Loss-of-Function Mutations in Human Regulator of G Protein Signaling RGS2 Differentially Regulate Pharmacological Reactivity of Resistance Vasculature

Hoa T.N. Phan,1 William F. Jackson, Vincent S. Shaw, Stephanie W. Watts, and Richard R. Neubig

Department of Pharmacology and Toxicology, Michigan State University, East Lansing, Michigan (H.T.N.P., W.F.J., V.S.S., S.W.W., R.R.N.)

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ABSTRACT

Regulator of G protein signaling 2 (RGS2) plays a role in reducing vascular contraction and promoting relaxation due to its GTPase accelerating protein activity toward Gαq. Previously, we identified four human loss-of-function (LOF) mutations in RGS2 (Q2L, D40Y, R44H, and R188H). This study aimed to investigate whether those RGS2 LOF mutations disrupt the ability of RGS2 to regulate vascular reactivity. Isolated mesenteric arteries (MAs) from RGS2−/− mice showed an elevated contractile response to 5 nM angiotensin II and a loss of acetylcholine (ACh)–mediated vasodilation. Reintroduction of a wild-type (WT) RGS2-GFP plasmid into RGS2−/− MAs suppressed the vasoconstrictor response to angiotensin II constriction response compared with RGS2 WT. In contrast, ACh-mediated vasodilation was restored by expression of RGS2 WT, D40Y, and R44H but not by RGS2 Q2L or R188H. Phosphorylation of RGS2 D40Y and R44H by protein kinase G (PKG) may explain their maintained function to support vasorelaxation. All mutants showed the expected LOF effects in suppressing vasoconstriction. Surprisingly, the D40Y and R44H mutants supported less relaxation to ACh, whereas relaxation mediated by the D40Y and R44H mutant proteins was equal to that with WT protein. Phosphorylation of RGS2 by PKG appears to contribute to this vasorelaxation. These results provide insights for precision medicine targeting the rare individuals carrying these RGS2 mutations.

SIGNIFICANCE STATEMENT

Regulator of G protein signaling 2 (RGS2) has been implicated in the control of blood pressure; rare mutations in the RGS2 gene have been identified in large-scale human gene sequencing studies. Four human mutations in RGS2 that cause loss of function (LOF) in cell-based assays were examined in isolated mouse arteries for effects on both vasoconstriction and vasodilation. All mutants showed the expected LOF effects in suppressing vasoconstriction. Surprisingly, the D40Y and R44H mutant RGS2 showed normal control of vasodilation. We propose that this is due to rescue of the mislocalization phenotype of these two mutants by nitric oxide–mediated/protein kinase G–dependent phosphorylation. These mechanisms may guide drug discovery or drug repurposing efforts for hypertension by enhancing RGS2 function.

Introduction

G protein–mediated signaling is critical for the regulation of cardiovascular function, including heart rate, cell growth, and vascular tone. Blockers of G protein–coupled receptor signaling (angiotensin receptor and β and α adrenergic receptor blockers) are among the best therapies for hypertension and heart failure. Regulator of G protein signaling (RGS) proteins accelerate the rate of GTP hydrolysis by active Gα and Gαq subunits, thereby reducing the amplitude and duration of G protein–coupled receptor/G protein signaling. Among more than 20 RGS proteins, RGS2 is highly expressed in vascular smooth muscle cells (VSMCs), where it is regulated by nitric oxide (NO) and controls vascular constriction and relaxation (Tang et al., 2003; Sun et al., 2005).

Biochemically, RGS2 negatively regulates Gαq, which transduces signals from a variety of vasoconstrictors. Consistent with a role in vascular control, RGS2 knockout mice are hypertensive (Heximer et al., 2003) and hypotensive human patients had reduced RGS2 mRNA in peripheral
blood mononuclear cells compared with controls (Semplicini et al., 2006). Furthermore, hypertensive patients were more likely to have the single nucleotide polymorphism (SNP) C1114G in the 3' untranslated region of the RGS2 gene, which correlated with lower RGS2 protein expression in cultured fibroblasts isolated from skin biopsy (Semplicini et al., 2006). Several rare coding SNPs were found in a Japanese hypertensive cohort at a frequency higher than normotensive controls but the sample size was too small to reach statistical significance (Yang et al., 2005). Subsequent studies confirmed loss of function (LOF) of these SNPs in vitro and defined mechanisms (Bodenstein et al., 2007; Gu et al., 2008). We recently identified four LOF mutations in human RGS2 (Phan et al., 2017) from a set of 16 variants of unknown significance found in the Genome Aggregation Database (Lek et al., 2016). Although they are biochemically characterized, the functional consequences of these mutations in the complex milieu of vascular tissue are unknown. Here, we used an ex vivo method in mesenteric arteries (MAs) to examine how these mutations affect vascular reactivity. We hypothesized that the LOF phenotype of RGS2 mutants identified in a cell-based assay would have LOF physiologic consequence in the MAs.

Materials and Methods

Ethical Approval. All experimental procedures were approved by the Michigan State University Animal Care and Use Committee and were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institutes of Health.

Animals. C57Bl/6 mice with wild type (WT) and RGS2<sup>−/−</sup> genotypes were obtained from Dr. Kendall Bumler (Washington University School of Medicine, St. Louis) (Osei-Owusu et al., 2015). Mice were provided access to food and water ad libitum in the Michigan State University animal facility on a 12-hour light/dark cycle. All of the experiments were performed using 3- to 5-month-old male mice. Mice were anesthetized by isoflurane, the MA was removed, and they were then euthanized by cervical dislocation.

Materials. Rp-8-pCPT-cGMPs (item 18445, CAS 208445-07-2) was purchased from Cayman Chemical. Angiotensin II (AngII; A9525), phenylephrine (PE; P6126), and acetylcholine (ACh; A6625) was purchased from Cayman Chemical. Angiotensin II (AngII; A9525), phenylephrine (PE; P6126), and acetylcholine (ACh; A6625) was purchased from Sigma-Aldrich (St. Louis, MO).

Pressure Myography. MAs were isolated free of adipose and connective tissue in a physiologic salt solution (PSS) containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl<sub>2</sub>·6H<sub>2</sub>O, 10 mM HEPES, and 10 mM dextrose, pH 7.4, at 4°C. MAs were mounted between two glass micropipettes in a custom-made cannulation chamber and were visualized on the stage of an inverted microscope (Zeiss Axiovert 35).

Immunofluorescence. After reversible permeabilization, MAs were fixed in 4% paraformaldehyde for 24 hours and rinsed with phosphate buffer (PB), pH 7.2. The arteries were permeabilized in DMSO 100% for 10 minutes, then incubated in blocking buffer/PE buffer containing 4% donkey serum (catalog number D9663, lot SLB99773V, Sigma-Aldrich) for 30 minutes, and then incubated with rabbit anti-GFP antibody (catalog number A6455, Life Technologies, Carlsbad, CA) at 1:500 dilution at 4°C overnight. The arteries were then incubated with 0.2% Triton X-100 in PBS and incubated in Cy3/anti-rabbit secondary antibody (code 711-165-152, Jackson ImmunoResearch, West Grove, PA) at 1:300 dilution at room temperature. The arteries were mounted using ProLong Gold Antifade Mountant with 4'6-diamidino-2-phenylindole (catalog number P36941, Life Technologies) and visualized using a 40× objective in an Olympus Fluoview 1000 laser scanning confocal microscope.

Cell Culture and Transfection. Cell lines were maintained in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Human embryonic kidney (HEK)-293 cells and Chinese hamster ovary (CHO)-K1 cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium/F-12 culture medium (catalog number 11039021, Gibco, Carlsbad, CA) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin and maintained at 37°C, 5% CO<sub>2</sub> in an incubator. MAs expressing RGS2-GFP were used for microscopy 24–48 hours after being transferred to culture medium.

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Confocal Microscopy. HEK-293 cells were seeded onto collagen-coated 35-mm glass-bottom Petri dishes (MatTek Corporation, Ashland, MA) and transfected with RGS2-GFP plasmid constructs along with plasmid DNA encoding eGFP<sup>Q209L</sup> or pDNA3.1 as the control. An Olympus FluoView1000 laser scanning confocal microscope with a 60× oil immersion objective using 488-nm excitation and 505- to 530-nm emission wavelengths for GFP was used for live cell imaging. This experiment was done 24 hours post-transfection. Representative images of at least 80 live cells per condition from three independent
experiments were acquired. The line scan analysis function in ImageJ (Schneider et al., 2012) was used to perform densitometric quantitation of protein expression.

**Ca**²⁺ **Mobilization Assay.** CHO cells were seeded into black, flat, clear-bottom 384-well plates (Greiner Bio-one, Kremsmünster, Austria) 24 hours post-transfection. Cells were allowed to attach overnight. After the media were removed, cells were loaded with 1 × Fluo-4 NW (Molecular Probes, Eugene, OR) in a loading buffer containing Hanks' basal saline solution and 20 mM HEPES, pH 7.4, supplemented with 2.5 μM probenecid. The cell plates were incubated for 30 minutes at 37°C, followed by 30 minutes at room temperature. The Fluo-4 NW was removed and loading buffer was added at 20 μl/well to the cell plate. A range of concentrations of AngII (catalog number a ligand plate for automated injection into the wells at 20 μl/well supplemented with 0.1% bovine serum albumin in loading buffer containing 2.5 μM probenecid. The cell plates were incubated for 30 minutes at 37°C, followed by 30 minutes at room temperature. The Fluo-4 NW was removed and loading buffer was added at 20 μl/well to the cell plate. A range of concentrations of AngII (catalog number A9525; Sigma) at 20 μl/well to the cell plate. A range of concentrations of AngII (catalog number A9525; Sigma) at 20 μl/well to the cell plate. A range of concentrations of AngII (catalog number A9525; Sigma) at 20 μl/well to the cell plate. The FDS2/C2 Cell kinetic fluorescence plate reader (Hamamatsu Photonics, Japan). Assay plates were placed in the plate reader and changes were measured.

**Statistical Analyses.** Data are presented as means ± S.E. Vessel diameter data were analyzed by the t test or two-way ANOVA followed by the indicated multiple-comparisons test. All statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad, San Diego, CA). Statistical significance was denoted by P < 0.05 or as indicated in the figure legends.

**Results**

**RGS2 Deficiency Enhances Vascular Contractility to PE and AngII and Causes Impairment of ACh-Mediated Vasorelaxation.** Vascular responses of RGS2-deficient arteries to vasoconstrictors and vasodilators have been extensively studied in different vascular beds, including the aorta, MA, renal interlobar, and uterine artery (Tang et al., 2003; Hercules et al., 2007; Osei-Owusu et al., 2012; Jie et al., 2016). Here we assessed the response of MA and aorta to PE, AngII, and ACh with the goals of: 1) validating the effect of RGS2 deficiency on vascular reactivity and 2) optimizing experimental conditions for studies of RGS2 mutant proteins in the context of vascular tissue. Second-order MAs and rings from the thoracic aorta from male RGS2²/² and RGS2²/² mice were used to determine how RGS2 protein affects vascular reactivity to different vasoactive substances. PE, which activates the Gαq-coupled α1 adrenergic receptor, caused a concentration-dependent contraction in RGS2²/² MAs and aorta; this response was significantly augmented in RGS2²/² aortic rings in wire myography (Fig. 1B) but not in pressurized MAs from RGS2²/² mice (Fig. 1A). The MAs isolated from RGS2²/² mice did show increased responsiveness to 5 nM AngII (28% ± 1.8% contraction in RGS2²/² vs. 10% ± 2.6% in RGS2²/², P < 0.01, t test; Fig. 1C; Supplemental Fig. 1A). In addition, the maximum of ACh-mediated vasodilation in RGS2²/² MA preconstricted with 5 μM PE was markedly impaired (48.6% ± 8.4% relaxation in RGS2²/² vs. 92.7% ± 6.2% relaxation in RGS2²/², P < 0.01 and P < 0.0001, respectively, in two-way ANOVA with the Sidak post-test; Fig. 1D). We chose to focus on testing the reactivity of MAs expressing RGS2 mutants to AngII and ACh in subsequent experiments because MAs contribute to vascular resistance, which determines arterial blood pressure (Christensen and Mulvany, 1993).

**Expression of RGS2-GFP in Cultured RGS2²/² MAs.** Using reversible permeabilization to deliver plasmids into intact arteries, we were able to express RGS2-GFP constructs in MAs. Immunofluorescent staining of GFP showed that RGS2-GFP was present in the MA between 48 and 72 hours after transfection control (Fig. 2A). The staining also showed that RGS2-GFP was present in the MA from RGS2²/² mice. Immunofluorescent staining of GFP showed that RGS2-GFP was present in the MA at ASPET Journals on October 14, 2023 molpharm.aspetjournals.org Downloaded from...
Human RGS2 Mutations Alter Vascular Reactivity

Human RGS2 Mutations Differentially Affect ACh-Evoked Vasodilation. Because freshly isolated RGS2−/− MAs show reduced relaxation to ACh, LOF mutations of RGS2 may behave similarly. To probe the function of these mutants in mediating ACh-induced vascular relaxation, we measured ACh-evoked vasodilation of MAs expressing WT and mutant RGS2. Using MAs preconstricted with PE (3 μM), we found that cultured RGS2-deficient MA (transfected with empty vector) showed a maximal relaxation of 52.8% (Fig. 4). This is similar to the response of freshly isolated RGS2−/− MAs (48.6% ± 8.4%; Fig. 1C) and much less than that of arteries expressing RGS2 WT (4.2% ± 1.3%, P < 0.05, one-way ANOVA, Dunnett post-test) (Fig. 3).

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Fig. 2. Reversible permeabilization permits transfection of RGS2-GFP into RGS2−/− MAs. Two to three days after reversible permeabilization (described in the Materials and Methods), arteries were stained for rabbit GFP and labeled with Cy3/anti-rabbit secondary antibody. The arteries were mounted to slides using Pro-Long Gold Antifade Mountant and visualized using confocal microscopy. (A) Expression of RGS2-GFP was detected compared with arteries exposed to empty vector. (B) RGS2-GFP was overexpressed in VSMCs but not endothelial cells (arrowheads). (C) Representative images showed whole vessel expression of RGS2-GFP. The expression level of the Q2L mutant in MAs was lower than that of RGS2-WT and other mutant RGS2 proteins. DAPI, 4′,6-diamidino-2-phenylindole. The round circle below the cell in the middle DAPI image is an artifact that appears to be a bubble.
Ser46 and Ser64 Phosphomimetic Mutants of D40Y and R44H RGS2 can Rescue Protein Plasma Membrane Localization and Function. In VSMCs, RGS2 has been shown to be an effector of the NO-cGMP pathway. RGS2 is phosphorylated by protein kinase G 1α (PKG1α), which is activated by cGMP; as a consequence, RGS2 membrane localization and GTPase accelerating protein (GAP) function is enhanced (Sun et al., 2005; Osei-Owusu et al., 2007). RGS2 phosphorylation at Ser46 and Ser64 by PKG1α increased plasma membrane (PM) localization and GAP activity of RGS2 protein (Tang et al., 2003; Osei-Owusu et al., 2007). Given that the two RGS2 mutants mentioned above (D40Y, R44H) had impaired PM localization in HEK-293 cells but supported a normal ACh-evoked relaxation response in the MAs, we hypothesized that phosphorylation of D40Y and R44H mutant proteins in VSMCs by NO-cGMP-PKG pathway activation upon ACh stimulation is able to rescue the impaired membrane localization and function of these mutants. The effects of site-specific phosphorylation on protein activity can be mimicked by mutation of Ser (S) or Thr (T) to Asp (D). Therefore, to model the effects of phosphorylation of Ser46 and Ser64 on RGS2 protein localization, we performed mutagenesis to make S46D and S64D mutations of tagged constructs encoding WT, D40Y, and R44H (RGS2 WT DD, D40Y DD, and R44H DD). The expression levels of the phosphomimetic mutants were similar to their nonphosphorylated proteins and similar to WT RGS2 (Supplemental Fig. 2). When coexpressed with Gq Q209L, D40Y, and R44H, mutant RGS2 remained in the nucleus, whereas RGS2 WT and the phosphomimetic mutants (RGS2 DD, D40Y DD, and R44H DD) localized to the PM (Fig. 5, A and B). In addition, the RGS2 D40Y and R44H mutants supported less Ca2+ inhibition compared with RGS2 WT (Fig. 5C), whereas RGS2 D40Y DD and R44H DD suppressed AngII receptor type 1–mediated Ca2+ mobilization equivalently to RGS2 DD (Fig. 5D).

Effect of PKG Inhibition on ACh-Mediated Relaxation in MAs Expressing the D40Y RGS2 Mutant. If phosphorylation helps maintain the function of the D40Y mutant RGS2, then inhibition of PKG with Rp-8-pCPT-cGMPS (30 μM) should prevent the rescue of relaxation.
PKG inhibition slightly reduced maximal relaxation of MAs expressing WT RGS2 from 99.6% ± 5.5% to 87.8% ± 4.2% (not significant) and shifted the ACh concentration-response curve a half-log to the right (logEC50 5.8 ± 0.06 vs. 6.3 ± 0.09, not significant; Fig. 5, E and F; Table 1). The relaxation response of RGS2−/− MAs transfected with empty vector was impaired (maximum relaxation 52.9% ± 14.3%) and was further depressed by PKG inhibition (maximum relaxation 38.4% ± 3.5%, not significant; Fig. 5, E and F; Table 1). However, PKG inhibition drastically reduced the dilation of MAs transfected with the D40Y mutant RGS2 (40.3% ± 6.7% vs. 98.4% ± 12.3%, P < 0.001; Fig. 5, E and F; Table 1).
In addition, we show for the first time that MAs from RGS2
were able to confirm the previously published effects of
differential relaxation supported by mutant RGS2 proteins may be
impaired ACh-mediated relaxation. The D40Y and R44H mutant alleles
in mice exhibit augmented vasoconstriction in mouse MAs.
Interestingly, only the Q2L and R188H mutations exhibited
behavior similarly to the MAs expressing D40Y.

**Discussion**

In this study, we examined the physiologic effects of human
RGS2 LOF mutations in a resistance artery. Four mutant
RGS2 proteins (Q2L, D40Y, R44H, R188H) were previously
identified as LOF mutations using a Ca 2+
mediating ACh-evoked relaxation, since overexpression of the
WT RGS2 in VSMCs was able to rescue the impaired ACh-mediated relaxation in RGS2 2−/−
arteries. In addition, mutants with different LOF mechanisms (Phan et al.,
2017) behaved differently (Fig. 4).

ACh mediates relaxation in part by activating the NO-
cGMP-PKG pathway in endothelium-intact arteries (Christopoulos and El-Fakahany, 1999; Sun et al., 2005). RGS2, as an effector of
PKG, has previously been shown to be phosphorylated at Ser46 and Ser64 (Tang et al., 2003), enhancing PM localization.
These phosphosites, interestingly, are located close
to the sites of the D40Y and R44H mutations in RGS2, as
shown in a model of the RGS2 N-terminal helix (Fig. 6)
interacting with lipids (Tikhonova et al., 2006). This
helix facilitates PM targeting (Heximer et al., 2001; Gu et al.,
2007). Introducing phosphomimetic mutants at two PKG1
phosphorylation sites of RGS2 (D40Y DD, R44H DD) in the
D40Y and R44H mutant RGS2 proteins enhanced PM localization
and function in suppressing Ca2+ release (Fig. 5, A, B, and D). Conversely, PKG inhibition substantially reduced
ACh-induced relaxation in RGS2 D40Y expressing RGS2 2−/−
MAs (Fig. 5F). These results support our hypothesis that the
normal ACh-mediated relaxation response of the D40Y and
R44H mutant RGS2 in MAs is due to phosphorylation
of mutant proteins by PKG. At the molecular level, this phos-
phorylation may rescue PM localization of the mutant
proteins. The RGS2 Q2L and R188H mutants may not be rescued
by phosphorylation since the mechanism of their LOF behav-
or is not related to PM localization.

Although the RGS2 D40Y and R44H mutant alleles supported
a normal relaxation response in resistance arteries, an intact endothelial NO-cGMP pathway was required for this
response. When endothelial function is compromised such as
in hypertension (Brandes, 2014) or in conditions wherein PKG
function is deficient (Michael et al., 2008), RGS2 proteins
expressed from these mutant alleles will likely be unphos-
phorylated, triggering aberrant Gq signaling and an im-
paired relaxation response.

**How Does Phosphorylation of RGS2 at Ser46 and Ser64 Rescue D40Y and R44H Membrane Localization?**
The RGS2-lipid bilayer interaction was impaired in the R44H
mutant according to the snorkeling-dependent stabilization
model (Gu et al., 2008). In the case of the D40Y mutant, the
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Human RGS2 Mutations Alter Vascular Reactivity

By overexpressing RGS2 in the MAs, we were able to probe the first two functions of these RGS2 mutants in regulating vascular reactivity of MAs. A limitation of this study is the lack of knock-in animal models to allow investigation of whether endogenous RGS2 null mutants affect whole-body blood pressure responses. In addition, we are unable to assess their effects on the central nervous system or the kidney. Nevertheless, our data provide a new understanding how human RGS2 mutants behave in VSMCs in the resistance vasculature. We also provide molecular evidence for functional differences among mutant alleles where the function of D40Y or R44H mutants can be rescued by PKG-mediated phosphorylation. These results provide critical insights for precision therapeutic approaches to individuals bearing particular RGS2 mutations and may facilitate drug discovery to target these rare individuals.

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Authorship Contributions

Participated in research design: Phan, Neubig.
Conducted experiments: Phan.
Contributed new reagents or analytic tools: Jackson, Watts.
Performed data analysis: Phan, Neubig.
Wrote or contributed to the writing of the manuscript: Phan, Jackson, Shaw, Watts, Neubig.

Note Added in Proof—Financial support was accidentally not included in the Fast Forward version published on October 23, 2019. The funding footnote has now been added.

References


Fig. 6. Modeled structure of full-length RGS2 with lipid membrane. The N-terminal amphipathic a-helix is oriented parallel with membrane, allowing interactions between amino acid side chains and components of lipid (Tikhonova et al., 2006). Mutated sites (D40, R44) are labeled in blue. Phosphorylated sites (S46, S64) are marked as spheres. The phosphate groups of lipids are in orange; the lipid acyl chains are in cyan.


**Address correspondence to:** Richard R. Neubig, Department of Pharmacology and Toxicology, Michigan State University, B423 Life Sciences Bldg., 1355 Bogue St., East Lansing, MI 48824. E-mail: rneubig@msu.edu