Doxorubicin induces cardiomyocyte dysfunction via a p38 MAP kinase-dependent oxidative stress mechanism

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Abstract

Doxorubicin, an anthracycline used for cancer therapy, is known to elicit an irreversible cardiotoxicity. Several mechanisms were postulated for its cardiac toxicity including generation of reactive oxygen species (ROS). This study was designed to determine the acute effect of doxorubicin on cardiac mechanical and intracellular Ca\textsuperscript{2+} properties in isolated ventricular myocytes. Contractile properties of male adult rat ventricular myocytes were analyzed including peak shortening (PS), time-to-PS (TPS), time-to-90\% relengthening (TR\textsubscript{90}) and maximal velocity of shortening/relengthening (±dL/dt). Intracellular Ca\textsuperscript{2+} transients and generation of ROS were measured with fura-2 and fluoroprobe 5-(6)-chloromethyl-2\textsuperscript{0},7\textsuperscript{0}-dichlorodihydrofluorescein diacetate, respectively. Acute (5 min) incubation of myocytes with doxorubicin (10\textsuperscript{\textminus}9–10\textsuperscript{\textminus}4 M) significantly prolonged TPS, TR\textsubscript{90} and intracellular Ca\textsuperscript{2+} transient decay rate without affecting PS, ±dL/dt, resting intracellular Ca\textsuperscript{2+} levels and electrically triggered intracellular Ca\textsuperscript{2+} rise. Interestingly, the doxorubicin-induced prolongation of TPS and TR\textsubscript{90} was ablated by treatment of the antioxidant Vitamin C (100 μM) or the p38 MAP kinase inhibitor SB203580 (10 μM). Both Vitamin C and SB203580 unmasked a doxorubicin-induced positive response in PS. Vitamin C itself enhanced basal ±dL/dt, whereas, SB203580 unmasked a doxorubicin-induced positive response of ±dL/dt. The doxorubicin-induced response of intracellular Ca\textsuperscript{2+} transients was essentially unaffected by Vitamin C. The role of ROS in doxorubicin-induced cardiac contractile response was confirmed with the ability of doxorubicin to enhance ROS generation, which was prevented by Vitamin C and SB203580. These data provide evidence that doxorubicin impairs cardiac contractile property in single myocytes through an oxidative stress-mediated pathway.

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1. Introduction

The anthracycline derivative doxorubicin (adriamycin) has been widely used to treat patients with neoplastic diseases. However, its use is limited by cardiac toxicity resulting in a late irreversible congestive cardiomyopathy [1,2]. Clinical manifestations of doxorubicin-induced cardiotoxicity encompass electrocardiographic abnormalities, such as QT prolongation, QT dispersion and occasionally after potential, followed by a life-threatening cardiomyopathy, namely, doxorubicin cardiomyopathy [3,4]. The precise mechanism(s) and time course of cardiac damages and whether they are due to direct effects of doxorubicin on the single cardiac myocytes are largely unknown. Several mechanisms have been postulated for the development of this deadly cardiotoxicity, such as free radical-induced myocardial injury, vasoactive amine release, myocyte damage induced by intracellular Ca\textsuperscript{2+} overload, impairment in myocardial adrenergic signaling/ regulation and cellular toxicity of doxorubicin metabolites, such as doxorubicinol [4–7]. Morphologically, doxorubicin exposure was shown to lead to acute and distinctive nuclear alterations associated with mild cytoplasmic changes in the
heart [2]. One of the commonly acknowledged mechanisms of doxorubicin-induced myocyte dysfunction is believed to be tissue oxidation via increased formation of reactive oxygen species (ROS), including superoxide anion \( \text{O}_2^- \) and other reactive oxygen intermediates [8,9].

The aim of the present study was to determine the effect of acute doxorubicin exposure on ventricular myocyte contractile function and the potential impact of the antioxidant Vitamin C and inhibition of the p38 mitogen-activated protein (MAP) kinase stress-signaling pathway. Our goal was to provide useful information towards pathogenesis of doxorubicin-induced cardiomyopathy so that optimal prevention and management against this detrimental cardiomyopathy may be achieved.

2. Research design and methods

2.1. Ventricular myocyte isolation

The experimental procedure used in this study was approved by the Animal Use and Care Committee at University of North Dakota. Briefly, adult male Sprague–Dawley rats (200–250 g) were anesthetized with ketamine/xylazine (5:3, 1.32 mg/kg i.p.). Hearts were rapidly removed and perfused (at 37 °C) with Krebs–Henseleit bicarbonate (KHB) buffer (mM: NaCl 118, KCl 4.7, MgSO41.2, KH2PO41.2, NaHCO325, N-[2-hydro-ethyl]-piperazine-N’-2-ethanesulfonic acid] (HEPES) 10, glucose 11.1, pH 7.4). The heart was then perfused for 20 min with KHB containing 223 U/ml collagenase (Worthington Biochemical Corp., Freehold, NJ, USA) and 0.5 mg/ml hyaluronidase (Sigma Chemical, St. Louis, MO, USA). After perfusion, the left ventricle was removed and minced. The cells were further digested with 0.02 mg/ml trypsin (Sigma) before being filtered through a nylon mesh (300 μm). Extracellular Ca\(^{2+}\) was added incrementally back to 1.25 mM. Freshly isolated rod-shaped (~70% of the total yield) myocytes were used within 12 h after isolation [10].

2.2. Measurement of cell shortening/relengthening and intracellular Ca\(^{2+}\) transient

Mechanical and intracellular Ca\(^{2+}\) properties of adult rat ventricular myocytes were assessed by an IonOptix Myocam system (IonOptix Incorporation, Milton, MA, USA) as described previously [10]. Cells were placed in a chamber mounted on the stage of an inverted microscope and superfused (at 30 °C) with a buffer, containing (in mM): 131 NaCl, 4 KCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 HEPES, at pH 7.4. The cells were field stimulated at a frequency of 0.5 Hz. Cell shortening and relengthening were assessed using the following indices: peak shortening (PS)—indicative of peak ventricular contractility, time-to-90% PS (TPS)—indicative of systolic duration, time-to-90% relengthening (TR90)—indicative of diastolic duration, maximal velocities of shortening (+dL/dt) and relengthening (−dL/dt)—indicative of maximal velocities of ventricular pressure rise/fall. Intracellular Ca\(^{2+}\) fluorescence was recorded with a dual-excitation single-emission photomultiplier system (IonOptix) in myocytes loaded with fura-2-AM (0.5 μM). Fluorescence emissions were detected between 480 and 520 nm after first illuminating the cells at 360 nm for 0.5 s then at 380 nm for the duration of the recording protocol (333 Hz sampling rate). The 360 nm excitation scan was repeated at the end of the protocol and qualitative changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]i) were inferred from the ratio of fura-2 fluorescence intensity (FFI) at two wavelengths (360 nm and 380 nm). Intracellular Ca\(^{2+}\) fluorescence properties analyzed included the baseline FFI, fluorescence decay rate as an indicative of intracellular Ca\(^{2+}\) clearing rate and electrically triggered intracellular Ca\(^{2+}\) rise. To test the acute effect of doxorubicin on cardiac mechanical and intracellular Ca\(^{2+}\) properties, cell shortening or intracellular Ca\(^{2+}\) transients were recorded before and 5 min after doxorubicin (10\(^{-9}\)–10\(^{-4}\) M) treatment. To evaluate the potential role of oxidative stress in the doxorubicin-induced cardiac response, ventricular myocytes were exposed to antioxidant Vitamin C (100 μM) or the p38 MAP kinase inhibitor SB203580 (10 μM) 5 min prior to and during the length of recording of doxorubicin dose response.

2.3. Measurement of ROS

Production of cellular ROS was evaluated by analyzing changes in fluorescence intensity resulted from oxidation of the intracellular fluoroprobe 5-(6)-chloromethyl-2’,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) [11]. In brief, isolated myocytes from each group were loaded with 1 μM of the non-fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (H2DCFDA, Molecular Probes, Eugene, OR, USA) at 37 °C for 30 min. The myocytes were rinsed and the fluorescence intensity was then measured using a fluorescent micro-plate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm (Molecular Devices, Sunnyvale, CA, USA). Untreated cells showed no fluorescence and were used to determine background fluorescence, which was subtracted from the treated samples. The final fluorescent intensity was normalized to the protein content in each myocyte group and was expressed as percent of the fluorescent intensity of the control myocytes.

2.4. Data analysis

A total of eight rats were used in this study. For each experimental series, data are presented as mean ± S.E.M. Statistical significance \( (p < 0.05) \) for each variable was estimated by two-way analysis of variance (ANOVA). A Dunnett’s test was used for post hoc analysis when required.
3. Results

3.1. Effect of doxorubicin on myocyte shortening mechanics in the absence or presence of Vitamin C and the MAP kinase inhibitor SB203580

Acute exposure (5 min) of doxorubicin ($10^{-9}$–$10^{-4}$ M) did not affect resting cell length in the absence or presence of Vitamin C (100 μm) or the MAP kinase inhibitor SB203580 (10 μM). Neither did doxorubicin alter cell survival during the span of its exposure to the myocytes (data not shown). The PS amplitude and maximal velocity of shortening/relengthening ($\pm dL/dt$) were not significantly altered by doxorubicin ($10^{-9}$–$10^{-4}$ M) after a 5 min exposure. Interestingly, both Vitamin C (100 μm) and SB203580 (10 μm) unmasked a positive contractile response in PS upon doxorubicin exposure with the exception of Vitamin C at the highest doxorubicin concentration. While Vitamin C itself significantly enhanced basal $\pm dL/dt$ in myocytes without doxorubicin exposure, it did not affect the response of doxorubicin on $\pm dL/dt$ with the exception of decreasing $+dL/dt$ at the highest concentration of doxorubicin. Unlike Vitamin C, SB203580 itself did not affect $\pm dL/dt$ in control myocytes, it unmasked a significant positive response of $\pm dL/dt$ upon doxorubicin exposure, similar to its effect on PS (Fig. 1). Although, acute administration of doxorubicin failed to alter PS and $\pm dL/dt$ in ventricular myocytes, it did prolong the duration of myocyte shortening (TPS) and relengthening (TR90) (Fig. 2). Our further study revealed that the doxorubicin-induced prolongation of TPS and TR90 were ablated by Vitamin C and SB203580, although, Vitamin C failed to prevent the prolonged TPS and TR90 at the highest concentration of doxorubicin. Fig. 2 also depicted that Vitamin C itself shortened both TPS and TR90, whereas, SB203580 itself shortened TPS but not TR90 in control myocytes.

3.2. Effect of doxorubicin on intracellular Ca$^{2+}$ transients in the absence or presence of Vitamin C

To examine the role of intracellular Ca$^{2+}$ homeostasis on doxorubicin-induced response on myocyte shortening, the
The effect of doxorubicin on intracellular Ca\(^{2+}\) transients was examined in myocytes in the absence or presence of antioxidant Vitamin C (100 \(\mu M\)). As shown in Fig. 3, doxorubicin (10\(^{-9}\)–10\(^{-4}\) M) did not affect the baseline intracellular Ca\(^{2+}\) levels and electrically triggered increase in intracellular Ca\(^{2+}\) but significantly prolonged the intracellular Ca\(^{2+}\) decay rate with a threshold between 10\(^{-7}\) and 10\(^{-6}\) M. Vitamin C did not alter doxorubicin-induced responses on intracellular Ca\(^{2+}\) transients although it elevated the baseline intracellular Ca\(^{2+}\) level in control myocytes (Fig. 3A) and elicited a rightward shift in the threshold of doxorubicin-induced prolongation of fluorescence decay rate (Fig. 3C).

### 3.3. Effect of doxorubicin on ROS generation

As shown in Fig. 4, ROS production was enhanced in doxorubicin (10\(^{-4}\) M)-treated myocytes, which was prevented by Vitamin C (100 \(\mu M\)) and the p38 MAP kinase inhibitor SB203580 (10 \(\mu M\)). These data suggest that the doxorubicin-induced cardiac mechanical dysfunction (prolongation of TPS and TR\(_{90}\)) may be mediated, at least in part, by doxorubicin-induced production of ROS in ventricular myocytes. The observation that doxorubicin-induced elevation in ROS generation was abolished by Vitamin C and SB203580 further substantiated a role of enhanced oxidative stress in doxorubicin-induced prolongation of TPS and TR\(_{90}\).
Fig. 4. Effect of doxorubicin (Dox, 10^{-7} M) on production of reactive oxygen species (ROS) in adult rat ventricular myocytes in the absence or presence of antioxidant Vitamin C (100 μM) or the p38 MAP kinase inhibitor SB203580 (10 μM). CM-H2DCFDA was used to monitor ROS generation. Mean ± S.E.M., n = 4–6 assays per data point, (*) p < 0.05 vs. respective control, (#) p < 0.05 vs. doxorubicin only.

4. Discussion

Our current study provided direct evidence that doxorubicin impaired ventricular contractile function in isolated adult rat ventricular myocytes through a p38 MAP kinase-dependent oxidative stress mechanism. The major mechanical interruptions in response to acute doxorubicin exposure found in our study were shown as prolonged duration of contraction and relaxation, both of which considered hallmarks of cardiomyopathies originated from several kinds of cardiac morbidities, such as heart failure, ischemia-reperfusion injury, hypertension and diabetes [12,13]. Our results also revealed that the doxorubicin-induced cardiac mechanical dysfunctions and elevation of ROS generation were attenuated, to a large extent, by Vitamin C and the p38 MAP kinase inhibition with SB203580. These data indicated the likely involvement of p38 MAP kinase and oxidative stress in the acute doxorubicin exposure induced prolongation of contraction and relaxation duration in isolated ventricular myocytes.

Our study showed that doxorubicin-induced ROS production, prolongation of contraction and relaxation duration (TPS and TR90), can be prevented by p38 MAP kinase inhibition and Vitamin C, suggesting that doxorubicin-elicited cardiac dysfunction is likely mediated via p38 MAP kinase-dependent accumulation of oxidative stress. The lack of protection for Vitamin C at the highest concentration of doxorubicin (10^{-4} M), although not known at this time, may simply reflect an overwhelmed cell injury elicited by this dose of doxorubicin. The data from Vitamin C and SB203580 is consistent with the notion of p38 MAP kinase activation being directly responsible for compromised cardiac contractile function [14] and observation that enhanced oxidative stress may directly lead to prolonged TPS and TR90 in isolated ventricular myocytes [13]. The involvement of oxidative stress in acute doxorubicin-induced cardiac dysfunction may also be supported by the report that doxorubicin-induced chronic cardiac toxicity is alleviated by the free radical scavenger metallothionein [15]. Interestingly, Vitamin C and p38 MAP kinase inhibition themselves significantly shortened baseline TPS and TR90, indicating that a tonic oxidative stress status in control myocytes may be sufficient to prolong the duration of contraction and relaxation. The doxorubicin-induced prolongation of TR90 is consistent with the prolonged intracellular Ca^{2+} transient decay rate. However, we are unable to interpret why doxorubicin-induced prolongation of intracellular Ca^{2+} transient decay was not reconciled with Vitamin C as it did for the prolonged TR90. Possible alteration of myofilament Ca^{2+} responsiveness or buffering effect of the fluoroscein dye fura-2 may contribute to such discrepant responses although further study is warranted. Several lines of evidence suggested the existence of an abnormal Ca^{2+} homeostasis in cardiac myocytes (such as prolonged intracellular Ca^{2+} transient decay seen in this study), which may explain mechanical abnormalities in doxorubicin-induced cardiomyopathy. Doxorubicin has been reported to prolong action potential duration, to inhibit gene transcription of sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA), to reduce sarcoplasmic reticulum (SR) Ca^{2+} load, and subsequently, Ca^{2+}-induced Ca^{2+} release in isolated ventricular myocytes [3,9]. The observation that acute doxorubicin exposure (5 min) in our current experimental setting failed to affect any other mechanical indices, such as PS, maximal velocity of shortening/relengthening (±dL/dt), resting and electrically-stimulated intracellular Ca^{2+} levels may suggest a significant difference in timing or sensitivity among various cardiac contractile components upon doxorubicin exposure. For example, proteins responsible for duration of cell shortening/relengthening (TPS and TR90), such as SR Ca^{2+} release channel, myosin ATPase, sarco(endo)plasmic reticulum Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger may be more sensitive to acute doxorubicin exposure than proteins responsible for cardiac contractility, such as actin, myosin and SR Ca^{2+} load.

Late stage doxorubicin-induced cardiac toxicity is associated with reduced cardiac contractility, decreased velocity of contraction/relaxation, intracellular Ca^{2+} overload, possibly through reduced myofilament Ca^{2+} sensitivity and impaired intracellular Ca^{2+} handling [4]. Isolated cardiac myocytes from doxorubicin-treated rabbits displayed reduced contractility, contraction/relaxation velocity and oxygen consumption. The decreased contractility of individual myocytes may be attributed to their low myosin content, and lead to decreased cardiac output following doxorubicin treatment [16]. Although, difference between acute and chronic doxorubicin exposure on cardiac toxicity is not fully clear, it is possible that certain components responsible for duration of contraction and relaxation (i.e., SR Ca^{2+} load, Ca^{2+}-induced Ca^{2+} release, SERCA pump and Na^+/Ca^{2+}-exchanger, etc.) are more sensitive to doxorubicin-induced oxidative damage than those elements regulating cardiac contractility or velocity of contraction/relaxation (e.g., actin, myosin, ATP level and myofilament Ca^{2+} sensitivity). It is worth mentioning that...
Doxorubicin-induced heart failure is not associated with β-adrenoceptor desensitization, unlike heart failure induced by pressure- or volume-overload [16]. One other interesting observation from our current study is that Vitamin C and p38 MAP kinase inhibition may unmask a doxorubicin-induced positive response in PS (similar for ±dL/dr in the presence of SB203580). Although the mechanism behind such “unmasking” effect is not clear, it is plausible to hypothesize that the doxorubicin-induced cardiac contractile response may consist of multiple components with one being p38 MAP kinase-dependent negative inotropic response and another being intrinsic positive inotropic response independent of p38 MAP kinase activation and oxidative stress.

In conclusion, our study demonstrates that doxorubicin prolongs duration of cell shortening/relengthening. The detrimental effect of doxorubicin is likely to be mediated through p38 MAP kinase-dependent accumulation of oxidative stress. While the precise natures of acute doxorubicin exposure-induced mechanical defects and cardiac protection from Vitamin C and p38 MAP kinase inhibition are still not clear, future studies should focus on the mechanism of action behind doxorubicin-induced ROS-dependent or independent regulation on cardiac contractile response. These approaches should be helpful in advancing our knowledge of the cellular effects and toxicological profiles of doxorubicin on heart function, in order to design better therapy against doxorubicin-induced cardiomyopathy.

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References


