EFFECTS OF HUMAN RGS2 PROTEIN MUTATIONS IN CARDIOVASCULAR DISEASE

By

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ABSTRACT

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Heart disease and stroke have remained the major causes of death and disability in the United States and the world despite continuing efforts in cardiovascular research and drug development. Hypertension places individuals at a higher risk for such diseases. Multiple gene candidates and genetic variants have been identified via genome-wide approaches with the hope to better understand the pathophysiology of hypertension and to develop more individualized therapeutic strategies. Often, however, the significance of identified mutant alleles is unknown.

Among those are genetic variants identified in regulator of G protein signaling 2 (RGS2). RGS2 has been strongly implicated in cardiovascular regulation. RGS2 selectively attenuates $G\alpha_q$-mediated signaling through its canonical GTPase-activating protein (GAP) activity, thereby suppressing vasoconstrictor action. It has also been shown to inhibit $G\alpha_s$-mediated signaling through direct actions on adenylate cyclase. Homozygous and heterozygous RGS2-deficient mice exhibit hypertension and are prone to heart failure. Some rare mutations in RGS2 identified in hypertensive human subjects result in either a low level of protein expression or functional deficiency. This evidence suggests that genetic changes affecting RGS2 protein expression or function may be novel risk factors contributing to the development of hypertension and exacerbation of heart failure.

Genome wide sequencing efforts have now identified over 100 missense mutations in the RGS2 coding sequence. These RGS2 mutations (in ~1.6% of individuals) represent 5 million

people in the US. Several mutations are found in a disease context, however, their functional significance and consequences on vascular function are generally unknown. In this thesis work, I experimentally elucidate the functional effects on GPCR-mediated signaling and consequences on vascular reactivity of these RGS2 mutants. Among 16 mutations tested, four mutations lead to loss-of-function proteins that fail to inhibit AT1R-mediated Ca^{2+} mobilization. These mutant proteins also showed deficits in attenuating angiotensin II-mediated vasoconstriction in mesenteric arteries. Furthermore, these mutant proteins differentially regulate acetylcholine-mediated relaxation in vascular smooth muscle cells through a pathway involving cGMP-PKG activation. I also provide evidence of an association between a loss-of-function mutation in RGS2 with high blood pressure and cardiovascular disease. This study provides a molecular and physiological understanding of different RGS2 alleles *in vitro* as the first step to identify key candidate alleles for further analysis in in vivo models and in human hypertension and heart failure.

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KEY TO ABBREVIATIONS

HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
MOPS	3-[N-morpholino] propane-sulfonic acid
DAPI	4′,6-diamidino-2-phenylindole
ACh	Acetylcholine
AC	Adenylyl cyclase
AF	Alexa Fluor
ACC	American college of cardiology
AHA	American heart association
ANOVA	Analysis of variance
ACE	Angiotensin converting enzyme
AngII	Angiotensin II
AT1R	Angiotensin II receptor type 1
ARB	Angiotensin receptor blocker
H295R	Angiotensin-II-responsive steroid-producing adrenocortical cell line
AVP	Arginine vasopressin
BCA	Bicinchoninic acid
BMI	Body mass index
CNS	Central nervous system
CTS	Cardiotonic steroid
CVD	Cardiovascular disease
PKG	cGMP-dependent protein kinase

CHO	Chinese hamster ovary
CRISPR	Clustered regularly interspaced short palindromic repeats
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
COX	Cyclooxygenase
dbGaP	Database of genotypes and phenotypes
DAG	Diacyl glycerol
DBP	Diastolic blood pressure
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
DMEM	Dulbecco's modified Eagle's medium
EDHF	Endothelium-derived hyperpolarizing factors
ENaC	Epithelial sodium channel
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
eIF2B	Eukaryotic translation initiation factor 2B
FBS	Fetal bovine serum
GPR68	G protein coupled receptor 68
GPCR	G protein coupled receptor
gnomAD	Genome aggregation database
GWAS	Genome wide association study
GO	Grand opportunity

GFP	Green fluorescent protein
GAP	GTPase-activating protein
GDP	Guanosine-5'-diphosphate
GTP	Guanosine-5'-triphosphate
HBSS	Hank’s basal saline solution
HEK	Human embryonic kidney
HA	Human influenza hemagglutinin
IP3	Inositol triphosphate
IRB	Institutional review board
LABA	Long-acting β (2)-adrenoceptor agonist
LOF	Loss of function
MBP	Maltose binding protein
MA	Mesenteric artery
METSIM	Metabolic syndrome in men (study in Finnish population)
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid derived suppressor cell
MI	Myocardial infarction
TES	N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
NHLBI	National Heart, Lung, and Blood Institute
NO	Nitric oxide
OGTT	Oral glucose tolerance test
PE	Phenylephrine

PI3K	Phosphoinositide 3-kinase
PLC	Phospholipase C
PM	Plasma membrane
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PGI2	Prostaglandin I2 (prostacyclin)
PDB	Protein data bank
RhoA	Rat sarcoma virus homolog family member A
RGS	Regulator of G protein signaling
RAS	Renin-angiotensin system
SR	Salt resistant
SS	Salt sensitive
STAT3	Signal transducer and activator of transcription 3
SNP	Single nucleotide polymorphism
SDS	Sodium dodecyl sulfate
SHR	Spontaneous hypertensive rat
SD	Standard deviation
SEM	Standard error of mean
SBP	Systolic blood pressure
T2D	Type 2 diabetes
US	United States
UTR	Untranslated region
TRPV6	Vanilloid transient receptor potential

VUS	Variant of uncertain significance
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell
WHR	Waist hip ratio
WT	Wild type

CHAPTER 1: INTRODUCTION

Genetics of hypertension

Hypertension is the leading cause of cardiovascular diseases such as coronary artery disease, left ventricular hypertrophy, cardiac arrhythmias, cerebral stroke and renal failure (Kjeldsen, 2018), which place patients at a higher risk of mortality and morbidity in the United State (US) and worldwide (Carey et al., 2018; Cifu and Davis, 2017). The prevalence of hypertension in the US is 45.6% according to the 2017 American College of Cardiology/American Heart Association guideline (Muntner et al., 2018). Furthermore, less than 50% of hypertensive adults in the US achieve appropriate control of blood pressure by taking antihypertensive medications (Muntner et al., 2018). This represents an enormous, persistent unmet medical need.

Arterial blood pressure is a quantitative trait. It is determined by multiple variants in many genes, as well as environmental factors and gene - environment interactions (Cowley, 2006). Human hypertension shows about 40-60% heritability (Nguyen et al., 2013). While rare Mendelian forms of hypertension have been characterized (Ehret and Caulfield, 2013b; Padmanabhan et al., 2017), defining the genetic structure of essential hypertension is challenging. Genetic analysis of hypertension has utilized a variety of approaches. Rat models such as the spontaneously hypertensive rat (SHR) and Dahl salt-sensitive/resistant (SS/SR) rats have contributed significantly to our understanding of hypertension (Lawler, 1987; Morrissey et al., 2011; Okamoto, 1964; Rapp, 2000; Sanders, 1992). The recent genome sequence of an SHR model (Atanur et al., 2010) provided a genomic catalog for molecular elucidation of hypertension physiology. While a large number of genomic quantitative trait loci have been defined for hypertension in rats (Rapp, 2000), only a few single-gene determinants of hypertension and related phenotypes have been deciphered (Atanur et al., 2010; Morrissey, 2011).

In humans, genome-wide association studies (GWAS) and meta-analysis published to date have identified over 300 variants associated with blood pressure traits. However, they only account for 6-8% of the heritability (Ehret, 2017). Furthermore, the inconsistency in finding loci linked to hypertension illustrates the heterogeneity of human hypertension (Lalouel, 2003; Samani, 2003). A large fraction of the susceptibility genes and variants in hypertension remains to be discovered (Zeller et al., 2011). Thus, analysis of genetic variants in hypertension alone is not strong enough to tackle this problem. Further investigation should take functions, biochemistry, molecular mechanisms of genetic variants in blood pressure regulation into consideration (Barlassina et al., 2002; Lalouel and Rohrwasser, 2001). Recent attention has turned to rare alleles including single nucleotide polymorphisms (missense, splice-site, regulatory) and insertion/deletions (indels) (Ehret and Caulfield, 2013a; Fu et al., 2013; Goldstein, 2009).

Hypertension physiology

The regulation of arterial pressure is dynamic and is orchestrated by determinants in different organs (Fig. 1-1). Arterial pressure is a function of cardiac output and peripheral resistance. The kidney determines cardiac output by regulating fluid and electrolyte balance. Peripheral resistance is governed by the structure and function of the vasculature. Both kidney and vascular function are influenced by neuroendocrine factors such as the sympathetic nervous system and hormones. A range of pressure sensors residing in the vasculature, neuroendocrine organs and kidney regulates these organs' functions, and determines arterial pressure output. Imbalance of one or more of these determinants will lead to essential hypertension.

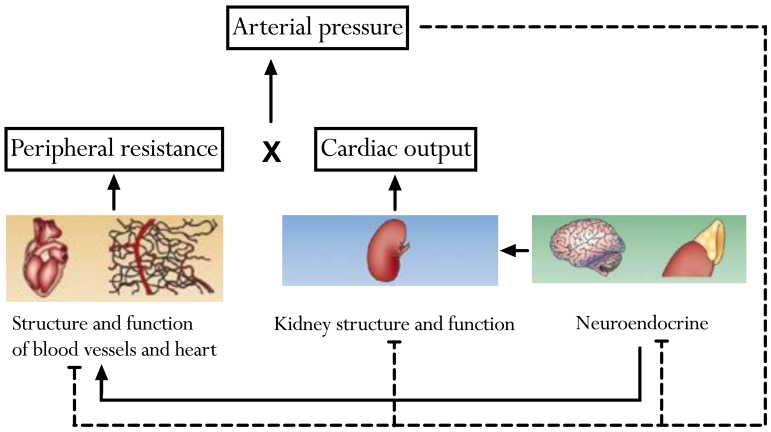

Figure 1-1. **Regulation of arterial blood pressure by the neuroendocrine system, kidney and cardiovascular system.**
Complex interactions among the cardiovascular, renal and neuroendocrine systems determine net arterial blood pressure by affecting total peripheral resistance and cardiac output (arrows). Negative feedback by arterial pressure contributes to this regulation by signaling through pressure sensors in the vasculature, kidney and neuroendocrine organs. Integrated control functions of these systems are shown as dashed lines. Reproduced with permission (Cowley, 2006).

G protein coupled receptor signaling

G protein-coupled receptors (GPCRs) comprise a large and diverse family of proteins whose structures feature seven trans-membrane helices with an extracellular N-terminus and an intracellular C-terminus. The main function of these receptors is to transduce extracellular stimuli into intracellular signals.

GPCRs couple to and transduce signals through a G protein heterotrimeric complex composed of three subunits, Gα, Gβ and Gγ. There are at least 20 different types of human Gα proteins, 5 types of Gβ proteins and 11 types of Gγ proteins (Hermans, 2003; Wong, 2003). There are four classes of heterotrimeric G-proteins, classified based on the Gα subtypes. They are Gq/11, Gi/o, Gs and G12/13. Activation of different class of G proteins results in different intracellular signaling cascades. Gαs stimulates adenylyl cyclase (AC), resulting in an increase in cyclic

adenosine monophosphate (cAMP) formation (Cabrera-Vera et al., 2003). Gαi/o inhibits AC causing a decrease in cAMP. Gαq/11 stimulates phospholipase C (PLC) leading to the formation of inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to IP3 receptors on the endoplasmic reticulum, causing a release of Ca^{2+} to the cytosol. cAMP, IP3, DAG, and Ca^{2+} are second messengers that bind to various proteins and mediate a wide array of functions (Newton et al., 2016). Gα12/13 activates the small G protein RhoA via Rho guanine nucleotide exchange factors (Cabrera-Vera et al., 2003). Gβγ regulates mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), PLC-β, AC and several small GTP-binding proteins. It also activates G-protein gated K+ channel (Krapivinsky et al., 1998) and a number of ion channels in vascular smooth muscle cells. This Gβγ complex mediates signaling involved in proliferation, apoptosis and ion channel activation (Cabrera-Vera et al., 2003; Schwindinger and Robishaw, 2001).

Agonist binding to a GPCR induces a conformational change in the receptor that triggers the activation of its cognate heterotrimeric G proteins. Upon activation, GDP in the inactive GDP bound Gα will be exchanged for GTP. The Gα-GTP is active and will dissociate from the Gβγ subunit. Both Gα-GTP and Gβγ, in turn, transduce signals to intracellular effectors (Premont et al., 1995). GPCR signaling is terminated when the GTP moiety in the activated Gα is hydrolyzed to GDP by the intrinsic GTPase activity of the Gα subunit. Gα, however, exhibits a slow rate of GTP hydrolysis *in vitro* (Siderovski and Willard, 2005). GTPase-activating proteins (GAPs) work to accelerate this hydrolysis and facilitate signal termination and reassembly of the trimeric G protein complex.

GPCR signaling in blood pressure regulation

Many genes in GPCR signaling pathways are candidates to study in essential hypertension due to their physiological functions in mediating vasoactive substance action and Na^+ reabsorption in nephrons (Morla et al., 2016). Genetic variants in several GPCR/G protein signaling components such as GNB3, GNAI2, RGS2, GRK4 have been implicated in hypertension (Bagos et al., 2007; Feldman and Gros, 2006; International Consortium for Blood Pressure Genome-Wide Association et al., 2011; Menzaghi et al., 2006; Siffert et al., 1998; Yang et al., 2015). Angiotensin II receptor and β-adrenergic receptor blockers are effective antihypertensive therapies, which indicates the significant contribution of these receptors in regulating blood pressure.

GPCR signaling in the vasculature

Vascular smooth muscle (VSM) controls the diameter of arteries and modulates peripheral resistance. This plays a major role in blood pressure regulation. Contraction of VSM can dramatically increase blood pressure due to the strong inverse relationship between vascular diameter and resistance (resistance ~ $1/r^4$). One of the hallmarks of essential hypertension is VSM hypercontractility. The compensatory mechanism of the heart to adapt to this pressure overload will, eventually, lead to left ventricular hypertrophy and reduced myocardial compliance (Soliman and Prineas, 2017).

The endothelium is the innermost layer of blood vessels. Upon activation by physiologic stimuli such as shear stress or pharmacologic stimuli such as acetylcholine, it produces and releases endothelium-derived relaxing factors such as NO, prostacyclin (PGI2) and endothelium-derived hyperpolarizing factors or EDHFs (Bernatova, 2014; Ozkor and Quyyumi, 2011). These factors are able to relax VSMCs and lead to vasodilation (Fig. 1-2).

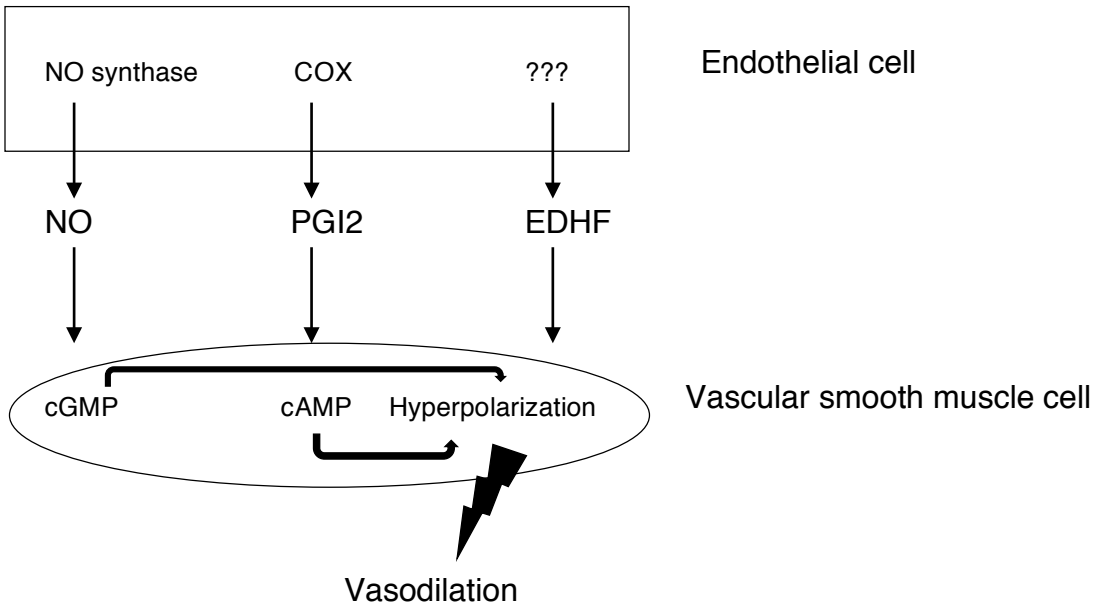

Figure 1-2. **Endothelium-mediated vasorelaxation mechanisms.**
Endothelial cells release NO, PGI2 and EDHF. These factors activate different targets in vascular smooth muscle cells in a paracrine manner, resulting in vascular relaxation (Ozkor and Quyyumi, 2011) *(reproduced with permission under the Creative Commons Attribution License)*.

Vasoconstrictors and vasodilators bind to and activate their cognate GPCRs expressed on both VSM and endothelial cells. Concerted regulation of these receptors is responsible for maintaining the balance between vasoconstriction and vasorelaxation (Fig. 1-3).

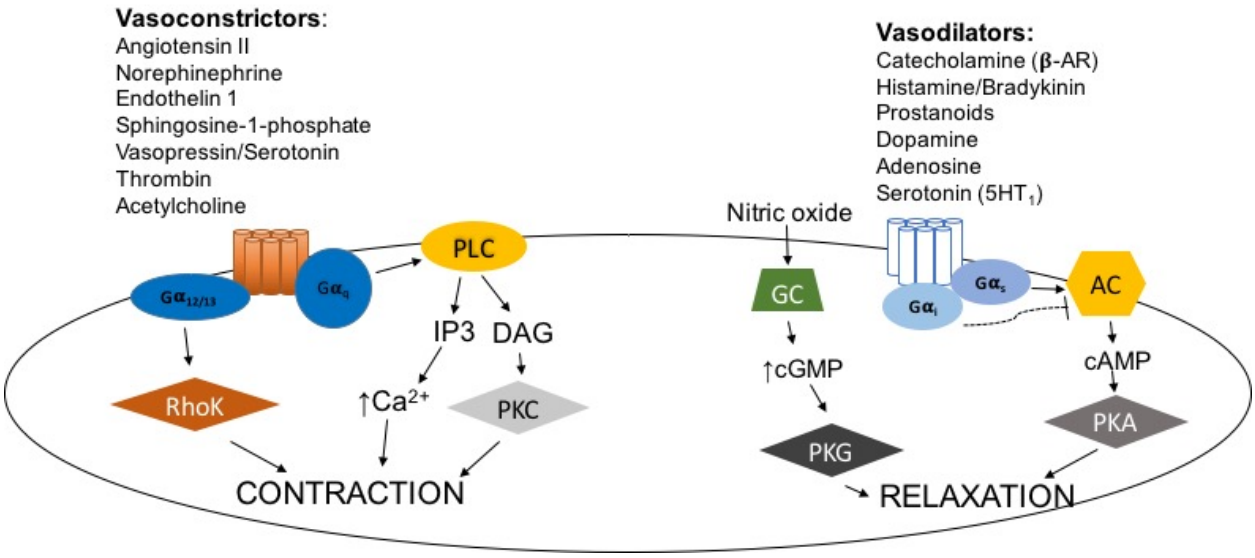

Figure 1-3. **GPCRs regulate vasoconstriction and vasodilation in vascular smooth muscle cells.** Vasoconstrictor agonists activate GPCRs that couple to G_q and $G_{12/13}$ to mediate VSMC contraction via increased intracellular Ca^{2+}, protein kinase C, and Rho kinase. Vasodilator agonists activate receptors that couple to Gs which, in turn, activates adenylate cyclase. Increased cAMP level results in protein kinase A-mediated vasodilation. Vasodilator action on endothelial cells leads to release of nitric oxide which increases cGMP and leads to protein kinase G-dependent relaxation of VSMC (Brinks and Eckhart, 2010).

GPCR signaling in the nervous system that regulates blood pressure

The link between the brain renin-angiotensin system (RAS) and hypertension has been extensively investigated. First, RAS system activation within the supraoptic and paraventricular hypothalamic nuclei stimulates the production and release of arginine vasopressin (Bonjour and Malvin, 1970; Coleman et al., 2009; Itaya et al., 1986; Littlejohn et al., 2013; Phillips, 1978; Szczepanska-Sadowska et al., 1998). Second, hindbrain and brain-stem actions of the RAS alter baroreflex function and sympathetic output (Grobe et al., 2008; Head and Mayorov, 2001).

Vasopressin release and sympathetic nervous system activation result in the development of hypertension in experimental animal models (Littlejohn et al., 2013; Osborn et al., 2007).

GPCR signaling in the kidney

The kidney plays an important role in maintaining solute and fluid homeostasis and also a source of RAS. It has been recognized as the most important determinant of blood volume and pressure (Wadei and Textor, 2012). In the distal nephron, sodium transport is tightly regulated to fine-tune sodium balance. Regulatory factors include endocrine, neuroendocrine and paracrine factors that activate different receptors, most of which are GPCRs (Morla et al., 2016). Classical GPCRs have been known for many years to regulate sodium transport. Angiotensin II and vasopressin are well known to increase Na^+ reabsorption. In contrast, bradykinin has an inhibitory effect upon the activation of receptors along the nephrons that eventually affects activities of solute transporters or sodium channels. More novel GPCRs have been recently discovered to regulate sodium reabsorption in the distal nephron (Morla et al., 2016). Examples are the protease-activated receptor 2, which is expressed ubiquitously in the nephron, and the 2-oxoglutarate receptor 1, expressed in pendrin-positive cells. These receptors favor sodium absorption. In contrast, activation of the sphingosin-1-phosphate receptors causes renal vasoconstriction and natriuresis. Together, this intricate signaling network contributes to a net effect on sodium and fluid retention that determines blood pressure.

Regulators of G protein signaling (RGS)

RGS proteins regulate the duration and amplitude of GPCR signaling by accelerating the intrinsic GTPase activity of Gα. The discovery of mammalian RGS proteins dated back to the mid-nineties (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996). This class of proteins was similar to a yeast SST2 protein found in the early 1980s to modulate sensitivity to pheromones (Chan and Otte, 1982) by interacting with and regulating yeast Gα proteins (Dohlman et al., 1995;

Dohlman et al., 1996). There are 20 human RGS proteins discovered to date, classified to 4 subtypes based on their structural similarity (Fig. 1-4). Among these proteins, RGS2, a small RGS protein belonging to the R4 subfamily, demonstrates selectivity toward Gαq (Heximer et al., 1997; Nance et al., 2013; Squires et al., 2018).

RGS protein	Sub-family	GAP for	Domains
RGS1	R4	Gi and Gq	
RGS2	R4	Gq >> Gi	
RGS3	R4	Gi and Gq	
RGS4	R4	Gi and Gq	
RGS5	R4	Gi and Gq	
RGS6	R7	Gi	
RGS7	R7	Gi	
RGS8	R4	Gi and Gq	
RGS9	R7	Gi	
RGS10	R12	Gi and Gq	
RGS11	R7	Gi	
RGS12	R12	Gi	
RGS13	R4	Gi and Gq	
RGS14	R12	Gi	
RGS15 = RGS3	-	-	
RGS16 (RGSr)	R4	Gi and Gq	
RGS17 (RGSZ2)	RZ	Gi >> Gq	
RGS18	R4	Gi and Gq	
RGS19 (GAIP)	RZ	Gi >> Gq	
RGS20 (RGSZ1)	RZ	Gi	
RGS21	R4		

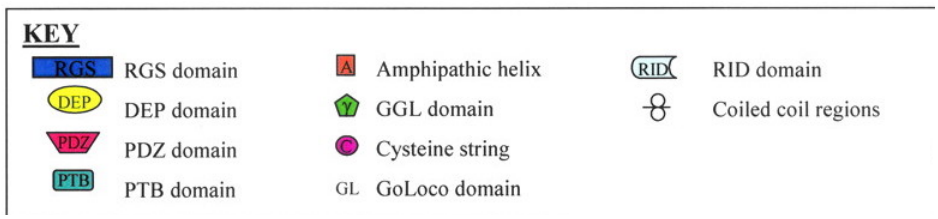

Figure 1-4. **Summary of RGS protein family members and their domain structure.**
The RGS domain (blue) is shared among RGS protein and plays as GAP for Gα (Riddle et al., 2005) *(reproduced with permission)*.

RGS2 in hypertension and cardiovascular diseases

As a selective inhibitor of Gαq signaling (Heximer et al., 1997), RGS2 suppresses signaling elicited by several vasoconstrictor agonists in vascular smooth muscle cells (VMSCs) including noradrenaline (Hague et al., 2005), angiotensin II (Li et al., 2005; Romero et al., 2006a), vasopressin (Zuber et al., 2007), and endothelin (Zhang et al., 2006). RGS2 has been tightly linked to the regulation of cardiovascular function. $RGS2^{-/-}$ mice are hypertensive and prone to heart failure due to prolonged Gαq signaling (Heximer et al., 2003b; Oliveira-Dos-Santos et al., 2000). The expression level of RGS2 is tightly linked to blood pressure control since even heterozygous knockout mice ($RGS2^{+/-}$) are hypertensive (Heximer et al., 2003b). A clinical study found that low protein levels of RGS2 in peripheral blood mononuclear cells was associated with non-responsiveness to anti-hypertensive treatment (Semplicini et al., 2010a).

RGS2 is also important in cardiac hypertrophy. RGS2 acting on Gαq-mediated signaling is a key component in suppressing cardiac hypertrophy (Zhang et al., 2006). $RGS2^{-/-}$ mice develop worsened heart failure in aortic banding models and adaptation mechanisms to cardiac stress are mediated by RGS2 suppressing Gαq-mediated signals in the heart (Takimoto et al., 2009).

The mechanism by which mice lacking RGS2 protein develop hypertension is not completely clear. Gurley et al (Gurley et al., 2010) performed kidney transplantation between $RGS2^{+/+}$ and $RGS2^{-/-}$ mice and showed that RGS2 deficiency in the kidney and its vasculature alone is sufficient to make the animals hypertensive. Conversely, transplantation of kidney from wildtype mice to global RGS2 knockout mice was able to rescue the hypertensive phenotype. RGS2 deficiency impaired kidney function and increased sodium retention by increasing renal vascular resistance and translocation of ENaC to the luminal wall (Osei-Owusu et al., 2015). In an

adrenal cell line, angiotensin II was found to upregulate RGS2 and RGS2 limited AngII-mediated aldosterone production (Romero et al., 2006a). However, systemic concentrations of aldosterone are not different between RGS2 wild type and knock out mice, potentially due to compensatory mechanisms under basal conditions (Tang et al., 2003b). Nevertheless, the kidney and its vasculature seem to play a major role in promoting hypertension in RGS2-deficient mice.

In VSMCs, RGS2 deficiency enhances contraction of different arterial beds stimulated by Gαq-mediated vasoconstrictors such as AngII, endothelin 1 or phenylephrine (Hercule et al., 2007; Osei-Owusu et al., 2012). RGS2 is also an effector of the NO/cGMP-dependent protein kinase Iα pathway (Sun et al., 2005b). RGS2 is phosphorylated by PKG1α which resulted in further inhibition of vasoconstrictor action, thus promoting smooth muscle relaxation.

RGS2 is also expressed in the endothelial cell layer of the vasculature. Acetylcholine-induced vasorelaxation is markedly impaired in global or endothelium-specific $RGS2^{-/-}$ mesenteric arteries as a result of impaired ACh-evoked EDHF- and NO-mediated relaxation (Osei-Owusu et al., 2012). Blocking Gi/o signaling with pertussis toxin can rescue the EDHF-mediated relaxation (Osei-Owusu et al., 2012), indicating a different mode of function of RGS2 in the endothelium in contrast to serving as a Gαq GAP as in vascular smooth muscle cells.

RGS2 may also interact with a number of other proteins beside Gαq. The amino terminal domain of RGS2 is thought to contribute to the inhibitory effect of RGS2 on a number of GPCRs such as α1-adrenergic (Hague et al., 2005), cholecystokinin 2 (Langer et al., 2009) or M1 muscarinic receptor (Bernstein et al., 2004) through a protein – protein interaction. RGS2 may also interact with Gαs, several adenylyl cyclase subtypes (Roy et al., 2006; Salim et al., 2003), the eukaryotic initiation factor eIF2B (Chidiac et al., 2014), the transcription factor STAT3 (Lee et al.,

2012), tubulin (Heo et al., 2006; Jiang et al., 2016), and TRPV6 channels (Schoeber et al., 2006). Although most studies used an overexpression approach, they highlight RGS2 as a versatile, dynamic protein that coordinates a wide range of intracellular signaling mechanisms.

RGS2 regulation and targeting for therapeutics

RGS2 expression is regulated by multiple mechanisms in the cardiovascular system (Tsang and Xiao, 2010). In the heart, it was suggested that myostatin stimulates RGS2 expression, thus preventing cardiac hypertrophy and heart failure (Biesemann et al., 2014). RGS2 mRNA is upregulated by Ang II in H295R cells through a calcium-dependent pathway (Romero et al., 2006a). RGS2 upregulation suppresses AngII-mediated aldosterone secretion which plays a key role in blood pressure regulation (Romero et al., 2006b). Low RGS2 protein expression in peripheral blood mononuclear cells also predicts less response to antihypertensive treatment (Semplicini et al., 2010b).

RGS2 downregulation has been observed in cancer. Epigenetic repression of RGS2 expression promotes bladder and pancreatic cancer progression (Wolff et al., 2012a; Ying et al., 2015). RGS2 mRNA expression is lower in breast cancer tissue than in normal tissues. In this case, it was suggested that RGS2 overexpression exerts an inhibitory effect on breast cancer cell growth (Lyu et al., 2015). RGS2 is specifically downregulated in androgen-independent prostate cancer cells in which RGS2 could function as a growth suppressor (Cao et al., 2006; Wolff et al., 2012b). Decreased RGS2 expression may contribute to chronic inflammation leading to metaplasia and eventually carcinogenicity in the skin keratinocytes (Udensi et al., 2011). RGS2 inhibits cell signaling in ovarian cancer (Hurst et al., 2009) and RGS2 mRNA is down-regulated in colorectal cancer (Jiang et al., 2010b). Low RGS2 mRNA expression also correlates with poor prognosis in

late stage colorectal cancer patients (Jiang et al., 2010a). Surprisingly, RGS2 mRNA expression was found to increase in myeloid derived suppressor cells isolated from tumors and regulates pro-angiogenic function of these cells in the tumor microenvironment. (Boelte et al., 2011).

RGS2 upregulation is also beneficial in asthma. In primary human airway smooth muscle cells, a combination of glucocorticoid and long-acting β (2)-adrenoceptor agonist (LABA) synergistically induce the expression of RGS2. This enhanced expression reduced intracellular free calcium flux elicited by histamine, methacholine, leukotrienes, and other spasmogens (Holden et al., 2011). Increased inflammatory cytokines and promoter polymorphisms that suppress RGS2 expression were found in patients with asthma or mice with airway inflammation (Jiang et al., 2014). Upregulation of RGS2 by digoxin or pirfenidone, respectively, was beneficial in heart failure (Sjögren et al., 2012) or pulmonary fibrosis treatment (Xie et al., 2016). Therefore, enhancing RGS2 protein levels could be beneficial in asthma or cancers.

Association between RGS2 polymorphisms and cardiovascular phenotypes in humans

There have been a number of polymorphisms found in the RGS2 gene at various allele frequencies and in different populations (gnomad.broadinstitute.org/gene/ENSG00000116741). Among them, common variants such as the intronic in/del variants (1891 to 1892 TC and 2138 to 2139 AA) and the 3'UTR polymorphism C1114G are associated with hypertension. Most SNPs in the coding region of RGS2 have very low allele frequencies. Although some of them were presented more in hypertensive subjects compared to normal subjects (Table 1-1), their implication in disease development is unknown. Prior evidence about the association of RGS2 polymorphisms with cardiovascular phenotypes such as hypertension, metabolic syndrome, carotid atherosclerosis and preeclampsia is summarized in Table 1-1.

Table 1-1. **RGS2 variants associated with cardiovascular phenotypes.**

Gene region	Polymorphism	Allele frequency	Study design	Population	Phenotype
Promoter	-391 C->G	GG: 54.1%	In vitro, ex vivo functional studies, epidemiological study (Freson et al., 2007)	White European	Higher protein/mRNA expression, Metabolic syndrome in men
		GG: 31.6% GC: 49%	Case control, treatment with antihypertensive drugs (He et al., 2015)	Chinese	Less responsive to treatment
	-638 A->G rs2746071	>40%	(Deja et al., 2014)	Poland	No association with HT and non-dipping phenomenon in patients with T1DM
		>20%	(Kamide et al., 2011; Li et al., 2010; Yang et al., 2005a; Zhang et al., 2013; Zhao et al., 2008)	Japanese, Chinese	No association with HT
		>10%	Case control (Kamide et al., 2011)	Japanese	Carotid atherosclerosis
Exon	Q2L, M5V, R44H	Rare	Case control, in vitro (Bodenstein et al., 2007b; Gu et al., 2008a; Yang et al., 2005a)	Japanese	Found predominantly in HT population
	Q50K		Case control (Riddle et al., 2006)	African American	2 HT, 1 NT
Intron	1891-1892TC in/del -rs34717272	>10%	Case control (Kamide et al., 2011)	Japanese	Carotid atherosclerosis
		>25%	(Hahntow et al., 2009; Kamide et al., 2011; Li et al., 2010; Riddle et al., 2006; Yang et al., 2005a; Zhang et al., 2013; Zhao et al., 2008)	Japanese, Chinese, Xinjiang Kazak, Surinamese, AA	Association with HT
	2138-2139AA in/del 1026 T->A	>25%	Case control (Riddle et al., 2006)	AA	Association with HT
		>10%	Case control (Kamide et al., 2011)	Japanese	Carotid atherosclerosis
			Case control (Yang et al., 2005a)	Japanese	HT

Table 1 1. (Cont'd)

3' UTR	C1114G-rs4606	Common	Case control, in vitro study (Semplicini et al., 2006a)	Italian	mRNA destabilization, HT
			Prospective study (Sartori et al., 2008)	Italian	Overweight or obesity in HT patients
			Case control (Lelonek et al., 2009a; Lelonek et al., 2009b)	Polish	No association with tilting or enhanced risk of severe clinical manifestation of syncope
			(Lelonek et al., 2009c)	Caucasian	Reduced number of episodes of syncope
			Population-based study (Kvehaugen et al., 2014; Kvehaugen et al., 2013)	Norwegian	Preeclampsia, recurrent preeclampsia, HT, development of HT after delivery
Near RGS2 gene	rs3767489		Longitudinal observational study (Watanabe et al., 2010)	Ohasama town (Japan)	Development of HT

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Hypotheses

Compared to RGS4 and RGS5, which are two closely related RGS proteins in the R4 subfamily, RGS2 has a longer amino terminus (Fig 1-5 A). This may explain a more complex protein interaction network between RGS2 and other proteins (previously discussed). RGS2 protein sequence is highly conserved across species (Fig. 1-5 B), which indicates that changes in its sequence may alter its function. In my studies, I focus on a set of 16 RGS2 human mutations found in the gnomAD database. These mutations were selected because they were found in at least two individuals in the Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA or had high frequency in the gnomAD database. The mutated residues are indicated by arrows in the alignment of RGS2 and RGS4 and RGS5 (Figure 1-5 A) or among RGS2 sequences from different species (Figure 1-5 B).

I hypothesized that human mutations in RGS2 protein alter expression or function of the protein and therefore may affect pharmacological reactivity of resistance arteries to vasoconstrictors and/or vasodilators. These effects may contribute to the development of hypertension.

Specific aims

Aim 1: Assess whether mutations in human RGS2 protein alter its expression, localization, and function.

a) Examine the effects of 16 human RGS2 mutations on RGS2 protein levels and stability in mammalian cells.

b) Assess the subcellular localization of RGS2 mutants.

c) Study the ability of RGS2 mutants to inhibit $G\alpha_q$-mediated Ca^{2+} mobilization.

Aim 2: Assess whether change-of-function mutations identified *in vitro* affect contraction and relaxation of isolated mouse mesenteric arteries.

Aim 3: Analyze whether there is an association of rare RGS2 mutations with cardiovascular phenotypes in human subjects.

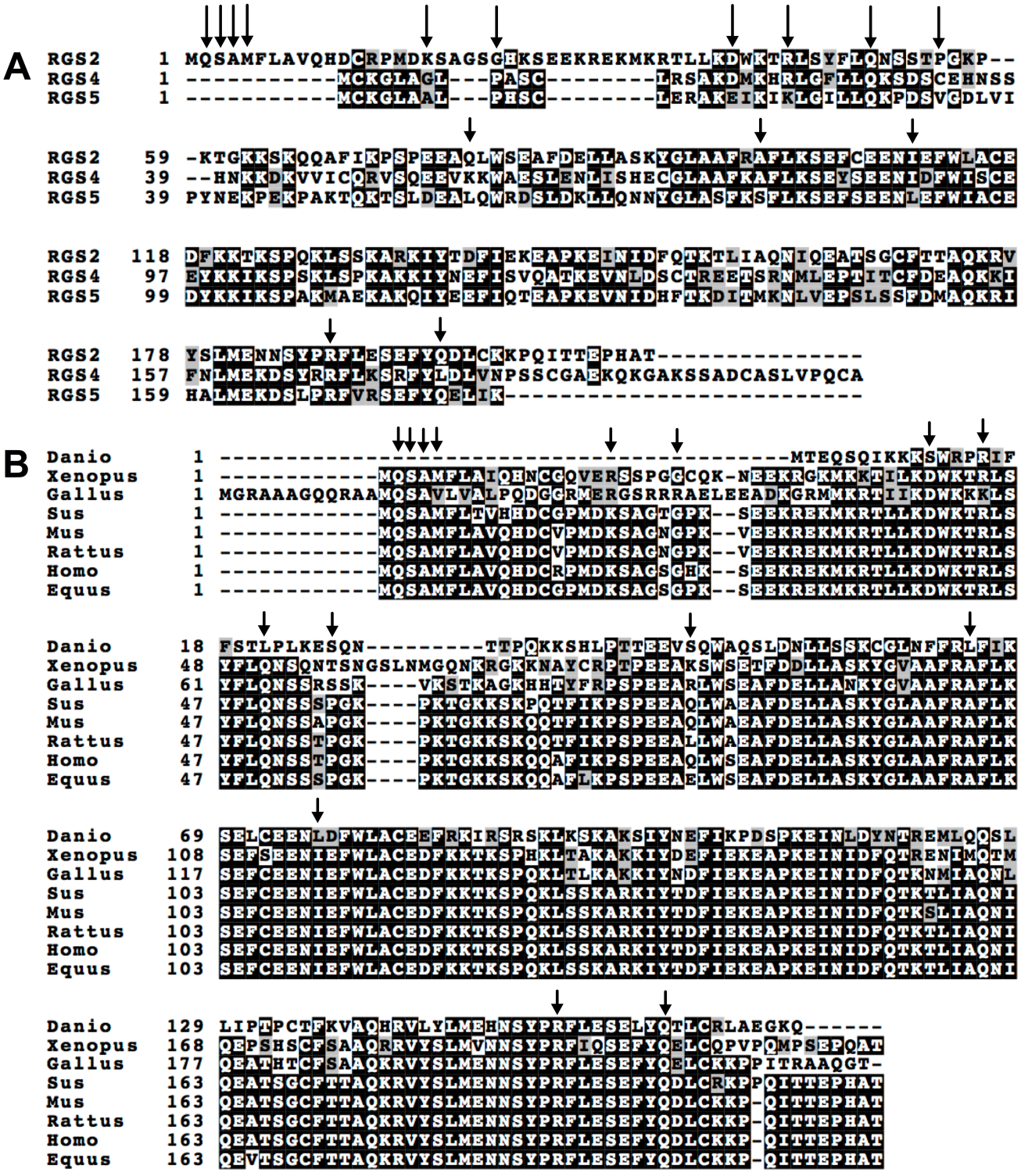

Figure 1-5. **Sequence alignment analysis.**
A. Sequence alignment of RGS2, RGS4 and RGS5 human proteins in the R4 RGS subfamily. B. Sequence alignment of RGS2 proteins across species. Arrows indicate the mutated residues tested. Black highlight indicates conserved residues, grey highlight indicates residues with the same charged/polar group, white highlight indicates missing or non-conserved residues.

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CHAPTER 2: HUMAN MISSENSE MUTATIONS IN REGULATOR OF G PROTEIN SIGNALING 2 AFFECT PROTEIN FUNCTION THROUGH MULTIPLE MECHANISMS

Abstract

RGS2 plays a significant role in alleviating vascular contraction and promoting vascular relaxation due to its GTPase accelerating protein (GAP) activity toward Gαq. Mice lacking RGS2 display a hypertensive phenotype and several RGS2 missense mutations have been found predominantly in hypertensive human subjects. However, the mechanisms whereby these mutations could impact blood pressure is unknown. Here, we selected 16 rare, missense mutations in RGS2 identified in various human exome sequencing projects and evaluated their ability to inhibit intracellular calcium release mediated by the angiotensin II receptor type 1 (AT1R). Four of them had reduced function and were further investigated to elucidate underlying mechanisms. Low protein expression, protein mis-localization, and reduced G protein binding were identified as likely mechanisms of the malfunctioning mutants. The Q2L mutant had 50% lower RGS2 than wild-type (WT) protein detected by Western blot. Confocal microscopy demonstrated that R44H and D40Y had impaired plasma membrane targeting; only 46 and 35% of those proteins translocated to the plasma membrane when co-expressed with $G\alpha_q$ Q209L compared to 67% for WT RGS2. The R188H mutant had a significant reduction in $G\alpha_q$ binding affinity (10-fold increase in K_i compared to WT RGS2 in a flow cytometry competition binding assay). This study provides functional data for 16 human RGS2 missense variants on their effects on AT1 receptor-mediated calcium mobilization and provides molecular understanding of those variants with functional loss *in vitro*. These molecular behaviors can provide insight to inform antihypertensive therapeutics in individuals with variants having reduced function.

Introduction

Heart and cerebrovascular diseases have remained the major causes of death and disability in the United States (Heron, 2016; Mozaffarian et al., 2016) despite relentless efforts in cardiovascular research and drug development. Hypertension is a multifactorial disorder that places individuals at a higher risk for such diseases. Multiple genome-wide association studies (GWAS) have discovered polymorphisms in genes and loci that are associated with hypertension (Adeyemo et al., 2009; Dominiczak and Munroe, 2010; Franceschini et al., 2014; Huan et al., 2015; Levy et al., 2009; Lind and Chiu, 2013; Newton-Cheh et al., 2009; Pan et al., 2015). However, current knowledge about hypertension genetics is still far from complete. Common variants identified in GWAS only explain a small fraction of the blood pressure variance landscape (Dominiczak and Munroe, 2010). This limitation highlights the need to study rare variants that may contribute to this complex disorder (Gibson, 2012; Schork et al., 2009).

G protein coupled receptors (GPCRs) play a critical role in vascular tone regulation (Brinks and Eckhart, 2010). Several GPCRs with preferential activation of heterotrimeric G proteins of the $G\alpha_q$ family (e.g. Angiotensin II, Endothelin and α1 adrenergic receptors) mediate vasoconstrictor responses in blood vessels and many antihypertensive drugs act to counteract their effects. Regulator of G protein signaling (RGS) proteins play a crucial role in modulating GPCR signaling through their GTPase-activating protein (GAP) activity toward Gα subunits. RGS2, in particular, has been strongly implicated in cardiovascular regulation due to its selectivity toward $G\alpha_{q,}$ resulting in diminished vasoconstrictor action (Heximer et al., 1997). Homozygous and heterozygous RGS2 knock out mice exhibit a hypertension phenotype attributed to prolonged Gαq-mediated vasoconstrictor signaling (Heximer et al., 2003) and a reduced NO/cGMP-mediated

vascular relaxation response (Sun et al., 2005; Tang et al., 2003). Mice lacking RGS2 are also prone to cardiac hypertrophy due to elevated Gαq signaling (Zhang et al., 2006). Increased sympathetic tone and altered renal mechanisms also contribute substantially to hypertension development in RGS2 knock-out mice (Gross et al., 2005; Gurley et al., 2010; Osei-Owusu et al., 2015).

The actual role of RGS2 in human hypertension is not well understood. A number of common variants in the promoter, introns and noncoding regions of RGS2 gene are associated with hypertension and suboptimal responsiveness to anti-hypertensive treatment in different ethnic groups (Freson et al., 2007; Riddle et al., 2006; Semplicini et al., 2006; Yang et al., 2005; Zhang et al., 2013). Several reported missense mutations in RGS2 were found predominantly in hypertensive subjects but with very low allele frequencies (Yang et al., 2005). Among those, the Q2L mutant allele was shown to have low protein expression due to rapid proteasomal degradation (Bodenstein et al., 2007; Park et al., 2015). The R44H mutant, on the other hand, showed less efficient plasma membrane targeting (Gu et al., 2008). Since these missense mutations of RGS2 had such low allele frequencies (less than 1%), it has been difficult to determine the functional significance of these mutations using epidemiological or informatics approaches.

The revolution in next-generation sequencing has revealed genetic information useful for prevention and clinical management (Rabbani et al., 2014). More commonly, healthcare professionals are presented with variants of uncertain significance (VUS), complicates the interpretation of genetic data (Ackerman, 2015; Gomez et al., 2016). Computational tools have been developed to predict variant effects with limited success (Schulz et al., 2015). Definitive characterization of VUS requires family segregation or functional studies. In alignment with this

common theme, many missense mutations of RGS2 have been released to public databases such as NHLBI GO Exome Sequencing project (http://evs.gs.washington.edu/EVS/) and Exome Aggregation Consortium (ExAC - http://exac.broadinstitute.org), now known as the Genome Aggregation Database (gnomAD - http://gnomad.broadinstitute.org) but the functional consequences and human phenotypes of these variants is largely unknown. We hypothesized that changes in the coding sequence of RGS2 may result in protein products that have altered function. In this study, we examined 16 mutations (Table 2-1) found in a Japanese population (Yang et al., 2005), the NHLBI exome sequencing project (Fu et al., 2013) and the gnomAD database (Lek et al., 2016) to determine whether they differ from WT RGS2 in their ability to regulate GPCR-mediated signaling. For those with reduced function, we determined the molecular characteristics contributing to those differences. Specifically, we investigated protein levels, protein localization and G protein binding activities of these mutants to further our understanding about the underlying mechanisms responsible for functional alteration of the mutants. Only a small fraction of variants showed altered function and it was due to several distinct mechanisms.

Table 2-1. **Missense mutations in the coding region of RGS2 examined in this study**

Mutation ID	**Missense variant**	**Allele count (gnomAD)**	**Codon change**	**Reference**
N/A	Q2L*	1/246268	CAA>CTA	(Lek et al., 2016; Yang et al., 2005)
rs141030117	Q2R	183/277230	CAA>CGA	(Lek et al., 2016; Yang et al., 2005)
rs145125159	S3G	95/277216	AGT>AGC	(Lek et al., 2016; Yang et al., 2005)
rs142499684	A4V	4/246268	GCT>GTT	(Lek et al., 2016)
rs193051407	M5V	158/277222	ATG>GTG	(Lek et al., 2016; Yang et al., 2005)
rs74466425	K18N	82/277236	AAG>AAC	(Lek et al., 2016)
rs148489044	G23D	184/277214	GGC>GAC	(Lek et al., 2016)
rs201233692	D40Y	236/276634	GAT>TAT	(Lek et al., 2016)
rs200339834	R44H*	35/276900	CGT>CAT	(Lek et al., 2016; Yang et al., 2005)
rs80221024	Q50K	238/277080	CAA>AAA	(Lek et al., 2016)
rs140811638	P55L	8/277186	CCT>CTT	(Lek et al., 2016)
N/A	Q78H	3/2055	CAG>CAC	(Yang et al., 2005)
rs139237239	A99G	6/277134	GCT>GGT	(Lek et al., 2016)
rs146862218	I110V	106/277132	ATT>GTT	(Lek et al., 2016)
rs369752935	R188H	8/277202	CGT>CAT	(Lek et al., 2016)
rs112707798	Q196R	36/246240	CAG>CGG	(Lek et al., 2016)

gnomAD: The Genome Aggregation Database - http://gnomad.broadinstitute.org
* RGS2 mutations were found in disease cohort (Yang et al., 2005a).

Materials and methods

DNA constructs

Mammalian expression vectors encoding the human, full-length, untagged or 3xHA tagged WT Angiotensin II type 1 (AT1) receptor and RGS2 in pcDNA3.1(+) were obtained from the University of Missouri-Rolla cDNA Resource Center (http://www.cdna.org). Other constructs were generated in our laboratory and the primer sequences for construct generation are available on request. RGS2 was amplified by PCR as attB1-2 fragments without a stop codon to allow subsequent cloning to an entry vector pDONR221 using Gateway® BP clonase (Invitrogen, Carlsbad, CA). RGS2-Q2L, RGS2-Q2R, RGS2-S3G, RGS2-A4V, RGS2-M5V, RGS2-K18N, RGS2-G23D, RGS2-D40Y, RGS2-R44H, RGS2-Q50K, RGS2-P55L, RGS2-Q78H, RGS2-A99G, RGS2-I110V, RGS2-R188H and RGS2-Q196R were generated in the entry vector by performing QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), using custom made primers (Supplemental Table 2-1). RGS2 WT and mutants were transferred into C-terminally V5 or GFP epitope-tagged expression vector (pcDNA™3.2DEST or pcDNA™DEST47, respectively) using Gateway® LR clonase (Invitrogen, Carlsbad, CA). A pMAL-C2H10T construct encoding the RH domain of human RGS2 (residues 72–203) was a kind gift from Dr. John J. Tesmer (University of Michigan). Mutagenesis for this vector was performed as described above. The open reading frame of all PCR and mutagenesis generated constructs was verified by sequencing at the RTSF Genomics Core at Michigan State University.

Cell Culture and Transfections

All cell lines were maintained in a humidified incubator at 37°C with 5% CO2. Human embryonic kidney (HEK)-293 cells were grown to 95% confluence in Dulbecco's modified

Eagle's medium (DMEM; GIBCO, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Chinese hamster ovary (CHO-K1) cells (ATCC, Manassas, VA) were grown to 95% confluence in F-12 nutrient mixture (GIBCO, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. HEK-293 and CHO-K1 cells were transiently transfected using, respectively, X-tremeGENE HP DNA Transfection Reagent (Roche Life Sciences) at 2 ml/mg of plasmid DNA and DNA-In® CHO (MTI-GlobalStem, Gaithersburg, MD) at 3 ml/mg of plasmid DNA according to the manufacturers' recommended protocols. Transfection mixes were prepared in Opti-MEM (GIBCO, Carlsbad, CA) and all transfections were performed under antibiotic-free conditions. Experiments were run 24-48 hours after transfection.

SDS-PAGE and Western Blot Analysis

Twenty-four hours post-transfection, cells were treated as indicated in the figure legend and harvested using modified RIPA lysis buffer containing 50mM Tris HCl pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2mM EDTA, supplemented with complete protease inhibitor (Roche Diagnostics, Indianapolis, IN). Protein concentration in the cell lysates was determined using the BCA assay (Pierce; Rockford, IL) and adjusted with an appropriate volume of Laemmli buffer (BioRad; Hercules, CA). Equal amounts of protein in each lane were resolved on a 12% SDS-PAGE gel for 1 hour at 180 V. Samples were transferred to an Immobilon-FL membrane (EMD Millipore, Darmstadt, Germany) for 1 hour at 100 V, 400mA on ice and subjected to Western immunoblot analysis. The membrane was blocked with Odyssey blocking buffer (PBS) (Licor Biosciences, Lincoln, NE) for 30 minutes at room temperature on an orbital shaker. The membrane was probed overnight at 4°C with primary antibody diluted in blocking buffer. Mouse anti-V5 antibody (Invitrogen, Cat. 46-0705, Lot # 1620500), rabbit anti-HA (Santa

Cruz, Dallas, TX; Cat. sc-805, Lot # D0413) and rabbit anti-actin (Santa Cruz, Dallas, TX; Cat. sc-1615-R, Lot # C2609) were used at a dilution of 1:5000, 1:1000 and 1:1000, respectively. The membrane was washed with PBS buffer supplemented with 0.1% Tween 20 (PBS-T) four times and probed for 1 hour at room temperature with IRDye–conjugated secondary antibody diluted in blocking buffer. IRDye 680RD donkey anti-mouse (Cat. 926-68072, Lot # C31216-02) and IRDye 800CW donkey anti-rabbit antibody (Cat. 926-32213, Lot # C40130-03) (1:10.000) were from LiCor. After four washes in blocking buffer, the protein bands were visualized using LiCor Odyssey FC scanner and images were scanned and analyzed using the Image Studio™ Lite (Licor Biosciences, Lincoln, NE).

Confocal microscopy

HEK293 cells were seeded into collagen-coated 35 mm glass-bottom Petri dishes (MatTek Corporation, Ashland, MA) and transfected with 0.5 μ g of plasmid DNA encoding RGS2-GFP with or without 0.5 μ g of plasmid encoding $G\alpha_q^{Q209L}$. Confocal microscopy was performed on live cells 24 hours after transfection using an Olympus FluoView 1000 laser scanning confocal microscope. Images represent single planes on the basal side of the cell obtained with a 60x oil objective using 488 nm excitation and 505–530 nm emission wavelengths for GFP. Shown are pictures representative of at least 100 live cells from 3 independent experiments. Densitometric quantitation of protein expression was performed in a blinded manner using the line scan analysis function of the ImageJ software package.

Ca^{2+} mobilization assays

Twenty-four hours after transfection, CHO cells were split into black, flat, clear bottom 384-well plates (Greiner Bio-one, Kremsmünster, Austria) and allowed to attach overnight. The

medium was aspirated and cells loaded with 1X fluo-4 NW (Molecular Probes, Eugene, OR) in a loading buffer consisting of Hank's basal saline solution supplemented with 20 mM HEPES, pH 7.4 and 2.5 μM probenecid following manufacturer's protocol. The cells were incubated for 30 min at 37°C, then 30 min at room temperature. Dye mix was removed and loading buffer was added to the cell plate. A concentration gradient of angiotensin II (Sigma, Cat. A9525) at 2x the final concentration was freshly prepared in loading buffer supplemented with 0.1% BSA for automated injection into the wells by a FDSS/μCell kinetic fluorescence plate reader (Hamamatsu Photonics, Japan). Assay plates were then placed onto the plate reader for measuring the changes of intracellular free calcium in response to the receptor activation.

Protein expression and purification

The RGS domain of WT and mutant RGS2 was expresses as a C-terminal fusion on bacterial maltose binding protein (MBP). Protein was purified as described previously (Shankaranarayanan et al., 2008). All proteins had similar purity and concentration (Supplemental Fig. 1). Biotinylated ΔN-Gαq encoding residues 35-359 of murine $G\alpha_q$ was a gift from Dr. John J. Tesmer (University of Michigan).

Flow Cytometry Protein-Interaction Assays (FCPIA)

The binding between $G\alpha_q$ with MBP-RGS2 and its point mutants were determined by a flow cytometry based assay (Nance et al., 2013; Roman et al., 2007). MBP-RGS2$^{72-203}$ was fluorescently labelled with amine reactive Alexa Fluor (AF) 532 NHS ester (Molecular Probes, Eugene, OR). Biotinylated murine $G\alpha_q$ was attached to xMAP LumAvidin beads (Luminex, Austin, TX). The indicated concentration of unlabeled MBP-RGS2 WT and mutants were used to compete with 80 nM of the Alexa Fluor (AF) 532 labelled WT MBP-RGS2. Binding was

performed as described (Nance et al., 2013) in a full skirt 96 well plate (Bioexpress). Samples were analyzed on a Luminex 200 flow cytometer, collecting at least 100 events per well. Data from at least 4 independent experiments of duplicate samples were fit by nonlinear regression using GraphPad Prism 7. K_i values were calculated using the Cheng-Prusoff equation.

Differential Scanning Fluorimetry Analysis

MBP-RGS2 proteins were cleaved using TEV protease (Promega, Madison, WI) and the RGS2 WT and mutant RGS domains were purified using Ni resin (Supplemental Fig. 2-1). Differential scanning fluorimetry analysis was performed in triplicate in 96 well-plates in final volume of 20 µl/well. Protein (7 µM final) was prepared in 20mM HEPES pH 8.0, 500 mM NaCl, 2mM DTT, 1x protein thermoshift dye (Applied Biosystems, Cat. 4461146) according to manufacturer's protocol. Samples were heated from 25 °C to 99 °C with ramp rate of 1°C in continuous mode in an ABI 7500-Fast Real time PCR (Applied Biosystems). Data from melting curves were analyzed in GraphPad Prism 7 using Boltzmann sigmoidal equation to obtain melting temperature.

Structure modelling

The structure of human RGS2 in complex with murine $G\alpha_q$ R183C (PDB - 4EKD) was modified to mimic the R188H mutant using the Pymol mutagenesis tool. The rotamer of the modified amino acid with least steric interference was chosen on the basis of least overlap of van der Waals radii.

Data Analysis and Statistics

All data were analyzed using GraphPad Prism 7.0 (GraphPad; La Jolla, CA). Dose response curves were fit using nonlinear regression (log(agonist) vs. response - Variable slope (four

parameters)). Datasets with three or more groups were analyzed with one-way analysis of variance with Bonferroni's post hoc test for multiple comparisons, unless indicated in figure legend. Data are presented as mean ± S.E.M, and a P value less than 0.05 was considered significant.

Results

Differential effects of human RGS2 mutants to inhibit AT1 receptor-mediated intracellular calcium release

In the current study, we investigated the biochemical properties of 16 SNPs in the coding region of RGS2 previously identified in humans (Figure 2-1A). As an initial screen to determine the function of RGS2 mutants, CHO cells were co-transfected with cDNAs encoding AT1R (1 μg) and WT or mutant RGS2 (0.75μg). At this ratio, the suppression of AT1R-stimulated Ca^{2+} release by RGS2 was sub-maximal, enabling the identification of both gain- and loss-of function mutations (Supplemental Fig. 2-2). In the absence of RGS2, AT1R stimulation by angiotensin II caused a rapid and transient peak of intracellular calcium $[Ca^{2+}]_i$ (Fig. 2-1 B, C). Maximal $[Ca^{2+}]_i$ obtained from the dose response curves in cells expressing receptor without RGS2 (Fig. 2-1 D, E) was set as 100%. WT RGS2 protein co-expression inhibits peak $[Ca^{2+}]_i$ by 48 ± 2% (Fig. 2-1 B, C, F) and increases the EC_{50} from 4.2±0.3 nM to 7.9±0.7 nM (Supplemental table 2-2) without changing receptor protein level (Supplemental Fig. 2-3). Four mutants including the Q2L, R188H, R44H and D40Y showed reduced function (Fig. 2-1 C, E, F). The 12 remaining mutants had comparable inhibitory effects to RGS2-WT. Therefore, they are considered to have normal function in this screen (Fig. 2-1 B, D, F). There was no significant change in EC_{50} mediated by RGS2 mutants compared to RGS2 WT (Supplemental table 2-2).

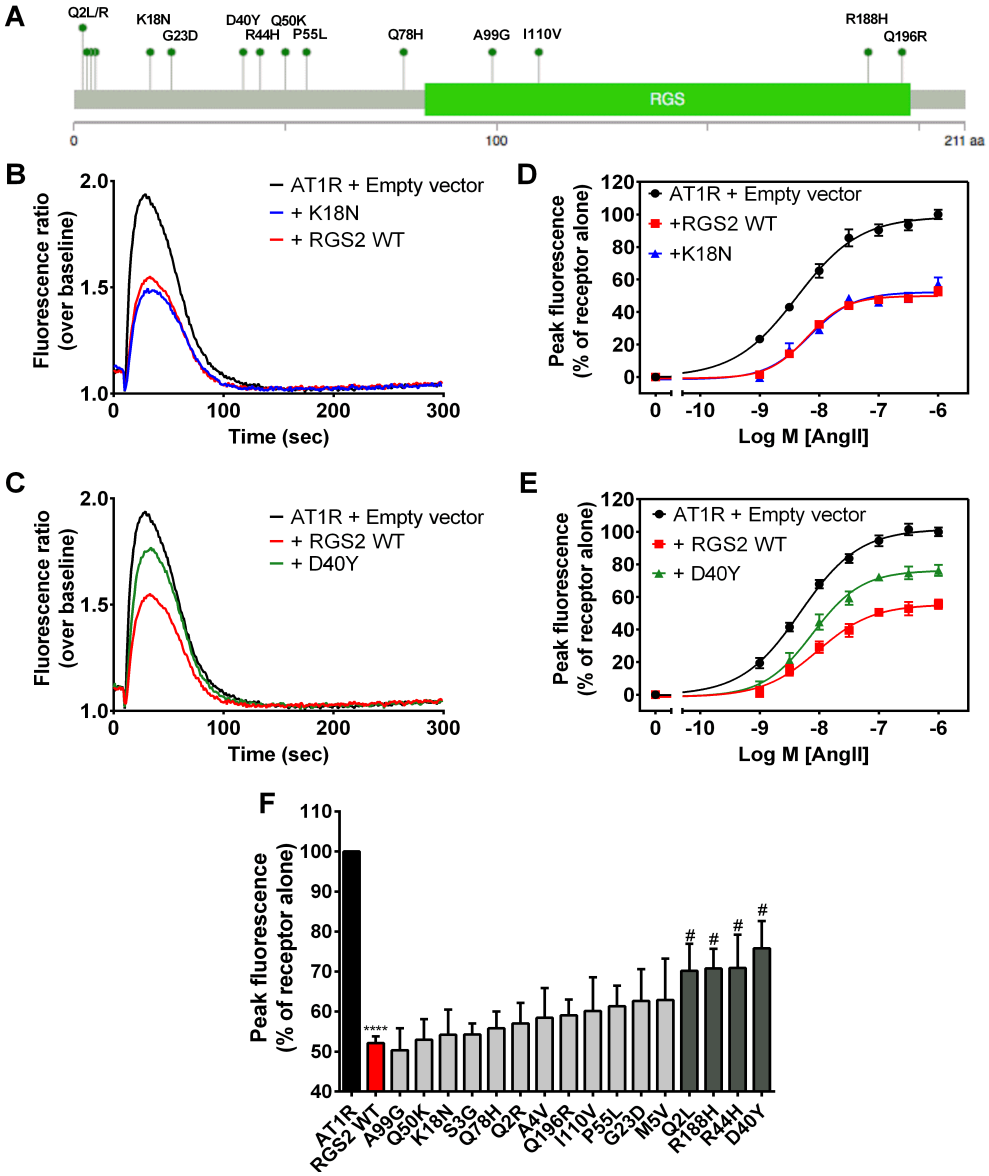


Figure 2-1. **Impact of RGS2 mutations on AT1 receptor-mediated intracellular calcium release.** A. RGS2 mutation map. Needle plot showing the 2D positions of 16 missense mutations on the human RGS2 protein. The plot was visualized by inputting amino acid changes of RGS2 protein to MutationMapper tool (cBioPortal). CHO cells transiently expressing AT1R receptor with or without RGS2 WT or RGS2 mutants were used for measurement of intracellular Ca^{2+}. B, C. Representative fluorescence traces (AngII 1μM) from cells expressing AT1R alone (black), cells co-expressing AT1R and RGS2 WT, K18N (normal function), or D40Y (decreased function). D, E. Concentration response curves. K18N inhibits AT1R-mediated Ca^{2+} as equally as RGS2 WT while D40Y showed reduced inhibition as compared to WT. F. Bar graph of maximal percentage inhibition mediated by RGS2 WT or mutants. Reduced function mutants are shown in dark grey (Q2L, R188H, R44H, D40Y) and mutants that did not show a significant difference from WT (uncorrected $p > 0.05$) are shown in light grey. **** $p < 0.0001$ compared to AT1R + Ctrl, # $p < 0.05$ compared to AT1R+RGS2 WT (n=6-8, Student t-test).

Transient protein expression levels of C-terminal V5 tagged RGS2

Differential protein expression could explain the different effects of these mutations. Specifically, the Q2L mutant has been shown to have low protein levels in HEK-293T (Bodenstein et al., 2007) and HeLa cells (Park et al., 2015) due to enhanced proteasomal degradation. In this study, we expressed C-terminal V5 tagged RGS2 WT and mutant constructs in CHO cells and total cell lysates were probed with V5 tag antibody using Western blot. Consistent with previous work, the Q2L protein levels were also low in CHO cells (60% reduction, $p < 0.05$, Fig. 2-2) due to rapid protein turnover ($t_{1/2}$ Q2L: 6 ± 1 min vs RGS2 WT: 17 ± 4 min) (Supplemental Fig. 2-4). None of the other 15 mutants showed low protein expression as compared to RGS2 WT (Fig. 2-2).

D40Y and R44H mutants are mis-localized

Two of the mutants with reduced function, D40Y and R44H, had disrupted plasma membrane (PM) localization (Fig. 2-3). This had previously been demonstrated for the R44H mutant (Gu et al., 2008). In HEK-293 cells transfected with C-terminal GFP-tagged RGS2, nearly 100% localized in the nucleus when it was expressed alone (Fig. 2-3 A, B, C, I). Co-transfection with $G\alpha_q$ Q209L induced 67% of WT RGS2 to translocate to the PM (Fig. 2-3 E, J, K). The Q2L mutant had a similar translocation level compared to RGS2 WT (Fig. 2-3 K). The R44H mutant, as expected, had less protein localizing to the PM (21 ± 2% less than WT RGS2, $p < 0.0001$, Fig. 2-3 G, J, K). The D40Y mutant was also less efficiently translocated with only 35% protein in the PM (32 ± 2% less than RGS2 WT, $p<0.0001$, Fig. 2-3 F, J, K). A fraction of total R188H-GFP protein formed multiple aggregates throughout the cytoplasm either with or without $G\alpha_q$ Q209L co-expression (white arrow – Fig. 2-3 D, H).

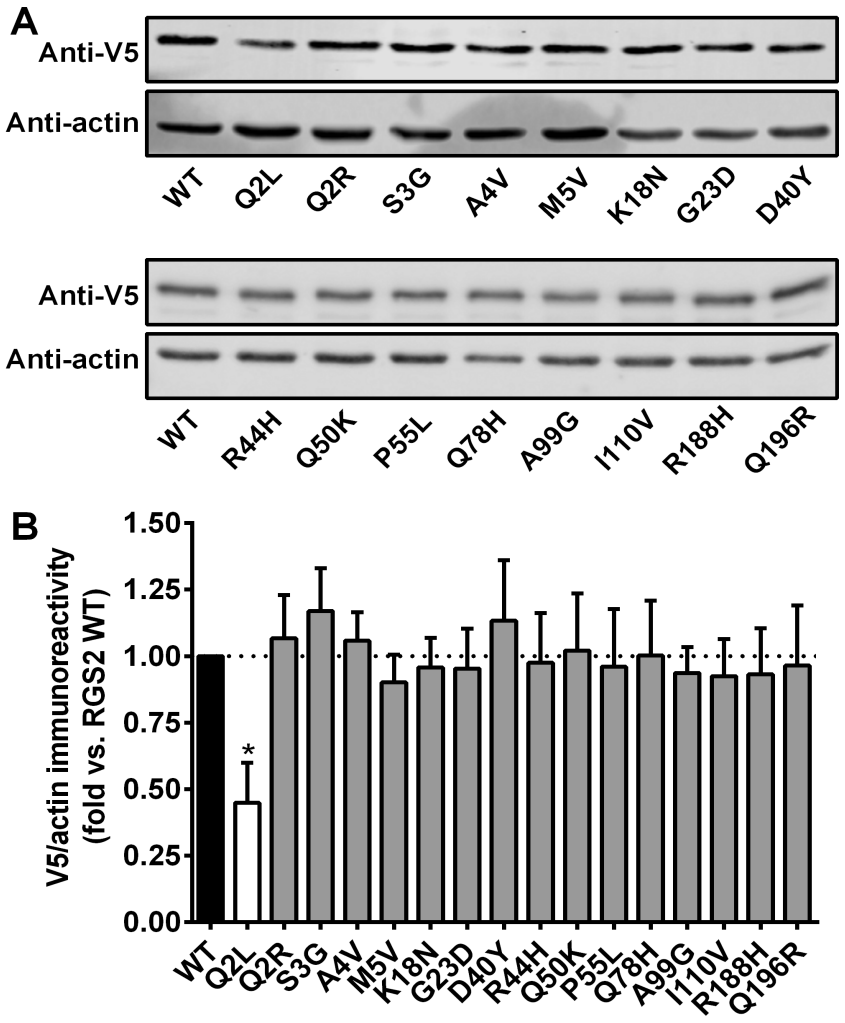


Figure 2-2. **Protein expression of RGS2 WT and mutants.**
CHO cells were transiently transfected with the V5-tagged (RGS2-V5) constructs; RGS2 protein levels were analyzed by anti-V5 Western blot. A. Representative Western blot of 10 independent experiments showing results with total cell lysates of CHO cells expressing RGS2 WT and RGS2 mutant proteins. B. Quantification of band intensities is normalized to the RGS2 WT protein level. Data are presented as mean ± SEM. * $p < 0.05$ (one-way ANOVA with Bonferroni post-test).

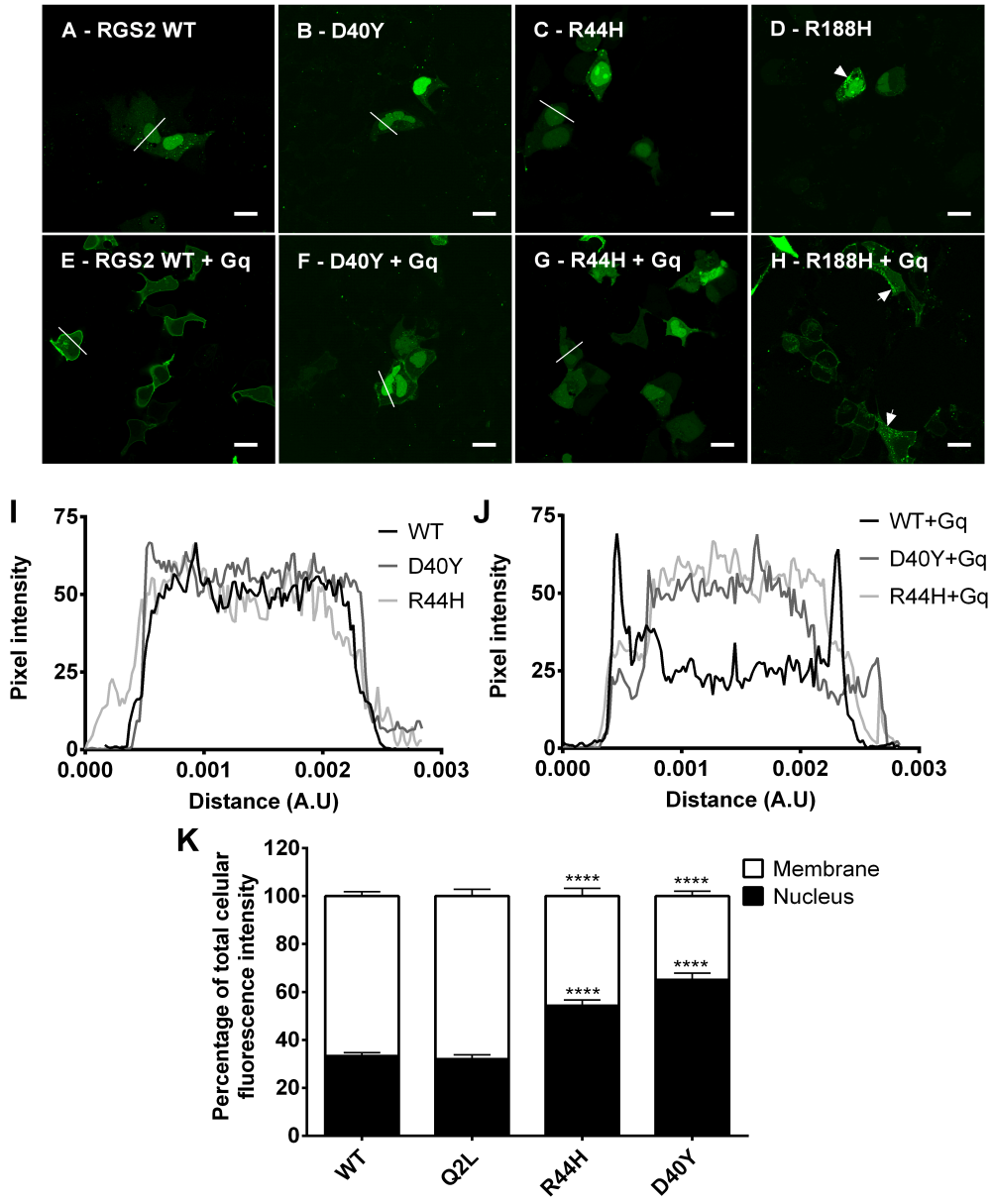


Figure 2-3. **Effects of RGS2 missense mutations on RGS2-GFP localization.**
A-C. When overexpressed in HEK293 cells, RGS2-GFP is localized to the nucleus. D, H. Unlike the other mutants, the R188H-GFP mutant showed punctate intracellular localization. E. Co-expression of WT-RGS2-GFP with constitutively active Gαq results in translocation of the RGS2-GFP to the plasma membrane. F, G. This translocation is impaired with D40Y and R44H; these 2 mutants remained in the nucleus when co-expressed with $G\alpha_q$ Q209L. I. Representative line scans across cells expressing WT, D40Y, R44H RGS2-GFP without $G\alpha_q$. J. Representative line scans across cells expressing WT + $G\alpha_q$, D40Y + $G\alpha_q$ or R44H + $G\alpha_q$. K. Localization of RGS2 in at least 100 cells determined by a blinded observer shows impaired membrane localization of D40Y and R44H. **** $p < 0.0001$, One-way ANOVA with Bonferroni post-test (scale bar: 20 μm).

Biochemical characteristics of RGS2 mutants: RGS2-Gα_q binding and thermal stability

Among the 16 RGS2 mutants investigated, there are 4 mutations located in the RGS domain of RGS2: A99G, I110V, R188H and Q196R. Only the R188H mutant was identified as having reduced function. We hypothesized that that mutation could interfere with the binding of RGS2 and $G\alpha_q$, thereby reducing the GAP activity of RGS2 toward $G\alpha_q$. Binding of fluorescently labelled MBP-RGS2 WT to biotinylated $G\alpha_q$ was measured by Flow cytometry protein-interaction assay (FCPIA). The K_d in saturation binding studies was 83 ± 5 nM. Competition binding measurements were performed, in which various concentrations of unlabeled MBP-RGS2 WT or mutants were mixed with 80 nM fluorescently labelled MBP-RGS2 WT then incubated with AlF_4^- activated $G\alpha_q$ bound to microspheres. Bound, labelled RGS2 was analyzed by flow cytometry. All MBP-RGS2 variants were further purified by gel filtration to ensure similar purity and then concentrated to approximately a 90 μM stock. K_i values of each mutant were calculated from IC_{50} values derived from the curves, using the Cheng-Prussoff equation in GraphPad Prism. Most RGS2 proteins (WT, Q196R, Q78H and A99G) had similar K_i values, 19, 20, 18, 15 nM, respectively. The R188H mutant, on the other hand, had a much lower affinity (K_i 233 nM, Fig. 2-4A).

We also measured the thermal stability of the R188H and Q196R mutants using differential scanning fluorimetry. Melting temperatures (T_m) of WT RGS2 and Q196R were similar, 46.8 and 49°C, respectively. R188H had a significantly lower T_m at 40 °C which reflects markedly lower protein stability ($p < 0.001$, Fig. 2-4B). R188H also had significantly higher basal fluorescence, indicating a less stable protein (Supplemental Fig. 2-5). This measure of protein stability, however,

did not correlate with protein stability in cells. The proteasome inhibitor MG-132 increased protein levels of R188H with a similar magnitude as that of RGS2 WT (Supplemental Fig. 2-6).

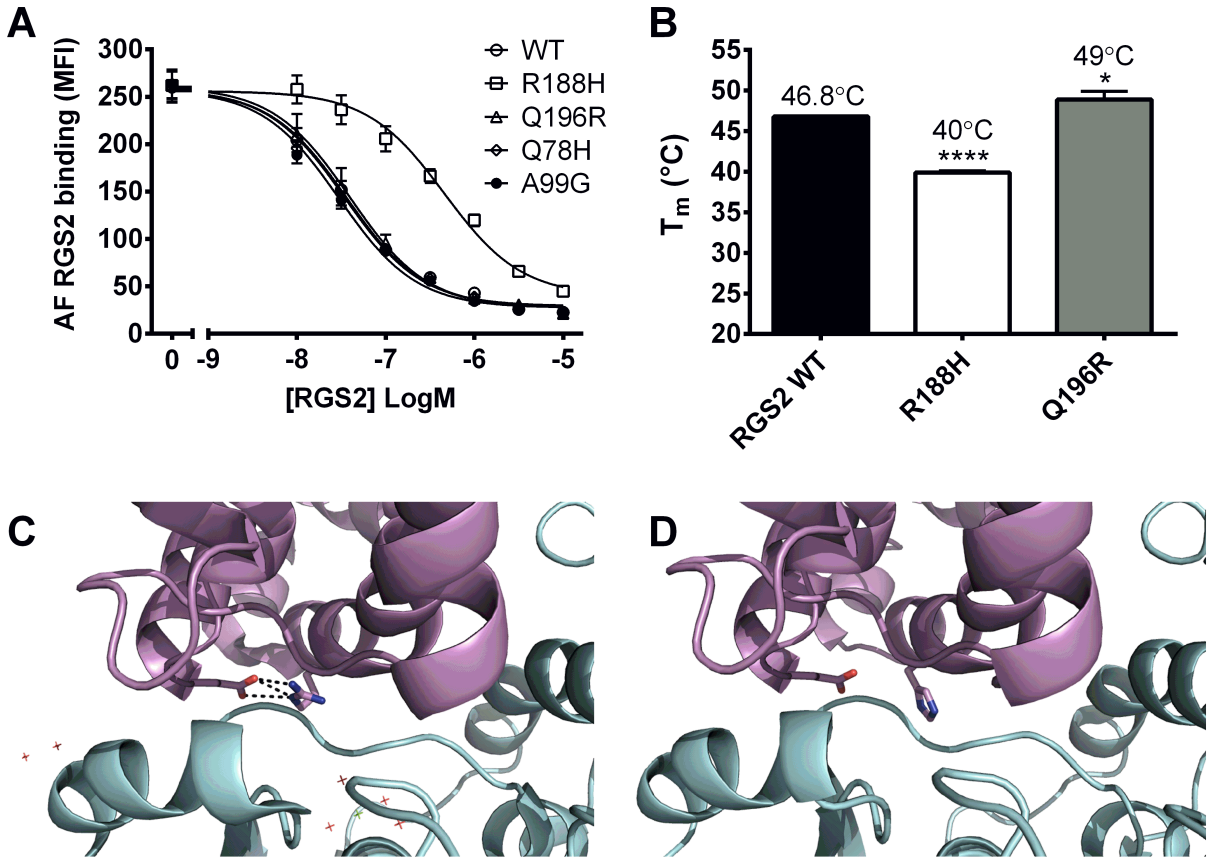


Figure 2-4. **Impaired $G\alpha_q$ binding by the R188H mutant.**
A. Binding affinities of RGS2 proteins with $G\alpha_q$ measured in a bead-based flow cytometry competition binding assay. As described in methods, $G\alpha_q$ was immobilized on beads and mutant proteins were used to compete for binding of AF532 labelled WT-RGS2. The R188H mutant exhibited a reduction in $G\alpha_q$ binding affinity comparing to RGS2 WT protein, demonstrated by the right ward shift in competition binding curve. The Q78H, A99G and Q196R had comparable binding affinities. B. Thermoshift analysis of the RGS spanning domains from RGS2 WT, R188H and Q196R. The bar graph shows the melting temperature of these proteins as mean ± SEM from 3 independent experiments. * $p<0.05$, ****$p<0.0001$ (One-way ANOVA with Bonferroni post-test). C, D. Proposed structural mechanism of the impaired $G\alpha_q$ binding and thermal instability of the R188H mutant RGS2. Structure of the RGS2 domain (violet) – $G\alpha_q$ complex (cyan) (Nance et al., 2013) is shown. C. The WT arginine at position 188 forms salt bridges with the glutamate residue at position 104. D. Histidine substitution at position 188 does not favor salt bridge formation.

Discussion

Over 70 rare non-synonymous RGS2 mutations have been identified in humans through multiple exome sequencing projects and these may contribute to a propensity for hypertension. Of these, 32 mutations are reported in at least 2 individuals, with the Q50K mutant having the highest allele frequency of 0.08% of overall population. Data are publicly available through the Genome Aggregation Database. In this study, we focused on 16 mutations (nonsynonymous polymorphisms) that were implicated in hypertension or found in multiple individuals in both the gnomAD and the NHLBI GO Exome Sequencing project databases. We identified 4 mutations that result in RGS2 proteins that display a functional deficit in inhibiting AT1R-mediated increases in intracellular calcium in CHO cells. Of these 4, the results with the D40Y and R188H mutants are novel while the Q2L and R44H mutants have been investigated in the past. Lower basal protein expression, impaired plasma membrane targeting, and G protein binding deficiency were identified as the mechanisms most likely responsible for the reduced function of these mutants.

Multiple $G\alpha_q$ coupled receptors have been used to probe RGS2 function *in vitro* such as M1 or M3 muscarinic receptors (Bodenstein et al., 2007; Gu et al., 2007; Gu et al., 2008) or the vasopressin receptor (Osei-Owusu et al., 2007). In this study, we chose the angiotensin II receptor because of its relevance in systematic regulation of vascular function and blood pressure (Cameron et al., 2016; de Kloet et al., 2015; Li and Zhuo, 2016). Moreover, RGS2 has been proposed to serve as a selective and potent regulator of AT1R, due to interaction through its amino terminal domains (Hercule et al., 2007; Heximer et al., 2003; Matsuzaki et al., 2011). As 12 of the RGS2 mutations included in the study are located in the RGS2 amino terminal region (Fig. 2-1 A), a functional screen against AT1R could reveal mutations that selectively disrupt RGS2 protein activity by

blocking RGS2-receptor coupling. However, we did not find such mutants in our screen (Fig. 2-1 F). We also did not identify any gain-of-function mutations, despite using conditions that would enable such identification (Supplemental Fig. 2-2).

Low expression of RGS2 protein resulting in prolonged GPCR signaling has been proposed for hypertension in the case of the rare Q2L missense mutation and the common C1114G polymorphism in the 3'-UTR of the RGS2 gene (Park et al., 2015; Semplicini et al., 2006). We further confirmed that the Q2L mutant RGS2 has low protein expression (Fig. 2-2) due to a rapid turnover rate (Supplemental Fig. 2-4 A, C). No other RGS2 mutants that we investigated had low cellular protein levels in our system (Fig. 2-2).

The amphipathic α helix at the amino terminus of RGS2 promotes plasma membrane association and affects protein function (Gu et al., 2007). The R44H mutation has been shown to impair RGS2 membrane targeting by interference with lipid bilayer association (Gu et al., 2008). In this study, we confirmed that effect of the R44H mutation (Fig. 2-3 C, G, J, K). We also identified another mutation in this α helix, D40Y, that exhibited reduced plasma membrane localization (Fig. 2-3 B, F, J, K) and function (Fig. 2-1 C, E, F). Unlike the R44H mutant in which both residues have a positive charge, the amino acid substitution in D40Y changes from the negatively charged aspartic acid to the hydrophobic tyrosine residue. This more dramatic alteration could explain the somewhat larger effect on $G\alpha_q$-dependent membrane localization (R44H: 46% of control vs D40Y: 35%, Fig. 2-3 K).

While protein expression levels and membrane localization indirectly affect the negative regulatory effects of RGS2 on $G\alpha_q$-coupled receptor signaling, the interaction between RGS2 and $G\alpha_q$ protein is absolutely critical for RGS2 function. The published crystal structure of the RGS2

domain/$G\alpha_q$ protein complex shows that the arginine residue at position 188 is close to the binding interface between two proteins (Nance et al., 2013). It may also form networked salt bridges (Donald et al., 2011) with the glutamate residue at position 104 to stabilize protein tertiary structure (Fig. 2-4C). When this arginine is mutated to a histidine residue, the histidine rotamer with the least steric hindrance may not be sufficiently close to E104 to form a salt bridge, potentially rendering RGS2 more flexible and less structurally stable (Fig. 2-4D). Besides the lower melting temperature (Fig. 2-4B), the initial fluorescence of the R188H mutant with the thermostability dye was noticeably higher than that of the WT and Q196R proteins (Supplemental Fig. 2-5). This suggests a disruption of basal protein folding. This misfolding could also explain the aggregation of the R188H-GFP in cells in the localization study. Disruption of the binding interface and structural instability properties of the R188H may explain the reduced binding affinity to $G\alpha_q$. Further investigation demonstrated that this mutant has slightly shorter half-life than WT RGS2 (16 ± 1.5 min vs 22 ± 1.3 min, N.S., Supplemental Fig. 2-3 B, D) and is also subjected to proteasomal degradation (Supplemental Fig. 2-6).

The regulatory function of RGS2 in cardiovascular homeostasis is not limited to its inhibitory effect toward $G\alpha_q$ signaling. RGS2 can also control protein synthesis through interaction with the eukaryotic initiation factor, eIF2B which has been implicated in protection against cardiac hypertrophy (Chidiac et al., 2014; Nguyen et al., 2009). RGS2 may also interact with $G\alpha_s$ and several adenylate cyclase subtypes to decrease cAMP production in cell-based assays (Roy et al., 2006; Salim et al., 2003). Overall, our study mainly focused on the canonical RGS2 effect to reduce $G\alpha_q$ signaling. How RGS2 mutants function with respect to these other mechanisms has not been tested so the 12 mutants with "normal" function here may be perturbed in other ways.

Some common polymorphisms in regulatory regions of RGS2 gene such as in-del mutations in the 3' UTR have been shown to contribute to cardiovascular diseases such as hypertension and responsiveness to anti-hypertensive treatment (He et al., 2015; Kvehaugen et al., 2014; Riddle et al., 2006; Semplicini et al., 2006; Zhang et al., 2013). In this study, we demonstrate that rare mutations in the protein coding region of RGS2 can affect protein function at least in 3 different ways (protein stability, localization, and G protein binding), and result in signaling deregulation. How the function of RGS2 mutants *in vitro* correlates with their activities in vascular tissue or in human physiology will need to be determined. The results from this study could guide the development of RGS2 transgenic animal models to derive further knowledge about the activity of rare RGS2 mutations *in vivo*. It will also provide a strategy to selectively target these mechanisms with directed repurposed or novel cardiovascular therapeutics.

APPENDIX

APPENDIX

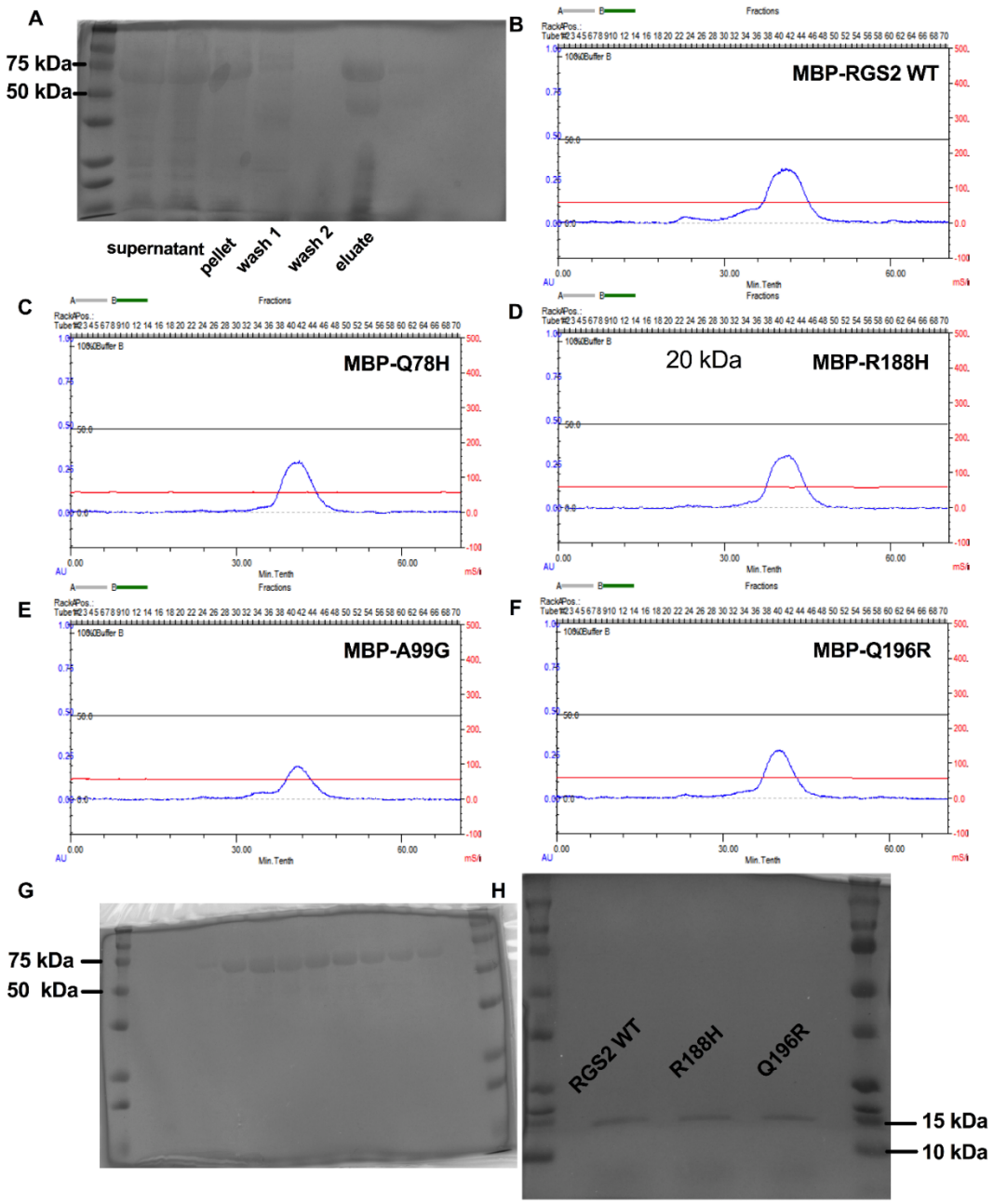
Supplemental Table 2–1. **RGS2 cloning and mutagenesis primers.**

cDNA	Mutation	Primer Sequence
RGS2		RGS2 Gateway forward 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGA AGGAGATAGAACCATGCAAAGTGCTATGTTCTTGGC 3'
		RGS2 Gateway reverse 5' GGGGACCACTTTGTACAAGAAAGCTGGGTATGTA GCATGAGGCTCTGTGGTG 3'
	Q2L	Forward 5' TGCTAAGTGCTATGTTCTTGGCTGTTC 3'
		Reverse 5' CGTGTTGAACAGCCAAGAACATAGCACTTAGC 3'
	Q2R	Forward 5' GAGATAGAACCATGCGAAGTGCTATGTTCTTGG 3'
		Reverse 5' CCAAGAACATAGCACTTCGCATGGTTCTATCTC 3'
	S3G	Forward 5' GATAGAACCATGCAAGGTGCTATGTTCTTGG 3'
		Reverse 5' CCAAGAACATAGCACCTTGCATGGTTCTATC 3'
	A4V	Forward 5' GAACCATGCAAAGTGTTATGTTCTTGGCTG 3'
		Reverse 5' CAGCCAAGAACATAACACTTTGCATGGTTC 3'
	M5V	Forward 5' GCAAAGTGCTGTGTTCTTGGCTGTTC 3'
		Reverse 5' GAACAGCCAAGAACACAGCACTTTGC 3'
	K18N	Forward 5' GCAGACCCATGGACAACAGCGCAGGCAGTGGC 3'
		Reverse 5' GCCACTGCCTGCGCTGTTGTCCATGGGTCTGC 3''
	G23D	Forward 5' GAGCGCAGGCAGTGACCACAAGAGCGAG 3'
		Reverse 5' CTCGCTCTTGTGGTCACTGCCTGCGCTC 3'
	R44H	Forward 5' GATTGGAAGACCCATTTGAGCTACTTC 3'
		Reverse 5' GTAAGAAGTAGCTTCAAATGGGTCTTCCAATC 3'
	Q50K	Forward 5' GAGCTACTTCTTAAAAAATTCCTCTACTCCTGGG 3'
		Reverse 5' CCCAGGAGTAGAGGAATTTTTTAAGAAGTAGCTC 3'
	P55L	Forward 5' CAAAATTCCTCTACTCTTGGGAAGCCCAAAACC 3'
		Reverse 5' GGT TTT GGG CTT CCC AAG AGT AGA GGA ATT TTG 3'
	Q78H	Forward 5' CCTGAGGAAGCACACCTGTGGTCAGAAGC 3'
		Reverse 5' GCTTCTGACCACAGGTGTGCTTCCTCAGG 3'
	A99G	Forward 5' GCTGCATTCAGGGGTTTTTTAAAGTCGG 3'
		Reverse 5' CCGACTTTAAAAAACCCCTGAATGCAGC 3'
	I110V	Forward 5' CTGTGAAGAAAATGTTGAATTCTGGCTGGCC 3'
		Reverse 5' GGCCAGCCAGAATTCAACATTTTCTTCACAG 3'
	R188H	Forward 5' CTCTTATCCTCATTTCTTGGAGTCAG 3'
		Reverse 5' CTGACTCCAAGAAATGAGGATAAGAG 3'
	D40Y	Forward 5' CCC TTT TAA AAT ATT GGA AGA CCC GTT TG 3'
		Reverse 5' CAA ACG GGT CTT CCA ATA TTT TAA AAG GG 3'
	Q196R	Forward 5' CAG AAT TCT ACC GGG ACT TGT GTA AAA AG 3'
		Reverse 5' CTT TTT ACA CAA GTC CCG GTA GAA TTC TG 3'

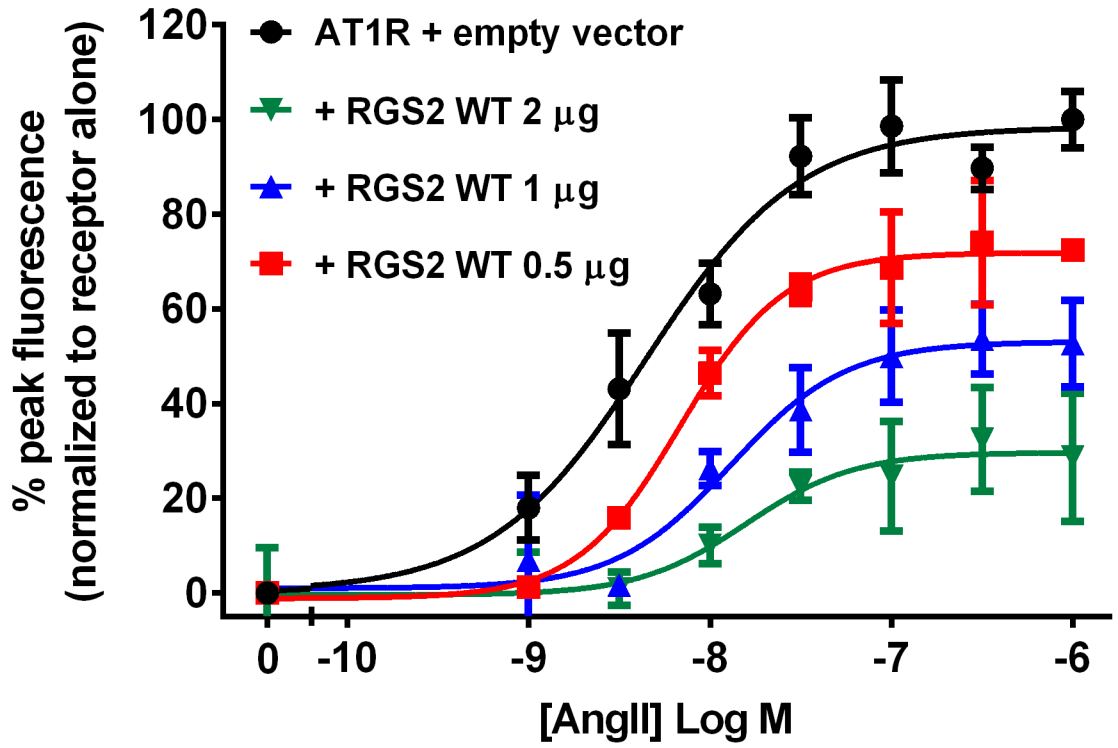
Supplemental Table 2–2. **Effect of RGS2 WT and mutant on AT1 receptor-mediated intracellular calcium release demonstrated by Max and EC_{50} derived AngII concentration response curves.**

(Data are presented as mean ± SEM, n≥6, * p< 0.05, *** p<0.001, compared to Ctrl; # p<0.05 compared to RGS2 WT)

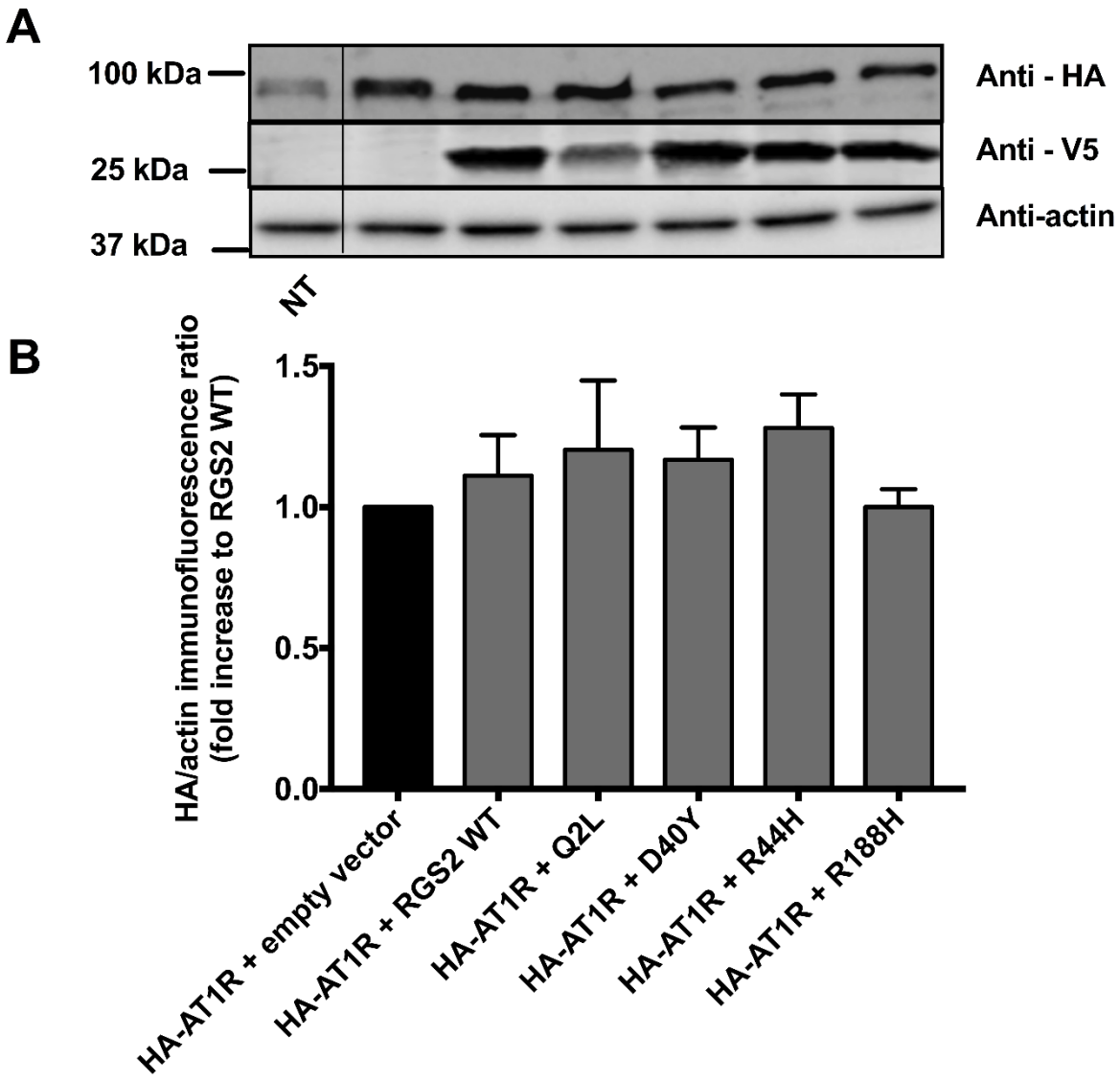
RGS2 mutant	Max (% of AT1R + Ctrl)	EC_{50} (nM)
Ctrl	100	4.2 ± 0.3
RGS2 WT	52.1 ± 1.4*	7.9 ± 0.7***
Q2L	70.2 ± 5.2#	7.0 ± 0.9
Q2R	57 ± 4.4	7.6 ± 0.5
S3G	54.3 ± 1.3	8.0 ± 0.4
A4V	58.5 ± 3.3	8.4 ± 1
M5V	62.9 ± 4.3	10.7 ± 2.4
K18N	54.2 ± 3	11.7 ± 1.2
G23D	62.7 ± 6.9	10.3 ± 1.5
D40Y	75.8 ± 3.1#	10.9 ± 2
R44H	70.9 ± 3.7#	8.4 ± 1.1
Q50K	53 ± 4.7	9.0 ± 2.3
P55L	61.4 ± 2.6	7.3 ± 0.8
Q78H	55.8 ± 3.3	7.5 ± 0.5
A99G	50.4 ± 5	9.2 ± 1.8
I110V	60.2 ± 5.1	9.6 ± 1.6
R188H	70.8 ± 4.3#	9.5 ± 1.5
Q196R	59.1 ± 3.1	7.2 ± 0.2



Supplemental Figure 2–1. **MBP-RGS2 protein purification.**
A. Coomassie staining of different fractions during Ni^{2+}-affinity chromatography. B-F. Size exclusion chromatography of MBP fusion - RGS2 WT, Q78H, R188H, A99G, and Q196R, showing similar protein purity. G. Coomassie staining of gel filtration fraction 37-44 (peak) of each protein, which were collected and concentrated to similar concentration at ~ 5 μM for FCPIA assay. H. Coomassie staining of cleaved RGS2 WT, R188H and Q196R.

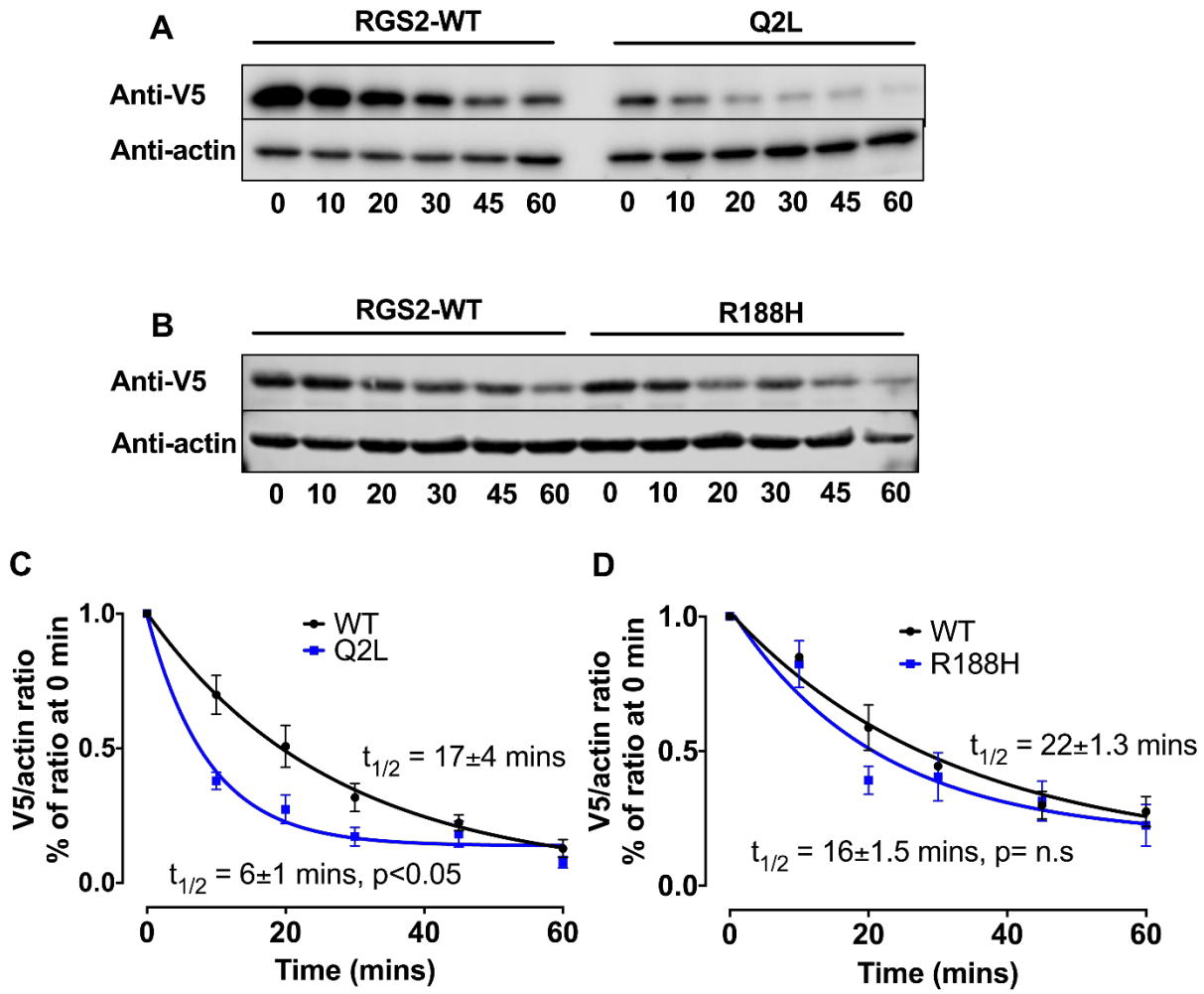


Supplemental Figure 2–2. **Increasing amount of RGS2-V5 increases suppression of AT1R-mediated Ca^{2+} mobilization.**
CHO cells were transfected with AT1R alone or with different amounts of V5-tagged RGS2 WT. 48 hours post transfection, cells were assayed for Ca^{2+} mobilization as described in Materials and Methods. Increasing amounts of RGS2 enhances suppression of AT1R-mediated Ca^{2+} mobilization. The amount used for testing mutated RGS2 (0.75µg) was chosen to enable the identification of both gain- and loss-of-function mutations. Data are presented as mean ± SEM, n=3.

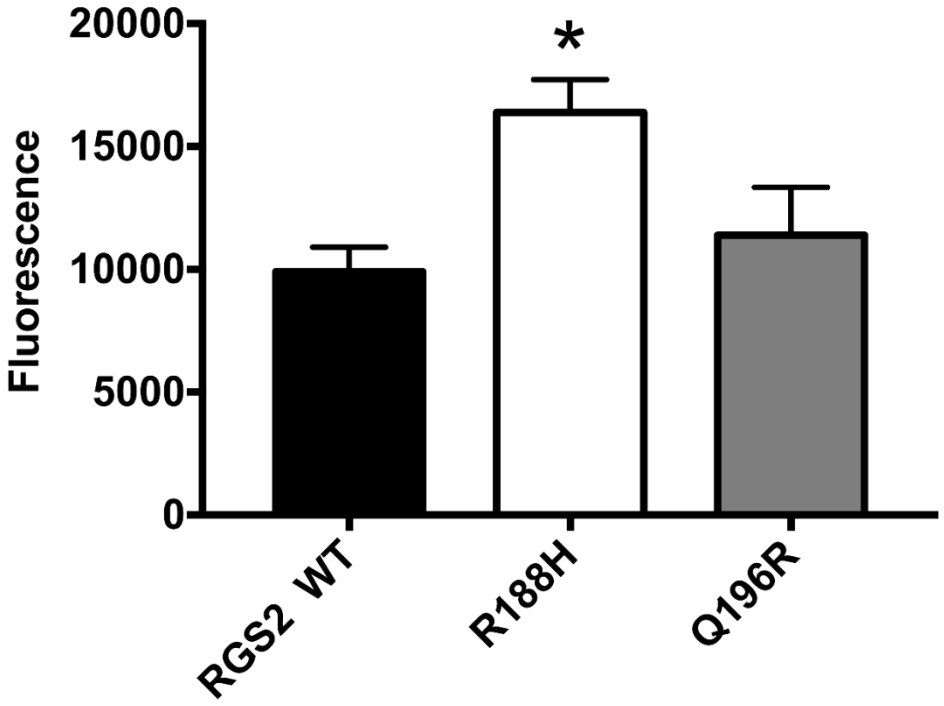


Supplemental Figure 2–3. **Co-expression with RGS2 WT or mutants does not change HA-AT1R protein expression. CHO cells transiently transfected with HA-AT1R with or without RGS2-V5 constructs.**

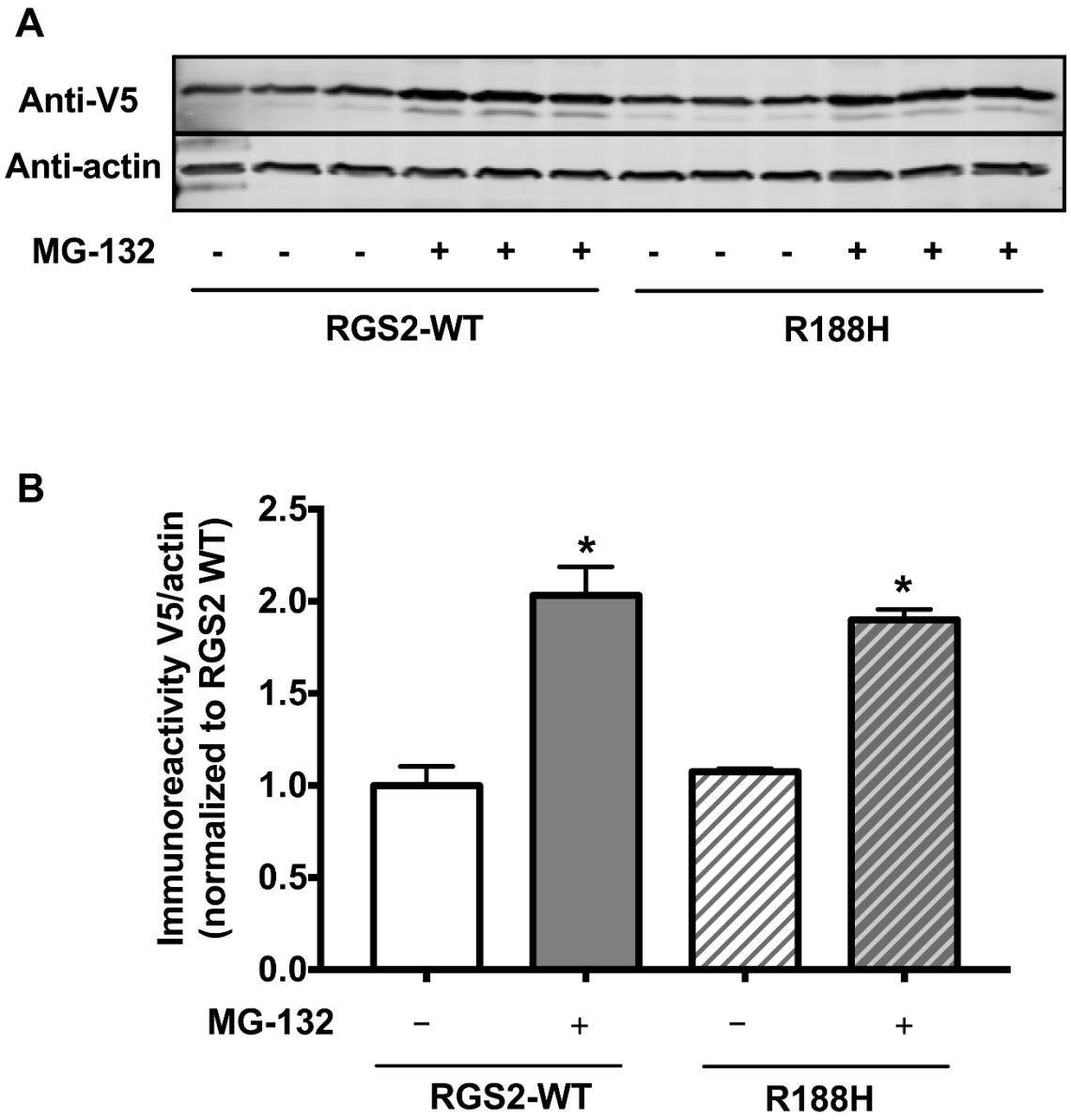
A. Representative Western blots demonstrating receptor level with or without RGS2-V5 co-expression. B. Quantified expression of HA-AT1R protein (relative to actin; n=3) normalized to cell lysate expressing receptor alone is shown. No significant change in receptor level was detected. HA-AT1R was used due to the fact there are no good commercially available AT1R antibodies (Elliott et al., 2013; Herrera et al., 2013). Complementary Ca^{2+} assays were run with the HA-tagged AT1R to demonstrate that it was functional and that RGS2 could suppress the Ca^{2+} mobilization (data not shown). NT: CHO cell lysate without HA-AT1R transfection (same gel).



Supplemental Figure 2–4. **Protein half-live of the Q2L and R188H.**
CHO cells transiently transfected with RGS2-V5 constructs were treated with cycloheximide (10 µg/ml) to inhibit protein translation. A, B. Representative Western blots of WT, Q2L and R188H after treatment with cycloheximide (n≥3). C, D. RGS2 decay curves. Effects of cycloheximide (10 µg/ml) treatment over a period of 60 minutes. RGS2 protein (relative to actin) at each time point was normalized to the corresponding 0 min time point.



Supplemental Figure 2–5. **Initial fluorescent intensity of RGS2 WT, R188H and Q196R in DSF analysis.**
Cleaved RGS2 WT, R188H and Q196R proteins were incubated with thermoshift dye as described in methods. Fluorescent intensities prior to heating were plotted. R188H displayed a significantly higher basal fluorescence compared to RGS2 WT and Q196R. *$p<0.05$, One-way ANOVA with Bonferroni post-test.



Supplemental Figure 2–6. **Effect of proteasomal inhibition on expression of R188H. CHO cells transiently transfected with RGS2-V5 constructs were treated with MG-132 to inhibit proteasomal degradation.**

A. Representative Western blots (n≥3) demonstrating effects of MG-132 (10 μM) treatment over a period of 3 hours on cellular levels of RGS2 WT and R188H protein. B. Quantified expression of each RGS2 protein relative to RGS2 WT without MG-132 treatment is shown. * $p<0.05$ using one-way ANOVA with Bonferroni posthoc test.

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CHAPTER 3: LOSS-OF-FUNCTION MUTATIONS IN HUMAN RGS2 DIFFERENTIALLY REGULATE PHARMACOLOGICAL REACTIVITY OF RESISTANCE VASCULATURE

Abstract

Regulator of G protein signaling 2 (RGS2) plays a significant role in reducing vascular contraction and promoting relaxation due to its GTPase accelerating protein activity toward Gαq, a G protein involved in vasoconstriction. Through a Ca^{2+} mobilization assay, we identified 4 human loss-of-function (LOF) mutations in RGS2 (Q2L, D40Y, R44H and R188H). The present 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 vasoconstriction. Reintroduction of a wild-type RGS2-GFP plasmid into $RGS2^{-/-}$ MAs suppressed the vasoconstrictor response to angiotensin II. RGS2 LOF mutants failed to suppress the angiotensin II constriction response compared to RGS2 WT. In contrast, ACh-mediated vasoconstriction was restored by expression of RGS2 WT, D40Y and R44H but not by RGS2 Q2L or R188H. Phosphorylation of RGS2 D40Y and R44H by PKG1α may explain their maintained function to support relaxation in MAs. This is supported by phosphomimetic mutants and suppression of vasorelaxation mediated by RGS2 D40Y by a protein kinase G inhibitor.

These results demonstrate that RGS2 attenuates vasoconstriction in MAs and that RGS2 LOF mutations cannot carry out this effect. Among them, the Q2L and R188H mutants supported less relaxation to acetylcholine while relaxation mediated by the D40Y and R44H mutant proteins was equal to that with WT protein. Phosphorylation of RGS2 by PKG1α appears to contribute to

this vasorelaxation. These results provide insights for precision medicine targeting this rare population carrying RGS2 mutations.

Introduction

G protein-mediated signaling is critical for the regulation of cardiovascular function, including heart rate, cell growth, and vascular tone. Blockers of GPCR signaling (angiotensin receptor blockers, beta and alpha 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 Gαi/o and Gαq subunits, thereby reducing the amplitude and duration of GPCR/G protein signaling. Among 20 RGS proteins, RGS2 is best known for its role to control vascular constriction and relaxation in vascular smooth muscle cells, where it is regulated by nitric oxide (Sun et al., 2005a; Tang et al., 2003a).

RGS2 negatively regulates Gαq, which transduces signals from a variety of vasoconstrictors. Consistent with a role in vascular control, RGS2 knockout mice were hypertensive (Heximer et al., 2003a) and hypertensive human patients had reduced RGS2 mRNA in peripheral blood mononuclear cells compared to controls (Semplicini et al., 2006b). Further, 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., 2006b). 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., 2005b). Subsequent study confirmed loss of function (LOF) of these SNPs in vitro and defined mechanisms (Bodenstein et al., 2007a; Gu et al., 2008b). We recently identified four LOF mutations in human RGS2 (Phan et al., 2017) from a set of 16 variants of unknown significance (VUS) found in the Genome Aggregation Database or gnomAD (Lek et al., 2016). Though

biochemically characterized, the functional consequences of these mutations in the complex milieu of vascular tissue are unknown. Here, we utilized 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 cell-based assay would have LOF physiological consequence in the MAs.

Experimental procedures

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 and RGS2$^{-/-}$ genotypes were obtained from Dr. Kendall Blumer at Washington University St. Louis (Osei-Owusu et al., 2015). Mice were provided access to food and water ad libitum in the MSU animal facility on a 12h light/dark cycle. All of the experiments were performed using 3–5 month old male mice. Mice were anesthetized by isoflurane, followed by removal of the mesentery and 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), acetylcholine (ACh) (A6625) were purchased from Sigma – Aldrich.

Pressure myography

Mesenteric arteries (MAs) were isolated free of adipose and connective tissue in a physiological salt solution (PSS) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, 10 mM HEPES, 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) as previously described (Diaz-Otero et al., 2016). Arteries

were pressurized and then equilibrated for 60 min in PSS containing 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2.2H_2O$, 1 mM $MgCl_2.6H_2O$, 10 mM HEPES, and 10 mM dextrose at 37°C. The baseline internal diameter and changes of diameter in response to vasoactive substances was recorded at 80 mm Hg and 37 °C using MyoVIEW II 2.0 software (Danish Myo Technology, Aarhus, Denmark). All MAs were challenged with 3μM PE to test their reactivity before experiments were performed. MAs that did not contract upon PE addition were discarded. Data are expressed as percentage of buffer baseline.

Wire myography

Methods were described previously (Russell and Watts, 2000) with modification. Aortas from $RGS2^{+/+}$ and $RGS2^{-/-}$ mice were isolated free of fat and connective tissue and cut into rings (2-3 mm in length). The aortic rings were mounted on two L-shaped stainless-steel wires inserted into the lumen and immersed in muscle baths filled with warm, aerated physiological salt solution (130 mM NaCl, 4.7 mM KCl, 1.18 mM KH_2PO4, 1.17 mM $MgSO_4.7H_2O$, 1.6 mM $CaCl_2.2H_2O$, 14.9 mM $NaHCO_3$, 5.5 mM dextrose, and 0.03 mM $CaNa_2EDTA$). One end of the preparation was attached to a stainless steel rod, the other was attached to a force transducer (FT03; Grass Instruments, Quincy, MA). The basal tension of the aortic ring was maintained at 0.5 g. Changes in isometric force after adding PE in a cumulative manner were recorded on a Grass polygraph (Grass Instruments).

Reversible permeabilization

Reversible permeabilization of isolated MAs to introduce RGS2 plasmids to the MAs was achieved by a modification of previous methods (Lesh et al., 1995; Welsh et al., 2002). Briefly, MAs from $RGS2^{-/-}$ mice were loaded with RGS2-GFP constructs by sequential incubation in the

following solutions (in mM): (1) 10 EGTA, 120 KCl, 5 Na_2ATP, 2 $MgCl_2$, 20 TES (pH 6.8; 120 min, 4 °C); and (2) 0.1 EGTA, 120 KCl, 5 Na_2ATP, 2 $MgCl_2$, 20 TES and 1 μg/ml of RGS2-GFP plasmids (pH 6.8; overnight, 4 °C). Subsequently, arteries were transferred to following solutions (in mM): (3) 0.1 EGTA, 120 KCl, 5 Na_2ATP, 10 $MgCl_2$, 20 TES (pH 6.8; 30 min, 4 °C); (4) 140 NaCl, 5 KCl, 10 $MgCl_2$, 5.6 glucose, and 2 MOPS (3-[N-morpholino] propane-sulfonic acid) (pH 7.1, 30 min, room temperature), then [Ca^{2+}] was gradually increased from 0.001 to 0.01 to 0.1 to 1.6 to 2.4 mM every 10 min by adding $CaCl_2$ to solution 4. Arteries were then incubated in DMEM/F-12 culture medium (Cat. 11039021, Gibco) supplemented with 50 U/ml penicillin, and 50 mg/ml streptomycin and maintained at 37 °C, 5% CO_2 in an incubator.

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 min, then incubated in blocking buffer - PB buffer containing 4% donkey serum (Sigma-Aldrich, St. Louis, MO, Cat # D9663, Lot # SLBQ9773V) for 30 min and then with rabbit anti-GFP antibody (Life Technologies, Carlsbad, CA, Cat. A6455) at 1:500 dilution at 4°C overnight. The arteries were then rinsed with 0.2%Triton X-100 in PBS and incubated in Cy3-anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, Code # 711-165-152, Lot # 131748) at 1:300 dilution at room temperature. The arteries were mounted using ProLong™ Gold Antifade Mountant with DAPI (Life Technologies, Carlsbad, CA, Cat. P36941) and visualized using a 40x 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_2. Human embryonic kidney (HEK)-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin. Chinese hamster ovary (CHO-K1) cells (ATCC, Manassas, VA) were maintained in F-12 nutrient mixture (GIBCO, Carlsbad, CA), with the supplementation mentioned above. X-tremeGENE HP DNA Transfection Reagent (Roche Life Sciences) was used to transfect HEK-293 cells at 2 ml/mg of plasmid DNA. DNA-In® CHO (MTI-GlobalStem, Gaithersburg, MD) was used to transfected CHO-K1 cells at 3 ml/mg of plasmid DNA according to the manufacturers' recommended protocols. Transfection mixes were prepared in antibiotic-free Opti-MEM (GIBCO, Carlsbad, CA). Experiments were run 24-48 hours after transfection.

Confocal microscopy

HEK293 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 $G\alpha_q^{Q209L}$ or pcDNA3.1 as control. An Olympus FluoView 1000 laser scanning confocal microscope with a 60x oil immersion objective using 488 nm excitation and 505–530 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 3 independent experiments were acquired. Densitometric quantitation of protein expression was performed in a blinded manner using the line scan analysis function of the ImageJ(Schneider et al., 2012).

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 removing the media, cells were loaded with 1X Fluo-4 NW (Molecular Probes, Eugene, OR) in a buffer containing Hank's basal saline solution (HBSS) supplemented with 20 mM HEPES, pH 7.4 and 2.5 μM probenecid. The cell plates were incubated for 30 min at 37°C, followed by 30 min at room temperature. The Fluo-4 NW was removed and loading buffer including HBSS supplemented with 20 mM HEPES was added at 20 μl/well to the cell plate. A concentration gradient of angiotensin II (Sigma, Cat. A9525) at 2x the final concentration was freshly prepared in loading buffer supplemented with 0.1% BSA for automated injection into the wells at 20 μl/well by a FDSS/μCell kinetic fluorescence plate reader (Hamamatsu Photonics, Japan). Assay plates were then placed onto the plate reader for measuring the changes of intracellular free calcium in response to the receptor activation.

Statistical analyses

Data are presented as means ± SE. Vessel diameter data were analyzed by Student's t-test or two-way analysis of variance 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 figure legends.

Results

RGS2 deficiency enhances vascular contractility to phenylephrine and angiotensin II and causes impairment of acetylcholine-mediated vasorelaxation

Vascular responses of RGS2-deficient arteries to vasoconstrictors and vasodilators have been extensively studied in different vascular beds including aorta, mesenteric artery, renal interlobar and uterine artery (Hercule et al., 2007; Jie et al., 2016; Osei-Owusu et al., 2012; Tang et al., 2003b). 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 $RGS2^{+/+}$ and $RGS2^{-/-}$ mice were used to determine how RGS2 protein affects vascular reactivity to different vasoactive substances. Phenylephrine, which activates the Gαq-coupled α1 adrenergic receptor, caused a concentration-dependent contraction in $RGS2^{+/+}$ MAs and aorta; this response was significantly augmented in $RGS2^{-/-}$ aortic rings in wire myography (Fig. 3-1B) but not in pressurized $RGS2^{-/-}$ MAs (Fig. 3-1A). The MAs isolated from $RGS2^{-/-}$ mice did show increased responsiveness to 5 nM AngII (Fig. 3-1C, 28 ± 1.8% contraction in $RGS2^{-/-}$ vs 10 ± 2.6% in $RGS2^{+/+}$, $p < 0.01$, Student t-test). Also, the maximum of ACh-mediated vasodilation in $RGS2^{-/-}$ MA pre-constricted with 3 μM PE was markedly impaired (Fig. 3-1D, 48.6 ± 8.4% relaxation in $RGS2^{-/-}$ vs 92.7 ± 6.2% relaxation in $RGS2^{+/+}$, ** $p < 0.01$, **** $p < 0.0001$, two-way ANOVA with Sidak post-test). We chose to focus on testing 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 et al., 1993).

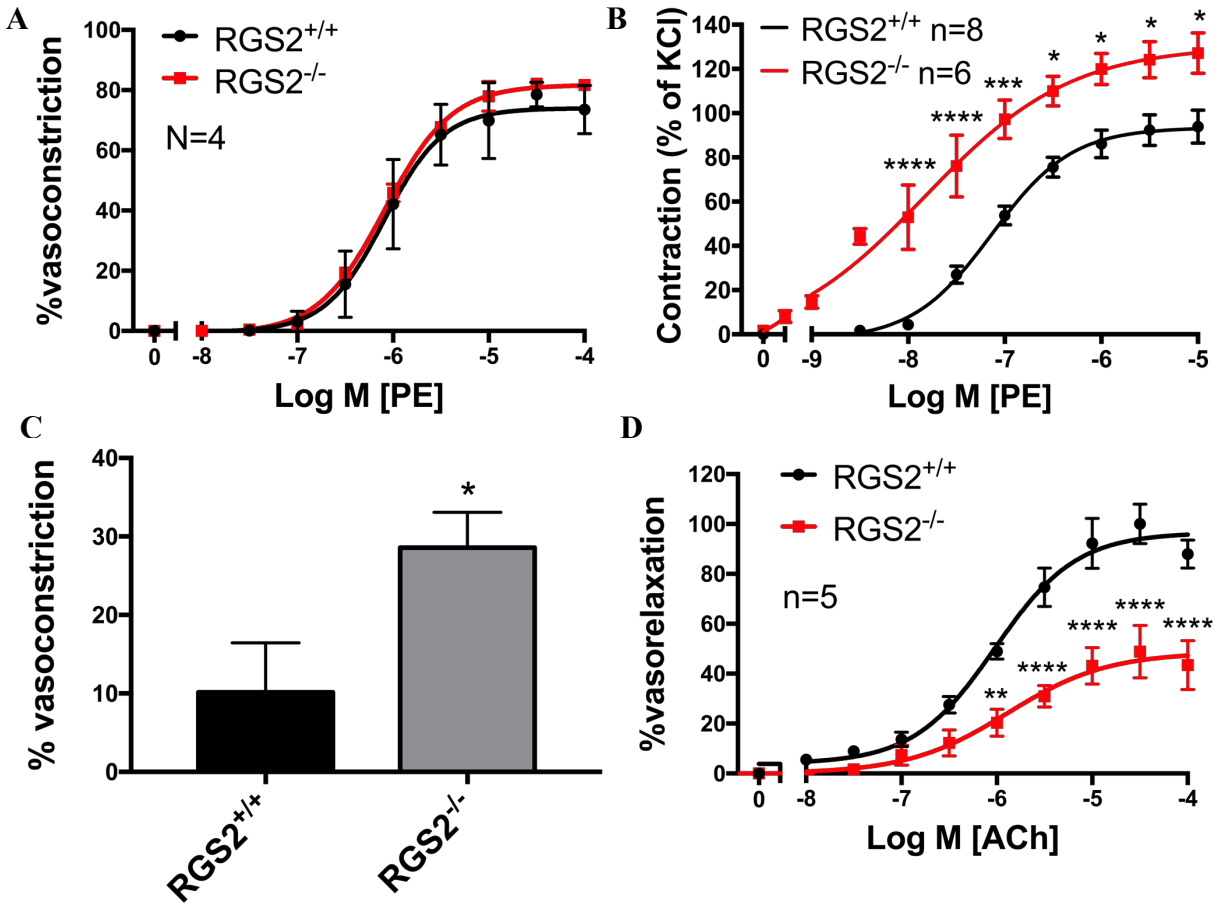

Figure 3-1. **Vascular reactivities of RGS2$^{+/+}$ and RGS2$^{-/-}$ arteries to the vasoconstrictors phenylephrine (PE) and angiotensin II (AngII) and the vasodilator acetylcholine (ACh).** A. Mesenteric arteries from RGS2$^{+/+}$ and RGS2$^{-/-}$ mice responded similarly to PE. B. Aorta isolated from RGS2$^{-/-}$ mice showed greater sensitivity to PE than aorta from RGS2$^{+/+}$ mice. C. Mesenteric arteries response to AngII. D. Concentration response of ACh-induced vasodilation expressed as percentages of increase in arterial diameter after constriction with PE ($3x10^{-6}$ M) (n=5). The data are expressed as the mean ± S.E; **, ***, ****, $p < 0.01$, 0.001, 0.0001, respectively, versus WT (two-way ANOVA) with Sidak post-test.

Expression of RGS2-GFP in RGS2$^{-/-}$ 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 is present in the MA between 48-72 hours after reversible permeabilization but absent with a pcDNA3.1 transfection control (Fig. 3-2A). The staining also showed that the Q2L-GFP appeared to have lower intensity compared to WT and other mutant RGS2 in the MAs which aligned with the low protein level of the Q2L mutant in CHO cells (Phan et al., 2017). Further characterization was done by performing z-stack imaging by confocal microscopy. RGS2-GFP was expressed in the vascular smooth muscle cell layer and virtually absent RGS2-GFP in the endothelial cell layer (Fig. 3-2B), based on the depth at z direction in the z-stack and morphology of cell nuclei (elongated nuclei in VSMCs and round nuclei in endothelial cells - Luo et al., 2016)

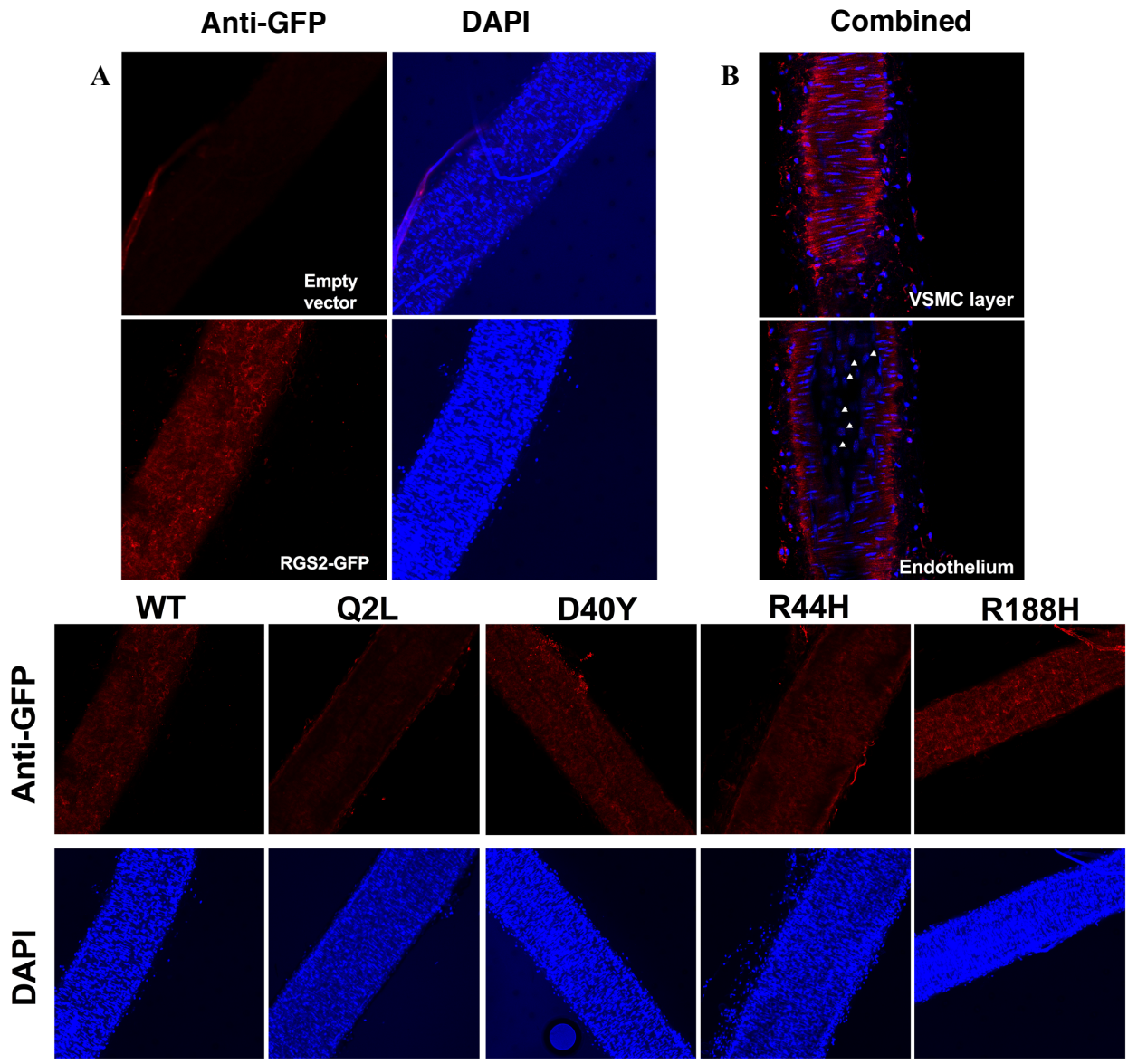

Figure 3-2. **Reversible permeabilization permits transfection of RGS2-GFP into RGS2$^{-/-}$ mesenteric arteries.**
2-3 days post reversible permeabilization (described in "Materials and methods"), arteries were stained for rabbit GFP, and labelled with Cy3-anti rabbit secondary antibody. The arteries were mounted to slide using ProLong™ Gold Antifade Mountant and visualized using confocal microscopy. A. Expression of RGS2-GFP is detected compared with arteries exposed to empty vector. B. RGS2-GFP was overexpressed in vascular smooth muscle cells but not endothelial cells (arrow heads). C. Representative images showed whole vessel expression of RGS2-GFP. Expression level of the Q2L mutant in the MAs is lower than that of RGS2-WT and other mutant RGS2 proteins.

RGS2 LOF mutations failed to suppress AngII-induced vasoconstriction

To examine the effect of RGS2 mutants in regulating vasoconstriction, we expressed plasmid constructs encoding RGS2 WT and mutants in $RGS2^{-/-}$ MAs by reversible permeabilization versus empty vector as control. For these experiments, we used a higher concentration of AngII for cultured MAs (50 nM instead of 5 nM in freshly isolated arteries) to be able to observe significant vasoconstriction effects of RGS2 WT and empty vector transfected arteries, potentially due to receptor tachyphylaxis (Buhrle et al., 1987) which commonly happens with AT1 receptors. MAs expressing empty vector or $RGS2^{-/-}$ MAs without RGS2 transfection, showed 34.3 ± 4.7% (Fig. 3-3A) and 30.0 ± 3.1% (Fig. 3-3B) contraction relative to baseline, respectively. Expression of RGS2 WT was able to suppress contraction in two independent experiments. In MAs expressing RGS2 WT, AngII caused a reduction in arterial diameter of only 4.2 ± 1.3% ($p < 0.01$, one-way ANOVA, Dunnett post-test, Fig. 3-3A) and 7.2 ± 2.6% ($p < 0.05$, one-way ANOVA, Dunnett post-test, Fig. 3-3B). Two normally functioning mutants, RGS2 M5V and A99G (Phan et al., 2017), resulted in a similar level of vascular contraction to RGS2 WT (6.8 ± 3.6% and 10.9 ± 4.2%, respectively, Fig. 3-3B). The RGS2 Q2L mutant gave reduced suppression of contraction compared to RGS WT (18.8 ± 6.6% versus 4.2 ± 1.3%), however, the difference was not statistically significant ($p = 0.09$). Arteries expressing RGS2 D40Y, R44H, and R188H mutants showed a significantly elevated contractile response to 50 nM AngII stimulation compared to arteries expressing RGS2 WT, 28.3 ± 7.7%, 25.7 ± 8.7% and 29.8 ± 5.5%, respectively, vs 4.2 ± 1.3% ($p < 0.05$, one-way ANOVA, Dunnett post-test).

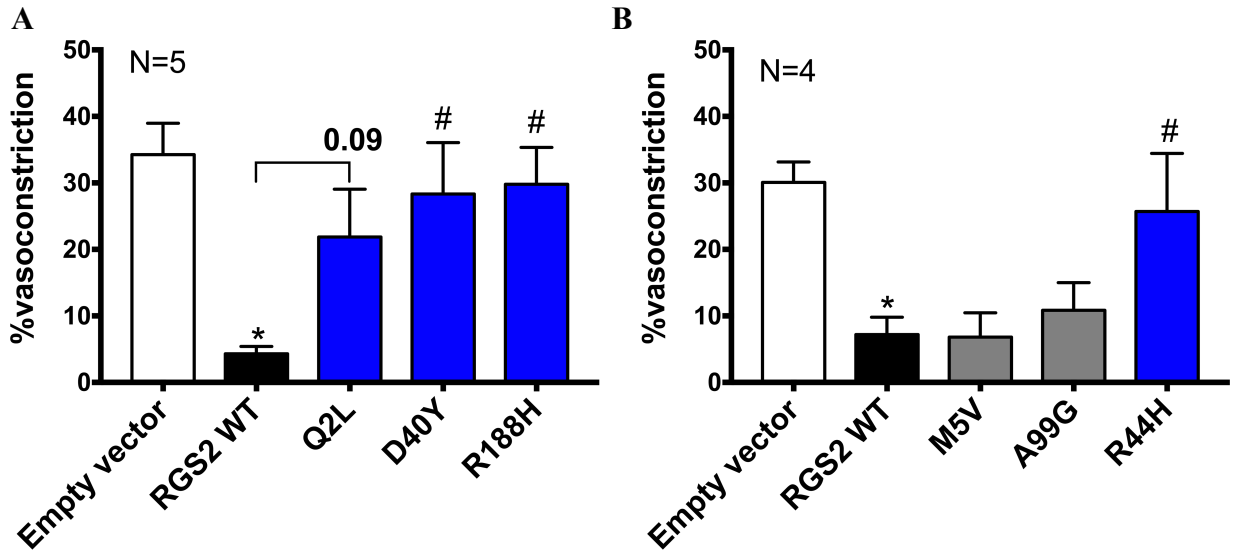

Figure 3-3. **Loss of function mutants of RGS2 failed to suppress AngII-mediated vasoconstriction.**
RGS2-GFP constructs were introduced to mesenteric arteries isolated from RGS2$^{-/-}$ mice by reversible permeabilization. RGS2 WT, M5V and A99G (2 normal function mutations (Phan et al., 2017)) expressing arteries reduced vascular contraction upon 50nM AngII stimulation. Q2L, D40Y, R44H and R188H expressing arteries showed elevated contractile response to 50nM AngII stimulation compared to RGS2 WT expressing arteries. * $p < 0.05$ compared to empty vector, # $p < 0.05$ compared to WT, one-way ANOVA with Dunnett post-test.

RGS2 LOF mutations differentially affect acetylcholine-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 pre-constricted with PE (3 μM), we found that cultured RGS2-deficient MA (transfected with empty vector) showed a maximal relaxation of 52.8 ± 14.3% (Fig. 3-4). This is similar to the response of freshly isolated RGS2$^{-/-}$ MAs (48.6 ± 8.4%, Fig. 3-1C) and much less than that of arteries expressing WT RGS2 (99.6 ± 5.5%, $p = 0.0001$, two-way ANOVA with Dunnett post-test, Fig. 3-4). Among the four LOF mutants, we found that the RGS2 Q2L and R188H did not rescue ACh-mediated relaxation the way RGS2 WT did (Q2L 75 ± 7%, $p < 0.05$;

R188H 63.8 ± 14.3%, $p < 0.001$, Dunnett post-test, Fig. 3-4A). Arteries expressing the D40Y and R44H mutant RGS2, however, showed an equivalent relaxation response to those with RGS2 WT (D40Y 98.4 ± 12.3%, R44H 93.98 ± 4.8%, Fig. 3-4B, 3-5E). It is notable that these latter two mutations reduce RGS2 function by perturbing membrane localization while the other two have different mechanisms (Phan et al., 2017).

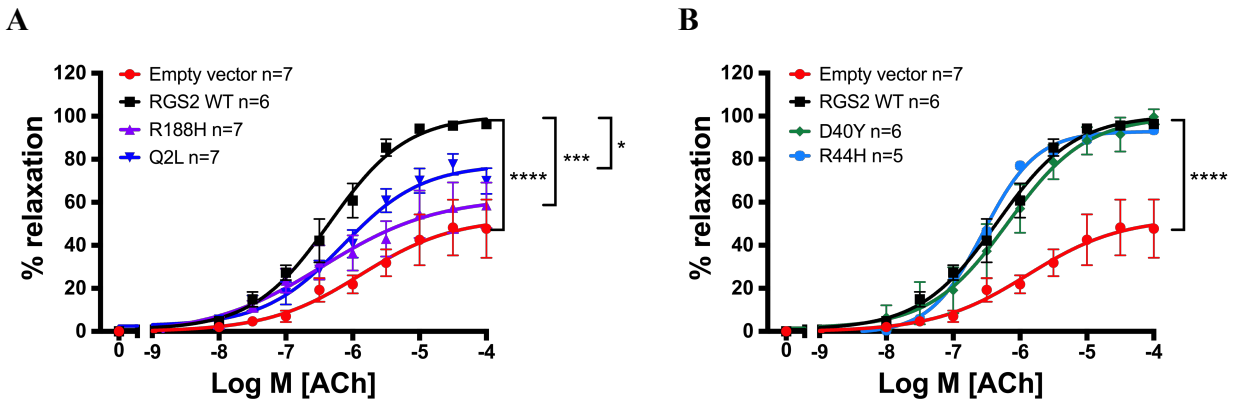

Figure 3-4. **Differential effects of RGS2 missense mutations on acetylcholine-mediated vasorelaxation.**
A. RGS2-GFP expression in RGS2$^{-/-}$ mesenteric arteries rescued ACh-mediated relaxation. RGS2 WT expressing arteries showed enhanced relaxation compared to empty vector transfected arteries. Q2L and R188H expressing arteries showed less relaxation compared to RGS2 WT expressing arteries. B. D40Y and R44H expressing arteries, however, showed comparable relaxation response to RGS2 WT. The data are expressed as the mean ± S.E; *, ***, ****, $p < 0.05$, 0.001, 0.0001, respectively, versus WT (two-way ANOVA) with Dunnett post-test.

Phosphorylation of D40Y and R44H at Ser46 and Ser64 rescues 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 PKG1α which is activated by cGMP and as a consequence, RGS2 membrane localization and GAP function is enhanced (Osei-Owusu et al., 2007; Sun et al., 2005b). RGS2 phosphorylation at Ser46 and Ser64 by PKG1α increased plasma membrane (PM) localization and GAP activity of RGS2 protein (Osei-Owusu et al., 2007; Tang et al., 2003b). Given that the two

RGS2 mutants mentioned above (D40Y, R44H) had impaired PM localization in HEK293 cells but normal ACh-evoked relaxation response in the MAs, we hypothesized that phosphorylation of D40Y and R44H mutant proteins in VSMCs by NO-cGMP-PKG1α 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, in order 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, R44H DD). When co-expressed with Gαq Q209L, D40Y and R44H mutant RGS2 remained in the nucleus while RGS2 WT and the phosphomimetic mutants (RGS2 DD, D40Y DD, R44H DD) localized to the PM (Fig. 5A, B). Additionally, the RGS2 D40Y and R44H mutants showed less Ca^{2+} inhibition compared to RGS2 WT (Fig. 3-5C) while RGS2 D40Y DD and R44H DD suppressed AT1R-mediated Ca^{2+} mobilization equivalently to RGS2 DD (Fig. 3-5D).

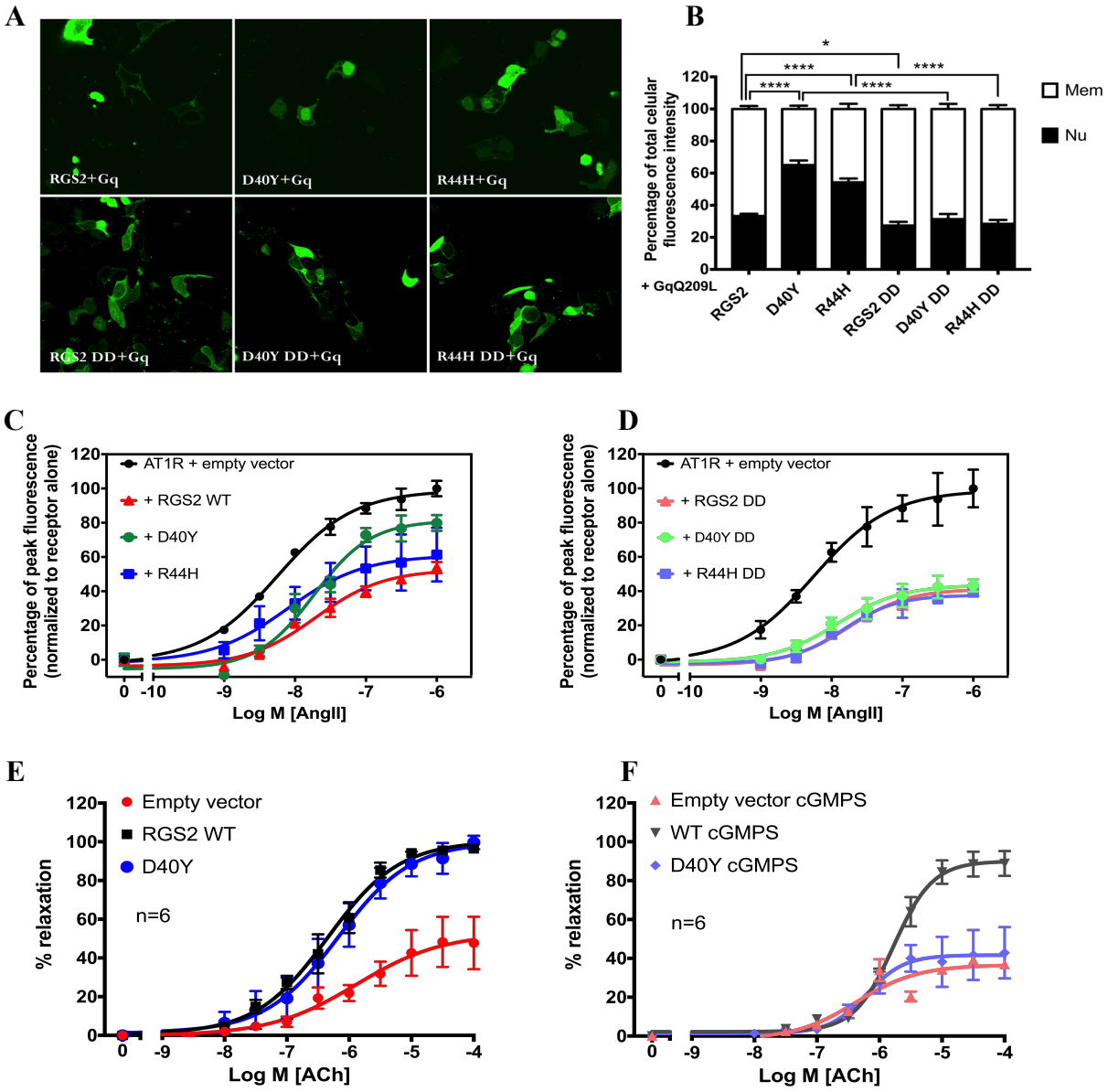

Figure 3-5. **Effects of phosphorylation on RGS2 localization and function.**
A. Representative confocal images showing that phosphomimetic mutations of D40Y and R44H rescued the mutants' membrane localization and function. When co-expressed with G*α*q Q209L, D40Y, R44H remained in the nucleus while RGS2 WT, RGS2 DD, D40Y DD, R44H DD localized to the plasma membrane. B. Quantification of protein localization of at least 80 live cells. C. LOF phenotype of the D40Y and R44H mutant RGS2 in Ca^{2+} assay. D. D40Y DD and R44H DD suppressed AT1R-mediated Ca^{2+} mobilization as equally as RGS2 DD. E. The D40Y mutant RGS2 supported normal ACh-mediated relaxation response F. PKG inhibition by 30 μM Rp-8-pCPT-cGMPS significantly reduced vascular relaxation in D40Y expressing MAs.

Effect of PKG inhibition on ACh-mediated relaxation in MAs expressing D40Y RGS2 mutant

Inhibition of PKG with Rp-8-pCPT-cGMPS (30 μM) altered relaxation responses of MAs. PKG inhibition slightly reduced maximal relaxation of MAs expressing WT RGS2 from 99.6 ± 5.5% to 87.8 ± 4.2% (n.s) and shifted the ACh-concentration response curve s to the right ($logEC_{50}$- 5.8 ± 0.06 vs -6.3 ± 0.09, n.s., Fig. 3-5E, F, Table 3-1). The relaxation response of $RGS2^{-/-}$ MAs transfected with empty vector was impaired (max relaxation 52.9 ± 14.3%), and was further depressed by PKG inhibition (max relaxation 38.4 ± 3.5%, n.s., Fig. 3-5E, F, Table 3-1). PKG inhibition drastically reduced the dilation of MAs transfected with D40Y mutant RGS2 (40.3 ± 6.7% vs 98.4 ± 12.3%, $p < 0.001$, Fig. 3-5E, F, Table 3-1). The marked reduction of relaxation response in MAs expressing the D40Y mutant is consistent with a model in which lack of phosphorylation and plasma membrane localization occurs when PKG1α is inhibited.

Table 3-1. **Relaxation of MAs expressing RGS2 WT and D40Y to ACh demonstrated by max and EC_{50} derived from ACh concentration – relaxation response curves.**
(Data are presented as mean ± SEM, ** $p< 0.01$, * $p<0.05$ compared to empty vector; ## $p<0.01$ compared to RGS2 WT + PKG inhibitor, &&& $p<0.001$ compared to D40Y).

RGS2 mutant/treatment	**Max**	**$LogEC_{50}$**
Empty vector (n=7)	52.9 ± 14.3	-5.9 ± 0.43
RGS2 WT (n=6)	99.6 ± 5.5**	-6.3 ± 0.09
D40Y (n=6)	98.4 ± 12.3*	-6.2 ± 0.19
Empty vector + PKG inhibitor (n=4)	$38.4 \pm 3.5^{\#\#}$	-6.3 ± 0.14
RGS2 WT + PKG inhibitor (n=4)	87.8 ± 4.2	-5.8 ± 0.06
D40Y + PKG inhibitor (n=4)	$40.3 \pm 6.7^{\#\#, \&\&\&}$	-6.2 ± 0.22

Discussion

In this study, we examined the physiological 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+} mobilization phenotypic assay in CHO cells (Phan et al., 2017). Here, we report that all four mutant proteins failed to effectively suppress AngII-mediated vasoconstriction in mouse MAs. Interestingly, only the Q2L and R188H mutations exhibited impaired ACh-mediated relaxation. The D40Y and R44H RGS2 mutants supported a normal relaxation response to ACh. We also provide mechanistic evidence that the differential relaxation supported by mutant RGS2 proteins may be attributed to phosphorylation of RGS2 by PKG1α in VSMCs. It appears that PKG-dependent phosphorylation can rescue PM localization in HEK293 cells and restore the vasorelaxation function of D40Y and R44H mutant RGS2 in MAs.

Freshly isolated mesenteric artery responses to AngII, ACh and PE

We were able to confirm the previously published effects of acetylcholine in WT and RGS2-deficient MAs (Osei-Owusu et al., 2012). Additionally, we show for the first time that MAs from $RGS2^{-/-}$ mice exhibit augmented vasoconstriction to AngII. This is consistent with previous literature showing that RGS2-deficient renal interlobar arterioles had an increased contraction in response to AngII (Hercule et al., 2007).

Acetylcholine-induced relaxation of mesenteric arteries expressing RGS2 mutant proteins

RGS2 promotes relaxation of VSMC in part by undergoing PKGIα-mediated phosphorylation and activation to inhibit contraction evoked by Gq/11-coupled vasoconstrictor receptors (Sun et al., 2005b; Tang et al., 2003b). It also promotes endothelial EDHF-dependent vasodilation by blunting Gi/o-dependent processes (Osei-Owusu et al., 2012). In our experiments,

the RGS2-GFP proteins were expressed in the VSMC layer but do not appear to be expressed at significant level in the endothelial cells (Fig. 3-2B). Therefore, we weren't able to assess the EDHF-dependent relaxation effect of RGS2 (Osei-Owusu et al., 2012) with our method. However, overexpression of RGS2 in VSMC is important in mediating ACh-evoked relaxation since over-expression of the WT RGS2 in VSMCs was able to rescue the impaired ACh-mediated relaxation in RGS2$^{-/-}$ arteries. Also mutants with different loss of function mechanisms (Phan et al., 2017) behaved differently (Fig. 3-4).

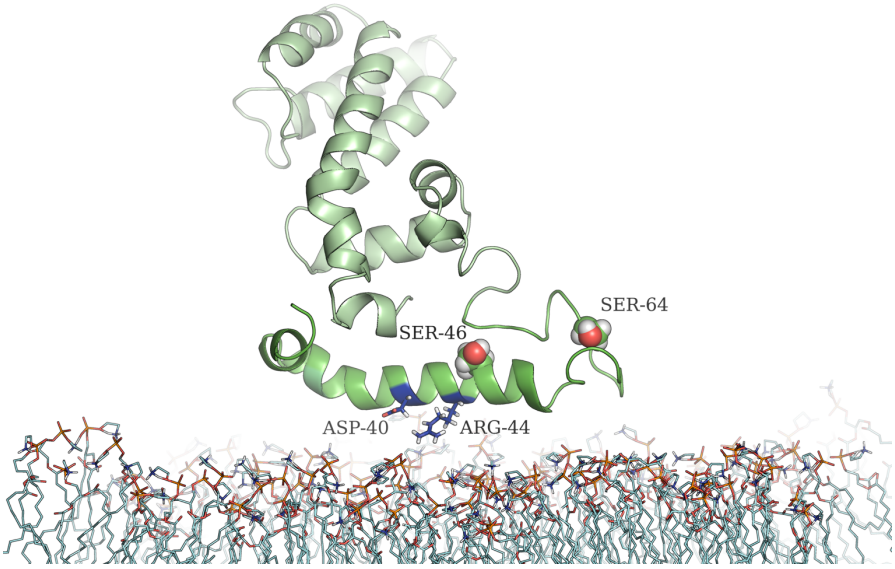

Figure 3-6. **Modelled structure of full-length RGS2 with lipid membrane.**
N-terminal amphipathic α-helix is orientated parallel with membrane, allowing interactions between amino acid side chains and components of lipid (Tikhonova et al., 2006). Mutated sites (D40, R44) are labeled in blue. Phosphorylated sites (S46, S64) are marked as spheres. The phosphate groups of lipids are in orange; the lipid acyl chains are in cyan.

Acetylcholine mediates relaxation in part by activating the NO-cGMP-PKG pathway in endothelium-intact arteries (Christopoulos and El-Fakahany, 1999; Sun et al., 2005b). RGS2, as an effector of PKG, has previously been shown to be phosphorylated at Ser46 and Ser64 (Tang et al., 2003b), 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. 3-6) interacting with lipids (Tikhonova et al., 2006). This helix facilitates PM targeting

(Gu et al., 2007; Heximer et al., 2001). Introducing phosphomimetic mutants at two PKG1α phosphorylation sites of RGS2 (D40Y DD, R44H DD) in the D40Y and R44H mutant RGS2 proteins enhanced PM localization and function in suppressing Ca^{2+} release (Fig. 3-5 A, B, D). Conversely, PKG1α inhibition substantially reduced ACh-induced relaxation in RGS2 D40Y expressing $RGS2^{-/-}$ MAs (Fig. 3-5F). 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 phosphorylation 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 behavior 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 PKG1α function is deficient (Michael et al., 2008), these mutant alleles will likely be unphosphorylated, triggering aberrant Gαq signaling and an impaired 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., 2008a). In the case of the D40Y mutant, the change from a negatively charged Asp residue to a hydrophobic Tyr residue may disrupt the local environment for the anchorage of the adjacent Trp41 to the PM (Gu et al., 2008a). Several

mechanisms have been proposed to explain how phosphorylation at these sites enhance recruitment of RGS2 to the plasma membrane. Salt bridge formation between the phosphorylated Ser residues and other Lys residues (Osei-Owusu et al., 2007) may stabilize the N-terminal amphipathic helix. This could explain how introduction of the Asp phosphomimetic in place of Ser at position 46 and 64 could stabilize the amphipathic α helix disrupted by D40Y and R44H mutations and rescue function.

RGS2 plays three roles in the vasculature *1)* a direct function as a GAP to negate vasoconstrictor actions through Gαq, *2)* acting as an effector of NO-cGMP-PKG signaling to promote vasodilation by modulating Gαq-mediated vasoconstrictor action, and *3)* promoting EDHF-mediated vasorelaxation (Osei-Owusu et al., 2012). By over-expressing 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 LOF mutants affect whole body blood pressure responses. Also, 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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CHAPTER 4: RGS2 MISSENSE MUTATION ENRICHED IN THE FINNISH POPULATION IS ASSOCIATED WITH HIGH BLOOD PRESSURE AND CARDIOVASCULAR DISEASE

Abstract

Hypertension is a major risk factor for cardiovascular disease, the leading cause of mortality worldwide. Common mutations in non-coding regions of the regulator of G protein signaling 2 (RGS2) gene have been found to associate with hypertension and metabolic syndrome in different ethnic groups. Multiple mutations in the coding region of this gene have also been found but the rarity of these alleles has precluded well-powered association analyses. Of these rare mutations in human RGS2, we previously showed that four exhibited loss-of-function in *in vitro* cellular assays. One of them, the D40Y mutation, is present at a minor allele frequency of 0.8% in Finns but is virtually absent from other populations. Here we examined genotypes and cardiovascular phenotypes from 19,292 individuals from the FinMetSeq study. We find a significant increase in systolic and diastolic blood pressure, pulse pressure and body mass index in D40Y minor allele carriers. These carriers also have a higher risk of coronary artery disease and myocardial infarction. Our data provide additional support for an important role of the RGS2 protein in cardiovascular regulation and in the genesis of human hypertension.

Introduction

More than 100 common mutations with minor allele frequency (MAF) > 5% that contribute to hypertension risk have been identified through genome-wide association studies (GWAS). However, these variants explain only ~3-5% blood pressure heritability (Seidel and Scholl, 2017). The hypothesis that low-frequency and rare variants may contribute to the missing information of hypertension genetics has been supported by numerous studies (Russo et al., 2018).

Since the discovery of the RGS protein family in the 1990s, much has been learned about their roles in physiological processes and disease states (Squires et al., 2018). Among them, RGS2 is unique in its potent inhibitory effect toward Gαq/11 (Heximer et al., 1997). This G protein family transduces signaling from most vasoconstrictor mediators including AngII, endothelin 1 and noradrenaline. RGS2 knockout mice are hypertensive (Heximer et al., 2003b; Tang et al., 2003b). Augmented and prolonged vasoconstriction and vascular hypertrophy was also observed in RGS2 knockout mice (Heximer et al., 2003b). RGS2 is also protective against cardiac hypertrophy by suppressing Gq/11-mediated signaling (Zhang et al., 2006). RGS2-deficient mice also exhibited cardiac hypertrophy and worsened heart failure in response to aortic banding (Takimoto et al., 2009). RGS2 has also been shown to play critical role in maintaining β cell mass and function which is important in diabetes (Dong et al., 2017). In human subjects, decreased RGS2 mRNA and protein expression levels were detected in peripheral blood mononuclear cells of hypertensive subjects compared to normotensive subjects (Semplicini et al., 2006a). Common variants in the RGS2 gene have been reported to associate with hypertension (Hahntow et al., 2009; Kamide et al., 2011; Li et al., 2010; Riddle et al., 2006; Yang et al., 2005a; Zhang et al., 2013; Zhao et al., 2008), metabolic syndrome (Freson et al., 2007), obesity (Sartori et al., 2008),

carotid atherosclerosis (Kamide et al., 2011), and preeclampsia (Kvehaugen et al., 2014; Kvehaugen et al., 2013) in a variety of ethnic groups. Rare mutations in the coding sequence of RGS2 were found at a greater frequency in hypertensive subjects, however, the numbers were too small to reach statistical significance (Riddle et al., 2006; Yang et al., 2005a).

We biochemically characterized a number of these mutations and identified four loss-of-function exonic variants in RGS2 (Phan et al., 2017). Among them, the D40Y mutation (rs201233692) is found at a relatively high frequency in the Finnish population (MAF 0.8%) and at a much lower frequency in the general population (0.08%) according to the gnomAD database (http://gnomad.broadinstitute.org accessed on 08/21/2018). Here, we report for the first time a cardiovascular phenotype associated with the rare D40Y mutation in RGS2 in a Finnish sample. Together with the previously documented loss of function behavior of this mutation in transfected cells (Chapter 2) and mesenteric artery (Chapter 3), our data indicate that RGS2 is an important protein in regulating human cardiovascular function. Disruption of RGS2 function appears to contribute to hypertension and cardiovascular disease.

Methods

Ethics statement

The METSIM and FINRISK studies were performed following the Helsinki Declaration and were approved by the ethics committees of the University of Kuopio and the National Institute for Health and Welfare, respectively. All participants gave written informed consent.

Study population

The FinMetsSeq study consisted of 9,957 and 9,335 Finns from the METSIM and FINRISK cohorts, respectively. These two cohorts have been described elsewhere (Borodulin et al., 2017; Laakso et al., 2017). In brief, the METSIM study is designed to investigate genetic and non-genetic factors for the risk of type 2 diabetes (T2D), cardiovascular disease (CVD), insulin resistance, and related traits in a cross-sectional and longitudinal setting. In total it includes 10,197 men randomly selected from the population register of Kuopio, Eastern Finland, aged 45 to 73 years at initial examination from 2005 to 2010. FINRISK is a large Finnish population survey on risk factors for chronic and non-communicable diseases. It began in 1972 and has been carried out every five years. Demographic information and a broad range of clinical phenotypes were collected through standard questionnaire, various laboratory tests or national registries for each participant in both cohorts. Diagnoses for coronary artery disease, stroke, and myocardial infarction were verified based on medical records. The cases with hypertension were diagnosed by systolic blood pressure (SBP) ≥140 mmHg, diastolic blood pressure (DBP) ≥90 mmHg, or by use of antihypertensive medications. FinMetSeq participants' samples were subjected to exome sequencing in Illumina HiSeq 2000 with a mean read depth of over 20× (submitted for publication). The characteristics of the study population are summarized in Table 4-1

Table 4-1. **Characteristics of the FinMetSeq study population.**

Trait description	**N**	**Trait abbreviation**	**Units**	**Mean**	**SD**
Gender	19292	Gender	-	4942 (Female, 25.62%)	
Age at baseline visit	19292	Age	years	53.4	11.5
Waist circumference	19274	Waist	cm	94.4	13.4
Waist/hip ratio	19272	WHR	-	0.934	0.091
Weight	19241	Weight	kg	80.4	15.2
Systolic blood pressure, mean of two measurements	19282	SBP	mmHg	138.0	18.7
Diastolic blood pressure (mmHg), mean of two measurements	19280	DBP	mmHg	84.0	11.1
Pulse pressure	19279	PP	mm Hg	54.0	15.3
Hip circumference (cm)	19274	Hip	cm	100.9	8.0
Body mass index: Weight/(height/100)^2	19239	BMI	kg/m^2	27.2	4.5
Bioimpedance: Fat mass (%)	11742	Fatmass	%	25.1	7.4
OGTT fasting plasma glucose (mmol/l)	11759	P_gl0	mmol/l	5.96	1.05
OGTT 2hr plasma glucose (mmol/l)	10980	P_gl120	mmol/l	6.50	2.46
OGTT fasting plasma glucose (mmol/l)	11753	P_ins0	mmol/l	9.40	11.9
OGTT 2hr plasma insulin (mmol/l)	10960	P_ins120	mmol/l	53.8	55.7

Single-variant association test

We tested for association strength between the D40Y mutation and thirteen cardiometabolic quantitative traits including SBP, DBP, pulse pressure, fasting and 2-hour plasma glucose and insulin levels in oral glucose tolerance test (OGTT), body weight, waist, hip, waist hip ratio (WHR), body mass index (BMI), and fat mass), as well as four disease endpoints including myocardial infarction (MI), hypertension, coronary artery disease (CAD), and stroke. We adjusted raw blood pressure traits for those participants who were taking blood pressure medication by 15 and 10 mmHg for systolic and diastolic blood pressure, respectively (Tobin et al., 2005). For quantitative traits, we applied a log transformation on traits which were significantly skewed. We used linear regression to adjust for age, age^2, T2D status, BMI, and cohort identity and inverse normalized the residuals. For dichotomous traits, we included gender, age, and age^2 as covariates. We carried out single-variant association tests with EPACTS (Efficient and Parallelizable Association Container Toolbox) v3.3.0 using a linear mixed model assuming an additive genetic effect (URL: https://genome.sph.umich.edu/wiki/EPACTS).

Results

Frequencies of RGS2 D40Y genotypes

Sample sizes for each continuous trait were different from each other because of missing values or adjustment for covariates. On average, genotype frequencies of rs201233692 were 98.14% G/G, 1.84% G/T and 0.01% T/T (Table 4-2).

Table 4-2. **Frequency and clinical characteristics of individuals carrying the RGS2 D40Y (rs201233692) genotypes in traits studied.**

Trait	**Genotypes: mean ± SD (n)**			**p-value**
	G/G	**G/T**	**T/T**	
Waist	94.4 ± 13.4 (18908)	95.7 ± 13.5 (363)	90.5 ± 23.8 (3)	0.763
WHR	0.93 ± 0.09 (18906)	0.94 ± 0.09 (359)	0.84 ± 0.08 (3)	0.341
Weight	80.4 ± 15.2 (18879)	81.4 ± 16.0 (359)	79.7 ± 37.6 (3)	0.146
SBP	138.0 ± 18.7 (18917)	141.5 ± 19.4 (362)	165.0 ± 40.3 (3)	**0.003**
DBP	84.0 ± 11.1 (18915)	85.1 ± 11.9 (362)	88.0 ± 6.56 (3)	*0.023*
PP	54.0 ± 15.3 (18914)	56.4 ± 16.1 (362)	77.0 ± 39.0 (3)	*0.029*
Hip	100.9 ± 8.0 (18908)	101.4 ± 8.3 (363)	106.5 ± 18.8 (3)	0.116
BMI	27.2 ± 4.5 (18877)	27.8 ± 4.6 (359)	28.7 ± 10.2 (3)	*0.018*
Fatmass	25.1 ± 7.4 (11529)	25.6 ± 7.1 (212)	33.7 (1)	0.673
P_gl0	5.96 ± 1.06 (11546)	5.94 ± 0.79 (212)	5.46 (1)	0.598
P_ins0	9.39 ± 12.0 (11540)	9.84 ± 8.17 (212)	9.1 (1)	0.219
P_ins120	53.5 ± 55.3 (10763)	67.2 ± 72.6 (196)	44.7 (1)	0.087
P_gl120	6.49 ± 2.45 (10783)	6.64 ± 2.87 (196)	6.71 (1)	0.628

Association of the D40Y RGS2 mutation with high blood pressure

Using a Bonferroni corrected p-value of 0.0038 (0.05/13) as our significance criterion to account for multiple testing, the inverse-normalized SBP was significantly higher in the G/T and T/T group compared to the G/G group ($p = 0.003$, Fig. 4-1A) after adjusting for covariates (Methods). In the G/G group, SBP was 138 ± 18.7 (n = 18917), while in mutant carrier groups, SBP was 141.5 ± 19.4 (n = 362) in G/T group and 165.0 ± 40.3 (n = 3) in T/T group. Without applying the Bonferroni correction, we identified association of three other traits with the G/T and T/T groups as follows. Diastolic blood pressure and pulse blood pressure tended to be higher in the G/T and the T/T group after adjustment for age, age^2, T2D status, BMI, and cohort identity ($p = 0.023$ and 0.029, respectively, Fig. 4-1 B, C). Body mass index also tended to be higher in the G/T and the T/T group ($p = 0.018$, Fig. 4-1D, Table 4-2).

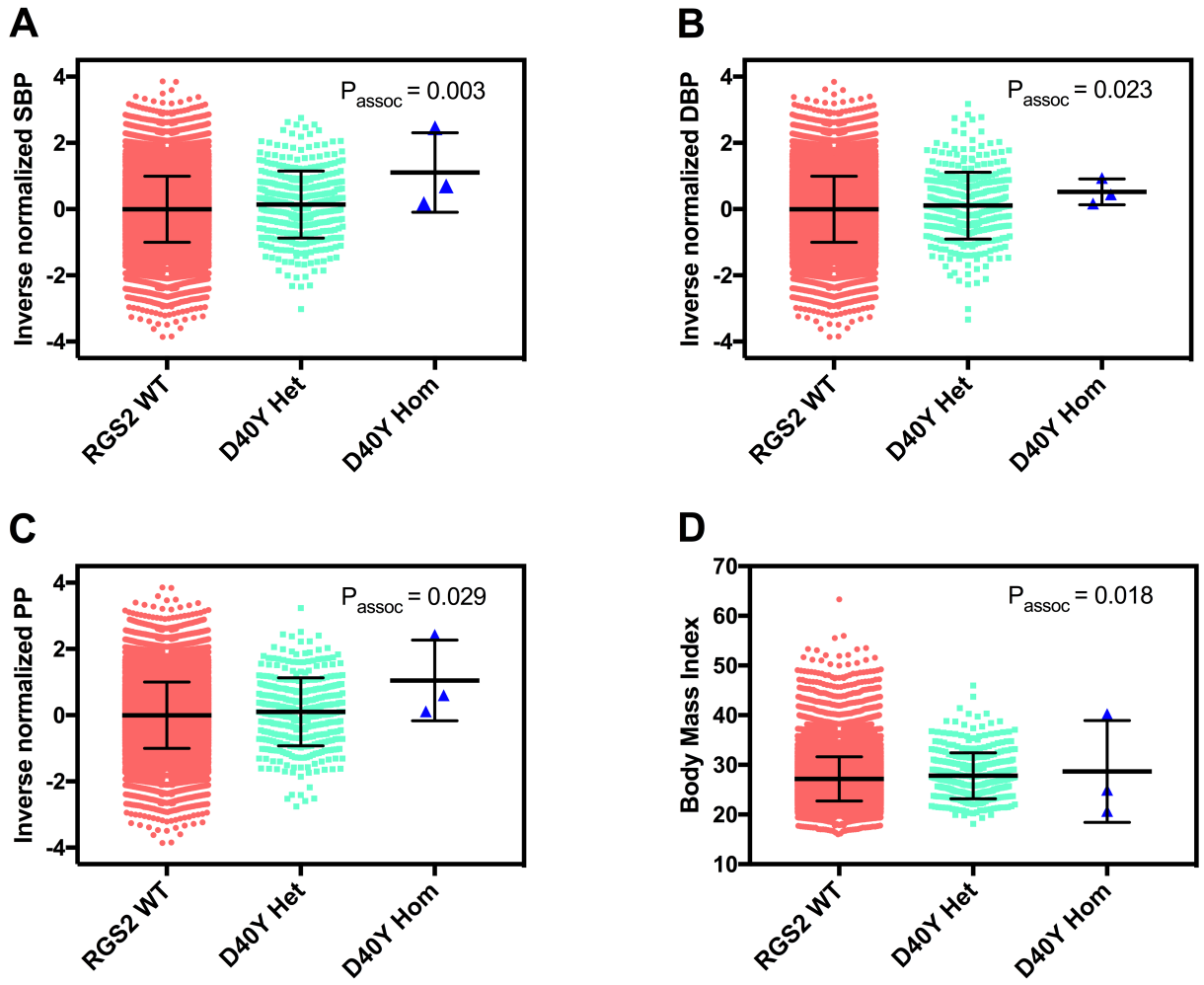


Figure 4-1. **Associations between RGS2 SNP rs201233692 systolic blood pressure (A), diastolic blood pressure (B), pulse pressure (C) and BMI (D).**
Bars show the adjusted means and SEs. P values were adjusted for age, sex, BMI, using mixed linear regression model assuming an additive effect. D40Y Het and D40Y Hom indicate heterozygote and homozygote D40Y mutant carriers, respectively.

Cardiovascular disease phenotypes

As the minor allele of rs201233692 was associated with higher blood pressure (systolic, diastolic and pulse pressure), we performed an analysis to investigate the association between this allele and cardiovascular clinical outcomes such as coronary artery disease (CAD), myocardial infarction (MI) and stroke. The rs201233692 genotypes were associated with CAD (OR = 1.52, p = 0.032) and MI (OR = 1.57, p = 0.013) but not a stroke (OR = 1.03, p = 0.885) as shown in Table 4-3.

Table 4-3. **Cardiovascular disease according to D40Y (rs201233692) genotypes.**

	Cases			**Controls**				
Disease	**G/G**	**G/T**	**T/T**	**G/G**	**G/T**	**T/T**	**Odds ratio**	**P value**
CAD	739	24 (3.1%)	0 (0%)	18187	339 (1.8%)	3 (0.02%)	1.52	0.032
MI	884	28 (3.1%)	0 (0%)	18042	335 (1.8%)	3 (0.02%)	1.57	0.013
Stroke	625	11 (1.7%)	1 (0.16%)	18301	352 (1.9%)	2 (0.01%)	1.03	0.885

Discussion

Using targeted single nucleotide variant testing from a Finnish population of 19,292 individuals, we identified an association of a biochemically documented RGS2 loss-of-function allele, D40Y (rs201233692), with high blood pressure, coronary artery disease and myocardial infarction. Specifically, the G/T and T/T genotypes of rs201233692 were significantly associated with higher systolic blood pressure. Carriers of the minor T allele tended to have higher diastolic and pulse pressure and BMI. The prevalence of CAD and MI was also higher in the population carrying the T allele. On the other hand, we found no association of this mutation with central adiposity measures or insulin level, nor did we find evidence for a relationship to the prevalence of stroke.

Mean SBP in carriers of the minor allele were 3 – 4 mm Hg higher than in non-carriers (141.5 ± 19.4 mmHg, n = 362 in G/T group and 165.0 ± 40.3 mmHg in T/T group vs. 138.0 ± 18.7 mmHg in G/G group). The effect size of this minor allele of RGS2 is larger than the modest effect size of 1 mmHg for SBP trait contributed by common variants identified through GWASs (Burrello et al., 2017; Ehret and Caulfield, 2013b). This supports a moderate effect of RGS2 protein on blood pressure regulation. Although identified through a looser statistical criterion, the D40Y mutant allele tended to associate with higher DBP, pulse pressure, and BMI (Fig. 4-1B, C, D).

Other reports of missense variants in RGS2 have yielded informative *in vitro* data but have not provided the significant clinical connection that we report here. Yang et al reported 5 missense mutations in RGS2 found in a Japanese hypertensive cohort (Yang et al., 2005a). Additionally, Riddle et al reported that the Q50K mutation in RGS2 was found in hypertensive subjects at a

higher frequency than normotensive subjects (Riddle et al., 2006). However, the small sample sizes in these studies limited the analysis of these rare mutations. A burden analysis that we undertook with data obtained from the NHLBI Exome Sequencing project was also limited by the small sample sizes (Supplemental method and supplemental figure 4-1). The Finnish population is genetically isolated (Lundmark et al., 2008) in a way that led to enrichment of rare variants or variants that are unique to this population (Nevanlinna, 1972). The almost exclusive presence of the D40Y mutant RGS2 in this population and the relatively large sample size of nearly 20,000 individuals enabled us to perform this analysis. This highlights the importance of identifying ethnic backgrounds in which variants are enriched to strengthen the analysis of rare variants and to mitigate the low abundance problem. Indeed, nearly 2% of the Finnish population carries the T allele.

Risks of CAD and MI were higher in the population carrying the D40Y minor allele (Table 4-3). Three common variants of RGS2, −638A>G, 1026T>A and 1891–1892delTC were found to associate with carotid atherosclerosis in a Japanese general or hypertensive population (Kamide et al., 2011). Another variant in RGS2, -391C>G, was found to associate with metabolic syndrome in White European men (Freson et al., 2007). RGS2 has also been shown to have protective role in inflammation in mice (George et al., 2017). Given that atherosclerosis is related to dysregulation in lipid profile and inflammation (Pant et al., 2014), these data suggest that the RGS2 protein may be involved in the development of atherosclerotic lesions.

Our study provides the first evidence that a coding mutation in RGS2 - a highly conserved gene (Riddle et al., 2006), contributes to hypertension and cardiovascular disease. However, a limitation of this study is that there are no other publicly available databases to assess replication

of our finding. Nevertheless, combining the strong experimental evidence of biochemical dysfunction produced by this mutation in HEK and CHO cells (Phan et al., 2017) and mutation-related alterations in vasconstriction and relaxation in mesenteric artery (Chapter 3), this finding directly strengthens support for a role of the RGS2 protein in human blood pressure and cardiovascular regulation.

APPENDIX

APPENDIX

Supplemental methods

Study samples

The Heart GO cohort of datasets listed below were requested from dbGaP database, following instructions for authorized data request available on the dataset websites. The study was approved by Michigan State University Institutional Review Board (Approval number: IRB# 15-1363). These datasets include exome sequencing data and phenotype data that enable genotype – phenotype analysis of a variety of cardiovascular disease including hypertension.

WHISP: Women's Health Initiative sequencing project. In the original WHI study, 161,808 postmenopausal women enrolled between 1993 and 1998. The WHI has two major parts: a partial factorial randomized Clinical Trial (CT) and a longitudinal cohort Observational Study (OS). The CT enrolled 68,132 postmenopausal women between the ages of 50-79 into trials testing three prevention strategies. The Observational Study (OS) examines the relationship between lifestyle, health and risk factors and specific disease outcomes. This component involves tracking the medical history and health habits of 93,676 women. Samples from **2150** phenotyped subjects presented in both arms of the WHI study underwent exome sequencing in 2010.

MESA: The Multi-Ethnic Study of Atherosclerosis (MESA) is a research study involving more than 6,800 men and women from six communities in the United States. Participants in MESA come from diverse race and ethnic groups, including African Americans, Latinos, Asians, and Caucasians. Residents of the study communities between the ages of 45 and 84 were selected and invited to participate. The MESA study is investigating the early stages of atherosclerosis, using a variety of imaging, genetic, biochemical and behavioral markers of disease. For GO-ESP, 423

MESA participants were selected for exome sequencing and sent to both sequencing centers for processing. A total of 423 samples passed Q/C metrics, 409 (97%) provided finished sequence data, and **404** individuals are represented in GO-ESP data in dbGaP.

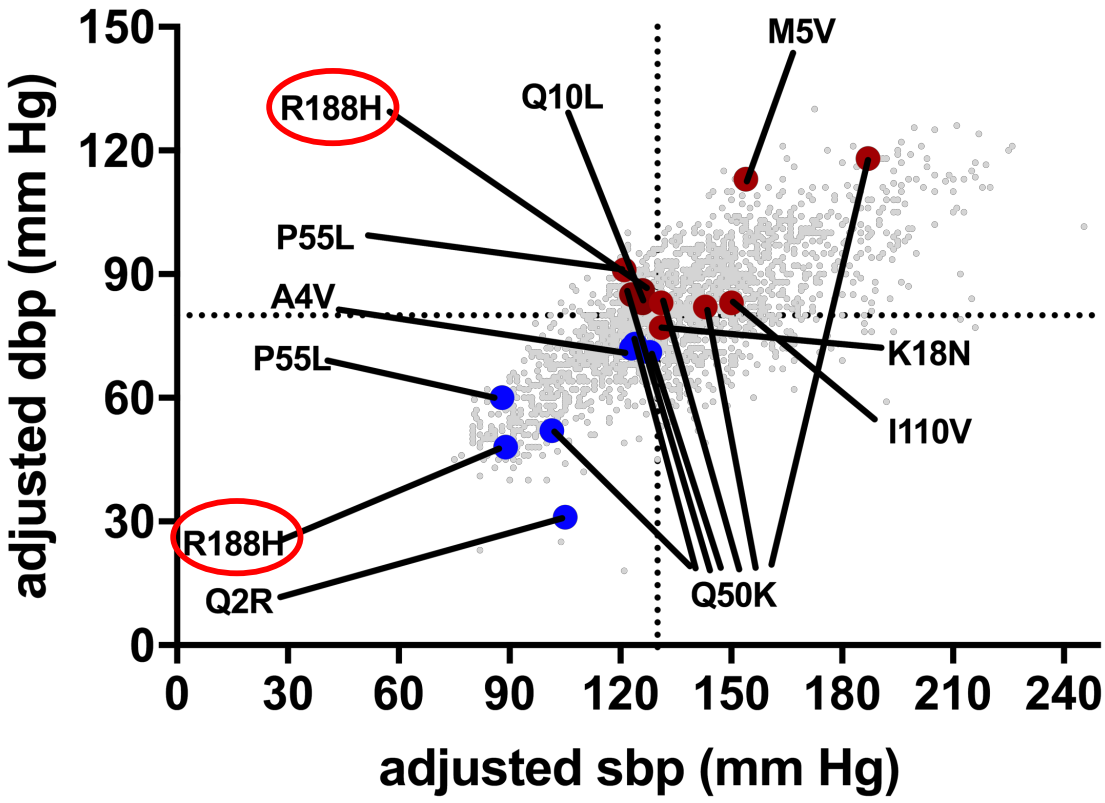
CHS: The Cardiovascular Health Study (CHS) is a prospective population-based cohort study of risk factors for CHD and stroke in adults 65 years and older, starting in 1990. For GO-ESP, a total of 376 CHS participants were selected for exome sequencing and sent to both sequencing centers for processing. A total of 239 samples passed Q/C metrics, 222 (93%) provided finished sequence data, and **210** individuals are represented in GO-ESP data in dbGaP.

JHS: The Jackson Heart Study (JHS) initial examination included men and women ages 35 to 84 during the period from 2000-2003. The JHS is uniquely positioned to answer key questions regarding the excess burden of CVD among African Americans and to address the critical shortage of minority examination The Second Exam occurred in 2005-2008 with repeat and novel measures of CVD risk. For GO-ESP, a total of 535 JHS participants were selected for exome sequencing and sent to both sequencing centers for processing. A total of 441 samples passed Q/C metrics, 422 (96%) provided finished sequence data, and **412** individuals are represented in GO-ESP data in dbGaP.

CARDIA: The Coronary Artery Risk Development in Young Adults (CARDIA) study started as a study of the distribution and evolution of risk factors for cardiovascular disease during young adulthood in black and white men and women during 1985-2011. For GO-ESP, a total of 209 CARDIA participants were selected for exome sequencing and sent to both sequencing centers for processing. A total of 209 samples passed Q/C metrics, 207 (99%) provided finished sequence data, and **204** individuals are represented in GO-ESP data in dbGaP.

Data analysis

Burden analysis was performed using the SKAT package in R. Hypertension was defined as SBP ≥ 130 mm Hg or DBP ≥ 80 mm Hg. We adjusted raw blood pressure traits for those participants who were taking blood pressure medication by 15 and 10 mmHg for systolic and diastolic blood pressure, respectively.



Supplemental Figure 4–1. **Systolic and diastolic blood pressure of the population studied were mapped.**
Red points represent carriers of RGS2 mutations that have hypertension. Blue points represent carriers of RGS2 mutations that have normal blood pressure. Grey points represent individuals that don't carry RGS2 mutations. There are two individuals carrying the LOF R188H mutant RGS2. One of them is borderline hypertensive while the other has normal blood pressure.

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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

The goals of my thesis work were to 1) elucidate the function of RGS2 mutant proteins in the context of vascular regulation and 2) determine whether rare mutations in human RGS2 contribute to the genetics of hypertension.

In Chapter 2, I identified four mutations in a subset of 16 mutations (25%) selected through publicly available exome sequencing database that result in RGS2 proteins that display a functional deficit in inhibiting AT1R-mediated increases in intracellular calcium in CHO cells. Of these four, the results with the D40Y and R188H mutants are novel while the Q2L and R44H mutants have been investigated in the past. I further investigated the mechanisms underlying the functional deficits of these mutations. The RGS2 Q2L protein had lower basal protein expression due to rapid proteasomal degradation. The D40Y and R44H mutant RGS2 had impaired plasma membrane targeting. The R188H mutant RGS2 was deficient in G protein binding. These mechanisms are most likely responsible for the reduced function of these mutants.

Computational, *in vitro*, *in vivo,* and human genetic analysis of human RGS2 variants

Currently, there are 110 RGS2 missense mutations identified and published in the gnomAD database (http://gnomad.broadinstitute.org/gene/ENSG00000116741, access Aug/29/2018). More and more prediction tools have been built to predict functional consequences of missense mutations, based on sequence conservation, structure and neural networks (Korvigo et al., 2018). We and other groups observed a strong destabilization of RGS2 protein by the Q2L mutation (Bodenstein et al., 2007b; Park et al., 2015; Phan et al., 2017). This deficit occurs despite benign predictions by tools such as Polyphen and SIFT, which are among the best prediction tools (Wei et al., 2010) (Table 5-1). I also found that the S3G, A99G, I110V to have normal function in the Ca^{2+} mobilization assay despite being predicted to be damaging. The A99G mutant RGS2 also

showed normal function in the context of mesenteric artery vasoconstriction (Fig. 3-3). Also, prediction tools only predict loss-of-function or gain-of-function but they cannot predict the severity of changes in protein function by mutations (Raraigh et al., 2018). For my dataset of 16 mutants, the predictions were correct 75% of the time. The false positive rate was 19% (i.e 3 mutants were predicted to be damaging by both SIFT and Polyphen but showed normal function) and the false negative rate was 6% (one mutation predicted as benign but having LOF behavior). Therefore, functional assays remain the ultimate way to study missense mutations in RGS2 or in other proteins. Higher throughput approach in mutagenesis (Heydenreich et al., 2017) and functional assays could help to achieve this goal in a fast and economical manner. Because the false negative rate is smaller than the false positive rate, future functional studies should focus on mutations that are predicted as damaging.

Table 5-1. **Functional prediction on human RGS2 rare missense mutations, using Polyphen-2 and SIFT. (LOF: loss of function, NF: normal function)**

Substitution	dbSNP ID	SIFT Prediction	Polyphen-2 Prediction	Functions			Substitution	dbSNP ID	SIFT Prediction	Polyphen-2 Prediction	Functions		
				Ca2+	Vasoconstriction	Relaxation					Ca2+	Vasoconstriction	Relaxation
Q2E	novel	TOLERANT	BENIGN				Q78H		TOLERANT	BENIGN	NF		
Q2L	novel	TOLERANT	BENIGN	LOF	LOF	LOF	F84S	novel	DAMAGING	PROBABLY DAMAGING			
Q2R	rs141030117	TOLERANT	BENIGN	NF			L87M	novel	DAMAGING	POSSIBLY DAMAGING			
S3G	rs145125159	TOLERANT	POSSIBLY DAMAGING	NF			A99G	rs139237239	DAMAGING	PROBABLY DAMAGING	NF	NF	N/A
A4V	rs142499684	TOLERANT	BENIGN	NF			S103L	novel	DAMAGING	PROBABLY DAMAGING			
A4G	novel	TOLERANT	BENIGN				F105I	novel	DAMAGING	PROBABLY DAMAGING			
M5V	rs193051407	TOLERANT	BENIGN	NF	NF	N/A	F105L	rs149228054	DAMAGING	PROBABLY DAMAGING			
M5I	novel	TOLERANT	BENIGN				I110V	rs146862218	DAMAGING	P+B	NF		
A8S	novel	TOLERANT	POSSIBLY DAMAGING				W113R	novel	DAMAGING	PROBABLY DAMAGING			
Q10L	novel	TOLERANT	BENIGN				E117V	novel	DAMAGING	PROBABLY DAMAGING			
Q10R	novel	TOLERANT	BENIGN				K120R	rs192640504	TOLERANT	PROBABLY DAMAGING			
D12Y	novel	DAMAGING	PROBABLY DAMAGING				T122N	novel	DAMAGING	PROBABLY DAMAGING			
M16V	novel	TOLERANT	BENIGN				A132V	novel	DAMAGING	PROBABLY DAMAGING			
D17N	novel	TOLERANT	B+P				T137S	novel	TOLERANT	BENIGN			
K18N	rs74466425	TOLERANT	BENIGN	NF			I140T	novel	DAMAGING	PROBABLY DAMAGING			
G23D	rs148489044	TOLERANT	BENIGN	NF			E143D	novel	TOLERANT	BENIGN			
H24P	novel	TOLERANT	BENIGN				P145S	novel	TOLERANT	PROBABLY DAMAGING			
K29R	novel	TOLERANT	POSSIBLY DAMAGING				I150V	novel	DAMAGING	PROBABLY DAMAGING			
D40Y	novel	DAMAGING	PROBABLY DAMAGING	LOF	LOF	NF	L157V	novel	TOLERANT	BENIGN			
T43N	novel	TOLERANT	BENIGN				N161Y	novel	DAMAGING	PROBABLY DAMAGING			
R44H	novel	DAMAGING	PROBABLY DAMAGING	LOF	LOF	NF	A165T	novel	TOLERANT	BENIGN			
S46N	novel	DAMAGING	PROBABLY DAMAGING				A165V	novel	TOLERANT	PROBABLY DAMAGING			
Y47C	novel	TOLERANT	BENIGN				T171A	novel	TOLERANT	BENIGN			
F48Y	novel	DAMAGING	BENIGN				T172A	novel	TOLERANT	BENIGN			
Q50K	rs80221024	TOLERANT	BENIGN	NF			A173V	novel	TOLERANT	PROBABLY DAMAGING			
P55L	rs140811638	TOLERANT	BENIGN	NF			Y178C	rs77790369	TOLERANT	PROBABLY DAMAGING			
G56R	novel	TOLERANT	BENIGN				P187A	novel	DAMAGING	PROBABLY DAMAGING			
K62Q	novel	TOLERANT	PROBABLY DAMAGING				R188H	novel	DAMAGING	P+B	LOF	LOF	LOF
S64G	novel	TOLERANT	BENIGN				R188P	novel	DAMAGING	PROBABLY DAMAGING			
A68T	novel	TOLERANT	BENIGN				Q196R	rs112707798	TOLERANT	BENIGN	NF		
F69S	novel	TOLERANT	BENIGN				C199W	novel	DAMAGING	PROBABLY DAMAGING			
I70V	novel	TOLERANT	BENIGN				P202T	novel	TOLERANT	BENIGN			
K71R	novel	TOLERANT	BENIGN				T205I	novel	DAMAGING	BENIGN			
S73A	novel	TOLERANT	BENIGN				T206I	novel	TOLERANT	BENIGN			
E76A	rs183691150	DAMAGING	PROBABLY DAMAGING				E207Q	novel	TOLERANT	P+B			
							P208S	novel	TOLERANT	BENIGN			

Opened questions

In the functional screen presented in Chapter 1, I only tested the inhibitory effect of RGS2 toward Gαq signaling by using the Ca^{2+} mobilization assay. RGS2 protein, however, is involved in multiple signaling networks beside its canonical Gq GAP activity. A growing body of literature has demonstrated that the amino-terminal domain of RGS2 interacts with many proteins. RGS2 binds to other effector proteins related to GPCR signaling such as Gαs (Roy et al., 2006) and several subtypes of adenylyl cyclase (Roy et al., 2006; Salim et al., 2003). These interactions with RGS2 result in decreased cAMP production. How these interactions affect GPCR signaling, whether mutations of RGS2 affect interactions between RGS2 proteins and Gs or adenylyl cyclase and its physiological consequences remained to be defined. RGS2 also binds to the epithelial Ca^{2+}

channel TRPV6 and regulates gating properties in a GPCR-independent manner (Schoeber et al., 2006). RGS2 was also shown to interact with tubulin and regulate neuronal cell differentiation (Heo et al., 2006). By binding β-tubulin, RGS2 may affect β-tubulin polymerization and chromosome segregation (Jiang et al., 2016). RGS2 can also control protein synthesis through interaction with the eukaryotic initiation factor eIF2B through residues 79-115, which has been implicated in protection against cardiac hypertrophy (Chidiac et al., 2014; Lee et al., 2017; Nguyen et al., 2009). How RGS2 mutants function with respect to these other mechanisms has not been tested. The 12 mutants with "normal" function in the Ca^{2+} assay may be perturbed in other ways.

In Chapter 3, I examined the physiological effects of the four human RGS2 LOF mutations (Q2L, D40Y, R44H, R188H) in mesenteric arteries (MAs). I found that all mutant proteins failed to effectively suppress AngII-mediated vasoconstriction in mouse MAs. Interestingly, only the Q2L and R188H mutations exhibited impaired ACh-mediated relaxation. The D40Y and R44H RGS2 mutants supported a normal relaxation response to ACh. This differential effect, again, was not predicted by prediction tools. I also provided mechanistic evidence that this differential effect may be attributed to phosphorylation of RGS2 by PKG1α in VSMCs. It appeared that PKG-dependent phosphorylation can rescue PM localization and restore the vasorelaxation function of D40Y and R44H mutant RGS2 in CHO cells and in MAs.

By using reversible permeabilization, I was able to express RGS2 only in VSMCs. One way endogenous RGS2 may support relaxation is through a Gαi/o-dependent mechanism in the endothelial cells (Osei-Owusu et al., 2012). A transgenic or knock-in animal model might be needed to evaluate whether LOF RGS2 mutations affect this relaxation mechanism. This animal model will also allow investigation of whether endogenous RGS2 LOF mutants affect whole body

blood pressure responses as well as an assessment of effects on the central nervous system or the kidney.

To better address these questions, we have developed RGS2-deficient and RGS2 Q2L knock-in rat models, using the CRISPR-Cas9 method. These models will allow us to perform analysis of RGS2 expression and function in tissues, measure vascular contractile responses, and assess *in vivo* cardiovascular function such as blood pressure, cardiac and vascular morphology. The rats with the Q2L mutant allele will also permit study of the pharmacological effects of different agents (e.g. digoxin and the clinically used proteasome inhibitor bortezomib) which have previously been shown to help restore expression and function of the mutant protein.

This intervention approach to selectively target patients with RSG2 deficits might have a broader application. The common C1114G polymorphism in the 3'UTR of RGS2 gene is associated with hypertension, obesity, and preeclampsia in several European populations (Kvehaugen et al., 2014; Kvehaugen et al., 2013; Sartori et al., 2008; Semplicini et al., 2006a). This polymorphism resulted in a lower level of RGS2 mRNA and reduced protein expression in peripheral blood mononuclear cells (PBMs) and cultured fibroblasts. This effect may be due to mRNA destabilization (Semplicini et al., 2006a). By increasing the RGS2 protein half-life, pharmacological interventions successfully tested in the Q2L rat model might be beneficial to the population carrying the C1114G mutation.

Among the LOF RGS2 mutations identified, I found that the D40Y mutant allele was enriched in Finnish population in the gnomAD database at a minor allele of 0.8%. Therefore, in chapter 4, we used targeted single nucleotide variant testing approach to test whether the D40Y mutant allele is associate with disease phenotype in a population of 19,292 Finns. We identified

the association of a biochemically documented RGS2 loss-of-function allele, the D40Y (rs201233692), with high blood pressure, coronary artery disease, and myocardial infarction. Specifically, the G/T and T/T genotypes of rs201233692 were associated with higher systolic blood pressure. Carriers of the minor T allele tended to have higher diastolic and pulse pressure and BMI. Prevalence of CAD and MI also tended to be higher in the population carrying the T allele. On the other hand, no association of this mutation was found with central adiposity measures or insulin level, nor was it related to the prevalence of stroke.

Role of RGS2 in cognitive function

Recently, Arey et al has shown that activation of Gαq signaling enhanced memory consolidation and slows cognitive decline in *C. elegans* (Arey et al., 2018). Nest shredding is one of goal-directed home cage behaviors which has been used as a test to evaluate rodent cognitive function. This behavior in mice mirrors the activities of daily living in humans, which relate to the ability to organize, plan and execute tasks (Jirkof, 2014). Reduced amounts of nestlet shredded during a fixed amount of time reflects cognitive impairment (Jirkof, 2014). Hypertension causes alterations in cerebral artery structure and function that can impair blood flow and results in cognitive decline (Pires et al., 2013). Our preliminary study to investigate the role of RGS2 in the development of vascular dementia suggested a protective role of RGS2 in nest shredding test (Fig. 5-1) and cerebral blood flow measurement (Fig. 5-2). RGS2 may also involve in several CNS diseases such as epilepsy (Namvar et al., 2017; Narla et al., 2016), and anxiety disorder (Hohoff et al., 2015; Koenen et al., 2009; Leygraf et al., 2006; Smoller et al., 2008). The gnomAD database contains data from other Finnish population studies focusing on Alzheimer's disease such as FINN – ADGEN study, Kuopio Alzheimer study. These data may provide information for analysis

regarding RGS2 mutations, especially the D40Y and the dementia phenotype. This direction can serve as a new research avenue to systematically understand the role of RGS2 in cognition or other function of the brain.

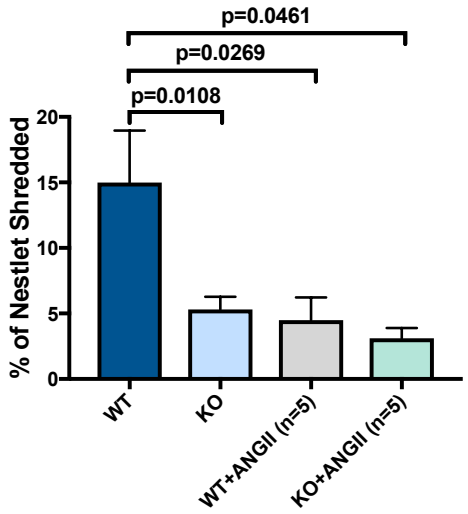

Figure 5-2. **RGS2 deficiency and AngII treatment affect a behavioral phenotype.**
There was a significant difference in the percent of nestlet shredded of all groups when compared to the untreated WT. Data are presented as mean ± SEM, n=5.

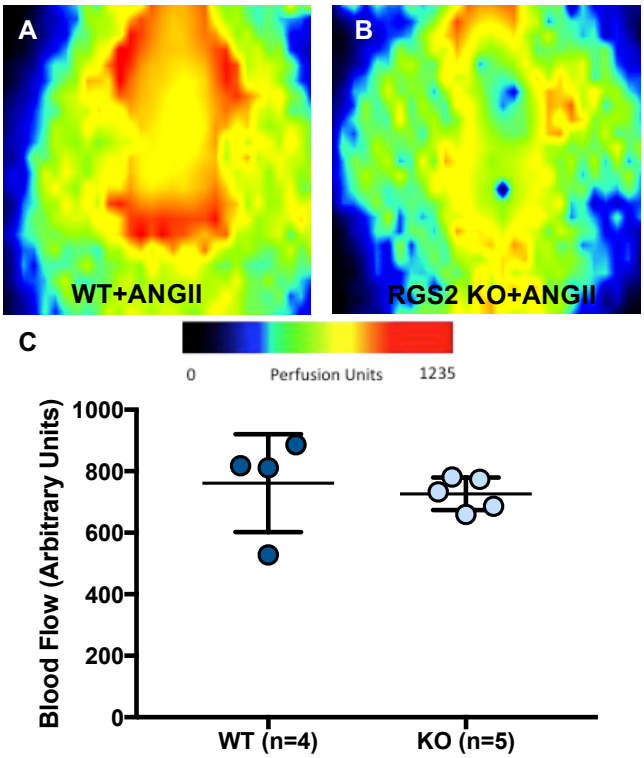

Figure 5-1. **Representative images of cerebral blood flow in RGS2$^{+/+}$ and RGS2$^{-/-}$ mice are shown in A and B. C.**
AngII infusion slightly decreases blood flow in the KO mice however, the results are not statistically significant (p=0.66). Data are presented as mean ± SEM, n=4 for WT, n=5 for KO.

Outstanding questions on RGS2 protein control of vascular function

RGS2 deficiency augments vasoconstriction of resistance arteries by affecting stretch-induced contraction or myogenic tone. Renal interlobar arteries isolated from RGS2 knockout mice exhibit enhanced constriction triggered by rises in transmural pressure (Hercule et al., 2007). How RGS2 regulates myogenic tone is unknown. Vascular smooth muscle cell stretching induces activation of stretch receptor including the transient receptor potential superfamily (Beech, 2005), vascular epithelial sodium channel (Kim et al., 2012), integrins (Chao and Davis, 2011), and the angiotensin type 1 receptor (Mederos y Schnitzler et al., 2011) and eventually results in myogenic response. Whereas RGS2 is known to negatively regulate vascular AT1R signaling, it remains possible that RGS2 regulates other mediators of vascular mechanotransduction. Recently, a Gαq-couple receptor GPR68 was found to be an essential flow sensor in arteriolar endothelium (Xu et al., 2018). In the airway, RGS2 was found to attenuate acid-induced GPR68-dependent increase in Ca^{2+} and MUC5AC secretion (Liu et al., 2013). I hypothesized that RGS2 may modulate myogenic tone, in part, through its effect on GPR68 receptor in arteriolar endothelium. Because both GPR68-deficient and RGS2-deficient resistance arteries showed impaired flow-mediated dilation, double knockout artery ($GPR68^{-/-}$ x $RGS2^{-/-}$) may show even more depressed dilation in myography assay. One may also manipulate RGS2 expression (overexpression or siRNA knockdown) in cells and perform high throughput shear assay (Xu et al., 2018) to evaluate whether RGS2 affect shear stress. It is notable that RGS2 inhibits Gq which couples to GPR68. If RGS2 modulates myogenic tone through GPR68, it may act through a different mechanism to enhance GPR68 function.

The ultimate goal of studying the role of RGS2 in cardiovascular disease is to apply what we know about RGS2 biochemical and genetic properties to clinical translation. Drug discovery efforts for RGS2 upregulators and stabilizers are actively being pursued. The LOF mutant RGS2 can be used to test efficacy of hits or lead compounds from these studies. The mechanism of action of these compounds may also be inferred depending on which mutant's function is rescued.

Overall, in my thesis work described here, I have identified four rare LOF mutations in human RGS2 with the potential cellular mechanisms underlying their phenotypes. The mislocalization phenotype of the D40Y and R44H could be rescued by PKG-dependent phosphorylation. These mechanisms may guide drug discovery or drug repurposing effort for hypertension by enhancing RGS2 function. One may restore the Q2L function by halting the proteasomal degradation. The D40Y and R44H function may be rescued by site-specific phosphorylation (PKG activators). I also established the association between the RGS2 D40Y mutant allele with hypertension and cardiovascular disease. This finding added to our knowledge about rare variants contributing to the development of hypertension.

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