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EFFECT OF DAUNORUBICIN ON SUBCELLULAR POOLS OF GLUTATHIONE IN CULTURED HEART CELLS FROM NEONATAL RATS

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Alterations in cellular GSH and its compartmentation were investigated as a possible mechanism of toxicity of the anthracycline derivative daunorubicin in neonatal heart cells. Cultured beating heart cells from neonatal rats were exposed to daunorubicin at therapeutically relevant concentrations and the resulting changes in cellular GSH as well as cytosolic and mitochondrial pools of GSH were determined. Toxicity was estimated as an increased permeability of the plasma membrane to cytosolic enzymes, e.g., lactate dehydrogenase.

Control heart cells were found to contain 12·2 ± 1·8 nmoles GSH/10^6 cells. Daunorubicin caused a rapid initial decrease followed by a transient increase in cellular GSH. The extent of the latter increase was dependent on the concentration of daunorubicin. High concentrations of daunorubicin gave only a slight increase followed by a pronounced decrease in cellular GSH.

By applying a digitonin-based method the effect of daunorubicin on the cytosolic and mitochondrial pools of GSH were separated. The concentration of cytosolic and mitochondrial reduced GSH was estimated to be 8·9 ± 1·5 nmoles/10^6 cells and 3·3 ± 0·6 nmoles/10^6 cells, respectively. The results indicate that daunorubicin caused a decrease of cytosolic GSH and, after a short lag period, a release of lactate dehydrogenase. No decrease of mitochondrial GSH occurred under these conditions indicating that daunorubicin influences selectively cytosolic GSH.

No lipid peroxidation products were detected in DRB-treated cells under conditions when lactate dehydrogenase was released. Likewise, addition of the iron-chelator desferrioxamin did not influence the release of lactate dehydrogenase, whereas dithiothreitol offered partial protection.

The results provide support for an oxidative mechanism in which the decrease in the cytosolic pool of GSH may be the causative factor of daunorubicin-induced toxicity. This decrease in GSH may affect the cytosolic NADPH and various redox groups on proteins, thereby altering the permeability of the plasma membrane and finally causing cell damage.

KEY WORDS: Daunorubicin, adriamycin, anthracycline, heart cells, GSH, GSSG, mitochondria.

ABBREVIATIONS: DRB, daunorubicin; ADR, adriamycin; TCA, trichloroacetic acid; GSH, reduced glutathione; GSSG, GSH disulfide; DTNB, 5,5′-dithiobis-C2-nitrobenzoic acid; LDH, lactate dehydrogenase; TBA, thiobarbituric acid; DEM, diethylmaleate; DTT, dithiothreitol; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; OPT, o-phtaldialdehyde.

INTRODUCTION

DRB is an anthracycline derivative and an antineoplasic agent which, like most other anthracyclines, is cardiotoxic.[1-3] Although several mechanisms have been proposed for the anthracycline cardiotoxicity [4-11] it is still essentially unknown. The current leading theory involves a one-electron reduction to a semiquinone followed by a subsequent autoxidation of this product and the formation of O_2^-.[4,5,12-17] The quinone

is thus functioning as a catalyst providing a continuous flow of electrons from reducing equivalents e.g., NADH and NADPH, via various flavoprotein dehydrogenases to molecular oxygen. The demonstration of a DRB-induced KCN-insensitive oxygen consumption in cultured heart cells,[18] as well as the recent demonstration that hyperoxia induced lipid peroxidation in ADR-treated heart cell cultures,[19] indeed provides support for a superoxide anion pathway *in vivo*. The $O_2^{\cdot-}$ may subsequently disproportionate to H_2O_2 either nonenzymatically or by a reaction catalyzed by superoxide dismutase,[20] Even though $O_2^{\cdot-}$ and H_2O_2 may lead to the generation of the highly reactive hydroxyl radical.[21] Moreover, H_2O_2 may also react directly with semiquinones producing hydroxyl radical or hydroxyl radical-like products.[22]

Due to the relatively low concentration of catalase in heart the enzyme primarely responsible for H_2O_2 inactivation in this tissue is GSH peroxidase.[17,23] This, together with the relatively low tissue concentration of reduced GSH [24, cf 25] tends to increase the susceptibility of heart to radical-generating drugs. The importance of the capacity for regeneration of reduced GSH in the protection of hepatocytes[26] and perfused liver[27] against ADR-mediated toxicity has been stressed in previous investigations. Using isolated hepatocytes and a recently described method to separate cytosolic and mitochondrial GSH[28] it was concluded that the toxic effects of ADR were preceded by a depletion of mitochondrial rather than cytosolic GSH.[29]

The present investigation concerns the role of GSH in DRB-induced toxicity in cultured heart cells from neonatal rats. It is shown that, in contrast to hepatocytes, DRB-induced toxic effects in neonatal heart cells are preceded by a decrease in cytosolic GSH with no apparent change in mitochondrial GSH. It is proposed that DRB acts through a direct lowering of the cytosolic GSH rather than through lipid peroxidation.

MATERIALS AND METHODS

Isolation of heart cells

The procedure for isolating beating heart cells from neonatal rats and the subsequent establishment of primary cultures of these cells have been described previously.[30] Plating of cells was achieved by transferring 1·5 ml of the cell suspension containing approximately $1{\cdot}5 \cdot 10^6$ cells/ml to a 35 × 10 mm Petri dish. After 24 hrs about $1 \cdot 10^6$ of the plated cells had developed into a confluent layer of beating myocytes as estimated visually. Growth medium was changed daily. After 3 days, when the cells were beating with a constant frequency of about 150 contractions per min, drug treatment was initiated.

DRB-treatment

DRB was dissolved directly in growth medium at the concentrations indicated. After 3 hrs the DRB-containing medium was replaced by normal medium without DRB. It has been shown previously that under these conditions the DRB added is taken up close to quantitatively.[18] The number of cells counted as described earlier[30] did not change significantly as a result of the treatment with DRB.

Determination of GSH

GSH was determined by two different methods, i.e., fluorimetrically[31] and enzymatically.[32] The two methods were routinely used in parallel and gave essentially the same results.

Separation of subcellular pools of GSH

Separation of mitochondrial and cytoplasmic pools of GSH was achieved by using digitonin as plasma membrane-permeabilizing agent. Digitonin dissolved in growth medium was added at various concentrations from a stock solution of 25 mg/ml; after the time indicated the medium was removed from the attached cells, and 0·5 ml was withdrawn for assay of cytosolic GSH as described above. Under these conditions cytosolic GSH was stable as indicated by the recovery of a known amount of GSH added with digitonin in a separate experiment. The remainder of the cells, including intact mitochondria, was scraped off in 0·5 ml 5% HPO_3 and mitochondrial GSH was determined. Marker enzymes for cytosol and mitochondria were LDH and citrate synthase, respectively.

Assays

LDH activity was estimated by following the decrease in absorbance at 340 nm using a medium compsed of 20 mM Tris-HCl (pH 7·3), 1 mM sodium puruvate and 0·1 mM NADH; the reaction was started by the addition of 100 μl sample. Citrate synthase was estimated as described[33] by following the absorbance change at 412 nm where the product of the reaction between DTNB and CoA has a strong absorption. Lipid peroxidation was determined as the TBA-reactive material formed in DRB-treated cells. The cells were first extracted with 10% TCA and the extract allowed to react with TBA after which the solution was extracted with butanol as described previously.[34]

Chemicals and miscellaneous

DRB (Cerubidin) was a gift from Ciba-Geigy (Basel, Switzerland). Growth medium and antibiotics were obtained from Gibco (Grand Island, N.Y., USA). Collagenase isolated from Clostridium histolyticum was obtained from Boehringer (Mannheim, FRG). All other biochemicals were obtained from Sigma Chem. Co. (St. Louis, Mo., USA).

RESULTS

The total amount of cellular GSH in cultured neonatal heart cells was 12.2 $\pm$ 1.8 (n = 3) and 12.4 $\pm$ 1.7 (n = 3) nmoles/10^6 cells as determined by the fluorimetric and enzymatic method, respectively. The content of GSH in the cells was influenced by the presence of daunorubicin. As shown in Figure 1, treatment of the cells with 1.7 μM DRB for 3 hours followed by a change to DRB-free medium gave an essentially unchanged or slightly increased GSH concentration after 20 hrs. However, in cells treated with 8.5 μM B the GSH concentration was slightly decreased initially

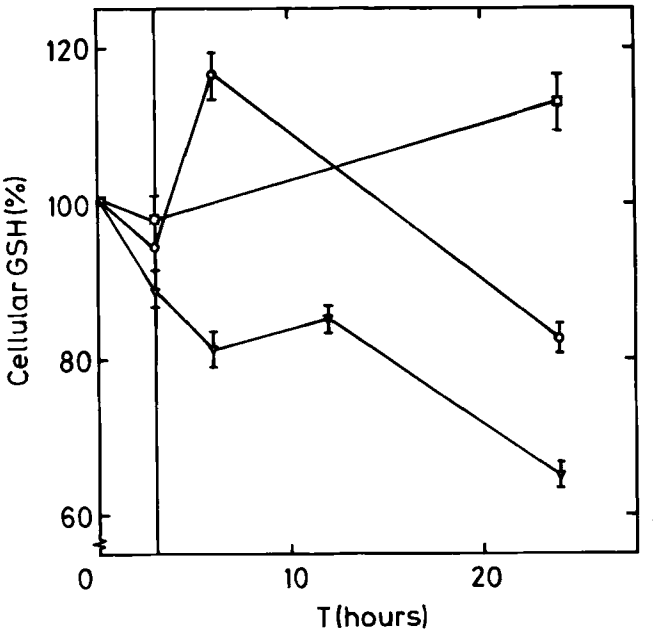

FIGURE 1 Measurement of GSH in DRB-treated heart cells in culture. Cells cultured for three days were treated with the indicated concentrations of DRB for three hours, as described in Methods. At the times indicated the culture medium was removed, the cells were scraped off the Petri dish and collected in 0·5% HPO_3; GSH was then determined as described in Methods. The initial concentration of GSH determined florimetrically, which corresponds to 100%, was 12·2 + 1·8 nmoles/10^6 cells. Additions were: □—□, (1·7 μM); ○—○, DRB (8·5 μM); ▽—▽, DRB (17 μM). Changes are expressed as % of the corresponding control in each point. The vertical line denotes the time when DRB-containing medium was exchanged for DRB-free medium. Every point represents the mean of three different plates ± S.D.

followed by an 18% increase relative to control after 10 hrs and finally a continuous decrease. Treatment with 17 μM DRB resulted in a more pronounced decrease with only a tendency for a recovery of GSH after 12 hrs. Essentially the same results (not shown) were obtained with the enzymatic method for GSH determination.[32] Both control and DRB-treated cells released substantial amounts of GSH, i.e. 0.7 ± 0.1 and 0.9 ± 0.2 nmoles/10^6 cells, respectively, to the surrounding medium (not shown). However, although this DRB-induced release was higher than in control cells, it was about 15-fold lower than the DRB-induced intracellular decrease in GSH. The transient variation in cellular GSH suggests that the cells resond to a DRB-induced oxidation and/or removal of GSH by increasing the net synthesis of GSH provided that the amount of DRB added is not too toxic. Indeed, exchange of DRB for a nontoxic amount of DEM, which relatively specifically reacts covalently with GSH,[35] also led to a transient decrease in GSH except that in this case the final GSH concentration was elevated 90% and kept constant (not shown).

Digitonin solubilization of the plasma membrane was used in order to separate the mitochondrial and cytoplasmic pools of GSH in the cultured heart cells. As shown in Figure 2 the bulk of cytosolic enzyme LDH was released to the surrounding growth medium after exposure of the cells to 200 μg digitonin/ml. Time of exposure to

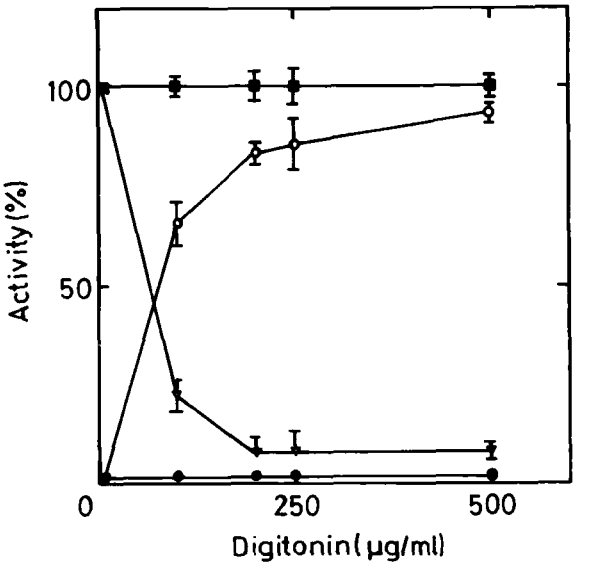

FIGURE 2 Release of cytoplasmic and mitochondrial marker enzymes after digitonin-treatment of cultured heart cells. The cells were treated with the indicated digitonin concentration for 15 minutes. LDH and citrate synthase activities were then determined both in the cells and in the surrounding medium. ▽—▽, LDH in the cells; ○—○, LDH in the surrounding medium; ■—■, citrate synthase in the cells; ●—●, citrate synthase in the surrounding medium. Each point represents the mean of three different plates ± S.D.

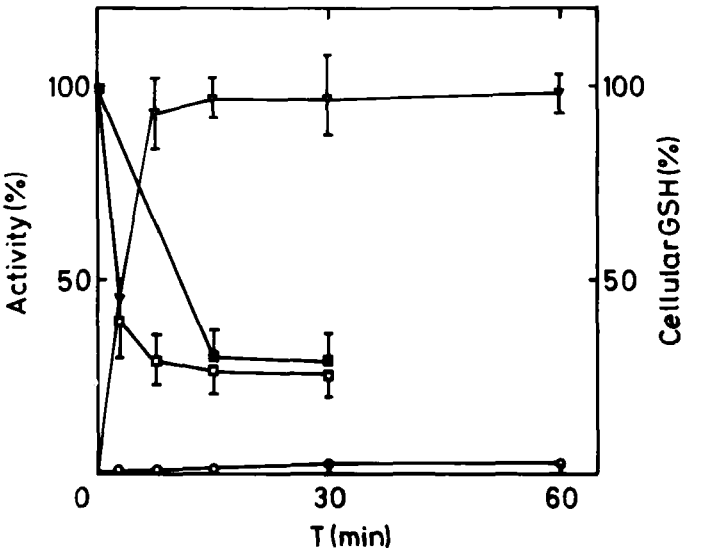

FIGURE 3 GSH release after digitonin-treatment of cultured heart cells. Conditions were essentially the same as in Fig.2 except that 250 μg/ml digitonin was used. ▽—▽, LDH released; ○—○, citrate synthase released; □—□ and ■—■, residual GSH as determined by the fluorimetric and enzymatic methods, respectively. Each point represents the mean of three different plates ± S.D.

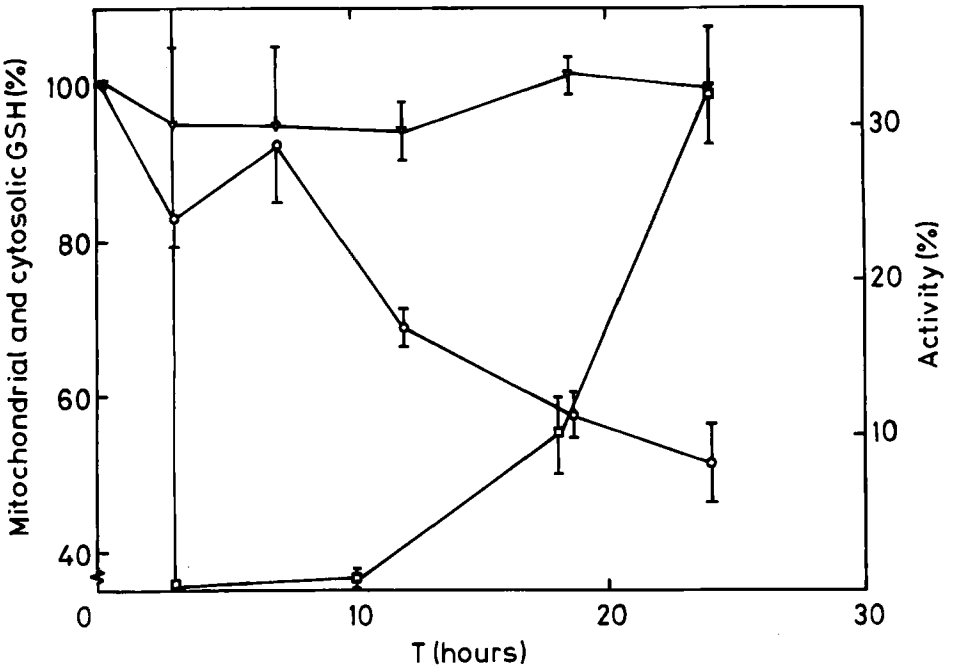

FIGURE 4 Subcellular GSH changes and LDH release in DRB-treated cells. Cell cultures were treated with DRB as described in fig.1 and at the times indicated the cells were analysed for mitochondrial and cytosolic GSH as in Fig.3 by the fluorimetric method. □—□, LDH released; ○—○, cytosolic GSH; ▽—▽, mitochondrial GSH. The vertical line denotes the time after which DRB-containing medium was exchanged for DRB-free medium. Each point represents the mean of three different plates ± S.D.

digitonin also influenced the release of LDH (cf. Figure 3); in the experiment shown in Figure 2 the cells were exposed to digitonin for 15 min. The decrease in cellular LDH and the appearance of medium LDH was close to quantitative although medium LDH, in contrast to remaining cellular LDH, had a slight tendency to increase at higher digitonin concentrations. Mitochondrial citrate synthase was essentially unaffected by digitonin even at higher concentrations (Figure 2) and could only be released by 1% Triton X-100. Total LDH and citrate synthase activities were 160 ± 11 and 32 ± 3 μmoles/min/10^6 cells, respectively (Figure 2). These results indicate that the plasma membrane can be made selectively permeable by digitonin in agreement with previous results obtained with hepatocytes,[28] suggesting that the method can be used to separate the mitochondrial and cytosolic pools of GSH in heart cells.

Indeed, treatment of heart cells with 250 μg digitonin/ml for 15 min led to a release of 73 ± 6% of the cellular GSH in parallel with LDH while 27 ± 6% of the cellular GSH as well as the citrate synthase activity remained in the cell fraction (Figure 3). These GSH values were obtained fluorimetrically. Determination of GSH enzymatically gave essentially the same results (Figure 3). Prolonged time of incubation with digitonin did not alter the distribution of GSH significantly. These results suggest that about one third of the total cellular GSH, i.e. 3.3 ± 0.6 nmoles/10^6 cells, is confined to the mitochondria and that 8.9 ± 1.5 nmoles/10^6 cells is confined to the cytosol.

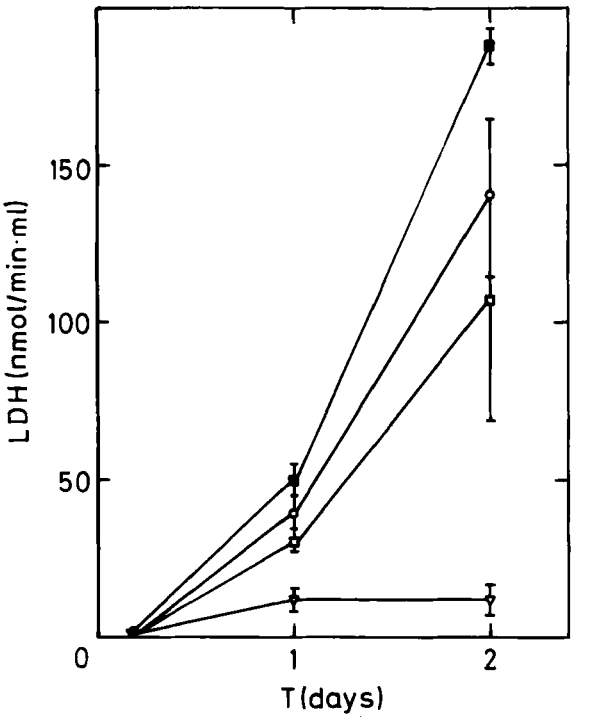

FIGURE 5 Effect of DTT on LDH release in DRB-treated heart cells. Cells were treated with DRB as described in Fig.1. DTT was added 24 hrs pior to DRB treatment and was present during the whole experiment. Additions were: ▽—▽, none; ■—■, 17 μM DRB; ○—○, 17 μM DRB plus 0·1 mM DTT; □—□, 17 μM DRB plus 0·7 mM DTT. Each point represents the mean of three different plates ± S.D.

By applying the digitonin method to DRB-treated heart cells it was possible to separate the effects of the drug on the mitochondrial and cytosolic pools of GSH. As shown in Figure 4, the DRB-induced and time-dependent release of LDH was accompanied by a marked decrease in cytosolic GSH whereas no significant effect on mitochondrial GSH was observed. The exposure of the cells to DRB apparently led to a rapid depletion of cytosolic but not mitochondrial GSH, which was followed by a brief recovery when DRB-free medium was introduced 3 hours later. However, after 7 hours the cytosolic GSH began to decline continuously and reached about 45 ± 6% of the original GSH level in 24 hours. As judged from the relative rates of release of LDH and decrease in cytosolic GSH the latter preceded the former, suggesting that less than a 30% decrease in the level of cytosolic GSH was sufficient to drastically alter the permeability of the plasma membrane of the heart cell.

It has previously been postulated that SH-donating agents may decrease anthracycline-induced lethality in mice.[24] Treatment of cultured heart cells with DTT prior to and during DRB-treatment indeed resulted in a decreased toxicity of DRB (Figure 5). The optimally protecting concentration of DTT was about 0.7–1 mM. However, no protection was provided by 1 mM desferrioxamin, a potent iron-chelating agent, and no lipid peroxidation products were detected by the TBA assay (not shown), suggesting that lipid peroxidation may not be the major cause of DRB-induced toxicity.

DISCUSSION

Although the morphological and functional aspects of anthracycline-mediated cardiotoxicity have been known for a long time, and several biochemical mechanisms for these effects have been proposed, the basic mechanism of the toxic action of anthracyclines is still a matter of controversy. The bulk of the experimental evidence is in favor of an oxidative mechanism in which the one-electron reduction of the quinone group of anthracyclines is followed by a rapid autooxidation and the generation of $O_2^{\cdot-}$ and subsequently H_2O_2.[4,5,12-18] The DRB-induced changes in cellular GSH may represent the difference between GSH consumption in e.g. redox reactions and other processes that remove GSH (such as direct inactivation of radicals and GSH transfer reactions) and the real rate of GSH synthesis. The perturbation of the balance between these processes may constitute an important pathway in DRB toxicity. The protective effect of DTT noted in the present investigation is consistent with this conclusion.

A specific role for the mitochondrion has previously been proposed in order to explain the anthracycline-mediated cardiotoxicity.[6,36] Even though effects of anthracyclines on cellular GSH in heart cells have been observed previously[19] no attempt has been made to distinguish these effects with respect to mitochondrial and cytosolic GSH, in spite of the fact that these pools apparently are essentially separate.[28,29,37] The particularly high concentration of mitochondria and the relative lack of defense systems against oxidative stress in heart may appear to be correlated to the heart-specific toxicity of anthracyclines. In hepatocytes, depletion of the mitochondrial GSH pool by the combined actions of ADR and BCNU was correlated to damage of isolated hepatocytes as judged by release of LDH and lipid peroxidation.[29] In contrast, the present results show that DRB-induced release of LDH from heart cells is parallelled by a decrease in cytosolic GSH. However, the mitochondrial GSH pool, which in heart cells contains about 3.3 nmoles/10^6 cells or about 30% of cellular GSH as compared to 3.2 nmoles/10^6 cells and about 10% in hepatocytes,[28,29] is not affected significantly under these conditions. These results clearly indicate that, at least in cultured neonatal heart cells, a relatively minor and selective change in the cytosolic GSH may be sufficient to affect the permeability of the plasma membrane.

Apart from the biochemical differences between hepatocytes and myocytes, the different conclusion drawn regarding the role of the mitochondrial pool of GSH as well as that of lipid peroxidation in these two cell types may be related to the different experimental conditions used. The heart cells used in this study were treated with low concentrations of DRB (maximally 17 μM) for a short period of time (3 hours) and the toxic effects were followed for a relatively long time (24 hours). In contrast, the hepatocytes were treated with high concentrations of ADR in combination with BCNU (111 μM and 150 μM, respectively) and toxicity was followed for a few hours.[29] Under these harsh conditions it is probably much more difficult to separate primary and secondary toxic effects.

The lack of detectable amounts of TBA-reacting materials in the heart cells after DRB-treatment may indicate either that these products are rapidly metabolized, or that lipid peroxidation does not constitute a major factor in anthracycline-induced cell toxicity. The lipid peroxidation detected in hepatocytes[29] may represent a late event in the toxic cascade again due to the harsh treatment used.

In conclusion, the results presented in this paper support the notion that changes in the status of the cytosolic pool of GSH may be responsible directly or indirectly,

in the latter case by influencing other cell constituents, e.g. NADPH, Ca^{2+} and proteins, for DRB-induced cell damage.

Acknowledgement

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