Mitochondrial dysfunction is an early indicator of doxorubicin-induced apoptosis

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Abstract

Generation of reactive oxygen species and mitochondrial dysfunction has been implicated in doxorubicin-induced cardiotoxicity. This study examined pro-apoptotic mitochondrial cell death signals in an H9C2 myocyte rat cell line and in isolated rat heart mitochondria exposed to doxorubicin. Mitochondrial and cellular viability were assessed using an MTT viability assay (formazan product formed by functional mitochondrial dehydrogenases) and calcein AM dye (fluoresces upon cleavage by cytosolic esterases). Mitochondrial dysfunction followed by cell death was observed using nM concentrations of doxorubicin. Significant doxorubicin-induced cell death was not apparent until after 6 h following doxorubicin exposure using the calcein AM assay. The involvement of apoptosis is evidenced by an increase in TUNEL (terminal (TdT)-mediated dUTP-biotin nick end labeling)-positive nuclei following doxorubicin treatment. Furthermore, doxorubicin administered to isolated mitochondria induced a rapid increase in superoxide production, which persisted for at least 1 h and was followed by increased cytochrome c efflux. In addition, caspase-3 activity was increased with doxorubicin administration in the H9C2 myocyte cell line. An oxidant-mediated threshold of mitochondrial death may be required for doxorubicin-induced apoptosis.
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1. Introduction

Doxorubicin is a powerful anthracycline antibiotic used to treat a multitude of human neoplasms; however, cardiac toxicity compromises the clinical usefulness of the drug [1,2]. It is believed that mitochondria play a significant role in this toxicity as isolated heart mitochondria and myocytes have been shown to give rise to increased levels of oxygen radicals after doxorubicin treatment [3–8]. The chronic side effects of doxorubicin are irreversible and devastating for the patient and include the development of cardiomyopathy and ultimately congestive heart failure [1,2]. Although recent evidence shows that less toxic doses of doxorubicin can be used effectively, heart failure in doxorubicin treated patients can go undetected for many years [9]. Therefore, it is essential to identify the mechanisms by which doxorubicin is cardiotoxic so that interventions to prevent the cardiotoxic effects of doxorubicin can be developed.

Doxorubicin-induced cardiomyopathy is partially caused by increased oxidant production in the heart [6,10–14]. The mitochondria are believed to be a primary sink for doxorubicin and specific dehydrogenase systems produce superoxide anions [7]. Single electrons are shuttled to doxorubicin, giving rise to oxygen radicals through the auto-oxidation of doxorubicin and semiquinones [7,15]. Moreover, Kotamraju et al. [6] recently showed that hydrogen peroxide produced from the superoxide anion might be the key oxidant responsible for doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes.

Mitochondria play a pivotal role in regulating apoptosis by mechanisms such as release of cytochrome c that results in the activation of caspases and subsequent cell death [16,17]. The mitochondria respond to several signals (i.e. reactive oxygen species, altered redox status, and increases in calcium levels), which trigger the release of caspase-activating proteins, such as cytochrome c and apoptosis-inducing factor [18]. The release of cytochrome c from the mitochondria into the cytosol can result in the activation of caspase-9, which is responsible for activating effector caspases, such as caspase-3, to induce cell death.

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This study examined doxorubicin-induced mitochondrial dysfunction and pro-apoptotic markers in a rat cardiac myocyte cell line and in isolated mitochondria from rat heart. In this study, we show that mitochondrial dysfunction may be an important early step in the regulation of doxorubicin-induced release of cytochrome c from the mitochondria. Our results show that when H9C2 cells are exposed to very low (nM) concentrations of doxorubicin, a rapid decline in mitochondrial reduction potential precedes cell death by approximately 6 h, implicating mitochondrial dysfunction as an early event in doxorubicin-induced cardiotoxicity. In addition, we show that doxorubicin treatment to isolated mitochondria causes a rapid increase in radical production, which can be maintained for over 1 h. Understanding the time-course of doxorubicin toxicity and the underlying causes of doxorubicin-induced cardiomyopathy may lead to specific therapeutic interventions that can attenuate these effects.

2. Methods

2.1. Cell culture

H9C2 cells (ATCC, Rockville, MD) were maintained in DMEM supplemented with 10% FBS in 95% air:5% CO_2 using standard culture methods. Cells were plated 24 h prior to doxorubicin treatment at a density of 150 cells/mm^2.

All studies were conducted in the presence of 10% FBS. Although growth factors and other possible confounding factors are present in the serum, removing the serum from the experiments could also confound interpretation through altering the baseline viability of the cultures and causing apoptotic changes independently of the doxorubicin treatment.

2.2. Viability assay

Cells were rinsed once with PBS followed by incubation at (37 °C) with 25 μM calcein AM and 1 mg/ml propidium iodide for 15 min. Fluorescence was measured at ex 485/em 530 and ex 480/em 620, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was determined by incubating cells with 0.25 mg/ml MTT in the culture media at 37 °C for 3 h. Formazan crystals were solubilized by overnight incubation in 50% *N,N*-dimethyl formamide, 20% sodium dodecyl sulfate, pH 4.8 and absorbance determined at 575 nm.

2.3. Mitochondrial isolation

Mitochondria were immediately isolated from the ventricles of a rat heart as previously described [19]. Briefly, tissue was weighed and minced in 10 volumes of isolation buffer (0.225 M mannitol, 0.075 M sucrose, 0.2% fatty acid free bovine serum albumin, pH 7.4). The tissue was homo-genized in a Potter-Elvehjem glass homogenizer and centrifuged for 7 min at 480×*g*. The resulting supernatant was stored on ice while the pellet was resuspended in 5 ml of isolation buffer and centrifuged for 7 min at 480×*g*. The combined supernatants were centrifuged for 7 min at 7700×*g*. The supernatant was decanted and the mitochondrial pellet was resuspended in 5 ml of wash buffer (0.225 M mannitol, 0.075 M sucrose, 0.001 M EGTA, pH 7.4) using a cotton swab so as not to break the mitochondria and then centrifuged for 7 min at 7700×*g*. The final mitochondrial pellet was resuspended in 1 ml of storage buffer (0.25 M sucrose, 0.002 M EDTA pH 7.4) and immediately used for experiments.

2.4. Superoxide anion measurement

Superoxide anion generation in isolated rat heart mitochondria was determined immediately following the isolation procedure. Briefly, mitochondria (0.5 mg/ml) were incubated with buffer (6 mM succinate, 70 mM sucrose, 220 mM mannitol, 2 mM, Hepes, 25 mM KH_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 5 μg/ml catalase, pH 7.4) and 10 μM doxorubicin at 37 °C. At the indicated time points, 40 μM acetylated cytochrome c was added and the change in absorbance at 550 nm was measured for 1 min at 37 °C with a Beckman spectrophotometer. Background absorbance for all groups was determined by the addition of SOD (1000 units/ml).

2.5. Markers for apoptosis

TUNEL (terminal (TdT)-mediated dUTP-biotin nick end labeling) staining was performed using ApoTag Plus kit (Intergen Company, Purchase, NY) according to the manufacturer's instructions. Caspase-3-like activity was measured using the synthetic peptide *N*-acetyl-DEVD-AMC (BD PharMingen, San Diego, CA). This assay detects activated caspase-3 and, to a lesser extent, caspase-6, -7, and -8. Although the caspase-3 assay can detect caspase-8, the difference in sensitivity for the two caspases in the assay (the assay is 20-fold more sensitive for caspase-3) argues that a substantial portion of the signal is caspase-3. Caspase-1 like activity was measured using the synthetic peptide *N*-acetyl-YVAD-AMC (BD PharMingen). Active caspases will cleave the AMC from the peptide and the free AMC will fluoresce. Standards of active caspase-3 and caspase-1 were also prepared. Briefly, 1 ml of assay buffer (20 mM HEPES, 10% glycerol, 2 mM DTT (2 μl of a 1 M solution) and 14 μl of peptide/ml of buffer) and 50 μl of sample were added to a microcentrifuge tube and protected from the light. Samples were incubated at 37 °C for 60 min (caspase-3) or 120 min (caspase-1); after which fluorescence was measured on a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Cytosolic cytochrome c was quantified using an
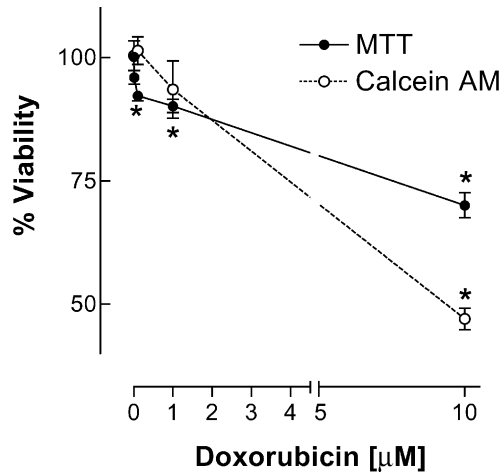
Fig. 1. Doxorubicin exposure reduces viability of H9C2 cells. H9C2 cells were exposed to very low (nM) and high concentrations (10 μM) of doxorubicin. Mitochondrial viability was determined using the MTT conversion assay and cellular viability was determined by the Calcein AM assay 20 h after addition of doxorubicin (see Methods). There was an immediate reduction (nm concentrations) in mitochondrial function determined by MTT conversion rate. Both the 100 nM, 1 μM, and 10 μM concentrations of doxorubicin are significantly ($P<0.05$) different from control. In contrast, the calcein assay (cellular viability) was not significant until 10 μM of doxorubiucin. Data were normalized to untreated cells as 100% and shown as mean±S.E. for 8 wells per group versus vehicle-treated cells. *$P<0.05$ vs. control.

ELISA kit from R&D Systems (Minneapolis, MN). The cytochrome *c* assay is calibrated against a highly purified natural rat cytochrome *c* protein isolated from rat heart. Based upon amino acid analysis, the molecular weight of this protein is 12.1 kDa and contains less than 5% of the reduced form of cytochrome *c*. This immunoassay recognizes both the oxidized and reduced forms of cytochrome *c*. Moreover, the cross-reactivity is approximately 7.2% and the limit of detection is 0.5 ng/ml, while the intra-assay and inter-assay precision is between 1% and 4%. Results were reported as arbitrary fluorescence units normalized to milligrams of protein.

2.6. Protein concentration

Cytosolic and mitochondrial protein concentrations were determined using the method developed by Bradford [20].

2.7. Statistical analysis

All experiments were performed successfully at least three times. Unpaired *t* tests were used for comparisons between two groups and analysis of variance with a Tukey post hoc test was used for multiple comparisons. Analysis of variance with repeated measure was performed when multiple time points were recorded from the same sample. All statistics were calculated using a statistical package from Prism (San Diego, CA). A *P* value of <0.05 was considered significant.

3. Results

3.1. Toxic effects of doxorubicin treatment in an H9C2 cultured myocyte cell line

We evaluated the role of oxidant production, mitochondrial dysfunction, and apoptosis in doxorubicin toxicity using a myocyte cell line as well as isolated heart mitochondria. Doxorubicin exposure resulted in dose-dependent toxicity in the H9C2 cardiac myocyte cell line as determined by both the MTT and calcein AM viability assay (Fig. 1). The conversion of MTT to a formazan product by mitochondrial dehydrogenases was used as an index of mitochondrial viability, and the calcein AM assay, which depends upon cleavage by cytosolic esterase activity, was used as an indicator of cellular viability. There was a significant reduction in mitochondrial function using nanomolar concentrations of doxorubicin although loss of viability required micromolar concentrations, indicating that low concentrations of doxorubicin are sufficient to cause significant mitochondrial dysfunction, but not a significant loss in cell number (Fig. 1). Greater reduction in mitochondrial function was seen with a concentration of 10 μM. This dose reduced MTT conversion by about 30%, which corresponded to a significant decline in cell viability, suggesting that this dose caused not only damage to the mitochondrial population but also may have induced an increased rate of apoptosis. We continued to use 10 μM concentration of doxorubicin in our experiments since similar levels are seen transiently in plasma following pharmaceutical use in patients. Time course analysis revealed a reduction in
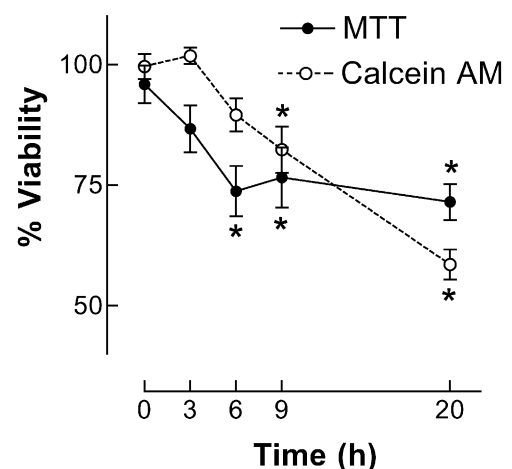

Fig. 2. Doxorubicin exposure of H9C2 cells results in inhibition of mitochondrial MTT conversion prior to cell death. H9C2 were exposed to 10 μM doxorubicin and viability was determined after 3, 6, 9, and 20 h. Mitochondrial function determined by the MTT viability assay was reduced more rapidly as compared to cellular viability. Data were normalized to untreated control cells at each time point and shown as mean±S.E. for 4–6 wells per group. *$P<0.05$ vs. control.

MTT conversion as early as 3 h in some experiments but reproducibly was lower 6 h following exposure to 10 μM doxorubicin (Fig. 2). In contrast, using the calcein AM assay, the toxic effects of 10 μM doxorubicin were not seen until 9 h of exposure (Fig. 2). The difference in the time course of these viability markers indicates that mitochondrial dysfunction is an early event in doxorubicin toxicity in these cells.

3.2. Evidence for doxorubicin-induced apoptosis in the H9C2 cell line

H9C2 cells were exposed to low (1 nM) and high concentrations (10 μM) of doxorubicin for 20 h. Exposure of H9C2 cells to 10 μM of doxorubicin for 20 h resulted in a 45% increase in TUNEL-positive cells (Fig. 3). This is notable as there was an overall decrease in total nuclei,
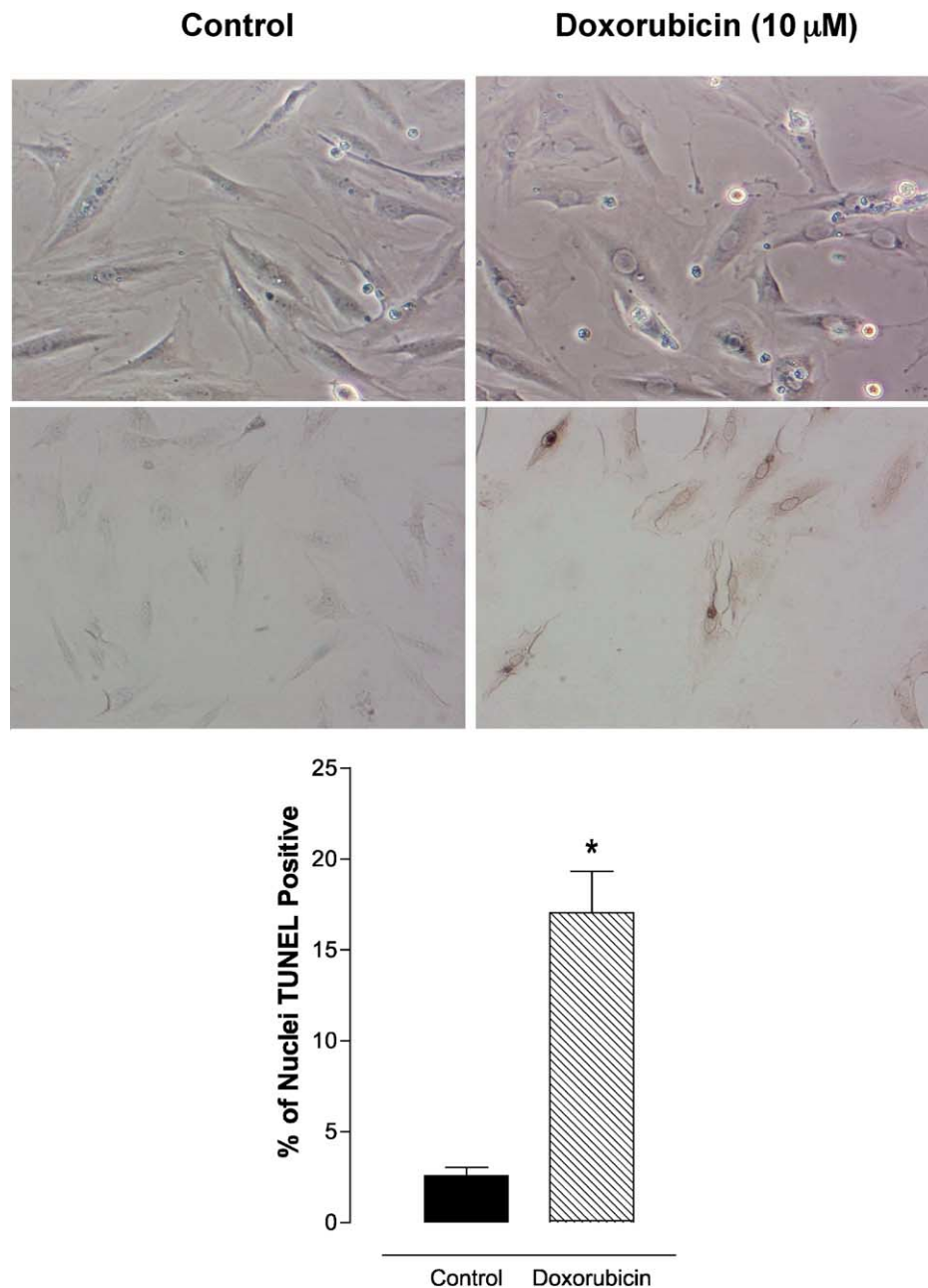

Fig. 3. Doxorubicin exposure increases cell death and TUNEL-positive nuclei in H9C2 cells. Top panels show photomicrographs of doxorubicin-treated H9C2 cells. H9C2 cells were plated at a density of 5000 cells per well in a 16-well slide and were exposed to 10 μM doxorubicin for 20 h. The top two panels are phase-contrast micrographs taken prior to TUNEL staining and the bottom two panels show the results of the TUNEL stain. Noticed marked swelling of H9C2 cells and an increase in the size of the nuclei occurred. The TUNEL analysis was quantified and bottom figure depicts the percent of total nuclei stained positive. Shown are mean±S.E. for n=8 wells per group. *P<0.001 vs. control.
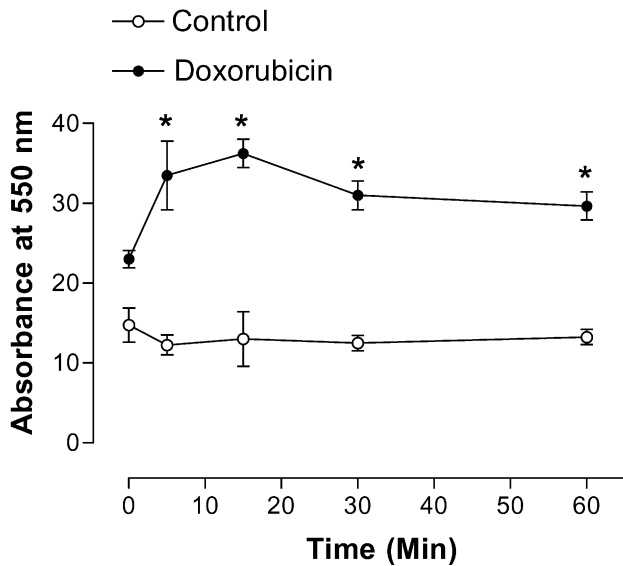
Fig. 4. Doxorubicin rapidly increased superoxide anion production in isolated rat heart mitochondria. Mitochondria were isolated from a Sprague–Dawley rat heart to obtain sufficient amounts of mitochondria to determine superoxide production. Following the isolation, mitochondria (0.5 mg/ml) were treated with 10 μM doxorubicin and cytochrome c reduction was monitored at the different time points. Superoxide production increased rapidly and was sustained for at least 1 h. Shown are mean±S.E. for $n=4$. *$P<0.05$ vs control.

of lower concentrations of doxorubicin (1 nM) on TUNEL staining. These results indicate that the increase in apoptotic nuclei occurs in a similar dose range as the loss in cell number, indicating the importance of apoptosis in doxorubicin toxicity in these cells.

3.3. Effects of doxorubicin on isolated rat mitochondria

We used mitochondria isolated from rat hearts, since using our cell line it would have been very difficult to isolate sufficient mitochondrial protein for assay analysis. Further, H9C2 cells are rat heart derived; therefore, the mitochondria are from a similar source. Doxorubicin exposure (10 μM) resulted in a remarkable increase in superoxide anion production by isolated rat heart mitochondia (Fig. 4). The increase in superoxide production was rapid (within 5 min) and peaked at 15 min following treatment. This increase in superoxide production persisted through 1 h of exposure. The very modest decline in superoxide production (15% after 30 min) is not statistically significant ($P=0.18$)

strongly suggesting the involvement of apoptosis in doxorubicin toxicity in these cells. In addition, profound swelling of myocytes and cell nuclei was observed upon doxorubicin administration. A further indication of apoptosis, 10 μM doxorubicin caused an increase in nuclei with chromatin condensation visualized by propidium iodide staining (data not shown). With 1 μM doxorubicin, only 5.6% of nuclei were TUNEL positive (data not shown). There was no effect
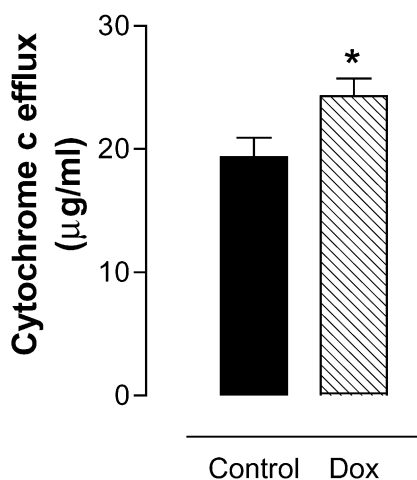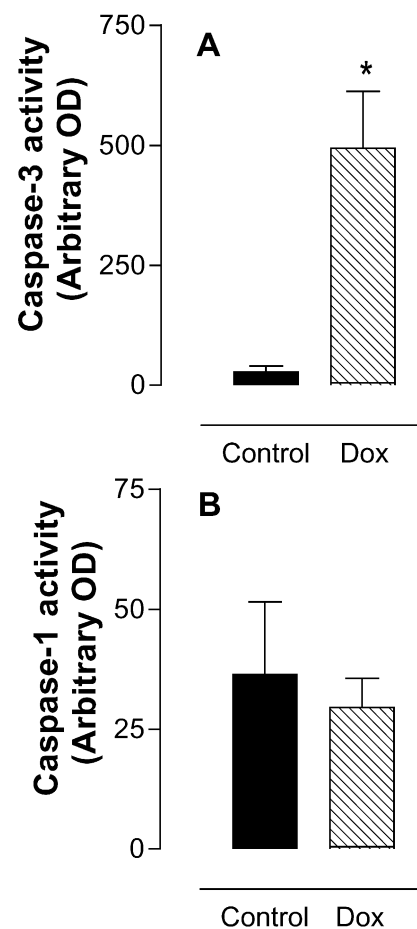

Fig. 5. Doxorubicin treatment increases cytochrome c efflux from isolated rat heart mitochondria. Mitochondria were incubated with doxorubicin (10 μM) for 3 h at 37 °C. Mitochondria were pelleted by centrifugation at $7700\times g$ for 7 min and cytochrome c concentration in the supernatant was determined using an ELISA. Shown are mean±S.E. for $n=4$. *$P<0.05$ vs. control.

Fig. 6. Doxorubicin treatment increases caspase-3, but not caspase-1, activities in H9C2 cells. Cells were treated with 10 μM doxorubicin for 6 h. Caspase-3 (A) and caspase-1 (B) activities were determined in the cytosolic fraction as described in Methods. Shown are mean±S.E. for $n=7$. *$P<0.01$ vs control.

and may represent normal variability in the assay. Alternatively, superoxide production may be decreasing from the peak in the doxorubicin-treated mitochondria and be due to destruction of mitochondria. These data demonstrate the intensity of the oxidative burst to produce superoxide anions due to doxorubicin treatment.

3.4. Markers of apoptosis

Doxorubicin treatment of isolated mitochondria showed a significant increase in cytochrome c efflux into the assay supernatant (Fig. 5), implicating mitochondrial dysfunction and activation of permeability transition due to doxorubicin toxicity. The high background levels of cytochrome c (Fig. 5) could be attributed to (1) significant levels of cytochrome c being present in the cytosol, (2) leakage (structural damage) during the isolation procedures, and (3) activation of permeability transition. However, the differences observed between control and the treatment group indicates a greater release of cytochrome c due to doxorubicin. Furthermore, caspase-3, a major "effector caspase", was significantly elevated in the H9C2 cardiac cell line following doxorubicin treatment (Fig. 6A). In contrast, caspase-1 activity (Fig. 6B), a mitochondrial independent pathway, was not affected by doxorubicin treatment. These results suggest that the mitochondrial-mediated pathway is important in inducing apoptosis under these conditions (see Discussion).

4. Discussion

Doxorubicin (adriamycin) is an anthracycline antibiotic that is used as a potent anticancer agent; however, it is exceptionally cytotoxic to the heart. Toxicity is believed to occur by doxorubicin-induced mitochondrial dysfunction and subsequent oxidant production. [21,22]. The heart is especially prone to oxidative damage due to high levels of enzymes with reductase and dehydrogenase properties, such as NADPH dehydrogenase and endothelial nitric oxide synthase (NOS), which play major roles in transferring electrons to generate oxidant species [5,6]. In our study, doxorubicin treatment of cultured myocytes induced apoptosis as determined by TUNEL staining of nuclei and caspase-3 activation. Interestingly, a rapid decline in mitochondrial reduction potential precedes cell death by approximately 6 h, implicating mitochondrial dysfunction as an early event in doxorubicin-induced cardiotoxicity. In support of this hypothesis, we also found that doxorubicin results in a very rapid (within 5 min) increase in superoxide production in isolated rat heart mitochondria and this is followed by release of the pro-apoptotic protein cytochrome c. Our experiments differ from the literature in that we examined rapid effects on mitochondrial function that were shown to precede myocyte death/apoptosis. Our results not only indicate that doxorubicin treatment can lead to oxidative stress, mitochondrial dysfunction, and apoptosis, but

describe a temporal relationship between these variables. Thus, very low (nM) concentrations of doxorubicin caused a rapid decline in mitochondrial reduction potential, which precedes cell death by approximately 6 h, implicating mitochondrial dysfunction as an early event in doxorubicin-induced myocyte apoptosis.

We showed that mitochondria isolated from rat heart treated with pharmacological levels of doxorubicin produce a ~ 4-fold greater amount of O_2^- compared to nontreated mitochondria and superoxide production persisted through 1 h of exposure. The rapid and sustained oxidant production could cause oxidative stress. Indeed, Luo et al. [23] showed that reactive aldehydes (malondialdehyde, hexanal and 4-hydroxy-non-2-enal) in rat plasma and heart tissues increased significantly following doxorubicin treatment. Importantly, changes occurred early, peaked around 2 h after doxorubicin administration, and the levels declined or returned to baseline value within 8–24 h [23]. Oxidative stress after doxorubicin treatment could therefore be directly responsible for the development of functional abnormalities also documented by echocardiography abnormalities in this study [23].

Which oxidant is responsible for oxidative stress continues to be a heavily debated topic. NO can rapidly scavenge superoxide produced in the mitochondria, because NO outcompetes the superoxide scavenging enzyme superoxide dismutase (SOD) for O_2^-. Therefore, O_2^- produced during doxorubicin toxicity and scavenged by NO may produce the strong oxidant peroxynitrite ($ONOO^-$). There are studies that support superoxide anion as the major cause for oxidative stress, whereas others suggest that hydrogen peroxides and peroxynitrite are more relevant oxidants inducing oxidative stress. In support of a primary role of superoxide production in doxorubicin toxicity, the overexpression of manganese superoxide dismutase has been shown to be cardioprotective in mice five days after doxorubicin treatment [14]. However, other studies suggest that doxorubicin-induced hydrogen peroxide formation is responsible for the toxicity [13]. Hydrogen peroxide production is also directly related to the production of the superoxide anion. Furthermore, hydrogen peroxide production could function as a gene regulator and increase transcription of eNOS [5,6], thereby increasing NO. Alternatively, studies have shown that doxorubicin interacts with eNOS (reductase enzyme moiety) and produce additional superoxide radicals [4–6,24]. We recently demonstrated that there is an increase in plasma iron levels after doxorubicin treatment, suggesting that hydroxyl radical formation is also a likely candidate for free radical damage [25]. Although there is debate over which specific radical is responsible for oxidative damage, it is likely a combination of all these oxidants and their respective reactions are involved in the pathophysiological events leading to cardiomyopathies.

Since oxidants are a major factor in reducing cellular ATP, glutathione, and other reducing equivalents, such as NADPH, they increase the susceptibility of the mitochon-

dria to undergo permeability transition. We show that mitochondrial dysfunction was prevalent in the early stages of doxorubicin treatment and may have been directly responsible for the apoptotic cell death observed. Moreover, we believe that a threshold of mitochondrial destruction needs to be reached before a cell will die. Very low levels of doxorubicin caused a reduction in mitochondrial function, but not cell death.

Additional in vitro studies will determine if the mechanism of doxorubicin action on apoptosis is the same using various doses for longer time periods in the H9C2 cells. These studies would use lower concentrations (e.g. 0.1–2 μM) to study the effects on cardiomyocyte apoptosis. However, long-term use of doxorubicin in cell culture and specifically mitochondria may be very difficult and different from its use in vivo. Chronic, low concentration studies in isolated mitochondria are not feasible due to difficulties in maintaining mitochondrial viability for longer than 2 h. In addition, in vivo and in vitro studies are needed using a low chronic dosage since other death pathways of apoptosis, such as the receptor-mediated pathway, may also be very relevant. In vivo studies use an acute dose of 20 mg/kg of doxorubicin in animals or lower chronic doses, which are all likely to increase plasma levels greater than 10 μM transiently and may only represent clinical conditions for a short period of time. However, some of the effects described in our study are rapid, and therefore could occur in patients since high concentrations could be reached transiently.

Others have also shown that doxorubicin can cause apoptosis in the rat heart [26]. In addition, apoptosis occurs in cell culture models and isolated myocytes exposed to doxorubicin [6,27,28]. The production of oxidant radicals, specifically hydrogen peroxides [6], is able to increase cytochrome c release and induce apoptosis. We found significant release of cytochrome c levels in myocytes treated with doxorubicin. Although apoptosis can occur via cytochrome c-independent mechanisms, it is well established that in most cell types, once cytochrome c is released it interacts with Apaf-1 and procaspase-9, leading to the generation of active caspase-9, which is capable of proteolytically activating caspase-3. Active caspase-3 can then initiate the apoptotic degradation phase [16]. We observed significant increases in caspase-3 activity, but not in caspase-1 activity, which is a well-known cytochrome c-independent caspase.

However, there are several other pathways that can activate caspase-3. For example, caspase-8 is able to activate caspase-3, and therefore this receptor-mediated pathway may also be intricately involved in the cell death observed. However, under these in vitro experimental conditions we believe that the activity of caspase-3 is mainly attributed to the mitochondrial pathway. We expect that investigating chronic doxorubicin toxicity in animals may give more insight on activation of receptor mediated pathways, since doxorubicin has also been shown to activate cytokines [29] and could have effected caspase-1 and caspase-8, which are thought to be primarily activated by receptor-mediated pathways. Since systemic alterations could occur after doxorubicin treatment, responses of receptor-mediated pathways need more investigation. Furthermore, caspase-12, an endoplasmic reticulum-mediated pathway, has received attention since it can be activated by endoplasmic reticulum-mediated stress and releases calcium, which activates caspase-12. Once caspase activation occurs, it may be responsible for cytoskeletal alterations that can result in cell shrinkage and negatively affect tension development [30], two events important for the development of chronic cardiomyopathies.

Therefore, other research efforts will further explore the use of specific antioxidants and caspase inhibitors to prevent apoptosis. Ebselen and lipoic acid could provide a sufficient water and lipid soluble antioxidant buffer to reduce oxidative stress and apoptosis. In addition, the prodomain of proximal caspases contains a *ca*spase *r*ecruitment *d*omain (CARD) for the activation or inhibition of caspases. For example, ARC (*a*poptosis *r*epressor with a *C*ARD) interacts with caspase-2 and -8 and functions as an inhibitor [31]. Alternatively, ARC has also been shown to block mitochondrial cytochrome c release and prevents caspase-3 activation, possibly by binding to voltage dependent anion channels in the mitochondria [32,33], presenting another avenue to inhibit caspase activation. In addition, we will attempt to use longer periods of doxorubicin treatment using lower concentrations to reflect the chronic use of doxorubicin by patients with lymphomas. These efforts to prevent mitochondrial dysfunction and release of cytochrome c from the mitochondria and/or inhibit caspase-3 activation may prove successful in preventing some of the cardiotoxic effects of doxorubicin.

Acknowledgements

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