Nox2 NADPH Oxidase Promotes Pathologic Cardiac Remodeling Associated with Doxorubicin Chemotherapy

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Abstract

Doxorubicin is a highly effective cancer treatment whose use is severely limited by dose-dependent cardiotoxicity. It is well established that doxorubicin increases reactive oxygen species (ROS) production. In this study, we investigated contributions to doxorubicin cardiotoxicity from Nox2 NADPH oxidase, an important ROS source in cardiac cells, which is known to modulate several key processes underlying myocardial response to injury. Nox2-deficient mice (Nox2−/−) and wild-type (WT) controls were injected with doxorubicin (12 mg/kg) or vehicle and studied 8 weeks later. Echocardiography indicated that doxorubicin-induced contractile dysfunction was attenuated in Nox2−/− versus WT mice (fractional shortening: 29.5 ± 1.4 versus 25.7 ± 1.0%; P < 0.05). Similarly, in vivo pressure-volume analysis revealed that systolic and diastolic function was preserved in doxorubicin-treated Nox2−/− versus WT mice (ejection fraction: 52.6 ± 2.5 versus 28.5 ± 2.3%; LVdP/dt min: −8,379 ± 416 versus −5,198 ± 527 mmHg s−1; end-diastolic pressure-volume relation: 0.051 ± 0.009 versus 0.114 ± 0.012; P < 0.001). Furthermore, in response to doxorubicin, Nox2−/− mice exhibited less myocardial atrophy, cardiomyocyte apoptosis, and interstitial fibrosis, together with reduced increases in profibrotic gene expression (procollagen IIIα1, transforming growth factor-β, and connective tissue growth factor) and matrix metalloproteinase-9 activity, versus WT controls. These alterations were associated with beneficial changes in Nox2 oxidase activity, oxidative/nitrosative stress, and inflammatory cell infiltration. We found that adverse effects of doxorubicin were attenuated by acute or chronic treatment with the AT1 receptor antagonist losartan, which is commonly used to reduce blood pressure. Our findings suggest that ROS specifically derived from Nox2 NADPH oxidase make a substantial contribution to several key processes underlying development of cardiac contractile dysfunction and remodeling associated with doxorubicin chemotherapy. Cancer Res; 70(22); 9287–97. ©2010 AACR.

Introduction

Doxorubicin remains one of the most widely used anti-neoplastic agents in the effective treatment of various cancers (1). Unfortunately, its chronic use is severely limited by dose-dependent cardiotoxicity characterized by progressive cardiac dilatation, contractile dysfunction, and ultimately congestive heart failure (2, 3). The chronic response to doxorubicin-induced myocardial injury involves complex reorganization of the extracellular matrix [mainly driven by matrix metalloproteinases (MMP)] and substantial alterations in cardiomyocyte biology, such as apoptosis and changes in cell growth, excitation-contraction coupling, and cytoskeleton organization (3).

The mechanisms by which doxorubicin causes deleterious structural and functional changes are poorly understood, although activation of several pathways has been proposed, including local release of vasoactive substances, mitochondrial dysfunction, lipid peroxidation, glutathione peroxidase depletion, reduced sarcoplasmic Ca2+-ATPase activity, and impaired myocardial energetics (1, 2). However, recent attention has focused on the potential involvement of myocardial reactive oxygen species (ROS) and oxidative stress, which are increased by chronic doxorubicin treatment and have been proposed to be at least partly responsible for the associated cardiotoxicity (4–5). Indeed, experimental doxorubicin-induced cardiac contractile dysfunction and adverse morphologic changes are inhibited by exogenous antioxidant treatment or overexpression of endogenous antioxidant enzyme, and similar protective effects are reported clinically (6–8).

Although, it is well established that oxidative stress modulates several key processes underlying doxorubicin-induced cardiotoxicity, such as extracellular matrix remodeling, cardiomyocyte apoptosis, and alteration of cardiac contractile properties (5, 9–13), the precise contribution of different ROS sources remains unknown. Identification of wide-ranging
cardiovascular actions of ROS has prompted detailed investigation of the roles of potential sources, including the mitochondrial electron transport chain, xanthine oxidase, and dysfunctional nitric oxide synthases (NOS; ref. 14). In this regard, recent work has identified a family of NADPH oxidases as important sources of myocardial ROS (10, 15, 16). The prototypic NADPH oxidase comprises a catalytic core (Nox2/p22phox), together with several cytosolic subunits (p47phox, p67phox, p40phox, and Rac) that associate on activation (14). Several Nox isoforms have been identified (Nox1 to Nox5); the major cardiac isoforms are Nox2 and Nox4, which are expressed in cardiomyocytes, fibroblasts, and endothelial cells (14, 15).

Recent studies indicate that NADPH oxidase–derived ROS play a pivotal role in cardiac pathophysiology. NADPH oxidase activity/expression is increased in experimental and clinical heart failure (15–17). Furthermore, NADPH oxidase–dependent ROS production regulates several key components of cardiac remodeling, such as myocyte hypertrophy, contractile dysfunction, apoptosis, and fibrosis (10, 11, 18). Interestingly, our previous studies using Nox2−/− mice revealed differential effects that were dependent on the remodeling stimulus. Nox2 NADPH oxidase was critical for cardiac hypertrophy and fibrosis in response to chronic angiotensin II (AngII) infusion (18). Similarly, development of cardiac contractile dysfunction, chamber dilatation, myocyte hypertrophy/apoptosis, and fibrosis after myocardial infarction were attenuated in Nox2−/− compared with wild-type (WT) mice (16). In contrast, after pressure overload, left ventricular (LV) hypertrophy occurred independently of Nox2, although Nox2 was essential for cardiac fibrosis and contractile dysfunction (10, 15). It therefore seems that Nox2 NADPH oxidase may influence individual components of the cardiac remodeling phenotype in a stimulus-specific manner.

The potential role of Nox2 NADPH oxidase in doxorubicin-induced cardiotoxicity has yet to be clearly established. However, doxorubicin increases myocardial NADPH oxidase activity in vivo (19) and stimulates MMP-2 expression/activity and apoptosis in vitro via NADPH oxidase–dependent activation of c-Jun NH2-terminal kinase (JNK)/extracellular signal-regulated kinase (ERK) and hydrogen peroxide, respectively (5, 20). Furthermore, production of several known stimuli of NADPH oxidases, which are important in cardiac remodeling (e.g., AngII, aldosterone, and endothelin-1), is increased by doxorubicin (21–23). More definitive evidence comes from a clinical study that identified several genetic polymorphisms of NADPH oxidases predisposing patients to increased risk of doxorubicin cardiotoxicity (4). The same group reported that doxorubicin-stimulated cardiac superoxide production was attenuated in Nox2−/− mice, which were resistant to doxorubicin-induced LV dilatation and contractile dysfunction (4, 24), although no further detailed analysis of remodeling was undertaken. The aim of this study was to precisely define the role of Nox2 NADPH oxidase in doxorubicin-induced cardiotoxicity and to investigate how it may modulate individual components of the remodeling phenotype in this setting.

### Materials and Methods

#### Experimental animals

A colony of Nox2−/− mice is established on a C57BL/6J background (25) in our institution. All experiments were performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (UK).

#### Doxorubicin treatment protocol

Male Nox2−/− and WT littermate controls (8–10 weeks) were injected with a cumulative dose of 12 mg/kg doxorubicin or equivalent volume of vehicle control via three weekly injections (4 mg/kg i.p. at 0, 7, and 14 days), and subsequent analyses were performed 8 weeks after the first injection. No mortality was associated with this dosing regimen. For ex vivo analyses, animals were sacrificed by sodium pentobarbitone overdose before hearts were excised and either frozen in liquid nitrogen and stored at −80°C or fixed in 10% neutral-buffered formalin solution for further studies. A separate cohort of mice was chronically treated with losartan (10 mg/kg/d in drinking water; pretreatment period of 7 days; ref. 26) and assessed 4 weeks after the first doxorubicin injection. A minimum of six animals per group were studied for all protocols.

#### Echocardiography and invasive assessment of cardiac function

Mice were anaesthetized with 1.5% isoflurane/oxygen, placed on a warming pad, and imaged in the supine position using a Vevo770 ultrasound system with high-frequency 45 MHz RMV707B scanhead (VisualSonics, Inc.). M-mode parasternal short-axis scans at papillary muscle level were used to quantify LV end-diastolic (LVEDD) and end-systolic volumes (IVC) was then briefly occluded, allowing construction of end-diastolic pressure-volume relations (LVEDD) and end-systolic diameters (LVESD) from which % fractional shortening was calculated (LVEDD − LVESD)/LVEDD*100.

Isoflurane was then increased to 2%, and the right carotid artery was cannulated with a high-fidelity 1.2-F pressure-volume catheter (SciSense, Inc.), aortic pressure was measured, and the catheter was advanced into the LV for recording of steady-state function. The abdominal inferior vena cava (IVC) was then briefly occluded, allowing construction of variably loaded pressure-volume loops, from which LV end-systolic (ESPVR) and end-diastolic pressure-volume relations (EDPVR) were determined. Absolute volume measurements were corrected for α (derived by simultaneous aortic outflow recording by echocardiography) and parallel conductance (hypertonic saline injection; ref. 27).

#### Assessment of cardiac remodeling

Following sacrifice, measurements of LV and right ventricular weights were taken and indexed to tibial length. All histologic analyses were performed on fixed (10% neutral-buffered formalin), paraffin-embedded LV sections (5 μm). Cardiomyocyte cross-sectional area was determined by fluorescence microscopy using a Nikon microscope and NIS-Elements software (Nikon).
Immunohistochemistry for 3-nitrotyrosine and CD45 was performed with rabbit (Millipore) and rat (BD Biosciences) polyclonal antibodies (1:1,000), respectively, using diaminobenzidine as the chromogen and nuclear counterstaining with hematoxylin. Data were quantified by blinded digital image analysis (NIS-Elements).

Cardiomyocyte apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Roche Diagnostics). TUNEL-positive myocyte nuclei were counted, and data were expressed as % total nuclei identified by 4′,6-diamidino-2-phenylindole staining in the same sections.

Real-time reverse transcription-PCR

Total RNA was extracted from LV homogenate using TRI reagent (Sigma-Aldrich), and cDNA was synthesized by reverse transcription (Applied Biosystems). mRNA expression of GATA-4, procollagen IIIα I, transforming growth factor-β3 (TGF-β3), connective tissue growth factor (CTGF), MMP-2, MMP-9, Nox2, and Nox4 was analyzed by real-time reverse transcription-PCR (RT-PCR) using fluorescent SYBR Green (Prism 7300, Applied Biosystems) and β-actin for normalization by the comparative Ct method. Primer sequences are shown in Supplementary Table S1.

Gelatin zymography

Activities of myocardial MMP-2 and MMP-9 were analyzed by gelatin zymography (16). Zymograms were digitized and densitometric values were digitally quantified (VisionWorksLS, Ultra-Violet Products) and normalized to the WT control.

NADPH oxidase activity

NADPH-dependent superoxide production was assessed in LV homogenate using lucigenin (5 μmol/L)–enhanced chemiluminescence (300 μmol/L NADPH; 100 μg protein; 37°C; ref. 15) performed in triplicate. Potential superoxide sources were assessed by experiments performed in the presence of Tiron (20 mmol/L; cell-permeable superoxide scavenger), diphenyleneiodonium (DPI; 10 μmol/L; inhibits NADPH oxidase and other flavoproteins), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 μmol/L; inhibits superoxide from dysfunctional NOS), oxypurinol (100 μmol/L; xanthine oxidase inhibitor), or rotenone (20 μmol/L; mitochondrial inhibitor).

Table 1. LV functional parameters in control and doxorubicin-treated WT and Nox2−/− mice

<table>
<thead>
<tr>
<th></th>
<th>WT control</th>
<th>WT DOX</th>
<th>Nox2−/− control</th>
<th>Nox2−/− DOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>11</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>511 ± 7</td>
<td>518 ± 16</td>
<td>491 ± 16</td>
<td>503 ± 14</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>4.12 ± 0.04</td>
<td>4.12 ± 0.07</td>
<td>4.24 ± 0.06</td>
<td>4.25 ± 0.06</td>
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<tr>
<td>LVESD (mm)</td>
<td>2.73 ± 0.07</td>
<td>3.07 ± 0.08†</td>
<td>2.77 ± 0.09</td>
<td>2.86 ± 0.15</td>
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<tr>
<td>FS (%)</td>
<td>34.4 ± 1.2</td>
<td>25.7 ± 1.0†</td>
<td>34.8 ± 1.4</td>
<td>29.5 ± 1.44†</td>
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<tr>
<td>Pressure-volume data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>MABP (mmHg)</td>
<td>79.5 ± 3.3</td>
<td>78.2 ± 2.7</td>
<td>79.6 ± 2.3</td>
<td>77.6 ± 3.0</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>105 ± 3.3</td>
<td>98 ± 2.1</td>
<td>100 ± 2.5</td>
<td>100 ± 2.8</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>6.6 ± 1.0</td>
<td>15.4 ± 2.4†</td>
<td>6.9 ± 1.5</td>
<td>4.9 ± 1.0†</td>
</tr>
<tr>
<td>LVDp/dtm (mmHg s⁻¹)</td>
<td>9,766 ± 386</td>
<td>6,114 ± 520‡</td>
<td>9,481 ± 475</td>
<td>8,965 ± 475</td>
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<tr>
<td>LVDp/dtm (mmHg s⁻¹)</td>
<td>−8,218 ± 337</td>
<td>−5,198 ± 527†</td>
<td>−8,600 ± 472</td>
<td>−8,379 ± 416‡</td>
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<td>r (ms)</td>
<td>6.0 ± 0.3</td>
<td>10.3 ± 0.6†</td>
<td>6.3 ± 0.5</td>
<td>5.7 ± 0.5†</td>
</tr>
<tr>
<td>LVEV (μL)</td>
<td>33.5 ± 3.3</td>
<td>63.2 ± 9.0⑥</td>
<td>32.8 ± 5.4</td>
<td>38.6 ± 3.8†</td>
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<tr>
<td>LVEDV (μL)</td>
<td>70.3 ± 7.1</td>
<td>77.6 ± 8.8</td>
<td>69.0 ± 5.5</td>
<td>69.9 ± 6.0</td>
</tr>
<tr>
<td>Stroke volume (μL)</td>
<td>43.2 ± 5.3</td>
<td>24.1 ± 3.2⑥</td>
<td>43.1 ± 4.3</td>
<td>38.3 ± 3.2†</td>
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<tr>
<td>Ejection fraction (%)</td>
<td>56.5 ± 3.5</td>
<td>28.5 ± 2.3†</td>
<td>58.9 ± 4.9</td>
<td>52.6 ± 2.5⑥</td>
</tr>
<tr>
<td>SW (mmHg μL g⁻¹)</td>
<td>37,964 ± 5,291</td>
<td>16,658 ± 3,448⑥</td>
<td>40,359 ± 3,286</td>
<td>29,074 ± 2,327</td>
</tr>
<tr>
<td>ESPVR</td>
<td>2.43 ± 0.42</td>
<td>1.17 ± 0.38*</td>
<td>2.64 ± 0.29</td>
<td>2.31 ± 0.20</td>
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<tr>
<td>EDPVR</td>
<td>0.040 ± 0.011</td>
<td>0.114 ± 0.012†</td>
<td>0.041 ± 0.008</td>
<td>0.051 ± 0.009†</td>
</tr>
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NOTE: Mean ± SEM.
Abbreviations: DOX, doxorubicin; FS, fractional shortening; LVEDV, LV end-diastolic volume; LVESP, LV end-systolic pressure; LVESV, LV end-systolic volume; MABP, mean arterial blood pressure; SW, stroke work.

*P < 0.05 versus control.
†P < 0.01 versus control.
‡P < 0.05, Nox2−/− doxorubicin versus WT doxorubicin.
§P < 0.1 versus control.
∥P < 0.001, Nox2−/− doxorubicin versus WT doxorubicin.
Isolated cardiomyocyte studies

Ventricular cardiomyocytes were isolated from male WT mice (aged 10–12 weeks) by collagenase digestion (28), plated at 50 cells/mm² in MEM containing 10% inactivated fetal bovine serum and antibiotics, and equilibrated for 1 hour. Cells were then incubated with or without doxorubicin (0.5 μmol/L; ref. 5) for 3 hours in the presence or absence of the AT1 receptor antagonist losartan (10 μmol/L, pretreatment for 30 minutes; ref. 29). Immediately after treatment, cardiomyocyte NADPH-dependent superoxide production was quantified using lucigenin-enhanced chemiluminescence and data were normalized to cell viability assessed by CellTiter-Fluor assay (Promega). The remainder of the cells were frozen in liquid nitrogen and stored at −80°C for real-time RT-PCR analysis of Nox2, Nox4, and TGF-β3 mRNA expression.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Bonferroni or Dunnett’s multiple comparison test, or unpaired Student’s t test, as appropriate. *P* < 0.05 was considered to be significant.

Results

Echocardiography

Data are summarized in Table 1. No differences were observed between WT and Nox2−/− controls. However, Nox2−/− mice showed better contractile function after doxorubicin treatment compared with WT mice, as indicated by higher % fractional shortening and lower LVESD. Heart rate and LVEDD were similar between groups.

LV pressure-volume analysis

Steady-state data are presented in Table 1. WT and Nox2−/− controls exhibited similar function, and consistent with echocardiography, doxorubicin-treated WT mice had impaired systolic function, reflected by decreased LVDp/dtmax stroke volume and ejection fraction, and increased LV end-systolic volume. LVDp/dtmin was also reduced in WT doxorubicin-treated mice, whereas LV end-diastolic pressure (LVEDP) and the isovolumic relaxation time constant τ were increased, indicating diastolic dysfunction. In contrast, none of these steady-state parameters were altered in Nox2−/− doxorubicin-treated mice. Furthermore, representative steady-state pressure-volume loops (Fig. 1A and C) clearly show that doxorubicin-induced decreases in stroke work (area of loop) observed in WT mice were preserved in Nox2−/− mice (Table 1).

Figure 1B and D shows representative data obtained under variable loading after IVC occlusion. The slope of the LV ESPVR was decreased in WT doxorubicin-treated mice, indicating reduced contractility, but preserved in Nox2−/− mice. In addition, the slope of the LV EDPVR (measures diastolic stiffness) was increased in WT, but not Nox2−/−, doxorubicin-treated mice (Table 1). Importantly, mean arterial blood pressure remained similar between groups.

Myocardial atrophy

Although there was no difference in body weight between WT and Nox2−/− controls (30.7 ± 0.7 versus 30.3 ± 0.8 g,
n = 30, 33), this was reduced by doxorubicin in both groups (WT: 24.9 ± 0.7 g, n = 33; P < 0.001 versus control; Nox2−/−: 27.6 ± 0.4 g, n = 37; P < 0.05 versus control), with a tendency toward a greater decrease in WT versus Nox2−/− mice. Morphometric data were therefore normalized to tibial length. Chronic doxorubicin treatment decreased LV/tibial length in WT, but not Nox2−/− mice (Fig. 2A). No differences in right ventricular/tibial length were observed between groups (data not shown). Consistent with this atrophic action of doxorubicin, cardiomyocyte cross-sectional area (Fig. 2B) was decreased in WT, but not Nox2−/−, mice. GATA-4 mRNA expression was similar between groups (Fig. 2C).

**Cardiomyocyte apoptosis**

Chronic doxorubicin treatment increased the number of TUNEL-positive cells in LV sections from WT versus Nox2−/− mice (158 ± 37% versus 23 ± 12%; P < 0.01; Fig. 2D) compared with controls. Representative examples of TUNEL-stained LV sections are shown in Supplementary Fig. S1.

**Cardiac fibrosis**

Interstitial fibrosis, assessed in LV sections by picrosirius red staining, was increased in WT versus Nox2−/− doxorubicin-treated mice (123 ± 20% versus 37 ± 23%; P < 0.01; Fig. 3A) compared with controls. Representative examples of picrosirius red–stained LV sections are shown in Supplementary Fig. S2. Consistent with this, mRNA expression of the profibrotic genes procollagen IIIα1, TGF-β3, and CTGF was attenuated in doxorubicin-treated Nox2−/− compared with WT mice (Fig. 3B–D).

**Myocardial MMP activity and expression**

Myocardial MMP-9 activity was increased by doxorubicin in WT, but not Nox2−/−, mice (Fig. 3F). Similarly, MMP-9 mRNA expression was elevated in WT, but not Nox2−/−, doxorubicin-treated mice (Fig. 3E). MMP-2 activity/expression remained similar between groups (data not shown).

**NADPH oxidase activity and expression**

LV NADPH-dependent superoxide production was increased in WT, but not in Nox2−/− doxorubicin-treated mice (Fig. 4A). Superoxide generation was inhibited by Tiron and DPI, but not by L-NAME, oxypurinol, and rotenone, in both WT and Nox2−/− mice (Fig. 4B shows WT data), indicating that the observed signal reflected NADPH oxidase activity.

Myocardial Nox2 mRNA expression was increased in doxorubicin-treated WT mice, but was not detected in Nox2−/− mice (Fig. 4C). Nox4 mRNA expression was also increased in doxorubicin-treated WT, but not Nox2−/−, mice (Fig. 4D).

**3-Nitrotyrosine staining**

LV 3-nitrotyrosine levels, used to assess in situ oxidative/nitrosative stress, were increased in doxorubicin-treated WT mice compared with controls, and this increase was attenuated in Nox2−/− mice (Fig. 4E). Representative examples of 3-nitrotyrosine–stained LV sections are shown in Supplementary Fig. S3.

**CD45 staining**

Immunohistochemistry showed that myocardial infiltration of CD45-positive leukocytes was increased in WT doxorubicin-treated mice (Fig. 5). Although there was a tendency toward increased leukocyte infiltration in Nox2−/− doxorubicin-treated mice, this was not significant.
mice, this was not significant and was clearly reduced compared with the WT group.

Losartan treatment

Chronic treatment with the AT1 receptor antagonist losartan resulted in normalization of echocardiographic fractional shortening (Fig. 6A) and LVESD (data not shown) in doxorubicin-treated WT mice; heart rate and LVEDD were unaffected (data not shown). Losartan also increased invasively assessed LVdP/dtmax,slo of the ESPVR (Fig. 6B and C), ejection fraction, stroke work, and LVdP/dtmin while decreasing τ and slope of the EDPVR (data not shown) in these animals. Furthermore, chronic losartan treatment prevented the development of myocardial atrophy in WT doxorubicin-treated mice, together with increases in NADPH-dependent superoxide production and mRNA expression of Nox2 and MMP-2 (Fig. 6D–F and H). Losartan also tended to reduce doxorubicin-induced increases in Nox4 mRNA expression, although this failed to reach significance (Fig. 6G). Similarly, in vitro studies in isolated WT cardiomyocytes showed that acute losartan treatment attenuated doxorubicin-induced increases in NADPH oxidase activity and mRNA expression of Nox2, Nox4, and TGF-β3 (Fig. 6I–L).

Discussion

This study clearly shows that Nox2 NADPH oxidase plays an important role in the development of doxorubicin-induced cardiac injury and remodeling. The major evidence for this was that genetic disruption of Nox2 protected against the development of cardiac contractile dysfunction, atrophic cardiomyocyte degeneration, apoptosis, interstitial fibrosis, and MMP-9 activation after chronic doxorubicin treatment. These actions on cardiac remodeling were associated with beneficial changes in NADPH oxidase activity, oxidative/nitrosative stress, and inflammatory cell infiltration.

Although doxorubicin is extremely effective against many tumors, its well-established cardiotoxic side effects severely limit its clinical use. The incidence of cardiotoxicity is low at doses <400 mg/m², rising to 7% at the commonly used upper limit (550 mg/m²), steeply increasing thereafter (4). In the present study, a cumulative doxorubicin dose of 12 mg/kg was used and mice were studied 8 weeks after first administration. This protocol was adopted to reflect common treatment regimens, which usually involve several cycles of chemotherapy, and to approximate the clinical chronology of chronic doxorubicin-induced cardiotoxicity, which typically develops over several months (21). It should be noted that doxorubicin exerts its antitumor action by inhibiting DNA topoisomerase II, thereby preventing unwinding of the double helix during replication, and by direct intercalation into the DNA, preventing resealing (20). Importantly, these pathways are not thought to involve ROS; indeed, our confirmatory studies in HeLa cells showed that knockdown of Nox2 using a specific small interfering RNA had no significant effect on the antitumor efficacy of doxorubicin (see Supplementary Fig. S4).

It is well established that doxorubicin increases myocardial ROS production, which has been implicated in the associated cardiotoxicity (4, 5). However, the sources of ROS and mechanisms by which they may modulate different components of the cardiotoxic remodeling phenotype are less clear.
Potential myocardial ROS sources include the mitochondrial electron transport chain, xanthine oxidase, dysfunctional NOS, and NADPH oxidases (14). In this study, we focused on NADPH oxidases, which are the only myocardial source whose primary function is ROS generation. NADPH oxidases are activated by several stimuli (e.g., AngII, cyclic stretch, and tumor necrosis factor-α) that are pivotal in cardiac remodeling and, importantly, are also capable of modulating other ROS sources (14). Indeed, NADPH oxidases may potentiate mitochondrial ROS generation and convert xanthine dehydrogenase to xanthine oxidase (30, 31). Furthermore, NADPH oxidase–derived ROS are known to promote oxidative degradation of tetrahydrobiopterin, leading to NOS uncoupling and superoxide production (32). However, NADPH-dependent superoxide production was unaffected by rotenone, oxypurinol, or L-NAME, suggesting that these alternate sources were not major contributors to doxorubicin-induced ROS generation in our study. Furthermore, our data clearly show that doxorubicin-stimulated NADPH-dependent superoxide production is critically dependent on Nox2, as increases observed in WT hearts were not seen in Nox2−/− mice. This pattern was repeated with 3-nitrotyrosine, indicating that in situ oxidative/nitrosative stress was only elevated in WT doxorubicin-treated hearts and was therefore dependent on Nox2. In addition, Nox2 mRNA expression was increased by doxorubicin in WT hearts and, as expected, was not detectable in Nox2−/− hearts. Our previous studies investigating Nox2 NADPH oxidase in cardiac remodeling found Nox4 expression to be increased in both WT and Nox2−/− mice after chronic pressure overload and myocardial infarction, together with parallel increases in NADPH oxidase activity (15, 16). However, in this study, although Nox4 mRNA expression was increased in doxorubicin-treated WT hearts, it remained unaltered in those from Nox2−/− mice. The reasons for this are unclear, although it may suggest potential cross-talk between the two isoforms and dependence of Nox4 on Nox2 in the setting of doxorubicin cardiotoxicity. Nonetheless, it seems that upregulation of both Nox2 and Nox4 may contribute to increased NADPH oxidase activity in doxorubicin-treated WT mice.

Doxorubicin is known to cause cardiac contractile dysfunction (2), and this was clearly evident in our WT mice. These animals developed systolic dysfunction as evidenced by reductions in fractional shortening, both pressure-based and volume-based indices (LV end-systolic volume and ejection fraction), and the slope of the ESPVR, which is acknowledged to be the gold standard measure of contractility (27). WT mice also showed impaired LV relaxation (reduced LVdP/dtmin and prolonged τ) and diastolic dysfunction (increased LVEDP and slope of the EDPVR). In contrast, all of these parameters were preserved in doxorubicin-treated Nox2−/− mice.

The marked attenuation of contractile dysfunction observed in Nox2−/− mice is likely to have occurred due to beneficial actions on different components of the remodeling phenotype. It seems that reduced cardiomyocyte atrophy and interstitial fibrosis may be the most important contributors, with apoptosis playing a secondary role by reducing LV...
mass and stimulating reparative extracellular matrix remodeling (3). In addition, it is possible that Nox2-derived ROS could exert adverse effects on contractile function through direct actions on excitation-contraction coupling, myofila-
ment calcium responsiveness, and cellular energetics (12, 13, 33). It is well established that doxorubicin cardiotoxicity is at least partly attributable to cardiomyocyte apoptosis (6). Indeed, in the present study, the incidence of TUNEL-positive apoptotic nuclei was reduced in hearts from doxorubicin-
−/− mice, suggesting that this may contribute to preserved contractile function in these animals. There
seems to be considerable debate in relation to the trophic actions of doxorubicin on the cardiomyocyte, with some studies reporting hypertrophy (7, 34), whereas others suggest an atrophic action (35, 36). Here, we clearly show that chronic doxorubicin treatment results in myocardial atrophy, as reflected by decreased morphometric LV/tibial length ratio and cardiomyocyte cross-sectional area. This was attenuated in Nox2−/− doxorubicin-treated mice, suggesting that preservation of cardiomyocyte mass may also have contributed to maintained contractile function. It should be noted that reduced cardiomyocyte apoptosis in Nox2−/− mice in response to doxorubicin is likely to have also influenced preservation of morphologic LV mass in these animals (37, 38), although the extent of its contribution in this context is unclear. It has been suggested that the atrophic action of doxorubicin may occur secondary to reductions in GATA-4, a key regulator of cardiac development, known to modulate myocardial expres-
sion of sarcomeric proteins such as α-myosin heavy chain and troponin I (39, 40). However, GATA-4 mRNA expression remained similar between groups, suggesting that this pathway was not involved in our study.

Contractile function is also known to be influenced by alterations in extracellular matrix remodeling and fibrosis. Indeed, chronic doxorubicin treatment was associated with increases in interstitial fibrosis and expression of profibrotic genes (procollagen IIIα1, TGF-β3, and CTGF) in WT, but not Nox2−/−, mice, and these changes are likely to be largely responsible for the differences in diastolic and relaxation properties (e.g., LVEDP, τ, and EDPVR) between groups. Furthermore, myocardial expression and activity of MMP-9, but not MMP-2, was increased in WT, but not Nox2−/−, doxorubicin-treated mice. Indeed, it is well established that MMP activation is redox sensitive (9), and several recent in vitro and in vivo studies have implicated NADPH oxidases, particularly those containing Nox2 (16, 41). Doxorubicin may also activate MMPs in a redox-sensitive manner. Myocardial activity and expression of MMP-2 and MMP-9 are increased acutely by doxorubicin in mice in vivo, and this is inhibited by the superoxide dismutase mimetic MnTMPyP (6). Similarly, MMP-2 and MMP-9 activity/expression are augmented by doxorubicin in H9c2 rat ventricular cardiomyocytes in association with elevated Nox1 expression, and attenuated by the flavoprotein inhibitor DPI (5). Indeed, in the present study, myocardial activity and expression of MMP-9, but not MMP-2, was increased in WT, but not Nox2−/−, doxorubicin-treated

Figure 5. Effect of doxorubicin on leukocyte infiltration in WT and Nox2−/− mice. A to D, representative LV sections stained for CD45. E, positive spleen control. F, negative secondary-only spleen control. G, mean quantification data (n = 9) as CD45-positive cells per high-power field (HPF); bars, SE.
* P < 0.05 versus control.
mice. In this regard, it has been suggested that doxorubicin-induced activation of MMP-2 and MMP-9 may involve different pathways. For example, a single bolus doxorubicin dose in mice induced early activation of myocardial MMP-2, which was followed by MMP-9 activation several days later (42). In addition, an *in vitro* study indicated that p38 mitogen-activated protein kinase may be responsible for doxorubicin-induced MMP-9 activation, whereas JNK/ERK may be involved in MMP-2 regulation (5). Doxorubicin cardiotoxicity is frequently characterized by LV dilatation, and MMP activation is thought to play an important role in this process (2, 3). However, in the present study, LV end-diastolic structural parameters assessed by both echocardiography and pressure-volume analysis remained similar between groups, despite activation of MMP-9. The reasons for this are unclear, although it may reflect a temporal relationship between MMP-9 and LV chamber dilatation or the involvement of other MMPs. It is also conceivable that LV dilatation may have been masked by the atrophic actions of doxorubicin on the cardiomyocyte, which were observed in WT, but not Nox2−/−, mice.

Doxorubicin is well known to induce myocardial inflammation (35). In the present study, chronic doxorubicin...
Treatment was associated with myocardial infiltration of CD45-positive cells in WT, but not Nox2−/− mice, suggesting a role for ROS derived specifically from Nox2 NADPH oxidase. Indeed, Nox2 is expressed in inflammatory cells, and their recruitment may influence redox-sensitive processes, such as MMP activation and extracellular matrix remodeling, through generation of cytokines and other factors (14). Interestingly, in mice lacking T cells (RAG1−/−), AngII-induced hypertension (known to be mediated by Nox2) is blunted and associated with reduced superoxide production and attenuation of endothelial dysfunction and cardiac hypertrophy (43). Not only does this indicate that T-cell Nox2 NADPH oxidase is necessary for AngII-induced hypertension, it also suggests that some of the protective effects against doxorubicin-induced cardiotoxicity observed in Nox2−/− mice in the present study may occur secondary to activation of Nox2 in inflammatory cells. However, it is interesting to note that although patients with chronic granulomatous disease (caused by defects in genes encoding the Nox2 NADPH oxidase complex) frequently develop recurrent infections, Nox2−/− mice do not due to compensation by other protective pathways (44, 45). Indeed, in our study, doxorubicin-treated Nox2−/− mice exhibited a tendency toward increased myocardial infiltration of CD45-positive cells, suggesting that the observed effects on cardiac remodeling may not be accounted for solely by differences in the inflammation.

It is well established that several of the actions of Nox2 NADPH oxidase in cardiac remodeling are mediated via activation of AngII (11, 18), production of which is increased by doxorubicin (21). Indeed, in the present study, chronic treatment with the AT1 receptor antagonist losartan prevented development of both contractile dysfunction and myocardial atrophy in response to doxorubicin. Furthermore, increases in NADPH oxidase activity and Nox2/Nox4 mRNA expression induced by doxorubicin both in vivo and in vitro were attenuated by cotreatment with losartan. These data suggest that AngII signaling may play an important role in doxorubicin-induced Nox2 activation, although it is likely that other key upstream mediators are also involved. Interestingly, Rac1-mediated activation of Nox2 NADPH oxidase is implicated in the proximal signaling involved in AngII-induced cardiomyocyte hypertrophy, ahead of downstream Akt activation (46), and doxorubicin cardiotoxicity is attenuated by statins that exert antioxidant effects via Rac1 inhibition (47). Signaling upstream of Rac1 translocation in neonatal cardiomyocytes may also involve protein kinase C, c-Src family tyrosine kinases, and proline-rich tyrosine kinase 2 (48–50). Nonetheless, data on NADPH oxidase–specific activation in the cardiomyocyte are scarce and further studies are clearly required to establish the precise mechanisms underlying doxorubicin-induced Nox2 activation, and these are currently the focus of our research.

In summary, these data suggest that ROS derived specifically from Nox2 NADPH oxidase make a significant contribution to several key processes underlying cardiac remodeling associated with doxorubicin chemotherapy. Selective targeting of NADPH oxidases may provide a novel therapeutic strategy against the cardiotoxic actions of doxorubicin, thereby maximizing its effectiveness as an antineoplastic agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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