Check for updates

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

Tomas G. Neilan, MD; Sarah L. Blake, MD; Fumito Ichinose, MD; Michael J. Raher, BSc; Emmanuel S. Buys, PhD; Davinder S. Jassal, MD; Elissa Furutani, BSc; Teresa Miriam Perez-Sanz, MD; Amanda Graveline, BSc; Stefan P. Janssens, MD, PhD; Michael H. Picard, MD; Marielle Scherrer-Crosbie, MD, PhD; Kenneth D. Bloch, MD

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

Downloaded from http://ahajournals.org by on April 20, 2024

The use of anthracyclines, including doxorubicin, as chemotherapeutic 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]

Clinical Perspective p 514

There are several mechanisms by which doxorubicin can induce production of ROS. For example, formation of doxorubicin-iron complexes may trigger a Fenton-type reaction[10] 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]

Received July 17, 2006; accepted May 11, 2007.

From the Cardiovascular Research Center (T.G.N., S.L.B., F.I., M.J.R., E.S.B., E.F., A.G., K.D.B.) and Cardiac Ultrasound Laboratory (T.G.N., D.S.J., T.M.P.-S., M.H.P., M.S.-C.) in the Cardiology Division of the Department of Medicine, and Department of Anesthesia and Critical Care (F.I., K.D.B.), Massachusetts General Hospital, Charlestown; and Center for Transgene Technology and Gene Therapy, Flanders Interuniversity Institute for Biotechnology, Cardiology Division, University Hospital Gasthuisberg, University of Leuven, Leuven, Belgium (S.P.J.).

Correspondence to Tomas G. Neilan, MD, Cardiac Ultrasound Laboratory, Division of Cardiology, Massachusetts General Hospital, 55 Fruit St, Boston, MA 02114. E-mail tneilan@partners.org

© 2007 American Heart Association, Inc.

Circulation **is available at http://www.circulationaha.org** **DOI: 10.1161/CIRCULATIONAHA.106.652339**

NOS enzymes produce NO from the catalytic conversion of L-arginine to L-citrulline in the presence of oxygen and NADPH.[14] 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.[15] Of the 3 NOS isoforms, NOS3 has the highest affinity for doxorubicin.[15] The deleterious role of NOS3 after the administration of doxorubicin has been observed previously in vitro.[12,16–18] However, N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of oxygenase domain of NOS3, has been shown to increase mortality after doxorubicin in mice,[4] 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 ($NOS3^{-/-}$) mice, and mice with a cardiomyocyte-specific overexpression of NOS3 (NOS3-TG). We found that, compared with WT mice, $NOS3^{-/-}$ 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, $NOS3^{-/-}$, and NOS3-TG[20] 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 the first acute protocol, 20 mg/kg of doxorubicin was administered as a single dose to WT (n=40), $NOS3^{-/-}$ (n=40), and NOS3-TG (n=15) mice. Cardiac function was measured by echocardiography at baseline and in surviving animals 5 days after doxorubicin, and survival up to 8 days was measured. In a second acute protocol, additional WT (n=12) and $NOS3^{-/-}$ (n=10) mice were challenged with doxorubicin, and invasive hemodynamic measurements were performed 5 days later. Untreated WT and $NOS3^{-/-}$ mice were used as controls (n=10 per group). In a third acute protocol, all 3 genotypes (n=5 per group) were injected with doxorubicin (20 mg/kg) or saline, and cardiac tissue was harvested 24 hours later for the measurement of cardiac cell death, ROS production, and gene expression, as well as NOS3 monomerization in WT and NOS3-TG mice. In a fourth acute protocol, NOS3-TG mice (n=3 per group) were treated with doxorubicin (20 mg/kg) or saline, and the production of ROS was measured in cardiac tissue incubated with and without L-NAME (1 mmol/L). In a chronic protocol, WT (n=20) and $NOS3^{-/-}$ (n=15) mice received doxorubicin (4 mg/kg) once a week for 5 weeks and underwent echocardiography at baseline and after 6, 12, and 16 weeks. Surviving animals were euthanized after 16 weeks.

Echocardiographic Measurements

Echocardiography was performed with the use of a 13-MHz linear array ultrasound probe (Vivid 7, GE Medical Systems, Milwaukee, Wis) in lightly sedated mice (ketamine, 50 mg/kg IP), as previously described.[21]

Invasive Hemodynamic Measurements

Mice were anesthetized with an intraperitoneal injection of morphine (2 mg/kg), etomidate (20 mg/kg), urethane (800 mg/kg), and pancuronium (2 mg/kg). Intubation, venous catheterization, and mechanical ventilation were performed. A 1.4F high-fidelity pressure catheter (1.4F, SPR 837, Millar Instruments Inc, Houston, Tex) was advanced into the left ventricle via the carotid artery, and systolic and diastolic function was evaluated, as previously described.[22]

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.[23]

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.[16] 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). Sections were washed in PBS and examined with a Zeiss Axiophot 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.[16] Mean fluorescence intensity of the digitized 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.[16] Briefly, cardiac tissue was preincubated in Krebs-HEPES buffer containing 10 μmol/L NADPH for 45 minutes. Tissues were then transferred into wells of a 96-well plate containing 300 μL of Krebs-HEPES buffer supplemented with 10 μmol/L NADPH and 10 μmol/L lucigenin (bis-*N*-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.[24] Membranes were blocked in 5% nonfat milk in Tris-buffered saline Tween-20 (TBST) (TBST milk) and then incubated with an anti-NOS3 antibody (BDTransduction, 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 (BDTransduction, 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′, 5′-GCACGTAGTCTTCGATCACTATC-3′), HO-1 (5′-GCCACCAAGGAGGTACACAT, 5′-GCTTGTTGCGCTCTATCTCC-3′), Bcl-xL (5′-

Downloaded from http://ahajournals.org by on April 20, 2024

GACAAGGAGATGCAGGTATTGG-3′, 5′-TCCCGTAGAGATCCACAAAAGT-3′), GATA-4 (5′-CCCTACCCAGCCTACATGG-3′, 5′-ACATATCGAGATTGGG GTGTCT-3′), and 18s rRNA (5′-CGGCTACCACATCCAAGGAA-3′, 5′-GCTGGAATTACCGCGGCT-3′). Changes in the gene expression normalized to levels of 18S rRNA were determined by the relative C_t 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_{ENDO}), 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_{ENDO} and SR were mildly decreased compared with baseline. Compared with WT mice, $NOS3^{-/-}$ had smaller cardiac dimensions and greater FS, EF, V_{ENDO}, 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

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

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

*$P<0.001$, †$P<0.05$, ‡$P<0.01$ vs WT doxorubicin group. Data within a group were analyzed by ANOVA for repeated measures, whereas analyses of data between groups were performed with an unpaired *t* test only if the primary analysis was significant.

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.[25] In hearts from saline-challenged mice, there were only very low levels of cell death in all 3

Downloaded from http://ahajournals.org by on April 20, 2024

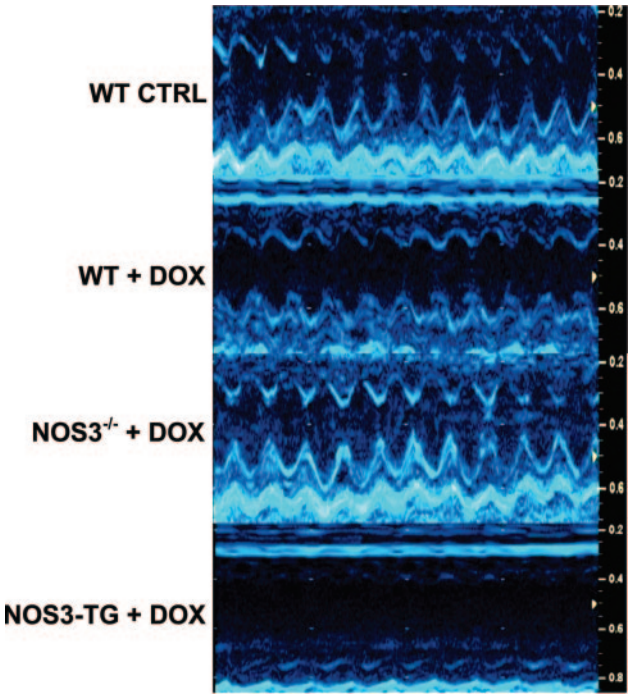

Figure 1. Cardiac function at baseline and 5 days after doxorubicin. Representative M-mode tracings are displayed from a control (CTRL) WT mouse and from WT, NOS3$^{-/-}$, and NOS3-TG mice 5 days after doxorubicin (DOX). Scale shown is in centimeters.

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.

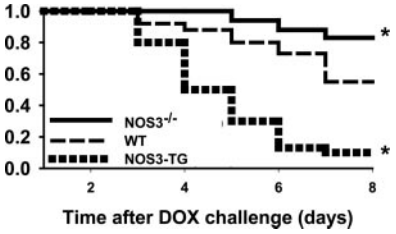

Figure 2. Effect of a single dose of doxorubicin (DOX) on mortality. A single dose of doxorubicin (20 mg/kg) was administered to WT, NOS3$^{-/-}$, and NOS3-TG mice, and survival was monitored for up to 8 days. *P<0.01 vs WT doxorubicin.

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

TABLE 2. Invasive Hemodynamic Measurements 5 Days After Doxorubicin Administration in WT and NOS3$^{-/-}$ Mice

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

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.

Downloaded from http://ahajournals.org by on April 20, 2024

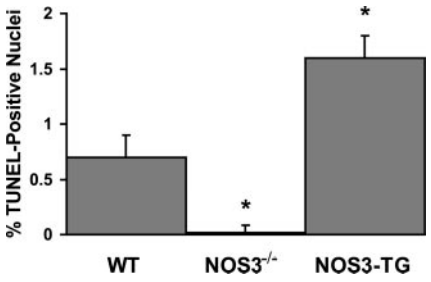

Figure 3. Cardiac cell death 24 hours after a single doxorubicin injection. WT, NOS3$^{-/-}$, and NOS3-TG mice received doxorubicin (20 mg/kg), and 24 hours later, hearts were harvested, fixed, embedded, and sectioned. Cardiac cell death was detected with the TUNEL assay, and nuclei were counterstained with DAPI. The percentage of TUNEL-positive nuclei is shown. *P<0.001 vs WT doxorubicin.

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).

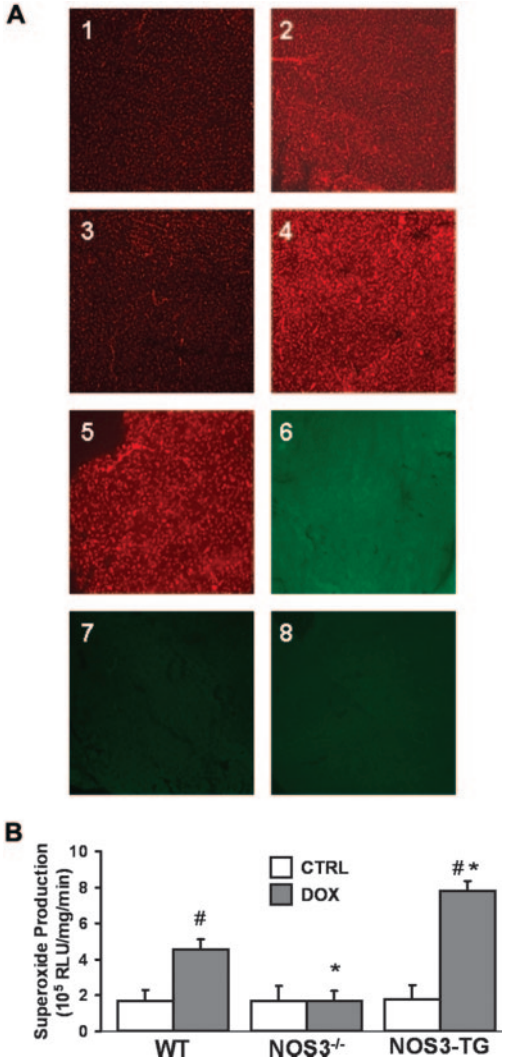

Figure 4. Production of ROS 24 hours after a single dose of doxorubicin. A, WT, NOS3$^{-/-}$, and NOS3-TG mice were injected with saline or doxorubicin (20 mg/kg). Twenty-four hours later, hearts were harvested. 1, Staining with DHE among saline-treated controls from all genotypes was identical, and a single representative control section is displayed. 2, DHE-stained heart from doxorubicin-treated WT mouse. 3, DHE-stained heart from doxorubicin-treated NOS3$^{-/-}$ mouse. 4, DHE-stained heart from doxorubicin-treated NOS3-TG mouse. 5, DHE-stained heart from doxorubicin-treated NOS3-TG mouse coincubated with L-NAME. 6, Staining with diaminofluorescein in a saline-treated NOS3-TG mice. 7, Diaminofluorescein-stained heart from doxorubicin-treated NOS3-TG mouse. 8, Diaminofluorescein-stained heart from doxorubicin-treated NOS3-TG mouse coincubated with L-NAME. B, WT, NOS3$^{-/-}$, and NOS3-TG mice were injected with saline (CTRL) or doxorubicin (DOX) (20 mg/kg), and 24 hours later hearts were harvested. Cardiac tissue was incubated in the presence of lucigenin, and superoxide-induced chemiluminescence was measured. Superoxide production is expressed as the relative light units (RLU) per milligram of cardiac tissue (dry weight) per minute. #P<0.001 vs saline-treated mice of the same genotype; *P<0.01 vs WT doxorubicin.

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).

Downloaded from http://ahajournals.org by on April 20, 2024

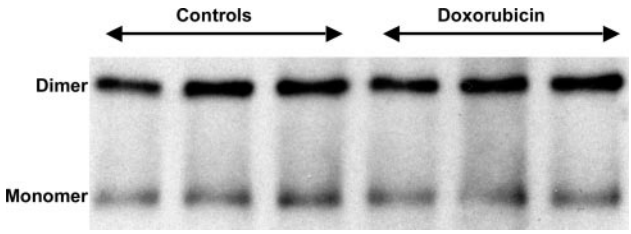

Figure 5. NOS3 dimerization state after doxorubicin administration. WT mice received saline (control, lanes 1 to 3) or doxorubicin (20 mg/kg, lanes 3 to 6), and cardiac tissues were harvested 24 hours later. Tissues were homogenized and centrifuged. Supernatants were fractionated by nondenaturing SDS-PAGE and transferred to membranes. NOS3 dimers and monomers were detected with an anti-NOS3 antibody. The expression of tubulin was measured in each lane to confirm equal loading of protein (data not shown).

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.

Discussion

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.

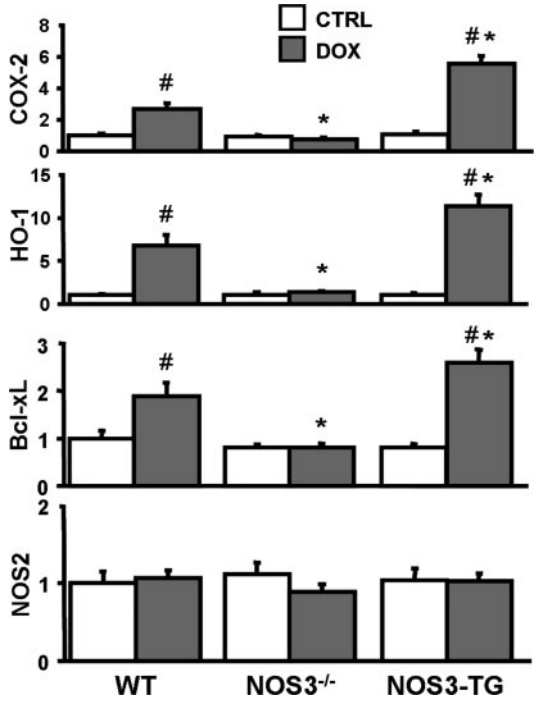

Figure 6. Changes in cardiac gene expression profile induced by doxorubicin. Relative COX-2, HO-1, Bcl-xL, and NOS-2 gene expression in WT, NOS3$^{-/-}$, and NOS3-TG mice after the administration of saline (CTRL) or doxorubicin (DOX) (20 mg/kg). Results are presented as fold change vs WT control. #$P<0.001$ vs saline-treated mice of the same genotype; *$P<0.01$ vs WT doxorubicin.

After a single dose of doxorubicin, conventional (EF and FS) and tissue-Doppler–derived (V_{ENDO} 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.[26,27] 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 cardiac dysfunction associated with doxorubicin administration is attributable, at least in part, to cardiac cell apoptosis.[18,23] 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.[9,17,18] Kalyanaraman and colleagues[18,28] 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.[29,30] 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-

Downloaded from http://ahajournals.org by on April 20, 2024

TABLE 3. Echocardiographic Indices at Baseline and at 6, 12, and 16 Weeks After the Initiation of 5 Serial Doses of Doxorubicin (4 mg/kg) in WT and NOS3$^{-/-}$ Mice

	Genotype	Baseline	6 Weeks	12 Weeks	16 Weeks	*P* (vs Time)
Heart rate, bpm	WT	623±10	620±10	588±10	570±15	0.04
	NOS3$^{-/-}$	620±11	618±12	616±9	590±13	0.12
LVIDd, mm	WT	3.2±0.1	3.5±0.1	3.9±0.1	4.2±0.1	<0.001
	NOS3$^{-/-}$	3.2±0.3	3.2±0.2	3.4±0.3	3.6±0.2†	0.03
LVIDs, mm	WT	1.4±0.1	1.6±0.1	2.5±0.1	3.0±0.1	<0.001
	NOS3$^{-/-}$	1.4±0.1	1.4±0.2	1.7±0.2‡	1.9±0.2‡	0.04
FS, %	WT	57±1	55±1	35±1	28±1	<0.001
	NOS3$^{-/-}$	57±1	57±1	51±2*	45±2*	0.001
EF, %	WT	78±1	77±3	47±2	42±2	<0.001
	NOS3$^{-/-}$	78±1	78±2	72±2*	64±2*	0.002
V_{ENDO}, cm · s^{-1}	WT	3.3±0.1	2.4±0.1	2.1±0.1	1.7±0.1	<0.001
	NOS3$^{-/-}$	3.3±0.1	3.1±0.2‡	2.9±0.2*	2.5±0.1*	0.003
SR, s^{-1}	WT	23±1	16±1	11±1	7±1	<0.001
	NOS3$^{-/-}$	24±1	21±1‡	19±1*	16±1*	0.001

For WT, n=20; for NOS3$^{-/-}$, n=15 at the initiation of the study. LVIDd indicates left ventricular internal dimensions in diastole; LVIDs, left ventricular internal dimensions in systole.

*P<0.001, †P<0.05, ‡P<0.01 vs WT doxorubicin group.

ide can be generated from the oxygenase domain instead of NO,[31] and this uncoupling may be associated with dissociation of NOS3 dimers.[32] 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.[33,34] 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.

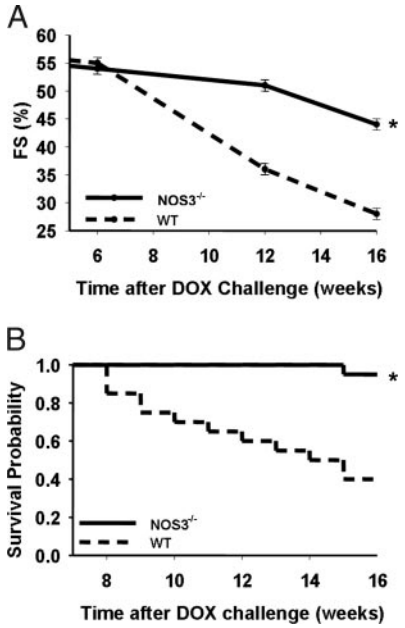

Figure 7. Cardiac dysfunction and mortality after serial doses of doxorubicin (DOX). Doxorubicin (4 mg/kg) was administered weekly over 5 successive weeks to WT and NOS3$^{-/-}$ mice, and mice were followed up for a total of 16 weeks. A, FS in WT and NOS3$^{-/-}$ after initiation of doxorubicin administration. B, Survival curves in WT and NOS3$^{-/-}$ after serial doses of low-dose doxorubicin. *P<0.01 vs WT doxorubicin.

Reactive nitrogen species including peroxynitrite, the reaction product of NO and superoxide, have been implicated in the pathogenesis of doxorubicin-induced cardiac dysfunction.[4,26] 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.[4] However, more recent evidence has suggested that deficiency of NOS2 may enhance doxorubicin-induced cardiac toxicity in mice.[36] 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.[4,26] However, it should be noted that peroxynitrite has been reported both to cause cardiac injury and to be cardioprotective.[4,37] 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.[18]

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-

Downloaded from http://ahajournals.org by on April 20, 2024

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 domain[12] or temporary reduction of cardiac NOS3 levels at the time of doxorubicin administration with the use of antisense or small interfering RNAs[40] 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.

Downloaded from http://ahajournals.org by on April 20, 2024

Acknowledgment

The authors would like to acknowledge the assistance of Masao Kaneki, MD, PhD.

Sources of Funding

The present study was supported by a Postdoctoral Fellowship Grant (Dr Neilan) and a Scientist Development Grant (Dr Scherrer-Crosbie) from the American Heart Association, as well as grants from the National Heart, Lung, and Blood Institute (HL-70896 and HL-71987).

Disclosures

Dr Janssens is a basic-clinical investigator of the Fund for Scientific Research–Flanders and is holder of a named chair financed by AstraZeneca. The remaining authors report no conflicts.

References

1. Singal PK, Iliskovic N. Doxorubicin-induced cardiomyopathy. *N Engl J Med*. 1998;339:900–905.
2. Minotti G, Cavaliere AF, Mordente A, Rossi M, Schiavello R, Zamparelli R, Possati G. Secondary alcohol metabolites mediate iron delocalization in cytosolic fractions of myocardial biopsies exposed to anticancer anthracyclines: novel linkage between anthracycline metabolism and iron-induced cardiotoxicity. *J Clin Invest*. 1995;95:1595–1605.
3. Yen HC, Oberley TD, Vichitbandha S, Ho YS, St Clair DK. The protective role of manganese superoxide dismutase against Adriamycin-induced acute cardiac toxicity in transgenic mice. *J Clin Invest*. 1996;98:1253–1260.
4. Pacher P, Liaudet L, Bai P, Mabley JG, Kaminski PM, Virag L, Deb A, Szabo E, Ungvari Z, Wolin MS, Groves JT, Szabo C. Potent metalloporphyrin peroxynitrite decomposition catalyst protects against the development of doxorubicin-induced cardiac dysfunction. *Circulation*. 2003;107:896–904.
5. Ito H, Miller SC, Billingham ME, Akimoto H, Torti SV, Wade R, Gahlmann R, Lyons G, Kedes L, Torti FM. Doxorubicin selectively inhibits muscle gene expression in cardiac muscle cells in vivo and in vitro. *Proc Natl Acad Sci U S A*.1990;87:4275–4279.
6. Favero TG, Zable AC, Abramson JJ. Hydrogen peroxide stimulates the Ca^{2+} release channel from skeletal muscle sarcoplasmic reticulum. *J Biol Chem*. 1995;270:25557–25563.
7. Yoshikawa T, Handa S, Suzuki M, Nagami K. Abnormalities in sympathoneuronal regulation are localized to failing myocardium in rabbit heart. *J Am Coll Cardiol*. 1994;24:210–215.
8. Adderley SR, Fitzgerald DJ. Oxidative damage of cardiomyocytes is limited by extracellular regulated kinases 1/2-mediated induction of cyclooxygenase-2. *J Biol Chem*. 1999;274:5038–5046.
9. Dowd NP, Scully M, Adderley SR, Cunningham AJ, Fitzgerald DJ. Inhibition of cyclooxygenase-2 aggravates doxorubicin-mediated cardiac injury in vivo. *J Clin Invest*. 2001;108:585–590.
10. Kotamraju S, Chitambar CR, Kalivendi SV, Joseph J, Kalyanaraman B. Transferrin receptor-dependent iron uptake is responsible for doxorubicin-mediated apoptosis in endothelial cells: role of oxidant-induced iron signaling in apoptosis. *J Biol Chem*. 2002;277:17179–17187.
11. Kunisada K, Tone E, Negoro S, Nakaoka Y, Oshima Y, Osugi T, Funamoto M, Izumi M, Fujio Y, Hirota H, Yamauchi-Takihara K. Bcl-xl reduces doxorubicin-induced myocardial damage but fails to control cardiac gene downregulation. *Cardiovasc Res*. 2002;53:936–943.
12. Vasquez-Vivar J, Martasek P, Hogg N, Masters BS, Pritchard KA Jr, Kalyanaraman B. Endothelial nitric oxide synthase-dependent superoxide generation from Adriamycin. *Biochemistry*. 1997;36:11293–11297.
13. Myers CE, McGuire WP, Liss RH, Ifrim I, Grotzinger K, Young RC. Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science*. 1977;197:165–167.
14. Palmer RM, Ashton DS, Moncada S. Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*. 1988;333:664–666.
15. Garner AP, Paine MJ, Rodriguez-Crespo I, Chinje EC, Ortiz De Montellano P, Stratford IJ, Tew DG, Wolf CR. Nitric oxide synthases catalyze the activation of redox cycling and bioreductive anticancer agents. *Cancer Res*. 1999;59:1929–19234.
16. Kalivendi SV, Kotamraju S, Zhao H, Joseph J, Kalyanaraman B. Doxorubicin-induced apoptosis is associated with increased transcription of endothelial nitric-oxide synthase: effect of antiapoptotic antioxidants and calcium. *J Biol Chem*. 2001;276:47266–47276.
17. Kalivendi SV, Kotamraju S, Zhao H, Joseph J, Kalyanaraman B. Doxorubicin-induced apoptosis is associated with increased transcription of endothelial nitric-oxide synthase: effect of antiapoptotic antioxidants and calcium. *J Biol Chem*. 2001;276:47266–47276.
18. Kotamraju S, Konorev EA, Joseph J, Kalyanaraman B. Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen: role of reactive oxygen and nitrogen species. *J Biol Chem*. 2000;275:33585–33592.
19. Vasquez-Vivar J, Kalyanaraman B, Martasek P, Hogg N, Masters BS, Karoui H, Tordo P, Pritchard KA, Jr. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci U S A*. 1998;95:9220–9925.
20. Janssens S, Pokreisz P, Schoonjans L, Pellens M, Vermeersch P, Tjwa M, Jans P, Scherrer-Crosbie M, Picard MH, Szelid Z, Gillijns H, Van de Werf F, Collen D, Bloch KD. Cardiomyocyte-specific overexpression of nitric oxide synthase 3 improves left ventricular performance and reduces compensatory hypertrophy after myocardial infarction. *Circ Res*. 2004;94:1256–1262.

21. Sebag IA, Handschumacher MD, Ichinose F, Morgan JG, Hataishi R, Rodrigues AC, Guerrero JL, Steudel W, Raher MJ, Halpern EF, Derumeaux G, Bloch KD, Picard MH, Scherrer-Crosbie M. Quantitative assessment of regional myocardial function in mice by tissue Doppler imaging: comparison with hemodynamics and sonomicrometry. *Circulation*. 2005;111:2611–2616.
22. Ullrich R, Scherrer-Crosbie M, Bloch KD, Ichinose F, Nakajima H, Picard MH, Zapol WM, Quezado ZM. Congenital deficiency of nitric oxide synthase 2 protects against endotoxin-induced myocardial dysfunction in mice. *Circulation*. 2000;102:1440–1446.
23. Neilan TG, Doherty GA, Chen G, Deflandre C, McAllister H, Butler RK, McClelland SE, Kay E, Ballou LR, Fitzgerald DJ. Disruption of COX-2 modulates gene expression and the cardiac injury response to doxorubicin. *Am J Physiol*. 2006;291:H532–H536.
24. Eissa NT, Yuan JW, Haggerty CM, Choo EK, Palmer CD, Moss J. Cloning and characterization of human inducible nitric oxide synthase splice variants: a domain, encoded by exons 8 and 9, is critical for dimerization. *Proc Natl Acad Sci U S A*. 1998;95:7625–7630.
25. Khaw BA, Silva JD, Petrov A, Hartner W. Indium 111 antimyosin and Tc-99m glucaric acid for noninvasive identification of oncotic and apoptotic myocardial necrosis. *J Nucl Cardiol*. 2002;9:471–481.
26. Weinstein DM, Mihm MJ, Bauer JA. Cardiac peroxynitrite formation and left ventricular dysfunction following doxorubicin treatment in mice. *J Pharmacol Exp Ther*. 2000;294:396–401.
27. Nozaki N, Shishido T, Takeishi Y, Kubota I. Modulation of doxorubicin-induced cardiac dysfunction in toll-like receptor-2-knockout mice. *Circulation*. 2004;110:2869–2874.
28. Konorev EA, Kotamraju S, Zhao H, Kalivendi S, Joseph J, Kalyanaraman B. Paradoxical effects of metalloporphyrins on doxorubicin-induced apoptosis: scavenging of reactive oxygen species versus induction of heme oxygenase-1. *Free Radic Biol Med*. 2002;33:988.
29. Kang YJ, Chen Y, Epstein PN. Suppression of doxorubicin cardiotoxicity by overexpression of catalase in the heart of transgenic mice. *J Biol Chem*. 1996;271:12610–12616.
30. Kang YJ, Chen Y, Yu A, Voss-McCowan M, Epstein PN. Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. *J Clin Invest*. 1997;100:1501–1506.
31. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation*. 2006;113:1708–1714.
32. Takimoto E, Champion HC, Li M, Ren S, Rodriguez ER, Tavazzi B, Lazzarino G, Paolocci N, Gabrielson KL, Wang Y, Kass DA. Oxidant stress from nitric oxide synthase-3 uncoupling stimulates cardiac pathologic remodeling from chronic pressure load. *J Clin Invest*. 2005;115:1221–1231.
33. Bachur NR, Gordon SL, Gee MV, Kon H. NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. *Proc Natl Acad Sci U S A*. 1979;76:954–957.
34. Iyanagi T, Yamazaki I. One-electron-transfer reactions in biochemical systems, III: one-electron reduction of quinones by microsomal flavin enzymes. *Biochim Biophys Acta*. 1969;172:370–381.
35. Deleted in proof.
36. Cole MP, Chaiswing L, Oberley TD, Edelmann SE, Piascik MT, Lin SM, Kiningham KK, St Clair DK. The protective roles of nitric oxide and superoxide dismutase in Adriamycin-induced cardiotoxicity. *Cardiovasc Res*. 2006;69:186–197.
37. Vinten-Johansen J. Physiological effects of peroxynitrite: potential products of the environment. *Circ Res*. 2000;87:170–172.
38. Aries A, Paradis P, Lefebvre C, Schwartz RJ, Nemer M. Essential role of GATA-4 in cell survival and drug-induced cardiotoxicity. *Proc Natl Acad Sci U S A*. 2004;101:6975–6980.
39. Kobayashi S, Lackey T, Huang Y, Bisping E, Pu WT, Boxer LM, Liang Q. Transcription factor GATA4 regulates cardiac BCL2 gene expression in vitro and in vivo. *FASEB J*. 2006;20:800–802.
40. Dykxhoorn DM, Novina CD, Sharp PA. Killing the messenger: short RNAs that silence gene expression. *Nat Rev Mol Cell Biol*. 2003;4:457–467.

CLINICAL PERSPECTIVE

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 NOS did not influence the production of reactive oxygen species after doxorubicin, suggesting that NOS3 mediates doxorubicin-induced reactive oxygen species production via the reductase domain. The observation that NOS3 contributes to the pathogenesis of doxorubicin-induced cardiac dysfunction may have clinical implications. Either pharmacological inhibition of the NOS3 reductase domain or temporary reduction of cardiac NOS3 levels at the time of doxorubicin administration with the use of antisense or small interfering RNAs may offer novel strategies to prevent cardiac dysfunction in patients receiving doxorubicin for cancer therapy.

Downloaded from http://ahajournals.org by on April 20, 2024