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

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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 nonredundant role in the pathogenesis of hypertension and to 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 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 protein kinase C, mitogen-activated protein kinase/extracellular signal–regulated kinase, 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 hyperresponsiveness 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 and major Gαs-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. (Circ Res. 2013;112:781-791.)

Key Words: animal model of cardiovascular disease ■ arterial stiffness ■ G-protein-coupled receptor signaling ■ hypertension ■ vascular smooth muscle ■ vessel wall remodeling

Blood vessels consist of 2 major cell types, endothelial and mural cells, such as pericytes and vascular smooth muscle cells (VSMC), which surround the endothelium. Regulator of G-protein signaling 5 (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 adults.

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 that confers the catalytic function for active Gα subunits. Members of the R4/B subfamily, which include, among others, RGS 2, 4, and 5, are the smallest RGS...
RGS5 Levels Are Reduced With Chronically Elevated Blood Pressure

The vasoactive peptide of the renin–angiotensin system, 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, WT mice were infused with AngII (1 mg/kg per day) for 21 days. As expected, this treatment resulted in a slow rise of MAP with a 38% increase at end point (untreated 82.7±2.8 vs treated 113.9±2.6 mm Hg; Figure 1). For the first 2 days, blood pressure remained unchanged. From day 2 onward, however, RGS5 levels were significantly reduced and further decreased over time. Interestingly, a decline in RGS5 levels preceded changes in blood pressure, which became apparent on day 7. Reduced RGS5 expression also was observed in a physiological model of renin–angiotensin-mediated hypertension (modified 2K1C) but not after L-NAME (N-nitro-l-arginine methyl ester) infusion (Online Figure I). Thus, arterial RGS5 levels inversely correlated 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 signaling, 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 angiotensin II (AngII)-releasing minipumps (Alzet, 1 mg/kg per day) for 6 hours and 1, 2, 7, and 21 days. Mean arterial blood pressure (MAP) was assessed in anesthetized mice (n=6); **P=0.001 AngII vs untreated). Aortas were harvested, and RGS5 mRNA expression was analyzed using quantitative polymerase chain reaction (n=4–6). ***P=0.001 control vs day 2 AngII; **P=0.03 control vs day 7 AngII; ***P=0.001 control vs day 21 AngII (t test).

**Figure 1.** Arterial regulator of G protein signaling 5 (RGS5) expression inversely correlates with blood pressure changes. Wild-type mice were left untreated (control) or implanted with angiotensin II (AngII)-releasing minipumps (Alzet, 1 mg/kg per day) for 6 hours and 1, 2, 7, and 21 days. Mean arterial blood pressure (MAP) was assessed in anesthetized mice (n=6); **P=0.001 AngII vs untreated). Aortas were harvested, and RGS5 mRNA expression was analyzed using quantitative polymerase chain reaction (n=4–6). ***P=0.001 control vs day 2 AngII; **P=0.03 control vs day 7 AngII; ***P=0.001 control vs day 21 AngII (t test).
with radiotelemetry devices and diurnal variations in blood pressure, heart rate, and locomotor activity measured over 72 hours. MAP in RGS5 KO mice is significantly higher than in WT controls during the day (WT 83.3±2.6 vs KO 115.3±5.1 mmHg; ΔMAP, 32 mmHg; \(P\leq0.0001\)). Murine blood pressure shows circadian variations and increases at night when mice become active. Interestingly, KO mice showed a significantly higher nocturnal blood pressure increase over WT mice (WT 102.0±2.5 vs KO 150.7±4.4 mmHg; \(\Delta\)MAP, 48.6 mmHg; \(P\leq0.0001\)), even though locomotor activity did 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 were recorded for both male and female RGS5 KO mice on 2 different genetic backgrounds (C3H and C57BL/6), whereas heterozygote deletion of RGS5 did not result in hypertension (Online Figure II). Moreover, acute treatment of RGS5-deficient mice with an AngII receptor 1 (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 per day) for 21 days increased blood pressure more profoundly in RGS5-deficient mice than in WT controls (ΔMAP WT 29.2±2.1 vs KO 51.4±3.1 mmHg; Figure 2C and 2D; \(P\leq0.0001\)), thus supporting a role for RGS5 within the renin–angiotensin signaling pathway. Loss of 1 RGS5 allele in heterozygote RGS5 mice is sufficient to increase blood pressure above that of WT mice under chronic AngII infusion. ATR1 receptor levels are unchanged in RGS5 KO mice, which supports a postreceptor regulatory mechanism (Online Figure II). Also, RGS2 and RGS4 expressions are unchanged in unchallenged RGS5 KO mice. Under AngII infusion, RGS2 is significantly upregulated in RGS5-deficient mice (Online Figure II; \(P\leq0.04\)). 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.24

**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 KO and WT 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 KO mice more profoundly than in WT controls (Figure 3A). These findings imply that RGS5 directly regulates vascular resistance, most likely by affecting vessel contractions. Significantly enhanced contraction in response to AngII was measured in mesenteric vessels from RGS5 KO mice (Figure 3B; \(P\leq0.0009\)). Enhanced vessel conductance and contractility also were observed with phenylephrin stimulation but not with endothelin or 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 VSMC derived from femoral arteries of WT and RGS5 KO mice were stimulated with AngII in a dose-dependent manner. Before stimulation with AngII, there was no difference in basal calcium levels in VSMC from WT and KO mice (Online Figure IV). Addition of AngII,
however, increased Fura-2 340/380 nm fluorescence in RGS5-deficient VSMC by 25% (Figure 3C). These findings establish a direct link between RGS5 expression and Ca2+-dependent vessel contraction, vascular resistance, and hypertension. Because vascular contractility and vessel wall remodeling are crucial in the development of hypertension, we hypothesized that chronic vasoconstriction in the absence of RGS5 also may cause structural alterations

Figure 3. Regulator of G protein signaling 5 (RGS5) regulates vascular resistance and vessel contractility.
A, Anesthetized wild-type (WT) and RGS5 knockout (KO) mice were acutely injected with increasing doses of angiotensin II (AngII). Mean arterial pressure (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 physiological salt solution (n=5–6; P=0.0009).
C, Changes in ratiometric 340/380 nm fluorescence (340/380 fluorescence) recorded in vascular smooth muscle cells (VSMCs) from femoral artery of WT and VSMCs 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 vs WT basal; **P<0.05; AngII-induced KO vs KO basal; ***P<0.05; AngII-induced KO vs 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 Regulator of G protein signaling 5 (RGS5) knockout mice. A, Mean arterial pressure (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).
in the vessel wall. To assess the passive mechanical wall properties, stress–strain relationships for arteries from 6-week-old WT and KO mice were determined. The stress–strain relationship for femoral arteries from RGS5 KO mice is significantly shifted to the left compared with WT (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) WT and KO mice was compared. In WT mice, changes in MAP between weeks 6 and 30 were not significant (6 weeks 82.3±5.4 vs 30 weeks 88.4±6.6 mm Hg). In contrast, blood pressure in KO mice increased significantly by 22% at 30 weeks as compared with measurements at 6 weeks of age (Figure 4A; 6 weeks 93.0±3.1 vs 30 weeks 119.3±7.3 mm Hg; $P<0.0001$). Age-related MAP differences in hypertensive RGS5-deficient mice may relate, in part, to increased stiffness of resistance arteries. Interestingly, although femoral arteries stiffen with age in WT and KO groups alike (Online Figure V), mesenteric arterial stiffness increases significantly ($P=0.01$) with age (>30 weeks) in KO 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.

A high PWV indicates lack of elasticity with a fast pulse wave moving along the aorta as measured from carotid to iliac arteries. To control for existing blood pressure differences between WT and KO groups, 6- and 30-week-old RGS5-deficient mice were acutely treated with ATR1 inhibitor before PWV recordings (Online Figure VI). Figure 4C shows that PWV is significantly enhanced in all KO groups compared with WT and also increased with age (6 weeks: WT 2.3±0.3 vs KO 3.5±0.6 m/s; 30 weeks: WT 2.4±0.5 vs KO 4.4±0.6 m/s; $P<0.0001$). Age-related decrease in aortic distensibility likely reflects 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 attributable to changes in extracellular matrix components and 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 became elevated in both strains. However, in RGS5 KO mice, blood pressure was increased by 50% compared with WT controls, indicative of an excessive adaptive response ($\Delta$MAP: WT 43.5±5.2 vs KO 86.2±6.1 mm Hg; Figure 5A). Moreover, medial areas of renal resistance arteries (size 200–300 μm) were significantly enlarged in 2K1C RGS5 KO mice, consistent with medial hypertrophy in more severe hypertension (Figure 5B; $P<0.0001$). Chronic hypertension also may lead to perivascular extracellular matrix changes and may contribute to inward remodeling of blood vessels. In kidneys of 2K1C RGS5 KO mice, the area of perivascular fibrosis compared with total vessel area was 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$_\text{q}$-dependent pathways, which activate protein kinase C (PKC) and other downstream kinases. Therefore, we first tested whether AngII-induced PKC signaling in hypertension involves RGS5. PKC inhibition with chelerythrine reduced elevated blood pressure in RGS5-deficient mice to levels of those in WT mice (WT untreated 82.6±3.8 vs KO chelerythrine 83.4±5.3 mm Hg). Moreover, chelerythrine abolishes AngII-induced hypertension in RGS5 KO mice (80.3±5.0 mm Hg; Figure 6A). Thus, MAP after PKC blockade changes more dramatically in KO mice as compared with WT controls ($\Delta$MAP; WT 52.0±4.8 vs KO 80.6±4.4 mm Hg), implying that RGS5 regulates vessel constriction via PKC signaling. This result was confirmed with the PKC inhibitor bisindolylmaleimide I (Online Figure VII).

AngII signaling also activates the mitogen-activated protein kinase (MEK)/extracellular signal–regulated kinase (ERK) pathway, and loss of RGS5 enhances AngII-stimulated ERK phosphorylation in VSMC because of prolonged G$_\text{q}$ signaling. MEK inhibition with specific inhibitors PD98059 (or U0126; Online Figure VII) reduces blood pressure in RGS5-deficient mice to WT levels (WT untreated 83.9±3.8 vs KO PD98059 84.0±4.7 mm Hg). PD98059 also abolishes AngII-induced hypertension in RGS5 KO mice (85.7±4.6 mm Hg; Figure 6B), leading to more dramatic changes in MAP in KO mice than in WT controls ($\Delta$MAP; WT 31.7±2.2 vs KO 76.7±3.4 mm Hg).

Rho-kinase activation is a common feature in hypertension, and chronic infusion with AngII increases Rho kinase activity in arteries. 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 KO mice to normotensive levels of control mice (Figure 6C; $\Delta$MAP; WT 28.7±2.1 vs KO 76.9±2.8 mm Hg; $P<0.0001$). This is the first evidence demonstrating that RGS5 controls blood pressure homeostasis via Rho kinase signaling. The role of RGS5 in 2 major GPCR signaling pathways involving MEK/ERK and 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 Kinase

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 KO background is significantly attenuated with MEK inhibition \((P=0.05);\) WT controls; Online Figure IX). Interestingly, the AngII-stimulated increases in Fura-2 340/380 nm fluorescence in RGS5 KO 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 kinase. Signaling through the mitogen-activated protein kinase pathway can activate MLC kinase,\(^32\) 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. AngII-induced phosphorylation of MLC is increased in primary VSMCs derived from RGS5 KO mice (Figure 7C). Increased Rho kinase activity in the absence of RGS5 also is evident by enhanced phosphorylation of myosin phosphatase target protein 1 at Thr853 (Online Figure XI). PD98059 had no significant inhibitory effect on MLC phosphorylation. However, fasudil and bisindolylmaleimide I reduce MLC activation in KO VSMCs to WT levels (Figure 7C and Online Figure XI). Consistently, AngII-stimulated ERK phosphorylation is inhibited by PD08056 and bisindolylmaleimide I, 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 were 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 analyze 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 KO 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 KO mice.
AngII (P < 0.0001). (C) fasudil, 20 mg/kg, on basal and AngII-induced MAP were each group are shown. Effects of (B) PD98059, 15 mg/kg, and drug bolus injections. Maximal MAP values and suppression for arterial pressure (MAP) was recorded 30 minutes before and after μ (WT) and RGS5 knockout (KO) mice was continuously infused a catheter in the femoral vein. A second group of wild-type, Chelerythrine, 10 mg/kg, was injected through Rho kinases. A protein kinase/extracellular signal regulated kinase, and 1 (ATR1) involving protein kinase C, mitogen-activated blood pressure downstream of angiotensin II (AngII) receptor

**Figure 6.** Regulator of G protein signaling 5 (RGS5) regulates blood pressure downstream of angiotensin II (AngII) receptor 1 (ATR1) involving protein kinase C, mitogen-activated protein kinase/extracellular signal regulated kinase, 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). Mean arterial pressure (MAP) was recorded 30 minutes 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 were assessed as described in (A) for each group n=3; *P<0.0001).

(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 that 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 exacerbate 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 2 previous studies that reported that deletion of the RGS5 gene causes hypotension. Here, we demonstrate a hypertensive phenotype in 2 different mouse strains. It is, therefore, unlikely that discrepancies arise because of genetic backgrounds. Furthermore, previous studies measured MAP indirectly using the tail-cuff method and recorded unusually high MAPs of 140 mm Hg for WT controls and 120 mm Hg for hypotensive KO mice. This could be related to the tail-cuff method that can affect blood pressure readings because of heat and restraint stress, which also may render comparisons between experimental groups invalid. In contrast, we used radiotelemetry for continuous direct blood pressure measurements and recorded 115.3±5.1 mm Hg as the day average MAP for RGS5 KO mice, which is clearly hypertensive when compared with physiological readings in WT controls (83.3±2.6 mm Hg). In addition, our study used a variety of complementary analyses, including blood flow and in situ signaling, that reinforced our finding of a hypertensive phenotype. For instance, hypertension in RGS5 KO mice is associated with arterial hypercontractility, resulting in increased peripheral vascular resistance. We also show that AngII induces higher Ca²⁺ transients in VSMCs isolated from RGS5 KO mice as compared with controls, indicating that RGS5 negatively regulates VSMC contraction. A similar effect has been shown in GPCR-stimulated human airway SMCs In which RGS5 knockdown resulted in higher intracellular Ca²⁺ levels, which in vivo translates to increased bronchial contractility. Our data also are supported by a recent finding that identified RGS5 as a target for the transcription factor peroxisome proliferator–activated receptor-γ. SMC–specific mutation of peroxisome proliferator–activated receptor-γ results in downregulation of RGS5 and enhanced myogenic tone of resistance arteries. Arteries, like the heart, adapt to hypertension by wall remodeling, a process that includes VSMC hyperplasia or hypertrophy, as well as alterations of extracellular matrix composition. Interestingly, resistance arteries in RGS5-deficient mice are stiffer than those in WT controls; differential vascular stiffening is first detectable in femoral arteries (at 6 weeks), followed by mesenteric arteries in aged KO mice (>30 weeks). A reduction in active and passive vascular compliance with aging is consistent with progressive hypertension and increased PWV, as observed in RGS5 KO mice compared with age-matched controls. Defective nitric oxide signaling in RGS5 KO mice also cannot be excluded. Moreover, renovascular complications, such as vessel hypertrophy and perivascular renal fibrosis, are aggravated in RGS5 KO mice. These findings are consistent with RGS5 protecting against cardiac hypertrophy and fibrosis in aortic banding models. RGS5 may even actively regulate transition of VSMC to a more synthetic phenotype under pathological
GAP function. PKC activation, in turn, stimulates multiple cardiac hypertrophy. As shown here, RGS5 inhibition inhibits MEK/ERK signaling in vivo, which protects against protein kinase. It previously has been shown that RGS5 downstream signaling pathways, including mitogen-activated mediated by Gα

AngII-mediated activation of PKC isoforms is classically Rho kinase. Specifically, a recent report links the RhoA guanine exchange factor, ARHGEF1, with AngII-induced activation of RhoA signaling in VSMCs, which bypasses Gα12/13. Instead, AngII activates ARHGEF1 via Gαi and Ca2+, involving phosphorylation through janus kinase 2. Interestingly, janus kinase 2 inhibition normalizes blood pressure in RGS5 KO mice and thus could provide a

Figure 7. Regulator of G protein signaling 5 (RGS5) regulates intracellular Ca++ levels and myosin light chain (MLC) activation. A, Changes in 340/380 nm fluorescence recorded in a vascular smooth muscle cell (VSMC) from femoral artery of a knockout (KO) mouse after preincubation with 25 μmol/L PD98059 followed by stimulation with angiotensin II (AngII) compared with 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 in (A) after preincubation with 50 μmol/L 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 MLC in femoral VSMC was quantified using in-cell Western technology (LI-COR Odyssey). Cells were stimulated with 1 μmol/L AngII for 10 minutes with and without PD98059, fasudil, or 10 μmol/L bisindolylmaleimide I (BIM) and fluorescence intensity was 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 were set to 100%. Original fluorescence recordings and basal levels of phosphorylated MLC (MLC-P) 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 mean arterial pressure (MAP). Inhibition of AngII receptor 1 (ATR1), protein kinase C, extracellular signal–regulated kinase, and Rho kinase reduces MAP in KO mice to WT basal levels, indicating a regulatory role for RGS5 in these pathways. MYPT-1 indicates myosin phosphatase target protein-1.

conditions because it previously has 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 affected by major signaling pathways downstream of ATR1 involving PKC, MEK, and Rho kinase. Specific inhibition of PKC, MEK, or Rho kinase signaling in RGS5 KO mice normalizes acute hypertension. AngII-mediated activation of PKC isoforms is classically mediated by Gαq coupling, which is a known target for RGS5 GAP function.14 PKC activation, in turn, stimulates multiple downstream signaling pathways, including mitogen-activated protein kinase. It previously has 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 Gαq-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 Gα12/13 by hitherto unrecognized interactions or alternatively regulated Rho kinase via Gαi proteins. Strong evidence exists for Gα12/13-independent Gαq-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 VSMCs, which bypasses Gα12/13. Instead, AngII activates ARHGEF1 via Gαi and Ca2+, involving phosphorylation through janus kinase 2.42 Interestingly, janus kinase 2 inhibition normalizes blood pressure in RGS5 KO mice and thus could 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., Ca²⁺ 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.⁵,⁸,¹⁴ Although 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 nonredundant 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 also are implicated in bronchial asthma by promoting airway smooth muscle hyperresponsiveness 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 in the pathogenesis and therapy of essential, age-related, and secondary causes of hypertension.

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**Disclosures**

None.
References


**Novelty and Significance**

**What Is Known?**

- Regulator of G-protein signaling 5 (RGS5) is a member of the extended RGS family that acts as a GTPase-activating protein 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 mitogen-activated protein kinase/extracellular signal-regulated kinase pathway.

**What New Information Does This Article Contribute?**

- Deletion of the RGS5 gene causes hypertension as measured by radiotelemetry 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 protein kinase C, mitogen-activated protein kinase/extracellular signal–regulated kinase, 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 G-protein-coupled receptors. RGS5 is abundant in arteries and it plays a major role in pathological vascular 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 angiotensin II (AngII) signaling in the context of blood pressure homeostasis. We found that loss of RGS5 results in vascular hyperresponsiveness 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.
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 C3HeB/Fe 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 (hprt).

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 36ºC as described previously. To test smooth muscle reactivity to vasoconstrictors arteries were exposed to increasing concentrations of PE (10^{-10}-10^{-4} M), AngII (10^{-11}-10^{-6} M), ATP (10^{-9}-10^{-4} M) or ET (10^{-10}-10^{-7} 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-Ca\(^{2+}\) physiological saline solution containing EGTA (2mM) bubbled with 95% \(\text{O}_2\), 5% \(\text{CO}_2\) 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 \times \text{internal diameter})/(2 \times \text{WT}); wall strain = (\text{internal diameter} – \text{internal diameter extrapolated to 5 mmHg pressure})/\text{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.10 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% CO\(_2\). 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% CO\(_2\) 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, \(\lambda_{ex}\) 340/380 nm, \(\lambda_{em}\) 510 nm) in a HEPES-buffered solution (HBS) containing (in mM): KCl 5.33, MgSO\(_4\) 0.41, NaCl 139, Na\(_2\)HPO\(_4\) 5.63, glucose 5, HEPES 20, glutamine 2, and Ca(NO\(_3\))\(_2\) 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 Ca\(^{2+}\) were added to RGS5 wild type and knockout vSMC to obtain \(R_{\text{min}}\). Media was then replaced with calcium free HBS supplemented with EGTA (3 mM) to obtain \(R_{\text{min}}\). Intracellular calcium [Ca\(^{2+}\)] (nM) was determined as described previously according to the equation: \[ [\text{Ca}^{2+}] = K_d \cdot b \cdot (R - R_{\text{min}})/(R_{\text{max}} - R) \] where \(R_{\text{min}} = 1.22 \pm 0.35 \text{ (WT)}\), 0.09 ± 0.06 (KO); \(R_{\text{max}} = 26.37 \pm 5.86 \text{ (WT)}\), 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 \(K_d\) (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 Na₃VO₄, 20 mM NaF, 1 mM Na₄P₂O₇, 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.¹ Three 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 Na₃VO₄, 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

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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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

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

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^{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 X. In-cell western for detection of MLC phosphorylation.** 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

**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

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<th>heart weight/body weight [mg/g]</th>
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<td>1001±126</td>
<td>4.6±1.2</td>
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</table>

**Supplemental References**


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