Cardiac Peroxynitrite Formation and Left Ventricular Dysfunction following Doxorubicin Treatment in Mice

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ABSTRACT

Selective cardiotoxicity of doxorubicin remains a significant and dose-limiting clinical problem. The mechanisms involved have not been fully defined but may involve the production of reactive oxygen species and/or alteration of cardiac energetics. Here, we tested the hypotheses that doxorubicin causes left ventricular dysfunction in mice and is associated with dysregulation of nitric oxide in cardiac tissue, leading to the accumulation of 3-nitrotyrosine (a biomarker of peroxynitrite formation). Animals were dosed with doxorubicin (20 mg/kg i.p.), and left ventricular performance was assessed in vivo using M-mode and Doppler echocardiography. Five days after doxorubicin administration, left ventricular fractional shortening, cardiac output, and stroke volume parameters were significantly reduced relative to control values (30.0 ± 3.6 vs. 46.1 ± 1.6%, 8.9 ± 0.9 vs. 11.5 ± 0.6 ml/min, and 21.2 ± 0.1 vs. 29.5 ± 0.1 µl for doxorubicin versus control, P < .05). Statistically significant (P < .05) increases in the immunoprevalence of myocardial inducible nitric oxide synthase (33 ± 18 versus 9 ± 2%, via quantitative image analysis) and 3-nitrotyrosine formation (56 ± 24 versus 0.3 ± 0.4%) were also observed after doxorubicin. Correlation analyses revealed a highly significant inverse relationship between left ventricular fractional shortening and cardiac 3-nitrotyrosine immunoprevalence (P < .01). No such relationship was observed for inducible nitric oxide synthase. Western blot analyses of cardiac myofibrillar fractions revealed extensive nitration of an abundant 40-kDa protein, shown to be the myofibrillar isoform of creatine kinase. These data demonstrate that alteration of cardiac nitric oxide control and attendant peroxynitrite formation may be an important contributor to doxorubicin-induced cardiac dysfunction. Furthermore, nitration of key myofibrillar proteins and alteration of myocyte energetics are implicated.

Doxorubicin (DOX) is an important antineoplastic agent commonly used for the treatment of breast and bladder cancers, Hodgkin’s lymphomas, and others (Blum and Carter, 1974; Hortobagyi, 1997). Despite its common use, the clinical utility of DOX is severely compromised by dose-limiting cardiotoxicities (Doroshow et al., 1979; Singal, 1987). Although the general phenomenon of cardiotoxicity is well known in humans and animals, dose dependencies and timing of DOX toxicity are often variable and difficult to predict. Many patients exhibit cumulative dose-dependent toxicities, but others experience acute life-threatening reactions or delayed cardiomyopathies manifesting months to years after the cessation of therapy (Singal, 1987).

A contemporary mechanism of DOX-induced cardiac dysfunction is based on tissue oxidation through increased cellular superoxide anion (O2−) formation (Yen et al., 1996; Hasi-noff, 1998). The anthracycline ring structure of DOX has been shown to undergo both enzymatic and nonenzymatic single-electron redox cycling, liberating O2 from molecular oxygen (Olson et al., 1981; Sarvazyan et al., 1995). Alternatively, this agent may impair myocyte energetics, through direct interaction with myofibrillar proteins and/or indirectly through oxidant production. Thus, although the clinical importance of DOX-related cardiac dysfunction and toxicity is generally recognized, the precise cellular events involved have not been established, and optimal therapeutic approaches for cardioprotection are not fully defined.

Recent evidence suggests that nitric oxide (NO) may play diverse roles in cardiac function and disease. Basal production of NO (via constitutive NO synthase (NOS) isoforms) modulates cardiomyocyte contractility and regulates blood flow distribution (Varin et al., 1999). In contrast, high levels of NO production (via inducible NOS, NOS-II) are associated with several forms of cardiac disease, including dilated cardiomyopathy and congestive heart failure (Haywood et al., 1996; Vejlstrup et al., 1998). The high concentrations of NO achieved through NOS-II induction may participate in further cardiomyocyte oxidative damage, apoptosis, and/or necrosis (Adams et al., 1999). Synthesis of NO in the vicinity of O2− results in the diffusion rate-limited production of peroxynitrite (ONOO−), a potent and aggressive cellular oxidant...
(Beckman and Koppenol, 1996; Ischiropoulos, 1998). One of the hallmarks of ONOO−-induced oxidation is the nitration of protein tyrosine residues, resulting in the formation of 3-nitro-tyrosine (3-NT). This modification has been implicated in a diverse array of disease settings, including acute lung injury, renal allograft rejection, and myocarditis (Kooy et al., 1995; MacMillan-Crow et al., 1996; Kooy et al., 1997).

Although NO and peroxynitrite are known cellular oxidants, their participation in DOX-induced cardiac toxicity has not been established, and no previous reports have evaluated their roles in this setting. Here, we tested the hypothesis that DOX administration leads to impaired cardiac performance in mice, as evaluated by noninvasive echocardiography of left ventricular (LV) function. Furthermore, we investigated the roles of NO control and myofibrillar energetics in this phenomenon.

**Materials and Methods**

**Animals.** Male CF-1 mice (Harlan, Indianapolis, IN) weighing 25 to 35 g were administered a single dose of DOX HCl (Bedford Laboratories, Bedford, OH) at 20 mg/kg i.p., and studied 5 days later. This time point was chosen as >5 final half-lives of elimination of DOX from both plasma and cardiac tissue in mice (van der Velden et al., 1990). All animal handling was approved by the Institutional Animal Care and Use Committee.

**Assessment of Cardiac Function.** Five days after DOX administration, in vivo cardiovascular function was determined using a Sonos 1000 echocardiography unit (Hewlett-Packard, Andover, MA). Mice were anesthetized by halothane inhalation (~1% halothane in 95% O2, 5% CO2). Normothermia was maintained with a heating pad. A 7.5-MHz pediatric probe placed in the parasternal, short-axis orientation recorded LV systolic and diastolic internal dimensions. Three loops of M-mode data were captured for each animal, and data were averaged from at least five beat cycles per loop. Parameters were determined using the American Society for Echocardiography leading-edge technique in a blinded fashion. These parameters allowed the determination of LV fractional shortening (FS) by the equation: FS = ([LVIDd − LVIDs]/LVIDd) × 100%, where LVID refers to the LV internal dimension at diastole (d) and systole (s). Ascending aortic flow waveforms were recorded using a continuous-wave Doppler flow probe oriented in a short-axis, suprasternal manner. Velocity-time integrals (VTIs) were calculated from these waveforms. After sacrifice, aortic root cross-sectional area was measured, and cardiac output (CO) was calculated by the equation: CO = heart rate × VTI × aortic cross-sectional area.

**General Histology and Immunohistochemistry.** Animals were sacrificed with 100 mg/kg pentobarbital sodium i.p. (Abbott Laboratories, Chicago, IL). The apical portion of the heart was bisected just distal to the mitral valve and immersed in 10% formalin. Tissues were paraffin-embedded and blocked according to standard procedures. Sections of 5 μm were evaluated using standard protocols for H&E staining and Masson’s trichrome for fibrosis. Sections were immunostained using polyclonal primary antibodies for NOS-II (1:200 dilution; Transduction Laboratories, Lexington, KY) or 3-NT (anti-3-NT, 1:200 dilution; Transduction Laboratories, Lexington, KY) as previously described (Miham et al., 1999a). Exposure of the tissue sections to 0.06% w/v diaminobenzidine followed by hematoxylin counterstaining provided visualization of immunoreactivity. Serial tissue sections from DOX-treated animals were used to validate antibody specificity for NOS-II (isotypic control, Fig. 2G) and 3-NT (antibody preadsorbed with 5 mM free 3-NT, Fig. 2H) as staining controls. Furthermore, Western blotting analyses were used to confirm antibody specificity, detecting 130-kDa NOS-II band from mouse macrophage isolate.

**Western Blot Analysis.** Cardiac myofibrillar fractions were prepared using a previously described protocol (Ventura-Clapier et al., 1987). Thirty micrograms of protein from the myofibrillar isolation was loaded onto 10% polyacrylamide gels (1.5 mm). Purified myofibrillar creatine kinase (M-CK; Sigma Chemical Co., St. Louis, MO) was preincubated with 80 μM tetranitromethane, a selective nitration agent (Sokolovsky et al., 1966), and used as a positive control (15 μg/lane). Proteins were electrophoresed under reducing conditions before transfer to nitrocellulose membranes. Protein bands were identified using FastBlot (Genotech Technologies, St. Louis, MO). Membranes were immunostained with anti-3-NT as described earlier. Antibody specificity was demonstrated using preadsorbed controls as described earlier. Migration of M-CK was confirmed by immunoblotting with polyclonal rabbit anti-M-CK antibody (1:200; Fitzgerald Industries, Concord, MA).

**Statistical Analysis.** Parameters between DOX and control groups were evaluated for significance using Student’s t test (SigmaStat; Jandel Scientific, San Rafael, CA). Comparisons of three or more groups were conducted by one-way ANOVA, with Student-Newman-Keuls post hoc analysis. Statistical associations between functional parameters and immunohistochemical data were evaluated using Spearman’s nonparametric correlation analysis. In all cases, results are expressed as mean ± S.E., and significance was defined as P < .05.

**Results**

Shown in Fig. 1 (top) are representative two-dimensional M-mode tracings of LV systole-diastole cycles from a control and a DOX-treated animal, respectively. The waveforms from the DOX-treated animal clearly demonstrate blunted anterior and posterior wall motion relative to the control animal, consistent with decreased contractile performance and reduced fractional shortening. Resting heart rate was not different between the two groups; however, LV fractional shortening and cardiac output were significantly reduced by 35 and 23%, respectively, relative to control animals (Fig. 1, bottom). Table 1 summarizes the remainder of cardiovascular parameters determined through noninvasive echocardiographic analysis and Doppler analysis. DOX treatment also resulted in significant reductions of end-diastolic dimension and stroke volume (14 and 28% reductions from control, respectively; P < .05), with no change in end-systolic dimension. Maximal aortic velocity was not statistically different between treatment groups.

Shown in Fig. 2 are representative photomicrographs of Masson’s trichrome and immunohistochemical staining. Trichrome staining demonstrated slight and diffuse evidence of interstitial fibrosis in DOX-treated animals and some disorder of cardiomyocytes (Fig. 2, A and B). No significant evidence of immune cell infiltration was observed (H&E staining, data not shown). Immunohistochemical staining showed statistically significant evidence of NOS-II expres-
significant negative correlation between LV performance and immunoreactivity for NOS-II or 3-NT. Figure 4 shows a highly statistical relationships between cardiac dysfunction and immunoreactivity of both NOS-II and 3-NT was significantly increased after DOX relative to control tissues. Pre-immune serum (isotypic control, Fig. 2G) and antibody specificity for NOS-II (isotypic control, Fig. 2G) revealed several nitrotyrosine bands from DOX-treated but not control cardiac tissue (lanes 3 and 5). Not all proteins detected by FastBlot staining (lane 2) demonstrated 3-NT immunoreactivity, indicating differential sensitivity to nitration. Lane 6 demonstrates the migration profile of nitrated M-CK (after exposure to 80 μM tetranitromethane, a selective protein tyrosine-nitrating agent), thus illustrating the identity of M-CK in the cardiac myofibrillar homogenate.

**Discussion**

DOX continues to be a mainstay chemotherapeutic agent, but cardiotoxicity remains a significant dose-limiting side effect requiring lifetime dosing limits. Although oxidative chemistries have long been implicated in the etiology of DOX-induced cardiotoxicity, the putative reactive oxygen species involved and/or the mechanism by which injury occurs remains poorly understood. Here, we demonstrated severe LV dysfunction in a well-established murine model of DOX cardiotoxicity (which mimics the human pathology) to test the hypothesis that NO-derived oxidative species, particularly ONOO⁻, are formed during and may participate in DOX-mediated cardiotoxicity.

Using echocardiographic techniques, we noninvasively assessed cardiac performance 5 days after a single dose of DOX. This time point was chosen as >5 final half-lives of elimination of DOX from both plasma and cardiac tissue in mice (van der Vijgh et al., 1990). Therefore, at the time of functional and immunohistochemical assessments, DOX was no longer present in the blood or cardiac tissues. We observed significant reductions in cardiac performance measuring a variety of independent parameters. Cardiac contractility, assessed by fractional shortening and stroke volume measures, was markedly reduced in the absence of heart rate changes. These changes are consistent with clinical observations, suggesting that this murine model appears appropriate for mechanistic evaluations.

NO is a well-established participant in the regulation of vascular tone and may play important roles in cardiac function and disease (Varin et al., 1999). In settings of cardiac injury and/or inflammation, elevated NO production can occur via induction of NOS-II (Haywood et al., 1996; Vejlstrup...
et al., 1998). High concentrations of NO achieved through NOS-II induction may participate in further cardiac oxidative damage, apoptosis, and/or necrosis (Adams et al., 1999). Using immunohistochemical analysis, we observed widespread induction of NOS-II in cardiac tissue from DOX-treated mice compared with control at 5 days after DOX dosing. This is the first experimental evidence that NOS-II induction may participate in DOX-related cardiotoxicity and, interestingly, was observed in cardiomyocytes in the apparent absence of immune cell infiltration. The prolongation of elevated NOS-II after the final elimination of DOX provides evidence that DOX-mediated oxidative stress may persist after the drug is cleared. Such induction processes may help explain the clinically observed time dissociation between drug administration and cardiac impairment in some patients.

Fig. 2. Representative photomicrographs demonstrating evidence of DOX-induced histopathology. A and B, Masson’s trichrome staining of 5-μm sections showing some evidence of interstitial, nonvascular collagen deposition (light blue, non-nuclear staining) in DOX-treated (B) but not control (A) mice. C and E, polyclonal rabbit anti-mouse antisera for NOS-II detected the minor presence of NOS-II in control (C) sections but significantly greater expression in DOX-treated sections (E). D and F, polyclonal rabbit anti-3-NT detected no protein nitration in control (D) but marked presence as a result of DOX treatment (F). G, preimmune, isotypic staining control of a serial section shown in E. H, control staining of serial section shown in F with anti-3-NT antibody preadsorbed with 5 mM free 3-NT, demonstrating antibody specificity (original magnification, 200×).

Fig. 3. Quantification of NOS-II and 3-NT immunoprevalence. Digitally captured images of myocardium from DOX-treated and control mice were evaluated for the extent of immunoreactivity. Filled columns, vehicle controls; open columns, DOX-treated mice; striped columns, isotypic controls (n = 4–10 independent samples). DOX immunoprevalence significantly increased compared with vehicle (✱) and isotypic controls (†).
consistent with the recent understanding that protein nitration may not be simply an irreversible, cumulative event in vivo. Recent evidence suggests that tyrosine nitration is a reversible process in a variety of tissues (Kamisaki et al., 1998), including cardiac tissue (M. J. Mihm and J. A. Bauer, unpublished observations). This evidence suggests that protein nitration may be a dynamic modulator of cellular function that may participate in both cellular homeostasis and disease.

Previous studies of oxidative cellular targets have demonstrated that although cardiac lipid peroxidation occurs in some settings (Miura et al., 1994), this parameter failed to correlate with tissue catalase status or DOX-induced mortality or heart weight changes (Baird et al., 1993). In contrast, we observed a high degree of correlation between cardiac dysfunction and protein oxidation. Thus, NO-related protein oxidative events may have particular importance, and inhibition of peroxynitrite may have therapeutic value in this setting. Drug therapy for the prevention of DOX-induced cardiotoxicity is currently limited to dexrazoxane (Dorr, 1996), an iron chelator that inhibits nonenzymatic redox cycling and production of \( \text{O}_2^- \). Although this agent has provided some benefit in breast cancer patients, in large-scale trials, the incidence of DOX-related cardiac events was reduced by only 50% (Swain, 1998). This is consistent with the likelihood that iron-related chemistries mediate only a portion of this toxicity and suggest that other processes are involved. Therefore, although dexrazoxane has shown promise in both experimental and clinical settings, the full benefit of iron chelation as cardioprotective therapy is not established and further mechanistic studies are warranted. Further studies investigating the capacity of selective NOS-II inhibitors and \( \text{ONOO}^- \) scavengers to prevent DOX-induced injury may provide additional mechanistic insight (as well as potential therapeutic benefit) and are ongoing in our laboratory.

Impaired energetic control is a well-established mediator and consequence of cardiac failure (Ingwall et al., 1985), although the mechanisms by which these changes occur remain poorly understood. Myocyte energetics are sensitive to oxidative conditions (Kowaltowski and Vercesi, 1999), and several studies have investigated the interaction between reactive oxygen species and cardiomyocyte organelles. Although most attention has focused on the influence of oxidants on mitochondrial function, evidence exists suggesting that the myofibrillar structure of the myocyte is most sensitive to DOX-induced oxidative insult (Nagineni et al., 1985). Similarly, we observed protein nitration in DOX-treated mice along the architecture of the myofibrillar structure (high-magnification light microscopy by oil immersion methods; data not shown). Given these observations, we used Western blotting analysis to investigate myofibrillar protein targets of \( \text{ONOO}^- \). Nitrination of several fibrillar proteins was observed, but the pattern of immunoreactive protein bands was not identical with general protein staining, indicating selectivity of the nitration events. The most prominent nitrated protein...
was M-CK, the monomeric myofibrillar isoform of creatine kinase. This isoform is a critical enzymatic controller of myocyte energetic and contractility and has been demonstrated to be a highly sensitive target of oxidative injury both in vitro and in vivo (Banerjee et al., 1991; Mekhfi et al., 1996). We recently observed significant inhibition of M-CK activity by exposure to nanomolar concentrations of ONOO⁻ in vitro (Mihm et al., 1999b). M-CK has been demonstrated to be impaired in settings of human heart failure, but the mechanisms involved are unknown. Our preliminary observation provides the first evidence of ONOO⁻–specific alterations of CK and provides a potential mechanism for CK impairment in cardiac failure.

Recent studies suggest that ONOO⁻ may not be the sole agent responsible for significant protein nitration in vivo, instead implicating neutrophil-mediated, enzymatically catalyzed H₂O₂–NO₂⁻ ion interactions as a mechanism of protein nitration (Sampson et al., 1998). Although these studies have demonstrated the reaction rate of this interaction is orders of magnitude slower than ONOO⁻–mediated tyrosine nitration, we examined the cardiac tissue from DOX-treated mice for evidence of immune cell infiltration. We observed no significant historical evidence of neutrophil accumulation in DOX-treated or control cardiac tissue, suggesting that ONOO⁻–mediated nitration remains the most plausible mechanism of nitration in this setting.

In summary, DOX caused significant LV dysfunction in mice, as measured by echocardiography, and the changes observed were similar to clinically observed events. Cardiac dysfunction was associated with a severe loss of NO control, evidenced by increased tissue levels of NOS-II and extensive myocyte protein nitration, suggesting involvement of the aggressive oxidant ONOO⁻. The extent of LV dysfunction was highly correlated with the extent of nitration rather than with NOS-II immunopositivity, suggesting that ONOO⁻ and attendant protein nitration may play a pivotal role in the observed dysfunction. Preliminary evidence demonstrates that the myofibrillar compartment, in particular M-CK, may be preferentially sensitive to modification by ONOO⁻. These findings suggest a novel mechanism of DOX-induced injury and provide additional mechanistic insight that may facilitate the development of novel therapeutic strategies, particularly those directed toward controlling ONOO⁻ and/or NOS-II induction.

**References**


**Doxorubicin-Induced Cardiac Peroxynitrite Formation**

Doxorubicin-induced cardiac peroxynitrite formation in chronic rejection of human renal allografts. *Proc Natl Acad Sci USA* **93:**11855–11858.