathione-S-transferase (GST) by daunorubicin

Conditions were as in Figure 1.

Table II

Specific activities of DT-diaphorase and glutathione-S-transferase in homogenates from cultured heart cells treated with 3-methyl-cholanthrene, Sudan III and daunorubicin

The cells were treated with 30 μM 3-methylcholanthrene, 20 μM Sudan III and 0.35 μM daunorubicin as described in Methods. The treatment lasted for two days.

Treatment	specific activity[1] (nmoles · min^{-1} · mg $prot^{-1}$)	
	DT-diaphorase	Glutathione-S-transferase
None	139 ± 9 (3)	41 ± 16 (3)
DMSO	127 ± 5 (3)	44 ± 15 (3)
3-methylcholanthrene	323 ± 15 (3)	41 ± 9 (3)
Sudan III	425 ± 41 (3)	58 ± 5 (3)
Daunorubicin	512 ± 44 (3)	103 ± 11 (3)

[1] numbers within parenthesis denote number of experiments

tion of daunorubicin of about 0.17 μM. At higher concentrations of daunorubicin glutathione-S-transferase was rapidly inactivated. Treatment of cells with Sudan III or 3-methylcholanthrene resulted in a selective induction of DT-diaphorase (Table 2).

The induction of DT-diaphorase and glutathione-S-transferase by daunorubicin may indicate that these enzymes participate in the metabolism of daunorubicin. In the case of the former enzyme this possibility may be tested by the inclusion of dicoumarol, a specific inhibitor of DT-diaphorase

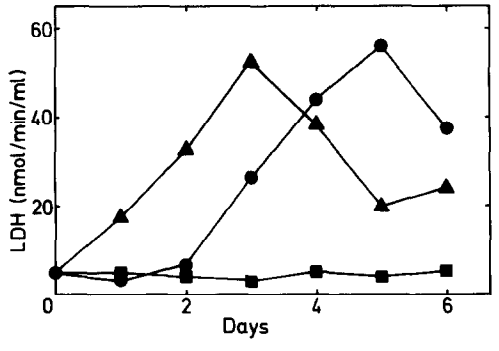

Fig. 3. Release of lactate dehydrogenase (LDH) from heart cells treated with daunorubicin and dicoumarol plus daunorubicin

Lactate dehydrogenase activity was measured spectrophotometrically at 340 nm as described in Methods. The concentration of daunorubicin was 0.35 μM and that of dicoumarol 1.1 mM. Every point is the mean of three different experiments. ●-●: daunorubicin-treated cells; ▲-▲: dicoumarol plus daunorubicin-treated cells; ■-■: control cells plus dicoumarol.

[21], in the culture medium. As shown in Fig. 3, release of cellular lactate dehydrogenase to the medium was estimated as a measure of cell injury, in the absence and in the presence of dicoumarol. In the presence of dicoumarol the cells were considerably more sensitive to daunorubicin indicating that DT-diaphorase indeed is involved in the removal of daunorubicin. Pretreatment of the cells with Sudan III, a known and potent inducer of DT-diaphorase [22], did not cause any further decrease in the sensitivity to daunorubicin as compared to the daunorubicin-treated control (not shown), suggesting that daunorubicin is a sufficiently potent inducer of DT-diaphorase in order to provide maximal protection under the conditions used.

4. Discussion

The present results indicate that administration of daunorubicin to cultured heart cells from neonatal rats markedly induce both DT-diaphorase and glutathione-S-transferase. Inhibition of DT-diaphorase by dicoumarol led to an increased toxic effect of daunorubicin, suggesting that DT-diaphorase indeed is involved in the metabolism of daunorubicin to the probably less toxic hydroquinone derivative [23]. Even though metabolites of daunorubicin have not been measured directly the work of others has implicated the formation of a reduced product [24]. This product, possibly a hydroquinone, may be formed by DT-diaphorase which is in agreement with the induction properties and proposed role of the enzyme in quinone metabolism [11]. The data obtained also indicate that maximal induction of DT-diaphorase is achieved at the low levels (0.35 μM) of daunorubicin used. This conclusion is further substantiated by the fact that pretreatment with Sudan III, a selective against daunorubicin. It is interesting to note that pretreatment with 3-methylcholanthrene also led to an induction of DT-diaphorase but not glutathione-S-transferase, suggesting that 3-methylcholanthrene or a metabolite of this compound (cf. ref. 25) is a selective inducer of DT-diaphorase.

The proposed role of glutathione-S-transferase is in agreement with the known importance of sulfhydryl compounds, e.g., glutathione, in decreasing

daunorubicin toxicity in vivo [26]. The inactivation of glutathione-S-transferase by higher concentrations of daunorubicin, demonstrated in the present investigation, emphasizes the possible role of glutathione-S-transferase in daunorubicin toxicity. In this context it should be pointed out that a glutathione-S-transferase, relatively specific for quinones, has been isolated [27]. It should be pointed out that definite evidence for the involvement of DT-diaphorase and glutathione-S-transferase in the metabolism of daunorubicin in heart cells requires identification of products. The use of the term induction also implies net synthesis of protein. Work is now in progress to answer these questions. Finally, the suggested roles and properties of DT-diaphorase and glutathione-S-transferase in daunorubicin metabolism indicate the future possibility of decreasing the cardiotoxic effect of anthracyclines by a selective induction of quinone-metabolizing enzymes prior to the administration of the drug.

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