

RhoA signaling and blood pressure: The consequence of failing to “Tone it Down”

Xue Bai, Rachel Dee, Kevin D Mangum, Christopher P Mack, Joan M Taylor

Xue Bai, Rachel Dee, Kevin D Mangum, Christopher P Mack, Joan M Taylor, Department of Pathology and Laboratory Medicine, McAllister Heart Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, United States

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Correspondence to: Joan M Taylor, PhD, Department of Pathology and Laboratory Medicine, McAllister Heart Institute, University of North Carolina at Chapel Hill, CB7525, 501 Brinkhous-Bullitt Bld, Chapel Hill, NC 27599, United States. jmt3x@med.unc.edu
Telephone: +1-919-8435511
Fax: +1-919-9666718

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Abstract

Uncontrolled high blood pressure is a major risk factor

for heart attack, stroke, and kidney failure and contributes to an estimated 25% of deaths worldwide. Despite numerous treatment options, estimates project that reasonable blood pressure (BP) control is achieved in only about half of hypertensive patients. Improvements in the detection and management of hypertension will undoubtedly be accomplished through a better understanding of the complex etiology of this disease and a more comprehensive inventory of the genes and genetic variants that influence BP regulation. Recent studies (primarily in pre-clinical models) indicate that the small GTPase RhoA and its downstream target, Rho kinase, play an important role in regulating BP homeostasis. Herein, we summarize the underlying mechanisms and highlight signaling pathways and regulators that impart tight spatial-temporal control of RhoA activity. We also discuss known allelic variations in the RhoA pathway and consider how these polymorphisms may affect genetic risk for hypertension and its clinical manifestations. Finally, we summarize the current (albeit limited) clinical data on the efficacy of targeting the RhoA pathway in hypertensive patients.

Key words: Hypertension; Blood pressure; RhoA; Smooth muscle contraction; Guanine nucleotide exchange factor; GTPase activating protein; Polymorphisms

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Core tip: Studies (primarily in pre-clinical models) indicate that the small GTPase RhoA and its downstream target, Rho kinase, play an important role in regulating blood pressure homeostasis. Herein, we summarize the underlying mechanisms and highlight signaling pathways and regulators that impart tight spatial-temporal control of RhoA activity. We also discuss known allelic variations in the RhoA pathway and consider how these polymorphisms may affect genetic risk for hypertension and its clinical manifestations. Finally, we summarize the current (albeit limited) clinical data on the efficacy of

targeting the RhoA pathway in hypertensive patients.

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INTRODUCTION

Although hypertension is a major risk factor for stroke, myocardial infarction, and kidney failure and contributes to over 350000 deaths annually in the United States^[1], we know surprisingly little about its development or the mechanisms by which it promotes cardiovascular disease. A number of antihypertensive drugs are available, but regimens are usually chosen empirically and multiple drugs that target different organ systems are frequently required for effective treatment. One reason for these difficulties is that blood pressure (BP) is a complex trait that is regulated by many organ systems and a large number of humoral factors. Thus, a better understanding of the molecular and genetic mechanisms that control BP under normal and pathologic conditions should lead to novel drug targets and/or to personalized therapies that are more effective and less toxic. Recent advances suggest that RhoA signaling plays a role in the development human hypertension. The focus of this review will be: (1) to highlight the mechanisms underlying RhoA-dependent regulation of BP; (2) to discuss how allelic variations in the RhoA signaling pathway affect genetic risk for hypertension and its clinical manifestations; and (3) to summarize the current (albeit limited) clinical data on the efficacy of targeting this pathway in hypertensive patients.

As a critical regulator of the actin cytoskeleton and acto-myosin contractility, the small GTPase, RhoA, regulates a variety of cellular processes including force development, endocytosis, exocytosis, adhesion, migration, proliferation, and differentiation^[2]. Like all GTPases, RhoA is regulated by guanosine triphosphate (GTP) binding and cycles between the active GTP-bound form and the inactive GDP-bound form. When GTP-bound, RhoA interacts with a variety of effector molecules that mediate its effects on the actin cytoskeleton including the Rho-associated coiled-coil domain containing protein kinases (ROCK I and II), the diaphanous-related formins (mDia1 and mDia2), protein kinase N, citron kinase, rhotekin, and rhotekin. With respect to regulation of BP, Rho kinases are arguably the most important effectors as evidenced by the findings that increased ROCK activity has been observed in spontaneously hypertensive rats and some hypertensive patient populations^[3,4] and ROCK inhibitors like Y-27632, Fasudil, and SAR407899 have been shown to reduce BP

in hypertensive animal models and patients^[5].

RHOA SIGNALING AND BP REGULATION

BP homeostasis is tightly controlled by many organ systems and humoral factors that regulate peripheral vascular resistance, sodium and water balance, and cardiac output. Below we summarize the role RhoA signaling in the regulation of BP highlighting recent findings that implicate this pathway in the development of hypertension.

RhoA and arteriole tone

Vascular resistance is a major determinant of BP and is controlled, in large part, by smooth muscle cell (SMC) contraction within small peripheral arterioles^[6-10]. Excitation-contraction coupling in SMC is mediated by the Ca^{2+} -dependent activation of myosin light chain kinase (MLCK), and SMC tension is directly proportional to myosin light chain (MLC) phosphorylation as this enables myosins molecular interaction with actin^[11,12]. Interestingly, besides promoting an increase in intracellular Ca^{2+} , many G protein-coupled receptor (GPCR)-coupled contractile agonists including angiotensin II (AngII), norepinephrine, and endothelin-1 (ET1) also stimulate RhoA activity in SMC and in intact arteries^[3,4,13]. Active RhoA leads to Rho-kinase (ROCK)-dependent inhibition of myosin phosphatase and results in elevated MLCK activity and enhanced sensitization to Ca^{2+} ^[3,14-16]. Importantly, several studies in animal models and patients (described in further detail below) indicate that RhoA-dependent pathways are involved in the increased vascular resistance associated with hypertension^[3-5,13,17].

Active RhoA also induces *de novo* formation of actin filaments that are necessary for force development and SMC contraction. Rho-dependent actin remodeling occurs by both ROCK-dependent and independent processes. The Rho effectors mDia 1 and 2 directly catalyze actin polymerization in cooperation with the actin binding protein, profilin, whereas ROCK stimulates actin polymerization by inhibiting the disassembly of actin polymers through LIM-kinase-dependent inhibition of cofilin (ROCK activates LIM-kinase 1 and LIM-kinase 2 by phosphorylation at threonine 508 or 505 respectively within the activation loop^[18-22]). ROCK also phosphorylates ERM proteins which enhances their tethering to integral plasma membrane proteins and promotes actin filament stabilization^[23].

Recent studies indicate that RhoA signaling also controls SMC contractile gene expression by regulating the nuclear translocation of the Myocardin-related transcription factors (MRTF-A and MRTF-B). Under conditions of low RhoA activity, monomeric (G)-actin binding to the MRTF N-terminus masks a nuclear localization sequence resulting in cytoplasmic sequestration of these serum response factor co-factors. The fall in cytoplasmic G-actin levels that occurs upon RhoA-mediated actin polymerization promotes MRTF

nuclear accumulation and promotes the expression of SM α -actin, SM γ -actin, SM myosin heavy chain, calponin, and SM22^[24,25]. Thus, not only does RhoA control SMC contractility, but it also regulates the levels of the SMC-specific contractile proteins that support this function. Moreover, elevated RhoA in endothelial cells impairs endothelial cell-mediated vasorelaxation as it decreases availability of the potent vasodilator, nitric oxide by reducing both eNOS expression and activity^[26-30]. In sum, signaling through RhoA enhances Ca²⁺ sensitivity, promotes actin remodeling and induces expression of contractile proteins and these responses are necessary for maintaining sustained SMC contractility and elevated vessel tone (Figure 1).

RhoA and kidney function

The kidneys play a major role in regulating BP by controlling sodium excretion and blood volume. In addition, since the kidneys are highly perfused organs receiving up to 25% of total cardiac output, increased contractility of renal arterioles can significantly increase total peripheral vascular resistance. In most vascular beds, arteriolar tone is controlled by autonomic innervation and circulating hormones. However, in pre-glomerular afferent arterioles, increased kidney perfusion (manifesting as increased renal BP) stimulates SMC contraction through the tubuloglomerular feedback and myogenic responses (see^[31] for review). The former mechanism is mediated by increased glomerular filtration and NaCl delivery from the loop of Henle to the macula densa (MD), a cluster of epithelial cells located at the junction between the distal convoluted tubule and the end of the thick ascending limb and adjacent to the abluminal SMCs of the afferent arterioles. Increased NaCl uptake by MD cells results in secretion of ATP and adenosine which stimulate afferent arteriole SMC contraction *via* P2Y4/P2Y6 and A2 GPCRs, respectively. The myogenic response is mediated by the activation of stretch-sensitive cation channels. Together these mechanisms stabilize renal blood flow to protect the sensitive glomerular capillaries from flow-induced trauma. Importantly, afferent arterioles express RhoA, ROCK I and II^[32], and several studies have convincingly demonstrated that the Rho/Rho kinase pathway influences both of these feedback mechanisms in response to increased kidney perfusion^[33-37]. The requirement of RhoA is likely due, at least in part, to its necessity for P2Y4/P2Y6 and A2 receptor-dependent contractility. Indeed, ATP (*via* P2Y4/Y6) and adenosine (*via* A2) stimulate RhoA activity in SMC and their pressor responses were prevented by pretreatment with the Rho-kinase inhibitor, Y-27632^[32].

Interestingly, recent evidence indicates that RhoA may play an additional role in other cell types within the kidney to impact volume homeostasis. In particular, RhoA activity in tubular epithelial cells can regulate sodium reabsorption and excretion primarily by altering the density and location of epithelial sodium channels (ENaCs) and the sodium-hydrogen exchanger

(NHE3)^[38]. *In vitro* studies in cultured epithelial cells indicated that the Na⁺ current through ENaCs was significantly increased by expression of wildtype or constitutively active RhoA (G14V) and suppressed by expression of dominant negative RhoA (T19N). The changes in current correlated with alterations in the density of ENaCs at the PM^[39] and mechanistic studies determined that RhoA signaling was essential for intracellular vesicle mediated transport of ENaCs to the apical cell surface^[40,41]. RhoA signaling also regulates the activity and subcellular localization of NHE3, a key regulator of sodium absorption in the proximal convoluted tubule. NHE3 associates with ezrin and cortical actin filaments at the plasma membrane and treatment with either the RhoA inhibitor, diphtheria toxin toxin B, or Y-27632 disrupted these interactions and promoted the internalization of NHE3 to sub-membrane compartments^[42,43]. Moreover, Nishiki *et al.*^[44] showed that spontaneously hypertensive rats exhibited elevated NHE3 activity and a exaggerated level of Na⁺ reabsorption when compared to normotensive controls and that Na⁺ reabsorption was normalized by treatment of the hypertensive animals with Y27632.

RhoA in the central and peripheral nervous system

The central nervous system (CNS) constantly assesses pressure levels in the vasculature and makes necessary signaling adjustments to prevent BP variability. The main mechanism by which the CNS monitors BP is through a rapid negative feedback loop termed the baroreceptor reflex. Baroreceptors are sensory neurons located primarily in the aortic arch and carotid sinuses that continuously respond to pressure-induced stretching of the vessels in which they reside. Impulses from baroreceptors are relayed *via* glossopharyngeal and vagus nerves to the nucleus tractus solitarius (NTS) in the brainstem^[45], which in turn relays the signal to the rostral ventrolateral medulla^[46] and increases or decreases parasympathetic and sympathetic stimulation to the heart and vessels accordingly. Interestingly, the CNS component of this feedback loop has been shown to be dependent on RhoA/Rho-kinase signaling. Rho-kinase inhibitors microinjected directly into the NTS or infection of this structure with an adenovirus expressing a dominant-inhibitory form of Rho-kinase reduces sympathetic nerve activity, heart rate, and BP in normotensive rats and these effects are even more pronounced in spontaneously hypertensive rats^[47,48]. Moreover, infusing the ROCK inhibitor, Y27632, into the neural cistern attenuated the BP increase that resulted from AII infusion into the same area of the brainstem^[49].

While RhoA's effects on the CNS are clear, a heretofore understudied area in this field is the extent to which RhoA regulates neurotransmitter release from the perivascular nerves which are known to play a major role in the control of resistance arteriole tone. While it has long been known that RhoGTPases have an important and conserved function in mediating neuronal

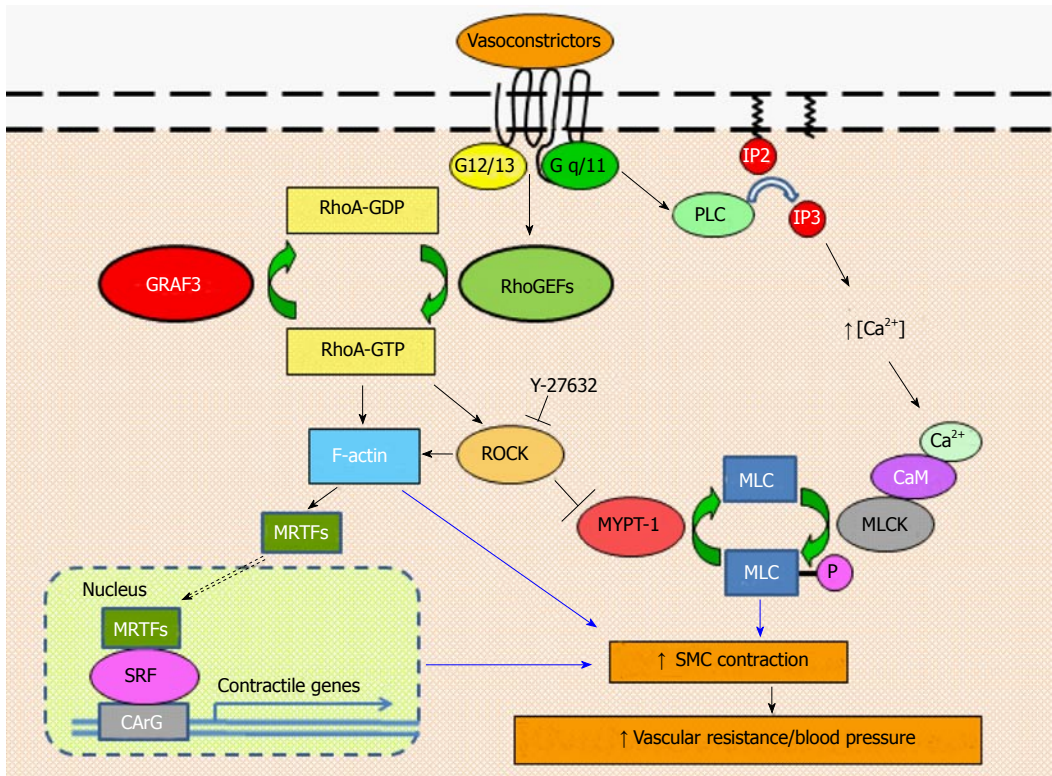


Figure 1 Schematic summarizing RhoA-dependent regulation of vascular smooth muscle contraction and blood pressure homeostasis. Excitation-contraction coupling in smooth muscle cell (SMC) is mediated by the Ca^{2+} -dependent activation of myosin light chain kinase (MLCK), and SMC tension is directly proportional to myosin light chain (MLC) phosphorylation (p) as this enables myosin's molecular interaction with actin. SMC contractility is also regulated by GPCR-coupled contractile agonist-mediated activation of the small GTPase RhoA. Downstream activation of Rho kinase (ROCK) inhibits myosin phosphatase target subunit 1 (MYPT-1), and results in increased levels of pMLC to promote smooth muscle contraction. RhoA also stimulates G-actin polymerization to filamentous actin (F-actin). Actin polymerization increases SMC tension and stimulates myocardin-related transcription factor (MRTFs) nuclear translocation which promotes SRF-dependent transcription of contractile genes. RhoGAPs (such as GRAF3) and RhoGEFs dynamically regulate RhoA activity to achieve blood pressure balance.

survival and death and that tight spatiotemporal control of RhoA is necessary for appropriate neuronal development (neurite outgrowth, growth cone dynamics) and regeneration, to our knowledge no studies have explored the consequence of Rho-kinase inhibition on peripheral nerve structure or function. Future studies to this end are warranted, because some studies in cells and invertebrate model systems indicate that Rho/Rho kinase signaling may limit the release of sympathetic (contractile) agents and promote the release of parasympathetic relaxation factors from motor neurons. For example, Yamaguchi *et al.*^[50] found that $\text{Ga}_{12/13}$ -mediated activation of RhoA/ROCK inhibited Ca^{2+} dependent exocytosis of the contractile neurotransmitter dopamine in PC12 cells. In support of these studies, an activating mutation in ArhGEF10, a RhoGEF highly expressed in the peripheral nervous system, was identified in patients who exhibited slowed nerve conduction velocities^[51,52]. On the other hand, Hiley *et al.*^[53] reported that release of the relaxation neurotransmitter, acetylcholine from cholinergic motor neurons in *C. elegans*, required the regulators of G protein signaling (RGS)-RhoGEF dependent activation of Rho A. Thus, it is formally possible that inhibition of RhoA in peripheral nerves could lead to an increase total peripheral resistance and BP. This concept requires

further exploration if RhoA/ROCK inhibitors are to be considered as future anti-hypertensive therapies.

RhoA in the myocardium

Several studies have shown that RhoA signaling has direct effects on cardiac function that increase cardiac output and BP. Transgenic mice that overexpressed either $\text{GDI}\alpha$ or dominant negative RhoA exhibited conduction defects and cardiomyocytes isolated from these mice exhibited decreased L-type Ca^{2+} channel currents that likely contributed to the decreased contractility observed *in vivo*^[54,55]. Vlasblom *et al.*^[56] showed that treatment of neonatal ventricular cardiomyocytes with Y27632 reduced the expression and activity of the sarcoplasmic reticulum Ca^{2+} ATPase, SERCA2a, thereby limiting the amount available for Ca^{2+} -induced Ca^{2+} release in the next cardiac cycle. In addition, RhoA-dependent pathways have been shown to be critical for phosphorylation and sensitization of cardiac troponin T complex to intracellular Ca^{2+} levels^[57]. Moreover, while not initially thought to be a major mechanism for modulating cardiac contractility, it is becoming clear that cardiac MLC phosphorylation can enhance muscle contractility by increasing Ca^{2+} sensitivity^[58] and that MLC phosphatase is a target for Rho kinase-dependent inhibition in the myocardium (like

in SMC). Indeed, Lauriol *et al.*^[59] showed that cardiac-restricted deletion of RhoA led to decreased contractility and this effect was correlated with decreased MLC activity. Other similarities between RhoA signaling in cardiomyocytes and SMC include the ability of RhoA-mediated signals to promote differentiation/maturation by promoting the expression of contractile genes^[60].

CONTROL OF RHOA GTPASE ACTIVITY

Rho proteins act as molecular switches that cycle between an inactive GDP-bound form and an active GTP-bound form and this cycle is under the direct control of three groups of regulatory proteins. Guanine dissociation inhibitors (GDIs) sequester RhoA into an inactive cytoplasmic fraction, guanine nucleotide exchange factors (GEFs) activate RhoA by facilitating exchange of GDP for GTP, and GTPase activating proteins (GAPs) promote RhoA's intrinsic GTPase activity to hydrolyze GTP to GDP and efficiently turn off (or tone down) RhoA-dependent signaling. The GEF and GAP protein families are quite large and structurally diverse and it is likely that additional differences in expression patterns and post translational modification allow for tissue-specific and tight spatio-temporal control of RhoA activity. The following section summarizes the known mechanisms for controlling RhoA activity in SMC.

RhoGEFs and BP control

GEFs activate small GTPases by increasing the GDP dissociation rate by several orders of magnitude which in turn promotes GTP-binding since GTP is in an approximately 10:1 molar excess to GDP in mammalian cells^[61]. To date, over 24 different Rho-selective GEFs have been identified. The common functional domain of RhoGEFs is the Dbl homology (DH) domain (also referred to as the RhoGEF domain), which typically serves as both the catalytic site and the major binding interface for RhoA (Figure 2). A pleckstrin homology (PH) domain is almost always found downstream of the DH domain and this unit serves to facilitate membrane binding and cooperates with DH domains to fully activate RhoA^[62]. Other common functional domains include the RGS domain that binds large G-proteins to couple the GPCR and Rho signaling pathways and the Postsynaptic Density 95, disk large, Zona occludens-1 (PDZ) domain that binds to Plexin-B1 and Lysophosphatidic acid (LPA) receptor to transmit Semaphorin 4D (57) and LPA signals^[63], respectively.

The major contractile agonists that stimulate RhoA activity in SMC include AII, phenylephrine (PE), ET1, and thromboxane A₂. The GPCRs for these ligands couple to various G α subunits, including G $\alpha_{12/13}$ and G $\alpha_{q/11}$, but each has distinct Gprotein coupling properties. For example, PE signals almost exclusively through G α_q and thromboxane through G $\alpha_{12/13}$ while other agonist-receptor interactions lead to more promiscuous G protein activation. These heterotrimeric GTPases in turn either

directly or indirectly activate a range of RhoGEFs. Therefore, it is likely that each agonist could stimulate somewhat overlapping but distinct set of RhoGEFs to enable fine tuning of the extent and duration of Ca²⁺ sensitization and thus engender precise spatial and temporal control of vessel tone.

RGS family of RhoGEFs (LARG, p115RhoGEF, and PDZRhoGEF)^[64] has received a lot of attention in the BP field because these proteins can interact with (and be directly activated by) G α_{12} and G α_{13} ^[65]. Indeed, activation of RhoA by many of the aforementioned contractile agonists is mediated, at least in part, by these RhoGEFs. However, studies performed in mice lacking the α subunits of G $\alpha_{q/11}$ or G $\alpha_{12/13}$ in smooth muscle convincingly showed that activation of these RhoGEFs are not simply due to direct binding to G $\alpha_{12/13}$ ^[13]. In fact G $\alpha_{12/13}$ depletion did not affect the pressor effects of AII, or PE, and only modestly reduced the pressor effects of ET1. Instead, depletion of G $\alpha_{q/11}$ completely abrogated PE-induced pressor responses and dramatically attenuated responses to AII. Subsequent studies from Guilluy *et al.*^[13] identified p115RhoGEF (p115) as the critical GEF that mediates AII-dependent RhoA activity in SMC and small arterioles and showed that smooth muscle specific deletion of p115 rendered mice resistant to AII-dependent hypertension. Interestingly, their mechanistic studies confirmed that p115 activation did not require G α_{12} /G α_{13} , but instead, was governed by G α_q -mediated, Janus tyrosine kinase-dependent phosphorylation of Tyr738 in the PH domain. Importantly, phosphorylation mimetic and deficient variants at Tyr738 elevated and reduced p115's GEF activity, respectively^[13]. As discussed in further detail below, phosphorylation-dependent activation of RhoGEFs has since emerged as a critical regulatory pathway. However, it should also be noted that AII-dependent activation of RhoA in SMC likely involves additional pathways as AII signaling in SMC has been linked to inhibition of p190Rho GTPase activating protein (see below^[66]); upregulation of LARG^[67], upregulation of PDZ-RhoGEF^[68], and G α_q /Ca²⁺/proline-rich tyrosine kinase 2 (PYK2) tyrosine kinase mediated phosphorylation/activation of PDZ-RhoGEF^[69]. Indeed, Ying *et al.*^[69] showed that Ca²⁺/PYK2-dependent activation of PDZ-RhoGEF was necessary for maximal AII induced RhoA activation.

Interestingly, p115 mutant mice also exhibited a partial reduction in DOCA/salt-induced hypertension but had normal basal BP and normal pressor responses to ET1 and PE; agents that also act through G α_q -dependent signaling pathways. Future studies are necessary to determine how PE and ET1/G α_q signals differ from those induced by AII. However, recent studies by the Somlyo laboratory shed some light on this phenomena as they found that there is functional overlap between p115 and LARG. Indeed using genetic mouse models, they found that while the time it took to reach maximal contraction was increased in SMC-

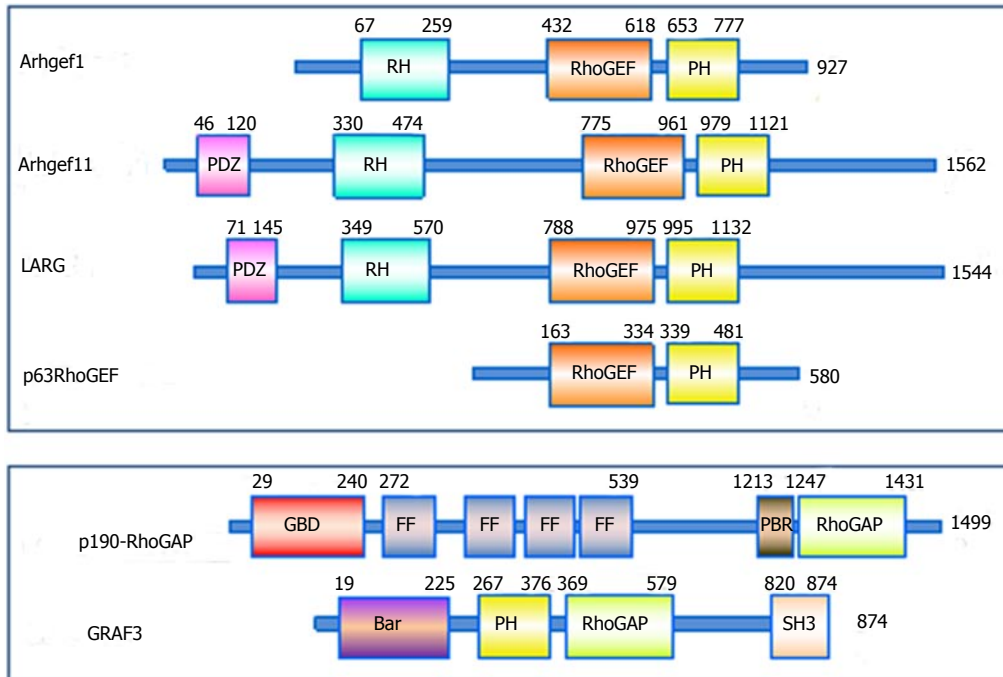


Figure 2 Multi-domain architecture of RhoGEF and RhoGAP proteins known to regulate smooth muscle cell phenotype. The catalytic domain of RhoGEFs is termed a Dbl homology (DH) domain, which serves as the major binding interface with Rho GTPases and catalyzes the dissociation of GDP from the GTPase. Pleckstrin homology (PH) domains are almost always downstream of the DH domain and these units cooperate to fully activate the GTPase. Other functional domains contained in specific RhoGEFs include the RH (Regulators of G protein Signaling Homology) domain and PDZ (Postsynaptic density 95, disk large, zona occludens-1) domain. RhoGAPs are also multi-domain containing proteins. The RhoGAP domain facilitates GTP hydrolysis and inhibits RhoA activity while other domains can regulate RhoGAP targeting and function. For example, BAR (Bin/amphiphysin/Rvs), PH, or polybasic region (PBR) domains direct lipid binding and promote membrane localization. Other domains are involved in protein-protein interactions such as GTP-binding domain (GBD), diphenylalanine motifs (FF) and the SH3 (SRC Homology 3) domains. The amino acid numbers are shown above each protein are based on the human orthologs (<http://www.ncbi.nlm.nih.gov/>).

specific double knockouts, maximal contraction of smooth muscle from PDZ-RhoGEF and LARG double knockdown tissues was similar to that of the single mutants^[70]. Thus it is formally possible that AII (but not ET1 and PE) induced hypertension is blocked by p115 knockout because AII has a relatively reduced capacity to activate LARG. Interestingly, Medlin *et al.*^[71] identified the vasoconstrictor agonist sphingosine-1-phosphate (S1P) as a potent activator of LARG in cultured vascular SMC. While this pathway has not yet been confirmed *in vivo* the finding that LARG knockout mice are resistant to salt-induced hypertension^[4] which leads to volume overload-induced stretching of vessels is consistent with the thesis that LARG may regulate RhoA activity and SMC contractility in response to mechanical forces^[72].

Besides activation of the RGS GEFs, several studies have linked $G_{\alpha_{q/11}}$ -dependent activation of RhoA to the Trio family of RhoGEFs (Trio, Duet, and p63-RhoGEF)^[73,74]. P63-RhoGEF is highly expressed in arterial smooth muscle and it has recently been shown to be important for the early phase of AII-dependent vessel contractility^[75] and for maximal pressor response to other vasoconstrictors such as PE and ET1 that act through $G_{\alpha_{q/11}}$ ^[76]. Moreover, another non-RGS RhoGEF termed lymphoid blast crisis (Lbc) has been shown to be critical for serotonin-dependent activation of RhoA and contractility in vascular SMC^[77]. In summary, specific vasoconstrictors can lead to activation of distinct

but overlapping sets of RhoGEFs (each with different activation kinetics, catalytic activities, and subcellular locales). A question that warrants further studies is to what extent GEFs might also govern control over the activation of specific RhoA effector subsets by various agonists.

Like p115, PDZRhoGEF and LARG are also activated by tyrosine phosphorylation. Focal adhesion kinase (FAK) as well as its related family member, PYK2 phosphorylate PDZRhoGEF^[69,78], while LARG was shown to be activated by FAK^[78] and Tec^[64,69]. Future identification of the specific sites of phosphorylation will aid in determining if there is a conserved mechanism by which these modifications promote GEF activity.

Control of RhoGEF expression is another important means of regulating RhoA activity in the vasculature. Because p115-RhoGEF, PDZ-RhoGEF, and LARG each play a role in BP regulation in rodents, it is no surprise that these GEFs are expressed in both conductance and resistance arteries of rats and mice^[4,68,69,79]. P63RhoGEF is also abundant in the peripheral vasculature^[80]. Interestingly, the expression of many of these RhoGEFs fluctuates as BP changes, suggesting that dynamic regulation of their expression is important for BP control. The most comprehensive study performed to date revealed that expression of each of the five RhoGEFs linked to Rho-A dependent vasoconstriction (p115, LARG, PDZ-RHOGEF, p63 RhoGEF, and Lbc)

are all down-regulated in cultured mesenteric artery SMC following treatment with AII for 48 h. Moreover, treatment with the Rho Kinase inhibitor fasudil prevented the AII-mediated suppression of p115, LARG and PDZ-RhoGEF indicating that RGS/RhoGEF expression is governed, at least in part, by negative-feedback signaling through the Rho/Rho kinase cascade. A similar decrease in RGS/RhoGEF expression was observed in mesenteric arteries from rats treated with AII for 14 d^[80]. Whether RhoGEF expression is altered in or contributes to hypertension in animal models is less clear. Ying *et al.*^[81] reported that aortic expression of all 3 RGS/RhoGEFs was higher in aortas from 12 wk old SHR than in normotensive rats. Similarly, a comprehensive microarray analysis revealed that LARG expression was upregulated in DOCA-salt hypertensive mice^[82]. In contrast, Hilgers *et al.*^[68] reported that mesenteric arteries from 14 d AII-treated rats exhibited decreased mRNA levels, but increased protein levels of PDZ RhoGEF. Thus, while it is clear that GEF expression is dynamic, the extent to which elevated expression of these factors contributes to the induction of hypertension and reduced expression to BP normalization is currently unresolved and requires further study.

RhoGAPs and BP control

GAPs inhibit Rho signaling by enhancing the intrinsic ability of Rho to hydrolyze GTP^[83,84]. More than 70 RhoGAPs have been identified in eukaryotes that can be divided into 23 subfamilies^[85]. Like the RhoGEFs, RhoGAPs are typically large multi-domain containing proteins and their diverse structures allow for dynamic and selective inhibition of small GTPase signaling (see Figure 2). Several Rho-selective GAPs including p190RhoGAP, ArhGAP1, Myr5, GRAF1, and GRAF3 have been shown to regulate RhoA in cultured vascular SMC^[86,87].

To our knowledge, GRAF3 is the only RhoGAP that has been implicated in the regulation blood pressure. The founding member of the GRAF (GTPase regulator associated with FAK-1) family, was originally identified by our group^[88-90] by screening an embryonic λ gt11 expression library for proteins that interacted with the carboxyl-terminal domain of FAK^[88]. The GRAF family's three members are defined by an N-terminal BAR (Bin/amphiphysin/Rvs) domain, a phosphatidylserine (PS)-binding PH domain, a central Rho-GAP domain, a serine/proline rich domain, and a C-terminal SH3 domain (Figure 1A). The GRAF1 SH3 domain was shown to specifically bind to a proline-rich region in the carboxy terminus of FAK and this protein-protein interaction was important for directing GRAF1 to the actin cytoskeleton^[88]. GRAF1 is expressed predominantly in the brain and striated muscle (cardiac and skeletal), and our studies in GRAF1-depleted *Xenopus* and mice revealed that GRAF1-dependent inhibition of RhoA activity promoted mammalian muscle growth

by facilitating myoblast fusion and injury repair^[90-93]. GRAF2 is more ubiquitously expressed^[94] and could partially compensate for the loss of GRAF1 during myotube formation supporting at least some functional redundancy within this family^[92]. Evolutionarily, GRAF3 is the youngest family member and is the most recently annotated. Interestingly, our genome wide analyses of chromatin structure in primary human SMC suggested that this gene was regulated in a smooth muscle-specific fashion. Indeed, we found that GRAF3 was highly and selectively expressed in SMC with particularly high expression in resistance vessels^[87]. After validating that GRAF3 functioned as a RhoA-specific GAP in these cells, we considered the possibility that GRAF3 might control BP homeostasis. Importantly, we found that homozygous GRAF3 knockdown mice showed a consistent and significant elevation in systolic, diastolic, and mean arterial BP (+ 20-30 mmHg). The observation that heterozygous GRAF3 knockdown mice exhibited a 15 mmHg increase in BP strongly supports a dose-dependent relationship between GRAF3 expression and BP. GRAF3-deficient mice exhibited significantly elevated pressor responses following treatment with AII, ET1, or PE and these effects were inhibited by treatment of GRAF3 deficient mice with Y-27632. Accordingly, RhoA activity and myosin light chain phosphorylation were elevated in GRAF3-depleted SMC *in vitro* and *in vivo*^[87].

The remarkable SMC-selective expression pattern of this Rho-selective GAP when coupled with the ability of ROCK inhibition to normalize the hypertensive phenotype of GRAF3-deficient animals strongly supports a model in which GRAF3 plays a major role in regulating BP homeostasis by limiting RhoA-mediated SMC contractility in resistance vessels^[87]. Interestingly, as discussed in further detail below, a large GWAS revealed that polymorphisms in the GRAF3 gene contribute to BP variation in humans^[95,96]. Thus future studies that strive to determine the mechanisms that control variations in GRAF3 expression and/or activity will likely lead to important insights into how to better control BP in the general population.

p190RhoGAP may also play a role in limiting RhoA-dependent arterial tone. p190RhoGAP contains an amino-terminal GTP-binding domain, a large middle domain with multiple protein-protein interaction motifs (diphenylalanine, FF motifs) a polybasic region, and a carboxy-terminal GAP domain^[97]. Knockdown of p190RhoGAP in SMC by siRNA increased RhoA/Rock activity^[98], and several studies have shown that p190RhoGAP is activated by phosphorylation of Y1105 by cAbl and Src tyrosine kinases^[66,99]. p190RhoGAP is a substrate for the tyrosine phosphatase, SHP-2, and SHP-2-dependent dephosphorylation of p190RhoGAP was shown to be important for the initial burst in RhoA activity in SMCs treated AII and ET1^[98]. Interestingly, ROCK-dependent phosphorylation at Ser1150 attenuated p190RhoGAP activity creating a positive feedback loop for further RhoA activation^[86]. Pho-

sphorylation of several C-terminal residues by ERK also suppresses p190RhoGAP activity during focal adhesion formation^[100]. Finally, although not yet shown in SMC, p190RhoGAP has also been shown to be regulated by phospholipid binding^[101]. Additional studies will be necessary to determine if p190RhoGAP plays an important role in BP regulation *in vivo*.

Regulation of GDIs

GDIs bind to GDP-bound GTPases and inhibit GDP dissociation. GDI binding also limits translocation of GTPases to the membrane effectively "locking" them in the inactive state. Indeed, studies have shown that GDIs can inhibit RhoA dependent Ca^{2+} sensitization in SMCs treated with α -adrenergic and muscarinic agonists^[102]. However, the extent to which RhoGDIs regulate BP or RhoA activity *in vivo* is unclear. One study showed that RhoGDI α knock out mice displayed a salt-dependent increase in BP, but this effect was attributed to an increase in Rac1 activity in the kidney^[103]. However, since SMC-specific Rac1 knockout mice were hypertensive and exhibited increased RhoA activity, it will be important to measure RhoA and Rac1 activity in SMC in RhoGDI α knock out mice^[104]. RhoGDIs have been shown to bind to and regulate RhoGEFs and RhoGAPs^[105], an effect that could indirectly influence RhoA activity and vessel tone. Thus, additional studies will be needed to assess RhoGDIs' role in RhoA dependent blood pressure regulation.

Direct regulation of RhoA

Additional control of RhoA signaling may be imparted by mechanisms that alter RhoA protein levels and/or alter functional post-translational modifications. Notably, protein ubiquitination followed by proteasome-dependent degradation is a major means of fine-tuning protein levels and Chen *et al.*^[106] reported that RhoA is a direct target of the Rho-BTB/Cullin-3 E3 ubiquitin ligase degradation pathway. Interestingly, the Sigmund laboratory found that Cullin-3 regulated vascular smooth muscle function and arterial BP through a RhoA/Rho-kinase pathway. Moreover, they found that a human hypertension-associated mutation in Cullin-3 in which exon 9 is deleted led to decreased Cullin-3 activity and reduced ubiquitin-mediated RhoA degradation^[107,108] (see below for further discussion of these and other genetic variants that influence RhoA signaling and human hypertension). Ubiquitination-dependent regulation of RhoA is also catalyzed by a distinct E3 ubiquitin ligase termed SMAD ubiquitin regulatory factor (Smurf1)^[109,110]. Interestingly, Smurf1-dependent degradation of RhoA in endothelial cells has been linked to the development of cerebral cavernous malformation (CCM) a disease that is accompanied by hyperpermeable blood vessels in the brain. CCM results from the homozygous inactivating mutations in one of three *ccm* genes. Crose *et al.*^[109] demonstrated that *ccm2* bound directly to Smurf1 and that this interaction

regulated RhoA degradation, likely explaining the common biochemical defect of elevated RhoA/ROCK signaling and increased permeability observed in *ccm* mutant endothelial cells^[109,110]. Whether this class of E3 ligases regulates RhoA in levels in SMC is currently unknown.

Signaling *via* nitric oxide and reactive oxygen species may add another level of spatial/temporal control of RhoA signaling in the vasculature. Levels of ROS increase in the vasculature under a number of pathological conditions including hypertension, and ROS-mediated activation of RhoA has been demonstrated in vascular smooth muscle^[81]. Interestingly, Aghajanian *et al.*^[111] have demonstrated that ROS can mediate direct activation of RhoA by reversible oxidation of reactive cysteines C16/C19 and that this acts in a similar fashion to GEFs in that it leads to nucleotide displacement and increased GTP binding. Because oxidation of RhoA does not impair RhoGEF binding it is possible that ROS-dependent oxidation might prime RhoA for vasoconstrictor-dependent activation. In contrast, the reactive vasodilator, NO has been shown to inhibit RhoA activation *via* post-translational modification. For example, treatment of SMC with the pharmacological NO donor, PAPA-NONOate promoted RhoA S-nitrosylation that reduced GTP binding and therefore inactivated RhoA^[112]. NO signaling may also limit RhoA activity in SMC by promoting cGMP-dependent phosphorylation of RhoA on sites that attenuate membrane targeting (and activation) of RhoA^[113,114]. However, the role that such post-translational modifications play *in vivo* has yet to be explored.

In summary, RhoA activity in SMC can be dynamically regulated by transcriptional and post-translational mechanisms that alter RhoA protein, its activators, and its inhibitors. Collectively these mechanisms play an important role in precise spatial-temporal control of vessel tone and BP homeostasis. Importantly, while several RhoAGEFs have been shown to be necessary for development of vasoconstrictor-induced hypertension, our recent results in GRAF3-depleted mice demonstrated for the first time that GAP-dependent control of RhoA activity in SMC contributes to the maintenance of basal BP^[87].

GENETIC REGULATION OF THE RHOA PATHWAY IN HUMAN HYPERTENSION

Hypertension is a devastating disease associated with significant morbidity and mortality due to detrimental pressure-related effects on the kidneys, heart, lungs, brain, and peripheral vasculature. Hypertension affects roughly 80 million people (approximately 32.6% of adults) in the United States alone and was predicted to be primarily responsible for 25% of deaths worldwide in 2010^[115]. Despite the fact that nearly 70 drugs (from 15 distinct classes of compounds) are approved

for treatment of hypertension in the United States, estimates project that reasonable BP control is achieved in only about half of hypertensive patients. This reality coupled with recent projections that the incidence of hypertension will increase to about 41% in the United States by 2030, indicate the urgent need for better screening and treatment modalities^[116]. Improvements in the detection and management of hypertension will undoubtedly be accomplished through a better understanding of the complex etiology of this disease.

One way to better predict patient response to therapy is to gain a more comprehensive understanding of the genes and genetic variants that influence BP regulation. Recent projections indicate that up to 60% of BP variation can be explained by genetic factors, but that no single gene exerts a principal effect. Thus, BP is considered to have a complex non-Mendelian mode of inheritance. Indeed a combination of classic positional cloning strategies in families with numerous affected members combined with more recent population-based GWAS studies have led to the identification of 25 rare mutations and 53 SNPs that are predicted to contribute to BP control^[117]. The aim of this section of is to highlight variants that impinge on the expression or activity of members of the RhoA signaling axis.

RhoA-related forms of monogenic hypertension

Virtually all known cases of monogenic hypertension are associated with volume expansion resulting from mutations in genes involved in renal salt handling or hormones that affect mineralocorticoid activity. However, although hypertensive patients with Gordon's Syndrome (pseudohypoaldosteronism type IIE) present with salt handling abnormalities, the high BP in these patients is caused by an autosomal dominant mutation in the Cullin-3 gene (see above). Interestingly, this E3 ligase helps target RhoA for proteosomal degradation and *in vitro* studies indicate that increased RhoA/ROCK signaling in vascular SMC may also play a role in Gordon's Syndrome patients^[118]. Exclusion of exon 9 abrogates the Cullin-3 dependent interactions between RhoBTB and the E3 ligase and as RhoBTB serves as a chaperone to recruit RhoA to this degradation complex, expression of exon 9-deficient Cullin-3 leads to aberrant RhoA accumulation^[107,108].

SNP/EQTls in RhoA-signaling molecules

Because Rho kinases are major RhoA effector proteins and because both animal and human studies have shown that treatment with Rho-kinase inhibiting compounds lowers BP, a number of case-controlled studies were designed to determine if genetic variants in these genes might influence the development of human hypertension. One group examined the effect of ROCK2 genetic variations on BP in 168 pairs of mono- and dizygotic twins. In this study, four variants were identified in ROCK2, the most notable of which was a nonsynonymous SNP in exon 10 that resulted

in a substitution of Thr with Asn at amino acid 431. Importantly, the Asn substitution was associated with increased systemic vascular resistance and BP and was predicted to account for 3%-5% of the BP variance between these patients^[119]. Another study in which 18 tag SNPs within the ROCK2 locus were genotyped in 586 normotensive controls and 607 hypertensive Caucasian patients identified a haplotype defined by four SNPs (rs965665, rs10178332, rs6755196, rs10929732) that was recessively associated with a lower risk of hypertension ($P = 0.003$). However, a subsequent study in a separate population of 1344 Chinese patients with coronary artery disease and hypertension and 1267 ethnically and geographically matched controls did not find an association between this haplotype and either BP or cardiovascular disease^[120,121]. Thus, future studies are necessary to determine the relevance of these SNPs with respect to BP control in the general population.

Recent studies have implicated artery stiffness in the pathology of HTN and this parameter has been shown to be a valuable predictor of end organ failure^[122-126]. Decreased vessel compliance elevates the mechanical load on the myocardium but also increases peripheral pulse-pressure in the microvasculature resulting in tissue damage in high flow organs such as the brain and kidneys. Until very recently, increased vascular stiffness during aging or the development of HTN was thought to result from changes in extracellular matrix content and composition (*i.e.*, elastin degradation, collagen deposition, *etc.*). However, new studies suggest that the intrinsic mechanical properties of VSMC (including RhoA-dependent formation of force-generating actin filaments, and increased cell adhesion to the extracellular matrix) may also play a role^[127,128]. Notably, Liao *et al.*^[129] identified two SNPs in ROCK2 that were in complete linkage disequilibrium and associated with arterial stiffness in 1483 un-selected patients from a Chinese population in Taiwan. Subsequent, *in vitro* studies revealed that both SNPs were functional. One SNP, rs978906, affected ROCK2 expression by interfering with microRNA(miR)-1183 binding to its 3' UTR, while the other, rs9808232, which was located in a protein-coding region, increased ROCK2 activity^[129].

As noted above, S1P is a major upstream activator of RhoA in SMC and has vasoconstrictive effects *in vivo*^[71,130]. Interestingly, Fenger *et al.*^[131,132] assessed the significance of 353 genetic variants contained within exons of genes in the metabolic sphingolipid network. Of these SNPs, 34 and 40 haplotypes were associated with changes in diastolic or systolic pressures respectively in their 2556 subjects. They found that while the BP effects could not be explained by any single gene, several 2-gene interaction pairs were highly correlated with BP variations. S1P is generated from ceramide in a process that involves two critical enzymes ceramidase (ASAH1) and sphingosine 1- kinase (SPHK1) and the most significant of the 2-gene interactions identified were contained in these genes^[131,132], further

supporting a role for RhoA signaling in the development of hypertension. It is likely that future gene interaction studies such as these will provide a powerful approach to both predict hypertension risk and possibly inform treatment options.

In the past decade, many GWAS studies have identified common genetic variations in coding and non-coding genomic regions that vary between individuals and are associated with changes in BP and several of these variants occur in genes linked to the Rho signaling cascade. Notably, one GWAS study that used hypertension as a dichotomous trait identified eight loci associated with BP, and two of these variants were located in RhoA-related genes. One of the target genes was the aforementioned RhoBTB1 which functions with the Cullin-3 complex to maintain low RhoA levels^[107,118]. Another SNP was found at the rhotekin-2 (RHTKN) locus. Although rhotekin was one of the first identified RhoA effector molecules (it has high affinity to Rho-GTP and is widely used in pull down assays for activated RhoA^[133]), how Rhotekin functions at a cellular level is still unclear. Nonetheless this association is provocative and clearly indicates that future studies are warranted. Two separate GWAS for BP variation and hypertension have identified significant association signals in the RhoA-interacting protein, plekstrin homology domain containing family A member 7 (PLEKHA7)^[134,135]. PLEKHA7 is highly expressed in the kidney and heart and localizes on the cytoplasmic surface of adherens junctions, where it interacts with junctional proteins cingulin and paracingulin to regulate the activity of Rho family GTPases, including RhoA^[136]. While the functional SNP(s) have yet to be identified, the finding that PLEKHA7 is required for the development of salt-induced hypertension *in vivo*, highlights the functional importance of this RhoA-interacting protein in BP regulation^[137].

Finally, two separate GWAS for BP and cardiovascular disease endpoints identified a novel BP associated locus containing two SNPs in perfect linkage disequilibrium (rs633185 and rs604723) within *GRAF3* gene (*ArhGAP42*). Both SNPs were associated with a significant reduction in BP with each copy of the minor allele^[95,96]. Of extreme importance, as noted above, we reported that mice in which *GRAF3* was depleted developed significant hypertension that was RhoA-dependent^[87]. Interestingly, the BP locus falls within the first intron of the *GRAF3* gene, indicating that one or both SNPs may affect expression of this Rho-GAP and result in altered SMC contractility. Indeed, data within the Genotype-Tissue Expression database indicated that *GRAF3* RNA levels in tibial artery samples were 3-fold higher in patients homozygous for the minor T allele compared to patients homozygous for the major C allele ($P < 1.5 \times 10^{-10}$,^[138]). Moreover, using allele-specific quantitative RT PCR on RNA isolated from human aortic SMC heterozygous at rs604723, we found that the minor T allele was associated with a significant increase in mRNA expression (Mangum and Mack, personal

communication) and we identified a novel cis element by which this allele upregulates *GRAF3* transcription. To our knowledge, this is only the second functional SNP identified in a GWAS study that has been linked to a causal gene and pathway (the first being rs5068 located within *NPPA/B*^[117]).

Collectively, these studies will likely have important implications in the future diagnosis and treatment of hypertension. For example, patients predicted to exhibit aberrantly high levels of RhoA signaling may respond better to anti-hypertensive regimens directly targeting vessel tone, compared to those that target blood volume. Moreover, they reveal that the RhoA signaling axis may provide highly selective targets for the treatment of human hypertension and related cardiovascular sequela.

PHARMACOLOGICAL REGULATION OF RHOA AND RHO-DEPENDENT PATHWAYS

Despite the importance of RhoA signaling in the development of hypertension, few treatments are currently available that target this signaling axis. However, some commonly used anti-hypertensives may interfere with RhoA signaling (Figure 3). For example, since RhoA-dependent regulation of vascular tone is a major contributor to AII-mediated increases in BP^[13,139], the highly utilized class of anti-hypertensives that target AII (*i.e.*, ACE inhibitors and AII receptor blockers) may exert some of their BP lowering effects by reducing RhoA activation. Moreover, although used to treat high cholesterol, HMG-CoA reductase inhibitors such as simvastatin and atorvastatin also have anti-hypertensive properties^[140] and their BP lowering effects have been attributed to their ability to block RhoA signaling. RhoA is known to be modified by covalent attachment of a geranylgeranyl isoprenyl to a C-terminal Cys, and this modification (which is blocked by simvastatin treatment) is required for membrane localization and activation of RhoA^[141].

While not yet included in standard of care treatment for hypertension, several pharmacologic agents have been developed for inhibiting Rho kinases. In general, kinases make good drug targets due to the relative ease of targeting specific molecules to the ATP-binding pockets of these enzymes. To date, most of the Rho kinase inhibitors utilized in animal studies and clinical trials target the ATP-binding pockets of both ROCK isoforms^[142-144]. Although not clinically used in the United States, studies abroad provide compelling evidence for the use of this therapeutic approach for BP control. One particularly effective ROCK inhibitor, fasudil, is currently used in Japan to treat cerebral vasospasm and clinical trials determined that fasudil was also effective in decreasing peripheral vascular resistance in hypertensive patients^[5]. However, despite their wide use in cells and animal disease models, neither

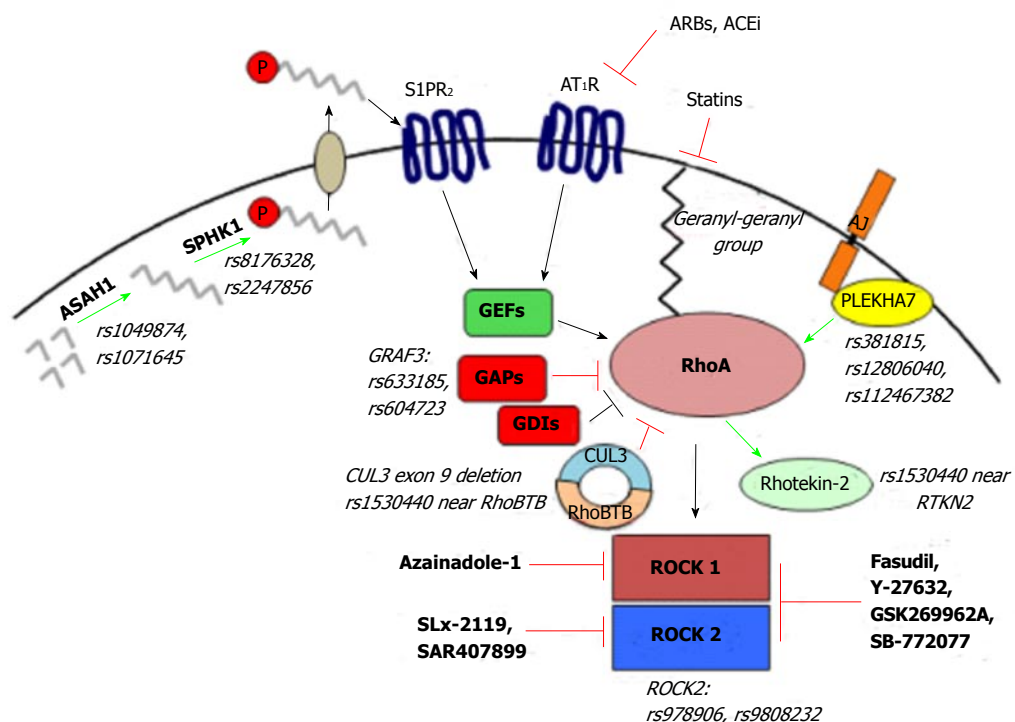


Figure 3 Pharmacologic and genetic regulation of the RhoA signaling axis. Schematic indicating the sites of action of pharmacological inhibitors (bold) of RhoA signaling molecules. Polymorphisms (SNPs/eQTLs) that could influence RhoA signaling are also shown. AJ: Adherens junction; A2R: Angiotensin type II receptor; ARBs: Angiotensin receptor blockers; ACEIs: Angiotensin converting enzyme inhibitors; ASAHI: Acid ceramidase; SPHK1: Sphingosine kinase 1.

fasudil nor Y-27632 exhibit suitable specificity for a therapeutic as they can inhibit the activity of several other kinases including PKC, PKA, and MLCK, at higher concentrations^[145,146]. These compounds also suffer from having short half-lives, which is a highly undesirable attribute of a drug designed to treat a longstanding disease^[147]. Thus, there is great need for development of additional potent, yet specific, ROCK inhibitors that can be safely used in patients^[148]. While a few such compounds have been developed recently with such attributes^[149-153], whether any these compounds exhibit the necessary selectivity and pharmacogenetic profiles required for BP management in patients requires further study. Moving forward, given the importance of RhoGEFs and RhoGAPs in the control of SM contractility and BP, we believe that it will be possible to engineer clinically-relevant small molecule regulators of these enzymes that could be used to develop new and effective anti-hypertensive therapies.

CONCLUSION: FUTURE POSSIBILITIES FOR PERSONALIZED TREATMENT OF HYPERTENSION

Current anti-hypertensive therapy is often empirically based and involves multiple drug regimens^[154,155]—an approach that is moderately effective at best as it frequently contributes to unwanted side effects and intolerance or non-adherence to medication. Accordingly, more effective and specific anti-hyper-

tensive agents are necessary. Moreover, based on the fact that BP is a highly variable trait among individuals, a better understanding of the genetic mechanisms regulating this disease is critical for a more personalized treatment plan for patients. Given the numerous regulatory and counter-regulatory mechanisms modulating the RhoA axis, this central axis provides an excellent opportunity for identifying genetic biomarkers that correlate with different levels of hypertensive risk and drug responses. Indeed, genetic variations in both upstream activators and downstream mediators of RhoA have been linked to BP regulation (Figure 3). Screening for such variants could potentially be used to tailor more effective individualized treatments. For example, one study showed that the BP lowering effects the ACE inhibitors or the angiotensin receptor blockers were more pronounced in patients carrying a GG genotype at the -391 RGS2 (Regulators of G-protein signaling 2) locus when compared to responses in GC or CC genotype carriers—while no differences were observed in the responses to calcium channel antagonists^[156]. Although RGS2 is known to couple to ATR1, the underlying mechanism by which these polymorphisms lead to altered sensitivity is currently unknown. Genetic differences in pharmacogenetics also play a role in response to anti-hypertensive agents, for example polymorphisms in the gene were associated with reduced BP-lowering effects of the β AR-blocker atenolol^[157]. Whether any of the aforementioned Rho-signaling SNPs influence specific responses to or bio-availability of anti-hypertensive treatments remains

a critical unexplored question. The clinical utility of targeting the RhoA pathway should also be further explored.

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