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Druggable targets in the Rho pathway and their promise for therapeutic control of blood pressure

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ABSTRACT

The prevalence of high blood pressure (also known as hypertension) has steadily increased over the last few decades. Known as a silent killer, hypertension increases the risk for cardiovascular disease and can lead to stroke, heart attack, kidney failure and associated sequela. While numerous hypertensive therapies are currently available, it is estimated that only half of medicated patients exhibit blood pressure control. This signifies the need for a better understanding of the underlying cause of disease and for more effective therapies. While blood pressure homeostasis is very complex and involves the integrated control of multiple body systems, smooth muscle contractility and arterial resistance are important contributors. Strong evidence from pre-clinical animal models and genome-wide association studies indicate that smooth muscle contraction and BP homeostasis are governed by the small GTPase RhoA and its downstream target, Rho kinase. In this review, we summarize the signaling pathways and regulators that impart tight spatial-temporal control of RhoA activity in smooth muscle cells and discuss current therapeutic strategies to target these RhoA pathway components. 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.

Contents

Abbreviations: ACE, angiotensin converting enzyme; A-II, angiotensin II; BP, blood pressure; DOCA, deoxycorticosterone-acetate; ET1, endothelin-1; GAP, GTPase activating protein; GDP, guanosine diphosphate; GEF, guanine nucleotide exchange factor; GTP, guanosine triphosphate; GWAS, genome-wide association studies; HTN, hypertension; L-NAME, L-N^G-Nitroarginine methyl ester; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; MRTF-A, myocardin related transcription factor-A; MRTF-B, myocardin related transcription factor-B; PAH, pulmonary arterial hypertension; PE, phenylephrine; ROCK, Rho Kinase or Rho-associated coiled-coil domain containing protein kinases; SHR, spontaneously hypertensive rat; SM, smooth muscle; SMC, smooth muscle cell; SRF, serum response factor.

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1. Introduction

Hypertension (HTN) is a major cardiovascular risk factor that significantly increases the incidence of stroke, myocardial infarction, heart failure, retinopathy, and kidney disease (Lim et al., 2012). Although HTN is one of the most modifiable cardiovascular risk factors, the number of individuals with HTN is increasing world-wide. Further amplifying the importance of HTN, the American Heart Association has recently revised its definition of Stage 1 HTN to include individuals with systolic blood pressure (BP) between 130 and 139 mmHg or diastolic BP between 80 and 89 mmHg. This change was prompted by

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studies demonstrating beneficial effects of lowering BP below the 120/80 mmHg threshold (Guo et al., 2013; Lewington, et al., 2003; Whelton et al., 2017) and effectively increased the number of Americans categorized as hypertensive from 32% to 46% (Whelton et al., 2017). It is also becoming clear that many people suffer from masked HTN (normal readings in the clinic, but hypertensive outside the clinic) and non-dipping HTN (steady BP through the day but no decrease in BP at night) (Booth et al., 2016; Peacock, Diaz, Viera, Schwartz, & Shimbo, 2014; Viera & Shimbo, 2014), suggesting that more intensive BP monitoring would identify additional at-risk individuals (Hinderliter, Voora, & Viera, 2018).

A number of relatively inexpensive first-line therapies are available to treat HTN including diuretics, angiotensin-converting enzyme (ACE) inhibitors, angiotensin II (A-II) receptor blockers, and calcium channel blockers. However, these drugs are usually prescribed empirically and are often ineffective. Indeed, over 50% of adults who are being treated for HTN still do not have their BP under control (S. Yoon, Fryar, & Carroll, 2015). Although treatment can be improved by multidrug regimens that target different BP control mechanisms, 13% of treated patients have drug-resistant HTN and remain hypertensive even after taking 3 or more medications, or require 4 medications for adequate BP control (Achelrod, Wenzel, & Frey, 2015; Persell, 2011). Taking multiple BP medications also increases the risk of unwanted side effects and drug-drug interactions. While incomplete health history and poor patient compliance contribute to the difficulties in treating HTN, our lack of understanding of the etiology of HTN is also a major factor.

Many of the difficulties of treating HTN stem from the fact that BP is an extremely complex trait regulated by many organ systems. Although the major determinants of BP are cardiac output and systemic vascular resistance, BP homeostasis requires proper regulation of heart and vasculature function by the autonomic nervous system, kidneys, and endocrine organs. The fact that these systems are tightly integrated by many feedback loops further complicates our understanding of the development of HTN and its treatment. Nearly all heritable genetic mutations that cause HTN affect kidney function and/or salt balance, but these variants only explain about 10% of HTN cases. More recent genome wide association studies (GWAS) have identified many genetic loci that correlate with relatively small differences in BP between populations. However, because most of these variations are within or near genes with no known connection to BP regulation, our understanding of how they affect the development of HTN is limited. A number of the genes identified by GWAS are highly expressed in endothelial and smooth muscle cells (SMCs), highlighting the importance of the vasculature as a major regulator of BP and as a target for potential therapies (Ehret et al., 2011; Ehret et al., 2016; Padmanabhan, Caulfield, & Dominiczak, 2015; Wain et al., 2011).

Recent advancements suggest that RhoA signaling in the vasculature is a particularly attractive target for therapeutic intervention in the treatment of HTN. Extensive studies have shown that RhoA signaling enhances Ca^{2+}-dependent, myosin-based force production in vascular SMCs and recent studies from our lab and others have implicated several components of the RhoA signaling pathway in the development of HTN in mouse models (Guilluy et al., 2010; Loirand & Pacaud, 2010; A. Wirth et al., 2008). Moreover, human genetic studies have identified BP-associated variants in several additional Rho-related genes further implicating this pathway in human HTN (Loirand, 2015). The goals of the current review are to summarize the data supporting the role of RhoA signaling in the development of HTN and to provide an overview of current and potential therapeutic targets within this pathway that could lead to better and perhaps novel HTN therapies.

2. Impact of the RhoA pathway on BP homeostasis

The 22 members of the Rho family of small GTPases can be divided into three major subfamilies, Rac, RhoA, and Cdc42. The RhoA family GTPases (RhoA, RhoB, and RhoC) are widely expressed and share 85% amino acid homology including a C-terminal cysteine residue that is the target of geranylgeranylation, a posttranslational modification that anchors RhoA family proteins to the plasma membrane. As discussed below, RhoA is by far the most studied member of this subfamily and has been shown to regulate a variety of cellular processes including (but not limited to) the modulation of actin and microtubule dynamics, cell force, cell shape and polarity, endocytosis, exocytosis, cell adhesion and migration, proliferation, and differentiation (Narumiya & Thumkeo, 2018). Because of its well-recognized role in mediating BP homeostasis, the remainder of this review will be focused on RhoA.

Like all GTPases, RhoA is regulated by guanosine triphosphate (GTP) binding and cycles between the active GTP-bound form and the inactive guanosine diphosphate (GDP)-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 limit) RhoA-dependent signaling. When GTP-bound, RhoA interacts with a variety of effector molecules that mediate its varied functions 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, rhophilin, and the rhotekins I and II among other enzymes (Narumiya & Thumkeo, 2018).

2.1. Rho A-dependent SMC contractility and peripheral vascular resistance.

Vascular resistance is a major determinant of BP and is controlled, in large part, by SMC contraction within small peripheral arterioles (Cowley, 2006; Davis et al., 2001; Davis & Hill, 1999; Hall, 2003; Lifton, Gharavi, & Geller, 2001). Importantly, studies in genetically engineered mice revealed that germline deletion of the Rho-specific GEF, LARG, significantly attenuated salt-induced HTN, while SMC-specific knockout of the related GEF, p115RhoGEF, inhibited the development of HTN in response to A-II. In addition, we recently showed that depletion of the SMC-selective, Rho-specific GAP, GRAF3 (ArhGAP42) in mice leads to basal HTN, increased pressor responses to A-II, endothelin-1 (ET1), and phenylephrine (PE), and elevated deoxycorticosterone-acetate (DOCA)-salt induced HTN. Collectively, these studies strongly support a critical role for RhoA in governing BP by modulating SMC tone.

Mechanistically, excitation-contraction coupling in SMC is mediated by Ca^{2+}-dependent activation of myosin light chain kinase (MLCK), and SMC tension is directly proportional to myosin light chain (MLC) phosphorylation at S19 (Fig. 1). By mechanisms that are still somewhat unclear, MLC phosphorylation enables myosin-actin cross-bridge cycling to enhance force generation (Budzyn, Marley, & Sobey, 2006; Etienne-Manneville & Hall, 2002). Besides promoting an inositol triphosphate-mediated increase in intracellular calcium, many circulating vasoconstrictors that activate G-protein coupled receptors including A-II, ET1, PE, and sphingosine-1-phosphate (S1P) also stimulate RhoA activity which further enhances Ca^{2+}-dependent SMC contractility (Guilluy et al., 2010; Loirand & Pacaud, 2010; A. Wirth et al., 2008) (Fig. 1). RhoA activation also regulates constriction of the pre-glomerular afferent arterioles that control kidney perfusion. In this tubuloglomerular feedback system, increased kidney perfusion (Carlstrom, Wilcox, & Arendshorst, 2015) results in increased NaCl delivery from the loop of Henle to the macula densa, a cluster of epithelial cells that are adjacent to the abluminal SMCs of the afferent arterioles. Increased NaCl uptake by macula densa cells results in the secretion of ATP and adenosine that stimulate RhoA activity in afferent arteriole SMC via the P2Y4/P2Y6 and A2 G-protein coupled receptors, respectively (Homma et al., 2014; Inscho, 2009; Nakamura et al., 2003; Roos, van Rodijnen, van Lambalgen, ter Wee, & Tangelder, 2006; Y. Shi, Wang, Chon, & Cupples, 2006; Yano et al., 1995). Since the kidneys typically receive

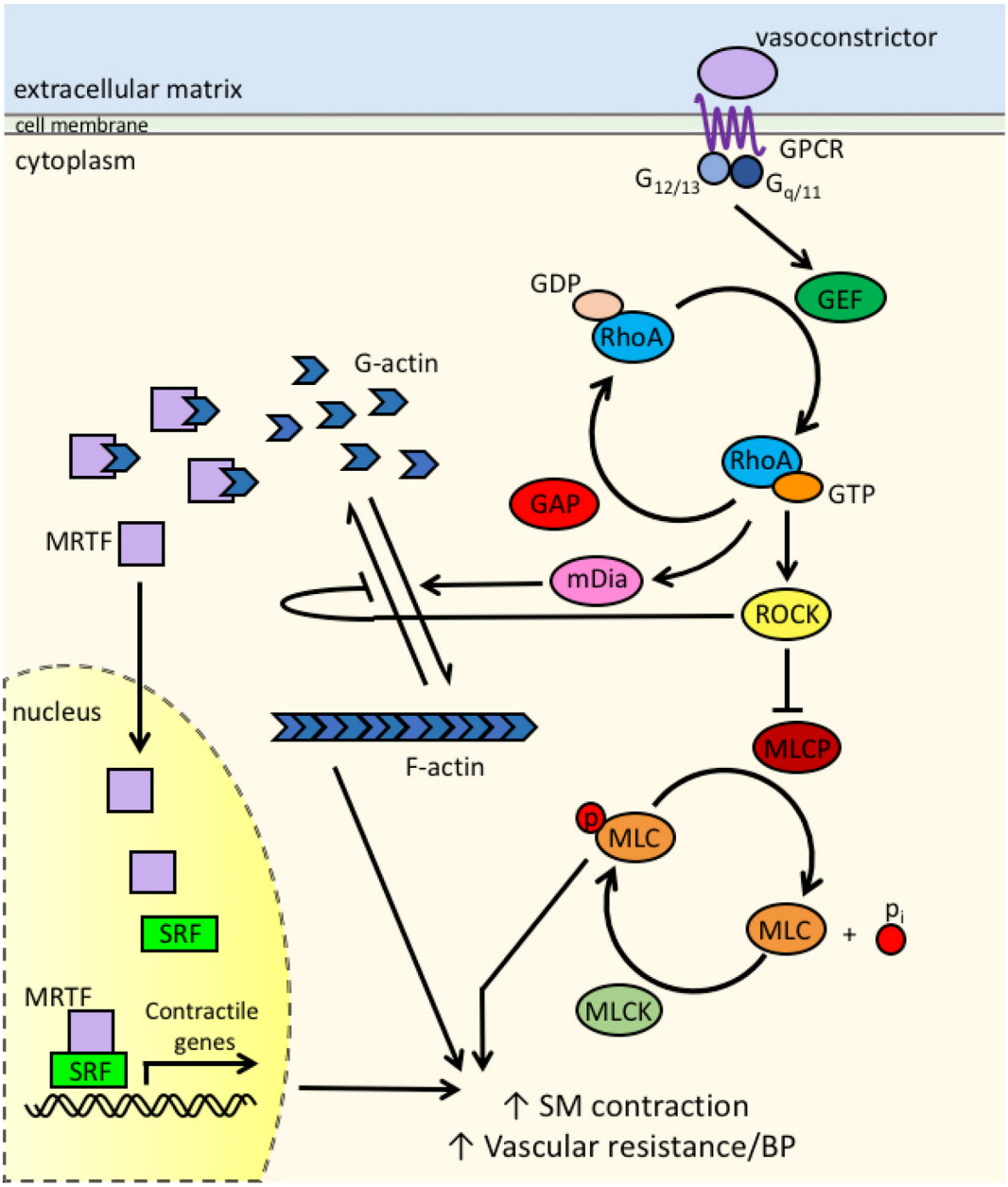

Fig. 1. RhoA pathway in smooth muscle cells (SMC). In addition to Ca^{2+} -dependent activation of MLCK (not shown here), vasoconstrictors such as AII, PE, and ET-1 stimulate G-protein signaling in SMC via GPCRs. G-proteins influence the activity of RhoA by activating GEFs, which facilitate the dissociation of GDP from RhoA, allowing GTP to bind. This activation is balanced by GAPs, which increase the efficiency of GTP hydrolysis. When RhoA is in its active, GTP-bound form, it's able to interact with any of it's downstream effectors, such as ROCK and mDia. ROCK inhibits MLCP, allowing for prolonged phosphorylation of MLC and enhanced SMC contraction, increased vascular resistance, and increased blood pressure. Simultaneously, mDia and ROCK promote the polymerization of F-actin from G-actin, releasing G-actin bound MRTF. Free MRTF translocates to the nucleus where it induces SRF-dependent transcription of contractile genes. The concurrency of these latter events further enhance SMC contraction and vascular resistance.

about 25% of total cardiac output, increased contractility of renal arterioles has a significant impact on systemic BP. Taken together these studies indicate that aberrant activation or loss of tight temporal control of the RhoA pathway in resistance vessels can lead high basal BP and exaggerated responses to vasoconstrictors.

RhoA regulates several effector molecules that impact SMC contractility (Fig. 1). Direct phosphorylation of MLC by ROCKs I and II promotes actin-myosin crossbridge cycling as does ROCK dependent inhibition of MLC phosphatase (MLCP). RhoA activity is also critical for *de novo* formation of actin filaments and formation of focal adhesions that are required for myosin-dependent force development and transmission, respectively. The Rho effectors mDia 1 and 2 are the most potent regulators of actin filament formation as these proteins function to directly catalyze actin polymerization in cooperation with the actin binding protein, profilin. ROCKs also inhibit actin de-polymerization by phosphorylating and activating LIM-kinase 1 and 2 (on Thr 508 or 505 respectively), which in turn, phosphorylate and inhibit the actin filament severing protein, cofilin (Ohashi et al., 2000; Sumi, Matsumoto, & Nakamura, 2001; Vardouli, Moustakas, & Stournaras, 2005; Yang et al., 1998). Finally, ROCK-dependent phosphorylation of ezrin-radixin-moesin (ERM) proteins promotes their tethering to integral plasma membrane proteins effectively stabilizing actin filaments and increasing force transmission (Matsui et al., 1998).

In addition to promoting acute changes in SMC contractility, recent studies indicate that RhoA signaling also controls the transcription of numerous contractile genes by modulating serum response factor (SRF) activity. SRF binds to CArG ($CC(A/T)_6GG$) cis elements located within the promoters of nearly all SMC contractile genes (including

SM myosin heavy chain, SM22, calponin, and SM α-actin). SRF activity is modulated by transcription cofactors of the myocardin family (Chang et al., 2003; Chen & Schwartz, 1996; Dalton & Treisman, 1992; Hill & Treisman, 1995; Mack, Thompson, Lawrenz-Smith, & Owens, 2000) and two such co-factors, myocardin transcription factor A and B (MRTF-A and MRTF-B) mediate strong trans-activation of SMC contractile genes (Hinson, Medlin, Lockman, Taylor, & Mack, 2007; Wang & Olson, 2004). We have previously demonstrated that RhoA promotes SMC contractile gene expression through actin polymerization-dependent regulation of MRTF-A and MRTF-B nuclear localization (Hinson et al., 2007; Lockman et al., 2004; Miralles, Posern, Zaromytidou, & Treisman, 2003; Sotiropoulos, Gineitis, Copeland, & Treisman, 1999; Staus, Blaker, Taylor, & Mack, 2007). Cytoplasmic monomeric G-actin is abundant when RhoA activity is low (for example in SMC under low tension (Albinsson, Nordstrom, & Hellstrand, 2004)), and under these conditions, G-actin binds to MRTF and masks an N-terminal nuclear localization sequence, resulting in cytoplasmic sequestration of these SRF co-factors. Upon RhoA activation, G-actin is recruited into growing F-actin filaments and MRTF-G-actin association decreases. As a consequence, MRTF nuclear localization sequence is un-masked, and MRTF accumulates in the nucleus and promotes SRF-dependent gene expression (Mack, 2011). Thus, signaling through RhoA in small arteriolar SMC enhances Ca^{2+} sensitivity, promotes actin remodeling and induces expression of contractile proteins each of which increase SMC tone and peripheral vascular resistance.

2.2. Non-vascular RhoA responses associated with BP homeostasis

Although Rho signaling components are relatively strongly expressed in vascular SMCs, nearly all, with the exception of the RhoGAP GRAF3 (see Section 5 below), are expressed in many other tissues. Thus, when evaluating Rho signaling molecules as targets of anti-HTN therapy, it is important to consider the potential impact of modulating Rho-signaling in other organ systems. Interestingly, with respect to BP regulation, studies using pre-clinical models indicate that attenuating RhoA signaling in the vasculature, kidney, myocardium, and CNS could all lead to the desired outcome of lowering BP. For example, blocking RhoA activity in endothelial cells can indirectly inhibit SMC contractility by increasing the secretion of the potent vasodilator, nitric oxide (Laufs & Liao, 1998; Ming et al., 2004; Wolfrum et al., 2004; Zhou & Liao, 2009). Some evidence suggests that blocking RhoA activity in tubular epithelial cells can alter sodium channel activity, limit Na^{+} reabsorption, and aid in maintaining blood volume homeostasis (Hayashi et al., 2004; Karpushev, Ilatovskaya, Pavlov, Negulyaev, & Staruschenko, 2010; Loirand & Pacaud, 2014; Nishiki et al., 2003; Pochynyuk et al., 2006; Staruschenko et al., 2004; Szaszi et al., 2000). Moreover, investigators have shown that inhibiting RhoA activity in the nucleus tractucs solitarius within the central nervous system reduced sympathetic nerve activity, heart rate, and BP in normotensive rats and these effects are even more pronounced in spontaneously hypertensive rats (Ito et al., 2003; Ito, Hirooka, Kimura, Shimokawa, & Takeshita, 2005). Likewise, while infusion of A-II into the neural cistern of rats promoted a significant rise in BP, co-infusion of A-II and the ROCK inhibitor, Y27632 did not (Sagara et al., 2007). On the other hand, some studies in cells and invertebrate model systems indicate that inhibition of Rho/Rho kinase signaling in motor neurons antagonized the secretion of parasympathetic relaxation factors (including acetylcholine) and promoted the secretion of sympathetic contractile agonists (including dopamine) (Hiley, McMullan, & Nurrish, 2006; Yamaguchi et al., 2000). Since perivascular nerves play a major role in the control of resistance arteriole tone, such outcomes may limit the therapeutic efficacy of RhoA/ROCK inhibitors as future anti-hypertensive therapies. Moreover, inhibition of RhoA in the heart leads to conduction defects (Wei et al., 2004; Yatani, Irie, Otani, Abdellatif, & Wei, 2005). Thus, as described in further detail below, targeting vascular SMC specific regulators of the RhoA pathway may provide a better avenue for pharmacological BP control.

3. Targeting the RhoA pathway for therapeutic BP control

In agreement with the pre-clinical animal studies highlighted above, several lines of evidence strongly implicate RhoA signaling in the development of human HTN. First, increased Rho-kinase activity has been observed in some hypertensive patient populations and as reviewed in Section 3.1 Rho kinase inhibitors have been successful in reducing systemic HTN in these cases, although current formulations exhibit relatively short-term effects (Feng, LoGrasso, Defert, & Li, 2016; Loirand, 2015; Zhou, Gensch, & Liao, 2011). Second, an autosomal dominant mutation in the E3 ligase, Cullin-3 (which targets RhoA), has been shown to cause high BP in patients with Gordon's Syndrome (pseudohypoaldosteronism type IIE). Importantly, the identical mutation in pre-clinical mouse models and led to decreased Cullin-3 activity, reduced ubiquitin-mediated RhoA degradation in vascular SMCs, and increased BP (Boyden et al., 2012; Ibeawuchi, Agbor, Quelle, & Sigmund, 2015). Third, many GWAS conducted over the past decade have identified common BP-associated genetic variations in coding and non-coding regions within or near genes linked to the RhoA signaling cascade. For example, in one study that used HTN as a dichotomous trait, two of the eight BP-associated loci were located in RhoA-related genes. One was within RhoBTB1, which functions with the aforementioned Cullin-3 complex to maintain low RhoA levels (Boyden et al., 2012; Pelham et al., 2012), while the other was within rhotekin-2 (RTKN2), a RhoA effector with a yet unknown function. Two separate GWAS identified a BP-associated locus within PLEKHA7 (Plekstrin Homology domain containing family A member 7) (Levy et al., 2009; Lin et al., 2011) which interacts with the junctional proteins cingulin and paracingulin to regulate several Rho family GTPases, including RhoA in the heart and kidneys (Citi, Pulimeno, & Paschoud, 2012). Importantly, PLEKHA7 was subsequently shown to be required for the development of salt-induced HTN in mice (Endres et al., 2014). Moreover, a few variants in ROCK II have been associated with the regulation of BP. Of particular interest was the identification of a common nonsynonymous ROCK II variant 431N (versus 431T) that was associated with an increase in BP in twins (Liao et al., 2015; Liu et al., 2013; Loirand & Pacaud, 2014; Rankinen et al., 2008; Seasholtz et al., 2006). This result was supported by Liao et al., who showed that the 431N variant had increased kinase activity and was associated with enhanced arterial stiffening, a vascular property strongly associated with HTN (Liao et al., 2015; Liu et al., 2013; Loirand & Pacaud, 2014; Rankinen et al., 2008; Seasholtz et al., 2006). This group identified a second variation in the ROCK II 3'UTR (rs9789060) that was also associated with increased stiffening and went on to show it affected ROCK II expression by interfering with miR-1183-dependent degradation of ROCK II mRNA levels. It is important to note that a third study failed to find an association between rs9789060 and BP (Liu et al., 2013). In another study on 586 normotensive and 607 hypertensive Caucasians, Rankinen et al. identified a minor allele locus within the ROCK II gene that lowered the risk of HTN by 85% (Rankinen et al., 2008). Finally, as discussed in further detail below, three separate GWAS for BP and cardiovascular disease endpoints identified a novel BP associated locus within the Rho-specific GAP, GRAF3/ArhGAP42 (Ehret et al., 2011; Kato et al., 2015; Li et al., 2016; Wain et al., 2011). Recent causality studies from our group demonstrated that GRAF3 is selectively expressed in SMC and is required for BP homeostasis in mice (Bai et al., 2013; Bai et al., 2017; Bai et al., 2017).

Collectively, these studies reveal that the RhoA signaling axis may provide tractable targets for the treatment of human HTN and related cardiovascular sequela. Indeed, some commonly used anti-hypertensives (i.e. ACE inhibitors, A-II blockers, and statins) likely function by interfering with RhoA signaling, supporting the clinical utility of inhibiting this pathway. (Brandes, 2005; Carbone et al., 2015; Guilluy et al., 2010; Kanaki et al., 2013). Nonetheless, despite the importance of the RhoA pathway in the pathogenesis of HTN and several other debilitating

diseases including amyotrophic lateral sclerosis, mental retardation, hepatocellular, lung, and colorectal carcinomas (Boettner & Van Aelst, 2002; Jansen, Gosens, Wieland, & Schmidt, 2018; Sahai & Marshall, 2002), surprisingly few treatments are available to directly target this pathway. In fact, of the nearly 300,000 ongoing clinical trials, only a handful involve compounds that target RhoA signaling components (clinicaltrials.gov). This could be due to the fact that many members of the RhoA pathway (with exception of Rho kinase) have traditionally been regarded as "undruggable". However, as described below, significant advancements in high resolution crystal structures, structure-function analyses and drug development technology are beginning to overcome these challenges and provide hope for the development of new therapies to target this critical pathway.

3.1. Targeting Rho kinase

The serine/threonine kinases, ROCK I and ROCK II, are the best studied RhoA effectors and have been implicated in a variety of diseases including HTN (Loirand, 2015). Since the development of kinase inhibitors has proven to be a very successful therapeutic approach, it is not surprising that ROCK has been an attractive target in the search for RhoA signaling inhibitors and that ROCK inhibitors are the furthest along in regard to clinical testing. Because ROCK I and II share 60% identity overall, 90% identity within the kinase domain, and 100% identity within the ATP binding pocket (Nakagawa et al., 1996) they share many substrates and promote many of the same downstream cell functions (Feng et al., 2016). Although ROCK I and ROCK II expression can vary somewhat between tissues, both of these kinases are widely expressed (Nakagawa et al., 1996; Wang et al., 2009). Differences in subcellular localization have been noted with ROCK I localizing more readily to microtubule-organizing centers (Chevrier et al., 2002) and catenin/E-cadherin containing complexes at the plasma membrane (Smith, Dohn, Brown, & Reynolds, 2012) and ROCK II to vimentin (Sin, Chen, Leung, & Lim, 1998) and actin fibers (Chen et al., 2002) (Schofield & Bernard, 2013; Wang et al., 2009). Isoform-specific inhibitors are being developed that could have differential effects depending upon the disease treated or end-point measured (Boerma et al., 2008; Hyun Lee et al., 2014; Loirand, 2015; Zanin-Zhorov et al., 2014; Zanin-Zhorov et al., 2017)(clinicaltrials.gov).

While over 30 common downstream targets of ROCK have been identified (Adducin, Diaphanous (mDia), LIM kinase (LIMK), NHE1, MARCKS, NF-L, CRMP2,FAK, c-Jun N-terminal kinase (JNK), MLC, MLCK, MLCP, ezrin/radixin/moesin (ERM), rhophilin, rhotekin, citron kinase, and Tau) (Feng et al., 2016; Loirand, 2015; Schofield & Bernard, 2013; Siehler, 2009), the most pertinent in regard to SM contraction and BP regulation is the myosin binding subunit (MYPT-1) of the MLCP complex. ROCK-dependent phosphorylation of MYPT-1 at T696, T853, and S854 inhibits MLCP activity resulting in increased MLC phosphorylation and hence increased contraction (Wang et al., 2009; Angela Wirth, 2010). ROCK has also been shown to directly phosphorylate MLC at S19.

As reviewed more thoroughly elsewhere (Feng et al., 2016; Loirand, 2015) over 170 ROCK inhibitors are in various stages of development (Table 1). Given the involvement of actin-based processes in many pathologies (adhesion, migration, cell division, force generation, etc.), ROCK inhibitors are being studied as treatments for a wide variety of disease states including central nervous system disorders (subarachnoid hemorrhage and vasospasm, cerebral stroke, spinal cord injury), neurodegenerative diseases (Alzheimer's disease, Parkinson's, Huntington disease, and amyotrophic lateral sclerosis [ALS]), cardiovascular disease (systemic HTN, pulmonary arterial HTN (PAH), spastic and stable angina, atherosclerosis, Raynaud syndrome), asthma, glaucoma, autoimmune diseases, cancer, erectile dysfunction, and kidney disease. Although the results of these studies and clinical trials provide important information on ROCK and its role in disease progression, we will focus our discussion on the major ROCK inhibitors that have been most commonly used to treat vascular diseases.

Fasudil (also known as HA-1077) was the first ROCK inhibitor described and was also the first to be tested clinically. This isoquinoline-based drug is classified as a class I ROCK inhibitor because it reversibly competes with ATP binding to the ROCK kinase domain. Fasudil inhibits both isoforms of ROCK with an IC_{50} of 1μM while hydroxyfasudil, the major active metabolite, is slightly more potent with an IC_{50} of ~0.7 μM (Nagumo et al., 2000). Like most other ROCK inhibitors of this class fasudil also inhibits other members of the broad AGC kinase family including PKA and PKC, albeit with less potency (IC_{50} of 5 μM and 37 μM, respectively) (Bain et al., 2007; Nagumo et al., 2000). Fasudil was shown to reduce BP by attenuating the Rho-mediated inhibition of MLCP in SMC (Nagumo et al., 2000), by increasing endothelial nitric oxide synthase expression (Takemoto, Sun, Hiroki, Shimokawa, & Liao, 2002), and by reducing circulating ACE and A-II levels (Ocaranza et al., 2011). Fasudil also inhibited pulmonary artery SMC proliferation, a process important for vessel stiffening, by a mechanism that likely involved downstream inhibition of c-Jun N-terminal kinase (JNK) and ERK-dependent activation of c-jun and c-fos expression (Chen, Dun, Miao, & Zhang, 2009).

Fasudil hydrochloride hydrate under the trade name Eril® was approved in Japan in 1995 to treat vasospasm-induced cerebral ischemia that can occur following surgery for subarachnoid hemorrhage. In 2002, Masumoto et al. showed that intracoronary fasudil was effective as an acute treatment of vasospastic angina (Masumoto et al., 2002), and additional clinical trials showed that long-term treatment with oral fasudil reduced stable effort-induced angina and improved exercise tolerance with no major adverse effects (Shimokawa et al., 2002; Vicari et al., 2005). In a rat model, moderate doses of fasudil decreased PAH while higher doses also decreased mean systemic arterial pressure (Jiang et al., 2007). By 2011, intravenous and inhaled fasudil were approved to treat PAH (Fukumoto et al., 2005; Nagaoka et al., 2005), and a new extended release formulation of fasudil, AT-877ER, was shown to reduce PAH in patients after three months of use (Fukumoto et al., 2013). Fasudil decreased forearm vascular resistance more dramatically in hypertensive patients than in normotensive controls (Masumoto et al., 2001). Fasudil has been shown to have beneficial effects on kidney function in diabetic rats (Komers, Oyama, Beard, & Anderson, 2011), suggesting that it might be useful for treating diabetic patients who are frequently hypertensive and have kidney disease. Ongoing phase III clinical trials are also assessing whether fasudil is an effective treatment for Reynaud's syndrome and carotid stenosis (clinicaltrials.gov). Fasudil has been approved in China, but not in the United States or Europe.

Several fasudil derivatives that are more potent and specific inhibitors of ROCK have been developed and are being used to treat glaucoma and ocular HTN. Ripasudil (Glanatec®), approved in Japan in 2014, affects the trabecular meshwork in the eye and reduces intraocular pressure by facilitating the outflow of aqueous humor through Schlemm's canal. The most common side effect of ripasudil treatment is conjunctival hyperemia, which subsides over time or with discontinued use (Garnock-Jones, 2014). Netarsudil (Rhopressa ®) has similar effects and indications, and in 2017, became the first ROCK inhibitor approved by the United States (Lu, Tsai, & Liu, 2017; Ren et al., 2016). Netarsudil also decreased the amount of aqueous humour produced, a feature attributed to additional effects of netarsudil as a norepinephrine transport inhibitor (Lu et al., 2017; Ren et al., 2016).

The classic ROCK inhibitor, Y-27632, is a pyridine-based class I inhibitor that has been tested in a number of animal and human disease models including HTN. In a landmark study, Uehata et al. demonstrated that Y-27632 decreased BP in spontaneously hypertensive rats (SHR), DOCA-salt treated rats, and rats made hypertensive by clipping of the renal artery (Uehata et al., 1997). Although mesenteric and cerebral arteries in SHR or DOCA/salt treated rats were more responsive to Y-27632 than those in normotensive rats (Asano & Nomura, 2003;

Table 1
ROCK inhibitors currently in clinical trials.

Drug	Disease	Phase	Status	ClinicalTrials.gov ID
Fasudil	Cerebral vasospasm, brain ischemia, stable angina, pulmonary HTN	NA	**Approved in Japan and China**	NA
	Raynaud/Scleroderma	3	Completed	NCT00498615
	Carotid Stenosis	2	Terminated	NCT00670202
	Atherosclerosis, Hypercholesterolemia	2	Completed	NCT00120718
	Amyotrophic Lateral Sclerosis	2	Uknown	NCT01935518
	Cardiovascular Disease	2	Recruiting	NCT03404843
Ripasudil	Glaucoma and ocular HTN	NA	**Approved in Japan**	NA
	Fuchs' Endothelial Dystrophy	4	Recruiting	NCT03249337
AR-12286	Chronic Angle-closure Glaucoma	2	Unknown	NCT02152774
	Advanced Glaucoma	2	Unknown	NCT02173223
	Glaucoma	2	Unknown	NCT02174991
	Exfoliation Syndrome, Ocular HTN, Open Angle Glaucoma	2	Completed	NCT01936389
AR-13324	Open-angle glaucoma, Ocular HTN	NA	**FDA approved**	NA
SAR-407899	Erectile dysfunction	2	Completed	NCT00914277
	Microvascular Coronary Artery Disease	2	Recruiting	NCT03236311
	Chronic Kidney Disease	1	Completed	NCT01485900
KD-025	Psoriasis Vulgaris	3	Completed	NCT02317627
	Psoriasis	2	Recruiting	NCT02852967
	Graft vs. Host Disease	2	Recruiting	NCT02841995
	Idiopathic Pulmonary Fibrosis	2	Recruiting	NCT02688647
	Fibrotic Disease	1	Recruiting	NCT03530995

ROCK inhibitor compounds and presumptive applications are outlined here, along with the phase of the study, the status of the study, and the registered clinical trials identifier number. NA, not applicable.

Weber & Webb, 2001), Y-27632 likely affected HTN by multiple mechanisms. For example, Y-27632 also reversed the decrease in renal sodium excretion observed in the SHR model (Nishiki et al., 2003), most likely by affecting the activity and location of Na+/H+ exchanger, NHE3 (Hayashi et al., 2004; Szaszi et al., 2000). In addition, local infusion of Y-27632 into the nucleus tractus solitarius of the brainstem caused a reduction in BP, heart rate, and renal sympathetic nerve activity, and the magnitude of these effects was greater in the SHR model (Ito et al., 2003; Ito et al., 2005). Subsequent studies by the same group demonstrated that fasudil had similar effects (Ito et al., 2003; Ito et al., 2005). Although Y-27632's poor potency and kinase selectivity limit its use clinically, Y-27632 derivatives with better pharmacologic properties are being developed and tested.

SAR407899 is a promising relatively new isoquinoline-based class I ROCK inhibitor that is significantly more potent than older generation drugs (IC_{50} between 122 and 280nM) (Lohn et al., 2009). *In vitro* studies demonstrated that SAR407899 inhibited myosin phosphatase phosphorylation, stress fiber formation, cell proliferation, and monocyte chemotaxis (Lohn et al., 2009). SAR407899 dose-dependently lowered BP in SHR, stroke-prone SHR, L-N^G-Nitroarginine methyl ester (L-NAME), and DOCA-salt rat models, and its effects in some models was superior to ACE inhibitors and calcium channel blockers (Lohn et al., 2009). SAR407899 inhibited pressor responses to PE, A-II, and vasopressin in rats more strongly than Fasudil or Y-27632, and it inhibited ET1-induced vasoconstriction of renal arteries isolated from diabetic rats more strongly than Y-27632 (Grisk et al., 2012). In spite of its efficient antihypertensive effects and the fact that long term treatment of rats with SAR407899 was well-tolerated, development of SAR407899 as an anti-hypertensive has been discontinued (Loirand, 2015). SAR407899 is still being tested in clinical trials as a treatment for kidney disease and microvascular coronary artery disease and it may prove useful for treatment of erectile dysfunction in diabetic and hypertensive patients where the eNO system is impaired (Guagnini, Ferazzini, Grasso, Blanco, & Croci, 2012).

Although ROCK inhibition has been shown to reduce BP and vascular resistance in many models, there are concerns about the suitability of ROCK inhibitors as viable long-term treatments for systemic HTN. Although most ROCK inhibitors seem to be fairly well-tolerated, the ubiquitous nature of ROCK I and ROCK II expression coupled with the relative lack of specificity of most ROCK inhibitors (especially the class I drugs) makes potentially unknown side-effects a significant drawback. While local delivery strategies can sometimes mitigate this concern (i.e. to the eye or specific vascular beds) (Bodor & Buchwald, 2000; Feng et al., 2016), systemic hypotension can be a serious problem for patients being treated in this manner. Another potential problem is that the systemic BP lowering effects of ROCK inhibitors frequently decrease after 7-10 days of chronic treatment (Feng et al., 2016). Moreover, ROCK inhibitors did not affect systolic BP in some long-term studies of salt-sensitive hypertensive Dahl rats (Loirand & Pacaud, 2014; Nishiki et al., 2003). The precise causes of this tolerance or inactivity are unknown, but likely involve compensation by the many feedback pathways that regulate BP. Finally many ROCK inhibitors have short half-lives, which is not ideal for the treatment of a chronic diseases like HTN (Surma, Wei, & Shi, 2011).

3.2. Targeting RhoA directly

Numerous failed attempts to identify small molecule inhibitors of H-Ras have led to the concept that small GTPases per se do not make good drug targets due to their globular structure and lack of surface moieties required for high affinity binding of small molecules (Jansen et al., 2018; Kristelly, Gao, & Tesmer, 2004; Shang et al., 2012). However, such direct targeting may be the most effective means to reduce signal output given that many disease-associated mutations of small GTPases enable GEF-independent activation (Olson, 2018; Porter, Papaioannou, & Malliri, 2016). Moreover, in the case of HTN, direct targeting could provide an added benefit over ROCK inhibitors, as such an approach would block additional downstream pathways implicated in SMC contractility (i.e. mDia1 and mDia2, MRTF etc).

3.2.1. GTP-binding inhibitors

Although direct targeting of the GTP binding site of RhoA (or related small GTPases) is challenging due to its high affinity for GTP (pico to nano-molar range) and the high concentration of GTP in cells (~0.5 mM), some studies support the validity of this approach. Indeed, using an *in silico* virtual docking approach followed by surface-plasmon resonance validation of synthesized chemicals, Deng et al. identified lead compounds that inhibited Rho-GTP binding in a dose-dependent fashion with IC_{50} values ranging from 1.24–2.05 μM (Deng et al., 2011). After further structural modifications to increase water solubility, one compound ((*E*)-3-(3-(ethyl(quinolin-2-yl)amino)phenyl)acrylic acid) was shown to both attenuate PE-induced contraction in thoracic aorta rings *ex vivo* and to reduce cerebral vasospasm in a subarachnoid hemorrhage model in rats (Ma et al., 2015). While the *in vivo* efficacy was similar to that of fasudil, this second generation inhibitor still exhibited

relatively low potency (IC_{50} 71 μM in the contractile assay). Future studies will be necessary to determine whether these or related compounds exhibit specificity for RhoA versus other Rho family GTPases and other GTP-binding proteins.

In support of the possibility of identifying GTP binding inhibitors that can specifically target particular Rho-related family members, simultaneous multiplex screening for small molecules in the 200,000 Molecular Libraries Screening Center Network library has already identified a CDC42 specific inhibitor as well as a broad Ras family inhibitor. These inhibitors prevented GTP binding in a dose-dependent fashion (as non-competitive allosteric inhibitors) and are active in cell based assays (Hong et al., 2013; Surviladze et al., 2010; Surviladze, Young, & Sklar, 2012). The subsequent application of this technology to screen the Prestwick Chemical Library of off patent and FDA approved drugs for inhibitors of eight Ras-related GTPases (but not RhoA, B, or C) led to the identification of R-enantiomers of naproxen and ketorolac (approved NSAIDs) as GTP binding inhibitors of Rac1 and Cdc42 (Oprea et al., 2015). Although not biochemically confirmed, *in silico* docking analyses predicted that these drugs bind to an allosteric site near the GTP binding site that alters Mg^{2+} ion coordination and results in stabilizing the GTPase in its GDP-bound form. To our knowledge, this approach has not been used successfully to identify RhoA specific inhibitors, but these results provide strong proof-of-concept for this approach.

3.2.2. Selective non-covalent RhoA modifiers

The potential for identifying allosteric modifiers to inhibit RhoA has been demonstrated by the fact that a number of bacterial toxins are highly potent (though non-selective) inhibitors of Rho family members. For example, *Histophilus somni* and *Vibrio parahemolyticus* produce toxins that inhibit Rho proteins by promoting the covalent attachment of an AMP molecule to tyrosine 34, while toxin B produced by *Clostridium difficile* induces glucosylation of nearby threonine 37. These residues lie within the regulatory switch-I domain of Rho family members and addition of these bulky modifications in this domain inactivate the Rho GTPases by multiple mechanisms that include inhibition of GTPase cycling (by blocking GEF and GAP association), inhibition of cytosol-membrane cycling (by blocking Rho GDI interactions) and inhibition of Rho effector coupling (Lemichez, 2017; Pommier & Cherfils, 2005). Similarly, *Clostridium botulinum* exoenzyme C3 transferase toxin inhibits RhoA, RhoB, and RhoC *in vitro* and *in vivo* by promoting ADP-ribosylation of asparagine 41 which blocks GEF binding (Jansen et al., 2018; Vogelsgesang, Pautsch, & Aktories, 2007). Such pathogenic agents have been useful as pharmacological tools to completely block Rho-dependent signaling pathways in cells and in pre-clinical animal models. However, their potential as therapeutic agents is limited because of their difficult delivery, their non-specific actions, and their sometimes covalent and irreversible effects (Jansen et al., 2018; Marchioni & Zheng, 2009). Nonetheless, the future exploitation of derivatives or mimetics of such enzymes could lead to the development of new inhibitors with tremendous clinical utility.

Finally, Rho family inactivation can be achieved by blocking plasma membrane targeting. For example, Rho GTPases are isoprenylated on carboxy-terminal Cysteine residues (within a so-called CAAX box) and this modification is important for membrane targeting and activation as evidenced by the fact that proteolytic cleavage of this site by *Yersinia* spp.-derived toxins effectively block Rho, Rac and CdC42 activation (Lemichez, 2017). Likewise, as RhoA is geranylgeranylated at this site, geranylgeranyl-transferase inhibitors and HMG-CoA-reductase inhibitors (which block both cholesterol and isoprenyl biogenesis) block RhoA membrane association and activation. In fact the clinical utility of this approach is highlighted by the fact that HMG-CoA reductase inhibitors (such as simvastatin and atorvastatin) used to treat high cholesterol, also have anti-hypertensive properties (Kanaki et al., 2013) and their BP lowering effects have been attributed to their ability to block RhoA signaling (Brandes, 2005; Kanaki et al., 2013; Strazzullo et al., 2007). However, the BP lowering effects of statins are modest (2 mmHg decrease systolic BP) and these isoprenoid pathway inhibitors display poor selectivity for individual Rho GTPases. Thus, further advancements in drug development are needed to realize the full potential of this approach.

4. RhoA GEFs and BP control

Another potential strategy for inhibiting RhoA signaling is inhibition of the GEFs that control RhoA activity. Over 24 Rho-specific GEFs have been identified, and to date, at least 5 of these have been implicated in regulating SMC differentiation and/or contractility. This list includes three members of the RGS-GEF subfamily (P115-RhoGEF, PDZ-RhoGEF, LARG), p63RhoGEF and lymphoid blast crisis (Lbc) (Cario-Toumaniantz et al., 2012; Hilgers, Todd Jr., & Webb, 2007; Jin et al., 2006; Wirth et al., 2008; Ying, Giachini, Tostes, & Webb, 2009). Each of these RhoGEFs contains a Dbl homology (DH) domain (also known as the RhoGEF domain) followed by a pleckstrin homology (PH) domain (Table 2). The DH domain serves as both the catalytic site and the major binding interface for RhoA, while the PH domain facilitates membrane binding and cooperates with DH domains to fully activate RhoA. Other common domains include a regulator of G-protein signaling (RGS) domain that binds heterotrimeric G proteins, or the density 95, disk large, zona occludes-1 (PDZ) domain that binds to specific transmembrane receptors (including the Lysophosphatidic Acid (LPA)receptor among others (Yamada, Ohoka, Kogo, & Inagaki, 2005). Importantly, these 5 GEFs are all highly expressed in conductance and resistance arteries of rats, mice, and humans (Cario-Toumaniantz et al., 2012; Hilgers et al., 2007; Jin et al., 2006; Wirth et al., 2008; Ying et al., 2009)(Genotype-Tissue Expression [GTEx] Portal, accessed on 07/12/2018).

The regulator of G-protein signaling (RGS) family of Rho GEFs (LARG, p115RhoGEF, and PDZRhoGEF) (Suzuki, Nakamura, Mano, & Kozasa, 2003) has received a lot of attention in the BP field because these proteins are activated by $G\alpha_{12}$ and $G\alpha_{13}$- coupled receptors which transduce signals mediated by major contractile agonists that include A-II, PE, ET1, and thromboxane A2 (Fukuhara, Chikumi, & Gutkind, 2001). p115RhoGEF (p115) is the critical GEF that mediates A-II-dependent RhoA activity in SMC and small arterioles, and importantly, Guilluy et al. showed that SM-specific deletion of p115 rendered mice resistant to A-II-dependent HTN (Guilluy et al., 2010). However, A-II–dependent activation of RhoA in SMC has also been linked to activation of LARG (Ying, Jin, Palmer, & Webb, 2006), PDZ-RhoGEF (Hilgers et al., 2007; Ying et al., 2009), and p63RhoGAP (Wuertz et al., 2010) and inhibition of p190RhoGAP (see below; (Bregeon, Loirand, Pacaud, & Rolli-Derkinderen, 2009)), suggesting significant overlap between these pathways. For example, Ying et al. showed that Ca^{2+}/PYK2-dependent activation of PDZ-RhoGEF was necessary for maximal A-II induced RhoA activation in SMC (Ying et al., 2009). Alternatively, p63-RhoGEF, which is highly expressed in arterial SM, was shown to be important for the early phase of A-II-dependent vessel contractility (Wuertz et al., 2010)

Table 2
Domain structure of RhoGEF proteins associated with blood pressure.

GEF protein	Length (AA)	Domain			
		PDZ	RH	Rho-GEF (DH)	PH
p115-RhoGEF	927		✓	✓	✓
PDZ-RhoGEF	1562	✓	✓	✓	✓
LARG	1544	✓	✓	✓	✓
p63-RhoGEF	580			✓	✓

The RhoGEF domain (also known as the Dbl homology [DH] domain), is the catalytically active domain of RhoGEFs and major binding site for RhoA GTPase. Immediately downstream of the RhoGEF domain is a pleckstrin homology (PH) domain, which works cooperatively with the RhoGEF domain to fully activate the RhoA GTPase. Other functional domains in these RhoGEFs are the Regulators of G protein signaling homology (RH) domain and the postsynaptic density 95, disk large, zona occludens-1 (PDZ) domain. Potential blood pressure therapeutics are being developed to target the interaction interface between RhoGEF domain and RhoA. Length is shown in amino acids (AA).

and for maximal pressor response to other vasoconstrictors such as PE and ET1 that act through $G\alpha_{q/11}$ (Momotani et al., 2011). Interestingly, p115 mutant mice exhibited normal pressor responses to ET1 and PE, but did not respond fully to A-II and had a partial reduction in DOCA/salt- induced HTN. On the other hand, LARG knockout mice were fully resistant to salt-induced HTN (Wirth et al., 2008), which is consistent with subsequent findings that LARG regulates RhoA activity and SMC contractility in response to mechanical forces—which are known to be applied to small vessels in this volume overload model (Guilluy et al., 2011). While not yet confirmed in pre-clinical animal models, the non-RGS Rho GEF termed lymphoid blast crisis (Lbc) is necessary for serotonin-dependent activation of RhoA and contractility in vascular SMC (Bear et al., 2010). Thus, specific vasoconstrictors can lead to activation of distinct but overlapping sets of RhoGEFs to enable the fine-tuning of vessel tone and BP homeostasis.

With respect to pharmacological treatments, these findings indicate that targeting a critical SM GEF might provide greater therapeutic benefit than targeting RhoA or Rho kinase, because it could lead to a more modest (and cell type restricted) reduction of RhoA activity and result in fewer side effects. Moreover, the use of validated inhibitors could provide 'personalized' approaches that would better target the underlying pathophysiology (i.e. to limit HTN due to volume overload or elevated sympathetic activity). On the other hand, known functional redundancies also suggest that it may be necessary to target multiple GEFs to achieve therapeutic efficacy.

4.1. Targeting RhoGEFs

GEF protein-dependent nucleotide exchange involves a multi-step reaction. Upon binding to the GDP-bound form of the GTPase, GEFs facilitate release of GDP resulting in the formation of a nucleotide-free GTPase–GEF transition complex. The reaction then terminates with the binding of GTP (which is in a 10:1 molar excess in the cell) and dissociation of the GEF from the now active GTPase. The implication of this multi-step process for drug discovery is that these transition-binding intermediates tend to have the characteristics of druggable hotspots (i.e. exposed hydrophobic surfaces, unpaired polar groups, deeply curved surfaces, etc.) while the unbound, inactive proteins do not (Beglov et al., 2018). Thus, it may be possible to identify small molecules that specifically target the GEF-GTPase interface (i.e. inhibit GEF binding or stabilize the GEF-GTPase transition state) or directly interfere with GEF activity.

4.1.1. Targeting the RhoA-GEF interface

Facilitated by high resolution crystal structures and sophisticated *in silico* screening, some recent drug discovery approaches support the possibility of targeting the RhoA-GEF interface. For example, Shang et al. virtually screened 4 million compounds for their ability to pack into the Rho-GEF binding surface groove of RhoA. One of the drugs identified (termed Rhosin) contains two aromatic rings tethered by a linker which wraps around RhoA AA58 (a critical Tryptophan that is essential for GEF binding) and prevents RhoA from interacting with LARG, DBL, LBC, p115RhoGEF and PDZ RhoGEF without interfering with RhoGAP, RhoGDI, or RhoA effector binding (Shang et al., 2012). Rhosin reversibly inhibited serum-induced RhoA, RhoB and RhoC activity, but did not inhibit other Rho GTPases (Rac1 or Ccd42) (Shang et al., 2012). Rhosin also significantly and dose-dependently inhibited several RhoA-dependent functions in cells including MLC and PAK phosphorylation and stress fiber and focal adhesion formation (Shang et al., 2012). Rhosin suppressed invasion and mammary sphere formation in breast cancer cells (Shang et al., 2012), and also mitigated the acquisition of drug resistance in cancer stem cells (Yoon et al., 2016). The ability of Rhosin to impact either BP homeostasis (or cancer growth) has not yet been tested in animal models. Since Rhosin exhibits a relatively low binding affinity, (K_d ~0.4 μM for RhoA binding in vitro) the identification of derivatives with better pharmacologic properties will be needed before Rhosin compounds can be tested clinically (Shang et al., 2012). Moreover, since Rhosin binds to RhoA and prevents all GEFs from binding to this site, this drug is likely to have more off-target effects than one that could interfere with specific GEF complexes.

The first proof-of-concept for targeting a specific GEF-GTPase interface was a series of elegant studies that unveiled the mechanism by which the fungal toxin, Brefeldin A (BFA), inhibited Arf1- dependent trafficking of proteins from the endoplasmic reticulum to the Golgi. Biochemical studies revealed that BFA selectively blocked the ability of the Arf1GEF, Sec7, from catalyzing GDP release. Importantly co-crystalization studies revealed that BFA binds to a hydrophobic pocket that does not exist in $Arf1^{GDP}$ but is created upon Sec7 binding. Since this energetically unfavorable hydrophobic pocket drives the conformational changes necessary for nucleotide dissociation, BFA binding effectively locks the complex in a GDP bound conformation (Cherfils & Melancon, 2005; Mossessova, Corpina, & Goldberg, 2003; Zeghouf, Guibert, Zeeh, & Cherfils, 2005). In spite of high sequence homology among the Arf1GEFs, only the Sec7 complex is targeted by BFA, suggesting that 'interfacial' small molecule inhibitors could be identified that specifically target RhoA-GEF interactions.

These studies have inspired a new line of investigation that seeks to exploit novel pockets at small GTPase-GEF interfaces. The most productive approach so far used NMR-based fragment screening combined with high resolution structure/functional analyses to identify small molecule inhibitors of kRas and its GEF, Sos1. These screens identified compounds that either block Sos1 binding to kRAS or that bind to a site adjacent to the functionally important switch I/II regions of KRas and (like BFA) block SOS1-dependent nucleotide exchange. A similar approach was used to identify a ligand that binds to the cavity adjacent to the switch II region of RhoA and inhibits the interaction between $RhoA^{GDP}$ and the LARG DH domain (Gao et al., 2014). Small molecules have also been identified that inhibit TrioGEF's interaction with Rac1 and RhoG and Cdc42's interaction with the GEF, intersectin-1 (Smithers & Overduin, 2016). Clearly much work needs to be done to realize the potential for such ligands. The inhibitors identified exhibit very low potency (in the high micromolar range) and their ability to block additional GEFs has not yet been evaluated. In fact, *in silico* evaluation of aforementioned SOS1 inhibitor suggests that the hydrogen bonds formed by this ligand in the Ras-SOS1 complex would likely be conserved in the complex of Ras and another cognate GEF, RasGRF1 (Gao et al., 2014). While challenging, further medicinal chemistry and molecular dynamics approaches should facilitate our ability to target RhoA's interaction with specific GEFs.

4.1.2. Targeting GEF activity

Another viable approach is to directly target the activity of specific RhoGEFs before they interact with the small GTPases. Using a high-throughput screen, Shang et al. identified a compound, Y16, that binds LARG at the junction between the DH and PH domains with a K_d of ~80 nM (Shang et al., 2013). Y16 prevented LARG from interacting with RhoA *in vitro* and reversibly attenuated serum-induced RhoA activity in NIH 3T3 cells and mammary sphere formation MCF7 cells with little to no toxicity (Shang et al., 2013). A separate fluorescent ligand-based screen of ten thousand compounds yielded five additional selective inhibitors of LARG-dependent RhoA GTPase activity, though their mechanisms of action have not yet been determined (Evelyn et al., 2009). Again, to date, many of these lead compounds exhibit low potency, but theoretically, combination therapy strategies which pair Y16 with some of these compounds and/or their derivatives may lead to a highly efficacious approach to limit LARG-mediated signaling.

Finally, since many GEFs are regulated by protein-protein interactions and post-translational modifications, it may be possible to identify drugs that block these mechanisms. In an excellent example of this approach, cAMP is a potent activator of the Rap1 GEFs, Exchange Proteins directly Activated by cAMP (EPAC1 and 2) and high throughput screens to identify small molecules that displace cAMP binding led to the discovery of several

inhibitors or partial agonists for these GEFs (Brown, Rogers, Aroonsakool, McCammon, & Insel, 2014; Liu et al., 2017; Parnell et al., 2017). Such studies support the concept that identification of small molecules that prevent physiological activation of specific GEFs could prove therapeutically useful. Along these lines, several studies have shown that RGSRhoGEFs are regulated by phosphorylation. Guilluy et al. demonstrated that A-II-dependent activation of p115GEF was mediated by phosphorylation of the PH domain (Tyr738) by Janus tyrosine kinase (Guilluy et al., 2010). Importantly, phosphorylation mimetic and deficient variants of Tyr738 elevated and reduced p115's GEF activity, respectively. PDZRhoGEF and LARG are also activated by tyrosine phosphorylation. Focal adhesion kinase (FAK) and its related family member proline-rich tyrosine kinase 2 (PYK2) phosphorylate and activate PDZRhoGEF (Chikumi, Fukuhara, & Gutkind, 2002; Ying et al., 2009), while FAK, Tec and the serine-threonine kinase p90 ribosomal kinase-2 each phosphorylate and activate LARG (Chikumi et al., 2002; Shi, Yang, Jin, Matter, & Ramos, 2018; Suzuki et al., 2003; Ying et al., 2009). Drugs that specifically inhibit these phosphorylation events could prove to be useful RhoA signaling inhibitors.

5. GTPase activating proteins (GAPs)

Although GEFs have classically been considered to be the major regulators of RhoGTPase activity, increasing evidence suggest the GAPs are also critically important. As their name implies, GAPs enhance the intrinsic GTPase activity of the Rho GTPases by several orders of magnitude thus decreasing the length of time that GTPases are in the active form (Bos, Rehmann, & Wittinghofer, 2007; Puetz, Lubomirov, & Pfitzer, 2009; Rittinger, Walker, Eccleston, Smerdon, & Gamblin, 1997). The 66 RhoGAPs in the human genome comprise a broad and diverse family that can be further subdivided based upon the presence of a variety of functional domains (Tcherkezian & Lamarche-Vane, 2007) (Table 3). By mediating interactions with different membrane and protein components these domains are critical for the selective function of the GAP proteins and the dynamic inhibition of small GTPase signaling. Several Rho-selective GAPs, including p190ARhoGAP, ArhGAP1, Myr5, GRAF1, and GRAF3, have been shown to inhibit RhoA activity in cultured vascular SMC and could be potential targets for therapeutic interventions that affect vascular function and BP (Bai et al., 2013; Mori et al., 2009). However, GRAF3 is the only RhoGAP that has been directly linked to the regulation of SM contractility and BP homeostasis (Bai et al., 2013; Ehret et al., 2011; Kato et al., 2015; Li et al., 2016; Wain et al., 2011).

5.1. GRAF3 and hypertension

We originally identified the founding member of the GRAF (GTPase Regulator Associated with FAK) family by screening an embryonic λgt11 expression library for proteins that interacted with the carboxyl-terminal domain of FAK (Hildebrand, Taylor, & Parsons, 1996; Taylor, Hildebrand, Mack, Cox, & Parsons, 1998; Taylor, Macklem, & Parsons, 1999). This family which is now known to comprise 3 members, GRAF1 (ArhGAP26), GRAF2 (ArhGAP10) and GRAF3 (ArhGAP42) contain 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 FAK-binding SH3 domain (Hildebrand et al., 1996). 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 (Doherty et al., 2011; Lenhart et al., 2014; Lenhart et al., 2015; Taylor et al., 1998). GRAF2 is more ubiquitously expressed (Ren et al., 2001) and could partially compensate for the loss of GRAF1 during myotube formation, supporting at least some functional redundancy within this family (Lenhart et al., 2014). Evolutionarily, GRAF3 is the youngest family member and is the most recently annotated. Importantly, we found that GRAF3 was highly and selectively expressed in SMC with particularly high expression in resistance vessels (Bai et al., 2013) suggesting that its expression levels might be a major determinant of RhoA activity, SM contraction, and vessel tone.

Table 3
Domain structure of RhoGAP proteins associated with blood pressure.

GAP protein	Length (AA)	Domain							
		BAR	PH	GBD	FF	PBR	GBD	Rho-GAP	SH3
GRAF3	874	✓	✓					✓	✓
P190-RhoGAP	1499			✓	✓✓✓✓	✓	✓	✓	

The GAP domain is the catalytically active domain of RhoGAPs. GRAF3 also contains a Bin/amphiphysin/RVS (BAR) domain, a pleckstrin homology (PH) domain, and a SRC homology 3 (SH3) domain, which sense and induce membrane curvature, aid in lipid binding, and promote protein-protein interactions, respectively. In addition to the catalytic GAP domain, p190-RhoGAP also contains a GTP-binding domain (GBD), four diphenylalanine motifs (FF), and a polybasic region (PBR). These regions aid in GTP binding, assist in protein-protein binding, and allow for membrane association, respectively. Potential blood pressure therapeutics involving these RhoGAPs focus on activating the GAP domain. Length is shown in amino acids (AA).

Over the last several years, studies from our laboratory confirmed that GRAF3 is a SMC-selective Rho GAP protein that imparts tight control of BP homeostasis by modulating vascular resistance. Specifically, we showed that mice with gene-trap-mediated reductions in GRAF3 levels exhibited significant basal HTN and elevated pressor responses to A-II, ET1, PE, and DOCA salt (Bai et al., 2013). Notably, the hypertensive phenotype in this model was fully reversed by treatment with ROCK inhibitors or by Cre-mediated re-expression of GRAF3 in SMC, strongly supporting the contention that GRAF3 control of SMC RhoA activity is necessary for homeostatic control of basal and pressor-induced BP. Mechanistically, our data supported a model wherein GRAF3 controls BP homeostasis by limiting RhoA-dependent MLC phosphorylation and blunting acute Ca^{2+}-mediated SMC contractility in resistance vessels. Moreover, depletion of endogenous GRAF3 from vascular SMC enhanced MRTF-A nuclear accumulation, and stimulated expression of contractile proteins including SM α-actin, SM-myosin heavy chain, calponin and SM-22, indicating that changing the levels of GRAF3 would likely have a long-lasting impact on vessel tone (Bai, Mangum, Kakoki, et al., 2017). Interestingly, GRAF3 mRNA was significantly upregulated in SMC cultures subjected to cyclic stretch and in isolated portal vein segments subjected to static stretch and we showed that these effects were mediated via the RhoA/MRTF/SRF pathway (Bai, Mangum, Kakoki, et al., 2017). Since similar physical forces are known to be increased in the vessel wall under hypertensive conditions (Albinsson et al., 2004), we postulated that GRAF3 might serve as a transcriptionally mediated negative feedback loop of the RhoA signaling axis. In support of this possibility, arterial GRAF3 mRNA levels were significantly increased in mice made hypertensive by L-NAME or DOCA-salt regimens (Bai et al., 2013). In fact, taking advantage of an elegant mouse model developed by the Smithies lab in which plasma volumes of mice range from ~50% below normal to ~50% above normal (Kakoki et al., 2013), we showed that GRAF3 expression increased in parallel to and was strongly correlated with plasma volume ($r^2 = 0.94$) (Bai, Mangum, Kakoki, et al., 2017). Taken together, these findings indicate that GRAF3 serves as a mechanical strain-sensitive rheostat that acts to prevent excessive feed-forward activation of the RhoA signaling axis in order to control SMC tone and BP.

Of clinical importance, three separate GWAS studies identified a blood-pressure associated allele within the GRAF3 locus. Notably, our recently published follow-up studies revealed that GRAF3 mRNA levels were approximately 3-fold higher in arteries from individuals homozygous for the protective allele and our human genetic data from well-characterized untreated patients confirmed that the protective allele was associated with a 5 mm Hg decrease in BP (Bai, Mangum, Dee, et al., 2017; Ehret et al., 2011; Kato et al., 2015; Wain et al., 2011). We went on to identify a novel mechanism for the BP-associated locus which mapped to the first intron of the GRAF3 gene (*ARHGAP42*) (Bai, Mangum, Dee, et al., 2017). Our studies revealed that rs604723 is the

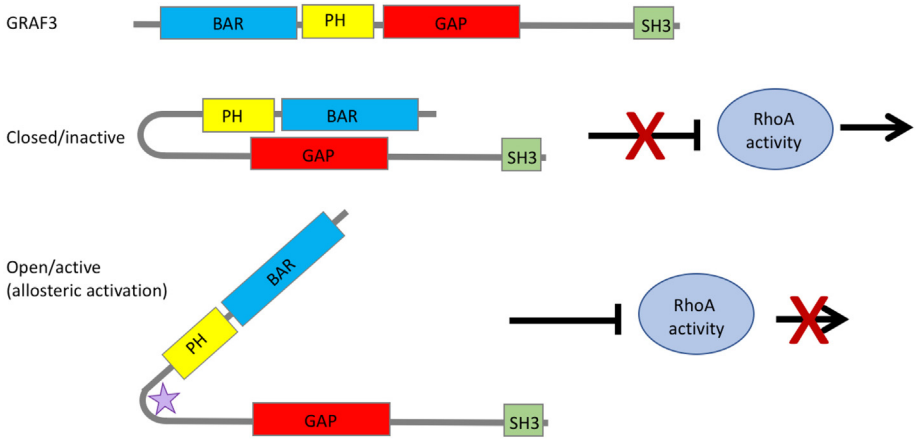

Fig. 2. Domain Structure and therapeutic strategy for targeting the SMC-specific RhoGAP GRAF3. Like other BAR-PH-GAP containing proteins, the BAR and PH domains of GRAF3 act as a functional unit to autoinhibit the GAP domain, preventing GRAF3's interaction with RhoA. One potential therapeutic strategy is to use allosteric activation (either post-translational modifications or small molecules, represented here by the purple star) to lock GRAF3 in its open conformation, enhancing hydrolysis of GTP to GDP, thereby decreasing the activity of RhoA and promoting SMC relaxation and decreased blood pressure.

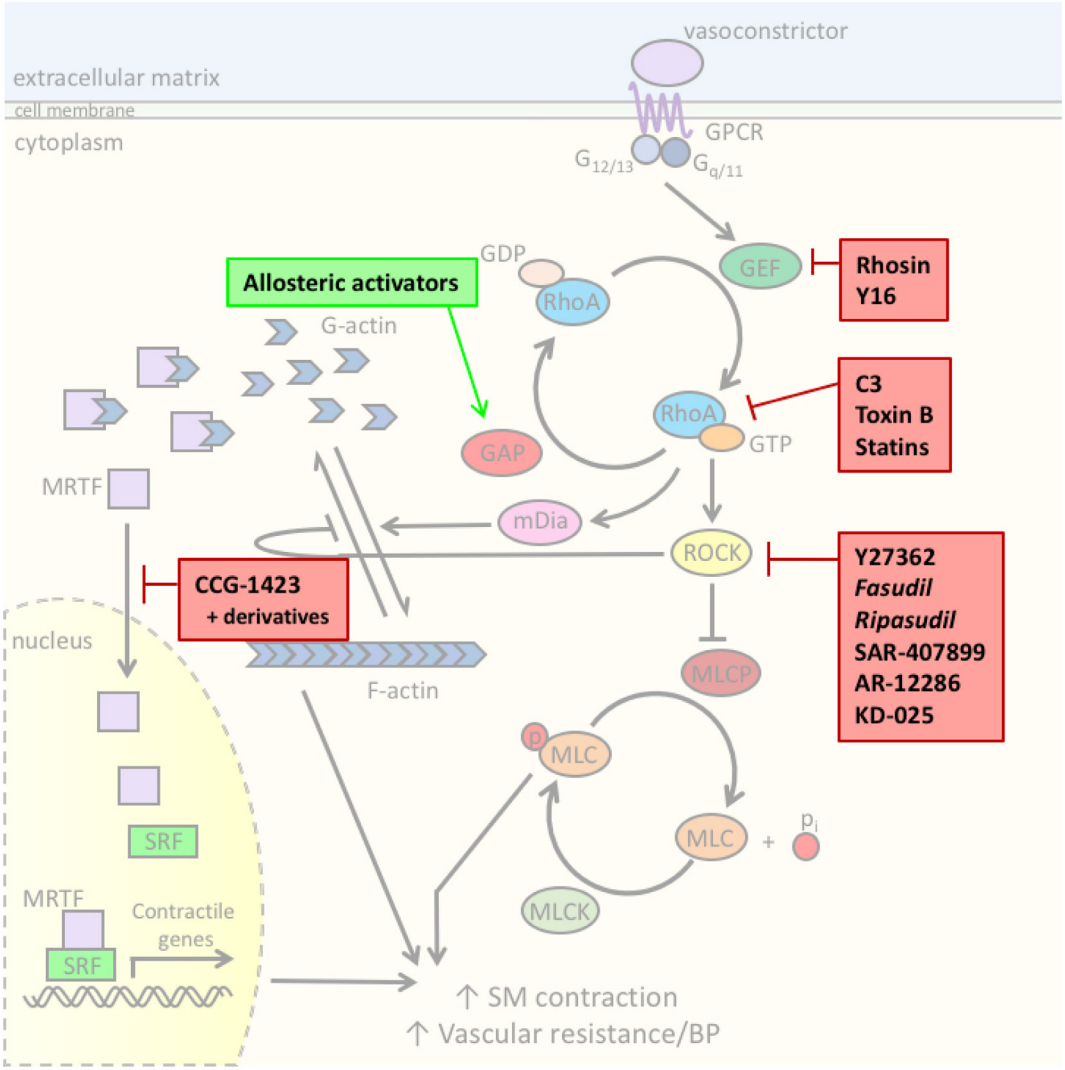

Fig. 3. Therapeutic potential of the RhoA pathway to treat HTN. There are many components of the SMC RhoA contractile pathway that could be targeted to reduce systemic blood pressure. While not an exhaustive list, shown here in bold boxes are compounds discussed in this article, including those that are proof-of-concept, in clinical development, or currently used in the clinic (italicized).

causative SNP at this locus and that the minor T allele variation increases *ARHGAP42* expression by promoting SRF binding to a SMC-selective intronic regulatory element. Our demonstration that the minor *ARHGAP42* allele is more highly expressed in HuAoSMCs, when coupled with similar data from human artery samples, strongly supports our hypothesis that this variation reduces BP by inhibiting RhoA-dependent constriction of resistance vessels. Our data add to a growing body of evidence that common noncoding variants alter cardiovascular risk by altering transcription factor binding and gene expression and support previous studies implicating RhoA signaling in the regulation of BP homeostasis in mice. Moreover, when coupled with the remarkable SMC-selective expression pattern of GRAF3, these studies provide strong evidence for GRAF3 as a novel therapeutic target for HTN.

5.2. Druggability of RhoGAPs

To date, targeting GAPs for therapeutic advances has been largely overlooked. This is due, in part, because GAPs have little to no effect on promoting GTP hydrolysis of oncogenic Ras and Rho mutants, and therefore would not be good targets for cancer treatment (Holderfield, 2018). GAPs have also been considered less attractive targets for antihypertensive therapies because the drug would need to enhance GAP activity and it is traditionally thought to be more difficult to develop small molecule activators than small molecule inhibitors. Nonetheless, due to the multi-domain nature and the varied physiological regulation of RhoGAP proteins, there are several possibilities for allosteric modulation of GAP activity. Indeed, the activities of several RhoGAPs including the GRAFs, OPHN1, β-chimerin, DLC1, and p50 Rho GAP are regulated by intramolecular auto-inhibition. For GRAF1 and similarly structured Oligophrenin and ASAP1, the BAR and PH domains physically associate with the GAP domain to sterically inhibit its function (Eberth et al., 2009; Jian et al., 2009). We and others have shown that this mechanism also controls the activity of GRAF3 (unpublished; (Luo et al., 2017)) (Fig. 2). Interestingly, FAK/Src-dependent phosphorylation of GRAF3 at Tyr376 modulated its GAP activity (Luo et al., 2017) and we have shown that a phosphomimetic 376E variant exhibited elevated activity (unpublished observations). By defining the structural interactions that control GRAF3 activity, these results could lead to the development of GRAF3 activators that target the BAR-PH/GAP interface that should be useful for reducing arterial tone.

6. Conclusions

In conclusion, the search for new approaches to control high BP remains dramatically important for reducing global health burden, and targeting RhoA-mediated SM contractility is a promising avenue (Fig. 3). Indeed, strong evidence from pre-clinical animal models indicate that modulating the activity of SM Rho-GEFs, Rho-GAPs, or ROCK has a major impact on systemic BP homeostasis. While new advances in drug development have led to potent and specific ROCK inhibitors that can be safely used in patients, whether any of these compounds exhibit the necessary selectivity and pharmacological profiles required for BP management in patients requires further study. Although efforts to target GEF and GAPs or the RhoA binding interface for these enzymes are lagging behind, recent advances in drug discovery indicate that it will likely soon be possible to engineer clinically-relevant small molecule regulators of these enzymes that could be effective anti-hypertensive therapies. To ensure success in this regard, we will need to continue to gain a better understanding of the mechanisms that regulate these enzymes. 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 that genetic variations in both upstream activators and downstream mediators of RhoA have been linked to BP regulation, screening for such variants could potentially be used to tailor more effective individualized treatment regimens.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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