G Protein Inactivator RGS6 Mediates Myocardial Cell Apoptosis and Cardiomyopathy Caused by Doxorubicin

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Running Title

RGS6 Mediates Doxorubicin Cardiomyopathy

Key words

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Additional Information

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Abstract
Clinical use of the widely used chemotherapeutic agent doxorubicin is limited by life-threatening cardiotoxicity. The mechanisms underlying Dox-induced cardiomyopathy and heart failure remain unclear, but are thought to involve p53-mediated myocardial cell apoptosis. The tripartite G protein inactivating protein RGS6 has been implicated in reactive oxygen species (ROS) generation, ATM/p53 activation and apoptosis in Dox-treated cells. Thus, we hypothesized that RGS6, the expression of which is enriched in cardiac tissue, might also be responsible for the pathological effects of Dox treatment in heart. In this study, we show that RGS6 expression is induced strongly by Dox in the ventricles of mice and isolated ventricular myocytes (VCM) via a post-transcriptional mechanism. While Dox-treated wild type (WT) mice manifested severe left ventricular dysfunction, loss of heart and body mass, along with decreased survival five days after Dox administration, mice lacking RGS6 were completely protected against these pathogenic responses. Activation of ATM/p53-apoptosis signaling by Dox in ventricles of WT mice was also absent in their RGS6−/− counterparts. Dox-induced ROS generation was dramatically impaired in both the ventricles and VCM isolated from RGS6−/− mice, and the apoptotic response to Dox in VCM required RGS6-dependent ROS production. These results identify RGS6 as an essential mediator of the pathogenic responses to Dox in heart, and they argue that RGS6 inhibition offers a rational means to circumvent Dox cardiotoxicity in human cancer patients.

Introduction
Doxorubicin (Dox), an anthracycline chemotherapeutic, is among the most effective and widely used drugs for treatment of human cancers (1, 2). Unfortunately, Dox induces life-threatening cardiotoxicity including cardiomyopathy and heart failure (1, 2). Patients receiving cumulative doses of Dox of 700 mg/m² have a 48% risk of developing heart failure (3). More than 50% of
childhood cancer survivors have been treated with anthracyclines and the 30 yr mortality rate of
initial survivors from cardiac death was 15 times higher than expected (4, 5). Irreversible
apoptotic death of VCM is a hallmark of pathological responses to heart injury and stress
underlying heart failure (6-8). One of the critical gaps in our knowledge is an understanding of
the pathogenic mechanisms responsible for Dox-induced heart injury. Multiple mechanisms
have been proposed including ROS-induced damage to heart cells and the very same
ATM/p53-apoptosis pathways believed to underlie the chemotherapeutic actions of Dox (1).
Indeed, genetic disruption or inhibition of p53 protects against Dox-induced myocardial cell
apoptosis and contractile dysfunction demonstrating a crucial role for p53 in the myopathic
response to Dox (9, 10).

Here we provide new evidence that the pleiotropic Regulator of G protein signaling (RGS) family
member RGS6 is a critically important upstream mediator of Dox-induced myocardial cell
apoptosis and cardiomyopathy. Our work was inspired by our discovery of a link between
RGS6 and apoptosis in cancer cells (11) and our finding that RGS6 mediates activation by Dox
of the ATM/p53-apoptosis pathway in mouse embryonic fibroblasts and MCF-7 breast cancer
cells via ROS (12), also strongly implicated in apoptotic signaling (13). These actions of RGS6
are entirely novel as they are independent of its canonical function as a GTPase-activating
protein (GAP) for heterotrimeric G proteins, necessary for RGS6-mediated control of cardiac
automaticity as we previously demonstrated (14). Given our finding that RGS6 is abundant in
heart, we hypothesized that RGS6 might mediate Dox-induced myocardial cell apoptosis and
cardiomyopathy.

Materials and Methods

An expanded Materials and Methods section is available as Online Supplementary Material.
**Mice-** We generated RGS6−/− mice as described previously (14). Experimental animals are age matched 3-6 month-old mice weighing approximately 25 – 35 grams and were backcrossed onto a C57BL6 background for five generations. Mice were monitored daily including weekends and holidays for signs of stress/discomfort, and were euthanized when they showed signs of sickness such as hunched posture, dyspnea, dehydration, or marked weight loss. All animal experiments were performed in agreement with the Guide for the Use and Care of Laboratory Animals.

**Ventricular function and other measurements in mice-** A well-established procedure (10, 15, 16) was used to induce ventricular dysfunction in mice. Briefly, saline or Dox (20 mg/kg body weight) was administered to WT and RGS6−/− mice by a single intraperitoneal injection. Body weight and survival of mice were recorded from day 0 (before saline or Dox administration) to day 5. This experiment was repeated several times; and each treatment group contained at least five mice to compensate for death of mice during treatment. On day 5, left ventricular function was assessed by invasive hemodynamic measurement as described previously (10, 15, 16). Briefly, a SPR-1000 Mikro-Tip® mouse pressure catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle via the right carotid artery. Pressure signals were acquired using a Pressure Control Unit (Millar Instruments) coupled to a Powerlab 4/30 SP analog-to-digital converter (AD Instruments, Mountain View, CA). Left ventricle pressure parameters were analyzed with Labchart 7.0 software (AD Instruments). Mice were euthanized and their hearts were collected and weighed after recordings were completed. Left legs were dissected from mice and subjected to boiling in 2% Na₂CO₃ for 30 min to clean the tibia of tissue. Lengths of water-rinsed and air-dried left tibias were measured using a dial caliper (Bel-Art Scienceware, Wayne, NJ) and used as denominator to normalize heart weight.
Staining for superoxide in hearts and for total ROS in VCM-DHE (dihydroethidium) staining was performed in heart frozen sections derived from paraformaldehyde (4% fresh)-perfused WT and RGS6⁻/⁻ mice treated with saline or Dox (10 mg/kg). Intracellular ROS in WT and RGS6⁻/⁻ VCM treated with saline or Dox was visualized using the cell-permeable oxidation-sensitive probe, CM-H₂DCFDA (5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate). All images were captured using a Zeiss fluorescence microscope.

Results

Loss of RGS6 prevents Dox-induced heart failure

To determine the role of RGS6 in Dox-induced cardiomyopathy, WT and RGS6⁻/⁻ mice were treated with saline or Dox followed by hemodynamic measurements of cardiac function five days later. This protocol is well established for inducing myopathic changes in hearts of mice that include left ventricular dysfunction (10, 15, 16). Dox-treated WT mice exhibited the expected severe left ventricular dysfunction compared to saline-treated control mice, manifested as lowered values of LVESP, LVDP, and ±dP/dt (Figs. 1A-1D). Remarkably, RGS6⁻/⁻ mice were completely protected from Dox-induced ventricular dysfunction (Figs. 1A-1D). Furthermore, RGS6⁻/⁻ mice were completely spared from Dox-induced loss of heart and body mass and death (Fig. 2), while Dox-treated WT mice lost approximately 15% heart mass (Fig. 2A), 20% body weight (Fig. 2B), and exhibited a mortality rate of nearly 50% (Fig. 2C). These findings provide the first evidence that RGS6 plays an essential role in mediating Dox-induced cardiomyopathy and heart failure.

RGS6 mediates Dox-induced activation of p53 and apoptosis in heart

Given the crucial role of p53 in Dox-induced myocardial apoptosis and our recent finding that RGS6 was required for activation of ATM, p53 and apoptosis in Dox-treated mouse embryonic fibroblasts and MCF-7 breast cancer cells (12), we next investigated the role of RGS6 in
apoptotic signaling in hearts from Dox-treated mice. Dox transiently induces p53, which leads to subsequent apoptosis in heart (10). Treatment of WT mice with Dox led to a rapid and robust induction of RGS6L, the predominant form of RGS6 expressed in mouse heart (14), in both ventricles (Fig. 3) and atria (Fig. S1). Similarly, Dox induced robust increases in the levels of total and phosphorylated p53 [p-p53(S15), the ATM phosphorylation site] in both tissues (Figs. 3, S1) that did not occur in hearts of RGS6−/− mice (Figs. 3, S1). Induction of RGS6 by Dox preceded phosphorylation and up-regulation of p53, despite the presence of basal levels of RGS6 (Figs. 3, S1).

We then investigated how loss of RGS6 impacted the ATM/p53-mediated apoptotic DNA damage signaling pathway in ventricles from Dox-treated mice. Dox induced the activating autophosphorylation of ATM and phosphorylation of the ATM substrates H2AX and Mdm2 (inactive phospho-Mdm2 is not detected by the antibody) in ventricles of WT mice. These responses were greatly diminished in RGS6−/− mice (Figs. 3A, S2). Because phosphorylation of p53 and Mdm2 inhibits ubiquitination and subsequent degradation of p53 by Mdm2 (17, 18), our results suggest that Dox promotes p53 up-regulation in ventricles by RGS6-dependent ATM activation. Dox promoted robust increases in the Bax/Bcl2 ratio and apoptosis (caspase-3 activation and PARP cleavage) in ventricles of WT mice, responses that were completely absent in RGS6−/− ventricles (Figs. 3A, S2). These findings demonstrate that RGS6 is an essential mediator of myocardial apoptosis underlying the pathogenic actions of Dox in the ventricle. This extends our preliminary observation that Dox-induced increases in TUNEL-positive cardiomyocytes in ventricles of WT mice were reduced in RGS6−/− mice (12). Thus, loss of RGS6 protects mice against Dox-induced ventricular apoptosis and myopathy.

**Dox acts directly on VCM to induce RGS6 and activate p53**
Dox transiently increased levels of RGS6, p53, and p-p53(S15) in VCM isolated from WT mice, effects apparent within 8 h and lasting up to 24 h (Fig. S3A). Dox-induced up-regulation of p53 was both RGS6-dependent, as it was not observed in RGS6−/− VCM, and was temporally correlated to up-regulation of RGS6, despite the presence of basal levels of RGS6 (Fig. S3A). Dox-induced phosphorylation of p53 in VCM from WT mice paralleled p53 up-regulation and was markedly reduced in RGS6−/− VCM (Fig. S3A). Therefore, Dox acts directly on VCM to induce RGS6, which promotes phosphorylation and consequent up-regulation of p53.

Given the rapid induction of RGS6 by Dox in ventricles and isolated VCM (Figs. 3A, S3A), we hypothesized RGS6 up-regulation might occur post-transcriptionally. To test this hypothesis, we first evaluated RGS6 mRNA levels in Dox-treated ventricles of WT mice, using quantitative RT-PCR as we described previously (14). RGS6 mRNA was not induced significantly in ventricles during the first 12 h of Dox treatment of mice (Fig. S3B). Further, blocking de novo protein synthesis with cycloheximide markedly reduced up-regulation of RGS6 and p-p53(S15) in VCM (Fig. S3C), demonstrating that Dox induces RGS6 at least in part by mechanisms requiring de novo protein synthesis.

**RGS6 mediates Dox-induced apoptosis in VCM via a ROS-dependent mechanism**

Oxidative stress, defined as excess of ROS compared to antioxidant defenses, is increased in heart failure and in hearts of Dox-treated mice (6, 16, 19). ROS are strongly implicated in apoptotic pathways (13), and we recently discovered that RGS6 mediates activation of ATM/p53-apoptosis signaling by a ROS-dependent mechanism in mouse embryonic fibroblasts and MCF-7 breast cancer cells (12), likely via the recently discovered oxidative activation of ATM (20). Thus, we investigated whether RGS6 fulfilled a similar role in the ventricle. Loss of RGS6 greatly impaired Dox-induced superoxide and total ROS generation in ventricles of mice and cultured VCM, respectively (Figs. 4A, 4B), demonstrating a key role for RGS6 in Dox-
induced ROS generation. Further, Dox induced activation of caspase-3 and apoptosis in cultured VCM by an RGS6-dependent mechanism that required ROS, as these responses were blocked by brief treatment of VCM with ROS scavengers including N-acetyl cysteine (NAC) and active pegylated forms of superoxide dismutase (SOD) or catalase (Figs. 4C, 4D). Thus, RGS6-dependent ROS generation is essential for Dox-induced VCM apoptosis.

Discussion

This study reveals a novel and essential role for RGS6 in mediating myopathic responses to Dox in heart. Mice lacking RGS6 were completely protected against Dox-induced heart failure and loss of heart mass as well as Dox-induced mortality. Moreover, RGS6 was required for the ability of Dox to induce apoptosis of ventricular myocardial cells, the hallmark pathologic response to heart injury and stress that underlies Dox-induced heart failure. The finding that RGS6 was required for Dox-induced apoptosis both in ventricles in vivo and in isolated VCM suggests this action of RGS6 was not strictly dependent on tissues beyond the heart. We showed previously that RGS6 is expressed highly in heart (14). We now show that Dox induces RGS6 expression in heart by a post transcriptional mechanism that is important for its ability to promote activation of p53, a key modulator of Dox-induced apoptosis (9, 10).

We provide new evidence that RGS6 functions as an essential upstream activator and integrator of ROS and ATM/p53-apoptosis pathways in heart. ROS production has been implicated in Dox-induced apoptosis and heart damage (1). Our results demonstrate that RGS6 is required for Dox-induced ROS generation and p53 activation both in ventricles in vivo and in isolated VCM, and that RGS6-dependent apoptosis in VCM is ROS-dependent. Recently we discovered that RGS6 promotes mitochondrial dependent apoptosis via ROS (11) and mediates Dox-induced activation of ATM and subsequent induction of p53 via ROS (12). These actions of RGS6 were independent of its GAP activity toward G proteins, pointing to an entirely novel
signaling function for a RGS protein family member. Although ROS can activate ATM by inducing DNA damage, RGS6-dependent ATM activation was independent of DNA damage (12), suggesting that RGS6 mediates the ROS-dependent oxidative activation of ATM described by Guo et al. (20). Our data support a model in which RGS6 up-regulation by Dox in heart induces ROS generation, which in turn promotes ATM/p53-apoptosis signaling via oxidative activation of ATM (Fig. S4).

We provide evidence for a new and crucial role for RGS6 in heart. While RGS6 in pacemaker cells of the heart controls automaticity by inactivating G proteins coupled to opening of GIRK channels (14), RGS6 in ventricular myocardial cells mediates Dox-induced myocardial apoptosis and heart failure. Expression of RGS proteins is normally under tight control, presumably to limit their strong negative impact on G protein signaling. Due to the G protein-independent nature of the proapoptotic actions of RGS6 (11, 12), it may represent a more viable target for drugs that would ameliorate Dox-induced cardiomyopathy. Indeed, inhibition of RGS6 would be expected to protect the heart against Dox-induced cell death and cardiomyopathy, while simultaneously enhancing parasympathetic tone (14), itself believed to be cardioprotective. Induction of RGS6 by Dox appears essential for its ability to mediate p53 activation and occurs by mechanisms requiring de novo protein synthesis, providing potential targets to prevent Dox-mediated induction of RGS6 and resulting pathologic sequelae. Identifying RGS6 as an essential signaling protein in the ROS/ATM/p53-apoptosis pathway in heart advances our understanding of the pathogenesis of Dox-induced cardiomyopathy and identifies RGS6 as a possible therapeutic target for cardio-protective adjuncts to Dox chemotherapy, which despite its life-threatening cardiotoxic actions remains one of the most effective and widely used drugs in cancer treatment.

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Non-standard Abbreviations and Acronyms

+dp/dt, maximal slope of the systolic pressure increment; -dp/dt, maximal slope of diastolic decrement; ATM, ataxia telangiectasia mutated; Dox, doxorubicin, GAP, GTPase-activating protein; LVDP, left ventricular developed pressure; LVESP, maximal left ventricular end-systolic pressure; PARP, poly ADP-ribose polymerase; RGS6, regulator of G protein signaling 6; ROS, reactive oxygen species; VCM, ventricular cardiomyocytes; WT, wild-type (RGS6+/+).

References


Figure Legend

Figure 1. **Loss of RGS6 prevents Dox-induced ventricular dysfunction.** Dox induced depression of (A) LVESP, (B) LVDP, (C) +dP/dt, and (D) –dP/dt in WT but not in RGS6–/– mice. Hemodynamic measurements of WT and RGS6–/– mice (n=5-8) were performed five days after saline or Dox (20 mg/kg ip) administration. *, p <0.05.

Figure 2. **Loss of RGS6 protects mice against Dox-induced toxicity.** Dox induced decreases in (A) heart weight, (B) body weight and (C) survival in WT but not in RGS6–/– mice. WT and RGS6–/– mice (n=5-14) were treated using the same protocol described in Figure 1. *, p <0.001.

Figure 3. **RGS6 is an essential mediator of Dox-induced increases in p53 and apoptosis in mouse ventricle.** WT and RGS6–/– mice were treated with Dox (10 mg/kg, ip) and levels of RGS6 and key apoptotic proteins were determined as described in Materials and Methods. (A) typical immunoblot (B) quantification of results from multiple ventricles. Values for each protein from untreated WT mice were set at 1 in B. *, p< 0.002; **, p< 0.001.

Figure 4. **RGS6 mediates Dox-induced ROS generation and ROS-dependent apoptosis.** Effects of RGS6 loss on Dox-induced (A) superoxide generation in ventricles of mice and (B) ROS production in cultured VCM. Effects of loss of RGS6 on Dox-induced (C) caspase-3 activity and (D) apoptosis in VCM. Mice were treated with Dox (10 mg/kg ip) for 12 hrs and VCM with Dox (0.5 µM) for 12 hrs (B) or 16 hrs with or without pre-incubation with ROS scavengers for 1 hr (C and D). Values of WT control were set at 1 in C and D. *, p < 0.001.
Figure 1

A

LVESP (mmHg)

B

LVDP (mmHg)

C

+dP/dT (mmHg/sec)

D

-dP/dT (mmHg/sec)

Treatment

Saline

Dox

Saline

Dox

WT

RGS6−/−
Figure 2

A
Heart weight / tibia length (mg/mm)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WT</th>
<th>RGS6^-/-</th>
</tr>
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<tbody>
<tr>
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<td></td>
<td></td>
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<tr>
<td>Dox</td>
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B
Body weight (% of Initial)

<table>
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<tr>
<th>Days of treatment</th>
<th>WT</th>
<th>RGS6^-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 1 2 3 4 5</td>
<td></td>
<td></td>
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<tr>
<td>Saline</td>
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<tr>
<td>Dox</td>
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C
Percent survival

<table>
<thead>
<tr>
<th>Time in Days after Dox treatment</th>
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<tbody>
<tr>
<td>0</td>
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<tr>
<td>WT mice</td>
</tr>
<tr>
<td>Dox</td>
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<tr>
<td>Saline</td>
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<tr>
<td>RGS6^-/- mice</td>
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<tr>
<td>Dox</td>
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<td>Saline</td>
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Figure 3

A

<table>
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<tr>
<th>Dox (h)</th>
<th>WT</th>
<th>RGS6−/−</th>
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<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
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<tr>
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<tr>
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<td>12</td>
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- RGS6L
- Total p53
- p53(S15)
- H2AX (S139)
- Total ATM
- p-ATM(S1981)
- Mdm2
- Bax
- Bcl-2
- PARP
- Cleaved PARP
- Pro-Caspase-3
- Cleaved Caspase-3
- Actin

B

Levels of proteins (normalized to actin)

- WT
- RGS6−/−
- Dox 0h
- Dox 4h
- Dox 8h
- Dox 12h

- RGS6L
- Total p53
- p53(S15)

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