Decreased Soluble Guanylate Cyclase Contributes to Cardiac Dysfunction Induced by Chronic Doxorubicin Treatment in Mice

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Abstract

Aims: The use of doxorubicin, a potent chemotherapeutic agent, is limited by cardiotoxicity. We tested the hypothesis that decreased soluble guanylate cyclase (sGC) enzyme activity contributes to the development of doxorubicin-induced cardiotoxicity.

Results: Doxorubicin administration (20 mg/kg, intraperitoneally [IP]) reduced cardiac sGC activity in wild-type (WT) mice. To investigate whether decreased sGC activity contributes to doxorubicin-induced cardiotoxicity, we studied mice with cardiomyocyte-specific deficiency of the sGC \( \alpha_1 \)-subunit (mice with cardiomyocyte-specific deletion of exon 6 of the sGC\( \alpha_1 \) allele [sGC\( \alpha_1^{−/−}\text{-CM} \]). After 12 weeks of doxorubicin administration (2 mg/kg/week IP), left ventricular (LV) systolic dysfunction was greater in sGC\( \alpha_1^{−/−}\text{-CM} \) than WT mice. To further assess whether reduced sGC activity plays a pathogenic role in doxorubicin-induced cardiotoxicity, we studied a mouse model in which decreased cardiac sGC activity was induced by cardiomyocyte-specific expression of a dominant negative sGC\( \alpha_1 \) mutant (DNsGC\( \alpha_1 \)) upon doxycycline removal (Tet-off). After 8 weeks of doxorubicin administration, DNsGC\( \alpha_1^{19/6} \), but not WT, mice displayed LV systolic dysfunction and dilatation. The difference in cardiac function and remodeling between DNsGC\( \alpha_1^{19/6} \) and WT mice was even more pronounced after 12 weeks of treatment. Further impairment of cardiac function was attenuated when DNsGC\( \alpha_1 \) gene expression was inhibited (beginning at 8 weeks of doxorubicin treatment) by administering doxycycline. Furthermore, doxorubicin-associated reactive oxygen species generation was higher in sGC\( \alpha_1 \)-deficient than WT hearts.

Innovation and Conclusion: These data demonstrate that a reduction in cardiac sGC activity worsens doxorubicin-induced cardiotoxicity in mice and identify sGC as a potential therapeutic target. Various pharmacological sGC agonists are in clinical development or use and may represent a promising approach to limit doxorubicin-associated cardiotoxicity. Antioxid. Redox Signal. 26, 153–164.

Keywords: cardiovascular, cardiomyopathy, chemotherapy, doxorubicin, cyclic nucleotides, soluble guanylate cyclase
Introduction

Adverse effects observed in chemotherapy patients receiving dexrazoxane, the only drug approved to attenuate doxorubicin-induced cardiotoxicity in patients, have restricted its use. This study illustrates that doxorubicin reduces cardiac soluble guanylate cyclase (sGC) activity and that reduced sGC activity exacerbates doxorubicin-induced cardiotoxicity. Increasing interest in sGC as a therapeutic target is driving clinical development of pharmacological sGC agonists, and the first drug in this class was recently approved to treat pulmonary hypertension. Because our data suggest that sGC protects against doxorubicin-induced cardiotoxicity, pharmacological sGC agonists could represent a novel therapeutic strategy to treat cardiotoxicity in patients undergoing chemotherapy.

Results

Doxorubicin administration reduces cardiac sGC activity

To examine the effect of doxorubicin treatment on sGC enzymatic activity in the heart, the increase in cGMP production induced by the NO donor diethylenetriamine NONOate (DETA-NO) was measured in cardiac extracts from wild-type (WT) mice 24 h after doxorubicin (20 mg/kg, intraperitoneally [IP]) or saline administration. Doxorubicin treatment reduced cardiac NO-stimulated sGC activity by ~20% (Fig. 1A).

sGC is a heterodimeric enzyme comprising a β1 subunit and either an α1 or α2 subunit, with the sGCα1β1 isoform being the predominant isoform in the heart (6). Immunoblot analysis did not reveal changes in sGCα1 or sGCα1β1 protein expression in hearts of doxorubicin-treated mice (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars), indicating that the doxorubicin-induced decrease in cardiac sGC activity is not due to altered sGC expression.

It is conceivable that the oxidative stress associated with doxorubicin administration results in direct oxidative modification of sGC, potentially leading to loss of its prosthetic heme moiety (2, 4, 26, 42, 48) and decreased enzyme activity. To explore this possibility, we administered the radical scavenger tempol to WT mice before treatment with doxorubicin (20 mg/ kg, IP) or saline and evaluated cardiac sGC activity in the presence of DETA-NO. Tempol administration prevented the doxorubicin-induced decrease in cardiac NO-stimulated sGC activity (Fig. 1B), suggesting that this decrease in activity is due to oxidative modification of sGC.

Our hypothesis that doxorubicin treatment results in oxidation of sGC was further corroborated by the observation that BAY 58-2667 (cinaciguat), an sGC agonist specifically targeting oxidized and heme-free sGC (42), increased sGC activity in cardiac extracts obtained from doxorubicin- and saline-treated WT mice to the same extent (Fig. 1C). Thus, whereas in hearts of doxorubicin-treated mice, activation of sGC by NO—requiring the heme group of sGC to be in a reduced state—was impaired, sGC activation by BAY 58-2667 was not.

Innovation

Adverse effects observed in chemotherapy patients receiving dexrazoxane, the only drug approved to attenuate doxorubicin-induced cardiotoxicity in patients, have restricted its use. This study illustrates that doxorubicin reduces cardiac soluble guanylate cyclase (sGC) activity and that reduced sGC activity exacerbates doxorubicin-induced cardiotoxicity. Increasing interest in sGC as a therapeutic target is driving clinical development of pharmacological sGC agonists, and the first drug in this class was recently approved to treat pulmonary hypertension. Because our data suggest that sGC protects against doxorubicin-induced cardiotoxicity, pharmacological sGC agonists could represent a novel therapeutic strategy to treat cardiotoxicity in patients undergoing chemotherapy.
FIG. 1. Cardiac NO-stimulated sGC activity is decreased in mice treated with DOX. (A) NO-stimulated sGC activity was lower in cardiac extracts from mice treated with DOX (20 mg/kg IP, 24 h) than from mice administered saline (n = 10 each). (B) Tempol administration before DOX or saline treatment prevented this decrease in cardiac NO-stimulated sGC activity (n = 9 and 8, respectively). (C) In addition, incubation with the sGC activator, BAY 58-2667, increased sGC activity in the cardiac extracts obtained from DOX- and saline-treated mice to the same extent (n = 10 each). Data are expressed relative to sGC activity in the contemporaneous saline-treated control group. *p < 0.05 versus saline. DOX, doxorubicin; IP, intraperitoneal; NO, nitric oxide; sGC, soluble guanylate cyclase.

Constitutive myocardial sGC−1 deficiency exacerbates doxorubicin-induced cardiac dysfunction

To evaluate whether the doxorubicin-induced decrease in sGC activity contributes to the development of cardiotoxicity, we studied mice with cardiomyocyte-specific reduction of sGC activity (sGC−1−/−CM). Cre-mediated deletion of exon 6 of sGCα1 in cardiomyocytes attenuated the ability of DETA-NO to activate sGC in the heart: the relative fold increase in cGMP synthesis was 0.64±0.09 versus 1.00±0.18 in sGC−1−/−CM and WT mice, respectively (n = 10 and 8, p < 0.05). Restriction of sGC−1 deficiency to the heart in sGC−1−/−CM mice was confirmed by polymerase chain reaction (PCR) analysis (Supplementary Fig. S2). Baseline echocardiographic parameters, including left ventricular (LV) end-systolic and end-diastolic internal diameters and fractional shortening (FS), were similar in sGC−1−/−CM mice and WT littermates (Supplementary Table S1).

Cardiotoxicity was induced in sGC−1−/−CM and WT mice (n = 14 for both) by administering doxorubicin (2 mg/kg IP, once weekly) for 12 weeks, as previously reported (51). Following 8 weeks of doxorubicin administration, echocardiography revealed that end-systolic internal diameter (LVIDES) was increased (Fig. 2A) and FS decreased (Fig. 2B) in both sGC−1−/−CM and WT mice compared with baseline measurements obtained before the first doxorubicin injection. However, after 12 weeks, doxorubicin-induced systolic dysfunction was significantly greater in sGC−1−/−CM mice than in WT littermates: LVIDES was greater (Fig. 2A) and FS lower (Fig. 2B) in sGC−1−/−CM than in WT mice. In addition, compared with baseline, LV end-diastolic internal diameter (LVIDD) was modestly increased after 12 weeks of doxorubicin treatment in sGC−1−/−CM mice (3.3±0.04 vs. 3.1±0.03 mm, respectively, p < 0.05), but not in WT mice (3.2±0.05 vs. 3.1±0.03 mm, p > 0.05). Invasive LV pressure–volume measurements confirmed more pronounced systolic dysfunction, as evidenced by the reduced ejection fraction (EF), in doxorubicin-treated sGC−1−/−CM versus WT mice (Table 1).

Similar to previous reports (10, 35, 36), doxorubicin administration in mice was associated with a decrease in heart rate (HR), assessed via invasive hemodynamic measurements (Table 1) and echocardiography (sGC−1−/−CM + doxorubicin vs. sGC−1−/−CM + saline; 426±18 vs. 505±18 beats per minute [bpm] and WT + doxorubicin vs. WT + saline; 428±17 vs. 531±13 bpm, p < 0.05 for doxorubicin vs. saline in both genotypes). HR did not differ between doxorubicin-treated sGC−1−/−CM and WT mice (p > 0.05).

Furthermore, doxorubicin administration reduced survival rates to a similar extent in sGC−1−/−CM and WT mice (71% vs. 86%, respectively, n = 14 for both, p > 0.05), as well as body weights (−23±3% vs. −19±2%, respectively, n = 10 and 12, p > 0.05).

Doxorubicin-associated cardiotoxicity is increased in mice with inducible cardiomyocyte-specific expression of a dominant negative sGC−1 mutant

To further assess whether reduced sGC activity plays a pathogenic role in doxorubicin-induced cardiotoxicity, a second mouse model with inducible cardiomyocyte-specific sGC−1 deficiency was generated and studied. Upon withdrawal of doxycycline from the diet, a dominant negative sGC−1 mutant (DNsGC−1, Tet-Off system) is expressed that competes with sGCα1 and sGCβ2 for binding to sGCβ1, thereby inhibiting the formation of the two catalytically active sGC heterodimers in the heart.

Four weeks after doxycycline removal from the diet, the ability of DETA-NO to activate sGC was attenuated in the
were similar in DNsGC
n
respectively, p
increased LVIDES and LVIDED (Fig. 3A, B) and decreased
weekly), systolic dysfunction and dilatation, characterized by
increased survival rates to a similar extent in DNsGC

in treatment. These results, TGF-

the greater doxorubicin-induced systolic dysfunction ob-

a

FIG. 2. Greater systolic dysfunction in sGC

1tg/−CM than WT mice after 12 weeks of DOX treatment. Echocardiographic analysis revealed decreased LV systolic function in sGC

1tg/−CM and WT mice (n=14 each) after 8 weeks of DOX administration, illustrated by an increased LVIDES (A) and a reduced FS (B). After 12 weeks of DOX treatment, systolic dysfunction was significantly greater in sGC

1tg/−CM than in WT mice. p < 0.05 versus baseline and a p < 0.05 versus WT (same time point). FS, fractional shortening; LV, left ventricular; LVIDES, left ventricular end-systolic internal diameter; sGC

1tg/−CM, mice with cardiomyocyte-specific deletion of exon 6 of the sGC1 allele; WT, wild-type.

hearts of mice with cardiomyocyte-specific expression of a
dominant negative mutation of sGC1 (DNsGC1βγγ) mice: the relative fold increase in cGMP synthesis was 0.59 ± 0.07
versus 1.00 ± 0.11 in DNsGC1βγγ and WT mice, respectively
(n = 8 each, p < 0.05). Baseline echocardiographic parameters
were similar in DNsGC1βγγ mice and WT littermates (Sup-

plimentary Table S2).

After 8 weeks of doxorubicin treatment (2 mg/kg IP, once
weekly), systolic dysfunction and dilatation, characterized by
increased LVIDES and LVIDED (Fig. 3A, B) and decreased
FS (Fig. 3C), were apparent in DNsGC1βγγ mice, but not
WT mice. After 12 weeks of doxorubicin treatment, LV
functional impairment and dilatation had further progressed
in DNsGC1βγγ mice. Overall, these data are consistent with
the greater doxorubicin-induced systolic dysfunction ob-

served in sGC1−/−CM mice and suggest that sGC may protect
against cardiac dysfunction associated with chronic doxorubi-
cin treatment.

Of note, treating mice with doxorubicin for 12 weeks de-
creased survival rates to a similar extent in DNsGC1βγγ and
WT mice (67% vs. 56%, respectively, n = 30 and 34,
p > 0.05), as well as body weights (−12 ± 0% vs. −15 ± 1%,
respectively, n = 18 and 17, p > 0.05).

Doxorubicin-induced cardiotoxicity in mice
with induced expression of mutated sGC1
is attenuated by reversal of sGC1 mutant expression

The observation that systolic dysfunction occurs within 8
weeks of doxorubicin treatment in DNsGC1βγγ, but not WT,
mice provided a model to test the therapeutic potential of re-
moving sGC activity inhibition by readministering doxycy-
cline. DNsGC1βγγ and WT mice were exposed to doxorubicin
for 12 weeks (2 mg/kg IP, once weekly), and after 8 weeks of
doxorubicin treatment, doxycycline was added to the diet of
both DNsGC1βγγ and WT mice for an additional 8 weeks,
restoring cardiac sGC activity in DNsGC1βγγ mice (Fig. 4A).

Cardiac function was assessed by echocardiography under 12
weeks of doxorubicin administration (T12 weeks) and 4 weeks
later (T16 weeks). After 12 weeks of doxorubicin treatment,
including 4 weeks of doxycycline administration, cardiac
dysfunction and dilatation were still more pronounced in
DNsGC1βγγ than in WT mice (Fig. 4B). However, 4 weeks
later, functional deterioration and adverse remodeling had
progressed further in WT mice, whereas cardiac dysfunction
dilation were reduced in DNsGC1βγγ mice (Fig. 4B). These
findings suggest that restoring sGC activity can attenu-
ate doxorubicin-induced LV dysfunction in mice.

Morphological changes associated with doxorubicin
administration were similar in DNsGC1βγγ
and WT hearts

To assess potential differences in the degree of doxorubicin-
induced cardiac atrophy between sGC1-deficient mice and WT
mice, we measured heart weights in both genotypes following
12 weeks of doxorubicin or saline administration. Doxorubicin
treatment decreased normalized heart weight by 18% and 24%
in sGC1−/−CM and WT mice, respectively, compared with
saline-treated controls (Supplementary Table S3, p > 0.05).
Similarly, in doxorubicin-treated DNsGC1βγγ and WT mice,
heart weight was decreased by 17% and 15%, respectively,
compared with saline-treated mice (Supplementary Table S3, p > 0.05).

To further explore the mechanisms underlying the differ-
ence in doxorubicin-induced cardiac dysfunction between
sGC1−/−CM and WT mice, we examined fibrosis, apoptosis, inflammation, and vascular density in hearts of
DNsGC1βγγ and WT mice following 12 weeks of doxorubi-
cin administration.

To investigate cardiac fibrosis, we measured collagen de-
position and expression levels of two key mediators of fib-
rosis: transforming growth factor (TGF)-β1 and connective
tissue growth factor (CTGF). CTGF is the profibrotic medi-
or of fibroblast proliferation, adhesion, migration, and the synthesis of extracellular matrix. Analysis of Sirius red-stained tissue sections
using circularly polarized light revealed comparable deposition
of thick, tightly packed, red birefringent collagen fibers as well
as thin, loosely assembled, green birefringent collagen fibers in
the two genotypes (Supplementary Table S4). Consistent with
these results, TGF-β1 and CTGF transcript levels were similar
in doxorubicin-treated DNsGC1βγγ and WT mice (Supple-
mentary Table S4).

In addition, the number of apoptotic cardiomyocytes was
similar in DNsGC1βγγ and WT mice after 12 weeks of doxo-
rubicin treatment. In accordance with these results, cardiac
Table 1. Greater Systolic Dysfunction in sGCz1−/−CM than Wild-Type Mice After 12 Weeks of Doxorubicin Administration

<table>
<thead>
<tr>
<th></th>
<th>WT + saline (n=6)</th>
<th>sGCz1−/−CM + saline (n=5)</th>
<th>WT + DOX (n=12)</th>
<th>sGCz1−/−CM + DOX (n=9)</th>
<th>Adjusted p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESV (µl)</td>
<td>19 ± 1</td>
<td>19 ± 1</td>
<td>26 ± 3</td>
<td>37 ± 3†</td>
<td>0.01</td>
</tr>
<tr>
<td>EDV (µl)</td>
<td>43 ± 2</td>
<td>41 ± 2</td>
<td>51 ± 2</td>
<td>58 ± 2†</td>
<td>0.07</td>
</tr>
<tr>
<td>EF (%)</td>
<td>60 ± 2</td>
<td>58 ± 3</td>
<td>55 ± 4</td>
<td>42 ± 4*</td>
<td>0.03</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>104 ± 5</td>
<td>96 ± 6</td>
<td>105 ± 5</td>
<td>102 ± 4</td>
<td>0.94</td>
</tr>
<tr>
<td>Ees (mmHg/µl)</td>
<td>3.6 ± 0.3</td>
<td>3.6 ± 0.4</td>
<td>4.0 ± 0.4</td>
<td>3.9 ± 0.3</td>
<td>1.00</td>
</tr>
<tr>
<td>Pees (mmHg)</td>
<td>91 ± 8</td>
<td>83 ± 4</td>
<td>97 ± 4</td>
<td>91 ± 5</td>
<td>0.67</td>
</tr>
<tr>
<td>Ees (mmHg)</td>
<td>2.5 ± 0.5</td>
<td>2.6 ± 0.3</td>
<td>3.3 ± 0.6</td>
<td>4.5 ± 1.7†</td>
<td>0.11</td>
</tr>
<tr>
<td>dp/dtmax (mmHg/s)</td>
<td>13,499 ± 1,433</td>
<td>11,995 ± 601</td>
<td>13,503 ± 678</td>
<td>10,387 ± 922*</td>
<td>0.03</td>
</tr>
<tr>
<td>dp/dtmin (mmHg/s)</td>
<td>−12,054 ± 1,809</td>
<td>−11,420 ± 950</td>
<td>−13,531 ± 846</td>
<td>−10,943 ± 991</td>
<td>0.21</td>
</tr>
<tr>
<td>Tau (ms)</td>
<td>5.1 ± 0.1</td>
<td>4.9 ± 0.2</td>
<td>5.6 ± 0.2</td>
<td>6.3 ± 0.4†</td>
<td>0.048</td>
</tr>
<tr>
<td>PRSW</td>
<td>83 ± 9</td>
<td>84 ± 6</td>
<td>70 ± 9</td>
<td>49 ± 7†</td>
<td>0.22</td>
</tr>
<tr>
<td>Ees (mmHg/µl)</td>
<td>7.4 ± 0.6</td>
<td>7.3 ± 1.4</td>
<td>4.0 ± 0.8</td>
<td>2.3 ± 0.3†</td>
<td>0.73</td>
</tr>
<tr>
<td>EDVVR (mmHg/µl)</td>
<td>0.22 ± 0.03</td>
<td>0.18 ± 0.02</td>
<td>0.16 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>0.56</td>
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<tr>
<td>HR (bpm)</td>
<td>576 ± 8</td>
<td>614 ± 12</td>
<td>528 ± 9†</td>
<td>530 ± 14†</td>
<td>1.00</td>
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Table Invasive hemodynamic measurements revealed increased systolic dysfunction in sGCz1−/−CM compared with WT mice after 12 weeks of DOX administration.

Multiplicity-adjusted p-values for comparison between WT + DOX and sGCz1−/−CM + DOX are reported. †p < 0.05 versus WT + saline and *p < 0.05 versus WT + doxorubicin.

bpm, beats per minute; DOX, doxorubicin; dp/dtmax, maximum first derivative of developed LV pressure; dp/dtmin, minimum first derivative of developed LV pressure; Ees, arterial elastance; EDVVR, end-diastolic pressure volume relationship; EDV, end-diastolic volume; Ees, end-systolic elastance; EF, ejection fraction; ESV, end-systolic volume; HR, heart rate; LV, left ventricular; MAP, mean arterial pressure; Pees, end-systolic pressure; Peds, end-systolic pressure; PRSW, preload recruitable stroke work; sGC, soluble guanylate cyclase; sGCz1−/−CM, mice with cardiomyocyte-specific deletion of exon 6 of the sGCz1 allele; Tau, time constant for isovolumic relaxation; WT, wild-type.

mRNA expression levels of antiapoptotic B cell lymphoma 2 (Bcl-2) and proapoptotic Bcl-2-associated X protein (Bax) did not differ between the two genotypes (Supplementary Table S4).

In addition, the number of infiltrated CD45-positive leukocytes, a measure of cardiac inflammation, was similar in hearts of DNsGCz1−/− and WT mice after 12 weeks of doxorubicin administration (relative to the number of cardiomyocyte nuclei; Supplementary Table S4).

Furthermore, vascular density was comparable in DNsGCz1−/− and WT hearts after 12 weeks doxorubicin treatment (Supplementary Table S4), suggesting that a lower density of capillaries, supplying cardiomyocytes with oxygen and nutrients, did not contribute to the greater cardiac dysfunction in doxorubicin-treated DNsGCz1−/− mice.

Doxorubicin-induced ROS generation is increased in sGCz1−/− mice

To consider the possibility that doxorubicin induced higher levels of oxidative stress in sGCz1−/− than in WT mice, we investigated the degree of doxorubicin-induced ROS generation in cardiomyocytes isolated from sGCz1−/− and WT mice. sGCz1−/− and WT cardiomyocytes were exposed to doxorubicin (50 µM) for 3 h and then incubated with chloromethyl derivate of 2’,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA). This cell-permeable compound is deacetylated by intracellular esterases and then oxidized by hydrogen peroxide (5) and other oxidants (20) to produce fluorescent 2’,7’-dichlorofluorescein (DCF). Three hours after exposure to doxorubicin, greater DCF fluorescence, and thus ROS production, was observed in sGCz1−/− than in WT cardiomyocytes (Fig. 5A).

Similarly, reactive nitrogen species-mediated tyrosine nitration was more pronounced in sGCz1−/− than WT hearts 24 h after doxorubicin administration (Fig. 5B). In addition, higher expression of malondialdehyde (MDA) was observed in DNsGCz1−/− than WT hearts after 12 weeks of doxorubicin treatment (Fig. 5C). MDA is a product of lipid peroxidation, frequently initiated by hydroxyl and hydroperoxyl radicals (3).

Taken together, these results suggest that doxorubicin-induced oxidative stress is more pronounced when cardiac sGC activity is reduced. This in turn could lead to enhanced sGC oxidation and decrease in activity and subsequent exacerbation of ROS levels via a yet to be identified negative feedback system.

Discussion

The long-term adverse cardiac effects of doxorubicin, which limit the use of this effective chemotherapeutic agent, emphasize the need for novel therapeutic approaches to prevent and treat cardiotoxicity associated with doxorubicin treatment. Our data identify sGC as a potential therapeutic target for doxorubicin-induced cardiotoxicity.

Twenty-four hours after doxorubicin administration, cardiac sGC activity was reduced in mice. Since cardiac sGC expression was not altered by doxorubicin treatment, the observed decrease in activity likely results from post-translational modification of sGC. Oxidative modification of sGC, leading to loss of its prosthetic heme group and generation of NO-insensitive sGC, was previously reported in cardiovascular tissues in a yet to be identified negative feedback system.
To determine whether reduced sGC activity contributes to the development of doxorubicin-induced cardiotoxicity, doxorubicin was administered to mice with a cardiomyocyte-specific deficiency in sGCα1. After 12 weeks of DOX administration, systolic dysfunction and dilatation were more pronounced in DNsGCα1<sup>−/−</sup> than in WT mice. *p<0.05 versus baseline and *p<0.05 versus WT (same time point). DNsGCα1<sup>−/−</sup> mice with cardiomyocyte-specific expression of a dominant negative mutation of sGCα1; LVID<sub>ES</sub>, left ventricular end-systolic internal diameter; LVID<sub>ED</sub>, left ventricular end-diastolic internal diameter; FS, fractional shortening.

FIG. 3. Greater DOX-induced LV dysfunction and dilatation in DNsGCα1<sup>−/−</sup> than in WT mice. After 8 weeks of DOX treatment, LVID<sub>ES</sub> and LVID<sub>DP</sub> were increased (A, B) and FS decreased (C) in DNsGCα1<sup>−/−</sup>, but not in WT, mice (n=20 each). After 12 weeks of DOX administration, systolic dysfunction and dilatation were more pronounced in DNsGCα1<sup>−/−</sup> than in WT mice. *p<0.05 versus baseline and *p<0.05 versus WT (same time point). DNsGCα1<sup>−/−</sup> mice with cardiomyocyte-specific expression of a dominant negative mutation of sGCα1; LVID<sub>ES</sub>, left ventricular end-systolic internal diameter; LVID<sub>ED</sub>, left ventricular end-diastolic internal diameter; FS, fractional shortening.

To determine whether reduced sGC activity contributes to the development of doxorubicin-induced cardiotoxicity, doxorubicin was administered to mice with a cardiomyocyte-specific deficiency in sGCα1. After 12 weeks of doxorubicin treatment, LV systolic dysfunction was more pronounced in sGCα1<sup>−/−</sup> than in WT mice. Similarly, mice with induced expression of a dominant negative mutated sGCα1 (DNsGCα1<sup>−/−</sup>) displayed greater LV systolic dysfunction and remodeling than WT mice after 12 weeks of doxorubicin treatment. Together, these data reveal a protective role for sGC against doxorubicin-induced cardiotoxicity.
Furthermore, we wanted to explore whether restoring sGC activity could attenuate established cardiac dysfunction. Since DNsGC\textsuperscript{a1tg/+} mice displayed LV dysfunction and remodeling after 8 weeks of doxorubicin administration, whereas WT mice did not, the impact of restoring cardiac sGC activity on doxorubicin-induced cardiotoxicity could be evaluated by initiating reversal of sGC\textsuperscript{a1} mutant expression in DNsGC\textsuperscript{a1tg/+} mice at this time point. Halting expression of mutated sGC\textsuperscript{a1} in DNsGC\textsuperscript{a1tg/+} mice after 8 weeks of doxorubicin administration, resulting in restored sGC activity, eventually reduced LV dysfunction. Four weeks after halting doxorubicin treatment, cardiac function was even less impaired in DNsGC\textsuperscript{a1tg/+} mice than in WT animals.

It is possible that mechanisms to compensate for reduced cardiac sGC activity are triggered in the DNsGC\textsuperscript{a1tg/+} mouse model. When expression of mutated sGC\textsuperscript{a1} is halted in these DNsGC\textsuperscript{a1tg/+} mice, the resulting increase in sGC activity in combination with potential compensatory mechanisms could lead to enhanced cGMP signaling in comparison with WT mice and may contribute to improvement of cardiac function beyond the level observed in doxorubicin-treated WT mice.

Despite the observation that doxorubicin-induced cardiac dysfunction was greater in sGC\textsuperscript{a1−/−CM} and DNsGC\textsuperscript{a1tg/+} mice than in WT littersmates, heart weights were similar in doxorubicin-treated sGC\textsuperscript{a1}-deficient and WT mice. In addition, cardiac Bcl-2 and Bax mRNA expression and cardiomyocyte apoptosis did not differ between DNsGC\textsuperscript{a1tg/+} and WT mice following 12 weeks of doxorubicin treatment. These results indicate that the increased cardiac dysfunction in doxorubicin-treated sGC\textsuperscript{a1}-deficient mice did not result from a greater loss of cardiomyocytes. In addition, cardiac fibrosis, inflammation, and vascular density did not differ between sGC\textsuperscript{a1} mutant and WT mice following 12 weeks of doxorubicin administration.

We cannot exclude that the degree of cardiac fibrosis, apoptosis, inflammation, or vascular density might differ at earlier or later time points. In addition, the difference in cardiac function between doxorubicin-treated sGC\textsuperscript{a1}-deficient and WT mice did not reflect an effect of sGC\textsuperscript{a1} deficiency on blood pressure, which was similar in doxorubicin-treated sGC\textsuperscript{a1−/−CM} and WT mice.

Oxidative stress has been suggested to play a prominent role in doxorubicin-associated cardiotoxicity. In mice overexpressing endothelial NO synthase (NOS3), doxorubicin-induced cardiotoxicity was greater than in WT mice due to abundant ROS production resulting from NOS3-mediated...
reduction of doxorubicin (34). We also observed increased 
doxorubicin-induced ROS production in sGC1-deficient 
cardiomyocytes and greater oxidative stress levels in sGC1- 
deficient hearts after in vivo exposure to doxorubicin.

It is possible that the more pronounced doxorubicin- 
inecled oxidative stress in sGC1-deficient cardiomyocytes 
leads to greater contractile impairment. Oxidative stress can 
alter expression, phosphorylation, or function of calcium 
regulatory proteins such as sarcoplasmic reticulum calcium 
ATPase 2A and ryanodine receptor 2 (1, 25, 54). Oxidative 
stress also induces structural modifications of sarcomeric 
proteins and disrupts mitochondrial function in cardiomyo- 
cytes (44). Additional studies are required to evaluate whe- 
ther increased oxidative stress levels in sGC1-deficient 
cardiomyocytes result in greater contractile impairment and, 
if so, which mechanisms are involved.

Further support for a protective role of NO-sGC-cGMP 
signaling in doxorubicin-induced cardiotoxicity was previ- 
ously provided by the observation that supplementing dietary 
nitrate reduced doxorubicin-induced cardiotoxicity (57). This 
cardioprotection was associated with a decrease in ROS 
generation, lipid peroxidation, and mitochondrial respiratory 
chain damage (57).

In addition, pharmacological inhibition of the cGMP- 
catabolizing enzyme, phosphodiesterase type 5 (PDE5), with 
sildenafil or tadalafil attenuated doxorubicin-associated car- 
diotoxicity in mice (17, 24). These PDE5 inhibitors reduced 
doxorubicin-induced oxidative stress, disruption of the 
mitochondrial membrane potential, apoptosis, and deple- 
tion of prosurvival proteins in the heart (17, 24). Because 
the ability of PDE5 inhibitors to augment cGMP levels is 
inherently limited by cGMP synthesis, strategies designed 
to enhance cGMP synthesis may be more effective in pre- 
venting doxorubicin-induced cardiotoxicity. Nitroglycerin 
and related NO donors are currently used to treat various 
cardiovascular diseases. However, nitrates produce off- 
target (cGMP-independent) effects and induce tolerance.

As many beneficial effects of NO are mediated through 
sGC, pharmacological agents directly targeting sGC could 
represent a novel mechanism-based approach to limit cardiol-

toxicity in patients undergoing doxorubicin-based chemo-
therapy. The therapeutic potential of targeting sGC to prevent 
or even reverse doxorubicin-induced cardiotoxicity is sup- 
sported by the observation that doxorubicin-induced LV 
dysfunction in DNsGC1 mutants specifically in 
cardiomyocytes of sGC1 (D529A, Asp to Ala) (55). Following 
implantation of microinjected FVB yzogotes into a pseudopregnant Swiss 
 foster mother, offspring were genotyped, and transgenic 
founders were backcrossed for >6 generations onto a C57BL/ 
6N background (Taconic, Hudson, NY).

To establish the DNsGC1 mice model, a first mouse line was generated in which expression of a mutated sGC1- 
encoding gene is driven by a doxycycline-responsive promoter element (tetracycline operator [TetO]). The dominant 
negative point mutation introduced into the sGC1-encoding gene results in an amino acid change in the catalytic region of 
sGC1 (D529A, Asp to Ala) (55). Following implantation of 
microinjected FVB yzogotes into a pseudopregnant Swiss 
 foster mother, offspring were genotyped, and transgenic 
founders were backcrossed for >6 generations onto a C57BL/ 
6N background (Taconic, Hudson, NY).

When doxycycline is removed from the diet (doxycycline- 
containing chow, 200 mg/kg; Harlan Laboratories, 
Huntingdon, United Kingdom) of dual heterozygous TetO- 
DNsGC1 mice and their WT controls (referred to as DNsGC1 mice), doxycycline-mediated repression of transcriptional 
activation is abolished (Tet-Off system), and tTA binds to 
activates transcription from the TetO promoter element, 
inducing expression of the sGC1 mutant specifically in 
cardiomyocytes. DNsGC1 mice and their WT controls 
(TetO-DNsGC1 littermates) were conceived and 
raised in the presence of doxycycline. When the mice were 4–6 weeks old, doxycycline was withdrawn, and 4 
weeks later, mice were used for experiments.

**Doxorubicin treatment regimen**

For acute studies, 8-week-old C57BL/6J mice (The Jack- 
on Laboratory) were injected with a single dose of doxo- 
rubicin (20 mg/kg IP, using a 2 mg/ml stock of doxorubicin 
hydrochloride obtained from Pfizer, New York, NY) or saline 
and sacrificed 24 h later. A subset of these mice were pro- 
vided with the radical scavenger, tempol (1-oxy-2,2,6,6- 
tetramethyl-4-hydroxipiperidine; Thermo Fisher Scientific, 
Waltham, MA), via drinking water (1 mM) 4 days before

**Materials and Methods**

**Experimental animals**

This study was carried out in strict accordance with the 
recommendations in the Guide for the Care and Use of Labo-

ratory Animals of the National Institutes of Health. 
Housing and all procedures involving experimental animals 
were approved by the Institutional Animal Care and Use 
Committees of Massachusetts General Hospital (Sub-
committee on Research Animal Care).

Cardiomyocyte-specific sGC1 mice were generated by 
crossing mice in which exon 6 of the sGC1 allele is flanked by 
two LoxP sites [sGC1fl/fl, generated by our group (7)] with 
mice expressing Cre recombinase under the control of an x-
myosin heavy chain promoter (2MHC-Cre+/–). In cardiomyo-
cytes of sGC1fl/flMHC-Cre+/– offspring (referred to as 
sGC1+/– mice), Cre recombinase induces deletion of exon 
6, encoding a portion of the catalytic domain of sGC1 required 
for enzyme activity. These mice were maintained on a C57BL/ 
6D background (The Jackson Laboratory, Farmington, CT) and 
sGC1fl/flMHC-Cre+/– littermates served as WT controls.

To establish the DNsGC1 mice model, a first mouse line was generated in which expression of a mutated sGC1- 
encoding gene is driven by a doxycycline-responsive promoter element (tetracycline operator [TetO]). The dominant 
negative point mutation introduced into the sGC1-encoding gene results in an amino acid change in the catalytic region of 
sGC1 (D529A, Asp to Ala) (55). Following implantation of 
microinjected FVB yzogotes into a pseudopregnant Swiss 
 foster mother, offspring were genotyped, and transgenic 
founders were backcrossed for >6 generations onto a C57BL/ 
6N background (Taconic, Hudson, NY).

This mouse line was crossed with a second mouse line 
expressing the gene encoding a tetracycline transactivator 
(tTA) protein under the control of an 2MHC promoter, kindly 
provided by Dr. D.A. Dichek (University of Washington 
Medical Center, Seattle, WA), and maintained on a C57BL/ 
6N background (Taconic, Hudson, NY).

When doxycycline is removed from the diet (doxycycline- 
containing chow, 200 mg/kg; Harlan Laboratories, 
Huntingdon, United Kingdom) of dual heterozygous TetO- 
DNsGC1 mice and their WT controls (referred to as DNsGC1 mice), doxycycline-mediated repression of transcriptional 
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(TetO-DNsGC1 littermates) were conceived and 
raised in the presence of doxycycline. When the mice were 4–6 weeks old, doxycycline was withdrawn, and 4 
weeks later, mice were used for experiments.
TRANSTHORACIC ECHOCARDIOGRAPHY

To measure LV systolic function and dimensions, sGCz1+/−CM mice were lightly sedated (ketamine, 100 mg/kg, IP) and echocardiography was performed using a 13 MHz ultrasound probe (Vivid 7; GE Healthcare, Wilmington, MA). DN sGCz1−/−, sGCz1−/−CM, and WT mice were administered a low dose of doxorubicin for 12 weeks (2 mg/kg IP, once weekly).

INVASIVE HEMODYNAMIC MEASUREMENTS

Mice were anesthetized by IP injection with ketamine (120 mg/kg), fentanyl (90 μg/kg), and rocuronium (10 mg/kg), intubated, and mechanically ventilated (FiO2 = 1, 10 μl/g, 120 breaths per minute). A fluid-filled catheter was introduced into the carotid artery to record arterial blood pressure and HR. After thoracotomy, a pressure–volume conductance catheter (PVR-1030; Millar Instruments, Houston, TX) was introduced through the apex into the LV, as described previously (19).

LV end-systolic and end-diastolic volumes and LV pressures were measured, and EF, arterial elastance, the maximum and minimum first derivative of developed LV pressure (dP/dt max and dP/dt min), and the time constant for isovolumic relaxation (Tau) were calculated. The preload recruitable stroke work, end-systolic elastance, and end-diastolic pressure–volume relationship were obtained by transiently occluding the inferior vena cava. All parameters were measured using LabChart (ADInstruments, Colorado Springs, CO).

sGC ACTIVITY MEASUREMENTS

sGC enzyme activity was measured as described previously (7). Cardiac tissues were homogenized and supernatants (containing 50 μg of protein) were incubated for 10 min at 37°C in a reaction mixture with DETA-NO (1 mmol/l) or BAY 58-2667 (100 μmol/l). cGMP in the reaction mixture was measured using a commercial radioimmunoassay (Cayman Chemical, Ann Arbor, MI). sGC enzyme activity was expressed as picomoles of cGMP produced per minute per milligram of protein in cardiac extract supernatant, normalized to the contemporaneous control group (saline-treated mice when sGC activity was measured in doxorubicin-treated mice and WT mice when sGC activity was measured in sGCz1-deficient mice).

HISTOLOGICAL AND IMMUNOBLOT ANALYSIS

Immunohistochemistry was performed as described previously (52). Images were obtained using an Axiosvert 200M imaging microscope (Zeiss, Oberkochen, Germany). To assess the degree of cardiac fibrosis, the area of collagen deposition was traced on Sirius red-stained tissue sections using circularly polarized light, allowing evaluation of tightly packed, red birefringent collagen and thin, loosely assem-
Statistical analysis

Values are presented as mean ± standard error of the mean. When comparing two groups, normal distribution of the data was assessed using the Shapiro–Wilks test, and an unpaired two-tailed t-test (parametric) or a Mann–Whitney test (non-parametric) was used. Serial echocardiographic data were analyzed by two-way analysis of variance (ANOVA) and Sidak’s multiple comparisons test. For these experiments, the required samples sizes were calculated to yield statistical power of ≥0.80 (α = 0.05). Analysis of pressure–volume measurements was performed via one-way ANOVA and Sidak’s multiple comparisons test (when passed the Shapiro–Wilk normality test) or the Kruskal–Wallis test and Dunn’s multiple comparisons test (when failed the Shapiro–Wilks normality test). Survival was analyzed via the Kaplan–Meier method.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

3-NT = 3-nitrotyrosine
ANOVA = analysis of variance
Bax = B cell lymphoma 2-associated X protein
Bcl-2 = B cell lymphoma 2
bpm = beats per minute
cGMP = cyclic guanosine 3’,5’-monophosphate
CM-H2DCFDA = chloromethyl derivate of 2’,7’-dichlorodihydrofluorescein diacetate
CO = carbon monoxide
CTGF = connective tissue growth factor
DCF = 2’,7’-dichlorofluorescein
DETA-NO = diethylenetriamine NONOate
DNsGCZ1gt+ = mice with cardiomyocyte-specific expression of a dominant negative mutation of sGCz1
DOX = doxorubicin
dP/dtmax = maximum first derivative of developed LV pressure
dP/dtmin = minimum first derivative of developed LV pressure
Ea = arterial elastance
EDPVR = end-diastolic pressure volume relationship
EDV = end-diastolic volume
Ees = end-systolic elastance
EF = ejection fraction
ESV = end-systolic volume
FS = fractional shortening
Gapdh = glyceraldehyde-3’-phosphate dehydrogenase
HR = heart rate
IP = intraperitoneal
IVSed = interventricular septal thickness at end-diastole
LV = left ventricular
LVdied = left ventricular end-diastolic internal diameter
LVIDes = left ventricular end-systolic internal diameter
LVpved = left ventricular posterior wall thickness at end-diastole
MAP = mean arterial pressure
MDA = malondialdehyde
MHC = myosin heavy chain
NO = nitric oxide
NOS3 = endothelial nitric oxide synthase
PCR = polymerase chain reaction
PDE5 = phosphodiesterase type 5
Peds = end-diastolic pressure
Prs = end-systolic pressure
PRSW = preload recruitable stroke work
ROS = reactive oxygen species
sGC = soluble guanylate cyclase
sGCz1-/-CM = mice with cardiomyocyte-specific deletion of exon 6 of the sGCz1 allele
Taur = time constant for isovolumic relaxation
tetO = tetracycline operator
TGF-ß1 = transforming growth factor-ß1
tTA = tetracycline transactivator
TTE = transthoracic echocardiography
WT = wild-type