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EFFECT OF DIFFERENT OXYGEN PRESSURES AND *N,N'*-DIPHENYL-*p*-PHENYLENEDIAMINE ON ADRIAMYCIN® TOXICITY TO CULTURED NEONATAL RAT HEART MYOCYTES

KARIN ÖLLINGER*† and ANDERS BRUNMARK‡

†Department of Pathology II, Linköping University, S-581 85 Linköping, Sweden
‡Department of Immunology, The Scripps Research Institute, La Jolla, CA, U.S.A.

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Abstract—The effect of different oxygen pressures and the antioxidant DPPD (*N,N'*-diphenyl-*p*-phenylenediamine) on Adriamycin (doxorubicin) cytotoxicity in highly purified cardiac myocytes was investigated to evaluate the involvement of free radicals in the mechanism of toxicity. Adriamycin exposure caused a time-dependent decrease in viability measured as intracellular potassium ion release or lactate dehydrogenase retention. Incubation of myocytes in 16, 172 or 834 μM oxygen during exposure to 200 μM Adriamycin for 6 hr killed 13, 42 and 56% of the cells in the respective cultures. DPPD prolonged viability in the latter two oxygen concentrations and protected against lipid peroxidation measured as production of malondialdehyde and 4-hydroxynonenal. Addition of superoxide dismutase decreased the Adriamycin-induced cell killing to 6% after a 4-hr incubation, as compared to 24% in cultures exposed to Adriamycin only. Adriamycin exposure decreased the concentration of reduced glutathione, and the toxicity of the drug was increased when glutathione reductase was inhibited by the addition of BCNU (1,3-bis-2-chloroethyl-1-nitrosourea). No significant effect on Adriamycin toxicity was observed after inhibition of glutathione synthesis by treatment with BSO (buthionine sulfoximine). It is concluded that free radicals play an important role in Adriamycin toxicity to heart myocytes, and that the cell killing mechanism is likely to be related to induction of lipid peroxidation.

Key words: heart cell cultures; glutathione; doxorubicin; lipid peroxidation; free radicals; antioxidants

Of all available chemotherapeutic substances, the anthracycline Adriamycin is the antineoplastic agent that exhibits the broadest spectrum of activity [1]. However, clinical use of Adriamycin is limited, because cardiomyopathy is induced by cumulative doses of the drug. The mechanism of anthracycline cardiac toxicity is not completely understood, but many studies have suggested that the effects are related to formation of free-radical, semiquinone drug intermediates [2–5]. In aerobic environments, a semiquinone may reduce molecular oxygen, leading to the formation of reacitve oxygen radicals, e.g. the superoxide anion radical and hydrogen peroxide. Superoxide radical production during Adriamycin exposure has been demonstrated in the following: (a) cardiac submitochondrial particles [6], in which complex I was found to be the major site of reduction of the anthracycline quinone; (b) heart sarcosomes, in which radical production is accompanied by a dose-related oxidation of NADPH, this effect being decreased by inhibition of NADPH cytochrome P450 reductase [5]; and (c) cardiac cytoplasmic protein preparations, in which the electron transfer reaction is catalysed by xanthine dehydrogenase and cardiac oxymyoglobin [7]. The superoxide dismutase and catalase enzymes exhibit lower activity in heart myocytes than in other cell types, and, consequently, these cells have a limited capacity to detoxify oxygen-derived radicals [8]. Moreover, heart tissue is characterized by both a high oxygen concentration and a high respiration rate, and is therefore prone to generate free radicals during Adriamycin exposure. A free-radical mechanism for Adriamycin heart damage is further suggested by the fact that *N*-acetylcysteine protects the Ca^{2+} pump of cardiac sarcoplasmic reticulum against radical-induced damage [9]. Also, the toxicity of Adriamycin has been shown to be reduced by iron-chelating agents, i.e. substances that prevent iron from catalysing the breakdown of hydrogen peroxide to hydroxyl radicals [10].

The antineoplastic activity of Adriamycin is related to intercalation of the drug in DNA, a process which inhibits DNA and RNA synthesis [11]. In this context, damage to DNA, e.g. strand breaks and strand cross-linking, has been detected after Adriamycin exposure, and such damage appears to require biological activation of Adriamycin presumably through a reduction process. A "site-specific free-radical concept" has been suggested by Bachur *et al.* [12] to explain nucleus DNA damage. The involvement of free radicals in the antineoplastic activity of Adriamycin is further indicated by the observation that SOD§ and catalase added to cell culture medium inhibits the Adriamycin-mediated killing of MCF-7 human breast cancer cells [10].

Heart tissue from neonatal rats contains several

* Corresponding author. Tel. (46) 13 22 46 50; FAX (46) 13 22 15 29.

§ Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; SOD, superoxide dismutase; DPPD, *N,N'*-diphenyl-*p*-phenylenediamine; BSO, buthionine sulfoximine; BCNU, 1,3-bis-2-chloroethyl-1-nitrosourea; MDA, malonedialdehyde.

cell types, but only two of these, i.e. (i) myocyte-like and (ii) fibroblast-like cells, have been observed in heart cell cultures. Heart myocytes do not undergo cell division *in vitro*, whereas fibroblasts proliferate readily and eventually dominate a culture [13]. Earlier studies have shown that the toxic effects of Adriamycin on cultured myocardial cells are mediated by free radicals [14, 15]; it should be noted that the different cell types in the cultures were not quantified in these studies.

In the present report, we examined the effect of Adriamycin on highly purified neonatal rat cardiomyocytes at different oxygen pressures, and the role of the antioxidant DPPD in protecting against Adriamycin toxicity.

MATERIALS AND METHODS

Chemicals. Adriamycin–HCl was a gift kindly provided by Farmitalia (Milan, Italy). All other chemicals were purchased and are listed here with their sources: GSH, GSSG, glutathione reductase, collagenase, collagen (type I), and SOD (Boehringer Mannheim, Mannheim, Germany); dialysed fetal calf serum, Hanks' balanced salt solution, Williams' culture medium, and HEPES (GIBCO Ltd, Paisley, U.K.); glutamine, penicillin, streptomycin, and Eagle's minimum essential medium (FLOW Laboratories, Irvine, U.K.); cytosine-β-D-arabinofuranoside, PIPES, EGTA, DPPD, insulin, luciferase-luciferin, and BSO (the Sigma Chemical Co., St Louis, MO, U.S.A.); deferoxamine B (Ciba-Geigy AG, Basel, Switzerland); LPO-586 (Bioxytech S.A., Bonneuil sur Marne, France); collagenase (Worthington Biochemical Corporation, New Jersey, U.S.A.); BCNU (Bristol-Myers, Copenhagen, Denmark); TRITC-phalloidin (Molecular Probes Inc., Junction City, OR, U.S.A.); Percoll (Kabi-Pharmacia, Uppsala, Sweden); all additional chemicals (Merck, Darmstadt, Germany). All of the chemicals were of the highest purity commercially available.

Animals. Neonatal male and female Sprague–Dawley rats were purchased from B & K Universal AB (Sollentuna, Sweden).

Preparation of heart myocytes. Two- to three-day-old rats were killed, and their hearts were removed and freed of connective tissue and then finely chopped and carefully washed twice in Hanks' balanced salt solution without Ca^{2+} or Mg^{2+} at 37°. The wash medium was decanted, and the chopped heart tissue suspended in 25 mL of Hanks' balanced salt solution supplemented with 20 mg of collagenase. The suspension was transferred to a closed glass incubator thermostatted at 37° and then stirred for 15 min. The cell-containing collagenase solution was decanted into tubes and centrifuged for 90 sec at 250 *g*. The resulting cell pellet was then resuspended in Eagle's minimum essential medium supplemented with 10% dialysed calf serum, 50 IU/mL penicillin, 50 UG/mL streptomycin, and 2 mM L-glutamine (referred to as Eagle's medium in the text). This procedure was repeated seven times to produce a pooled cell suspension; this pooled sample was filtered through a sterile Monodure sieve cloth (40 × 40 μM; Derma AB, Västra Frölunda, Sweden) to remove cell-clusters and any remaining tissue. Aliquots (1.5 mL) of the cell suspension were placed on 5 mL of Percoll that had been diluted to 1.05 g/mL with Eagle's minimum essential medium containing 10 mM HEPES, pH 7.2. The cells were centrifuged at 300 *g* for 150 sec and the pellets resuspended in fresh Eagle's medium. Cells were plated at a density of 105,000 cells/cm^2 in culture dishes (Costar, Cambridge, MA, U.S.A.) previously coated with 5.0 μg of type I collagen/cm^2. The cells were maintained in an atmosphere of 5% CO_2 and 95% air at 37°. After 24 hr the medium was changed to culture medium containing 10 μg of cytosine-β-D-arabinofuranoside per mL, and this medium was changed every second day.

Characterization of myocyte cultures. Myocytes were purified as described above and plated at a density of 400,000 cells/coverslip (Ø 22 mm). Control cells were plated before Percoll separation by washing the collagenase-released cells once with Eagle's medium.

After 24 hr and again after 5 days of culturing, the cells were extracted and fixed as previously described [16]. Extracted and fixed cells were stained with 3.3 μM TRITC-phalloidin for 18 min in darkness at room temperature. The stained cells were rinsed twice in phosphate buffer saline and mounted on glass slides. The slides were examined in a Leitz epifluorescence microscope using excitation filters for TRITC fluorescence. Myocytes have myofibrils with Z bands, whereas fibroblasts have actin fibres without Z bands, and these characteristics were used to distinguish the two cell types [17].

Determination of intracellular glutathione in cultured heart myocytes. Cell culture dishes were rinsed with ice-cold phosphate buffer saline and immediately frozen on a metal tray at −18° for 5 min. This procedure caused rupture of the cell membrane without effecting GSH levels. A solution of 0.1% (w/v) EDTA and 5 μM cysteine was then added to the dishes and the cells were detached with a rubber policeman. Samples for protein analysis were withdrawn, and the remaining solution was diluted with one volume of 80 mM $HClO_4$. The suspension was centrifuged at 400 *g* for 10 min, and the supernatant was used for HPLC analysis; these samples were stable for 5 days when stored at −18°. Separation of GSH and GSSG was performed on a Biophase ODS I 5 μ reverse-phase column (250 mm × 4.3 mm; Bioanalytical Systems Inc., West Lafayette, IN, U.S.A.). The mobile phase consisted of 0.1 M monochloroacetic acid dissolved in deionized triple-distilled water, pH 3.0, and was supplied by a Jasco 880-PU pump (Japan Spectroscopic Co. Ltd, Tokyo, Japan) at a rate of 1.0 mL/min. A BAS LC-4B Amperometric Detector (Bioanalytical Systems Inc., West Lafayette, IN, U.S.A.) with serially arranged dual Au/Hg electrodes was used to detect GSH and GSSG (R_t 5.2 and 11.8 min, respectively). The potential of the upstream electrode was −1.0 V, and that of the downstream electrode was +0.150 V. GSH and GSSG concentrations were calculated from standard curves and correlated to protein content. The respective detection limits for GSH and GSSG were 2×10^{-12} and 1.5×10^{-11} moles.

Determination of glutathione content in heart tissue. Hearts were washed in ice-cold Hanks' saline solution and then placed in 500 μL of 0.1% (w/v) EDTA solution supplemented with 5 μM cysteine and homogenized for 30 sec in a glass–glass homogenizer. An equal volume of 80 mM $HClO_4$ was added, and the suspension was centrifuged at 500 *g* for 20 min. The supernatant was analysed for glutathione content as described above.

Measurement of enzyme activity. Myocytes were detached by incubation in 0.25% trypsin for 3 min at 37°. The cells were then washed twice in ice-cold phosphate buffer saline, centrifuged at 300 *g* for 5 min, and the resulting pellet was resuspended in 0.05 M potassium phosphate buffer, pH 7.7. Samples were exposed to three cycles of freezing and thawing to rupture the cell membranes. Glutathione reductase [18] and glutathione peroxidase [19] activities were measured as previously described. When measuring glutathione peroxidase activity, *t*-butyl hydroperoxide was used as substrate to allow determination of both selenium-dependent and selenium-independent activity. The rate of NADPH oxidation in a sample containing all reagents except the enzyme preparation was used as a blank rate to correct for the oxidation of NADPH by the peroxide (i.e. non-enzymatic oxidation). Catalase activity was calculated from the first order rate constant $K = \log (S_0 S_t) \times 2.3/t$ where S_0 is the initial substrate concentration, S_t the final substrate concentration and t is the reaction time (3 min). Catalase activity is expressed as units that represent the calculated rate constant (K) per milligram protein [20]. SOD was determined as described by Fridovich [21]. Inhibition of CuZn-containing SOD was performed with 1 mM KCN. Reductase activity was determined as described by Williams and Kamin [22]. All enzyme activities were related to protein content determined by the method of Lowry *et al.* [23].

Incubation of heart myocytes with Adriamycin. All experiments were performed on 5–7-day-old cell cultures. Adriamycin was diluted with distilled water and stored in the dark at 4° for no longer than 3 weeks. The drug was added to myocytes in Eagle's minimum essential medium without serum and incubated in an atmosphere of 5% CO_2 and 95% air at 37°. At different time points, cell cultures were withdrawn and cell viability was measured as potassium ion release (see below) and lactate dehydrogenate activity [24]. Deferoxamine was added 3 hr before starting an experiment. DPPD, SOD, catalase and DMSO were added together with Adriamycin to the incubation mixture. Glutathione reductase was inhibited by adding 100 μM BCNU 30 min before Adriamycin was added [25]. This treatment reduced glutathione content in control cells by 40% and reduced the activity of glutathione reductase by 72%. Inhibition of glutathione synthesis was accomplished by adding 0.25 mM BSO to the cell culture medium 22 hr before the start of an experiment [26]. The BSO treatment decreased glutathione concentration by 93% as compared to the concentration in control cells, but did not affect cell viability.

Intracellular potassium content. The cell cultures were washed with cold K^+-free solution consisting of 0.8 mM NaH_2PO_4, 123.4 mM NaCl, 0.8 mM $MgSO_4$, 26.2 mM $NaHCO_3$, 2.7 mM $CaCl_2$ and 5.6 mM glucose [27]. Potassium ions were extracted from the cells by adding 250 μL of ultrapure water (Milli Q, Millipore) and analysed by using a potassium-ion selective electrode (Hitachi 717, Hitachi Ltd., Japan).

Incubation of heart myocytes in different concentrations of oxygen. To assess the effects of different concentrations of oxygen, myocytes were incubated at 37° in airtight chambers (FLOW Laboratories, Irvine, U.K.) in an atmosphere of N_2, air (21% oxygen) or O_2 (100% oxygen). Prior to this incubation, the incubation mixture was bubbled with the respective gas for 10 min before being added to the cell cultures. This procedure resulted in media with oxygen concentrations of 16, 172 and 834 μM in atmospheres of N_2, air and O_2, respectively, as measured according to Winkler [28].

Lipid peroxidation measurement. Malondialdehyde and 4-hydroxynonenal were measured in cell cultures after Adriamycin exposure by collecting 2×10^6 cells (detached with a cell scraper) in 500 μL of H_2O in glass test tubes placed on ice. The assay was performed using a colorimetric assay, LPO-586, manufactured by Bioxytech (Bonneuil sur Marne, France). The reagents were mixed with 200 μL of cell sample, and the mixture was incubated at 45° for 40 min and then centrifuged at 400 *g* for 10 min. The supernatant was monitored at 586 nm, and the concentration of MDA and 4-hydroxynonenal was calculated from standard curves. Interference from Adriamycin absorbance was compensated for by incubation of cell cultures with Adriamycin at 4° according to the method described by Graham *et al.* [29].

Statistical analysis. The results were evaluated with Student's *t*-test for matched pairs.

RESULTS

Characterization of heart myocyte cultures

After 24 hr of culturing, heart cells had attached and spread out on the bottom of culture dishes, and after 3–5 days intracellular contacts had been established and a functional electrical syncytium had developed. The cultured myocytes and fibroblasts were counted, as described in Materials and Methods, after 24 hr and on day 5 (Table 1). Percoll separation removed 91% of the fibroblasts with only a 26% loss of myocytes. On day 5, Percoll-separated cultures that had been subjected to cytosine-β-D-arabinofuranoside treatment contained 93% myocytes and 7% fibroblasts. Cytosine-β-D-arabinofuranoside prevented fibroblast proliferation without affecting myocyte morphology or beating frequency. Cultures grown without the mitotic inhibitor showed a 40–65% increase in the number of fibroblasts from day 1 to day 5 (results not shown). If implemented cytosine-β-D-arabinofuranoside treatment was discontinued after 10 days, myocyte beating continued for at least 11 weeks and no fibroblast proliferation was observed.

The concentration of reduced glutathione in newly excised heart tissue was 9.61 ± 0.46 nmol/mg protein, in agreement with earlier estimations

Table 1. Effect of Percoll separation on myocyte content in primary cultures

Culture	Number of cells per heart	% Myocytes after 24 hr	% Myocytes after 5 days
Control	2.31×10^6	51.1 ± 5.2	46.5 ± 12.0
Percoll purified	0.91×10^6	89.1 ± 2.2	93.6 ± 3.2

Heart cells were seeded on coverslips directly after collagenase digestion (=control) and after Percoll purification. After 24 hr or 5 days, the cells were stained with TRITC-associated phalloidin (marker for f actin) and the number of myocytes and fibroblasts were counted in a fluorescence microscope (100 cells on each coverslip). Values represent means ± SEM of five different cell preparations.

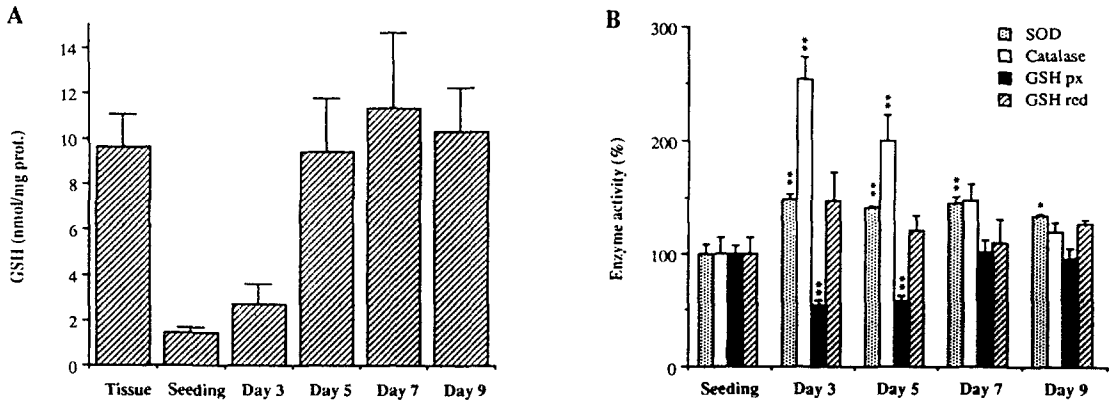

Fig. 1. Changes in GSH content and enzyme activities during culture of heart myocytes. (A) HPLC was used to measure reduced glutathione in heart tissue (=Tissue), in freshly-isolated cells (=Seeding) and in cell cultures cultured for 3–9 days; no oxidized glutathione was detected. (B) SOD, catalase, glutathione peroxidase (GSH px), and glutathione reductase (GSH red) activities were measured in freshly-isolated cells (=Seeding) and in cells cultured for 3–9 days (as described in the Materials and Methods). The values represent means ± SEM for five separate cell preparations.

Table 2. Viability of heart myocyte cultures of different ages exposed to 200 μM Adriamycin for 6 hr

Incubation	Day 3	Day 5	Day 7	Day 9
Control	95.8 ± 1.2	96.7 ± 0.3	96.9 ± 1.0	97.7 ± 0.7
200 μM Adr	32.7 ± 6.9	58.4 ± 3.0	59.7 ± 6.1	53.7 ± 7.2
200 μM Adr + DPPD	67.2 ± 6.0	87.8 ± 5.4	90.0 ± 4.3	88.8 ± 4.8

Viability of myocyte cultures of different ages measured as intracellular lactate dehydrogenase activity after exposure to 200 μM Adriamycin ±0.5 μM DPPD for 6 hr. Values are means ± SEM calculated from three different cell preparations.

[14, 30]; no oxidized glutathione was detected. During collagenase digestion, cellular glutathione concentration decreased by 85% to 1.46 ± 0.22 nmol/mg protein. After seeding, the concentration increased (Fig. 1A), and after 5 days in culture the cells contained the same amount of glutathione as heart tissue. This level remained unchanged for at least 4 days.

The activity of free radical detoxication enzymes was determined during a culturing period of 9 days (Fig. 1B). During the first 3 days, SOD activity increased from 4.3 ± 0.4 U/mg protein to 6.4 ± 0.2 U/mg protein and remained at this level for a total of at least 6 days. Cyanide inhibition of the CuZn-SOD isoform showed that the increase in activity was entirely due to CuZn-SOD (not shown). The activities of catalase and glutathione peroxidase showed an initial change characterized by increased catalase activity and lowered glutathione activity. After 7 days in culture, the activities of these

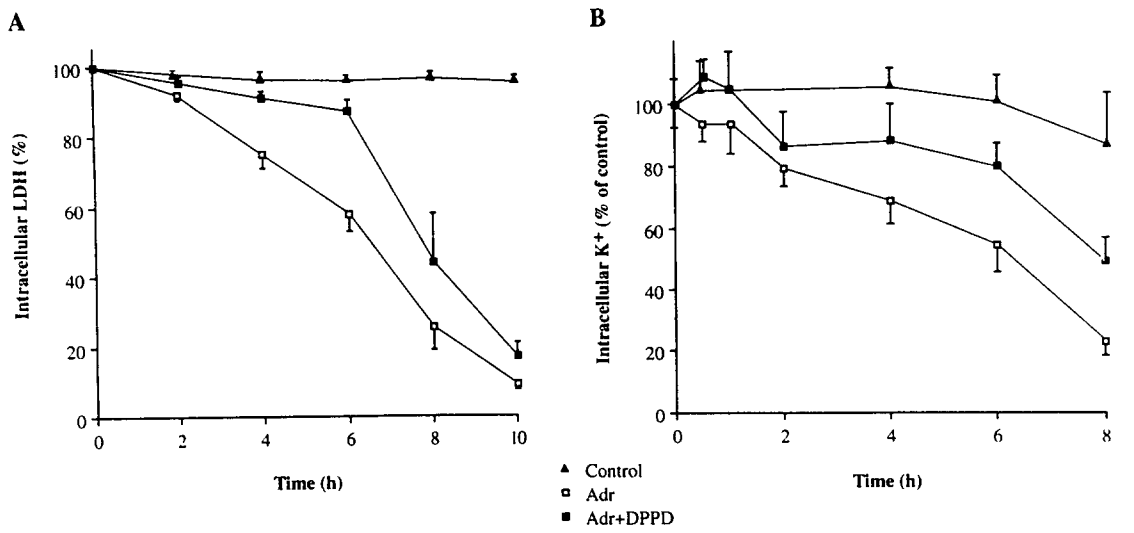

Fig. 2. Time course for myocyte killing during Adriamycin exposure. Myocytes were exposed to 200 μM Adriamycin in the absence (open squares) or presence (closed squares) of 0.5 μM DPPD. Control cultures were incubated in Eagle's minimum essential medium. (A) Time course showing viability determined as intracellular LDH activity. (B) Time course showing viability determined as release of K^+. Results shown represent means ± SEM for three to five separate cell preparations.

enzymes did not differ significantly from the values obtained at seeding (0.42 ± 0.05 K/mg protein and 74.3 ± 8.1 nmol/min × mg protein for catalase and glutathione peroxidase, respectively). During cultivation, glutathione reductase activity did not change significantly from the initial value of 4.9 ± 0.7 nmol/min × mg protein. On the other hand, NADPH cytochrome P450 reductase activity increased from 4.7 ± 0.2·10^{-3} to 11.2 ± 0.8·10^{-3} U/mg protein after 5 days and remained at this level for at least 4 more days (not shown).

Adriamycin-induced cytotoxicity and lipid peroxidation

Table 2 shows viability in 3-, 5-, 7- and 9-day-old myocyte cultures exposed to 200 μM Adriamycin for 6 hr; viability was measured as lactate dehydrogenase retention. No significant difference in toxicity was detected in cultures 5, 7 and 9 days old. So that the level of intracellular glutathione in the cultured cells would be the same as in heart tissue, all our experiments were performed 5–7 days after seeding (Fig. 1A).

Figure 2 shows the time course of cell killing, measured as lactate dehydrogenase retention (Fig. 2A) and as K^+ release (Fig. 2B), in cultures of neonatal rat heart myocytes exposed to 200 μM Adriamycin. The release of K^+ is a more sensitive indicator of cell viability, showing values ≤12 percentage points lower than the corresponding value for LDH activity.

DPPD is an antioxidant that prevents lipid peroxidation by reacting with lipid radicals [31]. Addition of DPPD to the incubation mixture delayed cell killing but could not prevent it (Fig. 2). This was probably not due to exhaustion of DPPD, since

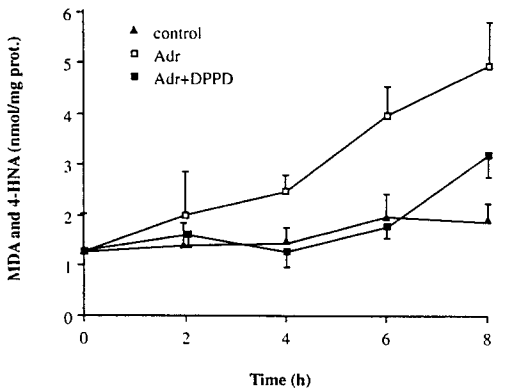

Fig. 3. Time course of lipid peroxidation in heart myocytes during Adriamycin exposure. Myocytes were exposed to 200 μM Adriamycin in the absence (open squares) or presence (closed squares) of 0.5 μM DPPD. Control cultures were incubated in Eagle's minimum essential medium. Lipid peroxidation was measured as MDA and 4-hydroxynonenal (4-HNA) at 568 nm.

treatment with a doubled concentration of DPPD (1 μM) did not provide more efficient protection (results not shown). Adriamycin exposure caused a time-dependent increase in lipid peroxidation, measured as accumulation of MDA and 4-hydroxynonenal (Fig. 3). The addition of DPPD decreased lipid peroxidation to control levels, and only after 8 hr could a minor increase in lipid peroxidation products be detected. Examination in a light microscope revealed morphological changes,

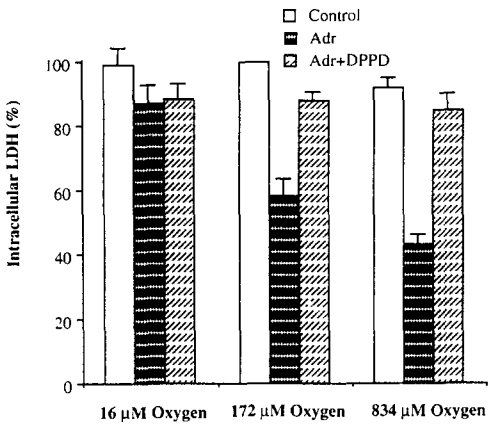

Fig. 4. Influence of oxygen pressure on Adriamycin toxicity to heart myocytes. Cell viability, measured as intracellular LDH retention, after 6 hr of exposure to 200 μM Adriamycin in the absence (grey bars) or presence (striped bars) of 0.5 μM DPPD. White bars represent control cells incubated under the same conditions but without Adriamycin and DPPD. 100% viability corresponds to control cells incubated in 172 μM oxygen for 6 hr. In 172 and 834 μM oxygen, the viability of cells incubated with Adriamycin and DPPD differed significantly ($P \leq 0.01$, 4 degrees of freedom) from the viability of cells incubated with Adriamycin but without DPPD. Values are means ± SEM for five experiments.

such as vacuoles around the nucleus and changes in beating frequency, in cells exposed to Adriamycin for 2–4 hr; cells incubated with Adriamycin alone or with Adriamycin and DPPD did not differ morphologically. The viability and morphology of cells incubated with 0.5 μM DPPD alone were not different from control cells.

Adriamycin toxicity at different oxygen concentrations

Cells exposed to 200 μM Adriamycin for 6 hr showed 87% viability, measured as lactate dehydrogenase retention, when incubated in 16 μM oxygen; in 172 μM oxygen cell viability was 58%, and in 834 μM oxygen, 42% (Fig. 4). The addition of 0.5 μM DPPD increased the viability of Adriamycin-exposed cells cultured in 172 and 834 μM oxygen to 88 and 85%, respectively. In cells exposed to Adriamycin in 16 μM oxygen, the addition of DPPD did not prevent cell killing but did result in cells with fewer vacuoles around the nucleus as compared to cells exposed to Adriamycin in 172 μM oxygen. Control cells incubated in 16 or 172 μM oxygen without Adriamycin did not show any loss of viability, whereas control cells incubated in 834 μM oxygen showed a 9% decrease in viability after a 6-hr incubation.

Effect of free-radical scavengers and deferoxamine on Adriamycin toxicity

Table 3 shows the viability of heart myocytes after incubation with 200 μM Adriamycin for 4 hr in the presence of the iron chelator deferoxamine or a free-radical scavenger, i.e. SOD, catalase or DMSO.

Table 3. Viability of Adriamycin-exposed heart myocytes in the presence of free radical scavengers

Treatment	Viability (%)
200 μM Adriamycin	75.6 ± 4.5
+0.5 μM DPPD	91.5 ± 1.6*
+SOD (50 U/mL)	94.0 ± 5.6*
+catalase (100 U/mL)	74.2 ± 3.2
+5% (v/v) DMSO	76.9 ± 3.9
+250 μM Deferoxamine	81.1 ± 4.5

Viability measured as intracellular lactate dehydrogenase retention after exposure to 200 μM Adriamycin for 4 hr. Values are means ± SEM calculated from four to five different cell preparations. * Represents values significantly different from those of cells exposed to Adriamycin alone, $P \leq 0.01$ (4 degrees of freedom).

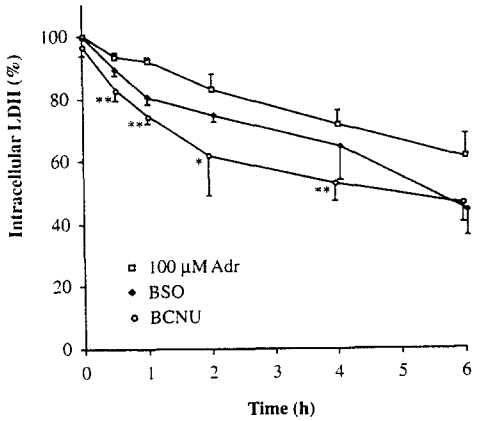

Fig. 5. Time course showing the viability of Adriamycin-exposed cultured heart myocytes pretreated with BSO or BCNU. Myocyte cultures pretreated with 0.5 mM BSO for 22 hr or with 300 μM BCNU for 30 min were exposed to 100 μM Adriamycin and viability was measured as intracellular LDH activity. Results are given as means ± SEM for five separate cell preparations. *$P \leq 0.05$, **$P \leq 0.02$ represent values significantly different from those obtained for cells incubated with Adriamycin alone (4 degrees of freedom).

Only SOD was found to significantly inhibit the killing of myocytes. However, after 6 hr of incubation, this protection decreased and cell viability was not significantly different from that in the absence of the enzyme. The intracellular activity of SOD was not increased during incubation with SOD and heat-inactivated SOD did not protect against Adriamycin toxicity. It was not possible to use a higher concentration of any of the scavengers due to viability loss in control cells.

Effect of enzyme inhibitors on cell viability during Adriamycin exposure

To evaluate the ability of intracellular glutathione to protect cells against Adriamycin toxicity, we

Table 4. Intracellular GSH content in cardiomyocytes after Adriamycin exposure

Treatment	Control (% GSH)	100 μM Adr (% GSH)
None	100	74.8 ± 4.3
DPPD	99.0 ± 13.9	77.5 ± 8.7
BSO	6.7 ± 3.7	ND*
BCNU	59.6 ± 11.2	40.6 ± 7.3

* Not detected

Levels of intracellular reduced glutathione in cultures of heart myocytes before and after exposure to 100 μM Adriamycin for 30 min. An HPLC/EC detector was used to measure glutathione. Values are expressed as a percentage of GSH concentration compared to control cells; 100% corresponds to 9.6 nmol GSH/mg protein. Values are means ± SEM for five separate myocyte preparations.

inhibited glutathione reductase and γ-glutamylcysteine synthetase (i.e. glutathione synthesis) in 5-day-old cell cultures. Inhibition of glutathione reductase was brought about by pretreatment with BCNU for 30 min. Figure 5 shows the time course of cell killing for BCNU-treated myocytes exposed to 100 μM Adriamycin: after 4 hr 47% of the BCNU-pretreated cells had been killed, as compared to 28% of the control myocytes. The experiment was repeated using 7-day-old cell cultures without any significantly different result.

In order to deplete intracellular glutathione, myocytes were incubated for 22 hr with 0.5 mM BSO, an inhibitor of γ-glutamylcysteine synthetase. This treatment reduced glutathione concentration to 7% of that in controls without any loss of viability or change in glutathione reductase activity. Furthermore, as also indicated in Fig. 5, the time course for the viability of BSO-pretreated cells incubated with 100 μM Adriamycin was lower throughout the time span of the experiments, although no statistically significant differences could be established.

Decreases in glutathione concentration caused by Adriamycin treatment

Intracellular GSH decreased by 25% during the first 30 min of exposure to 100 μM Adriamycin (Table 4). Since cultures exhibited only a minor decrease (8% K^+ release) in cell viability during the first 30 min, the loss of GSH at this time cannot be attributed to cell rupture. The presence of the antioxidant DPPD did not affect GSH levels in control cells and did not prevent a GSH decrease in Adriamycin-treated cells. No GSSG could be detected in controls or Adriamycin-exposed cells. Depletion of intracellular glutathione by BSO pretreatment for 22 hr lowered GSH concentration to 0.64 nmol/mg protein. Exposure of these cells to 100 μM Adriamycin for 30 min decreased viability to 89.3% and GSH concentration to below the detection limit. No GSSG was detected in BSO-pretreated cells after Adriamycin exposure. Inhibition of glutathione reductase provides information about the importance of regeneration of oxidized glutathione, mainly reflecting peroxide breakdown by glutathione peroxidase. BCNU pretreatment reduced the glutathione level by 40%, and Adriamycin incubation decreased viability by 87% and also lowered glutathione concentration by an additional 19 percentage points. Thus, BCNU-pretreated myocytes did not decrease their glutathione content more than control cells upon Adriamycin exposure.

DISCUSSION

The method for purification of neonatal rat heart myocytes presented here yields myocytes of high purity. Cultures obtained by using this method should be suitable for studies of free-radical-mediated heart myocyte damage, since fibroblast overgrowth is minimized and most cellular systems aimed at protecting against free radicals remain at levels comparable to those existing in newly-excised cardiac myocytes. The latter point is illustrated by the fact that although there was a decrease in intracellular glutathione concentration after preparation, the level returned to normal after 5 days. Furthermore cultured myocytes as compared to freshly isolated myocytes, exhibited a slight increase in the activity of SOD, catalase and NADPH cytochrome P450 reductase, and a decrease in glutathione peroxidase activity. However, when cultures of different ages were exposed to Adriamycin for 6 hr (Table 2), 3-day-old cultures were much more sensitive (67% cells killed) than 5- to 9-day-old cultures (40–46% killed). Notably, it was the low level of intracellular glutathione in 3-day-old cultures that corresponded to this observation, rather than changes in the activity of enzymes protecting against free radicals. The myocytes in 3-day-old cell cultures had not yet developed contact, which might be another explanation for the increased Adriamycin toxicity in these young cultures.

Three main findings in our study indicate that free radicals are important factors in Adriamycin-induced damage to heart myocytes. First, the toxicity of Adriamycin seems to be oxygen-dependent: in 16 μM oxygen Adriamycin-mediated cell killing was one-third of that occurring in 172 μM oxygen (air), whereas 834 μM oxygen enhanced cell killing. Secondly, Adriamycin cell killing can be delayed by the addition of SOD. It is, however, unlikely that an enzyme the size of SOD could cross the cell membrane of an intact cell. We therefore propose that extracellular SOD can affect the intracellular equilibrium of the superoxide/hydroperoxyl radical system. Hydroperoxyl radicals can diffuse through the plasma membrane and dismutate to hydrogen peroxide extracellulary in an SOD-catalysed reaction. It is also possible that Adriamycin is able to redox cycle at or near the cell surface without actually entering the cell. Adriamycin toxicity towards the cell membrane has been described by other investigators [10]. Furthermore, superoxide radicals seem to be the damaging species, since addition of SOD decreases toxicity. Hydrogen peroxide is the product of superoxide dismutation, and catalase-catalysed breakdown of this compound did not

protect against Adriamycin toxicity, which implies that the damage had already occurred before hydrogen peroxide was formed. The third finding that suggests the involvement of free radicals in Adriamycin toxicity is that DPPD protected Adriamycin-exposed myocytes from death for a period of up to 6 hr and also completely abolished lipid peroxidation.

The loss of SOD and DPPD protection after 6 hr of Adriamycin exposure may imply that there is a free-radical-independent mechanism that contributes to cell killing; this mechanism might be related to the intercalating ability of Adriamycin, which could affect protein production [32]. It is also possible that the antioxidant protection used is not sufficient to protect against free radical production during time-periods greater than 6 hr.

Lipid peroxidation has previously been proposed as the main mechanism for Adriamycin cardiotoxicity [15] and an increase in serum neutral lipid peroxides has been shown earlier in Adriamycin administration in rats [33]. The causal relation between lipid peroxidation and cell death has, however, been questioned. On the one hand, lipid peroxidation could be a consequence of cell death, and it could therefore be argued that the presence of MDA is not evidence for lipid peroxidation as a cell-killing mechanism. On the other hand, if lipid peroxidation is the consequence of cell death, addition of an antioxidant molecule would be expected to prevent accumulation of MDA but not to have an effect on cell killing [34].

Although glutathione is a scavenger of free radicals, it is probably more important as a cofactor in detoxification of peroxide by glutathione peroxidase. Intracellular steady-state levels of GSH may decrease due to redox cycling and subsequent radical generation by quinoid compounds [35, 36]. Furthermore, in mice, Adriamycin administration has been found to lower the level of cardiac GSH in a dose-dependent manner, and this effect was prevented by administration of *N*-acetylcysteine and vitamin E [37]. In the present study, the inhibition of glutathione reductase by BCNU pretreatment led to potentiation of cell killing, whereas depletion of intracellular glutathione by pretreatment with BSO did not. These somewhat conflicting results resemble those found in isolated, glutathione-depleted hepatocytes [38], where exposure to adriamycin did not potentiate cell killing whereas BCNU treatment did. Thus, our findings indicate that intracellular glutathione concentration can be decreased considerably without affecting viability, and also suggest that the function of glutathione reductase, i.e. its ability to reduce oxidized glutathione, is of importance in the toxic mechanism of Adriamycin.

Specific heart toxicity after Adriamycin administration has been proposed to be due to a combination of redox cycling of the drug producing oxygen-free radicals and low activity of the free-radical-protective enzymes in the heart tissue [8]. Several sites for enzymatic reduction and redox cycling of Adriamycin in the heart have been reported, e.g. at complex I of heart mitochondria [6], the sarcotubular membranes [5] and heart nuclei [39]. Interestingly, heart mitochondria, but not hepatocyte mitochondria, have been found to contain an NADH reductase that can catalyse redox cycling of Adriamycin and increase the production of free radicals in heart tissue [40]. These findings, together with the results of the present study, show that generation of free radicals causes severe cellular damage to myocardial cells, and this specific effect might explain Adriamycin toxicity in heart tissue. Moreover, when comparing similarly cultured myocytes and hepatocytes exposed to 200 μM Adriamycin, it was found that toxicity was three times greater in the myocyte cultures and that these cells were altered morphologically whereas the hepatocytes were not (K. Öllinger, unpublished results).

Our results suggest that the mechanism of cell injury caused by Adriamycin is oxygen-dependent and DPPD- and SOD-sensitive and initially involves redox cycling of the drug, which in turn leads to the generation of free radicals, proximally or in the cell membranes, and finally to lipid peroxidation and cell death.

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