G-protein Inactivator RGS6 Mediates Myocardial Cell Apoptosis and Cardiomyopathy Caused By Doxorubicin

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Abstract

Clinical use of the widely used chemotherapeutic agent doxorubicin is limited by life-threatening cardiotoxicity. The mechanisms underlying doxorubicin-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 doxorubicin-treated cells. Thus, we hypothesized that RGS6, the expression of which is enriched in cardiac tissue, might also be responsible for the pathologic effects of doxorubicin treatment in heart. In this study, we show that RGS6 expression is induced strongly by doxorubicin in the ventricles of mice and isolated ventricular myocytes via a posttranscriptional mechanism. While doxorubicin-treated wild-type (WT) mice manifested severe left ventricular dysfunction, loss of heart and body mass, along with decreased survival 5 days after doxorubicin administration, mice lacking RGS6 were completely protected against these pathogenic responses. Activation of ATM/p53 apoptosis signaling by doxorubicin in ventricles of WT mice was also absent in their RGS6−/− counterparts. Doxorubicin-induced ROS generation was dramatically impaired in both the ventricles and ventricular myocytes isolated from RGS6−/− mice, and the apoptotic response to doxorubicin in ventricular myocytes required RGS6-dependent ROS production. These results identify RGS6 as an essential mediator of the pathogenic responses to doxorubicin in heart, and they argue that RGS6 inhibition offers a rational means to circumvent doxorubicin cardiotoxicity in human patients with cancer. Cancer Res; 73(6); 1662–7. ©2012 AACR.

Introduction

Doxorubicin, an anthracycline chemotherapeutic, is among the most effective and widely used drugs for treatment of human cancers (1, 2). Unfortunately, doxorubicin induces life-threatening cardiotoxicity including cardiomyopathy and heart failure (1, 2). Patients receiving cumulative doses of doxorubicin 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-year mortality rate of initial survivors from cardiac death was 15 times higher than expected (4, 5). Irreversible apoptotic death of ventricular myocytes is a hallmark of pathologic 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 doxorubicin-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 doxorubicin (1). Indeed, genetic disruption or inhibition of p53 protects against doxorubicin-induced myocardial cell apoptosis and contractile dysfunction showing a crucial role for p53 in the myopathic response to doxorubicin (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 doxorubicin-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 doxorubicin of the ATM/p53 apoptosis pathway in mouse embryonic fibroblasts (MEF) 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 showed (14). Given our finding that RGS6 is abundant in heart, we hypothesized that RGS6 might mediate doxorubicin-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<sup>−/−</sup> mice as described previously (14). Experimental animals were age-matched 3- to 6-month-old mice weighing approximately 25 to 35 grams and were backcrossed onto a C57BL6 background for 5 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 carried out 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 doxorubicin (20 mg/kg body weight) was administered to WT and RGS6<sup>−/−</sup> mice by a single i.p. injection. Body weight and survival of mice were recorded from day 0 (before saline or doxorubicin administration) to day 5. This experiment was repeated several times; and each treatment group contained at least 5 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, an SPR-1000 MikroTip mouse pressure catheter (Millar Instruments) 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). 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 minutes to clean the tibia of tissue. Lengths of water-rinsed and air-dried left tibias were measured using a dial caliper (Bel-Art Scienceware) and used as denominator to normalize heart weight.

Staining for superoxide in hearts and for total ROS in ventricular myocytes

Dihydroethidium staining was conducted in heart frozen sections derived from paraformaldehyde (4% fresh)-perfused WT and RGS6<sup>−/−</sup> mice treated with saline or doxorubicin (10 mg/kg). Intracellular ROS in WT and RGS6<sup>−/−</sup> ventricular myocytes treated with saline or doxorubicin was visualized using the cell-permeable oxidation-sensitive probe, CM-H<sub>2</sub>DCFDA [5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate]. All images were captured using a Zeiss fluorescence microscope.

Results

Loss of RGS6 prevents doxorubicin-induced heart failure

To determine the role of RGS6 in doxorubicin-induced cardiomyopathy, WT and RGS6<sup>−/−</sup> mice were treated with saline or doxorubicin followed by hemodynamic measurements of cardiac function 5 days later. This protocol is well established for inducing myopathic changes in hearts of mice that include left ventricular dysfunction (10, 15, 16). Doxorubicin-treated WT mice exhibited the expected severe left ventricular dysfunction compared with saline-treated control mice, manifested as lowered values of left ventricle end systolic pressure (LVESP), left ventricular development pressure (LVDP), and +dp/dt<sub>max</sub> (Fig. 1A-D). Remarkably,
RGS6<sup>−/−</sup> mice were completely protected from doxorubicin-induced ventricular dysfunction (Fig. 1A–D). Furthermore, RGS6<sup>−/−</sup> mice were completely spared from doxorubicin-induced loss of heart and body mass and death (Fig. 2), whereas doxorubicin-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 doxorubicin-induced cardiomyopathy and heart failure.

**RGS6 mediates doxorubicin-induced activation of p53 and apoptosis in heart**

Given the crucial role of p53 in doxorubicin-induced myocardial apoptosis and our recent finding that RGS6 was required for activation of ATM, p53, and apoptosis in doxorubicin-treated MEFs and MCF-7 breast cancer cells (12), we next investigated the role of RGS6 in apoptotic signaling in hearts from doxorubicin-treated mice. Doxorubicin transiently induces p53, which leads to subsequent apoptosis in heart (10). Treatment of WT mice with doxorubicin 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 (Supplementary Fig. S1). Similarly, doxorubicin induced robust increases in the levels of total and phosphorylated p53 [p-p53(S15), the ATM phosphorylation site] in both tissues that did not occur in hearts of RGS6<sup>−/−</sup> mice (Fig. 3; Supplementary Fig. S1). Induction of RGS6 by doxorubicin preceded phosphorylation...
and upregulation of p53, despite the presence of basal levels of RGS6 (Fig. 3; Supplementary Fig. S1).

We then investigated how loss of RGS6 impacted the ATM/p53-mediated apoptotic DNA damage signaling pathway in ventricles from doxorubicin-treated mice. Doxorubicin 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−/− ventricles (Fig. 3A; Supplementary Fig. S2). Because phosphorylation of p53 and Mdm2 inhibits ubiquitination and subsequent degradation of p53 by Mdm2 (17, 18), our results suggest that doxorubicin promotes p53 upregulation in ventricles by RGS6-dependent ATM activation. Doxorubicin 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 (Fig. 3A; Supplementary Fig. S2). These findings show that RGS6 is an essential mediator of myocardial apoptosis underlying the pathogenic actions of doxorubicin in the ventricle. This extends our preliminary observation that doxorubicin-induced increases in terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)-positive cardiomyocytes in ventricles of WT mice were reduced in RGS6−/− mice (12). Thus, loss of RGS6 protects mice against doxorubicin-induced ventricular apoptosis and myopathy.

**Doxorubicin acts directly on ventricular myocytes to induce RGS6 and activate p53**

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

Given the rapid induction of RGS6 by doxorubicin in ventricles and isolated ventricular myocytes (Fig. 3A; Supplementary Fig. S3A), we hypothesized RGS6 upregulation might occur posttranscriptionally. To test this hypothesis, we first evaluated RGS6 mRNA levels in doxorubicin-treated ventricles of WT mice, using quantitative reverse-transcription PCR as we described previously (14). RGS6 mRNA was not induced significantly in ventricles during the first 12 hours of doxorubicin treatment of mice (Supplementary Fig. S3B). Further, blocking de novo protein synthesis with cycloheximide markedly reduced upregulation of RGS6 and p-p33(S15) in ventricular myocytes (Supplementary Fig. S3C), showing that doxorubicin induces RGS6, at least in part, by mechanisms requiring de novo protein synthesis.

RGS6 mediates doxorubicin-induced apoptosis in ventricular myocytes via a ROS-dependent mechanism

Oxidative stress, defined as an excess of ROS compared with antioxidant defenses, is increased in heart failure and in hearts of doxorubicin-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 MEFs and MCF-7 breast cancer cells (12), likely via oxidative activation of ATM (Supplementary Fig. S4). Thus, we investigated whether RGS6 fulfilled a similar role in the ventricle. Loss of RGS6 greatly impaired doxorubicin-induced superoxide and total ROS generation in ventricles of mice and cultured ventricular myocytes, respectively (Fig. 4A and B), showing a key role for RGS6 in doxorubicin-induced ROS generation. Further, doxorubicin induced activation of caspase-3 and apoptosis in cultured ventricular myocytes by an RGS6-dependent mechanism that required ROS, as these responses were blocked by brief treatment of ventricular myocytes with ROS scavengers including N-acetyl cysteine (NAC) and active PEGylated forms of superoxide dismutase (SOD) or catalase (Fig. 4C and D). Thus, RGS6-dependent ROS generation is essential for doxorubicin-induced ventricular myocytes apoptosis.

Discussion

This study reveals a novel and essential role for RGS6 in mediating myopathic responses to doxorubicin in heart. Mice lacking RGS6 were completely protected against doxorubicin-induced heart failure and loss of heart mass as well as doxorubicin-induced mortality. Moreover, RGS6 was required for the ability of doxorubicin to induce apoptosis of ventricular myocardial cells, the hallmark pathologic response to heart injury and stress that underlies doxorubicin-induced heart failure. The finding that RGS6 was required for doxorubicin-induced apoptosis both in ventricles in vivo and in isolated ventricular myocytes 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 doxorubicin induces RGS6 expression in heart by a posttranscriptional mechanism that is important for its ability to promote activation of p53, a key modulator of doxorubicin-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 doxorubicin-induced apoptosis and heart damage (1). Our results show that RGS6 is required for doxorubicin-induced ROS generation and p53 activation both in ventricles in vivo and in isolated ventricular myocytes and that RGS6-dependent apoptosis in ventricular myocytes is ROS-dependent. Recently, we discovered that RGS6 promotes mitochondrial dependent apoptosis via ROS (11) and mediates doxorubicin-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 an 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 and colleagues (20). Our data support a model in which RGS6 upregulation by doxorubicin in heart induces ROS generation, which in turn promotes ATM/p53 apoptosis signaling via oxidative activation of ATM (Supplementary 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 doxorubicin-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. Because of the G-protein–independent nature of the pro-apoptotic actions of RGS6 (11, 12), it may represent a more viable target for drugs that would ameliorate doxorubicin-induced cardiomyopathy. Indeed, inhibition of RGS6 would be expected to protect the heart against doxorubicin-induced cell death and cardiomyopathy while simultaneously enhancing parasympathetic tone (14) itself believed to be cardioprotective. Induction of RGS6 by doxorubicin appears essential for its ability to mediate p53 activation and occurs by mechanisms requiring de novo protein synthesis, providing potential targets to prevent doxorubicin-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 doxorubicin-induced cardiomyopathy and identifies RGS6 as a possible therapeutic target for cardioprotective adjuncts to doxorubicin chemotherapy, which despite its
life-threatening cardiotoxic actions remains one of the most effective and widely used drugs in cancer treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Grant Support
This work was supported by AHA-SDG 11SDG7580008 (J. Yang); NIH grants CA161882 (R.A. Fisher); and HL079031, HL62494, HL70250, and HL113001 (M.E. Anderson).

Received September 5, 2012; revised November 27, 2012; accepted December 17, 2012; published OnlineFirst January 21, 2013.

References

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Stewart
Study supervision: R.A. Fisher

Acknowledgments
The authors thank Dr. Chantal Allamargot of the University of Iowa Central Microscopy Research Facility for assistance with histology/microscopy of hearts and Dr. John Koland for his helpful suggestions.

www.aacrjournals.org Cancer Res; 73(6) March 15, 2013 1667

Published OnlineFirst January 21, 2013; DOI: 10.1158/0008-5472.CAN-12-3453
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