Regulator of G Protein Signaling 5 Controls Blood Pressure Homeostasis and Vessel Wall Remodeling

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ABSTRACT

**Rationale:** Regulator of G protein signaling 5 (RGS5) modulates G protein coupled receptor signaling and is prominently expressed in arterial smooth muscle cells. Our group first reported that RGS5 is important in vascular remodeling during tumor angiogenesis. We hypothesized that RGS5 may play an important role in vessel wall remodeling and blood pressure regulation.

**Objective:** To demonstrate that RGS5 has a unique and non-redundant role in the pathogenesis of hypertension and identify crucial, RGS5-regulated signaling pathways.

**Methods and Results:** We observed that arterial RGS5 expression is downregulated with chronically elevated blood pressure after angiotensin II (AngII) infusion. Using a knockout mouse model, radiotelemetry and pharmacological inhibition, we subsequently showed that loss of RGS5 results in profound hypertension. RGS5 signaling is linked to the renin-angiotensin system and directly controls vascular resistance, vessel contractility and remodeling. RGS5-deficiency aggravates pathophysiological features of hypertension such as medial hypertrophy and fibrosis. Moreover, we demonstrate that PKC, MEK/ERK and Rho kinase signaling pathways are major effectors of RGS5-mediated hypertension.

**Conclusions:** Loss of RGS5 results in hypertension. Loss of RGS5 signaling also correlates with hyper-responsiveness to vasoconstrictors and vascular stiffening. This establishes a significant, distinct and causal role of RGS5 in vascular homeostasis. RGS5 modulates signaling through the angiotensin II receptor 1 (ATR1) and major $G_{q,c}$-coupled downstream pathways including Rho kinase. So far, activation of RhoA/Rho kinase has not been associated with RGS molecules. Thus, RGS5 is a crucial regulator of blood pressure homeostasis with significant clinical implications for vascular pathologies such as hypertension.

**Keywords:** Hypertension, vessel wall remodeling, G protein coupled receptor signaling, animal model of cardiovascular disease, arterial stiffness, vascular smooth muscle

**Non-standard Abbreviations:**
2K1C  2 kidney-1 clip  
ATP  adenosine 5'-triphosphate  
AngII  angiotensin II  
ATR1  angiotensin II receptor, type 1  
BIM  bisindolylmaleimide I  
ECM  extracellular matrix  
ERK  extracellular signal regulated kinase  
ET  endothelin  
GAP  GTPase activating protein  
GPCR  G protein coupled receptor  
JAK2  janus kinase 2  
L-NAME  Nω-nitro-l-arginine methyl ester  
MAP  mean arterial blood pressure  
MEK  mitogen activated protein kinase kinase  
MLC  myosin light chain  
MYPT1  myosin phosphatase target protein-1  
PAS  periodic acid Schiff  
PKC  protein kinase C  
PE  phenylephrine  
PPARγ  peroxisome proliferator-activated receptor γ  
PWV  pulse wave velocity  
RGS  regulator of G protein signalling  
vSMC  vascular smooth muscle cells.
INTRODUCTION

Blood vessels consist of two major cell types, endothelial and mural cells such as pericytes and vascular smooth muscle cells (vSMC) which surround the endothelium. RGS5 is expressed in mural cells and has emerged as a crucial modulator of vascular pathology in cancer. For instance, we have demonstrated that RGS5 is highly upregulated in angiogenic tumor pericytes. Loss of RGS5 results in pericyte maturation and normalization of tumor vasculature. Moreover, we showed a crucial role for RGS5 in regulating vascular barrier function in tumors, and in brain capillaries during ischemia, and also provided the first genetic evidence that RGS5 is involved in vascular wall remodeling in the adult.

A striking feature of RGS5 expression is its dynamic nature in various physiological and pathological states, which indicates a role in adaptive processes. This is consistent with RGS5 being a member of the extended family of RGS molecules which are modulators of G protein-coupled receptors (GPCRs). G protein signaling pathways rely on rapid “on-off” kinetics and RGS molecules act as GTPase-activating proteins (GAP) for heterotrimeric G proteins and as such regulate duration and intensity of signaling events. They contain a highly conserved carboxyl-terminal RGS domain which confers the catalytic function for active Gα subunits. Members of the R4/B subfamily, which include amongst others RGS 2, 4, and 5, are the smallest RGS proteins in size and contain only short peptide sequences flanking the RGS box. Nevertheless, there is increasing evidence that RGS molecules interact with other cellular proteins downstream of GPCRs and exert effects beyond their GAP activity. Importantly, recent data from transgenic and knockout mice highlight a critical role for some RGS molecules in cardiovascular function. For instance, RGS2-deficient mice develop hypertension and consequent cardiac hypertrophy. Conversely, cardiac-specific RGS4 or RGS5 over-expression in transgenic mice has a protective effect in late stage heart failure by counter-regulating hypertrophic stimuli or preventing hypertrophy in response to pressure overload, respectively.

So far, RGS5 has been shown to accelerate GTP hydrolysis of Gαi and Gαq in vitro and attenuate the MAP kinase pathway. Interestingly, RGS5 is overexpressed in arteries compared with veins and its expression dynamically changes after aortic banding. This indicates that RGS5 may regulate hemodynamic adaptation in arteries. Furthermore, RGS5 is downregulated in arteries from hypertensive rats which implies a more direct involvement in regulation of vascular tone. In humans, rgs5 gene polymorphism has been associated with hypertension. In earlier studies RGS5-deficient mice have been shown to be hypotensive relative to wild type controls. This remains an unexpected finding and difficult to reconcile with our current understanding of the function of RGS5 as negative regulator of vasoconstriction.

Here, we demonstrate that loss of RGS5 results in hypertension, a finding which is supported by complementary functional assays. This establishes a non-redundant crucial role for RGS5 in vascular function and blood pressure homeostasis.

METHODS

Detailed Methods are provided in the Online Supplement. Hemodynamic measurements in conscious mice were conducted with telemetry probes (DSI). For blood pressure measurement in anesthetized mice, a Millar 1.4 French probe and transducer (ADInstruments) was used. Blood flow in femoral arteries was assessed with a transit-time ultrasonic system (Transonic Systems). AngII infusion (Alzet) and abdominal aortic constriction (modified 2K1C) were used to generate hypertension. Femoral vSMC were isolated and cytosolic calcium was monitored using the fluorescent indicator Fura-2AM. In cell western assays were performed using the Odyssey Infrared Imaging System (LI-COR Biosciences).

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RESULTS

RGS5 levels are reduced with chronically elevated blood pressure.

The vasoactive peptide of the renin-angiotensin system, angiotensin II (AngII) and its GPCRs, play a major role in cardiovascular regulation and the pathogenesis of hypertension. To test the hypothesis that RGS5 levels change during vessel wall remodeling, wild type mice were infused with AngII (1 mg/kg/day) for 21 days. As expected, this treatment results in a slow rise of mean arterial blood pressure (MAP) with a 38% increase at endpoint (untreated 82.7±2.8 versus treated 113.9±2.6 mmHg, Figure 1). For the first 2 days after minipump implantation, aortic RGS5 expression and blood pressure remain unchanged. From day 2 onwards, however, RGS5 levels are significantly reduced and further decrease over time. Interestingly, a fall in RGS5 levels precedes changes in blood pressure which become apparent on day 7. Reduced RGS5 expression was also observed in a physiological model of renin-angiotensin-mediated hypertension (modified 2 kidney-1 clip model, 2K1C) but not after L-NAME (Nω-nitro-l-arginine methyl ester) infusion (Online Figure I). Thus, arterial RGS5 levels inversely correlate with changes in blood pressure in AngII-mediated hypertension. We hypothesized that under physiological conditions RGS5 provides negative feedback to AngII signaling. However, under chronic conditions for instance excessive or prolonged AngII signalling, loss of RGS5 results in pathologically high blood pressure.

RGS5 controls blood pressure.

To investigate whether RGS5 is causally involved in blood pressure regulation, RGS5-deficient mice were implanted with radiotelemetry devices and measured variations in blood pressure, heart rate, and locomotor activity over 72 hours. Mean arterial blood pressure in RGS5 knockout mice is significantly higher than in wild type controls during day time (wild type 83.3±2.6 versus knockout 115.3±5.1 mmHg, ΔMAP, 32 mmHg, P≤0.0001). Murine blood pressure shows circadian variations and increases at night when mice become active. Interestingly, knockout mice show a significantly higher nocturnal blood pressure increase over wild type mice (wild type 102.0±2.5 versus knockout 150.7±4.4 mmHg, ΔMAP, 48.6 mmHg, P≤0.0001) even though locomotor activity does not differ between groups (Figure 2A and Online Figure II). Heart rates are also identical between experimental groups (Online Figure II). Similar differences in MAP are recorded for both male and female RGS5 knockout mice on 2 different genetic backgrounds (C3H and C57BL/6) whereas heterozygote deletion of RGS5 does not result in hypertension (Online Figure II). Moreover, acute treatment of RGS5-deficient mice with an ATR1 antagonist normalizes blood pressure, demonstrating that signaling through ATR1 is a major effector mechanism in RGS5-mediated hypertension (Figure 2B). We also found that chronic infusion of AngII (1 mg/kg/day) for 21 days increases blood pressure more profoundly in RGS5-deficient mice than wild type controls (ΔMAP wild type 29.2±2.1 versus knockout 51.4±3.1 mmHg, Figure 2 C, D, P≤0.0001), thus supporting a role for RGS5 within the renin-angiotensin signaling pathway. Loss of one RGS5 allele in heterozygote RGS5 mice is sufficient to raise blood pressure above wild type under chronic AngII infusion. ATR1 receptor levels are unchanged in RGS5 knockout mice which supports a post-receptor regulatory mechanism (Online Figure II). Also, RGS2 and 4 expressions are unchanged in unchallenged RGS5 knockout mice. Under AngII infusion, RGS2 is significantly upregulated in RGS5-deficient mice (Online Figure II, P=0.04). Nevertheless, loss of RGS5 is sufficient to cause hypertension and may result in hyper-responsiveness to vasoconstrictor-mediated responses. This finding is consistent with a critical role of RGS5 in terminating vasoconstrictor-mediated GPCR signaling.

RGS5 regulates vascular flow and contractility.

AngII-mediated blood pressure control is complex and involves multiple organs including brain, kidney and the vascular wall. Circulating aldosterone and renin concentrations in RGS5 knockout and wild type mice are not significantly altered (Online Table I). Given the important role of RGS5 in angiogenic vessel remodeling, we hypothesized that RGS5 directly modulates vascular tone. To address this, hindlimb blood flow was measured by an ultrasonic transit time flow probe. Acute
injection of the vasoconstrictor AngII reduces femoral artery blood flow in RGS5 knockout mice more profoundly than in wild type controls (Figure 3A). These findings imply that RGS5 directly regulates vascular resistance, most likely by affecting vessel contractions. Indeed, significantly enhanced contraction in response to AngII was measured in mesenteric vessels from RGS5 knockout mice (Figure 3B, \( P=0.0009 \)). Enhanced vessel conductance and contractility were also observed with phenylephrin (PE) stimulation but not with endothelin (ET) or adenosine 5'-triphosphate (ATP, Online Figure III) indicating selectivity of RGS5-regulated effects in these vascular beds.

Vessel contractility is in part mediated by alterations in cytosolic free calcium (Ca\(^{2+}\)) concentrations. To assess the role of RGS5 in pressor-induced calcium release, Fura-2 loaded vSMCs derived from femoral arteries of wild type and RGS5 knockout mice were stimulated with AngII in a dose-dependent manner. Prior to stimulation with AngII, there was no difference in basal calcium levels in vSMC from wild type and knockout mice (Online Figure IV). Addition of AngII, however, increased Fura-2 340/380 nm fluorescence in RGS5-deficient vSMCs by 25\% (Figure 3C). These findings establish a direct link between RGS5 expression and Ca\(^{2+}\)-dependent vessel contraction, vascular resistance and hypertension. Since vascular contractility and vessel wall remodeling are crucial in the development of hypertension, we hypothesized that chronic vasoconstriction in the absence of RGS5 may also cause structural alterations in the vessel wall. To assess the passive mechanical wall properties, stress-strain relationships for arteries from 6 week-old wild type and knockout mice were determined. The stress-strain relationship for femoral arteries from RGS5 KO mice is significantly shifted to the left compared with wild type (Figure 3D, \( P=0.01 \)), indicating that femoral arteries of RGS5 KO mice are stiffer; this may facilitate the hypertensive phenotype which is largely controlled by enhanced smooth muscle contractility.

**Age-dependent vascular stiffening in RGS5-deficient mice.**

To assess the impact of RGS5-deficiency over time, blood pressure in young (6 weeks) and old (30 weeks) wild type and knockout mice was compared. In wild type mice, changes in MAP between week 6 and 30 are not significant (6 weeks 82.3±5.4 versus 30 weeks 88.4±6.6 mmHg). In contrast, blood pressure in knockout mice increases significantly by 22\% at 30 weeks as compared to measurements at 6 week of age (Figure 4A, 6 weeks 93.0±3.1 versus 30 weeks 119.3±7.3 mmHg, \( P\leq0.0001 \)). Age-related MAP differences in hypertensive, RGS5-deficient mice may relate in part to increased stiffness of resistance arteries. Interestingly, whilst femoral arteries stiffen with age in wild type and knockout groups alike (Online Figure V), mesenteric arterial stiffness increases significantly \((P=0.01)\) with age (>30 weeks) in knockout mice but not in age-matched controls (Figure 4B).

Pulse wave velocity (PWV), the rate of propagation of pressure waves within a vessel, is another indirect measurement of vessel aging and an independent cardiovascular risk factor in hypertension.\(^{25}\) A high PWV indicates lack of elasticity with a fast pulse wave moving along the aorta as measured from carotid to iliac arteries.\(^{26}\) To control for existing blood pressure differences between wild type and knockout groups, 6 and 30 week-old RGS5-deficient mice were acutely treated with ATR1 inhibitor prior to PWV recordings (Online Figure VI). Figure 4C shows that PWV is significantly enhanced in all knockout groups compared to wild type, and also increased with age (6 weeks: wild type 2.3±0.3 versus knockout 3.5±0.6 m/s; 30 weeks: wild type 2.4±0.5 versus knockout 4.4±0.6 m/s, \( P\leq0.0001 \)). Age-related decrease in aortic distensibility likely reflects both changes in active arterial tone and structural alterations specific for the vessel wall of RGS5-deficient mice.

**Increased vascular damage in RGS5-deficient, hypertensive mice.**

Enhanced arterial wall stiffening due to changes in extracellular matrix (ECM) components and/or growth-mediated wall thickening are important pathological aspects of hypertension. To examine the impact of RGS5 deficiency on renin-angiotensin-mediated hypertension, 2K1C mice were examined. After 4 weeks, blood pressure becomes elevated in both strains. However, in RGS5 knockout mice blood pressure is increased by 50\% compared to wild type controls indicative of an excessive adaptive response (\( \Delta \text{MAP} \) wild type 43.5±5.2 versus knockout 86.2±6.1 mmHg, Figure \( \Delta \text{MAP} \) wild type 43.5±5.2 versus knockout 86.2±6.1 mmHg, Figure
Moreover, medial areas of renal resistance arteries (size 200-300 µm) are significantly enlarged in 2K1C RGS5 knockout mice consistent with medial hypertrophy in more severe hypertension (Figure 5B, \(P<0.0001\)). Chronic hypertension may also lead to perivascular ECM changes and contribute to inward remodeling of blood vessels. Indeed, in kidneys of 2K1C RGS5 knockout mice, the area of perivascular fibrosis compared to total vessel area is strongly enhanced in small renal vessels (size 50-70 µm) in the left kidney (Figure 5C, \(P=0.001\)). Collectively, these findings indicate that loss of RGS5 aggravates hypertension-induced vascular damage.

RGS5 regulates blood pressure involving PKC, MEK/ERK and Rho kinase signaling pathways.

AngII signaling is predominantly mediated through \(G_{\alpha_q}\) dependent pathways which activate protein kinase C (PKC) and other downstream kinases.\(^{27}\) Therefore, we first tested whether AngII-induced PKC signaling in hypertension involves RGS5. PKC inhibition with chelerythrine reduces elevated blood pressure in RGS5-deficient mice to those of wild type levels (wild type untreated 82.6±3.8 versus knockout chelerythrine 83.4±5.3 mmHg). Moreover, chelerythrine abolishes AngII-induced hypertension in RGS5 knockout mice (80.3±5.0 mmHg, Figure 6A). Thus, MAP after PKC blockade changes more dramatically in knockout mice as compared to wild type controls (ΔMAP wild type 52.0±4.8 versus knockout 80.6±4.4 mmHg) implying that RGS5 regulates vessel constriction via PKC signaling. This result was confirmed with the PKC inhibitor bisindolylmaleimide I (BIM, Online Figure VII).

AngII signaling also activates the MEK/ERK pathway and loss of RGS5 enhances AngII-stimulated ERK phosphorylation in vSMC due to prolonged \(G_{\alpha_q}\) signaling.\(^{28}\) Indeed, MEK inhibition with specific inhibitors PD98059 (or U0126, Online Figure VII) reduces blood pressure in RGS5-deficient mice to wild type levels (wild type untreated 83.9±3.8 versus knockout PD98059 84.0±4.7 mmHg). PD98059 also abolishes AngII-induced hypertension in RGS5 knockout mice (85.7±4.6 mmHg, Figure 6B) leading to more dramatic changes in MAP in knockout mice than in wild type controls (ΔMAP wild type 31.7±2.2 versus knockout 76.7±3.4 mmHg).

Rho activation is a common feature in hypertension\(^{29}\) and chronic infusion with AngII increases Rho kinase activity in arteries.\(^{30}\) A regulatory role for RGS5 in Rho kinase signaling has not been reported before. Interestingly, Rho kinase-specific inhibitors (HA1077, fasudil hydrochloride or Y27632, Online Figure VII) reduce AngII-induced hypertension in RGS5 knockout mice to normotensive levels of control mice (Figure 6C, ΔMAP wild type 28.7±2.1 versus knockout 76.9±2.8 mmHg, \(P<0.0001\)). This is the first evidence demonstrating that RGS5 controls blood pressure homeostasis via Rho kinase signaling. The role of RGS5 in two major GPCR signaling pathways involving MEK/ERK and Rho kinases was confirmed in conscious mice chronically infused with AngII and treated with inhibitors (Online Figure VIII).

RGS5 differentially regulates downstream signaling of MEK/ERK and Rho kinases.

To further investigate the role of RGS5 in vessel constriction/remodeling involving MEK/ERK and Rho kinase, AngII-induced calcium release was measured with inhibitors. Figure 7A shows that the increase in Fura-2 340/380 nm fluorescence in femoral vSMC induced by AngII on a RGS5 knockout background is significantly attenuated with MEK inhibition (\(P=0.05\), wild type controls see Online Figure IX). Interestingly, the AngII-stimulated increases in Fura-2 340/380 nm fluorescence in RGS5 knockout vSMC are also reduced with Rho kinase inhibition (Figure 7B) which has been shown to regulate G protein-dependent activation of cellular Ca\(^{2+}\) entry.\(^{31}\)

Myosin light chain (MLC) is another important regulator of vessel contraction and relaxation, and a potential target for MEK and Rho kinases. Signaling through the MAP kinase pathway can activate MLC kinase (MLCK)\(^{32}\) which in turn phosphorylates and activates MLC. Rho kinase phosphorylates and inactivates MLC phosphatase which also results in activation of MLC kinase and MLC.\(^{33}\) First, we investigated whether MLC activation is enhanced in the absence of RGS5. Indeed, AngII-induced phosphorylation of MLC is increased in primary vSMC derived from RGS5 knockout.
mice (Figure 7C). Increased Rho kinase activity in the absence of RGS5 is also evident by enhanced phosphorylation of myosin phosphatase target protein 1 (MYPT1) at Thr853 (Online Figure XI). PD98059 had no significant inhibitory effect on MLC phosphorylation. However, fasudil and BIM reduce MLC activation in knockout vSMC to wild type levels (Figure 7C and Online Figure XI). Consistently, AngII-stimulated ERK phosphorylation is inhibited by PD08056 and BIM but not fasudil (Online Figure XI). These data show that vessel contraction involving Ca\(^{2+}\) transients is regulated via MEK/ERK and Rho kinase signaling whereas MLC activation is predominantly mediated by the Rho kinase pathway. In the absence of RGS5, AngII-induced responses involving both pathways are enhanced leading to increased vasoconstriction and hypertension; inhibition of key signaling mediators upstream (ATR1) and downstream of RGS5 (PKC, MEK/ERK or Rho kinase) normalizes blood pressure (Figure 7D).

Long term effects of MEK/ERK or Rho kinase inhibition in RGS5 knockout mice.

To analyse whether MEK or Rho kinase inhibition reduces long term vascular abnormalities, 2K1C mice were treated with PD98059, fasudil or vehicle 2 weeks after renal constriction for a total of 2 weeks. Consistent with acute results, both inhibitors suppress hypertension induced by chronic renin-angiotensin signaling in RGS5 knockout mice to normotensive control levels (Figure 8A). Interestingly, MEK/ERK inhibition abolished hypertrophy in renal arteries, resulting in an 80% reduction of medial thickness in knockout mice (Figure 8B). In contrast, inhibition of Rho kinase signaling substantially reduced perivascular fibrosis in kidneys with minimal effects on vessel hypertrophy over a 2 week treatment period (Figure 8C). Thus, our results indicate that hypertension and increased vascular damage with altered RGS5 signaling can be prevented by blocking cooperative functions of MEK/ERK or Rho kinase signaling.

DISCUSSION

Our study establishes RGS5 as a critical modulator of blood pressure and vascular tone. Endogenous arterial RGS5 levels decrease with chronic AngII stimulation, implying that dynamic regulation of RGS5 represents an adaptive process which controls blood pressure homeostasis. This is strongly supported by our finding that ablation of the \(rgs5\) gene causes hypertension which is enhanced with physical activity and diurnal factors. Moreover, we demonstrate here that in the absence of RGS5, chronic infusion of the potent vasoconstrictor, AngII, and creation of renal artery stenosis in the 2K1C model exacerbates hypertension. Blockade of ATR1 rapidly abolishes the hypertensive effects. This indicates that hypertension in RGS5 KO mice is likely to be caused by alterations in AngII signaling thus linking RGS5 to modulation of the renin-angiotensin system.

These findings contrast with two previous studies which reported that deletion of the \(rgs5\) gene causes hypotension.\(^{21-22}\) Here, we demonstrate a hypertensive phenotype in two different mouse strains. It is therefore unlikely that discrepancies arise due to genetic backgrounds. Furthermore, previous studies measured MAP indirectly using the tail cuff method and recorded unusually high mean arterial blood pressures of 140 mmHg for wild type controls\(^{21-22}\) and 120 mmHg for “hypotensive” knockout mice.\(^{22}\) This could be related to the tail cuff method which can affect blood pressure readings due to heat and/or restraint stress which may also render comparisons between experimental groups invalid.\(^{34}\) In contrast, we used radiotelemetry for continuous, direct blood pressure measurements and recorded 115.3±5.1 mmHg as day average MAP for RGS5 knockout mice which is clearly hypertensive when compared to physiological readings in wild type controls (83.3±2.6 mmHg). In addition, our study uses a variety of complementary analyses including blood flow and in situ signaling that reinforce our finding of a hypertensive phenotype. For instance, hypertension in RGS5 knockout mice is associated with arterial hypercontractility, resulting in increased peripheral vascular resistance. We also show that AngII induces higher Ca\(^{2+}\) transients in vSMCs isolated from RGS5-knockout mice as compared to controls indicating that RGS5 negatively regulates vSMC contraction. A similar effect has been shown in GPCR-stimulated human airway
SMCs where RGS5 knockdown results in higher intracellular Ca\(^{2+}\) levels which in vivo translates to increased bronchial contractility.\(^{35,36}\) Our data are also supported by a recent finding which identified RGS5 as target for the transcription factor peroxisome proliferator-activated receptor (PPAR)\(\gamma\). SMC-specific mutation of PPAR\(\gamma\) results in downregulation of RGS5 and enhanced myogenic tone of resistance arteries.\(^{37}\)

Arteries, like the heart, adapt to hypertension by wall remodeling, a process which includes vSMC hyperplasia or hypertrophy as well as alterations of ECM composition.\(^{38}\) Interestingly, resistance arteries in RGS5-deficient mice are stiffer than wild type controls; differential vascular stiffening is first detectable in femoral arteries (at 6 weeks) followed by mesenteric arteries in “aged” knockout mice (>30 weeks). A reduction in active and/or passive vascular compliance with aging is consistent with progressive hypertension and increased PWV as observed in RGS5 knockout mice compared to age-matched controls. Defective NO signaling in RGS5 knockout mice can also not be excluded. Moreover, renovascular complications such as vessel hypertrophy and perivascular renal fibrosis are aggravated in RGS5 knockout mice. These findings are consistent with RGS5 protecting against cardiac hypertrophy and fibrosis in aortic banding models.\(^{35}\) RGS5 may even actively regulate transition of vSMC to a more synthetic phenotype under pathological conditions as it has previously been shown to inhibit GPCR-induced hypertrophic responses in cultured vSMC whereas loss of RGS5 induces hypertrophy.\(^{28}\)

Excessive AngII-mediated contractility and vessel remodeling in the absence of RGS5 are effected by major signaling pathways downstream of ATR1 involving PKC, MEK and Rho kinase. Specific inhibition of either PKC, MEK or Rho kinase signaling in RGS5 knockout mice normalizes acute hypertension. AngII-mediated activation of PKC isoforms is classically mediated by G\(\alpha_q\) coupling which is a known target for RGS5’s GAP function.\(^{14}\) PKC activation in turn stimulates multiple downstream signaling pathways including MAP kinases. It has previously been shown that RGS5 inhibits MEK/ERK signaling in vivo which protects against cardiac hypertrophy.\(^{13}\) As shown here, RGS5 inhibition of MEK/ERK signaling is equally important to prevent hypertension, and may at least in part operate through the G\(\alpha_q\)-PKC signaling axis. Surprisingly, RGS5 also regulates Rho kinase activity. To our knowledge, this is the first report of an involvement of RGS5 in Rho kinase signaling. RGS5 may be linked to G\(\alpha_{12/13}\) by hitherto unrecognized interactions, or alternatively regulate Rho kinase via G\(\alpha_q\) proteins. Strong evidence exists for G\(\alpha_{12/13}\)-independent, G\(\alpha_q\)-mediated mechanisms of RhoA activation.\(^{39-41}\) For instance, a recent report links the RhoA guanine exchange factor Arhgef1 with AngII-induced activation of RhoA signaling in vSMC which bypasses G\(\alpha_{12/13}\). Instead, AngII activates Arhgef1 via G\(\alpha_q\) and Ca\(^{2+}\) involving phosphorylation through JAK2.\(^{42}\) Interestingly, JAK2 inhibition normalizes blood pressure in RGS5 knockout mice and could thus provide a link between RGS5 and RhoA/Rho kinase (Online Figure XII). Furthermore, our data indicate that MEK/ERK and Rho kinase pathways function most likely independently but cooperatively (e.g. Ca\(^{2+}\) increase) to increase blood pressure in the absence of RGS5. This is consistent with a model in which activation of multiple, independent signaling pathways lead to blood pressure changes in response to AngII. Antagonizing one of them is sufficient to normalize blood pressure. Interestingly, hypertension after chronic activation of the renin angiotensin system in 2K1C mice is also blocked by either MEK/ERK or Rho Kinase inhibitors. Morphologically, however, MEK/ERK inhibitors reduce vessel hypertrophy whereas Rho kinase inhibition ameliorates perivascular fibrosis over a 2 week treatment period indicating differential effects.

RGS5 is expressed in multiple organs and cell types involved in blood pressure regulation such as the nervous system, kidney, heart, and vSMC.\(^{5,8,43-44}\) Whilst we demonstrate acute and chronic changes in the vascular wall, the relative contribution of the sympathetic nervous system in regulating the hypertensive phenotype needs to be established.

It is becoming evident that the RGS5 molecule is an exquisite and non-redundant modulator of GPCR signaling with emerging clinical implications. For instance, loss of RGS5 has been shown to be crucially involved in cardiac remodeling and heart failure.\(^{13}\) Reduced RGS5 levels are also implicated in bronchial asthma by promoting airway smooth muscle hyper-responsiveness after
prolonged exposure to β2-adrenergic receptor agonists.35-36 Here we demonstrate a critical role for RGS5 in hypertension. Importantly, our study establishes a significant, distinct and causal role of RGS5 in blood pressure and vascular homeostasis. Moreover, we have identified crucial signaling mechanisms by which RGS5 effects are mediated. Supported by genetic linkage studies,18 our results strongly argue that further mechanistic exploration of RGS5 function will yield critical insights into the pathogenesis and therapy of essential, age-related and secondary causes of hypertension.

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DISCLOSURES
None.

REFERENCES


FIGURE LEGENDS

**Figure 1.** Arterial RGS5 expression inversely correlates with blood pressure changes. Wild type mice were left untreated (control) or implanted with AngII releasing minipumps (Alzet, 1 mg/kg/day) for 6 h, 1, 2, 7 and 21 days. MAP was assessed in anesthetized mice (n=6), ***P≤0.001 AngII versus untreated. Aortas were harvested, and RGS5 mRNA expression analyzed using qPCR (n=4-6), *P=0.04 control versus day 2 AngII, **P=0.03 control versus day 7 AngII, ***P≤0.001 control versus day 21 AngII, t-test.

**Figure 2.** RGS5 knockout mice develop AngII-mediated hypertension. A, Wild type (WT) and RGS5 knockout (KO) mice were implanted with radio-telemetry devices and MAP/activity recorded over 72h (n=8-12), *P≤0.0001. Average MAP values for 3 consecutive days and nights are shown, *P≤0.0001. Systolic/diastolic blood pressure is shown in Online Figure II. B, WT and KO mice with telemetry implants were acutely treated with ATR1 antagonist (20 mg/kg) and MAP recorded for 2 hours (n=6). *P≤0.001. C, WT, heterozygote RGS5 knockout (HET) and KO mice were implanted with telemetry devices. After a recovery period of 10 days (day 0), baseline MAP was recorded for 30 min/day for 3 days. On day 4, AngII pumps were implanted i.p. (1 mg/kg/day) and blood pressure recorded daily for 30 min (n=3-4), *P≤0.0001. D, Difference between baseline blood pressure and maximal blood pressure *P≤0.0001.

**Figure 3.** RGS5 regulates vascular resistance and vessel contractility. A, Anesthetized wild type (WT) and RGS5 knockout (KO) mice were acutely injected with increasing doses of AngII. MAP and blood flow in femoral arteries were measured to calculate vessel conductance (n=6, *P≤0.0001). B, Mesenteric arteries were mounted on a wire myograph and exposed to increasing concentrations of AngII. Contractions are expressed as a percentage of the contraction evoked by high potassium PSS (n=5-6, P=0.0009). C, Changes in ratiometric 340/380 nm fluorescence (340/380 fluorescence) recorded in a vSMC from femoral artery of WT and a vSMC from femoral artery of a KO mouse after stimulation with AngII as indicated. Mean of changes in 340/380 fluorescence after exposure to AngII as indicated (n=13-15 for each group, *P<0.05 AngII-induced WT versus WT basal, **P<0.05 AngII-induced KO versus KO basal, ***P<0.05 AngII-induced KO versus AngII-induced WT at same concentration). Basal levels for WT and KO were identical and set to 1.0. C, Stress-strain relationships for femoral arteries from 6 week-old WT and KO mice (n=6, *P=0.01).

**Figure 4.** Age-related increase in blood pressure and decrease in vascular distensibility in RGS5 knockout mice. A, MAP was measured acutely in anesthetized wild type (WT) and RGS5 knockout (KO) mice at 6 or 30 weeks of age (n=6, *P≤0.0001). B, Stress-strain relationships for mesenteric arteries from WT controls aged 6 weeks (young) or >30 weeks (old, n=4-6) and corresponding KO groups (n=6, *P=0.01). C, Pulse wave velocity (PWV) was measured from the aortic arch to the aortic bifurcation in 6 and 30 week-old anesthetized mice (n=6, *P≤0.0001).

**Figure 5.** Pathological features of hypertension are enhanced in RGS5 knockout mice. A, Wild type (WT) and RGS5 knockout (KO) mice were subjected to abdominal aortic banding (2K1C) and MAP acutely measured in anesthetized mice after 4 weeks (n=12, *P≤0.0001). B, 4 µm sections of left kidneys from 2K1C WT and KO mice were stained with Periodic acid-Schiff (PAS) reagents and medial hypertrophy quantified. The ratio of medial to total vessel area (x100) was determined from digital images of renal resistance arteries (200-300 µm, n=12, *P≤0.0001, **P =0.02, t-test). Original magnification, 20x, scale bar: 50 µm. C, Collagen was stained with picrosirius red as marker for perivascular fibrosis and the ratio of area of picrosirius red stain to total vessel area (%) was quantified from digital images of small renal arteries (50-70 µm, n=12, *P≤0.001, **P =0.01, t-test). Original magnification 100x, scale bar: 10 µm.

**Figure 6.** RGS5 regulates blood pressure downstream of ATR1 involving PKC, MEK/ERK and Rho kinases. A, Chelerythrine, 10 mg/kg, was injected through a catheter in the femoral vein. A second group of wild type (WT) and RGS5 knockout (KO) mice was continuously infused with AngII through the jugular vein (3 nmol/kg, 2 µl/sec). MAP was recorded 30 min before and after drug bolus
injections. Maximal MAP values and suppression for each group are shown. Effects of B, PD98059, 15 mg/kg, and C, fasudil, 20 mg/kg, on basal and AngII-induced MAP was assessed as described under A (for each group n=3, *P<0.0001).

Figure 7. RGS5 regulates intracellular Ca++ levels and myosin light chain (MLC) activation. A) Changes in 340/380 nm fluorescence recorded in a vSMC from femoral artery of a knockout (KO) mouse after pre-incubation with 25 μM PD98059 followed by stimulation with AngII, compared to a vSMC from femoral artery of a KO mouse in the absence of PD98059. Mean of changes in 340/380 fluorescence after exposure to AngII and PD98059 as indicated (ratio, n=10-14 for each group, *P<0.05). B) Changes in 340/380 fluorescence recorded as described under A after pre-incubation with 50 μM fasudil followed by stimulation with AngII. Mean of changes in 340/380 fluorescence after exposure to AngII and fasudil as indicated (ratio, n=11-14 for each group, *P<0.05 for all groups). C, Phosphorylation of myosin light chain (MLC) in femoral vSMC was quantified using in-cell western technology (LI-COR Odyssey). Cells were stimulated with 1 μM AngII for 10 min with and without PD98059, fasudil or 10 μM BIM and fluorescence intensity quantified (n=3, *P<0.05, **P<0.002, t-test, mean±SD). Results are expressed as percentage relative responses to WT AngII-treated cells which was set to 100%. Original fluorescence recordings and MLC-P basal levels are shown in Online Figure X. D, RGS5 is a negative regulator of vasoconstriction. In the absence of RGS5 (KO) AngII-mediated constriction is enhanced leading to higher MAP. Inhibition of ATR1, PKC, ERK and Rho kinase reduces MAP in KO mice to WT basal levels indicating a regulatory role for RGS5 in these pathways.

Figure 8. Differential vascular effects of MEK and Rho kinase inhibition. A, Wild type (WT) and knockout (KO) controls or 2K1C mice were treated with vehicle (DMSO), PD98059 (15 mg/kg, i.p. twice per week) or fasudil (20 mg/kg, i.p. twice per week) for 2 weeks. MAP was recorded in anesthetized mice (n=3-6, *P<0.0001). B, Sections of left kidneys from 2K1C WT and KO mice and treatment groups were stained with PAS reagent and medial hypertrophy quantified. The ratio of medial to total vessel area (x100) was determined from digital images of renal resistance arteries (100-300 μm, n=6), *P<0.005, **P<0.0001, t-test. Original magnification, 20x, scale bar: 50 μm. C, The ratio of area of picrosirius red stain to total vessel area (%) was quantified from digital images of small renal arteries (25-50 μm, n=6, *P<0.001, **P<0.0001, t-test). Original magnification 100x, scale bar: 10 μm.
Novelty and Significance

What Is Known?

- Regulator of G protein 5 (RGS5) is a member of the extended RGS family that acts as a GTPase-activating protein (GAP) for heterotrimeric G proteins.

- RGS5 is highly expressed in arteries and RGS5 knockout mice have been reported to be hypotensive based on the tail cuff method of blood pressure assessment.

- RGS5 protects against cardiac hypertrophy, which involves signaling through the MEK/ERK pathway.

What New Information Does This Article Contribute?

- Deletion of the RGS5 gene causes hypertension as measured by radio-telemetry in conscious, freely moving mice.

- RGS5 signaling is linked to the renin-angiotensin system and directly controls vascular resistance and vessel contractility involving major pathways such as PKC, MEK/ERK and Rho kinase.

- RGS5 plays a protective role in vascular remodeling and fibrosis.

- Rho kinase is an unexpected mediator of RGS5 signaling and specific inhibition controls blood pressure and fibrosis in RGS5-deficient mice.

Proteins of the RGS5 family have emerged as potent regulators of GPCRs. RGS5 is abundant in arteries and it plays a major role in pathological vessel remodeling in tumors. The reported hypotensive phenotype in RGS5 knockout mice has been difficult to reconcile with our current knowledge of RGS5 function. Using state-of-the-art and complementary methodologies we now show that RGS5 negatively regulates AngII signaling in the context of blood pressure homeostasis. We found that loss of RGS5 results in vascular hyper-responsiveness and hypertension and that lack of RGS5 significantly contributes to vascular stiffening and age-related hypertension. Our studies show that RGS5 controls multiple pathways downstream of AngII signaling, including the Rho kinase. These findings provide new insights into the pathogenesis of essential, age-related and secondary causes of hypertension.
Figure 1

[Graph showing MAP and RGS5 mRNA levels over time with control and AngII treatment.]

MAP [mmHg]

RGS5 mRNA

MAP [mmHg]

Relative RGS5 mRNA

control 6 h d 1 d 2 d 7 d 21

AngII
Figure 3
Figure 4

A

MAP [mmHg]

WT
KO

**

WT young
WT old

6 weeks 30 weeks

B

stress [kPa]

WT young
WT old

0.00 0.25 0.50 0.75 1.00

strain

C

PWV [m/s]

WT
KO

**

KO young
KO old

6 weeks 30 weeks

0.00 0.25 0.50 0.75 1.00

strain
Figure 5

A

ΔMAP [mmHg]

WT

KO

B

WT

KO

control

2K1C

C

WT

KO

control

2K1C

Medial/total vessel area x100

WT

KO

WT 2K1C

KO 2K1C

Picrosirius red/total vessel area [%]

WT

KO

WT 2K1C

KO 2K1C
Figure 7

A

PD98059

KO

KO + PD98059

0
10
20
30
40

time [min]

0.5
1.0
1.5
2.0

340/380 fluorescence

10nM AngII

100nM AngII

1μM AngII

B

fasudil

KO

KO + fasudil

0
10
20
30
40

time [min]

0.5
1.0
1.5
2.0

340/380 fluorescence

10nM AngII

100nM AngII

1μM AngII

C

% change in phosphorylation

WT

KO

AngII

+ + + + + + + +

PD98059

- - - - - - - -

fasudil

- - + - - - + +

BIM

- - - + - - + +

D

% change in % change in

ATR1

candesartan

Gq

Gq

Gq

chelerythrine,

BIM

PD98059,

U0126

enhanced vasoconstriction
Figure 8

A

MAP [mmHg]

WT

KO

control
2K1C + DMSO
2K1C + PD98059
2K1C + fasudil

B

WT

KO

2K1C + DMSO
2K1C + PD98059
2K1C + fasudil

C

WT

KO

2K1C + DMSO
2K1C + PD98059
2K1C + fasudil

Medial/total vessel area x100

Picrosirius red/total vessel area [%]

WT

KO

DMSO
PD98509
fasudil

2K1C

2K1C
Regulator of G Protein Signaling 5 Controls Blood Pressure Homeostasis and Vessel Wall Remodeling
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Regulator of G protein signaling 5 controls blood pressure homeostasis and vessel wall remodeling

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Detailed Methods

Mice. RGS5 knockout mice were generated on a mixed genetic background (129 x C57BL/6) and bred on a C3HeBFe or C57BL/6 background for more than 30 generations.¹ Female mice were analysed between 8-14 weeks unless stated otherwise. All mice were kept under specific pathogen-free conditions at the University of Western Australia. All experimental protocols involving mice were approved by the Animal Ethics Committee of the University of Western Australia. Aldosterone and renin (RIA for AngI) concentrations were measured by Royal Perth Hospital Pathology Laboratories in plasma obtained by terminal exsanguination of wild type and RGS5 knockout mice.

Drugs. The following drugs and doses were used in in vivo studies: angiotensin II (Sigma), 5 µg/kg in acute experiments, 1 mg/kg/day in minipumps; ATR1 antagonist (candesartan, Astra Zeneca), 20 mg/kg; chelerythrine chloride (Calbiochem), 10 mg/kg; bisindolylmaleimide I (BIM, Calbiochem), 10 mg/kg; PD98059 (Tocris), 15 mg/kg; U0126 (Calbiochem), 10 mg/kg; fasudil hydrochloride (Tocris), 20 mg/kg; L-NAME (Sigma), 0.5 g/L in drinking water; Y27632 (Calbiochem), 30 mg/kg. The optimal dose for each drug was determined empirically in vivo (Online Figure XIII).

Gene expression analyses. RNA from aortas was prepared using Trizol reagent (Invitrogen). Gene expression was quantified by qPCR using the Rotor Gene Real-Time PCR detection system. Primer sequences are as published² or were obtained from a public source (PrimerBank).³ All reactions were normalized to hypoxanthine-guanine phosphoribosyltransferase (hpri).

Histology and Immunohistochemistry. For histological analyses, formalin-fixed tissue was embedded in paraffin and 4 µm sections were stained with Periodic acid-Schiff (PAS) or picrosirius red following standard protocols. Images were recorded on a Nikon Ti-E microscope and quantified using NIS software modules (version 3.0).

Hemodynamic measurements. The telemetry probe (PA-C10, Data Sciences International, DSI) was implanted into mice to record blood pressure, heart rate and activity in the conscious animals as published.⁴ Under general anesthesia (100 mg/kg ketamine, 20 mg/kg xylazine), the carotid artery was exposed via a skin incision on the neck and a catheter inserted. The catheter tip was advanced into the
aortic arch and secured with sutures. Through the same ventral throat incision a subcutaneous tunnel was formed across the right pectoral area and enlarged to form a pocket along the right flank. The telemetry transmitter was placed into the pocket close to the right hind limb. Mice were treated with analgesics for 3 days (buprenorphine, 0.1 mg/kg) and were recovered for 7-10 days after surgery before recordings commenced. Data were collected using the DataQuest ART system, version 4.1 (DSI). Continuous (15 min sampling/hour) day and night recordings (12 h light to dark cycle) were collected over 72 h. Intra-arterial blood pressure under general anesthesia (urethane, 1.1 g/kg) was recorded using a transducer-tipped catheter. The right common carotid artery was catheterized with a Millar 1.4 French blood pressure probe and transducer (ADInstruments). The catheter was connected to a Millar transducer amplifier and PowerLab data acquisition system (PowerLab 16/35 and Lab Chart Pro Data Acquisition Software, ADInstruments). Vasoactive drugs were applied via intravenous bolus injections through a catheter in the femoral vein. AngII was continuously infused through the jugular vein using an infusion pump (AL-2000, World Precision Instrument Inc.). For determination of blood flow in femoral arteries, a transit-time ultrasonic system (Transonic Systems) was used. Under general anaesthesia (urethane, 1.1 g/kg), the femoral artery was exposed and a specific probe (0.5 mm PS series) placed around the artery. Flow was recorded with a T206 flowmeter (Transonic Systems).

**Pulse wave velocity.** Pulse wave velocity was measured as described with the following modifications: under general anesthesia (100 mg/kg ketamine, 20 mg/kg xylazine), a Millar micro-tip pressure transducer (1.4-Fr, ADInstruments) was inserted via the left carotid artery into the aortic arch for measuring intra-arterial blood pressure (proximal pressure wave). A Transonic probe (0.5 mm PS series) was positioned around the aorta at the iliac bifurcation to record the pressure wave outside the vessel (distal pressure wave). The length of the aorta between the two probes was measured to calculate PWV in the aortic segment according to the Bramwell-Hill equation which is commonly used in clinical studies. RGS5 knockout mice were pre-treated with a bolus injection of ATR1 antagonist (20 mg/kg) to normalize blood pressure to wild type levels.

**Minipump implantation.** Mice were anesthetized (100 mg/kg ketamine, 20 mg/kg xylazine). Osmotic minipumps (Alzet model 1004, BioScientific) were implanted i.p. following the manufacturer’s instructions. Mice were treated with analgesics for 3 days after minipump implantation (buprenorphine, 0.1 mg/kg).

**Models of hypertension.** 2K1C model of hypertension: Renal artery stenosis was introduced using a modified version of the 2K1C model. Briefly, mice were anaesthetized (100 mg/kg ketamine, 20 mg/kg xylazine) and the kidneys exposed via midline incision and deflection of the gut. Surgical silk was used to position an intra-renal snare (clip) on the descending aorta below the left kidney using a 27G (0.4 mm diameter) needle as a spacer to reduce aortic diameter by 40%. Mice were treated with analgesics for 3 days after surgery (buprenorphine, 0.1 mg/kg). Blood pressure was assessed 4 weeks later using a transducer-tipped catheter (Millar probe, ADInstruments). The left kidney is located above the clip and was therefore used as “unclipped” kidney in histology. L-NAME model of hypertension: mice received L-NAME (0.5 g/L) in drinking water for 12 days. Blood pressure was recorded before and on day 12 after L-NAME treatment using a transducer-tipped catheter (Millar probe).

**Assessment of active and passive vessel wall properties.** Mesenteric artery reactivity: First order mesenteric arteries 1-2mm in length were mounted on a 4 channel wire myograph (Danish Myo Technology, Denmark), and bathed in physiological saline at 36°C as described previously. To test smooth muscle reactivity to vasoconstrictors arteries were exposed to increasing concentrations of PE (10⁻¹⁰⁻¹⁻⁰⁴ M), AngII (10⁻¹¹⁻¹⁻⁰⁶ M), ATP (10⁻⁹⁻¹⁻⁰⁴ M) or ET (10⁻¹⁰⁻¹⁻⁰⁷ M), each applied cumulatively. Contractions were expressed as a percentage of the contraction evoked by high potassium physiological saline solution (PSS, isotonic replacement of Na⁺ with K⁺). Concentration-response curves were constructed using Prism (v.5.0; GraphPad Software, San Diego, CA, USA) and sigmoidal curves fitted to the data where applicable using the least squares method. Passive mechanical wall properties were assessed in femoral and mesenteric arteries (3-4 mm long) mounted
on a pressure myograph (Living Systems Instrumentation) as described previously. Briefly, arteries were continuously superfused at 15 mL/min with zero-Ca²⁺ physiological saline solution containing EGTA (2mM) bubbled with 95% O₂, 5% CO₂ at 36°C. Each artery was pressurised from 0 to 200 mmHg in 10 mmHg increments. Arterial dimensions (length, outside diameter and wall thickness (WT)) were measured at each 10 mmHg increment. Wall stress and strain were derived: wall stress (kPa) = (intraluminal pressure × internal diameter)/(2 × WT); wall strain = (internal diameter – internal diameter extrapolated to 5 mmHg pressure)/internal diameter extrapolated to 5 mmHg pressure.

Isolation of primary vSMC. Primary vascular smooth muscle cells were isolated using the enzyme digestion method adapted from Ray et al. Arteries from mice were dissected and cleaned of fat and connective tissue. Pooled arteries were rinsed in DMEM containing 1% antimitotic antibiotics (Invitrogen), cut into small pieces and incubated in collagenase (1.4 mg/ml, type II, Worthington Biochemical Corporation) solution for 6 h at 37°C, 5% CO₂. Cells were washed in culture media (high glucose DMEM supplemented with 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, Invitrogen), plated and maintained at 37°C in 5% CO₂ for 7-10 days. The media was changed every 2-3 days. Cells were passaged every 4-5 days and used within the first 2 passages.

Calcium measurement. Cytosolic calcium was monitored using the fluorescent indicator Fura-2AM (Fura-2, 1 μM, exc 340/380 nm, em 510 nm) in a HEPES-buffered solution (HBS) containing (in mM): KCl 5.33, MgSO₄ 0.41, NaCl 139, Na₂HPO₄ 5.63, glucose 5, HEPES 20, glutamine 2, and Ca(NO₃)₂ 2.5 (adjusted to pH 7.4 with NaOH), at 37 °C as described previously. Ratiometric Fura-2 340/380 nm fluorescence was measured using a Hamamatsu Orca ER digital camera attached to an inverted Nikon TE2000-U microscope. Metamorph 6.3 was used to quantify the signal by manually tracing cells. An equivalent region not containing cells was used as background and was subtracted. Fura-2 340/380 nm fluorescence was plotted relative to the pre-treatment fluorescence assigned a value of 1.0. Fluorescent ratios recorded over 3 min were averaged 7 min following addition of increasing concentrations of AngII (10 nM, 100 nM, 1 μM) and reported as a percentage from the baseline pre-treatment average. Inhibitors, PD9859 and fasudil (Tocris), were used at 25 and 50 μM, respectively. Baseline calibrations: 5 μM ionomycin and 5 mM Ca²⁺ were added to RGS5 wild type and knockout vSMC to obtain Rₘ₃₉. Media was then replaced with calcium free HBS supplemented with EGTA (3 mM) to obtain Rₐₙ₉. Intracellular calcium [Ca²⁺] i (nM) was determined as described previously according to the equation: [Ca] = Kₑ.b.(R₉ₐₐₙ₉)/(R₉ₐₐₙ₉-R) where R₉ₐ₉ = 1.22 ± 0.35 (WT), 0.09 ± 0.06 (KO); R₉ₐₙ₉ = 26.37 ± 5.86 (WT), 16.39 ± 3.49 (KO); b (fluorescent intensity during illumination at 380nm with 0mM calcium and 5mM calcium) = 2.52 ± 1.27 (WT), 1.55 ± 0.50 (KO) and Kₑ (dissociation constant) = 224 nM as determined previously.

In-cell western assay. Femoral vascular smooth muscle cells were seeded in clear bottom, black walled 96-well tissue culture trays (BD Biosciences). After 16-24 h serum starvation, cells were pre-incubated with inhibitors (PD98059 25 μM, fasudil 50 μM, Tocris, BIM 10 μM, Calbiochem) for 30 min and subsequently stimulated with AngII (1 μM, Sigma) for 10 min. For the in-cell western the Odyssey Infrared Imaging System (LI-COR Biosciences) was used according to the manufacturer’s instructions. Briefly, following treatment cells were fixed in 4% formaldehyde for 20 min at room temperature. Fixed cells were permeabilized with 0.1% triton-X/PBS, before blocking for 90 min. Phospho-MLC (Thr18/Ser19, Cell Signaling Technologies) and α-tubulin (Sigma) were diluted 1:50 and 1:500 respectively in blocking buffer and added to the cells overnight at 4°C. No primary antibodies were added to wells to calculate the background. The cells were washed and incubated with anti-mouse IRDye 680 (1:500) and anti-rabbit IRDye 800 (1:200) secondary antibody for 1 h at room temperature. The level of phosphorylation minus background was measured and quantified using the Li-Cor ICW module and normalized to levels of tubulin.

Ex vivo phosphorylation studies and western blot analysis. Intestinal tract and associated mesentery was removed from anaesthetized mice and incubated at 37°C in MOPS-buffered PSS plus
1 mM Na$_3$VO$_4$, 20 mM NaF, 1 mM Na$_4$P$_2$O$_7$, with or without PKC/ERK/Rho kinase inhibitors (BIM 10 μM, PD98059 25 μM, fasudil 50 μM,) for 30 min, followed by stimulation with AngII (1 μM) as described. First order mesenteric arteries (5 arteries per experimental group) were rapidly removed (within 2 min) and flash frozen in liquid nitrogen. Pooled arteries were ground to a fine powder in liquid nitrogen and incubated in ice-cold 0.1% Triton lysis buffer (10 mM HEPES, pH 7.4, 50 mM sodium pyrophosphate, 50 mM NaF, 5 mM EDTA, 5 mM EGTA, 100 μM Na$_3$VO$_4$, 50 mM NaCl, 0.1% Triton X-100, 500 μM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin) for 1 h. Insoluble material was removed by centrifugation. Equal amounts of protein were loaded on a SDS-polyacrylamide gel and transferred to a membrane. Nonspecific binding was blocked with 5% nonfat dry milk for 1 h. The blots were incubated overnight with antibodies against pERK/panERK (Cell Signaling Technologies), pMLC (Thr18/19, Cell Signaling Technologies), panMLC (Santa Cruz Biotechnology), pMYPT1 (Thr850, Millipore) and panMYPT1 (Santa Cruz Biotechnology), followed by anti-mouse or anti-rabbit-HRP conjugated secondary antibodies (Vector). Immunoreactive bands were visualized with SuperSignal chemiluminescent substrate (Pierce) and quantified by densitometry using the Quantity One software (Bio-Rad Laboratories).

**Statistical analysis.** All data are presented as mean±SEM unless otherwise stated, numbers of mice/group (n) are provided in figure legends. Comparison between groups was performed using 1-way or 2-way ANOVA followed by the Tukey post hoc test for significance unless otherwise stated. A $P$ value <0.05 was considered significant.

**Online Figure I**

**Online Figure I. RGS5 expression in hypertension.** A, Mice were submitted to abdominal aortic constriction for 28 days (2K1C) and MAP (*$P$<0.001 control versus 2K1C) and RGS5 mRNA expression levels (**$P$=0.004, control versus 2K1C, t-test) assessed (n=3). B, Mice were treated with L-NAME in drinking water for 12 days and MAP (n=6, *$P$<0.0001 control versus 2K1C)) and RGS5 mRNA expression (n=5-6, not significant) measured.
Online Figure II. Hypertension in RGS5 knockout mice. A, Blood pressure recording (systolic and diastolic) for a period of 24 h in wild type (WT, n=8), and B, in RGS5 knockout mice (KO, n=12). Data were extracted from MAP telemetry recordings shown in Figure 2. C, Mice were implanted with

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Online Figure II. Hypertension in RGS5 knockout mice. A, Blood pressure recording (systolic and diastolic) for a period of 24 h in wild type (WT, n=8), and B, in RGS5 knockout mice (KO, n=12). Data were extracted from MAP telemetry recordings shown in Figure 2. C, Mice were implanted with
radio-telemetry devices (DSI) and heart rates (bpm, beats per minute) recorded over 72 hours (n=8-12). D, WT and mice heterozygote for the RGS5 deletion (HET) were implanted with radio-telemetry devices and MAP and activity recorded over 72 hours (n=5). E, Average MAP values recorded on 3 consecutive days in free-moving, conscious C3H males, RGS5 knockout males on a C3H background (WT 83.2±3.3 versus KO 111.5±5.5 mmHg) (left), C57BL/6 females and RGS5 knockout females on a C57BL/6 background (wild type 84.3±3.5 versus knockout 113.1±3.7 mmHg) (middle), and C57BL/6 males and RGS5 knockout males on a C57BL/6 background (WT 83.3±2.8 versus KO 113.3±3.7 mmHg) (right), (n=6 for all groups, *P<0.0001). F, ATR1a/b mRNA expression levels in aorta from untreated mice (control) or mice treated with AngII for 21 days (AngII minipumps, i.p., 1 mg/kg/day) (left), ATR1a/b protein expression and quantification in untreated WT and KO aortas (right). G, RGS2 mRNA expression in aorta from untreated mice (control) or mice treated with AngII for 21 days (AngII minipumps, i.p., 1 mg/kg/day, n=4-6, *P=0.04 AngII-induced WT versus AngII-induced KO, **P=0.02 untreated KO versus AngII-induced KO). H, RGS4 mRNA expression in aorta from untreated mice (control) or mice treated with AngII for 21 days (AngII minipumps, i.p., 1 mg/kg/day, n=4-6).
Online Figure III. Contractility of RGS5-/- resistance vessels. A, Anesthetized wild type (WT) and RGS5 knockout (KO) mice (n=3) were acutely injected with increasing doses of PE (0.2, 2, 20, 100 μg/kg, *P<0.001), ATP (0.06, 0.64, 6.40 and 64 μM/kg), and ET (2.5, 25, 250 and 1000 pM/kg). MAP and blood flow in femoral arteries were measured to calculate vessel conductance. B, Mesenteric arteries from WT and KO mice (n=5-6) were mounted on a wire myograph and exposed to increasing concentration of PE (10⁻¹⁰⁻¹⁰⁻⁴ M, *P=0.02), ATP (10⁻⁹⁻¹⁰⁻⁴ M) or ET (10⁻¹⁰⁻¹⁰⁻⁷ M). Contractions are expressed as a percentage of the contraction evoked by high potassium PSS.
Online Figure IV

![Graph showing intracellular calcium levels for WT and KO vSMC.](image)

**Online Figure IV. Baseline levels of cytosolic calcium in vSMC.** Wild type (WT, n=4) and RGS5 knockout (KO, n=3) vSMC were calibrated as described in Online Materials. No significant difference in basal calcium levels were recorded between groups prior to AngII stimulation.

Online Figure V

![Graph showing stress-strain relationships for femoral arteries from WT and KO groups.](image)

**Online Figure V. Vascular stiffness increases with age in femoral arteries of both experimental groups.** Stress-strain relationships for femoral arteries from wild type (WT) controls aged 6 weeks (young) or >30 weeks (old) (n=4-6, upper panel, *P<0.0001) and corresponding knockout (KO) groups (lower panel, n=6, *P<0.0001).
**Online Figure VI.** Normalization of blood pressure with αATR1 treatment in RGS5 mice. Prior to pulse wave velocity analyses, RGS5 knockout mice (KO) were acutely treated with ATR1 antagonist (20mg/kg, KO + αATR1) which reduces blood pressure to normotensive levels (WT, wild type 84±3.9 mmHg, KO untreated 116.4±4.5 mmHg, KO treated 86.7±3.6 mmHg). One of four experimental groups (n=6) is shown as representative example, *P<0.0001.
Online Figure VII. RGS5 regulates blood pressure downstream of ATR1 involving PKC, MEK/ERK and Rho kinases. A, Bisindolylmaleimide I (BIM), 10 mg/kg, was injected i.v. through a catheter in the femoral vein. A second group of wild type (WT) and RGS5 knockout (KO) mice was continuously infused with AngII through the jugular vein (3 nmol/kg, 2 μl/sec). MAP was recorded 30 min before and after drug bolus injections. Maximal MAP values and suppression for each group are shown. Effects of B, U0126, 10 mg/kg, and C, Y27632, 30 mg/kg, on basal and AngII-induced MAP was assessed as described under A (n=3, *P<0.0001).
Online Figure VIII. Assessment of MEK and Rho kinase pathway inhibition in conscious mice. A, Wild type (WT) and knockout (KO) mice were implanted with telemetry devices. After 10 days recovery, baseline MAP was recorded for 30 min/day for 3 days. On day 4, AngII infusion pumps were implanted i.p. (1mg/kg/day) for 28 days and blood pressure recorded (n=4). Mice were i.p. injected with PD98059 (15mg/kg) or fasudil (20mg/kg). B and C, Differential blood pressure reduction in WT and KO mice after B, acute PD98059 (ΔMAP WT 35±5.1 versus KO 74.8±6.3 mmHg) or C, fasudil treatment (ΔMAP WT 38.3±4.8 versus KO 70.1±7.1 mmHg), n=4, *P≤0.0001.
Online Figure IX. **RGS5 regulates intracellular Ca\textsuperscript{2+} levels involving MEK and Rho kinase pathways in wild type vSMCs.** 

**A,** Changes in 340/380 nm fluorescence recorded in a vSMC from femoral artery of a wild type (WT) mouse after pre-incubation with 25 μM PD98059 followed by stimulation with AngII, compared to a vSMC from femoral artery of a WT mouse in the absence of PD98059. Mean of changes in 340/380 fluorescence after exposure to AngII and PD98059 as indicated (ratio, n=10-14 for each group, *P=0.05). **B,** Changes in 340/380 fluorescence recorded in a vSMC from femoral artery of a WT mouse after pre-incubation with 50 μM fasudil followed by stimulation with AngII, compared to a vSMC from femoral artery of a WT mouse in the absence of fasudil. Mean of changes in 340/380 fluorescence after exposure to AngII and fasudil as indicated (ratio, n=11-14 for each group, *P=0.05 for all groups).
Online Figure X

A, Vascular SMCs derived from femoral arteries of 6-8 mice/group were seeded in triplicates on clear bottom, black walled 96-well plates and stained for pMLC to determine basal levels; tubulin served as control. Specific antibody staining is visualized as pseudo-colored fluorescence (LI-COR Odyssey) (left). Representative wells from 3 independent experiments are shown. Quantification of results, expressed as % change in phosphorylation to WT cells which was set to 100% (right). Results are not significant.

B, Vascular SMCs derived from femoral arteries of 6-8 mice/group were seeded in triplicates on clear bottom, black walled 96-well plates, stimulated with 1 μM AngII for 10 min in the absence or presence of inhibitors as indicated and stained for pMLC; tubulin served as a control. Specific antibody staining is visualized as pseudo-colored fluorescence (LI-COR Odyssey). Representative wells from 2-3 independent experiments are shown.
Online Figure XI. RGS5-controlled signaling in mesenteric arteries. A-C, Mesenteric arteries (n=5) were left untreated and incubated in buffer or stimulated ex vivo with 1 μM AngII in the presence or absence of inhibitors followed by lysate collection and immunoblotting with the indicated antibodies. A, MYPT1-P and MYPT1, quantification of 2 independent experiments, *P<0.05, **P<0.002. B, MLC-P and MLC, and C, ERK-P and ERK.
Online Figure XII

Online Figure XII. Normalization of basal hypertension in RGS5 knockout mice by JAK2 inhibition. **A.** In vivo dose-response assessment for JAK2 inhibitor (A490). Wild type (WT) and knockout (KO) mice were injected i.v. with escalating doses of JAK2 inhibitor through a catheter in the femoral vein (1, 10, 100 mg/kg). **B.** JAK2 inhibitor, 10 mg/kg, was injected through a catheter in the femoral vein. A second group of WT and KO mice was continuously infused with AngII through the jugular vein (3 nmol/kg, 2 µl/sec). MAP was recorded 30 min before and after drug bolus injections. Maximal MAP values and suppression for each group are shown (for each group n=3, *P≤0.0001).
Online Figure XIII. In vivo dose response assessment for signaling inhibitors. Wild type (WT) and knockout (KO) mice were injected i.v. through a catheter in the femoral vein with the following inhibitors: A, PKC inhibitors chelerythrine chloride (1, 10, 100 mg/kg) and BIM (1, 10, 50 mg/kg); B, MEK inhibitors PD98059 (1, 10, 30 mg/kg) and U0126 (1, 10, 50 mg/kg); C, Rho kinase inhibitors fasudil hydrochloride (2, 20, 100 mg/kg) and Y27632 (3, 30, 100 mg/kg). MAP was recorded 30 min before and after drug bolus injections. Maximal MAP values and suppression for each dose are shown (n=3 for all groups).
Online Table I: Renal/cardiac-specific parameters in RGS5 wild type and knockout mice

<table>
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<th>mouse strain</th>
<th>number of mice</th>
<th>plasma aldosterone [pmol/L]</th>
<th>plasma renin concentration [ng AngI/ml/h]</th>
<th>heart weight/body weight [mg/g]</th>
<th>age [weeks]</th>
</tr>
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<tbody>
<tr>
<td>RGS5 wild type</td>
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<td>755±332</td>
<td>923±98</td>
<td>4.9±0.8</td>
<td>12</td>
</tr>
<tr>
<td>RGS5 knockout</td>
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<td>850±571</td>
<td>1001±126</td>
<td>4.6±1.2</td>
<td>12</td>
</tr>
</tbody>
</table>

Supplemental References

11. Viola HM, Arthur PG, Hool LC. Transient exposure to hydrogen peroxide causes an increase in mitochondria-derived superoxide as a result of sustained alteration in l-type ca2+ channel function in the absence of apoptosis in ventricular myocytes. *Circ Res*. 2007;100:1036-1044.