Characterizing the Role of RGS5 in the Regulation of Vascular Smooth Muscle Cell Function

By

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A thesis submitted in conformity with the requirements for the degree of Masters of Science in Physiology at the University of Toronto

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ABSTRACT

Regulators of G-protein signaling (RGS) modulate G-protein coupled receptor (GPCR) activity in vascular smooth muscle cells (VSMCs). One such protein, RGS5, has been shown to have selective expression in VSMCs and pericytes, and can inhibit signaling from Gαq and Gαi subunits. Using an RG55 knockout model, we assessed the functional effect of RGS5 in the constriction and dilation of resistance arterioles. Furthermore, we examined the intracellular lipid interaction of RGS proteins to identify the determinants regulating the biologic function of RGS5. Surprisingly, loss of RGS5 function in mesenteric arterioles had no effect on constriction and dilation of resistance arterioles. Cultured VSMCs showed increased basal ERK1/2 phosphorylation and increased VASP signaling in response to SNP treatment in RG55KO VSMCs as compared to wild type controls, with no effect on cell proliferation. These data suggest RGS5 may integrate multiple intracellular pathways with competing effects on VSMC contraction.
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Unless otherwise noted below, all experiments and data presented in this thesis were collected and analyzed by the author.

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List of Abbreviations

ACE – Angiotensin converting enzyme
ACh – Acetylcholine
AngII – AngiotensinII
AT1R – Angiotensin receptor type 1
AT2R – Angiotensin receptor type 2
BP – Blood pressure
CV – Cardiovascular
CVD – Cardiovascular disease
DAG – Diacylglycerol
GPCR – G-protein coupled receptor
EDHF – Endothelial derived hyperpolarizing factor
ERK1/2 – Extracellular regulated kinase 1/2
ES – Embryonic stem cells
ETA – Endothelin receptor type A
ET-1 – Endothelin 1
GAP – GTPase activating protein
GDP – Guanine diphosphate
GTP – Guanine triphosphate
IP3 – 1,4,5 inositol triphosphate
KO – Knockout
MLC – Myosin light chain 20
MLCK – Myosin light chain kinase
MLCP – Myosin light chain phosphatase
NO – Nitric oxide
NTD – N-terminal domain
PE – Phenylepherine
PIP2 – Phosphatidylinosital 4,5-bisphosphate
PKA – Protein kinase A
PKC – Protein kinase C
PKG – Protein kinase G
PLC - Phospholipase
RGS – Regulator of G-protein signaling
S1P – Sphingosine-1-phosphate
S1PR – Sphingosine-1-phosphate receptor
SNP – Sodium nitroferricyanide
VASP – Vasodilator stimulated protein
VSMC – Vascular smooth muscle cell
WT – Wild type
Chapter 1: Introduction

1.a Cardiovascular physiology and blood pressure regulation

Among the current health and disease paradigms in Canada and the developed world, chronic CV disease, and more specifically heart disease, is a leading cause of morbidity and mortality (Burt, Whelton et al. 1995; Whitworth 2003). Persistently high blood pressure (hypertension) is a key risk factor for the development of heart and other cardiovascular diseases. Hypertension is defined as a systemic blood pressure equal to or exceeding 140 mmHg/90 mmHg (systolic/diastolic). As of 2005, Statistics Canada reported over 1.7 million Canadians over the age of 65 were living with hypertension – approximately two out of three of whom are female. In total, over 4 million Canadians over the age of 12 live with hypertension. In addition to this, the number of Canadians with “high-normal” blood pressure (130-139/80-89) is rising and everyday these individuals are at risk of moving into the hypertensive group. These numbers and the burden they cause on the health care system are staggering, however, what is truly alarming is how fast these figures are rising and how few of these cases can be explained and properly treated (Chobanian, Bakris et al. 2003). Approximately 90-95% of the clinically presented cases of hypertension are termed primary hypertension, which generally refers to hypertension of unknown medical origin (Carretero and Oparil 2000). Numerous studies have linked hypertension to increased body mass index and
age, with treatment strategies focusing on bettering lifestyle factors to combat the illness. These strategies include reducing body weight, sodium intake, and alcohol consumption, as well as increasing physical activity and cessation of smoking for smokers (Chobanian, Bakris et al. 2003). Unfortunately, in many of these cases, changes in lifestyle factors have limited efficacy in reducing these patients’ blood pressures (Miura and Nakagawa 2005), forcing physicians to employ pharmacologic therapies. These data point to the importance of basic research into understanding the mechanisms that are critical for controlling systemic blood pressure.
Normal blood pressure maintenance requires the interplay of the shown cellular, tissue and organ systems. The two primary components of the determinants of blood pressure can be divided into cardiac and vascular mechanisms (left and right sides respectively), with the focus of this thesis being on the vascular aspect; specifically, on the role of vessel tone and arteriole radius on maintenance of systemic blood pressure. This diagram also illustrates the potential for a variety of compensatory mechanisms from factors directly involved in modulating vessel tone and in tissues entirely independent of the vasculature.
**1.a.1 The resistance vasculature in blood pressure physiology**

Integrative cardiovascular physiology (CV) is a broad field requiring a detailed understanding of the communication between a wide number of vital systems, organs and tissues. Notwithstanding the significant contribution of renal and central nervous system function to the control of systemic blood pressure, the peripheral vasculature is often the end effector organ of such physiologic regulation and therefore a large amount of work has been dedicated to understanding the signaling pathways that control its function (Grassi and Heistad 2009; Leibovitz, Ebrahimian et al. 2009; Resch, Wiest et al. 2009). Arterioles are the primary site of regulation of peripheral vascular tone. They are situated, in terms of size as well as anatomical location, between the large conduit arteries, such as the aorta, carotid and femoral arteries, and the fragile capillary beds. The resistance arterioles are unique in their mural composition, containing large muscular medial layers and relatively high ratios of wall thickness to lumen size. These differ from conduit arteries, which have large amounts of elastin in addition to SMCs in the medial layers, and the capillaries, which have no smooth muscle cell medial layers at all. This structural feature of capillaries, aside from facilitating rapid diffusion of nutrients and waste products to and from the surrounding tissue, leaves the vasculature sensitive to sudden increases in flow and pressure (West and Mathieu-Costello 1995). As such, sudden increases or decreases in cardiac output would cause extensive and potentially irreversible damage to these delicate vascular beds. Therefore the role of the resistance vasculature is both to maintain normal flow rates to
downstream tissues over a wide range of systemic pressures, as well as assist in the establishment and regulation of systemic pressure.

1.a.ii Assaying resistance artery function

To date there have been numerous studies examining how modifications in these vessels and their function can effect overall systemic pressure (Lee and Smeda 1985; Laurant, Touyz et al. 1997; Resch, Wiest et al. 2009). Furthermore, a number of current and potential treatment strategies for dealing with hypertension are directed at adjusting the responses of these blood vessels (Khan, Hemmelgarn et al. 2009). In order to assess their function, a number of assays are employed, and among these is a broad group of experiments termed myography (McPherson 1992; Wright and Angus 2000). Briefly, a myograph uses a variety of strategies for measuring (‐graph) the smooth muscle (myo‐) dependant vasoconstriction or the interplay of signals between endothelium and SMC that leads to vasodilation. There are varying types of myographs, with modifications ranging from fundamental measuring parameters to superficial aesthetics of the apparatus itself (Angus and Wright 2000). The primary methodology used in this thesis was the pressure myograph. In pressure myography, isolated intact blood vessels are cannulated, pressurized and maintained at physiological or near‐physiological temperatures. The vessels are maintained using physiological buffer and are stretched to near‐physiological lengths. As such, all efforts are taken to reproduce a near comparable physiological environment for the vessel. Conversely, in wire
myography, isolated vessel rings are mounted between wires that are connected to force transducers. These assays measure the force of constriction from isolated rings and quantify the data as a measure of vessel function. Unfortunately, the state of the vessel at the time of measurement, as well as the type of force being exerted upon it, are far from physiological. This chief criticism is the main factor behind the choice to use pressure myography in our case (Buus, VanBavel et al. 1994). Pressure myography, however, is by no means a “perfect” physiological assay. The system employs pressure with the absence of flow, thus removing important factors such as shear and its downstream effects on vascular signaling. This criticism makes pressure myography a limited tool. Furthermore, in instances where vessels are markedly shorter, such as pulmonary arterioles, and thus difficult to cannulate onto adjacent micropipettes, wire myography is preferred as vessel disc segments can be significantly shorter than those used for pressure myography.

1.b. VSMC contraction and vasoconstriction

Using pressure myography it is possible to measure and quantify vasoconstriction and dilation, and through this to assay vascular smooth muscle cell (VSMC) function and physiology. Briefly, the medial layer of resistance arterioles, as already stated, is rich in SMCs: a specialized cell capable of contraction. This contractile mechanism differs from skeletal or cardiac muscle in its relatively slower response to stimulus and ability to maintain a longer overall period of contraction. It is mediated by a number of intracellular proteins, however Gαq mediated signaling comprises the
majority of the driving force behind SMC contraction. The signaling induced by Gαq coupled receptors is a complicated interconnected series of partnered proteins and secondary messengers. Briefly, upon activation via an extracellular agonist, Gαq activity leads to activation of the membrane bound protein, phospholipase-C (PLC). PLC propogates the signal through the formation of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG) from the precursor phosphatidylinositol 4,5-bisphosphate (PIP2). At this point, the signaling becomes further complicated due to multiple pathways being activated in tandem. IP3 binding to receptors on the sarcoplasmic reticulum mobilizes the calcium stored therein, increasing intracellular calcium levels. Calcium binds to Calmodulin, forming a complex, and activates myosin light chain kinase (MLCK). MLCK phosphorylates myosin light chain 20 (MLC), which leads to contraction of the smooth muscle cell. Occurring in parallel to the above pathway, accumulation of DAG leads to activation of protein kinase C (PKC) and through this the Rho/RhoK signaling pathways (Shimokawa and Takeshita 2005). These signals ultimately lead to the inhibition of myosin light chain phosphatase (MLCP), the antagonist to MLCK and contractility. As a result, Gαq activation leads to the phosphorylation of MLC and contraction of the SMC through two different pathways.

Vasodilatory signaling from ligands such as adenosine, catecholamines, and glucocorticoids also occur through GPCRs, and in arterioles, cause VSMC relaxation both directly at the level of the VSMC and also through the endothelium. The studies presented herein focus on the contractile role of VSMCs, the regulation of that contractile response, and the role of this mechanism in the regulation of systemic
vascular pressure. Furthermore, the studies presented examine the precise mechanisms behind the regulation of these contractile signals and establishes connections between biochemical abnormalities and clinical implications. It should also be noted that contractile activity is not the only component of VSMC function. SMCs are unique in their ability to shift their phenotypes from one of contractile to proliferative and migratory activity (Owens 1995). This shift, as well as the migration of SMCs into the intimal layer, is implicated in the development of diseases involving vascular legions, such as atherosclerosis and re-stenosis (Ross 1993). In fact, a number of vascular agonists that affect contractile response, have also been shown to be involved in the determination of VSMC phenotype (Somlyo and Somlyo 1994). As such, the pathways studied become much more complex and interwoven, and require careful attention to experimental setup and the establishment of proper controls. Furthermore, the existence of these alternate functions creates yet another layer of inquisition into the role of VSMC specific proteins. Ultimately, assessment of vascular contractile response to these receptors may potentially leave out the importance of this

1.c Heterotrimeric G-proteins in VSMC function

The role of G-proteins both at the level of cardiac, vascular and renal tissues has been continually characterized over years of investigation (Lefkowitz 1996; Cho, Harrison et al. 2004; Callera, Tostes et al. 2007). Changes in GPCR signaling may result in large sudden shifts in cardiac output, and of particular relevance to this thesis, changes
in VSMC contractile function and vascular resistance. These changes can go on to affect systemic pressure and through that, lead to a number of clinical complications as described earlier.

Although the role of G-proteins in the normal function of these systems has been shown to be essential, there still remain aspects to their molecular mechanisms that are unclear. One such region of interest is in the exact roles for the number of g-proteins and g-protein coupled receptors (GPCRs) present in both the heart and the vasculature. Indeed, in the vasculature, GPCR signaling is responsible for the bulk of contractile activity of vascular smooth muscle cells (VSMCs). Currently, of the established vasoconstrictors a large number of them work via GPCRs. Of these GPCRs, the large majority signal through Gαq and Gαi, both targets of the R4 subfamily of RGS proteins. These include alpha-adrenergic receptors, angiotensin 1 receptors (AT1R, AT2R), endothelin-1 receptors (ETA), and sphingosine-1-phosphate (S1PR1-5) (Demoliou-Mason 1998; Waeber, Blondeau et al. 2004). The prevalence of Gαq coupled receptors, their presence on VSMCs and their coupling to known vasoconstrictors establishes the importance of this family of G-proteins in regulating vascular pressure. Furthermore, the majority of medications currently prescribed to treat hypertension target GPCRs, as well as the production of and binding of GPCR agonists (Lefkowitz 1996; Carretero and Oparil 2000; Chobanian, Bakris et al. 2003). These include angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor (AT1R, AT2R) blockers, as well as alpha- and beta-adrenergic receptor blockers. Two key GPCRs, the AT1 and AT2 receptors, have been implicated as key players in a number of conditions, including hypertension, cardiac
remodeling, and cardiac hypertrophy (Zhang, Zhang et al. 2003; Zhu, Zhu et al. 2003). Furthermore, GPCR activity has been a target for the understanding and treatment of type 2 diabetes through recent studies in the impaired function of pancreas islet cells (Ahren 2009). Leukocyte function and migration (Irukayama-Tomobe, Tanaka et al. 2009), retinal physiology and rhodopsin function (Ahuja, Crocker et al. 2009) and neuronal signaling (Yu, Arttamangkul et al. 2009) are but a few examples of the multiple diverse functions of GPCR signaling. In addition, investigation into the regulation of GPCR signaling activity has revealed the necessity of these regulatory mechanisms (Gros, Benovic et al. 1997; Heximer, Knutsen et al. 2003).

The mammalian vasculature is a highly active physiological player in the greater scheme of overall organism health. Within the vessel wall, the endothelium, vascular smooth muscle cells and perivascular innervations are responsible for the bulk of vascular responses and systemic effects through complex communication and signaling crosstalk. These interactions however are beyond the scope of this thesis, which will instead focus on GPCR-mediated pathways within vascular smooth muscle cells (VSMCs). VSMCs are responsible for the contractile or dilatory response of the vessel wall, and are further characterized by the ability to alter their phenotypes from one of modulating vessel tone, to a proliferative and angiogenic phenotype (Owens 1995; Manabe and Nagai 2003). It has been demonstrated that G-protein signaling, through GPCRs, is responsible for a number of these changes in cellular activity and contractility. In normal quiescent arteries, there is an equilibrium of signaling between the two layers leading to a basal level of tone throughout the vasculature.
1.c.1 Bayliss effect (myogenic response) and the role of G-proteins/GPCRs as mediators of this effect

The myogenic response is the intrinsic ability of resistance arterioles to contract in response to increased transmural pressure (Bayliss 1902; Falcone, Davis et al. 1991). This VSMC dependent mechanism is believed to be responsible not only for the establishment of resting vessel tone but also required for the maintenance of constant flow over a range of pressure changes (Schubert, Lidington et al. 2008). It is from this resting membrane tone that further vasodilator and vasoconstrictor signaling act, thus rendering maintenance of this vascular homeostasis essential for normal circulatory function. Currently, the mechanisms initiating the myogenic response are poorly understood due to the multitude of interacting factors that lead to pressure induced contraction, as well as vasoconstriction in general. What is accepted, however, is that the myogenic response is a VSMC specific characteristic and is most prevalent in the arterial resistance vasculature, which is replete with SMCs. Furthermore, S1P signaling, ERK activation and activation of the Rho/Rho kinase signaling pathway and MLCP inhibition have been established as essential mediators of the myogenic response (Bolz, Vogel et al. 2003; Schubert, Lidington et al. 2008). These S1P receptors (types 2 and 3, on VSMCs) are GPCRs and act through a variety of Gα subunits including Gαq, Gαi and G12/13. Existing evidence supporting specificity of RGS5 for these receptors as well as established specificity for Gαq and Gαi provide the rationale for signaling and functional assays of S1P activity in RGS5 knockout vessels and primary cultured VSMCs (Cho, Harrison et al. 2003). As such, the arterial SMC specific expression of RGS5, as well as
S1P specificity and general capacity for G\(\alpha q\) and G\(\alpha i\) targeting, suggest a role for RGS5 in regulating the myogenic contractile response in arterioles.

1.d The GTPase cycle of Heterotrimeric G-proteins

At the most fundamental level, signals outside cells must be transmitted inwards to elicit a biological response. G-protein coupled receptors are the largest family of surface receptors, with over 800 currently identified genes in the human genome to date (Cotton and Claing 2009). Furthermore, G-proteins can respond to a number of agonists, ranging from chemokines, hormones and neurotransmitters to mechanical stretch and the movement of matrix proteins. In the case of vasoactive agonists, ligands bind to the extracellular domain of a family of receptor proteins termed seven transmembrane (7TM) proteins, which are coupled at their intracellular domains with a heterotrimeric G-protein, creating a G-protein coupled receptor (GPCR) (Bourne, Sanders et al. 1990). Binding of the extracellular agonist induces conformational changes in the 7TM receptor, which activates the coupled heterotrimeric G-protein. In the inactivated state, the three subunits (alpha, beta, gamma) of the heterotrimeric G-protein are tightly bound and maintain close proximity with the 7TM receptor, with a GDP occupying the binding pocket of the G\(\alpha\) subunit. Upon binding of the extracellular ligand and conformational change in the receptor, the GDP is replaced with a GTP, and the G-protein signaling becomes activated. The signal is then transmitted into the cell through the action of the activated G-protein and it’s downstream signaling partners and secondary messengers. More specifically, it is the alpha subunit of the
heterotrimeric G-protein (beta and gamma being the other two subunits) that is responsible for the variation in downstream signaling. Upon activation, Gα and Gβγ activate separate signaling partners resulting in a chain of intracellular signaling. This signaling is brought to an end by the hydrolysis of the GTP bound to Galpha. In vivo this signaling is tightly controlled and displays a rapid ON/OFF motif, while the intrinsic GTPase activity of the Gα subunit is too slow to allow this. In the absence of the rapid termination, the signal continues beyond physiological levels. Though in some cases this may be innocuous, data from Heximer et al (2003) has shown a hypertensive phenotype due in part to this overactive G-protein signaling. As such, due to the high number of vasoconstrictors signaling through GPCRs, the G-protein regulatory mechanisms of VSMCs are essential in maintaining normal systemic pressure.

Gα subunits are divided into four subtypes: Gαi/0, Gαq/11, Gαs, and Gα12/13. Each Gα subunit associates with a number of different downstream signaling partners and as such affects a variety of intracellular responses. The focus of this thesis will be on Gαi/0 and Gαq/11 in VSMCs. Vascular GPCRs coupled to Gαq and Gαi have long been characterized as a highly vasoactive class of receptors. Specifically, Gαq activity leading sequentially to PLC activation, intracellular calcium release, and contraction of VSMCs to produce vasoconstriction of arteries. In addition, the activation of Gαi results in the inhibition of adenyl cyclase and reduction of intracellular cAMP levels. In the endothelium, Gαi is coupled to GPCRs coupled to known vasodilators such as Bradykinin (Liao and Homcy 1993). In VSMCs, the reduction in cAMP levels results in reduced PKA activity and attenuation of Gαs mediated vasodilatation.
1.e The RGS superfamily

Signaling by neurotransmitters and hormones through G-protein coupled receptors is a common mechanism in most mammalian organ systems. This signaling, as described above, is dependant on the hydrolysis of GTP, bound to the G-alpha subunit. Alpha subunits have intrinsic GTPase activity- but it is very slow. In vivo, the hydrolysis of this GTP is known to occur significantly faster than that which is observed in vitro (Siderovski, Hessel et al. 1996; Watson, Linder et al. 1996). This GTPase activation is partly the result of the activity of a family of proteins termed the Regulators of G-protein Signaling (RGS). This family of more than 35 proteins, characterized by a 120 amino acid GTPase activating domain, is responsible for catalyzing the hydrolysis of GTP by the Gα subunit to speed this process up to 2000-fold (Ross and Wilkie 2000). One possible manifestation of a lack of RGS protein activity is in increased sensitivity of a GPCR to its associated agonist. This increased sensitivity would result in a leftward shift in the dose response curve for said agonist. Alternately, or in addition to this, the agonist may induce a more pronounced cellular response at any given concentration. This would be evident in an upward shift in dose response curves, or more likely, a stronger response at peak concentrations.

These GTPase activating proteins (GAPs) form a family of nearly 30 different peptides, with diverse expression profiles between organ systems and tissues. Within this large group of proteins, there are distinct subfamilies of RGS proteins sharing
further sequence homology. Briefly, these subfamilies are distributed across a wide variety of tissues and organ systems and as such play a number of roles. The subfamilies include RZ, R4, R7, and R12. In the absence of these GAPs, the signaling produced from GPCRs can be greatly affected (Heximer, Knutsen et al. 2003; Cifelli, Rose et al. 2008). In studying the function of any one RGS protein it is important to understand the potential expression and functional overlap between it and the other family members. Thus, provided below is a brief description of each RGS subfamily.

1.e.i RGS7-like (R7) Subfamily

This family of RGS proteins contains RGS6, RGS7, RGS9 and RGS11 and all of their assorted splice variants. These proteins are unique due to a common 64 amino acid Ggamma-like (GGL) domain as well as a Dishevelled/EGL-10/Pleckstrin domain (DEP) (Snow, Krumins et al. 1998; Chatterjee, Liu et al. 2003). These distinctive domains have been identified as key factors in the regulatory role of RGS proteins of this subfamily, and are responsible for allowing a number of intracellular signaling activities and protein-protein interactions.

1.e.ii RGS12-like (R12) Subfamily

The proteins of the R12 subfamily arise from the many splice variants of the Rgs10, Rgs12 and Rgs14 genes. The proteins of this subfamily are less understood than those of the previously described R7 class. The members of the R12 family of proteins vary greatly in size with RGS10 containing 173 amino acids and RGS12 containing 1447 residues. Existing literature in this field identifies a number of domain-specific

1.e.iii Rz-like (Rz) Subfamily

The members of the Rz subfamily are encoded by the Rgs17, Rgs19 and Rgs20 genes. As is observed with the other RGS subfamilies, a wide variety of proteins arise from these genes as a result of splice variants. The function of these proteins varies from other RGS subfamilies in that they are strongly affected by phosphorylation of both themselves and their binding partners (Glick, Meigs et al. 1998; Ogier-Denis, Pattingre et al. 2000).

1.e.iv RGS4-like (R4) Subfamily

The members of the R4 family are composed of little more than an RGS box domain and short N-terminus and C-terminus extensions, making these proteins the smallest and simplest RGS proteins in terms of structure. This subfamily is the largest of the RGS subgroupings, containing RGS1, 2, 3, 4, 5, 8, 13, and 16, with each of these proteins transcribed from a distinct gene (Sierra, Gilbert et al. 2002). Furthermore, with the exception of RGS2 (Heximer, Watson et al. 1997), every member of this subfamily is able to bind and inhibit Gαi and Gαq/11 signaling. In addition, with the exception of RGS3, the RGS proteins of the R4 subfamily all contain an NTD alpha-helix (Bansal, Druey et al. 2007). Of interest to our laboratory are three specific members of the R4 subfamily: RGS2, RGS4, and RGS5. The focus of this thesis is on RGS5, and to a lesser extent RGS2, both of which are key proteins identified for the study of vascular
homeostasis and blood pressure regulation (Grayson, Ohms et al. 2007). Indeed, there are phenotypes presented from gene-knockout animal models for each of the above proteins (hypertensive RGS2 knockout (Heximer, Knutsen et al. 2003), and hypotensive RGS5 knockout (Cho, Park et al. 2008; Nisancioglu, Mahoney et al. 2008)). The existing literature and preliminary data from our group on members of the R4 group are outlined below and comprise the majority of the rationale for the studies described herein.

1.e.iv.1 RGS5

Our preliminary data shows that RGS5 is the most highly expressed RGS protein in peripheral VSMCs and pericytes (Zhang and Heximer, unpublished data). RGS5 has often been cited as a potential target for anti-hypertensive therapy (Grayson, Ohms et al. 2007) and more recently, a potential key player in the modulation of vascular remodeling (Manzur and Ganss 2009). RGS5 has a striking expression profile that is concentrated primarily in the VSMCs of arterial vasculature in adult animals. Briefly, rgs5 gene expression peaks in the embryonic period, and falls subsequently in a vascular bed-specific manner to maintaining significant selective expression in certain blood vessels of adult animals (Cho, Kozasa et al. 2003). This adult expression is significantly higher in arterial SMCs when compared to accompanying veins, suggesting a role for RGS5 in the regulation of arterial SMC signaling activity (Li, Adams et al. 2004). Data from studies in mice lacking intact RGS2 – another member of the R4 subfamily - has
revealed a marked hypertensive phenotype when compared to age-matched wild type controls (Heximer, Knutsen et al. 2003). This hypertension is partly the result of prolonged vasoconstrictor signaling through Gαq-coupled receptors. Also of interest, Li et al (2004) demonstrated the presence of RGS5 in most vascular beds in non-human primates, including iliac, mammary and renal arteries. Furthermore, examination of peripheral atherosclerotic plaques revealed RGS5 expression in the medial SMCs accompanied by a pronounced downregulation in SMCs of the atherosclerotic plaque. Recent studies by other groups have demonstrated an induction of RGS5 during tumor growth, and neovascularization. These findings suggest a potential role for RGS5 as a regulator of vascular remodeling, and a potential candidate for further study as an intrinsic anti-atherosclerotic factor.

As described earlier, GPCR mediated contractile pathways in VSMCs are dependant on Gαq and Gαi mediated signaling (Petitcolin, Spitzbarth-Regrigny et al. 2001). RGS5 has been demonstrated to have specificity for both of these subunits (Zhou, Moroi et al. 2001). Although the focus of this thesis has been the contractile machinery of the VSMC, this is not the only pathway in which Gαq and Gαi signaling are active. Numerous cellular activities including proliferation, migration and cell-cell adhesion are mediated by signaling through these subunits (van Biesen, Luttrell et al. 1996; Ai, Kuzuya et al. 2001). Briefly, Gαq activates PLC as described earlier, however both Gαq and Gαi have been shown to be involved in mitogen activated protein (MAP) kinase pathways. These pathways are highly active in SMCs in varying functions, from focal adhesions to Wnt signaling. As such, the specificity of RGS5 for both subunits suggests a potential role
in many of these processes. One specific VSMC function that has recently been identified as involving ERK signaling, and is of particular interest to us, is the myogenic response (Pitson, Moretti et al. 2003; Lidington, Peter et al. 2009).

As stated previously, the current literature examining the *in vivo* role of RGS5 describes a hypotensive phenotype in RGS5 knockout mice as well as dilated aorta tissue sections (Cho, Park et al. 2008; Nisancioglu, Mahoney et al. 2008). Specifically, Cho et al (2008) report increased basal ERK1/2 and basal and stimulated VASP phosphorylation in immunoblot assays of cultured smooth muscle cells treated with a number of agonists as a potential mechanism for the reported blood pressure differences between wt and KO animals. It is important to mention that telemetric investigation of the blood pressure phenotype in waking RGS5KO mice (Figure 2) could not confirm the hypotensive phenotype, acquired using tail-cuff measurements of mouse blood pressure. Specifically, invasive blood pressure measurements taken using carotid catheterization and wireless telemetry reveal a normotensive phenotype in RGS5 knockout mice as compared to wild type controls (Heximer, 2009, unpublished). This method of invasive waking blood pressure measurement is superior to measurements conducted using a tail-cuff plethysmography on a number of levels. Chief among these factors is the use of a catheter allowing direct internal measurement of mean arterial pressure from within the carotid artery, as opposed to tail cuff measurements, which must be discarded and repeated in the event of animal movement or weak pressure readings. Moreover, carotid catheter telemetry allows animals to behave normally and move freely while
measurements are being taken, while plethysmography requires the manual manipulation of animals into restraints, a source of agitation and subsequent heightened sympathetic nervous activity. It is currently unclear whether external factors, specifically stressors driving sympathetic activity, play a role in the apparent discrepancy between ours and the published results. There is potential for an affected mechanism at the level of the nervous response in the RGS5KO mice, hindering the increase in sympathetic activity observed in wild type animals. However, examinations into these signaling pathways are beyond the scope of this thesis and must be addressed in a separate study. Furthermore, a number of vascular specific mechanisms may be involved in the modulation of resistance vessel tone and systemic pressure, resulting in a global compensatory effect and unchanged whole animal phenotype.
FIGURE 2: There is no difference in mean arterial pressure between wild type and RGS5KO mice. Wild type and RGS5KO mice were implanted with a carotid catheter telemetry device allowing for continual measurement of mean arterial pressure over a period of 48 hrs. The data shown is the average mean arterial pressure taken from 6 wild type and 5 knockout animals. (Figure is from Zhang et al, Unpublished)
Work described herein uses pressure myography to identify the intracellular pathways regulated by RGS5 in VSMCs and to examine the potential discrepancy between the published literature and our own physiologic data. Current literature from studies conducted on other RGS5 knockout mouse models describe a hypotensive phenotype as compared to wild type controls. In addition, Cho et al have described increased VASP phosphorylation in assays of intracellular signaling conducted on cultured RGS5 knockout smooth muscle cells. These results have led to a proposed role for RGS5 as a negative regulator of vasodilator signaling in the arterial vasculature.

Acetylcholine (ACh), acting through endothelial cells, is an established potent dilator of resistance arterioles. Briefly, the endothelial factors (NO, EDHF, and Prostaglandins) act on VSMC GPCRs coupled to G\(\alpha_s\) subunits. This category of G\(\alpha\) subunit acts through the PKA pathway and through a number of signaling effects such as inhibition of MLCK and reduction of intracellular Calcium levels. Since our model is a whole-animal knockout and these endothelial-dependent processes occur through GPCRs and G\(\alpha\) subunits, there is the possibility that RGS5 may act at some point in this signaling pathway. The majority of the rationale for the proposed role of RGS5 as a mediator of vasodilator signaling arises from the published data demonstrating increased VASP phosphorylation in RGS5 knockout VSMCs.

VASP, or vasodilator-stimulated phosphoprotein, is phosphorylated in response to vasodilator signaling and is often used as a readout of cyclic nucleotide (PKA and PKG activity (Chen, Daum et al. 2004). These kinases, both established downstream signaling partners of GPCRs and G\(\alpha\) signaling have been linked to many different intracellular
signaling pathways in a number of cell types. Increased VASP phosphorylation in the
RGS5 knockouts, would point to a potential role of RGS5 in the attenuation of
vasodilator signaling. However the Ena/VASP family of proteins is involved in a number
of matrix-related cellular processes (Reinhard, Jouvenal et al. 1995) and as such, its role
as a marker for vasodilator signaling activity may be an underestimate of its true biologic
potential. Modulation in VASP phosphorylation has also been connected to changes in
migratory and cell-cell adhesion activity in tissue culture experiments (Bear, Svitkina et
al. 2002).
FIGURE 3: Schematic figure depicting RGS5 promoter activity as well as origin of arterial tissue. Left panel, Illustrates the gradient of RGS5 gene promoter activity, which has been assayed using X-Gal staining and real time quantitative RT-PCR (Zhang and Heximer, unpublished). Right panel, Displays the correlation between shifts in embryonic tissue origin and promoter activity for each portion of the arterial vasculature.
**RGS5 reporter/knockout mouse model**

Our experiments utilized isolated resistance arteries from our RGS5 reporter/knockout mouse (Deltagen laboratories). This animal model was developed using a LacZ-neo cassette, inserted into exon 2 of the Rgs5 gene (Figure 3). *In vivo*, the endogenous Rgs5 promoter will drive LacZ transcription, which will allow for staining and visualization of RGS5 expressing cells. This model is effective as a reporter of cells and tissues wherein RGS5 plays a role.
FIGURE 4: Schematic representing LacZ-Neo cassette insert into RGS5 gene. Insertion of a LacZ-Neomycin resistance cassette into exon 2 disrupts translation of the functional RGS5 gene. Furthermore, this strategy allows transcription of the insert to be driven by the endogenous promoter, *in vivo*. This construct is unique in its insertion of the gene construct into exon 2, where existing RGS5KO mouse models employ LacZ inserts into exon 1. The 3′ probe sequence used for genotyping original ES cell lines is shown in blue.
1.e.iv.2 RGS protein amphipathic helix domain structure and function

The amphipathic helix of RGS proteins is essential for intracellular localization and proper function

All members of the RGS superfamily contain the characteristic 120-amino acid RGS-box domain, however it is becoming evident that additional domains in the peptides are essential for normal intracellular trafficking and function. Among these additional domains is the N-terminal amphipathic helix present in every R4 family RGS protein. For example, Heximer et al (2001) demonstrated the important role of the RGS2 N-terminus in determining subcellular localization and function (Heximer, Lim et al. 2001). Additionally, Zeng et al (1998) have demonstrated the necessity of the N-terminal domain of RGS4 for proper effector association and GAP activity (Zeng, Xu et al. 1998). Both of these groups demonstrated the necessity of the N-terminal domain for proper RGS function. It was later established by Bernstein et al (2000) that the essential role of the N-terminal domain required the formation and binding of an amphipathic alpha-helix (Bernstein, Grillo et al. 2000).

The importance of the NTD amphipathic α-helix in RGS function has been established over continued study. We here used biochemical assays to determine the importance of this prototypical amphipathic helix domain in RGS2 so that we might better understand its function in RGS5 and other R4 group proteins. A recent study conducted on a hypertensive Japanese cohort demonstrated a conserved mutation in the RGS2 gene (Yang, Kamide et al. 2005). Briefly, a single nucleotide polymorphism of
1115G>A, resulting in a mutation of arginine-44 to histidine (R44H), was identified in the above-mentioned cohort of hypertensive Japanese individuals. We undertook investigations to address how this affects N-terminal domain (NTD) structure and interaction with the plasma membrane using a series of spectroscopic analyses. Previous studies using this technique were successful in identifying the secondary structure of the NTD of RGS4 (Bernstein, Grillo et al. 2000). These studies demonstrated that the association with the plasma membrane, which is necessary for proper function of the RGS protein, occurred through a NTD α-helix.

1. Rationale

RGS5 as a regulator vascular tone and VSMC signaling

Our lab and others have demonstrated a vascular smooth muscle cell-specific expression pattern for RGS5. RGS5 has been shown to inhibit Gαi and Gαq coupled signaling, and more specifically when overexpressed in VSMCs it can inhibit signaling via the vasoconstrictive agonist receptors for AngII, ET-1 and S1P. Surprisingly, however, recent studies by Cho et al. (Cho, Park et al. 2008) and Nisancioglu et al. (Nisancioglu, Mahoney et al. 2008) have demonstrated hypotension in RGS5 knockout mice, implicating its potential role as an inhibitor of vasodilatory signaling. To date, there is no explanation for the apparent discrepancy between the biochemical actions of RGS5
and its biologic role in vivo. This work aims to investigate several VSMC-specific signaling pathways in RGSSKO mice in order to determine the contribution of RGSS to blood pressure regulation in vivo.

RGS protein amphipathic helix domain structure and function

Our lab and others have previously demonstrated the necessity for intact amphipathic helix domain structure for proper function of members of the R4 subfamily of RGS proteins (Bernstein, Grillo et al. 2000; Gu, He et al. 2007; Gu, Tirgari et al. 2008). Furthermore, due to the heavy sequence conservation in the NTD helices of proteins of this family, investigations in this field can lead to greater understanding of all R4 proteins. Previous data has shown that human mutations exist within the NTD amphipathic helix of RGS2 and that these may have important functional consequences. This project set out to determine whether specific modulation of basic amino acid charges in the amphipathic helix domain was sufficient to alter the membrane affinity of the RGS amino terminal domain.
1. Hypotheses

Hypothesis. RGS5 signaling regulates VSMC function and vascular tone.

Hypothesis 2. The amphipathic helix is critical for lipid bilayer interaction and function of R4 subfamily RGS proteins as inhibitors of Gαq vasoconstrictor signaling.
Chapter 2: Materials and Methods

2.a RGS5 Reporter Knockout Mouse

Deltagen laboratories conducted gene knockout procedures. A LacZ-neomycin cassette was inserted into the second exon of the RGS5 gene in mouse embryonic stem cells, which were then implanted into C57BL/6 mice. As a result, *in vivo* expression of the RGS5-LacZ gene insert was driven by the wild type RGS5 promoter. (web link; http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=markerDetail&key=33891)

2.a.i Breeding and genotyping of RGS5KO mouse

All experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), Institutional Guidelines and the Canadian Council on Animal Care. Genotyping was conducted as described by Deltagen laboratories (www.informatics.jax.org/external/ko/deltagen/459.html). Loss of gene expression was verified in knockout animals by RT-PCR as described in Cifelli, et al (2008). Animals used in experiments were RGS5KO and wild type littermate controls from RGS5 heterozygous-heterozygous matings.
2. b Pressure Myography

Mice were anesthetized using isoflurane and sacrificed by cervical dislocation. Intact large and small intestines were isolated from the lower gastrointestinal tract and placed in cold (4°C) physiological MOPS (3-(N-morpholino)propanesulfonic acid) buffer. All subsequent steps occur at 4°C until stated otherwise. Intact vessels are isolated using a combination of blunt and sharp dissection methods, at which point they are stored in fresh buffer until cannulation and perfusion.

Intact arteries were placed in small vessel pressure myograph chambers (Living Systems, Virginia, USA) containing 5 ml MOPS buffer. The vessels were cannulated and secured to glass micropipettes using surgical suture. Vessels are initially pressurized to 40 mmHg and then slowly increased to 120 mmHg. The resulting bowed vessel was separated until straightened between the micropipettes, at which point pressure was returned to 40 mmHg. The vessel bath was then warmed to 37°C and allowed to equilibrate within the chamber for 30 minutes.

Phenylepherine (PE), Acetylcholine (ACh) and Sphingosine-1-phosphate (S1P) dose responses were conducted at 40mmHg pressure and 37°C. For experiments examining myogenic response, pressure was increased using a Servo-pump (Living Systems, Virginia, USA) routed through a pressure monitor examining inflow and outflow pressure (Living Systems, Virginia, USA).

Vessel diameter was visualized using a Nikon TMS microscope connected to a Panasonic CCTV camera and displayed on a conventional black and white monitor. The
image was routed through a video dimension analyzer (Living Systems, Virginia, USA) and diameter data was recorded using Acqknowledge software on a laboratory computer. Diameter data was saved from the laboratory computer and analyzed using Microsoft Excel. The formulae used to analyze raw diameter data were designed to present contractile or dilatory responses of vessels in reference to the baseline diameter prior to commencing each experiment. As such, for experiments using vasoconstrictor agonists, baseline diameter was taken as the diameter of the vessel at 40 mmHg, prior to the addition of any vasoconstrictor. For vasodilatory studies, measurements were analyzed in order to display the amount of ACh mediated vasodilation occurring in the presence of 1mM PE concentration within the organ bath. The myogenic vasoconstrictor response was assessed by comparing the diameters of each vessel in the presence and absence of Calcium at each pressure step.

**2.b.i Myogenic response:** Once arteries are heated and equilibrated to 40 mmHg, pressure is reduced to 20 mmHg and increased in stepwise increments of 20 mmHg up to 120 mmHg. The vessels are maintained at each pressure step for 5 min (20, 40, 60 mmHg) and 7 min (80, 100, 120 mmHg). The diameter data for active myogenic tone was combined with the passive vessel tone data below as follows:

\[
\frac{(\text{Passive diameter at pressure step}) - (\text{Active diameter at pressure step})}{(\text{Passive diameter at pressure step})}
\]
Passive myogenic tone: Arteries are washed with Ca2+ free MOPS buffer (37°C) 4 times at 8-10 minute intervals. Pressure is then reduced to 20 mmHg and increased in 20 mmHg stepwise increments to 120 mmHg. The vessel was allowed to remain at each pressure step for 1 min.

2.b.ii PE dose response: For each dose, 1ml of MOPS buffer was removed from the organ bath and replaced with PE doses prepared in 1 ml of MOPS buffer. The concentrations were as follows: 1, 3, 10, 30, 100, 300, 1000, 3000, 10000, 30000 (nM). PE doses were heated to 37°C and added in 5 min intervals. Diameter data was analyzed using the following formula:

\[
\frac{\text{[(Baseline Diameter) - (Stable reading of diameter at PE dose)]}}{\text{(Baseline Diameter)}}
\]

2.b.iii ACh dose response: Before ACh addition, arteries are initially constricted to 1 mM PE to establish background vascular tone. Each dose of ACh is prepared containing 1 mM PE to maintain constant PE concentration within the organ bath. ACh concentrations were increased as follows: 1, 3, 10, 30, 100, 300, 1000, 3000, 10000, 30000 (nM). Diameter data was analyzed using the following formula:

\[
\frac{\text{[(Stable reading of diameter at ACh dose) - (Diameter change in response to PE)]}}{\text{(Diameter change in response to PE)}}
\]
2. b.iv S1P dose response: S1P doses were prepared following established protocols from the research laboratory of Steffen Bolz. Briefly, S1P was dissolved in 4% BSA to a concentration of 1 X 10^{-4} \mu M. This was used as the peak concentration while the remaining 4 doses were prepared using 1 in 10 dilution. Diameter data was analyzed using the following formula:

\[
\frac{((\text{Baseline Diameter}) - (\text{Diameter at S1P dose}))}{(\text{Baseline Diameter})}
\]

2. c Aortic Smooth Muscle Cell Culture Experiments

All tissue culture experiments were conducted on primary isolated aortic smooth muscle cells from wild type and RGS5 knockout mice. Briefly, aortas from aortic root to renal branch were isolated from wild type and knockout animals and processed using elastase, collagenase and mechanical degradation until surrounding connective tissue was removed. Unless otherwise noted, cells are grown in Complete Media (DMEM/F-12, 10% FBS, P/G/S antibody, PDGF). Cells were grown in a hera cell 150 incubator and experiments and procedures were conducted in a class IIa biological safety cabinet.

2. c.i ERK Signaling Assay

Primary cultured wild type and RGS5 knockout aortic smooth muscle cells were plated in 6-well plates. Cells were serum starved for 24 hours prior to treatment and then treated with AngII, ET-1, S1P, PDGF and SNP, or vehicle control. Cells were incubated for 10 minutes and harvested on ice using an SDS-glycerol lysis buffer.
2.c.ii VASP Signaling Assay

Primary cultured smooth muscle cells were plated on 6-well plates and serum starved for 24 hours prior to treatment with 1.5mM SNP or vehicle control. Once treated, treated and control wild type and knockout cells were harvested using an SDS-glycerol lysis buffer at 10-, 45-, and 90-minute intervals.

2.c.iii Cell Proliferation Assay

Primary cultured smooth muscle cells were plated in 6-well plates at a concentration of $1 \times 10^5$ cells per well. Sufficient wells were plated to allow 2 wells per genotype, per day for a total of 14 wells per genotype. All cells were plated simultaneously and allowed to grow in parallel. Two wells from each genotype were harvested each day and counted using a hemocytometer. The resultant numbers were averaged between wells for each genotype. Shown is the average cell count at each time point (n=3) with error bars depicting standard error from the mean.

2.d RGS2 N-terminal domain helix secondary structure and peptide-lipid association

RGS2 peptide-lipid interactions and secondary structure assays were conducted using synthesized NTD peptides from the Hospital for Sick Children: Advanced Protein Technology Centre (Toronto, ON).

2.d.i Tryptophan Fluorimetry

Tryptophan fluorescence spectra of RGS2 WT, R44H, and L45D N-terminal domain peptides were measured using an AVIV ratio spectrofluorometer ATF105.
(Lakewood, NJ). The NTD Peptide samples were then resuspended in RBS to a final concentration of 0.2 µM. Extruded unilaminar liposomes (Encapsula NanoSciences, Nashville, TN) were made from bovine brain lipids (Avanti Polar Lipids) and were diluted in PBS. Liposomes were added to peptide solution for 5 min before measurement to allow consistent lipid association. For each lipid concentration, fluorescence emission spectra after 295 nm excitation were recorded at 2 nm steps from 310 to 400 nm. Similarly generated liposome and PBS alone control emission spectra were subtracted from peptide spectra to account for nonpeptide, background fluorescence emission. In experiments involving trifluoroethanol (TFE), solutions with peptides were thoroughly mixed and incubated for 5 min before measurement.

2.d.ii Circular Dichroism

Peptide secondary structure was assessed using an AVIV Circular Dichroism Spectrometer model 202. Wild-type RGS2, L45D, and R44H mutant peptides were analyzed with or without liposomes. Unilaminar liposomes for CD studies were made as described previously (Bernstein et al., 2000). In brief, a 3:2 solution of dipalmitoylphosphatidylcholine/dipalmitoylphosphatidylglycerol (Avanti Polar Lipids) in chloroform was dried under nitrogen and resuspended in PBS. Lipids in solution were sonicated for 5 min with 20-s pulses and chilled on ice. Liposomes were made fresh for each experiment. Peptides (7-21 µM) were diluted in PBS with and without lipids or TFE, and spectra were measured from 190 to 260 nm in 1-nm increments averaged over 4 s after a 5-min incubation period. The spectra of lipids and PBS alone were subtracted
from sample measurements to account for nonpeptide, background fluorescence emission.

2.e Statistical methods

Unless otherwise stated data are presented as mean +/- standard error of the mean (SEM). One-way, two-way and repeated measures ANOVA were used as indicated in the Figure legends. In order to determine the significance of cell signaling assays, student’s t-tests were conducted, with significance being assigned to results with p-values of less than 0.05.
Chapter 3: Results

3.a Pressure Myography

Loss of RGS5 does not alter myogenic responsiveness

Myogenic Response (MR): The bayliss effect, or myogenic response, is a mechanism inherent to VSMCs, which causes contraction in response to increases in transmural pressure and cellular stretching. In order to examine whether RGS5 plays a role in mediating the myogenic response, isolated arterioles were exposed to increasing pressure from 20 mmHg to 120 mmHg, in stepwise increments of 20 mmHg. These experiments were repeated in calcium-free physiological MOPS buffer and the resultant ratio was determined to be the active myogenic tone. The measurements taken in calcium free buffer allow for the examination of compliance independent of the contractile activity of the VSMCs. If RGS5 is playing a role in the mediation of the myogenic response, we expect to observe one or both of the following: initiation of contraction at lower pressure steps or increased overall contraction at any or all pressure steps. The results show a slightly increased myogenic response in the RGS5 knockout as compared to wild type, manifesting in increased sensitivity (lower set point) to pressure while maintaining similar overall constriction at peak pressure (120 mmHg) (Figure 5). Passive vessel tone was analyzed separately in order to investigate whether the matrix composition of RGS5 knockout vessels varies as compared to wild type vessels. There was no significant difference in passive tone between wild type and RGS5 knockout mesenteric vessels (Figure 6). Significance of myogenic response curves and
passive tone curves were assessed using two-way ANOVA with repeated measures. Vessel diameter was also analyzed in order to ensure there is no variation between vessels used for pressure myography experiments, and no significant difference was observed (Figure 7). For vessel diameter, student’s t-test was used to assess whether there was a significant difference in vessel diameter between wild type and knockout vessels.
FIGURE 5: No significant difference in myogenic response of wild type and RGS5 knockout mesenteric arterioles. Cannulated mesenteric resistance arterioles were maintained in MOPS physiological buffer at 37°C. Intramural pressure was increased in 20 mmHg stepwise increments for 5 min (20, 40, 60 mmHg) or 7 min (80, 100, 120 mmHg) periods, and stable reading of vessel diameter was recorded. Significance was assessed by two-way ANOVA with repeated measures. Error bars shown are standard error from the mean. Data described is calculated using the following formula:

\[
\frac{\text{[Passive diameter at pressure step) − (Active diameter at pressure step)]}}{\text{Passive diameter at pressure step}}
\]
FIGURE 6: No significant difference in vascular wall compliance between wild type and RGS5KO mesenteric resistance arterioles. Following (3 X 10 min) washing of mesenteric resistance arterioles with Calcium-free MOPS physiological buffer, cannulated vessels are exposed to stepwise increases in intralumenal pressure. Resultant curves display the compliance of wild type and RGS5KO vessels. Error bars shown are standard error from the mean. Significance was assessed by two-way ANOVA with repeated measures. The data shown is calculated as follows:

\[
\frac{(\text{Diameter at pressure step}) - (\text{Diameter at 20 mmHg})}{(\text{Diameter at pressure step mmHg})}
\]
FIGURE 7

FIGURE 7: No significant difference in vessel diameter between wild type and RGS5KO vessels used in pressure myography. Diameters of vessels (at 40 mmHg) used for pressure myography were recorded throughout and compiled. The error bars shown represent the standard error from the mean. Significance was ascertained using a student’s t-test, with significance assigned to p-values of less than 0.05. (n=8 KO, n=12 WT)
Shingosine-1-phosphate (S1P): S1P, a sphingolipid and bioactive cell signaling mediator, binds to two subtypes of GPCRs on VSMCs: S1P2 and S1P3 (Waeber, Blondeau et al. 2004). These receptors are in turn coupled to both Gαi and Gαq-bound heterotrimers. As such, the variety of downstream signaling effects S1P induces are plentiful and of these, vasoconstriction is a closely examined one. Furthermore, S1P signaling has been shown to be essential for the myogenic response (Bolz, Vogel et al. 2003). Importantly, RGS5 has been shown to specifically target S1P signaling in VSMCs (Cho, Harrison et al. 2003). Taken together, these points suggest that RGS5 may be an important regulator of the S1P-mediated component of the myogenic response. Our studies in resistance arterioles from RGS5 knockout mice were aimed at testing this possibility. To examine whether RGS5 is acting on the S1P receptor coupled Gαq-subunit, isolated resistance arterioles were treated with increasing doses of S1P, a vasoconstrictor and key regulator of the myogenic response. If RGS5 displays specificity for the S1P receptor, we expect to see one of or both of the following: increased sensitivity of the vessel to exogenously applied S1P and increased overall constriction in response to S1P. RGS5 knockout vessels demonstrated no significant difference in contractile response compared to wild type vessels (Figure 8). Significance was determined using a two-way ANOVA with repeated measures.
FIGURE 8: No significant difference in response to S1P in wild type and RGS5KO mesenteric resistance arterioles. Cannulated mesenteric resistance arteries were maintained at 40 mmHg and 37°C, and treated with increasing concentrations of sphingosine-1-phosphate. Significance was determined using a two-way ANOVA with repeated measures. The error bars shown represent the standard errors from the mean. The data shown was calculated as follows:

\[
\left(\frac{\text{Baseline Diameter} - \text{(Diameter at S1P dose)}}{\text{Baseline Diameter}}\right) \times 100
\]
**Loss of RGS5 does not alter vasoconstrictor or vasodilator responses in resistance arterioles**

Phenylepherine (PE) Dose Response: Phenylepherine is widely used in studies of α-adrenergic signaling. Briefly, VSMCs contain a number of catecholamine receptors that are essential in mediating sympathetic signaling in mammalian organisms. The cellular effects of these catecholamines, which include epinephrine, norepinepherine and dopamine, are dependant upon the type of cell surface receptor (GPCRs with varying Gα subunits) they bind. Of these, alpha-adrenergic receptors are Gαq coupled and as such induce PLC activation and a subsequent increase in intracellular calcium. This mechanism, as previously described, is essential for the initiation of calcium dependant SMC contraction, and in vessels, calcium dependant vasoconstriction. Consequently, nearly all known potent vasoconstrictors act through Gαq coupled receptors. In order to examine whether RGS5 is acting in a general Gαq vasoconstrictor pathway, isolated resistance arterioles were treated with increasing doses of PE, a potent vasoconstrictor and alpha-adrenergic agonist. α-adrenergic receptors follow the classical Gαq-mediated pathway described above which culminates in phosphorylation of MLC by MLCK. Changes in vessel diameter were recorded and analyzed to assess vasoconstrictor response. If RGS5 attenuated Gαq mediated signaling, we would expect to see enhanced contractile activity in the knockout vessels as compared to wild type controls. The results show no significant difference in vasoconstriction to PE between wild type and RGS5 knockout vessels (Figure 9). Significance was determined using two-way ANOVA with repeated measures.
FIGURE 9: No difference in phenylepherine mediated contraction between wild type and RGS5KO mesenteric resistance arterioles. Cannulated arterioles were maintained at 40 mmHg and 37°C, while they were treated with increasing concentrations of phenylepherine. Significance was determined using a two-way ANOVA with repeated measures. Error bars shown represent the standard error from the mean. The data shown was calculated as follows:

\[
\frac{[(\text{Baseline Diameter}) - (\text{Stable reading of diameter at PE dose})]}{\text{(Baseline Diameter)}} \times 100
\]
Acetylcholine (ACh) Dose Response: In order to examine whether RGS5 is acting at some point in smooth muscle cell relaxation, isolated resistance arterioles were treated with ACh, in order to induce NO dependant vasodilation. If the current reports are correct we may have expected to observe enhanced vasodilatory activity in RGS5-deficient arterioles. However, there was no significant difference in vascular response to ACh between wild type and RGS5 knockout vessels (Figure 10) suggesting that RGS5 did not play a significant role in endothelial-dependent vasodilation. Significance was calculated using two-way ANOVA with repeated measures.
FIGURE 10

FIGURE 10: No significant difference in ACh mediated relaxation of PE-induced constriction between wild type and RGS5KO mesenteric resistance arterioles.

Cannulated arterioles were constricted with phenylepherine (1µM) and maintained at 40 mmHg and 37°C. Vessels were then treated with increasing concentrations of acetylcholine. Significance was determined using a two-way ANOVA with repeated measures. Error bars shown are standard error from the mean. The data shown was calculated as follows:

\[
\frac{[(\text{Stable reading of diameter at ACh dose}) - (\text{Diameter change in response to PE})]}{(\text{Diameter change in response to PE})} \times 100
\]
3. b Western immunoblots

*Downstream signaling pathways are altered in RGS5-deficient primary cultured smooth muscle cells*

ERK signaling assay: Extracellular regulated kinases 1 and 2 are ubiquitously utilized proteins for assessing vasoactive signaling due to their role in the propagation of extracellular signaling through GPCRs (Leroy, Missotten et al. 2007). Specifically, this signaling has been connected to \( G_{\alpha q} \) and \( G_{\alpha i} \) signaling, and as described earlier, results in a number of intracellular effects ranging from migration and adhesion to proliferation. Current literature presents increased ERK1/2 phosphorylation in vehicle treated cultured RGS5 knockout VSMCs. We define these ERK phosphorylation results from vehicle treated groups as basal ERK phosphorylation, as they are the basal levels of phosphorylation without the addition of agonists (stimulated). We undertook experiments to examine ERK1/2 signaling in primary cultured VSMCs in order to understand the discrepancy between our whole animal data and the published results. We expected to see either increased basal and/or stimulated ERK1/2 levels in RGS5KO primary cultured cells when compared to controls. Cells were treated with S1P, AngII, ET-1, SNP and PDGF and resulting immunoblots were analyzed for phosphoERK/totalERK ratio. (S1P, AngII, ET-1 and SNP results are shown; Figure 11) Results demonstrate no significant difference in stimulated ERK1/2 phosphorylation between RGS5KO and wild type cells, however basal ERK1/2 phosphorylation in RGS5KO cells appeared higher if not equal to the levels observed in wildtype cells. As such, vehicle treated lanes were
pooled and significantly higher levels of ERK1/2 phosphorylation was observed in the
RGS5 knockout smooth muscle cells in the absence of agonists, as compared to wild
type controls (Figure 12).
FIGURE 11

FIGURE 11: No significant difference in ERK1/2 phosphorylation when stimulated by vasoactive agonists. Primary cultured wild type and RGS5KO aortic smooth muscle cells were treated with S1P, AngII, ET-1, and SNP, incubated for 10 min, and harvested. Lysates were analyzed by western immunoblot for ERK1/2 phosphorylation. Results shown are phosphorylated ERK bands, normalized to total ERK and analyzed using ImageJ. Error bars depict the standard error from the mean. Significance was assessed using a student’s t-test. (n= 4, for each treatment group)
FIGURE 12: RGS5KO primary cultured VSMCs display increased basal levels of ERK phosphorylation. Although no difference was observed in stimulated ERK1/2 phosphorylation levels, basal ERK1/2 phosphorylation in RGS5KO cells appeared to be the same as or higher than wild type levels. Vehicle treated bands from all ERK1/2 signaling assay treatment groups (shown in Figure 11) were compiled and analyzed using ImageJ and significance was determined using a student’s t-test. Error bars shown are standard error from the mean.
VASP signaling assay: VASP, as described earlier, is a widely utilized protein in the measurement of PKA and PKG activity. Furthermore, as its name implies, VASP is phosphorylated in response to vasodilator stimulus. Previous studies have demonstrated increased VASP phosphorylation in response to SNP addition, in conjunction with observed hypotension, in RGS5 knockout mice. These results comprised the bulk of the rationale for the current proposed role for RGS5 as a regulator of vasodilator signaling. In order to assess whether our primary cultured VSMCs were displaying similar characteristics to those in the literature, we conducted western immunoblots examining VASP phosphorylation in response to SNP treatment. Cells were treated with SNP (4mM) for 10, 45 and 90 min to determine whether RGS5 was affecting NO induced (vasodilatory) signaling. We expected increased VASP phosphorylation in the RGS5 knockout cells in response to SNP treatment, as the literature reports. Our results show increased VASP phosphorylation in RGS5 knockout VSMCs as compared to wild type controls after 45 and 90-minute incubation periods. (Figure 13)
FIGURE 13: RGS5KO cultured VSMCs display higher levels of VASP phosphorylation than wild type controls. Primary cultured VSMCs were treated with 1.5mM SNP for 10, 45 and 90 minute incubation periods and harvested. Lysates were analyzed by western immunobloting for VASP phosphorylation. Data shown is phosphorylated VASP bands normalized to total VASP and then analyzed by ImageJ software. Lanes corresponding to vehicle treated cells are labeled C (Control) and lanes corresponding to SNP treated cells are labeled T (Treatment), while WT and KO refer to wild type and RGS5KO cells, respectively. The numerical values correspond to SNP incubation time prior to addition of lysis buffer. Error bars represent standard error from the mean, and significance was determined using a student’s t-test. (n=4)
3. c Cell proliferation assay

*RGS5 knockout primary cultured smooth muscle cells do not display any difference in cellular proliferation when compared to wild type controls*

In order to assess whether RGS5 affects overall smooth muscle cell ERK signaling, we assessed proliferation of cultured arterial smooth muscle cells. G-protein pathways, as previously described, are not limited to contractile signaling, and can activate proliferative signaling through the MAP kinase signaling pathway. This assay provided two potential insights into the function of RGS5: we were able to examine whether RGS5 attenuates growth factor signaling leading to proliferation, as well as assess whether the increase in basal ERK 1/2 phosphorylation observed in western immunoblots also affects alternate MAP kinase signaling pathways. Our expected results were enhanced proliferative activity in RGS5KO cells compared to wild type, specifically in the rate of cell growth. However, results showed no significant difference in proliferation rate between wild type and RGS5 knockout cells (Figure 14). There was a slight discrepancy in peak cell concentration however, consistent in all experimental trials.
FIGURE 14

There is no significant difference in growth rate between wild type and RGS5KO primary cultured SMCs. $1.0 \times 10^5$ cells were plated in 14 wells for each genotype (28 total wells). 2 wells per genotype were counted each day using a hemocytometer and an average was taken. Error bars depict standard error from the mean, and significance was assessed at specific time points using a student’s t-test.
3.4 R44H Mutation Does Not Disrupt the Helix Forming Capability of the RGS2 NTD Helix.

The aspartic acid in the previously published mutant L45D is a known helix breaker and thus the L45D mutant does not associate with the PM because of its inability to form a helix. To test whether the R44H mutant's inability to associate with the PM is due to the same mechanism, we compared the secondary structure of RGS2, R44H, and L45D peptides in the presence and absence of anionic liposomes using circular dichroism. As expected, in the absence of lipids, all of the peptides show a disordered random coil CD signature. In the presence of anionic lipids, however, both RGS2 and R44H mutant peptides show characteristic -helix formation with a molar ellipticity minima at 222 nm (Figure 15), consistent with the non-helix-breaking nature of histidine. In contrast, CD spectra of L45D show a random coil spectrum even in the presence of liposomes (Heximer et al., 2001). Together, these data indicate that the R44H NTD, despite retaining the ability to form a proper α-helix, was unable to form a stable interaction with the lipid bilayer.
FIGURE 15: R44H mutant peptides form α-helices in the presence of lipids. R44H mutation shows normal helix forming potential in the presence of negatively charged phospholipids. The indicated mutant and wild type peptides corresponding to residues 34 to 57 in PBS were incubated either with or without lipids and CD spectra were
collected as described under *Materials and Methods*. Black circles represent control spectrum of peptides in PBS solution; White circles represent spectrum of peptide solution containing 1.4 mM lipid liposomes. Shown is a representative experiment from three independent experiments. (Figure from Gu, Tirgari et al, 2008, *Molecular Pharmacology*)
The Amphilic Helix Domain of the R44H Mutant Does Not Stably Associate with Purified Liposomes

The NTD amphiphatic helix is required for interaction of RGS2 with negatively charged phospholipids on the inner leaflet of the plasma membrane. Mutations that disrupt amphipathicity are predicted to disrupt its localization and function. Because the R44H mutation specifically changes an arginine residue adjacent to the hydrophobic face of the RGS2 helix to histidine, we proposed that this mutation would result in a reduction in RGS2-lipid bilayer affinity. Tryptophan spectroscopy has been used to study the interaction of tryptophan-containing amphipathic -helical peptides with lipid bilayers (Burstein, Vedenkina et al. 1973). This assay measures changes in the fluorescence emission spectrum of a tryptophan residue on the hydrophobic face of an amphipathic helix. As the local environment of the tryptophan changes from polar (solution) to hydrophobic (lipids), there is a blue shift in the spectrum maxima (Burstein, Vedenkina et al. 1973). Spectra were corrected for background as described previously. Corrected spectra from wild-type RGS2 shows the maxima shift with increasing concentrations of lipids, whereas the R44H spectra is unaffected by the presence of lipids. Likewise, L45D, a mutant peptide previously shown not to interact with the PM or form an α-helix in the presence of lipids (Heximer et al., 2001) shows no change in its tryptophan properties in this assay. One possible explanation for the differences in tryptophan spectroscopy of the R44H mutant is that the long hydrophobic side chain of arginine 44 could contribute to an increased local hydrophobic environment of tryptophan when the peptide is in a helical formation. To address this possibility, we tested whether the tryptophan
spectrum was altered by TFE, a helix-promoting solvent, and in the absence of liposomes. As shown in figure 17, the addition of TFE results in a marked increase in helix formation of the wild-type RGS2 peptide without inducing a blue shift in the tryptophan spectra. Although we cannot exclude the possibility that the Arg44 residue contributes to the hydrophobic environment of the nearby Trp41, such an interaction cannot explain the profound blue-shift observed in the presence of liposomes. Accordingly, these data suggest that the spectral changes of tryptophan observed in the presence of liposomes are due to its insertion into the hydrophobic core of the lipid bilayer. (Figure 16) These results, in conjunction with those from circular dichroism analysis lead to the proposed “snorkeling” mechanism for NTD amphipathic helix-lipid association. (Figure 18)
FIGURE 16: R44H mutant peptides do not interact with lipid bilayers. R44H mutation results in decreased penetration of the hydrophobic face of the RGS2 helix into the lipid bilayer core. RGS2 helix domain peptides corresponding to residues 34 to 57 from WT,
R44H, and L45D were incubated with increasing amounts of purified unilamellar liposomes from brain lipid extracts. Tryptophan fluorescence emission spectra of Trp41 on the hydrophobic face of the RGS2 amphipathic helix were collected with lipid concentrations ranging from 0 to 0.4 mg/ml. Shown is a representative experiment from three independent experiments. (Figure from Gu, Tirgari et al, 2008, Molecular Pharmacology)
FIGURE 17

FIGURE 17: TFE addition to R44H peptides causes $\alpha$-helix formation without any associated blue shift in the tryptophan spectra. Tryptophan Fluorimetry and Circular Dichroism studies were conducted on the R44H mutant peptide in the presence of trifluoroethanol and the absence of lipids in order to assess whether the local environment of the tryptophan residue contributing to the blue shift associated with lipid interaction. The results demonstrate no blue shift in the tryptophan emission
spectra, in spite of prominent \(\alpha\)-helix formation. Data shown is one representative experiment of three independent experiments. (Figure is from Gu, Tirgari et al, 2008, *Molecular Pharmacology*)
“snorkeling” mechanism by which NTDs stably interact with lipid bilayers. Figure 18A describes the high degree of sequence homology as well as the heavily conserved highlighted basic residues. These residues are believed to be required for stable RGS2 NTD $\alpha$-helix interaction with a lipid bilayer, as described in figure 18B. We propose that the R44H mutation hinders NTD $\alpha$-helix interaction with the lipid bilayer due to its shorter and sterically unfavourable side-chain, making it incapable of “snorkeling”.

(Figure from Gu, Tirgari et al, 2008, Molecular Pharmacology)
Chapter 4: Discussion

General discussion

Blood pressure regulation is essential for maintenance of normal cardiovascular function in all mammalian organisms. It has already been established that G-proteins are integral in the control of blood pressure and flow at the level of the heart, the kidneys and the vasculature, and furthermore, that the fine tuning of these G-protein signals by RGS proteins is essential for normal function. When the signaling required for proper function becomes compromised, the results include a number of vasculopathies and CVDs including hypertension, an established risk factor for numerous serious clinical complications such as myocardial infarction, and stroke. The current literature has firmly established a role for RGS2 as a potent regulator of VSMC contractile activity through its ability to inhibit $\mathrm{G} \alpha q$ signaling. Existing literature proposes a role for RGS5 as a regulator of VSMC activity, but as a mediator of vasodilatory signaling, and not as a mediator of $\mathrm{G} \alpha q$ vasoconstrictor signaling. The data described herein provides both further support and new insights into the study of RGS proteins and their potential biologic roles as regulators of VSMC function.

4.4 RGS5 discussion

Interestingly, the results described in this thesis do not support a direct role for RGS5 as a key regulator of vascular tone or systemic pressure. Both PE and ACh dose response curves, as well as myogenic response and S1P dose response curves, show no significant difference between wild type and RGS5 knockout vessels. Furthermore,
whole animal studies previously conducted in our lab support these observations (Zhang and Heximer, unpublished). Though these observations are contrary to the experimental hypothesis, as well as the earlier literature in support of the rationale for this study, these results may be indicative of a more complex role in previously under-examined mechanisms, such as SMC migration/interaction and myoendothelial coupling. Our current results all support the conclusion that the absence of RGS5 has little or no effect in the regulation of VSMC contraction and dilation in response to the agonists tested. Of course, this does not rule out the possibility that RGS5 specifically regulates another GPCR-mediated pathway that was not tested in this work. Interestingly, the literature also describes a lean-body habitus in RGS5 knockout mice compared to wild type controls, which is also not evident in our findings. As these knockouts were made with different targeting constructs this may indicate differences between different genetic RGS5KO models, where the site of genetic manipulation is has different effects on the expression of genes nearby on the same chromosome. Alternatively, the genetic backgrounds of the different strains may not be identical - all of our animals were backcrossed > 8 generations into a C57Bl/6 background.

Although we did not observe the hypotensive phenotype reported in the literature, a number of observations did match the existing data and are likely to be specific for the loss of RGS5. Particularly the observation of increased basal ERK 1/2 phosphorylation, as well as an increase in VASP phosphorylation in RGS5 knockout cells as compared to wild type (Cho, Park et al. 2008). As described earlier, both of these intracellular signaling proteins are involved in cytoskeletal modulation, while ERK has
been specifically shown to be involved in the activation of the myogenic contractile response (Lidington, Peter et al. 2009). This combination of altered cell signaling is intriguing as it may describe a compensatory mechanism by which increased myogenic response and/or agonist-mediated contraction is being tempered by increased VASP signaling. Consistent with this possibility, no phenotype was observed in the whole animal or isolated arteriolar preparations, despite our observation of altered signaling in cultured primary RGS5KO VSMCs.

4.a.i Myogenic Response in RGS5 knockout resistance vessels

In our attempts to elucidate the role of RGS5, we focused our attention on a VSMC specific mechanism – the myogenic response. Although the findings from the pressure myography studies showed no significant difference between genotypes, there was a slightly higher, albeit non-significant increase in myogenic response in the RGS5 knockout vessels. These preliminary results, as well as data supporting a potential role for RGS5 in mediating S1P signaling (and thus the myogenic response) (Cho, Harrison et al. 2003; Peter, Lidington et al. 2008), were the rationale for the dose response experiments examining vascular application of S1P. The resultant dose response curves, however, were not significantly different. Nevertheless, we must consider that these studies were carried out in mesenteric resistance arteries, which show a relatively weak response to S1P compared to other vascular beds and thus it is possible that investigating myogenic and S1P responses in other vascular beds may reveal more intriguing findings than those observed in mesenteric vessels. We also considered
literature that established a connection between myogenic responses and constriction induced by vasoactive agonists. This data, from de Wit et al, describes an increase in norepinepherine-mediated constriction due to the action of the myogenic response (de Wit, Bolz et al. 1998). Thus, since RGS5 may act on both G-protein mediated pathways (S1P and NE) we postulated a leftward shift (greater sensitivity) in RGS5KO during the PE dose response as compared to wild type, corresponding with the discrepancy observed between RGS5KO and wild type myogenic response curves. This too is not evident from our findings as the PE dose responses show no difference between experimental groups. Therefore, these results, despite existing literature to the contrary, suggest the absence of RGS5 does not significantly alter the myogenic response or constrictor activity in response to S1P. However, there are a number of studies that have examined alternate pathways for modulation of vascular tone, and more specifically, the triggering and modulation of the myogenic response (Davis and Hill 1999) These include pathways implicating stretch activated cation channels that allow calcium entry, or mechanisms implicating the trafficking of globular actin and it’s dynamic polymerization in SMCs, while numerous mechanisms are proposed supporting the Rho/ROK/MLCP signaling that results in sustained contraction (Cipolla, Gokina et al. 2002; Fanchaouy, Bychkov et al. 2007; Schubert, Lditiongton et al. 2008). Many of these same signaling pathways act through G-protein coupled receptors and a number of those include Gαi and Gαq coupled heterotrimeric G-proteins. As a result there is potential for RGS5 to play a role in many cellular mechanisms. In the experiments presented herein, S1P and AngII receptor activity was assessed as they have been identified as both targets of R4 family
RGS proteins as well as potential mediators of the myogenic response (Wang, Liu et al. 2002; Mederos y Schnitzler, Storch et al. 2008; Sharif-Naeini, Folgering et al. 2009). Although we do not observe any significant difference in myogenic response between wild type and knockout vessels, there could potentially be a compensatory mechanism through which the constrictor response is being dulled. The higher levels of VASP phosphorylation in our knockout cells show an effect of the absence of RGS5 at an end point of the vasodilatory signaling pathway, namely PKG activity. Thus, using increased VASP phosphorylation as a marker for increased PKG activity, our results support a potential increased vasodilatory mechanism active in these vessels.

As described earlier, the myogenic response has been demonstrated to be closely regulated by the activity of S1P through the S1P receptor, a GPCR, and Ca\(^{2+}\) independent vasoconstriction (Schubert, Lidington et al. 2008). As a result, the observation of an increased, although non-significant, initial constriction at a lower intravascular pressure (lower set-point) caused us to consider testing S1P responsiveness in these same vessels. Our hypothesis predicted a leftward shift in the S1P dose response curve from the RGS5 knockout vessels as compared to wild type controls, indicating an increase in VSMC sensitivity at the level of the GPCR. Surprisingly, the S1P dose responses for the experimental groups were not significantly different. As such, the current evidence supports no role for RGS5 in regulating S1P vasoconstrictor signaling through the S1P2 or S1P3 receptor.
4.a.ii Cell proliferation and migratory signaling in RGS5KO VSMCs

As previously illustrated, rates of proliferation of cultured VSMCs from wild type and RGS5KO mice were assessed. The results from the proliferation assays are intriguing as they present a potential for alternate pathways in which RGS5 may be acting. Prior to discussing this possibility, it is important to note that AngII and S1P signaling, both of which have been shown to be targeted by RGS5, are not only responsible for vasoconstrictor activity but also have been implicated in cell motility and proliferative activity (Hobson, Rosenfeldt et al. 2001; Watterson 2004; Cotton and Claing 2009). More specifically, recent findings concerning S1PR5 have shown a potential link between the function of centrosomes in mitotic proliferation and S1P (Gillies, Lee et al. 2009). As such, the absence of any difference in S1P mediated vasoconstriction does not necessarily remove the potential for RGS5 to be mediating S1PR signals, and may in fact point to highly specialized S1P signaling pathways in VSMCs that are entirely isolated from contractile signals.

In order to address whether or not RGS5 affects mitotic activity, cell proliferation assays were conducted. The results of these experiments demonstrated no role or effect of RGS5 in terms of growth rate, however an interesting observation was the fluctuation in peak cell concentrations. Although no statistical significance was found between points of curves, this modulation at the upper plateau of the growth curve could be a result of modified cell size or cell-cell contact characteristics and affected SMC contact inhibition. The results described herein do not support a role for
RGS5 in mediating S1P dependant vasoconstriction, but do however demonstrate a change in S1P mediated signaling, suggesting a role for RGS5 in one of the many processes this molecule affects. Another interesting finding is that of Chitaley and colleagues (2003) which identifies VASP as a substrate for PKC (Chitaley, Chen et al. 2004). PKC, which is a downstream signaling partner to GPCRs through DAG activity, is an active player in the Rho/ROK signaling pathway. As such, a role for RGS5 in regulating migratory or adhesion signaling might be apparent in changes in Rho activity or the activity of agonists affecting its functions. The results from ERK1/2 phosphorylation assays further support this conclusion as basal ERK1/2 phosphorylation is heightened in knockout VSMCs. Indeed it has been established that S1P acts in an autocrine and paracrine manner, supporting the hypothesis that increased basal ERK1/2 may be a result of changes in autocrine/paracrine S1P signaling in cultured cells (Rosen and Goetzl 2005). The above results showing increased VASP phosphorylation serve as a further indicator of affected cell-cell interaction signaling. The initial rationale derived from existing literature hypothesized that RGS5 knockout blood vessels would demonstrate prolonged vasodilator signaling or heightened response to vasodilator drugs. The myography results however are contrary to this, with wild type and RGS5 knockout vessels behaving similarly in acetylcholine dose response experiments. Vasodilator stimulated protein is believed to be involved in a number of cellular mechanisms, with established connections to cell matrix modification and migration (Bear, Loureiro et al. 2000; Machesky 2000). These observations, made in fibroblasts, describe attenuated cell migratory activity in VASP knockout cells. As such, if RGS5 plays a role in VASP
phosphorylation, further experiments examining the migratory activity of RGS5 knockout VSMCs would be prudent to help elucidate its \textit{in vivo} role.

\textit{4.a.iii Limitations of RGS5 study}

Although the LacZ reporter-knockout model is effective in identifying tissues where RGS5 promoter activity is high, it cannot be used to specifically determine the half-life, cellular localization, or binding characteristics of the RGS5 protein, since the addition of the LacZ insert may compromise all of these factors. Furthermore, LacZ expression and subsequent coloration in response to X-Gal staining has been associated with a threshold effect, which may serve to confound observed expression location. Quantitative Real Time PCR studies establish the presence of this threshold effect. For example, carotid vessels, which display no X-Gal staining, contain six thousand molecules of RGS5 mRNA per two nanograms of RNA, compared to descending aorta RGS5 levels which contain 170 thousand molecules per 2 nanograms of RNA. Thus, the presence of RGS5 mRNA with the associated absence of X-Gal staining, demonstrates a threshold effect.

An additional limitation concerning the verity of the RGS5KO at the genetic level concerns the cellular response to the absence of RGS5. As with any genetically manipulated biological model, the possibility exists for compensatory activity by similar genes or genes in related pathways. While we cannot rule out the possibility of parallel pathway compensatory mechanisms, our RT PCR analysis of RGS5 deficient vessels showed no upregulation of any of the other RGS family mRNA levels.
An additional limitation concerns the use of mesenteric arterioles for the pressure myography assays. The mesenteric vasculature is useful in its relative ease of isolation and analysis however in whole animal physiology, these vessels do not provide a significant contribution to the establishment of peripheral resistance and maintenance of systemic pressure. Due to the technical challenges associated with the isolation and measurement of these vessels however, mesenteric vessels were chosen. Finally, our X-Gal staining assays showed notable RGS5 promoter activity in the mesenteric arterioles, however these levels were relatively lower than the strongest staining, which we observed in the aorta, iliac and renal arteries. Ultimately, further analysis using more pertinent vessels to blood pressure regulation may reveal a more prominent effect of a lack of RGS5 in vascular function. An additional limitation, as described earlier, concerns changes in cell size, which may be responsible for the variation in peak cell number observed in proliferation assays. Further experiments examining variations in cell size and shape may elucidate this question.

4.a.iv Future studies for RGS5

The results from these studies have been quite helpful in answering the question: Does RGS5 plays a role in mediating VSMC contractility or relaxation in order to control vascular pressure? In completing these studies, we have effectively removed this pathway among the list of potential roles for RGS5. Unfortunately, the biologic role
of RGS5 in arterioles still remains unclear, however there are a number of studies and currently unexamined cellular mechanisms that await attention.

One such area is that of the interaction and signaling that occurs between VSMCs and endothelial cells. Recent studies have demonstrated an important role for myoendothelial junctions in this interaction. Myoendothelial coupling occurs through trans-internal elastic lamina (trans-IEL) endothelial cell protrusions, which may be another area wherein g-protein signaling is playing a role. Briefly, myoendothelial coupling appears to increase as the vasculature moves from conduit to resistance arteries. Furthermore, Ca\(^{2+}\) signaling (“sparks” and “pulsars”), which is influenced by changes in transmural pressure, has been localized to regions closely associated with these protrusions (Ledoux, Taylor et al. 2008). Intriguingly, trans-IEL protrusion number appears to be inversely related to the pattern of RGS5 expression we have observed in our LacZ staining studies (Siegl, Koeppen et al. 2005). Briefly, the regions of highest LacZ staining are in key conduit arteries and regions experiencing high levels of intravascular pressure or turbulent flow, namely the aortic arch, descending aorta, and renal and femoral arteries. Expression is completely absent however in the carotid artery, with the exception of the carotid bulb. These points of expression, combined with results showing abrogation of leaky tumour vasculature in RGS5 knockout mice suggest a structural/adhesion-mediating role for RGS5. More specifically, RGS5 may be involved in mediating the interaction of VSMCs with neighboring VSMCs and/or endothelial cells. Further evidence to support this line of research is evident in the work of Ladds and colleagues. Briefly, studies by Ladds and colleagues (2009) examined RGS transcript
levels in the human myometrium, and the shifts in their levels through pregnancy. This is intriguing as the process of pregnancy and birthing is made possible through a shift in SMC phenotype and function, from proliferative to contractile at term, and it is at this time point that it appears RGS5 levels fluctuate (Ladds, Zervou et al. 2009). This area of research is particularly striking as it allows further consideration of RGS5 as a mediator of vascular development, and a regulator of SMC differentiation and interaction.

Furthermore, it is established that in order to shift from a proliferative (during pregnancy) to contractile (during labour) phenotype, SMCs must modify their ECM and intercellular adhesion qualities, allowing the myometrium to contract as a syncitium. As such, a shift in RGS levels, as described by Ladds et al, could be involved in fine tuning the exact timing or spatial arrangement of this SMC contractile syncitium. These potential mechanisms from studies in myometrial SMCs can be related to new literature discussing the vascular role of RGS5. As discussed earlier, current literature from Hamzah and colleagues (2008) describes normalized tumour vasculature in RGS5 knockout mouse models. The authors proceed to further review the potential for RGS5 as a mediator of vascular remodeling and correlate it’s cardiovascular role in conjunction with RGS2 and RGS4, the other known cardiovascular RGS proteins (Manzur and Ganss 2009). The normalization of tumour vasculature could be a result of a number of factors including variations in matrix protein composition, modified SMC migration and adhesion characteristics, as well as changes in myoendothelial coupling.

In closing, although the current data does not define a conclusive role for RGS5 in the vasculature, it is clear that RGS5 does not play a role in regulating arterial tone in
response to vasoactive agonists. As described earlier, there may be an effect at the level of myogenic response that is being masked by an increased vasodilatory response in knockout arteries. This negative data adds to our understanding of the phenotype of the RGS5 KO model. With the definition that no clear vascular phenotype was identified, future research can be conducted in additional areas of vascular research as outlined. Thus, our negative outcomes have contributed to improved understanding of RGS proteins in vascular biology.

4.b RGS NTD Amphipathic Helix secondary structure discussion, and future directions

RGS2 has long been identified as a potent mediator of vasoconstrictor signaling through its attenuation of Gαq activity. It is unique in the RGS superfamily for this Gαq specificity. More recent data, as described earlier, has linked mutations in RGS2 to cases of hypertension in humans. We examined one mutation, R44H, which occurred within the NTD amphipathic alpha-helix of RGS2, in order to determine a potential mechanism wherein RGS2 is involved in the development of hypertension. Studies conducted in our lab in cultured cells showed reduced RGS2 mutant Gαq signaling attenuation, demonstrated by increased Calcium influx in mutant-transfected cells. These results confirmed a functional detriment resulting from the R44H mutation. In order to determine the mechanism for this, spectroscopic analysis of the secondary structure and lipid interaction of the NTD alpha helix was conducted. Interestingly, the point mutation in the hydrophobic core of the amphipathic helix did not abolish alpha-helix
formation, as is evident by the circular dichroism data. However, analysis of tryptophan fluorimetry data revealed reduced alpha helix-lipid bilayer interaction, leading to the development of a snorkeling model of RGS2 NTD interaction with the plasma membrane. The arginine residue in wild type RGS2 NTD amphipathic helices are essential for lipid interaction as the long nonpolar sidechains stably interact with the non-polar core of the lipid bilayer while allowing the conjugated polar head groups to interact with the polar regions of the phospholipid bilayer and cytosol. The mutation of arginine to a larger and sterically non-ideal histidine residue hinders the ability of the helix to interact and as such disrupts overall plasma membrane association without disrupting helix formation. It is interesting to note that proper membrane association is essential to normal RGS2 function, promoting further studies into NTD characteristics of other R4 subfamily members.
Chapter 5: References


