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Regulator of G Protein Signaling 6 Protects the Heart from Ischemic Injury

Boyd R. Rorabaugh, Bandana Chakravarti, Nathaniel W. Mabe,[1] Sarah L. Seeley, Albert D. Bui,[2] Jianqi Yang, Stephanie W. Watts, Richard R. Neubig, and Rory A. Fisher

Department of Pharmaceutical and Biomedical Sciences, Raabe College of Pharmacy, Ohio Northern University, Ada, Ohio (B.R.R., N.W.M., S.L.S., A.D.B.); Department of Pharmacology, Carver College of Medicine, University of Iowa, Iowa City, Iowa (B.C., J.Y., R.A.F.); and Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan (S.W.W., R.R.N.)

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ABSTRACT

Gαi-coupled receptors play important roles in protecting the heart from ischemic injury. Regulator of G protein signaling (RGS) proteins suppress Gαi signaling by accelerating the GTPase activity of Gαi subunits. However, the roles of individual RGS proteins in modulating ischemic injury are unknown. In this study, we investigated the effect of RGS6 deletion on myocardial sensitivity to ischemic injury. Hearts from RGS6 knockout ($RGS6^{-/-}$) and RGS6 wild-type ($RGS6^{+/+}$) mice were subjected to 30 minutes of ischemia and 2 hours of reperfusion on a Langendorff heart apparatus. Infarcts in $RGS6^{-/-}$ hearts were significantly larger than infarcts in $RGS6^{+/+}$ hearts. $RGS6^{-/-}$ hearts also exhibited increased phosphorylation of β_2-adrenergic receptors and G protein–coupled receptor kinase 2 (GRK2). Mitochondrial GRK2 as well as caspase-3 cleavage were increased significantly in $RGS6^{-/-}$ hearts compared with $RGS6^{+/+}$ hearts after ischemia. Chronic propranolol treatment of mice prevented the observed increases in ischemic injury and the GRK2 phosphorylation observed in $RGS6^{-/-}$ hearts. Our findings suggest that loss of RGS6 predisposes the ventricle to prodeath signaling through a β_2AR-GRK2–dependent signaling mechanism, and they provide evidence for a protective role of RGS6 in the ischemic heart. Individuals expressing genetic polymorphisms that suppress the activity of RGS6 may be at increased risk of cardiac ischemic injury. Furthermore, the development of agents that increase RGS6 expression or activity might provide a novel strategy for the treatment of ischemic heart disease.

Introduction

Ischemic heart disease is the primary cause of death worldwide (Laslett et al., 2012). Current treatments for acute myocardial ischemia focus on reperfusion therapy using fibrinolytic drugs and coronary catheter-based interventions. Coronary reperfusion is essential for the survival of myocardial tissue. However, the identification of endogenous signaling pathways that are either detrimental or cardioprotective (Cohen and Downey, 2011) to the ischemic heart could lead to development of novel pharmacotherapies to supplement reperfusion procedures and reduce cardiac injury in patients with ischemic heart disease.

G protein–coupled receptors play important roles in protecting the heart from ischemic injury. Adenosine, acetylcholine, bradykinin, opioids, and many other endogenous hormones and neurotransmitters activate G protein–coupled receptor signaling pathways that have cardioprotective effects in the ischemic heart (Eisen et al., 2004). $G\alpha_i$ signaling reduces myocardial infarct size, suppresses ischemia-induced apoptosis, and, in some cases, enhances postischemic recovery of contractile function (Schultz et al., 1998; Waterson et al., 2011; Köhler et al., 2014). Regulator of G protein signaling (RGS) proteins suppress $G\alpha_i$ signaling by increasing the rate of hydrolysis of Gα-bound GTP. We previously reported that disruption of interactions between RGS proteins and $G\alpha_{i2}$ enhances $G\alpha_i$ signaling in cardiac myocytes and protects the heart from ischemic injury (Waterson et al., 2011; Parra et al., 2014). However, it is unknown which RGS proteins modulate this cardiac response to ischemia. RGS6 is one of only a few RGS proteins that have been identified at the protein level in the ventricle (Stewart et al., 2012; Yang et al., 2013). Thus, we examined the effect of RGS6 deletion on myocardial sensitivity to an ischemic insult. We anticipated that deletion of RGS6 would result in a cardioprotective phenotype similar to that observed in mice expressing RGS-insensitive $G\alpha_{i2}$. Surprisingly, we found that deletion of RGS6 worsens ischemic injury, indicating that RGS6 expression is cardioprotective. These data suggest that RGS6 may provide a novel therapeutic target for the treatment of ischemic heart disease.

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[1]Current affiliation: Department of Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, North Carolina.
[2]Current affiliation: Ohio University College of Osteopathic Medicine, Athens, Ohio.

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ABBREVIATIONS: AR, adrenergic receptor; EDP, end diastolic pressure; ERK, extracellular signal–regulated kinase; GRK2, G protein–coupled receptor kinase 2; RGS, regulator of G protein signaling.

Materials and Methods

Mice. The generation of RGS6 knockout ($RGS6^{-/-}$) mice was previously described (Yang et al., 2010). $RGS6^{+/-}$ mice were crossed to produce RGS6 wild-type ($RGS6^{+/+}$) and $RGS6^{-/-}$ animals, which were subsequently used as breeders to produce $RGS6^{+/+}$ and $RGS6^{-/-}$ mice used in experiments. Mice were backcrossed onto the C57BL6 background for 12 generations. All mice used in this study were 12–16 weeks of age. Mean body weights for male $RGS6^{+/+}$ and $RGS6^{-/-}$ animals were 28.4 ± 1.0 g and 28.2 ± 0.9 g, respectively. Female body weights were 22.3 ± 0.7 g for $RGS6^{+/+}$ mice and 23.0 ± 0.5 g for $RGS6^{-/-}$ mice. This investigation conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of Ohio Northern University.

Langendorff Isolated Heart Experiments. Mice were anesthetized with a single intraperitoneal injection containing sodium pentobarbital (100 mg/kg) and heparin (70 mg/kg). Hearts were quickly removed and mounted on a Langendorff isolated heart apparatus. Contractile function of the left ventricle was measured as previously described (Waterson et al., 2011). Hearts were equilibrated for 55 minutes prior to 30 minutes of ischemia and 2 hours of reperfusion. Hearts were paced at 500 beats per minute throughout the experiment except during ischemia, and pacing was resumed after 3 minutes of reperfusion. After 2 hours of reperfusion, hearts were stained with 1% triphenyltetrazolium chloride and infarct size was measured as previously described (Waterson et al., 2011).

Preparation of Mitochondrial Lysates. Mitochondrial lysates were prepared as previously described (Chen et al., 2013). Briefly, hearts were perfused on a Langendorff isolated heart apparatus and subjected to 30 minutes of ischemia and 30 minutes of reperfusion. Ventricular tissue was diced with a razor blade and then homogenized with 30 strokes of a Dounce homogenizer in mannitol-sucrose-HEPES buffer, which contained 210 nM mannitol, 70 mM sucrose, 5 mM HEPES (pH 7.5), and 1 mM EDTA. The homogenate was centrifuged at 4°C for 10 minutes at 600*g*. The supernatant was transferred to another tube and recentrifuged for 10 minutes at 600*g*. The supernatant was subsequently centrifuged again at 5500*g* for 20 minutes at 4°C. The resulting mitochondrial pellet was resuspended in 1 ml mannitol-sucrose-HEPES buffer without EDTA and centrifuged at 5500*g* for 20 minutes. The pellet was resuspended in 125 μl RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA Sigma phosphatase inhibitor cocktail 2, Sigma phosphatase cocktail 3, and Sigma protease inhibitor; Sigma-Aldrich, St. Louis, MO). A 10-μl aliquot of the lysate was used to measure protein concentration, and the remainder was used for Western blotting.

Chronic Treatment with Propranolol. Male mice were treated for 9 weeks with water alone or with propranolol (0.5 g/l) dissolved in their drinking water. This propranolol dose was chosen based on previous work demonstrating that 0.5 g/l in drinking water produces in vivo blockade of β-adrenergic receptors (ARs) in the mouse heart (Asai et al., 1999; Dash et al., 2001; Adlam et al., 2012).

Western Blots. Hearts were isolated and the atria were removed. Ventricular tissue was immediately frozen in liquid nitrogen and stored at −80°C. The tissue was homogenized in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0), containing protease (p8340) and phosphatase (number 3) inhibitor mixtures (Sigma-Aldrich), and was quantified and probed as we previously described (Stewart et al., 2015).

Statistical Analysis. Data are reported as the mean ± S.E.M. Male and female data were analyzed separately. Infarct sizes were compared by the *t* test. Preischemic and postischemic parameters of contractile function were analyzed by two-way analysis of variance with time (preischemic versus postischemic recovery as repeated measures) and genotype ($RGS6^{+/+}$ versus $RGS6^{-/-}$) as factors. Bonferroni post hoc analysis was used to identify significant differences between specific groups. Western blots to measure expression of G protein–coupled receptor kinase 2 (GRK2) and β2-AR phosphorylation were analyzed using the *t* test. GraphPad Prism software (GraphPad Inc., San Diego, CA) was used for all statistical analyses, and *P* values ≤ 0.05 were considered statistically significant.

Results

RGS6 Protects the Heart from Infarction. Hearts from $RGS6^{-/-}$ and $RGS6^{+/+}$ mice were exposed to 30 minutes of ischemia and 2 hours of reperfusion. Infarcts in both male and female $RSG6^{-/-}$ hearts were significantly larger than their respective wild-type controls (Fig. 1), indicating that deletion of RGS6 worsened myocardial ischemic injury. Deletion of RGS6 had no effect on preischemic contractile function of male or female hearts. Hearts completely stopped beating during the 30-minute ischemic period. Developed pressure, +dP/dT, –dP/dT, and coronary flow rate were significantly lower after 1 hour of reperfusion compared with their preischemic values in both sexes (Table 1). However, parameters of postischemic recovery of contractile function were unaffected by deletion of RGS6, with the exception of end diastolic pressure (EDP), which was significantly elevated in female $RGS6^{-/-}$ hearts compared with female $RGS6^{+/+}$ hearts.

EDP was monitored throughout the 30-minute ischemic episode. All hearts, regardless of genotype, exhibited a time-dependent increase in EDP during the 30-minute ischemic episode (Fig. 2), indicating that the myocardium had developed a state of contracture and was unable to relax. However, EDP was significantly greater in male $RGS6^{-/-}$ hearts compared with male $RGS6^{+/+}$ hearts. A similar effect was observed in female hearts (Fig. 2).

The data shown in Figs. 1 and 2 and Table 1 indicate that RGS6 expression produces the same cardioprotective effect in both male and female mice. Since there was no apparent difference between the sexes, subsequent experiments designed to identify the mechanism by which RGS6 protects the ischemic heart were performed only in males.

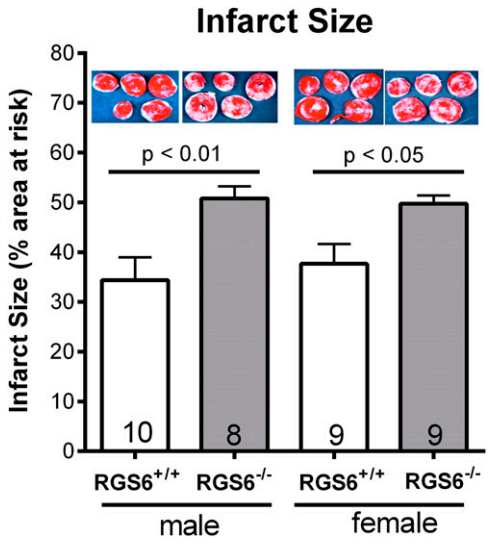

Fig. 1. Effect of RGS6 on infarct size after 30 minutes of ischemia. Hearts isolated from male and female $RGS6^{-/-}$ and $RGS6^{+/+}$ mice were subjected to 30 minutes of ischemia and 120 minutes of reperfusion. Infarct sizes were measured by triphenyltetrazolium chloride staining. Male and female hearts were analyzed separately by the *t* test. Data represent the mean ± S.E.M of 8–10 hearts.

TABLE 1
Parameters of preischemic and postischemic recovery of contractile function in RGS6$^{+/+}$ and RGS6$^{-/-}$ hearts
Data represent the mean ± S.E.M.

Parameter	No. of Rats	Developed Pressure	+dP/dT	−dP/dT	EDP	Flow Rate
		mmHg	*mmHg/s*	*mmHg/s*	*mmHg*	*ml/min per g*
Male[a]						
Preischemic						
RGS6$^{+/+}$	10	107 ± 3	4107 ± 181	−3328 ± 126	3 ± 1	30 ± 2
RGS6$^{-/-}$	8	104 ± 3	3872 ± 89	−3293 ± 72	4 ± 1	27 ± 1
Recovery						
RGS6$^{+/+}$	10	42 ± 5*	1670 ± 209*	−1289 ± 145*	22 ± 2*	19 ± 2*
RGS6$^{-/-}$	8	34 ± 3*	1246 ± 109*	−1089 ± 109*	24 ± 1*	15 ± 1*
Female[b]						
Preischemic						
RGS6$^{+/+}$	9	116 ± 4	4141 ± 218	−3370 ± 135	3 ± 0.7	27 ± 2
RGS6$^{-/-}$	9	121 ± 5	4118 ± 238	−3454 ± 142	2 ± 0.5	27 ± 3
Recovery						
RGS6$^{+/+}$	9	37 ± 3*	1397 ± 137*	−1137 ± 86*	8 ± 3	17 ± 2*
RGS6$^{-/-}$	9	34 ± 3*	1178 ± 125*	−1017 ± 98*	21 ± 5*,**	15 ± 2*

Preischemic contractile function was measured prior to the onset of ischemia. Postischemic recovery of contractile function was measured after 1 hour of reperfusion. Male and female data were analyzed separately by two-way analysis of variance with genotype (RGS6$^{+/+}$ versus RGS6$^{-/-}$) and time (preischemic versus postischemic recovery as a repeated measure) as factors. Bonferroni post hoc analysis was used to identify significant differences between specific groups.

[a]In male hearts, time (preischemic versus recovery) had significant effects on developed pressure [$F = 375_{(1, 16)}$, $P < 0.0001$], +dP/dT [$F = 272_{(1, 16)}$, $P < 0.0001$], −dP/dT [$F = 354_{(1, 16)}$, $P < 0.0001$], EDP [$F = 209_{(1, 16)}$, $P < 0.001$], and coronary flow rate [$F = 148_{(1, 16)}$, $P = 0.04$].

[b]In female hearts, time (preischemic versus postischemic recovery) had significant effects on developed pressure [$F = 585_{(1, 16)}$, $P < 0.0001$], +dP/dT [$F = 402_{(1, 16)}$, $P < 0.0001$], −dP/dT [$F = 469_{(1, 16)}$, $P < 0.0001$], EDP [$F = 17_{(1, 16)}$, $P < 0.001$], and coronary flow rate [$F = 5_{(1, 16)}$, $P = 0.04$].

*$P < 0.001$ (significant difference compared with preischemic value); **$P < 0.05$ (significant difference compared with the recovery of EDP in RGS6$^{+/+}$ hearts).

RGS6 Deletion Alters β_2-AR Signaling. Hearts derived from RGS6$^{-/-}$ mice exhibit enhanced bradycardia in response to carbachol, indicating that they have increased sensitivity to cholinergic stimulation (Posokhova et al., 2010, 2013; Yang et al., 2010). In addition, muscarinic receptor blockade produces a significantly larger increase in heart rate in RGS6$^{-/-}$ versus RGS6$^{+/+}$ mice (Posokhova et al., 2010), suggesting that RGS6$^{-/-}$ mice likely have an increase in sympathetic tone that compensates for their increased myocardial sensitivity to parasympathetic stimulation. Consistent with such a compensatory increase in sympathetic tone, we found no differences in basal heart rate (Yang et al., 2010) or mean blood pressure (unpublished data) in RGS6$^{-/-}$ mice despite their increased parasympathetic sensitivity. Analysis of ventricular tissue (which was flash frozen in liquid nitrogen without exposure to ischemia) demonstrated that β_2-AR phosphorylation was increased in RGS6$^{-/-}$ hearts (Fig. 3A). Ritodrine (a β_2-AR–selective agonist) induced a small but significant inotropic effect in RGS6$^{+/+}$ hearts (Fig. 3B). In contrast, no significant inotropic response was observed in RGS6$^{-/-}$ hearts at the ritodrine concentrations that were tested (Fig. 3B). Increased β_2-AR phosphorylation and the lack of a significant ritodrine-induced inotropic effect are consistent with β_2-AR desensitization (Hausdorff et al., 1990). The observation that isoproterenol-induced (Fig. 3C) and phenylephrine-induced (Fig. 3D) inotropic responses were not altered by RGS6 deletion indicates that RGS6 deletion specifically altered β_2-AR signaling and did not suppress signaling through β-ARs in general (β_1-AR, β_2-AR, and β_3-AR) and α_1-ARs, respectively. Furthermore, chronic treatment of mice with propranolol abolished the difference in infarct size between RGS6$^{+/+}$ and RGS6$^{-/-}$ hearts (Fig. 4), providing functional evidence for the involvement of β-AR signaling in promoting hypersensitivity to ischemic injury in RGS6$^{-/-}$ hearts.

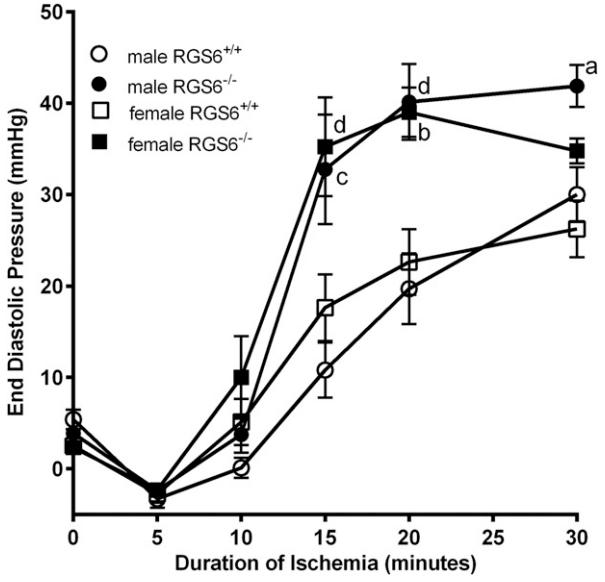

Fig. 2. Deletion of RGS6 increases the development of contracture during a 30-minute episode of ischemia. EDP was measured throughout the 30-minute ischemic period. Data from male and female hearts were analyzed separately by repeated-measures two-way analysis of variance with duration of ischemia and genotype as factors. Bonferroni post hoc analysis was used to identify differences between groups at specific time points. There were significant effects of the duration of ischemia [$F = 108_{(5, 80)}$, $P < 0.0001$], genotype [$F = 11_{(1, 16)}$, $P < 0.005$], and an interaction between the duration of ischemia and genotype [$F = 11_{(5, 80)}$, $P < 0.0001$] on EDP in male hearts. There were also significant effects of the duration of ischemia [$F = 79_{(5, 75)}$, $P < 0.0001$], genotype [$F = 8_{(1, 15)}$, $P < 0.05$], and an interaction between the duration of ischemia and genotype [$F = 5_{(5, 75)}$, $P < 0.001$] on EDP in female hearts. Data represent the mean ± S.E.M of 8–10 separate hearts. [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.005$; [d]$P < 0.001$.

RGS6 Deletion Increases Basal GRK2 Expression and Phosphorylation. GRK2 is an important regulator of β_2-AR signaling. Chronic stimulation of β-ARs increases the expression of GRK2 (Iaccarino et al., 1998) and enhances

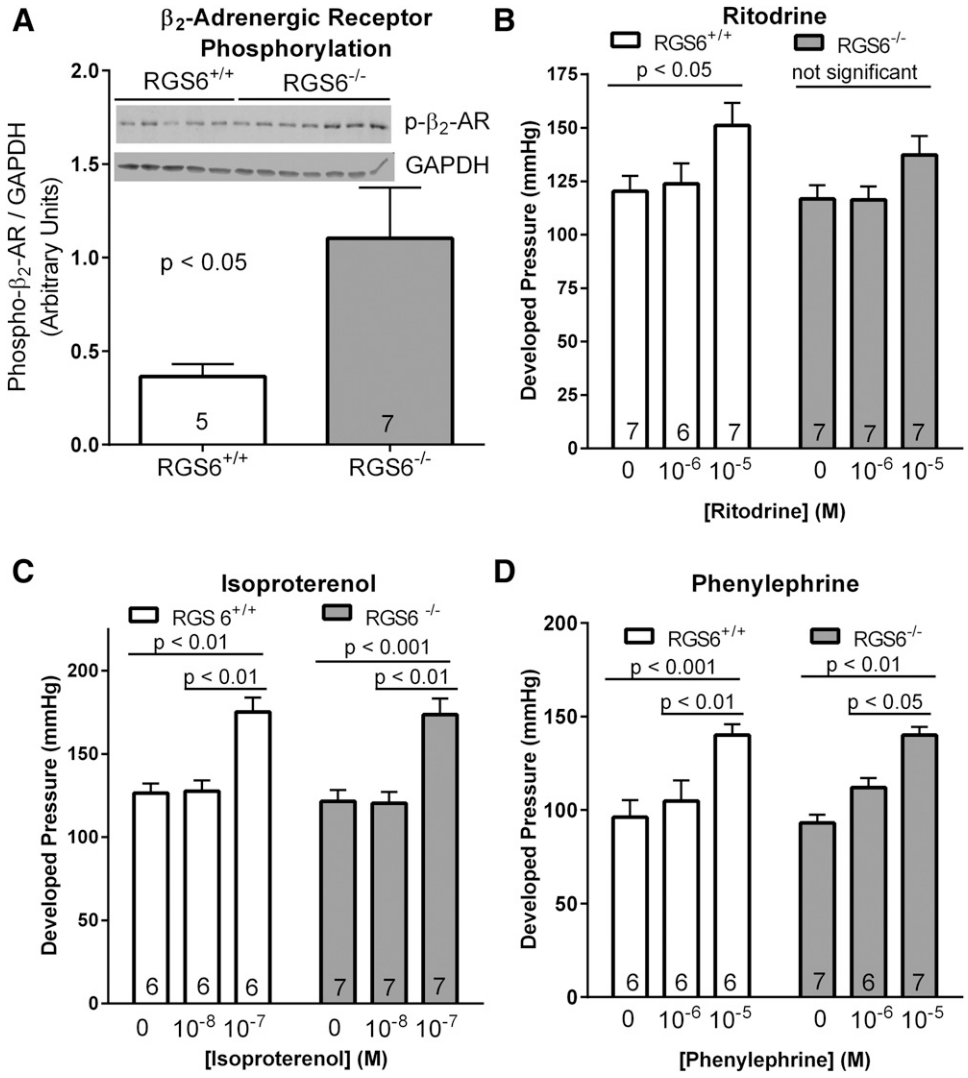

Fig. 3. RGS6 deletion increases β_2-AR phosphorylation and alters β_2-AR signaling. (A) Ventricular tissue from male mice was homogenized and blotted for phosphorylated (Ser^{355}/Ser^{356}) β2-AR (p-β_2-AR) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Blots were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). p-β2-AR band intensities were normalized to those of GAPDH, and data were analyzed using the *t* test. (B–D) Inotropic responses to ritodrine (B), isoproterenol (C), and phenylephrine (D) were measured on a Langendorff isolated heart apparatus in hearts that were not subjected to ischemia. Each agonist was infused by a syringe pump for 5 minutes through a side arm located immediately upstream of the heart. Hearts were continuously paced at 500 beats per minute, and contractile force of the left ventricle was measured by an intraventricular balloon. Data were analyzed by two-way analysis of variance and Bonferroni's post hoc analysis with genotype and drug concentration as factors. Data represent the mean ± S.E.M of five to seven hearts.

myocardial sensitivity to ischemic injury (Hu et al., 2006). GRK2-dependent phosphorylation of β_2-ARs leads to receptor desensitization (Hausdorff et al., 1990) and also causes a shift in β_2-AR signaling from Gαs coupling to Gαi coupling (Zhu et al., 2012). In addition to regulating β_2-AR signaling, GRK2 also modulates proapoptotic signaling pathways in the ischemic heart. Under conditions of ischemia, extracellular signal–regulated kinase (ERK)–dependent phosphorylation of GRK2 promotes GRK2 translocation to mitochondria where it enhances calcium-dependent opening of the mitochondrial permeability transition pore (Chen et al., 2013). This results in the activation of proapoptotic signaling proteins and triggers the death of cardiac myocytes (Chen et al., 2013). We found that deletion of RGS6 significantly increased GRK2 expression (Fig. 5A). GRK2 phosphorylation was also increased in $RGS6^{-/-}$ hearts (Fig. 5B). This effect was reversed by chronic treatment of mice with propranolol (Fig. 5C).

RGS6 Deletion Increases Mitochondrial GRK2 and Phoshpo-GRK2 as well as Cleavage of Caspase-3 after an Ischemic Insult. Previous work has demonstrated that myocardial ischemia induces ERK-dependent translocation of GRK2 to the mitochondria where it activates caspase-3, and that this leads to myocyte apoptosis (Chen et al., 2013). Thus, we examined the potential role of this signaling pathway in promoting ischemic injury in $RGS6^{-/-}$ hearts. Mitochondrial GRK2 phosphorylation (Fig. 6A) and GRK2 expression (Fig. 6B) were similar in $RGS6^{+/+}$ and $RGS6^{-/-}$ hearts in the absence of ischemia. However, an ischemic insult significantly increased both phospho-GRK2 (Fig. 6A) and total GRK2 (Fig. 6B) in $RGS6^{-/-}$ heart mitochondria compared with $RGS6^{+/+}$ heart mitochondria. The integrity of the mitochondrial fraction was verified in Fig. 6D. We also found that cleaved caspase-3 was significantly increased in $RGS6^{-/-}$ hearts (compared with $RGS6^{+/+}$ hearts) after an ischemic insult (Fig. 6C). These data provide evidence that RGS6 expression protects the ischemic heart by suppressing the GRK2-dependent activation of proapoptotic signaling proteins in the mitochondria.

Discussion

The primary finding of this study is that expression of RGS6 protects the heart from ischemic injury. In light of the expression of RGS6 in the mouse ventricle (Yang et al., 2013), the ability of RGS6 to suppress $G\alpha_i$ signaling (Hooks et al., 2003), and previous work demonstrating that disruption

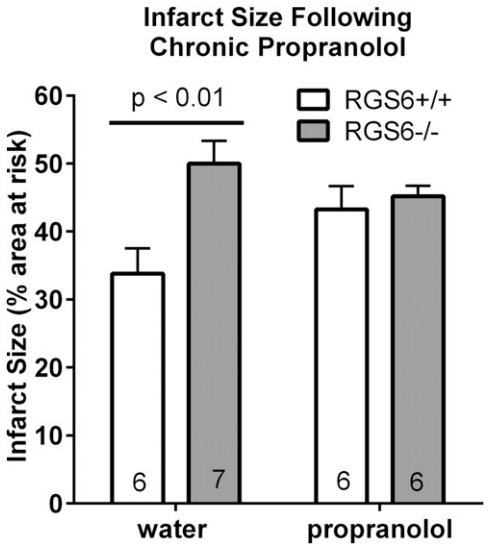

Fig. 4. Infarct size after chronic treatment with propranolol. Male RGS6$^{+/+}$ and RGS6$^{-/-}$ mice were treated for 9 weeks with water alone or with propranolol (0.5 g/l) dissolved in their drinking water. Hearts were isolated and subjected to 30 minutes of ischemia and 2 hours of reperfusion prior to the measurement of infarct sizes by triphenyltetrazolium chloride staining. Data were analyzed by two-way analysis of variance with drug treatment (propranolol versus water) and genotype (RGS6$^{+/+}$ versus RGS6$^{-/-}$) as factors. There was a significant effect of genotype [$F = 8.3_{(1,\ 21)}$, $P = 0.009$]. Tukey post hoc analysis indicated a significant difference ($P < 0.01$) between RGS6$^{-/-}$ and RGS6$^{+/+}$ hearts given water alone. There was also a significant interaction between genotype and propranolol [$F = 5.1_{(1,\ 21)}$, $P = 0.035$], indicating that propranolol did not have the same effect on both genotypes. No effects of propranolol were observed on either heart size or mouse behavior.

of interactions between Gα_{i2} and endogenous RGS proteins enhances Gα_{i2} signaling and protects the heart from ischemic injury (Waterson et al., 2011; Parra et al., 2014), we anticipated that genetic deletion of RGS6 would protect the heart from ischemia. Thus, the finding that RGS6 deletion potentiates ischemic injury was unexpected. mRNA transcripts encoding at least 16 different RGS proteins have been identified in cardiac myocytes and fibroblasts (Zhang and Mende, 2011), and several have been found in the heart at the protein level (Jean-Baptiste et al., 2005; Chakir et al., 2011; Stewart et al., 2012; Miao et al., 2016). However, to our knowledge, this is the first study to provide evidence that a specific RGS protein (RGS6) plays a role in the myocardial response to an ischemic insult.

The observation that GRK2 expression (Fig. 5A) and GRK2 phosphorylation (Fig. 5B) are increased in RGS6$^{-/-}$ hearts is consistent with increased infarct sizes (Fig. 1) in RGS6$^{-/-}$ hearts and with previous work demonstrating that increased GRK2 signaling worsens ischemic injury (Brinks et al., 2010). Increased GRK2 expression and phosphorylation are also consistent with our findings that β2-AR phosphorylation was increased (Fig. 3A) and that the inotropic effect of β2-AR stimulation was diminished in RGS6$^{-/-}$ hearts (Fig. 3B), suggesting that β2-ARs were modestly desensitized. GRK2-dependent phosphorylation of β_2-ARs causes these receptors to shift from coupling to Gαs to Gαi (Zhu et al., 2012). Gα_{i2} and Gα_{i3} are both expressed in the heart and are activated by cardiac β_2-ARs (Xiao et al., 1999). Importantly, these G$_{\alpha i}$ isoforms have divergent effects on the ischemic heart. Gα_{i2} signaling is cardioprotective (Waterson et al.,

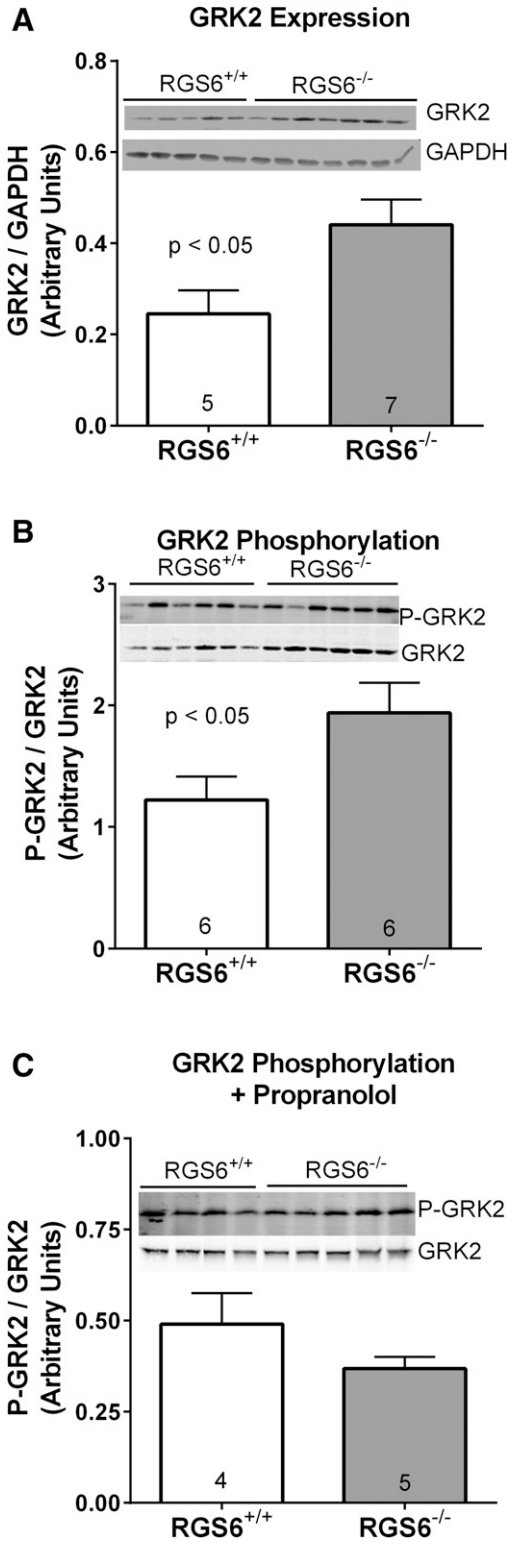

Fig. 5. Deletion of RGS6 increases basal expression and phosphorylation of GRK2. Hearts from male mice were isolated and immediately flash frozen. (A) Ventricular tissue was homogenized and blotted for GRK2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (B and C) The effect of chronic β-AR blockade on GRK2 phosphorylation was investigated by giving mice drinking water alone (B) or water containing propranolol (0.5 g/l) (C) for 9 weeks. Hearts were isolated and immediately flash frozen. Ventricular tissue was homogenized and blotted for phospho-GRK2 and total GRK2. Blots were quantified by densitometry using ImageJ software, and data were compared using the *t* test. Data represent the mean ± S.E.M of four to seven hearts.

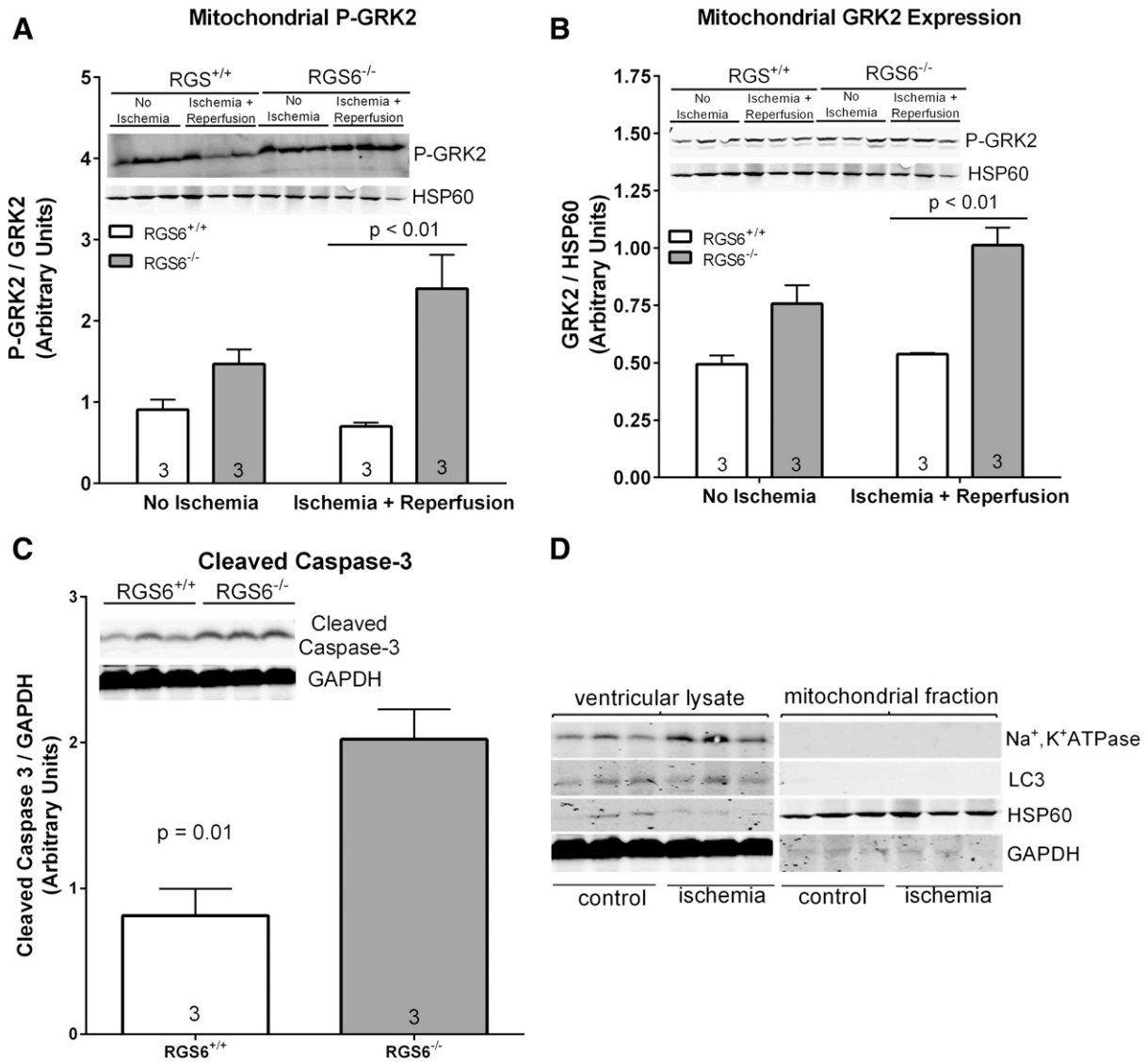

Fig. 6. Mitochondrial GRK2 phosphorylation and cleavage of caspase-3 are enhanced in RGS6$^{-/-}$ hearts after an ischemic insult. (A and B) Male RGS6$^{+/+}$ and RGS6$^{-/-}$ hearts were mounted on a Langendorff isolated heart system and either subjected to 30 minutes of ischemia and 30 minutes of reperfusion (ischemia + reperfusion) or continuously perfused for the same amount of time (no ischemia) prior to mitochondrial isolation and blotting for mitochondrial GRK2, phospho(Ser^{570})-GRK2, and HSP60. In (A), two-way analysis of variance (factors = genotype and ischemia versus no ischemia) indicated a significant effect [$F = 23_{(1, 8)}$, $P < 0.01$] of genotype and an interaction between genotype and ischemia [$F = 6_{(1, 8)}$, $P = 0.05$] on mitochondrial phospho-GRK2. In (B), there was also a significant effect of genotype [$F = 39_{(1, 8)}$, $P < 0.0005$] on mitochondrial GRK2 expression and a significant effect of ischemia [$F = 6_{(1, 8)}$, $P < 0.05$] on GRK2 expression. (C) Cleaved caspase-3 was measured in ventricular homogenates after 30 minutes of ischemia and 2 hours of reperfusion. Blots were quantified by densitometry using ImageJ software. Caspase-3 cleavage was significantly increased ($P < 0.01$, analyzed by the t test) in RGS6$^{-/-}$ hearts compared with RGS6$^{+/+}$ hearts after an ischemic insult. Data in (A) to (C) represent the mean ± S.E.M of three hearts. (D) The purity of the mitochondrial preparation was validated by blotting mitochondrial lysates and ventricular homogenates from RGS6$^{+/+}$ hearts for sodium-potassium ATPase (cell membrane marker), LC3 (lysosomal marker), heat shock protein 60 (mitochondrial marker), and glyceraldehyde-3-phosphate dehydrogenase (cytosolic marker).

2011; Kohler et al., 2014; Parra et al., 2014), whereas Gα_{i3} signaling worsens ischemic injury (Kohler et al., 2014). In vitro assays indicate that RGS6 catalyzes the hydrolysis of Gα_{i3}-bound GTP at nearly twice (1.9-fold) the rate that it catalyzes the hydrolysis of Gα_{i2}-bound GTP (Hooks et al., 2003). If this selectivity toward Gα_{i3} also occurs in the intact heart, then RGS6 deletion would be expected to enhance signaling through Gα_{i3} to a greater extent than it enhances cardioprotective signaling through Gα_{i2}. Thus, it is possible that RGS6 protects the heart from ischemia by suppressing β2-AR–induced Gα_{i3} signaling. Alternatively, the increased myocardial sensitivity to ischemic injury in RGS6$^{-/-}$ hearts might result from increased GRK2 activity in the mitochondria. Previous work has demonstrated that under conditions of ischemia or oxidative stress, ERK-dependent phosphorylation of GRK2 causes GRK2 to be translocated to the mitochondria where it promotes opening of the mitochondrial permeability transition pore, leading to apoptosis and myocyte death (Chen et al., 2013). Our findings that RGS6$^{-/-}$ hearts exhibit overexpression of GRK2, increased basal phosphorylation of GRK2, and increased sensitivity to ischemic injury suggest that RGS6 expression protects the heart from ischemic injury by suppressing signaling through this pathway. The observations that RGS6 deletion increases the

presence of phospho-GRK2 and total GRK2 expression in mitochondrial lysates and increases caspase-3 cleavage are also consistent with this mechanism.

It is well established that myocardial injury can occur as a result of both ischemia and reperfusion (Fröhlich et al., 2013; Jivraj et al., 2015). Both male and female RGS6$^{-/-}$ hearts demonstrated significantly elevated EDP relative to male and female RGS6$^{+/+}$ hearts during the 30-minute ischemic insult (Fig. 2). This state of contracture progressed more rapidly and to a greater extent in RGS6$^{-/-}$ hearts compared with hearts from RGS6$^{+/+}$ mice. Importantly, this difference between RGS6$^{-/-}$ hearts and RGS6$^{+/+}$ hearts was evident prior to the onset of reperfusion. The fact that RGS6$^{-/-}$ hearts developed significantly greater contracture than RGS6$^{+/+}$ hearts during ischemia (prior to reperfusion) suggests that the increased myocardial injury observed in RGS6$^{-/-}$ hearts was likely the result of increased sensitivity to ischemia-induced injury rather than increased sensitivity to reperfusion-induced injury.

RGS6$^{-/-}$ hearts are hypersensitive to cholinergic stimulation (Posokhova et al., 2010, 2013; Yang et al., 2010), yet they have normal basal heart rates (Yang et al., 2010). This suggests that RGS6$^{-/-}$ mice compensate for increased sensitivity to parasympathetic stimulation by increasing the activity of sympathetic neurons. The observations that β2-AR phosphorylation was increased and that β2-AR–induced responses were functionally desensitized in RGS6$^{-/-}$ hearts are consistent with chronic β2-AR signaling. The finding that increased basal GRK2 phosphorylation and myocardial hypersensitivity to ischemic injury were reversed by chronic propranolol treatment provides evidence that RGS6$^{-/-}$ hearts are hypersensitive to ischemic injury as a result of excessive β-AR signaling.

Our study used the Langendorff isolated heart model to investigate the effect of RGS6 deletion on the ischemic heart. However, our assessment of signaling pathways involved in RGS6-mediated cardioprotection was performed primarily in hearts that were isolated and immediately flash frozen in liquid nitrogen. Thus, our observations of increased β_2-AR phosphorylation (Fig. 3A), increased GRK2 expression (Fig. 5A), increased GRK2 phosphorylation (Fig. 5B), and increased caspase-3 cleavage (Fig. 6C) in RGS6$^{-/-}$ hearts reflect their status under in vivo conditions. On the basis of these findings, we speculate that RGS6$^{-/-}$ hearts would have also exhibited increased sensitivity to ischemic injury if myocardial ischemia had been induced in vivo.

Cardioprotective stimuli that decrease infarct size do not always enhance postischemic recovery of contractile function (Cohen et al., 1999; Sumeray et al., 2000; Schulz et al., 2001; Perrin et al., 2003). Some investigators have attributed this observation to "myocardial stunning," which is characterized by contractile dysfunction without infarction. Myocardial stunning may result from the generation of reactive oxygen species, which damage contractile proteins (Bolli and Marban, 1999), or from calcium overloading in cardiac myocytes (Kumar et al., 2009), which induces degradation of contractile proteins by calcium-dependent proteases (Bolli and Marban, 1999). Importantly, these processes do not destroy cardiac myocytes, and the contractile function can recover with prolonged reperfusion time as new protein synthesis enables the damage to be repaired. In addition, postischemic coronary flow rates are typically much lower than preischemic coronary flow rates. This decrease in coronary flow (and subsequent decrease in the delivery of oxygen and nutrients) can suppress cardiac contractile function and mask the true contractile capacity of the myocytes. Importantly, our data indicate that RGS6 expression protects the heart from infarction but does not prevent postischemic contractile dysfunction within the 1-hour reperfusion period that was measured. One limitation of the Langendorff isolated heart model is that cardiac contractile function cannot be monitored over the long term. Thus, the extent to which RGS6 deletion would affect contractile function days or weeks after ischemia remains unknown.

RGS6$^{-/-}$ mice are resistant to doxorubicin-induced production of reactive oxygen species and apoptosis, suggesting that RGS6 might mediate the cardiotoxic effect of doxorubicin that is commonly observed in patients with cancer who are treated with this drug (Yang et al., 2013). It should be noted that the proapoptotic role of RGS6 after doxorubicin treatment of cardiac myocytes, mouse embryonic fibroblasts, and MCF-7 breast cancer cells occurs through a mechanism that is independent of its interaction with G proteins (Huang et al., 2011; Yang et al., 2013). Thus, the involvement of RGS6 in both G protein–dependent and G protein–independent signaling mechanisms could account for its ability to either decrease or increase myocardial injury in the contexts of ischemia or doxorubicin treatment, respectively.

Single nucleotide polymorphisms have been identified in the human gene encoding RGS6 (Sibbel et al., 2011). Posokhova et al. (2013) identified a RGS6 single nucleotide polymorphism (Genbank accession number NM_004296.5) in which cytosine 37 is deleted, resulting in a frame shift and a premature stop codon. This mutation results in a loss of RGS6 function and is associated with increased heart rate variability in humans who are heterozygous for this polymorphism. The prevalence of this polymorphism is unknown. However, our data suggest that carriers of this polymorphism or other polymorphisms that result in a loss of RGS6 function may be at increased risk of cardiac ischemic injury.

RGS proteins represent a potential class of new therapeutic drug targets (Sjögren and Neubig, 2010). Our data demonstrate that a deficiency of RGS6 worsens ischemic injury, but it is unclear whether overexpression of RGS6 to quantities above endogenous levels would have a cardioprotective effect. Prior work has demonstrated that RGS2 and RGS6 expression can be enhanced in the ventricles by treatment with cardiotonic steroids and doxorubicin, respectively (Sjögren et al., 2012; Yang et al., 2013). Despite the cardiotoxic effects of these agents, these studies establish the proof of concept that pharmacological intervention can be used to increase the expression of RGS protein in the ventricles. The development of agents that increase RGS6 expression could potentially provide a new approach to treating ischemic heart disease.

Authorship Contributions

Participated in research design: Rorabaugh, Fisher.

Conducted experiments: Rorabaugh, Chakravarti, Mabe, Seeley, Bui, Yang, Watts.

Performed data analysis: Rorabaugh, Chakravarti.

Wrote or contributed to the writing of the manuscript: Rorabaugh, Watts, Neubig, Fisher.

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Address correspondence to: Boyd R. Rorabaugh, Department of Pharmaceutical and Biomedical Sciences, Raabe College of Pharmacy, Ohio Northern University, 525 South Main Street, Ada, OH 45810. E-mail: b-rorabaugh@onu.edu