Disruption of Nitric Oxide Synthase 3 Protects Against the Cardiac Injury, Dysfunction, and Mortality Induced by Doxorubicin

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Background—Flavoprotein reductases are involved in the generation of reactive oxygen species by doxorubicin. The objective of the present study was to determine whether or not one flavoprotein reductase, endothelial nitric oxide synthase (nitric oxide synthase 3 [NOS3]), contributes to the cardiac dysfunction and injury seen after the administration of doxorubicin.

Methods and Results—A single dose of doxorubicin (20 mg/kg) was administered to wild-type (WT) mice, NOS3-deficient mice (NOS3−/−), and mice with cardiomyocyte-specific overexpression of NOS3 (NOS3-TG). Cardiac function was assessed after 5 days with the use of echocardiography. Doxorubicin decreased left ventricular fractional shortening from 57±2% to 47±1% (P<0.001) in WT mice. Compared with WT mice, fractional shortening was greater in NOS3−/− and less in NOS3-TG after doxorubicin (55±1% and 35±2%; P<0.001 for both). Cardiac tissue was harvested from additional mice at 24 hours after doxorubicin administration for measurement of cell death and reactive oxygen species production. Doxorubicin induced cardiac cell death and reactive oxygen species production in WT mice, effects that were attenuated in NOS3−/− and were more marked in NOS3-TG mice. Finally, WT and NOS3−/− mice were treated with a lower dose of doxorubicin (4 mg/kg) administered weekly over 5 weeks. Sixteen weeks after beginning doxorubicin treatment, fractional shortening was greater in NOS3−/− than in WT mice (45±2% versus 28±1%; P<0.001), and mortality was reduced (7% versus 60%; P<0.001).

Conclusions—These findings implicate NOS3 as a key mediator in the development of left ventricular dysfunction after administration of doxorubicin. (Circulation. 2007;116:506-514.)

Key Words: cardiomyopathy • free radicals • heart failure • nitric oxide • nitric oxide synthase

The use of anthracyclines, including doxorubicin, as chemotherapy agents is limited by cardiac injury.1 However, the mechanisms by which doxorubicin induces cardiac injury and dysfunction are incompletely understood. A number of doxorubicin-induced biochemical changes have been identified that can damage cardiac myocytes. Some of these include cellular toxicity from metabolites of doxorubicin,2 generation of reactive oxygen species (ROS),3 production of reactive nitrogen species,4 selective inhibition of cardiac muscle gene expression,5 impaired calcium homeostasis causing intracellular calcium overload,6 and disturbance of myocardial adrenergic signaling.7 The cardiac toxicity associated with doxorubicin administration is mediated, at least in part, by induction of cardiac cell apoptosis.8,9

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There are several mechanisms by which doxorubicin can induce production of ROS. For example, formation of doxorubicin-iron complexes may trigger a Fenton-type reaction10 with a resultant increase in ROS levels.11 Another mechanism through which doxorubicin forms ROS involves the reduction of a quinine moiety in doxorubicin to a semiquinone intermediate by flavoprotein reductases;12 this reduced form of doxorubicin generates superoxide anions in the presence of molecular oxygen.13 A variety of flavoprotein reductases have been implicated in the formation of ROS after doxorubicin administration, including cytochrome P450 reductases, mitochondrial NADH dehydrogenase, and nitric oxide (NO) synthases (NOS).12

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NOS enzymes produce NO from the catalytic conversion of L-arginine to L-citrulline in the presence of oxygen and NADPH. Three NOS isoforms exist: neuronal (nNOS, or NOS1), inducible (iNOS, or NOS2), and endothelial (eNOS, or NOS3). Doxorubicin binds to all 3 NOS isoforms, leading to inhibition of NOS activity and reduction of the anthracycline. Of the 3 NOS isoforms, NOS3 has the highest affinity for doxorubicin. The deleterious role of NOS3 after the administration of doxorubicin has been observed previously in vitro. However, N\textsuperscript{6}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of oxygenase domain of NOS3, has been shown to increase mortality after doxorubicin in mice, suggesting that NO synthesis is protective against doxorubicin-induced cardiac toxicity.

Therefore, to define the role of NOS3 in the development of anthracycline-induced cardiac injury, we compared the impact of doxorubicin administration on cardiac structure and function in wild-type (WT) mice, NOS3-deficient (NOS3\textsuperscript{-/-}) mice, and mice with a cardiomyocyte-specific overexpression of NOS3 (NOS3-TG). We found that, compared with WT mice, NOS3\textsuperscript{-/-} had reduced, and NOS3-TG had increased, cardiac dysfunction and injury after doxorubicin.

Methods

Study Design

All experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. Male and female WT, NOS3\textsuperscript{-/-}, and NOS3-TG mice from a C57BL/6 background were studied between 10 and 12 weeks of age (The Jackson Laboratory, Bar Harbor, Me). Doxorubicin was administered via intraperitoneal injection. Acute and chronic protocols were used. In Laboratory, Bar Harbor, Me). Doxorubicin was administered via

Cardiac Cell Death

Cardiac cell death was detected with the use of the terminal deoxynucleotidyltransferase–mediated dUTP nick-end labeling (TUNEL) technique (DeadEnd Fluorometric TUNEL System, Promega Corporation, Madison, Wis), as previously described.

Detection of ROS

ROS generation was examined by 2 independent methods. Tissue superoxide production was estimated by dihydroethidium (DHE) staining of cardiac tissue sections, as previously described. To test the effect of inhibiting the NOS3 oxygenase domain on ROS production, cardiac sections from NOS3-TG animals, treated with saline or doxorubicin, were coincubated with DHE and L-NAME, (1 mmol/L). Tissues were washed in PBS and examined with a Zeiss Axioshot microscope for green fluorescence. To examine the ability of doxorubicin and L-NAME to modulate cardiac NO production, frozen cardiac sections from NOS3-TG animals, treated with doxorubicin or saline, were stained with diaminofluorescein (DAF- FM DA, Calbiochem) in the presence or absence of L-NAME, as previously described. Mean fluorescence intensity of the digitzed image was measured with Image J software (version 1.35, NIH) for quantification.

Superoxide production was measured by lucigenin-enhanced chemiluminescence in freshly harvested cardiac tissue from mice 24 hours after challenge with saline or doxorubicin as previously described. Briefly, cardiac tissue was preincubated in Krebs-HEPES buffer containing 10 \( \mu \)mol/L NADPH for 45 minutes. Tissues were then transferred into wells of a 96-well plate containing 300 \( \mu \)L of Krebs-HEPES buffer supplemented with 10 \( \mu \)mol/L NADPH and 10 \( \mu \)mol/L lucigenin (bis-2-methylacridinium nitrate). Addition of superoxide dismutase (200 U/mL) to the reaction buffer abolished the chemiluminescence signal, confirming the specificity of the assay (data not shown). To test the effect of inhibiting the NOS3 oxygenase domain, doxorubicin-induced superoxide production was determined by measuring lucigenin-enhanced chemiluminescence production in cardiac tissue from doxorubicin-challenged NOS3-TG animals (n = 3 per group) in the presence and absence of L-NAME (1 mmol/L).

NOS3 Protein Expression and Monomerization

Hearts were harvested from WT mice 24 hours after doxorubicin or saline treatment. NOS3 dimerization was measured by low-temperature SDS-PAGE, as previously described. Membranes were blocked in 5% nonfat milk in Tris-buffered saline Tween-20 (TBST) (TBST milk) and then incubated with an anti-NOS3 antibody (BD Transduction, Bedford, Mass; 1:2500) in TBST milk. The bound antibody was detected with the use of horseradish peroxidase–labeled anti-mouse antibody (Cell Signaling, Charlottesville, Va; 1:1000) in TBST milk and visualized with the use of chemiluminescence ECL Plus (Amersham Biosciences Corp, Piscataway, NJ). To confirm equal loading of protein, membranes were subsequently incubated with anti-tubulin antibody (BD Transduction, 1:1000 in TBST milk), and bound antibody was detected as above.

Measurement of Gene Expression

Total RNA was extracted from the left ventricle with the use of TRIzol reagent (Invitrogen), and cDNA was synthesized with the use of Moloney murine leukemia virus reverse transcriptase (Invitrogen, La Jolla, Calif). NOS2, NOS3, cyclooxygenase-2 (COX-2), heme oxygenase-1 (HO-1), Bcl-xL, GATA-4, and 18S ribosomal RNA (rRNA) transcript levels were measured by real-time polymerase chain reaction with the use of an ABI Prism 7000 (Applied Biosystems, Inc, Foster City, Calif) and primers for NOS2 (TaqMan, Applied Biosystems), NOS3 (TaqMan, Applied Biosystems), COX-2 (5'-TGAGCAACTATTCCAAACCAGC-3'), HO-1 (5'-GCCACAAAGGAAGGATCAGCAT-3'), Bcl-xL (5'-
GACAAGGAGTAGCCATATTGG-3', 5'-TCCCGTGAGAGATCCAAAAGT-3'), GATA-4 (5'-CCTACACCGCTACATG-3', 5'-ACATATCGGAGATGGG TTGCT-3'), and 18s rRNA (5'-CGCTGACACATCAGGAA-3', 5'-GCTGGAATTACCGG-GCT-3'). Changes in the gene expression normalized to levels of 18s rRNA were determined by the relative C method (Applied Biosystems).

**Statistical Analysis**

All data are expressed as mean±SEM. The primary hypothesis tested was whether genetic manipulation of cardiac NOS3 expression would influence the cardiac dysfunction induced by administration of doxorubicin. The comparisons within genotypes between baseline and after doxorubicin administration are presented to confirm that doxorubicin induced cardiac dysfunction under the conditions employed in our studies. Statistical significance between 2 measurements was determined by the 2-tailed paired or unpaired Student t test, as appropriate. Repeated measures were analyzed by an ANOVA for repeated measures, and pairwise comparisons were made only if the ANOVA was significant (SAS Institute Inc, Cary, NC). Analysis of survival rates after doxorubicin was performed with the log-rank test. Probability values of <0.05 were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Echocardiography and Survival After a Single Doxorubicin Administration**

Echocardiographic indices were similar in WT, NOS3−/−, and NOS3-TG mice at baseline (Table 1). Values at baseline and after doxorubicin administration did not differ between males and females of any of the genotypes; therefore, results from both genders were pooled for subsequent studies. Five days after a single injection of doxorubicin, echocardiography was performed in surviving mice (WT, n=32; NOS3−/−, n=38; NOS3-TG, n=5). After doxorubicin, the left ventricular (LV) internal dimension in systole was increased in WT mice (Figure 1), whereas fractional shortening (FS), ejection fraction (EF), peak endocardial systolic velocity (V_{EsdO}), and strain rate (SR) were decreased (Table 1). Five days after doxorubicin was administered to NOS3−/− mice, LV internal dimension in systole, FS, and EF were unchanged, and V_{EsdO} and SR were mildly decreased compared with baseline. Compared with WT mice, NOS3−/− had smaller cardiac dimensions and greater FS, EF, V_{EsdO}, and SR after doxorubicin. In contrast, 5 days after doxorubicin challenge, LV internal dimensions increased to a greater extent in NOS3-TG mice than in WT and NOS3−/− mice. The doxorubicin-induced increase in cardiac dimensions in NOS3-TG mice was associated with a marked reduction in cardiac function (Table 1, Figure 1). The severity of the doxorubicin-induced reduction in cardiac function in NOS3-TG mice was likely underestimated because only 5 of 15 mice survived 5 days after doxorubicin administration. Moreover, when WT, NOS3−/−, and NOS3-TG mice were followed to 8 days after doxorubicin administration, the greatest mortality was observed in NOS3-TG, and the least mortality was seen in NOS3−/− mice (Figure 2).

**Table 1. Echocardiographic Parameters at Baseline and 5 Days After a Single Dose of Doxorubicin (20 mg/kg) in WT, NOS3−/−, and NOS3-TG Mice**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Baseline</th>
<th>Day 5</th>
<th>P (vs Baseline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>WT 619±15</td>
<td>571±18</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 618±14</td>
<td>570±15</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>NOS3-TG 621±12</td>
<td>500±21†</td>
<td>0.04</td>
</tr>
<tr>
<td>LVIDd, mm</td>
<td>WT 3.2±1.0</td>
<td>3.2±1.0</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 3.2±1.0</td>
<td>3.1±1.0</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>NOS3-TG 3.2±1.0</td>
<td>3.4±1.0‡</td>
<td>0.01</td>
</tr>
<tr>
<td>LVIDs, mm</td>
<td>WT 1.4±1.0</td>
<td>1.7±1.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 1.4±1.0</td>
<td>1.4±1.0‡</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>NOS3-TG 1.4±1.0</td>
<td>2.2±1.0‡</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FS, %</td>
<td>WT 57±2</td>
<td>47±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 57±2</td>
<td>55±1†</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>NOS3-TG 57±1</td>
<td>35±2*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>EF, %</td>
<td>WT 78±2</td>
<td>66±3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 78±2</td>
<td>74±2‡</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>NOS3-TG 77±2</td>
<td>52±3*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>V_{EsdO}, cm·s⁻¹</td>
<td>WT 3.2±0.2</td>
<td>2.2±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 3.3±0.2</td>
<td>2.9±0.1‡</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>NOS3-TG 3.3±0.3</td>
<td>1.7±0.2*</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SR, s⁻¹</td>
<td>WT 26±1</td>
<td>15±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 25±1</td>
<td>20±2‡</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>NOS3-TG 25±2</td>
<td>10±1*</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are mean±SEM. LVIDd indicates left ventricular internal dimensions in diastole; LVIDs, left ventricular internal dimensions in systole.

**Invasive Hemodynamic Measurements After Doxorubicin**

Invasive hemodynamic measurements did not differ between untreated WT and NOS3−/− mice except for an elevated LV end-systolic pressure in NOS3−/− (Table 2). Five days after a single dose of doxorubicin (20 mg/kg), invasive hemodynamic measurements were performed in surviving WT (n=10) and NOS3−/− (n=10) mice. After doxorubicin, LV end-diastolic pressure increased and LV end-systolic pressure decreased in WT mice. The maximum rate of change of pressure (dp/dt\_{MAX}), the minimum rate of change of pressure (dp/dt\_{MIN}), and dp/dt\_{MAX} divided by the instantaneous pressure (dp/dt\_{MAX}/IP) were reduced in WT mice after doxorubicin compared with untreated WT mice. Doxorubicin treatment also decreased LV end-systolic pressure, dp/dt\_{MAX}, dp/dt\_{MIN}, and dp/dt\_{MAX}/IP in NOS3−/− mice (Table 2). However, compared with WT mice, NOS3−/− mice after doxorubicin had a lower LV end-diastolic pressure and greater dp/dt\_{MAX}, dp/dt\_{MIN}, and dp/dt\_{MAX}/IP.

**Doxorubicin-Induced Cardiac Cell Death**

Doxorubicin causes cardiac cell death, which can be detected with a TUNEL assay. In hearts from saline-challenged mice, there were only very low levels of cell death in all 3
genotypes (2 ± 1 per 100,000 cells; data not shown). Twenty-four hours after WT mice received a single injection of doxorubicin, the frequency of TUNEL-positive cardiac nuclei increased to 0.7 ± 0.2% (Figure 3). In contrast, in NOS3−/− mice treated with doxorubicin, the frequency of TUNEL-positive nuclei increased only modestly to 0.02 ± 0.005%, whereas NOS3-TG had a marked increase in TUNEL-positive nuclei to 1.6 ± 0.2% in response to doxorubicin.

Reactive Oxygen Species

There was no difference in staining with DHE between genotypes after saline challenge. Twenty-four hours after doxorubicin, there was an increase in DHE staining in cardiac sections from WT (Figure 4A, compare panels 1 and 2) and NOS3-TG (panel 4) mice but not NOS3−/− mice (panel 3). Incubation of cardiac sections from doxorubicin-challenged NOS3-TG mice with L-NAME did not attenuate DHE staining (panel 5). The same concentration of L-NAME inhibited NO production in cardiac sections from saline-challenged NOS3-TG mice (compare panels 6 and 8; mean fluorescence intensity, control 16 ± 3% versus 7 ± 2% doxorubicin; P < 0.05). Of note, doxorubicin also inhibited NO production in NOS3-TG mice (panel 7; mean fluorescence intensity, control 16 ± 3% versus 8 ± 3% L-NAME; P < 0.05).

There was no difference in lucigenin-enhanced chemiluminescence between the 3 genotypes after saline challenge. Twenty-four hours after administration of doxorubicin, there was an almost 3-fold increase in chemiluminescence in the hearts of WT mice (Figure 4B). In contrast, there was no increase in chemiluminescence in NOS3−/− mice. Furthermore, in NOS3-TG mice challenged with doxorubicin, there was a 5-fold increase in chemiluminescence after 24 hours. In

![Figure 1. Cardiac function at baseline and 5 days after doxorubicin. Representative M-mode tracings are displayed from a control (CTRL) WT mouse and from WT, NOS3−/−, and NOS3-TG mice 5 days after doxorubicin (DOX). Scale shown is in centimeters.](image1)

![Figure 2. Effect of a single dose of doxorubicin (DOX) on mortality. A single dose of doxorubicin (20 mg/kg) was administered to WT, NOS3−/−, and NOS3-TG mice, and survival was monitored for up to 8 days. *P < 0.01 vs WT doxorubicin.](image2)

### Table 2. Invasive Hemodynamic Measurements 5 Days After Doxorubicin Administration in WT and NOS3−/− Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control</th>
<th>Doxorubicin</th>
<th>P (vs Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>WT 621 ± 23</td>
<td>559 ± 34</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 618 ± 31</td>
<td>578 ± 35</td>
<td>0.27</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>WT 3 ± 1</td>
<td>6 ± 1</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 4 ± 1</td>
<td>4 ± 1</td>
<td>0.47</td>
</tr>
<tr>
<td>LVESP, mm Hg</td>
<td>WT 120 ± 7</td>
<td>102 ± 5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 141 ± 6</td>
<td>123 ± 6†</td>
<td>0.002</td>
</tr>
<tr>
<td>dP/dt_{max}, mm Hg · s⁻¹</td>
<td>WT 15654 ± 874</td>
<td>8966 ± 975</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 16765 ± 963</td>
<td>13962 ± 810*</td>
<td>0.01</td>
</tr>
<tr>
<td>dP/dt_{min}, mm Hg · s⁻¹</td>
<td>WT 11456 ± 876</td>
<td>7884 ± 712</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 12532 ± 902</td>
<td>10641 ± 899†</td>
<td>0.05</td>
</tr>
<tr>
<td>dP/dt_{max}/IP, s⁻¹</td>
<td>WT 278 ± 11</td>
<td>154 ± 10</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>NOS3−/− 268 ± 13</td>
<td>194 ± 11†</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Control indicates untreated mice; doxorubicin, mice that received doxorubicin (20 mg/kg); LVEDP, left ventricular end-diastolic pressure; and LVESP, left ventricular end-systolic pressure.

*P < 0.001, †P < 0.05, ‡P < 0.01 vs WT doxorubicin group.
comparison to doxorubicin-challenged WT mice, NOS3−/− mice had reduced cardiac superoxide production, and NOS3-TG mice had increased production. Coincubation with L-NAME did not alter lucigenin-enhanced chemiluminescence in heart tissues from doxorubicin-challenged NOS3-TG mice (data not shown).

Doxorubicin Does Not Induce NOS3 Monomerization
Administration of doxorubicin did not induce NOS3 monomerization in the hearts of WT mice (Figure 5). Similarly, there was no evidence of increased NOS3 monomerization in the hearts of NOS3-TG after treatment with doxorubicin (data not shown).

Doxorubicin-Induced Changes in Cardiac Gene Expression
There were no differences in ventricular levels of mRNAs encoding COX-2, HO-1, Bcl-xL, NOS2, and GATA-4 among genotypes after saline challenge (Figure 6; GATA-4 not shown). Doxorubicin induced COX-2, HO-1, and Bcl-xL gene expression in WT mice. Levels of COX-2, HO-1, and Bcl-xL mRNAs were unchanged in NOS3−/− mice after doxorubicin challenge. The doxorubicin-induced COX-2, HO-1, and Bcl-xL gene expression was greater in NOS3-TG than in WT mice. Doxorubicin did not alter GATA-4 (data not shown) or NOS2 mRNA levels in any of the genotypes (Figure 6).

Cardiac Dysfunction and Mortality After Serial Doses of Doxorubicin
Doxorubicin (4 mg/kg) was administered weekly over 5 weeks to WT and NOS3−/− mice, and mice were followed up by serial echocardiography for an additional 11 weeks. Conventional echocardiographic indices of LV systolic function (FS, EF) were unchanged in WT and NOS3−/− mice 6 weeks after the initiation of doxorubicin (Table 3). In WT mice, V_{endo} decreased from 3.3±0.1 to 2.4±0.1 cm·s⁻¹ (P<0.001) 6 weeks after the initiation of doxorubicin, whereas SR decreased from 23±1% to 16±1% (P<0.001). In contrast, in NOS3−/− mice, 6 weeks after initiation of doxorubicin, both V_{endo} and SR were unchanged (Table 3).

In WT and NOS3−/− mice surviving to 16 weeks, FS decreased (Figure 7A), as did EF, V_{endo}, and SR (Table 3). However, the reductions in FS, EF, V_{endo}, and SR caused by doxorubicin in NOS3−/− were less marked than in WT mice (P<0.01 for all).
of doxorubicin. NOS3-deficient mice. Moreover, doxorubicin-induced superoxide production in the hearts of WT but not in NOS3−/− mice. The superoxide production in the hearts of WT but not in NOS3−/− mice was greater in NOS3−/− mice than in WT mice, and this was associated with a marked increase in mortality. The superoxide production in the hearts of WT but not in NOS3−/− mice was greater in NOS3−/− mice than in WT mice, and this was associated with a marked increase in mortality. The superoxide production in the hearts of WT but not in NOS3−/− mice was greater in NOS3−/− mice than in WT mice, and this was associated with a marked increase in mortality.

The cardiac dysfunction associated with doxorubicin administration is attributable, at least in part, to cardiac cell apoptosis. We measured cardiac cell death using the TUNEL assay, which detects cells undergoing apoptosis or necrosis. TUNEL-positive nuclei were readily detectable in WT, NOS3−/−, and NOS3-TG mice within 24 hours after administration of doxorubicin. The preservation of cardiac function in NOS3−/− mice was associated with a reduction in the frequency of TUNEL-positive cells in cardiac sections. Moreover, the frequency of cell death was greater in NOS3-TG than in WT mice, and this was associated with more marked doxorubicin-induced cardiac dysfunction. Taken together, these results suggest that NOS3 deficiency may protect against doxorubicin-induced cardiac dysfunction at least in part by preventing cardiac cell death.

Doxorubicin-induced cardiac cell apoptosis has been attributed to the production of ROS. Kalyanaraman and colleagues have reported that agents that scavenge ROS protect against doxorubicin-induced cardiac apoptosis. Similarly, cardiac-specific overexpression of antioxidant genes protected mice from doxorubicin-induced cardiac dysfunction. We observed that doxorubicin administration led to the superoxide production in the hearts of WT but not in NOS3-deficient mice. Moreover, doxorubicin-induced superoxide production was greater in NOS3-TG than in WT mice. These results strongly suggest that doxorubicin induces cardiac ROS production via a NOS3-dependent mechanism.

After a single dose of doxorubicin, conventional (EF and FS) and tissue-Doppler-derived (Vpbox and SR) echocardiographic indices of LV systolic function were depressed to a greater extent in WT than in NOS3−/− mice. NOS3-TG mice had greater increases in LV dimensions and a more marked reduction in systolic function after doxorubicin than did WT or NOS3−/− mice. Invasive hemodynamic measurements confirmed that NOS3−/− mice were protected from doxorubicin-induced cardiac dysfunction. Mortality, after a single high dose of doxorubicin, was markedly greater in NOS3-TG mice than in WT and was reduced in NOS3−/− mice. Because of the high mortality that we observed in NOS3-TG mice 5 days after a single dose of doxorubicin and the marked reduction in cardiac function detected by echocardiography, invasive hemodynamic measurements were not performed in this genotype, and chronic studies were limited to NOS3−/− and WT mice. Administration of a single dose of doxorubicin to mice has been validated extensively in the study of doxorubicin-induced cardiac dysfunction in vivo. However, patients are usually treated with multiple lower doses of doxorubicin over a more prolonged period. Therefore, we investigated whether or not NOS3−/− mice would be protected in a model wherein a lower dose of doxorubicin was given repeatedly over 5 weeks. With the use of tissue-Doppler imaging, subtle LV dysfunction was detected in WT mice as early as 6 weeks after the initiation of doxorubicin therapy, whereas NOS3−/− mice were protected from early doxorubicin-induced cardiac dysfunction. Sixteen weeks after initiating doxorubicin administration, systolic function was impaired to a greater extent in WT than in NOS3−/− mice and was associated with a marked increase in mortality.

The present study reveals a critical role for NOS3 in the development of cardiac injury after the administration of doxorubicin in mice. Genetic disruption of NOS3 protected against the doxorubicin-induced cardiac dysfunction, injury, and mortality. Furthermore, cardiomyocyte-specific overexpression of NOS3 exacerbated the pathological response to doxorubicin.

**Discussion**

Sixteen weeks after initiation of doxorubicin administration, 60% (12/20) of WT mice had died (Figure 7B), whereas fewer (1/15) NOS3−/− mice had died.

The cardiac dysfunction associated with doxorubicin administration is attributable, at least in part, to cardiac cell apoptosis. We measured cardiac cell death using the TUNEL assay, which detects cells undergoing apoptosis or necrosis. TUNEL-positive nuclei were readily detectable in WT, NOS3−/−, and NOS3-TG mice within 24 hours after administration of doxorubicin. The preservation of cardiac function in NOS3−/− mice was associated with a reduction in the frequency of TUNEL-positive cells in cardiac sections. Moreover, the frequency of cell death was greater in NOS3-TG than in WT mice, and this was associated with more marked doxorubicin-induced cardiac dysfunction. Taken together, these results suggest that NOS3 deficiency may protect against doxorubicin-induced cardiac dysfunction at least in part by preventing cardiac cell death.

Doxorubicin-induced cardiac cell apoptosis has been attributed to the production of ROS. Kalyanaraman and colleagues have reported that agents that scavenge ROS protect against doxorubicin-induced cardiac apoptosis. Similarly, cardiac-specific overexpression of antioxidant genes protected mice from doxorubicin-induced cardiac dysfunction. We observed that doxorubicin administration led to the superoxide production in the hearts of WT but not in NOS3-deficient mice. Moreover, doxorubicin-induced superoxide production was greater in NOS3-TG than in WT mice. These results strongly suggest that doxorubicin induces cardiac ROS production via a NOS3-dependent mechanism.

When NOS3 is deprived of l-arginine or cofactors, superox-
ide can be generated from the oxygenase domain instead of NO, and this uncoupling may be associated with dissociation of NOS3 dimers. An alternative mechanism by which doxorubicin can induce NOS3 to generate superoxide involves conversion of doxorubicin by the NOS3 reductase domain to an unstable semiquinone intermediate, which in turn produces superoxide in the presence of oxygen. Prior in vitro studies suggested that the doxorubicin-induced superoxide production by NOS3 was not affected by L-NAME. We observed that doxorubicin-induced cardiac superoxide production was neither blocked by L-NAME nor associated with monomerization of NOS3 in the hearts of WT and NOS3-TG. Taken together, these findings suggest that the NOS3 reductase domain is responsible for cardiac superoxide generation in response to doxorubicin administration.

Reactive nitrogen species including peroxynitrite, the reaction product of NO and superoxide, have been implicated in the pathogenesis of doxorubicin-induced cardiac dysfunction. Doxorubicin is thought to increase cardiac reactive nitrogen species by induction of NOS2 expression, and NOS2 deficiency or inhibition has been reported to protect against doxorubicin-induced cardiac dysfunction. However, more recent evidence has suggested that deficiency of NOS2 may enhance doxorubicin-induced cardiac toxicity in mice. We did not detect an increase in NOS2 mRNA levels at 1 (Figure 6) or 5 days (data not shown) in the hearts of WT, NOS3−/−, and NOS3-TG mice treated with doxorubicin. These observations suggest that induction of NOS2 gene expression is not responsible for the increased cardiac cell death and impaired LV function that we detected after doxorubicin administration. It is possible that NO and superoxide, both generated by NOS3, could impair cardiac function via peroxynitrite formation, potentially accounting for the profound sensitivity of NOS3-TG mice to doxorubicin. However, it should be noted that peroxynitrite has been reported both to cause cardiac injury and to be cardioprotective. Moreover, it has been suggested that doxorubicin-induced cardiac injury is mediated by hydrogen peroxide, the product of superoxide and superoxide dismutase, and not peroxynitrite. Mouse models have suggested important roles for a variety of gene products in doxorubicin-induced cardiac dysfunction. Protective gene products, including COX-2, HO-1, Bcl-xL, and GATA-4, appear to act by preventing doxorubicin-
induced cardiac cell apoptosis.23,27,38 We tested the hypothesis that the decreased cardiac cell death observed in doxorubicin-treated NOS3−/− mice was associated with increased expression of protective genes. There were no differences in cardiac COX-2, HO-1, Bcl-xL, and GATA-4 mRNA levels between genotypes after saline challenge and the expression of genes encoding COX-2, HO-1, and Bcl-xL was induced in the ventricles of doxorubicin-treated WT but was not induced in NOS3−/− mice. Moreover, in NOS3-TG mice, the accentuated cardiac dysfunction after doxorubicin administration was associated with greater increases in protective gene expression than were detected in WT mice. Doxorubicin did not alter cardiac GATA-4 gene expression in any of the genotypes at 24 hours. It is likely that GATA-4 mRNA levels change at later times after doxorubicin challenge, as has been reported previously.38,39 Taken together, these findings strongly suggest that NOS3 deficiency prevents doxorubicin-induced cardiac dysfunction via a mechanism that does not require induction of protective genes. Induction of COX-2, HO-1, and Bcl-xL gene expression in WT and NOS3-TG mice after doxorubicin likely represents an attempt by the cardiac cells to limit injury.

The observation that NOS3 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction may have clinical implications. Either pharmacological inhibition of the NOS3 reductase domain12 or temporary reduction of cardiac NOS3 levels at the time of doxorubicin administration with the use of antisense or small interfering RNAs40 may offer novel strategies to prevent cardiac dysfunction in patients receiving doxorubicin for cancer therapy.

In summary, we observed that genetic disruption of NOS3 protected mice from the cardiac injury, dysfunction, and mortality associated with doxorubicin administration. Cardiomyocyte-specific overexpression of NOS3 rendered mice more sensitive to doxorubicin-induced cardiac dysfunction. Doxorubicin-induced levels of cardiac ROS synthesis and cardiac cell apoptosis were greatest in mice engineered to have high levels of cardiac NOS3 expression and least in NOS3-deficient mice. These findings implicate NOS3 as a key mediator of doxorubicin-induced cardiac dysfunction in mice and should promote further research designed to determine whether or not cardiac NOS3 can be targeted therapeutically to reduce anthracycline-induced cardiotoxicity in patients.

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Disclosures

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References

We designed the study to determine whether or not disruption of nitric oxide synthase 3 (NOS3) would influence the observed cardiac injury and dysfunction after administration of doxorubicin. We found that genetic disruption of NOS3 reduced the cardiac dysfunction and mortality after doxorubicin. We also found that cardiomyocyte-specific overexpression of NOS3 increased the cardiac injury and dysfunction after doxorubicin. Disruption of NOS3 resulted in a decrease in reactive oxygen species and cardiac cell apoptosis after doxorubicin. Furthermore, we found that blocking the oxygenase domain of NOS3 resulted in a decrease in reactive oxygen species and cardiac cell apoptosis after doxorubicin. Disruption of NOS3 may offer novel strategies to prevent cardiac dysfunction in patients receiving doxorubicin for cancer therapy.
Disruption of Nitric Oxide Synthase 3 Protects Against the Cardiac Injury, Dysfunction, and Mortality Induced by Doxorubicin


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