Daunorubicin-Induced Apoptosis in Rat Cardiac Myocytes Is Inhibited by Dexrazoxane

Douglas B. Sawyer, Ryuji Fukazawa, Margaret A. Arstall, Ralph A. Kelly

Abstract—The clinical efficacy of anthracycline antineoplastic agents is limited by a high incidence of severe and usually irreversible cardiac toxicity, the cause of which remains controversial. In primary cultures of neonatal and adult rat ventricular myocytes, we found that daunorubicin, at concentrations $\leq 1 \mu\text{mol/L}$, induced myocyte programmed cell death within 24 hours, as defined by several complementary techniques. In contrast, daunorubicin concentrations $\geq 10 \mu\text{mol/L}$ induced necrotic cell death within 24 hours, with no changes characteristic of apoptosis. To determine whether reactive oxygen species play a role in daunorubicin-mediated apoptosis, we monitored the generation of hydrogen peroxide with dichlorofluorescein (DCF). However, daunorubicin (1 $\mu\text{mol/L}$) did not increase DCF fluorescence, nor were the antioxidants $N$-acetylcysteine or the combination of $\alpha$-tocopherol and ascorbic acid able to prevent apoptosis. In contrast, dexrazoxane (10 $\mu\text{mol/L}$), known clinically to limit anthracycline cardiac toxicity, prevented daunorubicin-induced myocyte apoptosis, but not necrosis induced by higher anthracycline concentrations ($\geq 10 \mu\text{mol/L}$). The antiapoptotic action of dexrazoxane was mimicked by the superoxide-dismutase mimic porphyrin manganese(II/III)tetrakis(1-methyl-4-peridyl)porphyrin (50 $\mu\text{mol/L}$). The recognition that anthracycline-induced cardiac myocyte apoptosis, perhaps mediated by superoxide anion generation, occurs at concentrations well below those that result in myocyte necrosis, may aid in the design of new therapeutic strategies to limit the toxicity of these drugs.

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Key Words: cardiotoxicity ■ dexrazoxane ■ superoxide anion ■ antioxidant

The anthracycline antibiotics have been a mainstay of cancer chemotherapy for more than 20 years, although the development of a dilated cardiomyopathy often limits the cumulative dose that can be delivered.1,2 There appear to be 2 types of clinical cardiotoxicity, which are not necessarily mechanistically related. One type occurs rapidly; is characterized by electrocardiographic changes, arrhythmias, and a reversible decrease in ventricular contractile function; and can occur after a single dose of anthracycline.3 The more common delayed form is characterized by a largely irreversible decrease in ventricular contractile function, the incidence of which is in direct proportion to the cumulative anthracycline dose.1 At cumulative doses of 430 to 600 mg/m² of doxorubicin, the prevalence of ventricular contractile dysfunction rises to 60% of patients.4

However, the mechanism(s) by which anthracyclines cause irreversible myocardial injury remains unclear. Data have been presented that implicate free-radical formation,5,6 lipid peroxidation,7 mitochondrial impairment,8,9 alterations in calcium handling,10 and direct suppression of muscle-specific gene expression.11-13 The quinone moiety of the anthracyclines is known to act as a catalyst for the formation of reactive oxygen species, including superoxide anion and hydrogen peroxide.8 The unique sensitivity of the myocardium to anthracyclines may be due to the low levels of catalase and superoxide dismutase (SOD) found in cardiac myocytes.14 Therefore, approaches to prevention of anthracycline-induced cardiac injury have centered on the use of antioxidants to minimize the generation of free radicals, with mixed results.15-17 Recently, simultaneous administration of the iron chelator dexrazoxane with anthracyclines has been shown to markedly reduce the risk of cardiomyopathy in patients.18 Despite its documented clinical utility, the mechanisms by which dexrazoxane prevents cardiac damage remain unknown.

The chemotherapeutic benefits of anthracyclines appear to be derived from induction of programmed cell death (apoptosis) in malignant tissues.19,20 Therefore, we examined whether the anthracycline daunorubicin could induce programmed cell death in isolated ventricular myocytes. At concentrations relevant to those achieved clinically (1 $\mu\text{mol/L}$), daunorubicin was found to induce myocyte apoptosis. Only at higher concentrations ($\geq 10 \mu\text{mol/L}$) did daunorubicin induce necrosis in isolated cardiac myocytes. Both dexrazoxane and the
SOD mimetic manganese(II/III)tetrakis(1-methyl-4-peridyl)porphyrin (MnTMPyP) had no effect on daunorubicin-induced necrosis, yet did inhibit apoptotic cell death in cardiac myocytes.

Materials and Methods

Chemicals
Daunorubicin, α-tocopherol, ascorbic acid, SOD, fumonisin B₁, N-acetylcyesteine (NAC), and pyrrolidinedithiocarbamate (PDTC) were from Sigma. MnTMPyP was from Calbiochem. Dexrazoxane was from Pharmacia.

Isolation and Culture of Cardiac Myocytes
Neonatal rat ventricular myocytes were isolated from 1-day-old Sprague-Dawley pups as previously described. Primary adult myocyte isolates were isolated from male Sprague-Dawley rats (225 to 275 g) as previously described.21 Adult myocytes were cultured in DMEM supplemented with 7% FCS (Gibco BRL) as previously described.21,22 Culture medium was removed and centrifuged at 3000 g for 5 minutes to exclude trypan blue. For detection of DNA fragmentation using the comet assay, cells were plated at a density of 1000/mm². Neonatal myocytes were cultured at 37°C, 5% CO₂ in DMEM supplemented with 7% FCS (Gibco BRL) for 48 to 72 hours, with addition of fresh medium before treatment with daunorubicin. Using this method, we routinely obtained cultures with <5% nonmyocytes. Calcium-tolerant adult rat ventricular myocytes were isolated from male Sprague-Dawley rats (225 to 275 g) as previously described.21 Primary adult myocyte isolates were cultured in defined medium consisting of DMEM with 25 mmol/L HEPES and 44 mmol/L NaHCO₃, with 4.2 mmol/L l-glutamine (Gibco BRL), supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mg/mL BSA, 2 mmol/L l-carnitine, 5 mmol/L creatine, 5 mmol/L taurine, 0.1 mmol/L triiodothyronine, and 100 mmol/L insulin from bovine pancreas (Sigma). All culture dishes used for adult myocytes were pretreated with laminin (Gibco BRL) as previously described.21 Adult myocytes were cultured for 18 to 24 hours before treatment with daunorubicin. After incubation with daunorubicin for 24 hours, cells were fixed in 4% formaldehyde in PBS before staining for quantification of apoptotic cell death.

Detection of Apoptosis and Necrosis

Quantification of DNA Fragmentation by Gel Electrophoresis
Myocyte-membrane integrity was measured using the ability of cells to exclude trypan blue. For detection of DNA fragmentation using gel electrophoresis, DNA was isolated from neonatal myocytes. Culture medium was removed and centrifuged at 3000g for 5 minutes to collect any detached myocytes. Adherent cells were lysed with a hypotonic lysis buffer (10 mmol/L Tris- HCl [pH 7.4], 10 mmol/L EDTA, 0.5% Triton X-100) and then pooled with the pellet made up of detached cells. After incubation at 4°C for 10 minutes, lysates were centrifuged at 10 000g for 15 minutes. Supernatants were incubated with RNase A for 1 hour at 37°C, followed by proteinase K for 1 hour at 37°C. DNA was precipitated overnight at 4°C with 0.1 volume of 5 mol/L NaCl and 1 volume of isopropanol and then electrophoresed on a 2.5% agarose gel and visualized under UV light after staining with ethidium bromide.

Comet Assay
The comet assay was also used to detect DNA fragmentation in individual cells. Briefly, cells were suspended by treatment with trypsin and pooled with suspended cells in PBS. Cells were then embedded in 1% low–melting-temperature agarose in PBS and layered onto frosted slides in a sandwich between layers of 1% low–melting-temperature agarose in PBS. Cells were then stained with 200 µg/mL 4',6-diamino-2-phenylindole (DAPI)–Stained Cells
Fixed cells were stained for 30 minutes in PBS containing 10 µmol/L 99 Hoescht 33258 as well as 2 U/mL Texas Red-conjugated phalloidin (Molecular Probes) to stain filamentous actin.

Flow Cytometric Analysis of Propidium Iodide (PI)–Stained Cells
Flow cytometric analysis of cells fixed in 70% ethanol/PBS and stained with PI (Sigma) was performed to quantify the percentage of cells undergoing apoptosis. This method is based on the observation that cells undergoing apoptosis when fixed in ethanol and stained with PI have a hypodiploid quantity of DNA and localize in a broad area below the G₀/G₁ peak on a PI histogram. Briefly, cells were suspended as described above, with care to include any cells that had already detached and fixed in 70% ethanol. This fixation is necessary to allow small fragments of DNA to leave the cell. If flow cytometry could not be performed on the same day, then samples were stored at −20°C in ethanol for no longer than 1 week. Cells were resuspended both with PBS and then suspended in PBS with PI (20 µg/mL) and RNase A (5 Kunitz units/mL, Sigma). Analyses were performed after a minimum 30-minute incubation at room temperature. Ten thousand cells from each sample were counted using a Becton Dickinson FACSscan (excitation, 488; emission, 590). Gating was performed on the cells to exclude very small debris with an enhancement factor was calculated for each group of experiments as (OD₄₀₅ – background) / (OD₃₄₂ – background)×100, after subtraction of background OD₃₄₂.

ELISA for Histone-Bound DNA Fragments
Apoptosis was also quantified in neonatal ventricular myocytes using a commercially available ELISA for histone-bound DNA fragments (Boehringer). Plates were plated in a 24-well culture dish and grown to confluence. Lysis of cells was done according to the manufacturer's directions after 2 washes in PBS. Samples were run in triplicate, with optical density (OD) measured at 405 nm. The apoptosis enhancement factor was calculated for each group of experiments as (OD treatment/OD control)×100, after subtraction of background OD₄₀₅.

Myocyte Oxidative Stress
2',7'-Dichlorofluorescin (DCF) was used as a measure of the oxidative stress induced by daunorubicin. Flow cytometry was also used to measure levels of reactive oxygen species in myocytes. Myocytes were incubated in the presence or absence of daunorubicin and 100 µmol/L H₂O₂. After H₂O₂ treatment, there was evidence of DNA fragmentation and comet formation under alkaline conditions, but no comets were seen under neutral conditions, as would be expected for single-stranded DNA breaks.

Terminal Deoxynucleotidyl Transferase–Mediated Nick-End Labeling (TUNEL) Assay
Apoptosis was detected in adult myocytes using the TUNEL assay. Labeling (3' end) of DNA with digoxigenin was done and stained with peroxidase substrate via a horseradish peroxidase-conjugated anti-digoxigenin antibody using a commercially available kit (Apoptag, Oncor) following the manufacturer’s directions with the modification of diluting the terminal deoxynucleotidyl transferase enzyme 8-fold. Cells were counterstained with methyl green (Sigma) and magnified at ×400. In these experiments, many cell fragments were seen on the coverslips that stained positively. Cells were counted as apoptotic as long as they retained some cytoplasm surrounding a positively stained nucleus or nuclear fragment. For each experimental condition, contiguous visual fields were counted to accumulate data on at least 200 total myocytes per condition per experiment.

Detection of Chromatin Condensation With Hoescht 33258
Neonatal myocytes were also stained with Hoescht 33258 for detection of chromatin condensation characteristic of apoptosis. Fixed cells were stained for 30 minutes in PBS containing 10 µmol/L Hoescht 33258 as well as 2 U/mL Texas Red-conjugated phalloidin (Molecular Probes) to stain filamentous actin.

2',7'-Dichlorofluorescin (DCF) was used as a measure of the oxidative stress induced by daunorubicin. Flow cytometry was also used to measure levels of reactive oxygen species in myocytes. Myocytes were incubated in the presence or absence of daunorubicin...
in the culture medium for 3 hours, with or without 1 hour of preincubation with antioxidants. DCF diacetate (DCFDA) was added for the last hour. DCFDA was prepared in ethanol and diluted into myocyte culture medium to a final concentration of 5 mmol/L. DCFDA is taken up by cells and on deacetylation forms a nonfluorescent DCF. On oxidation, this becomes DCF.27 Cells were trypsinized but not fixed, and the mean fluorescence was measured for 10,000 cells using a Becton Dickinson FACScan with excitation at 488 nm and emission at 530 nm (±21 nm). Background fluorescence increased following addition of daunorubicin because of the fluorescence of the anthracycline, with some overlap in the emission spectra for the 2 fluorochromes.25 Thus, specific background for each concentration of daunorubicin was subtracted to obtain a corrected DCF fluorescence.

Statistical Analysis
All data are presented as mean±SEM. Comparison of groups was done using ANOVA, followed by Dunnett multiple-comparison test. Statistical significance was achieved when \( P < 0.05 \).

**Results**

**Daunorubicin Induces Concentration-Dependent Apoptosis and Necrosis in Neonatal and Adult Ventricular Myocytes**

Isolated neonatal ventricular myocytes undergo morphological changes consistent with apoptosis when exposed to 1 \( \mu \)mol/L daunorubicin for 24 hours (Figure 1A and 1B). A significant fraction of the daunorubicin-treated myocytes shrank, although most remained attached to coverslips (Figure 1B). Staining with Hoescht 33258 does not clearly show chromatin condensation because of the intrinsic fluorescence of daunorubicin bound to the DNA in these cells. However, the single-cell electrophoresis ("comet") assay for apoptosis demonstrates double-stranded DNA fragmentation,23 as evidenced by the "tail" of nucleic acid, indicative of apoptosis in myocytes treated with 1 \( \mu \)mol/L daunorubicin (Figure 1C and 1D).

Figure 1. Daunorubicin (1 \( \mu \)mol/L) causes apoptosis in isolated neonatal ventricular myocytes. A and B, Neonatal myocytes stained with Hoechst 33258 and Texas Red-phalloidin. A, Control myocytes. B, Myocytes exposed to 1 \( \mu \)mol/L daunorubicin for 24 hours. C and D, Comet assay for detecting apoptosis is illustrated for control myocytes (C) and for myocytes exposed to 1 \( \mu \)mol/L daunorubicin for 18 hours (D). Single cells are embedded in agarose and electrophoresed and stained with the fluorescent agent YOYO-1. Single-cell electrophoresis shows the presence of "comets," characteristic of DNA fragmentation, in cells treated with 1 \( \mu \)mol/L daunorubicin. No comets are seen in myocytes not exposed to daunorubicin. Photomicrographs shown are representative of at least 3 experiments for each condition (bar=40 \( \mu \)m for panels A and B, 100 \( \mu \)m for panels C and D).
DNA fragmentation on gel electrophoresis indicative of apoptosis was also observed (Figure 2A). Untreated neonatal myocytes, or those exposed to 0.1 µmol/L daunorubicin for 18 hours, exhibited little DNA fragmentation, whereas 1 µmol/L daunorubicin produced clear DNA fragmentation. These and subsequent experiments were done with 18-hour treatments of daunorubicin to look for evidence of DNA fragmentation before onset of morphological changes.

Myocytes exposed to 1 µmol/L daunorubicin for 18 hours excluded trypan blue, similarly to untreated myocytes (Figure 2B), as expected for cells early in apoptosis. However, myocytes exposed to 10 µmol/L daunorubicin had increased uptake of trypan blue. Importantly, and in contrast to myocytes exposed to lower concentrations of the anthracycline, there was no detectable increase in DNA laddering either at 6 or 18 hours with 10 µmol/L daunorubicin (6 hour data not shown). This loss of membrane integrity with 10 µmol/L daunorubicin, in the absence of DNA fragmentation at these higher concentrations of drug, is consistent with necrosis.

When apoptotic cell death in neonatal myocytes exposed to 1 µmol/L daunorubicin was quantified by flow cytometry (Figure 3), the percentage of apoptotic cells increased from 5% to 15% (n=11 independent experiments, P<0.001; Figure 3C). The baseline rate of apoptosis is variable, from <1% in some experiments to as much as 9%. In all experiments, there was a higher percentage of apoptosis in the daunorubicin-treated cells. These data were supported by less quantitative assays as well. Using the comet assay, the percentage of apoptotic cells increased from ~1% in untreated myocytes to 13% in myocytes exposed to 1 µmol/L daunorubicin. This concentration of drug, when apoptosis was quantified by ELISA assay for histone-bound DNA fragments, resulted in an “apoptosis enhancement factor” of 18 (range 2.4 to 55.6, n=3 independent experiments). While each of these methods gave a similar magnitude of induction of apoptosis by 1 µmol/L daunorubicin, flow cytometry showed the least variability, likely because of the large number of cells that can be analyzed in each experiment.

We also verified that a similar phenomenon occurred in the adult ventricular myocyte phenotype. Adult myocytes in primary culture in defined medium were exposed to 1 µmol/L daunorubicin for 18 hours and examined for evidence of DNA fragmentation with the TUNEL technique (Figure 4). Exposure to 1 µmol/L daunorubicin increased the fraction of TUNEL-positive myocytes from 6% to 22% (n=3, P<0.005; Figure 4B). Exposure to 10 µmol/L daunorubicin also increased the number of apoptotic myocytes in adult ventricular myocyte primary isolates. However, as in neonatal myocytes, the majority of cells became rounded up and failed to exclude trypan blue, indicating that there was also necrotic cell death at this higher concentration of drug (data not shown).

Daunorubicin, Oxidative Stress, and Apoptosis
Apoptosis induced in neoplastic cells by anthracyclines had been shown to be mediated by the activation of ceramide synthase, at least in some cell lines, and is inhibitable by the fungal toxin fumonisin B1. However, pretreatment of ventricular myocytes for 3 hours with fumonisin B1 (25 µmol/L) did not protect against daunorubicin-induced apoptosis, as assessed by the comet, ELISA, or flow cytometric assays (data not shown; n=3 independent experiments), indicating that activation of ceramide synthase does not appear to be required for anthracycline-induced apoptosis in cardiac myocytes.

Anthracyclines are also known to catalyze the formation of superoxide anion, hydrogen peroxide, and hydroxyl radical, and these reactive oxygen species are thought to mediate, at
least in part, the cardiac toxicity of anthracyclines. We examined whether the antioxidants NAC (100 μmol/L), α-tocopherol (1 mmol/L) or ascorbic acid (1 mmol/L), could affect daunorubicin-induced apoptosis when administered 3 hours before the anthracycline. We loaded myocytes with DCFDA to confirm that these antioxidants were decreasing the level of reactive oxygen species in both the absence and presence of daunorubicin. Exposure of myocytes to antioxidants in the absence of daunorubicin caused a clear decrease in DCF fluorescence, consistent with a decline in the level of reactive oxygen species (Figure 5A). Even in the presence of daunorubicin, both antioxidant treatments maintained a level of DCF fluorescence that was lower than that observed in control myocytes not exposed to antioxidants. However, neither the antioxidant NAC nor the combination of α-tocopherol and ascorbic acid ameliorated the increase in apoptotic-cell death induced by 1 μmol/L daunorubicin (Figure 5B). Thus, it would appear that daunorubicin-induced apoptosis in myocytes does not occur through increases in DCF-detectable reactive oxygen species. Moreover, as shown in Figure 5B, treatment of cells with either NAC or the antioxidant PDTC (100 μmol/L), which also decreased the level of DCF fluorescence, caused a significant increase in myocyte apoptosis over baseline, control levels, even in the absence of daunorubicin.

**Daunorubicin-Induced Apoptosis and Superoxide Anion**

The ability of both PDTC and daunorubicin to induce apoptosis in cardiac myocytes suggested a potential mechanism of action. It is known that PDTC chelates copper and thereby inactivates the copper-dependent cytosolic and extra-
cellular forms of SOD.\textsuperscript{28} We examined whether superoxide anion could be a mediator of daunorubicin-induced apoptosis by determining whether the superoxide anion dismutase-mimetic porphyrin MnTMPyP\textsuperscript{29} or exogenous SOD could inhibit anthracycline-induced programmed cell death. While exogenous SOD had no effect on apoptosis (data not shown), MnTMPyP (50 \textmu M) markedly diminished daunorubicin-induced apoptosis, as determined by flow cytometric analysis of PI-labeled myocytes (Figure 6A).

We next examined whether dexrazoxane, used clinically to prevent anthracycline-induced cardiomyopathy in humans,\textsuperscript{18} could inhibit daunorubicin-induced apoptosis in vitro. Dexrazoxane is used typically at 10:1 molar ratios with anthracyclines to prevent cardiac toxicity in both experimental animal preparations and in humans.\textsuperscript{18,30} As shown in Figure 6A, myocyte apoptosis induced by 1 \textmu M daunorubicin, quantified by measuring the hypodiploid fraction or flow cytometry of PI-labeled cells, was prevented when 10 \textmu M dexrazoxane added to the medium of neonatal myocytes at the same time as anthracycline. There was a significant drop in myocyte-membrane integrity caused by 10 \textmu M daunorubicin (*P<0.01) that was not altered by the presence of dexrazoxane. Data are mean\pm SE for 4 independent experiments.

**Discussion**

**Anthracyclines and Apoptosis**

The mechanism(s) by which the anthracyclines cause cardiotoxicity has been the subject of much investigation and debate for more than 2 decades. The data presented here suggest that...
this largely permanent loss of contractile function may occur, in part, through initiation of programmed cell death of ventricular myocytes. Early in vitro work with cultured cardiac myocytes had shown direct cardiotoxicity of anthracyclines at concentrations that approximated those relevant clinically. In retrospect, the results of these investigators appear to suggest that these drugs initiated programmed cell death of myocytes. For example, Tobin and Abbott31 found that 1.7 μmol/L doxorubicin produced death of some myocytes in primary culture, and ultrastructural analysis showed nuclear fragmentation, segregation, and chromatin clumping, characteristics considered typical for cells undergoing programmed cell death. However, Lampidis et al32 found effects of doxorubicin on cultured neonatal rat ventricular myocytes over a broad concentration range (0.1 to 100 μmol/L). They described rapid myocyte degeneration at the upper end of this concentration range, but at concentrations of 1.8 μmol/L and below, myocytes continued to beat spontaneously for >24 hours. This is similar to our finding that there is a switch from overt myocyte necrosis to initiation of programmed cell death that occurred at daunorubicin concentrations between 1 and 10 μmol/L. Newman et al33 also found that a 24-hour exposure to 1.8 μmol/L daunorubicin caused no decrease in viability of neonatal myocytes, as assessed by trypan blue exclusion, although there was a significant accumulation of cardiac muscle-specific enzymes in cell culture media, indicative of some myocyte damage.

Many apparently distinct effects of anthracyclines on cardiac myocytes have been found, and it is unclear what role each of these play in the cumulative toxicity of these drugs. Anthracyclines at concentrations at or below 1 μmol/L induce increases in myocyte intracellular calcium, probably mediated by a cAMP-dependent protein kinase10 and by activation of L-type calcium channels.34 Importantly, doxorubicin at similar, clinically relevant concentrations has been shown by Kurabayashi et al11,12,35 in a series of reports to inhibit the transcription of a number of striated muscle-specific genes, as well as to activate specific genetic responses that are independent of stress-related activator protein-1, protein kinase A, or protein kinase C activation. Recently, using an mRNA differential-display strategy, these investigators demonstrated a rapid decline in mRNA of nuclear genes coding for proteins involved in myocyte energy production, as well as of a previously unrecognized negative regulator of cardiac-specific gene expression termed CARP (for cardiac Adriamycin-responsive proteins).36 These authors speculate that this sequence of events could have been initiated by a signal-transduction cascade triggered by oxygen radicals.37 It is possible that myocyte apoptosis is a consequence of 1 or more of these electrophysiological or transcriptional events triggered by the anthracyclines.

Although programmed cell death has been clearly documented now to participate in the tumoricidal action of anthracyclines, apoptosis has not been recognized in the heart with these drugs. Zhang et al38 have reported evidence of apoptosis in the kidney and intestines, but not the heart, of spontaneously hypertensive rats after treatment with doxorubicin. Our results suggest that programmed myocyte cell death should occur relatively early after anthracycline administration. When Unverferth et al39 obtained myocardial biopsies within 24 hours of doxorubicin administration, the most striking features they identified were mitochondrial swelling and nucleolar contraction. It is tempting to speculate that the chromatin condensation occurring in these myocytes represented a transient wave of programmed cell death in the myocardium. Incremental small decreases in ventricular systolic function might not be readily apparent clinically, and the delay in the clinical appearance of cardiomyopathy in these patients is likely a function of the imperfect correlation between left-ventricular function and symptomatic heart failure. Indeed, when sensitive provocative testing has been performed, such as exercise echocardiography, decrements in contractile function have been evident even at low cumulative anthracycline doses.40 Finally, it has been noted recently that a rise in serum levels of cardiac-specific troponin can be detected following a single dose of anthracycline.41 This observation is consistent with the concept that actual myocyte death, and not only changes in myofibrillar organization, myocyte-specific gene expression, or cellular electrophysiology, may occur at low cumulative drug concentrations.

**Anthracyclines and Reactive Oxygen Species**

The inhibition of daunorubicin-induced apoptosis in myocytes by the iron chelator dexrazoxane and the SOD mimic MnTMPyP indicates that reactive oxygen species play a role in the triggering of apoptosis in myocytes. Early investigations into the mechanisms contributing to anthracycline-induced cardiotoxicity suggested that drug-induced oxygen-radical cascades played a role in the cardiotoxic effects of these drugs. Biochemical and biophysical measurements in cardiac mitochondrial preparations showed that the quinone moiety of the anthracyclines was reduced by 1 electron at complex I of the mitochondrial electron transport chain to form superoxide anion.42,43 At anthracycline concentrations from 20 to 200 μmol/L, there was a rapid increase in production of superoxide anion, with subsequent increases in H₂O₂ and OH⁻ by submitochondrial particles.44,45 In the intact heart, formation of OH⁻ peaks at Adriamycin concentrations of 1 μmol/L and is inhibited by treatment with exogenous SOD, catalase, and dexrazoxane.46 Iron acts as a cofactor in the formation of reactive oxygen species catalyzed by the quinone group of anthracyclines, and thus dexrazoxane may bind iron and displace it from iron-anthraclycline complexes.47 Iron also acts as a cofactor in the formation of OH⁻ by the Fenton reaction from H₂O₂ and dexrazoxane may be working at this level as well.

H₂O₂ alone appears less important for the induction of myocyte apoptosis. Although Sarvazyan25 was able to show rapid increases in DCF fluorescence indicative of significant oxidative stress in single ventricular myocytes treated with Adriamycin doses at or above 80 μmol/L, we were unable to detect any increase in DCF fluorescence in myocytes treated with 1 μmol/L daunorubicin at 3 hours. This suggests that at these lower, clinically relevant concentrations, the increase in formation of H₂O₂ in the myocyte may be relatively small, although we cannot exclude the possibility that a longer exposure to daunorubicin at these concentrations might result in a measurable increase in DCF fluorescence. Moreover,
treatment of myocytes with NAC or the combination of α-tocopherol and ascorbic acid caused an easily detectable decrease in DCF fluorescence, consistent with their “antioxidant” effects, yet neither of these antioxidant treatments was able to prevent daunorubicin-induced apoptosis. Although interpretation of the NAC experiments is complicated by independent effects of NAC on myocyte apoptosis, together, these antioxidant experiments suggest that H2O2 is not an essential mediator of the apoptotic signal induced by daunorubicin.

In contrast to H2O2, superoxide anion is poorly quenched by NAC.48 In addition, at neutral pH in the presence of metal ions, cysteine and other sulfhydryl compounds exhibit autoxidation behavior and, at concentrations such as those used here for NAC, can produce superoxide anion at quantities measurable by SOD-inhibitable reduction of cytochrome c.49 Thus, the increase in apoptosis observed in the presence of NAC may also have been due to generation of superoxide anion or other reactive oxygen species. Likewise, superoxide may also be a mediator for the increased apoptosis caused by PDTC, since PDTC is known to increase the level of superoxide anion both by the inhibition of Cu/Zn-SOD and by autoxidation.24 Interestingly, both NAC and PDTC have been shown to cause apoptosis in vascular smooth muscle cells through a mechanism that was attributed to the antioxidant properties of these compounds.50 Given the multiple effects of these compounds, including inhibition of nuclear factor κB,51 further investigation is necessary before firm conclusions can be drawn regarding the mechanisms of NAC- and PDTC-induced apoptosis.

The ability of the SOD-mimetic MnTMPyP to inhibit daunorubicin-induced apoptosis is the strongest evidence that superoxide anion mediates a selective apoptotic signal. The narrow window of daunorubicin concentrations that result in induction of apoptosis without necrosis may be due to a limited range of drug concentrations in which superoxide anion is selectively increased without an appreciable rise in the formation of additional cytotoxic oxygen species, such as H2O2 and OH-. However, the fact that both an iron chelator and an SOD mimic gave the same result suggests that daunorubicin-induced apoptosis may involve downstream products of superoxide. Interestingly, overexpression of manganese-SOD in mice protected against some of the early mitochondrial damage after Adriamycin treatment, as well as the level of serum markers of myocyte death, although myocyte apoptosis was not examined in this study.52

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