

The Rho exchange factor Arhgef1 mediates the effects of angiotensin II on vascular tone and blood pressure

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Hypertension is one of the most frequent pathologies in the industrialized world. Although recognized to be dependent on a combination of genetic and environmental factors, its molecular basis remains elusive. Increased activity of the monomeric G protein RhoA in arteries is a common feature of hypertension. However, how RhoA is activated and whether it has a causative role in hypertension remains unclear. Here we provide evidence that Arhgef1 is the RhoA guanine exchange factor specifically responsible for angiotensin II-induced activation of RhoA signaling in arterial smooth muscle cells. We found that angiotensin II activates Arhgef1 through a previously undescribed mechanism in which Jak2 phosphorylates Tyr738 of Arhgef1. Arhgef1 inactivation in smooth muscle induced resistance to angiotensin II-dependent hypertension in mice, but did not affect normal blood pressure regulation. Our results show that control of RhoA signaling through Arhgef1 is central to the development of angiotensin II-dependent hypertension and identify Arhgef1 as a potential target for the treatment of hypertension.

High blood pressure is an important health concern and a major risk factor for cardiovascular morbidity and mortality¹. Both the pathogenesis of arterial hypertension and the molecular mechanisms involved in blood pressure control remain poorly understood. Hypertension is associated with several physiological and biochemical changes in the vessel wall, including excessive vessel contraction and hypertrophy and hyperplasia of smooth muscle cells, resulting in increased peripheral vascular resistance. Central to these processes is the renin-angiotensin-aldosterone system, a prime candidate as a causative factor in the development and maintenance of hypertension. Angiotensin II (Ang II), considered the main mediator of this system, acts directly on vascular smooth muscle as a potent vasoconstrictor, thereby regulating vascular tone. Ang II also alters renal sodium and water absorption through its ability to stimulate synthesis and secretion of aldosterone. Furthermore, it enhances thirst and stimulates the secretion of vasopressin. Consequently, Ang II has a crucial role in both the acute and chronic regulation of blood pressure. The Ang II type 1 (AT1) receptor, a G protein-coupled receptor, mediates most of the physiological and pathophysiological actions of Ang II^{2,3}.

Increasing evidence implicates overactivation of the monomeric G protein RhoA as a critical component of the pathogenesis of hypertension in several experimental models^{4–7} and in humans⁸. RhoA is a molecular switch that cycles between an inactive, GDP-bound form and an active, GTP-bound form⁹. The active, GTP-bound form triggers activation of its effector Rho kinase, which phosphorylates the myosin phosphatase target subunit (MYPT) and thereby inhibits the activity of myosin light chain phosphatase. This inhibition results in Ca²⁺ sensitization of contractile proteins, which underlies the tonic

component of vascular smooth muscle contraction¹⁰. Pharmacological analyses using Rho kinase inhibitors suggest that RhoA-dependent pathways are involved in the increased vascular resistance associated with hypertension^{7,11}. Inhibition of the AT1 receptor reduces the upregulation of RhoA and Rho kinase activity in hypertensive rats^{12,13}, and *in vivo* long-term infusion of Ang II increases the activity of RhoA and Rho kinase in arteries¹⁴, suggesting a causative role for Ang II-induced activation of RhoA and Rho kinase in the development of hypertension.

These observations lead to the hypothesis that inhibiting RhoA activation could reduce the development of Ang II-dependent hypertension. Because guanine nucleotide exchange factors (GEFs) mediate the activation of Rho proteins by exchanging GDP for GTP^{15,16} and are themselves activated by upstream signals, such as G protein-coupled receptors, tyrosine kinase receptors and adhesion receptors, we first aimed to identify the GEF(s) responsible for Ang II-induced RhoA activation in vascular smooth muscle cells (VSMCs). After identifying Arhgef1 as the GEF that specifically mediates Ang II-dependent RhoA activation, we uncovered the mechanism by which Arhgef1 is activated by Ang II signaling in VSMC, and provided evidence that this activation has a causal role in the development of hypertension.

RESULTS

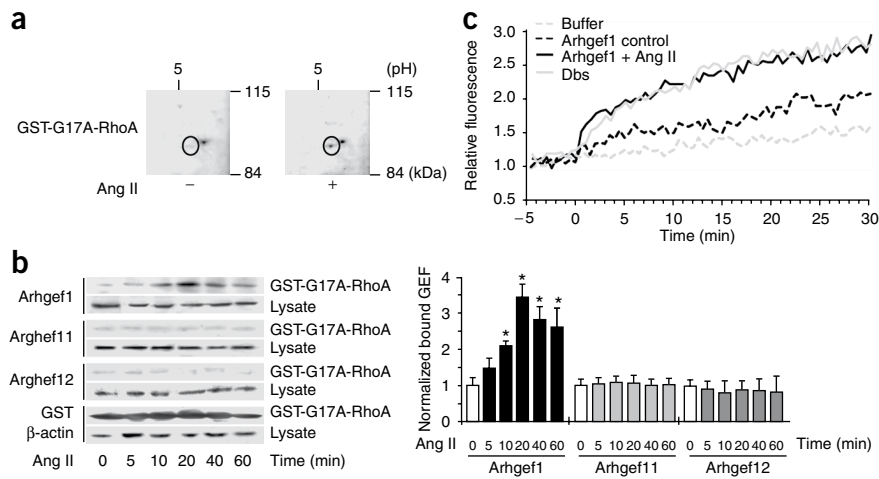
Arhgef1 is activated by Ang II in VSMCs

We first developed a proteomics approach to identify RhoA GEFs activated by Ang II. We used the G17A-RhoA mutant, which preferentially binds to active RhoA GEFs¹⁷, to identify interacting proteins in VSMCs with and without Ang II treatment. After incubating nucleotide-free

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Figure 1 The RhoA exchange factor Arhgef1 is activated by Ang II in rat aortic smooth muscle cells and mediates Ang II-induced RhoA activation. **(a)** Representative Coomassie blue-stained two-dimensional gels of proteins from unstimulated VSMCs (–) and VSMCs stimulated by Ang II (0.1 μ M, 40 min) (+), precipitated with the G17A-RhoA mutant, which preferentially binds to active RhoA GEF. Circles show Arhgef1. **(b)** Immunoblot of proteins from unstimulated and Ang II-stimulated VSMCs precipitated with G17A-RhoA, using antibodies to Arhgef1, Arhgef11 and Arhgef12. Graph shows densitometric analysis of immunoblots (mean \pm s.e.m. of four independent experiments; * P < 0.01 versus unstimulated condition). White bars indicate the unstimulated condition. **(c)** *In vitro* guanine nucleotide exchange activity on RhoA of Arhgef1 immunoprecipitated from cells stimulated by Ang II (0.1 μ M, 40 min) and from control cells. Arhgef1 was added at 0 min. Enhancement of fluorescence intensity indicates guanine nucleotide exchange on RhoA. Guanine nucleotide exchange activity is expressed as relative fluorescence, with the initial fluorescence intensity set as 1. The Rho GEF Dbs is used as positive control; curve labeled buffer corresponds to the basal exchange activity on RhoA in the absence of a GEF. Curves are representative of four independent experiments.



G17A-RhoA with VSMC lysates, we precipitated G17A-RhoA and analyzed coprecipitated proteins by two-dimensional electrophoresis. The protein whose coprecipitation was most consistently induced by Ang II had a molecular weight of 110 kDa and was identified as the RhoA-specific GEF Arhgef1 (also known as p115 or Lsc) (**Fig. 1a** and **Supplementary Table 1**). Arhgef1 belongs to the Dbl-Rho GEF family, characterized by a pleckstrin homology domain adjacent and C-terminal to the catalytic Dbl homology domain¹⁶. Arhgef1, Arhgef11 (PDZ-Rho GEF) and Arhgef12 (Larg) constitute the small

subfamily of regulator of G-protein signaling (RGS)-containing Rho GEFs¹⁵. To confirm this result, we performed identical experiments in which we blotted proteins that coprecipitated with G17A-RhoA with an antibody to Arhgef1. Ang II enhanced the association between endogenous Arhgef1 and G17A-RhoA, indicating increased Arhgef1 activity (**Fig. 1b**). This effect was specific, as we did not detect Ang II-induced activation of Arhgef11 or Arhgef12 (**Fig. 1b**).

Ang II-induced Arhgef1 activation was further confirmed using an *in vitro* guanine nucleotide exchange assay on RhoA. Whereas similar

Figure 2 Ang II-induced Arhgef1 activation is mediated by Tyr738 phosphorylation of Arhgef1. **(a)** Autoradiography and corresponding Coomassie blue gel showing phosphorylation of endogenous Arhgef1 immunoprecipitated (IP) from VSMCs after Ang II stimulation (20–60 min) and the effect of the AT1 receptor antagonist losartan (1 μ M). **(b)** Autoradiography and corresponding Coomassie blue gel showing phosphorylation of immunoprecipitated WT-Arhgef1 and phosphoresistant Arhgef1 mutants expressed in unstimulated (–) and Ang II-stimulated VSMCs (+). WT-Arhgef1 and Arhgef1 mutants were expressed as fusion proteins with the AU1 tag and immunoprecipitated with antibody to AU1. **(c)** Immunoblot (WB) analysis of Ang II-induced phosphorylation of the Rho kinase target MYPT in VSMCs expressing AU1-tagged WT-Arhgef1 and Arhgef1 mutants. Expression of the different Arhgef1 variants was determined by immunoblotting with antibody to AU1. **(d–g)** *In vitro* exchange assay on RhoA of WT-Arhgef1 and various mutants immunoprecipitated with antibody to AU1 from control or Ang II-stimulated VSMC. Arhgef1 was added at 0 min. The curve labeled buffer indicates the spontaneous exchange rate on RhoA in the absence of a GEF. Guanine nucleotide exchange activity is expressed as relative fluorescence, with the initial fluorescence intensity set as 1. Curves are representative of four independent experiments. **(h)** Proteins that bound to GST-G17A-RhoA were pulled down from lysates of rat aortic smooth muscle cells expressing AU1-tagged WT-Arhgef1, Y738F-Arhgef1 or Y738E-Arhgef1. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and GST. Graph shows densitometric analysis of the immunoblots (mean \pm s.e.m. of five independent experiments; * P < 0.001 versus unstimulated condition). White bars indicate the unstimulated condition.

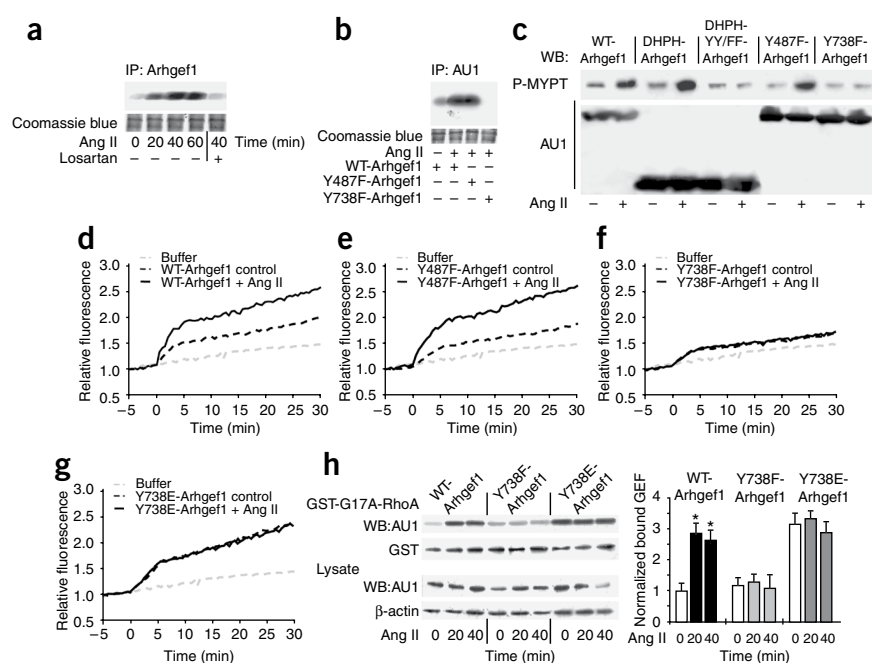
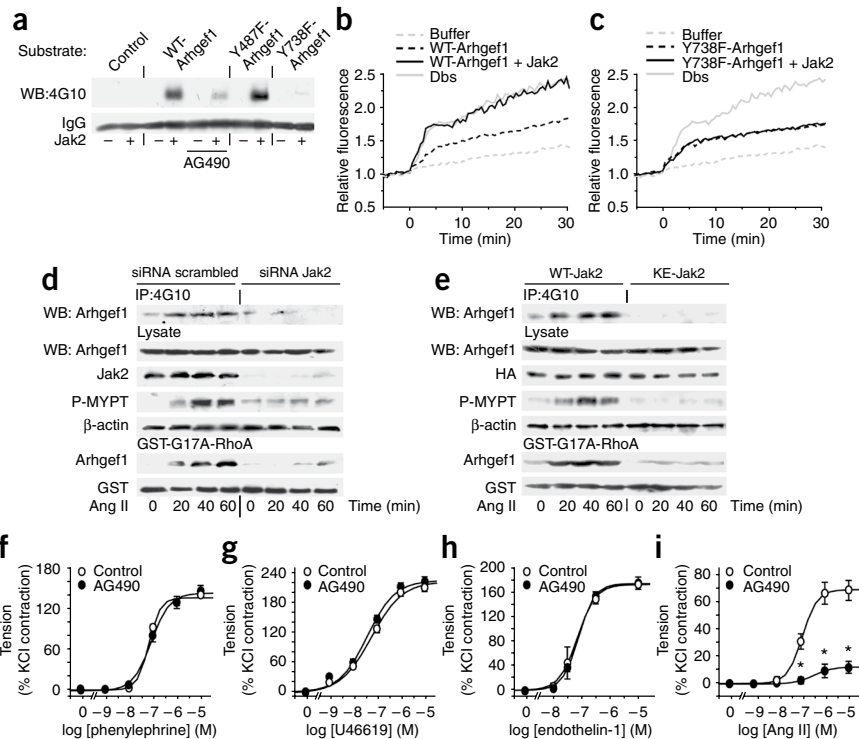


Figure 3 Ang II-induced Tyr738 phosphorylation of Arhgef1 is mediated by Jak2. **(a)** *In vitro* phosphorylation of immunoprecipitated AU1-tagged WT-Arhgef1 and Arhgef1 mutants by Jak2, analyzed by immunoblotting (WB) with 4G10 antibody that recognizes phosphotyrosine. IgG indicates Ponceau red staining of the band corresponding to the AU1-specific IgG used for immunoprecipitation. Control is using cells transfected with an empty plasmid. Jak2-mediated phosphorylation was inhibited by AG490 (20 μ M). **(b,c)** *In vitro* exchange assay on RhoA of WT-Arhgef1 **(b)** and Y738F-Arhgef1 **(c)** immunoprecipitated and *in vitro* phosphorylated by Jak2. The GEF was added at 0 min. The Rho GEF Dbs was used as a positive control; the curve labeled buffer indicates the spontaneous exchange rate on RhoA in the absence of a GEF. Guanine nucleotide exchange activity is expressed as relative fluorescence, with the initial fluorescence intensity set as 1. Curves are representative of four independent experiments. **(d,e)** Immunoblot analysis of immunoprecipitated (IP) phosphotyrosine proteins, lysates and proteins pulled down with GST-G17A-RhoA of VSMCs treated with scrambled siRNA or siRNA targeting Jak2 **(d)** and VSMCs expressing WT-Jak2 or the kinase-dead KE-Jak2 mutant **(e)**. The cells were unstimulated or were stimulated with Ang II for 20, 40 and 60 min. HA, hemagglutinin. **(f–i)** Cumulative concentration-response curves for the action of the indicated vasoconstrictors on rat aortic rings in the absence and in the continuous presence of AG490 (20 μ M). Contraction is expressed as a percentage of the maximal KCl (60 mM)-induced contraction. Data shown are the mean \pm s.e.m. of 4–8 independent experiments; * P < 0.001 versus control.



amounts of Arhgef1 were immunoprecipitated from cells treated or not with Ang II, increased Arhgef1 exchange activity was observed after Ang II stimulation (Fig. 1c). We then used small interfering RNA (siRNA) to test the role of Arhgef1 in the activation of RhoA signaling (Supplementary Fig. 1 and Supplementary Table 2). Knockdown of Arhgef1 inhibited Ang II-induced activation of RhoA. In contrast, we did not observe a requirement for Arhgef1 in RhoA signaling induced by potent vasoconstrictors other than Ang II, such as the thromboxane analog U46619, endothelin-1 or noradrenaline (Supplementary Fig. 1).

Ang II induces phosphorylation of Tyr738 of Arhgef1

We next sought to identify the molecular mechanism linking Ang II to Arhgef1 activation. We first ruled out a role of $G\alpha_{13}$ in Ang II-induced Arhgef1 activation because we did not detect any interaction between Arhgef1 and $G\alpha_{13}$ after Ang II stimulation, and overexpression of the RGS domain of Arhgef1, the region of the protein that specifically binds $G\alpha_{13}$, did not alter Ang II-induced RhoA signaling (Supplementary Fig. 2a–d). We noted that the increased exchange activity of Arhgef1 was retained after immunoprecipitation from Ang II-stimulated cells (Fig. 1c), suggesting that Arhgef1 might be stably modified. Because phosphorylation has been described as a mechanism regulating the activity of other GEFs¹⁵, we investigated the phosphorylation status of Arhgef1. Ang II treatment of VSMCs induced phosphorylation of endogenous Arhgef1 on tyrosine residues, which was prevented by the AT1 receptor antagonist losartan (Fig. 2a). Examination of the sequence of human Arhgef1 revealed the presence of two potential sites of tyrosine phosphorylation: Tyr487, in the Dbl homology domain, and Tyr738, in the pleckstrin homology domain. Substitution of these tyrosine by phenylalanine residues and transfection of the resulting phosphoresistant Arhgef1 mutants

(Y487F-Arhgef1 and Y738F-Arhgef1) into VSMCs indicated that Ang II-induced Arhgef1 phosphorylation occurred on Tyr738 (Fig. 2b).

To investigate the functional role of Tyr738 of Arhgef1, we analyzed the effect of Ang II on phosphorylation of the Rho kinase target MYPT in cells expressing various Arhgef1 mutants. Ang II increased MYPT phosphorylation in cells expressing WT-Arhgef1 (wild type), DHPH-Arhgef1 (truncated Arhgef1 containing only the Dbl homology and pleckstrin homology domains) or Y487F-Arhgef1, but not in cells expressing DHPH-Arhgef1 with both Tyr487 and Tyr738 substituted by phenylalanine residues (DHPH-YY/FF-Arhgef1) or Y738F-Arhgef1 (Fig. 2c). We next assessed the ability of Arhgef1 mutants with substitutions at Tyr487 and Tyr738 to stimulate guanine nucleotide exchange on RhoA *in vitro*. Whereas the Tyr487 mutation did not modify the exchange activity of Arhgef1 (Fig. 2d,e), the activity of the Y738F-Arhgef1 mutant was lower than that of WT-Arhgef1 and was not stimulated by Ang II (Fig. 2f). By contrast, the activity of the phosphomimetic Y738E-Arhgef1 mutant under basal conditions was higher than that of WT-Arhgef1, but was stimulated no further by Ang II (Fig. 2g). Pull-down assays with G17A-RhoA to characterize the activity of these Arhgef1 mutants in cells confirmed that Y738F-Arhgef1 was not activated by Ang II, whereas Y738E-Arhgef1 was constitutively active (Fig. 2h). Taken together, these data show that activation of Arhgef1 by Ang II was the consequence of Tyr738 phosphorylation on Arhgef1.

Jak2 mediates Ang II-induced Arhgef1 phosphorylation

A pharmacological analysis using inhibitors of kinases known to be activated by AT1 receptor stimulation¹⁸ led to the identification of the tyrosine kinase Jak2 as the kinase potentially mediating Ang II-induced phosphorylation and activation of Arhgef1. We first showed that the Jak2 inhibitor AG490 prevented Ang II-induced phosphorylation of

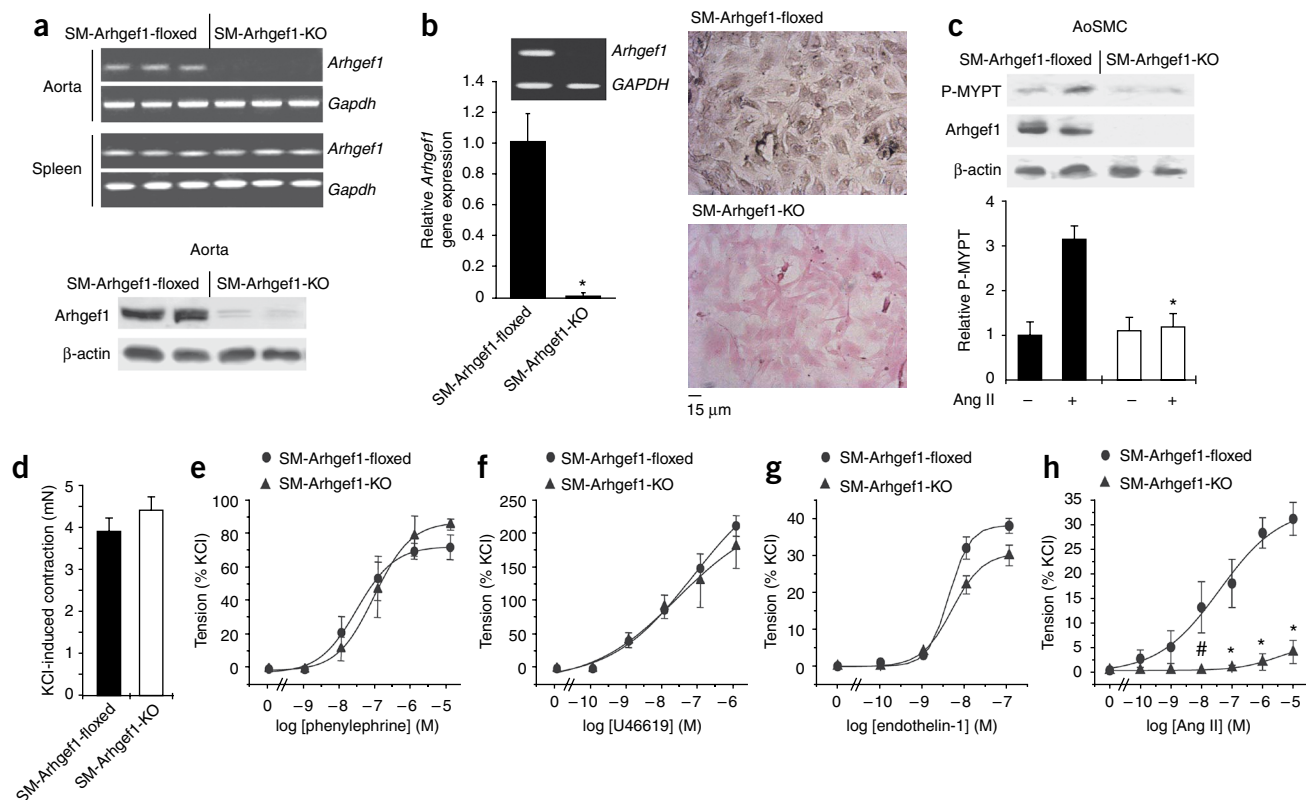


Figure 4 Smooth muscle cell-specific *Arhgef1* inactivation prevents Ang II induced RhoA signaling activation and contraction in arteries. **(a)** PCR analysis (top) of *Arhgef1* and *Gapdh* gene expression in aorta and spleen of SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice (three representative mice per group). Immunoblots (bottom) show *Arhgef1* protein expression in the aorta of SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice (two representative mice per group). **(b)** PCR analysis of *Arhgef1* gene expression (left) and immunostaining of *Arhgef1* (right) in VSMCs isolated from the aortas of SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice. *Arhgef1* gene expression was expressed relative to its level in VSMCs from SM-*Arhgef1*-floxed mice. Data shown are the mean \pm s.e.m. of VSMC cultures from three mice per group; * $P < 0.001$. **(c)** Immunoblot analysis of lysates of aortic smooth muscle cells (AoSMC) from SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice, unstimulated (–) or stimulated with Ang II (+). Graph shows the densitometric analysis of phospho-MYPT blots. Results are normalized to MYPT phosphorylation in unstimulated SM-*Arhgef1*-floxed AoSMCs (means \pm s.e.m. of five mice per group; * $P < 0.001$ versus Ang II-stimulated SM-*Arhgef1*-floxed AoSMCs). **(d)** Amplitude of the contraction induced by 60 mM KCl in aortic rings from SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice (means \pm s.e.m. of ten mice per group). **(e–h)** Cumulative concentration-response curves for the action of the indicated vasoconstrictors on aortic rings from SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice. Contraction is expressed as a percentage of the maximal KCl (60 mM)-induced contraction. Data shown are the means \pm s.e.m. (five to ten mice per group); # $P < 0.01$; * $P < 0.001$.

Arhgef1 (**Supplementary Fig. 3**). We confirmed this result using an *in vitro* kinase assay, which showed that recombinant Jak2 could phosphorylate WT-*Arhgef1* (**Fig. 3a**). This phosphorylation was impaired by the Jak2 inhibitor AG490 (**Fig. 3a**). Recombinant Jak2 also phosphorylated the Y487F-*Arhgef1* mutant but not the Y738F-*Arhgef1* mutant, suggesting that Jak2 phosphorylates *Arhgef1* on Tyr738. We next tested whether Jak2 phosphorylation led to an increase in the catalytic activity of *Arhgef1*. Greater exchange activity on RhoA was observed *in vitro* with Jak2-phosphorylated WT-*Arhgef1*, whereas the stimulating effect of Jak2 was lost with the Y738F-*Arhgef1* mutant (**Fig. 3b,c**).

To test the role of endogenous Jak2 in Ang II-induced *Arhgef1* and RhoA signaling activation, we used siRNA-mediated Jak2 silencing in VSMCs. Jak2 knockdown inhibited Ang II-induced phosphorylation of *Arhgef1* and MYPT as well as *Arhgef1* activation as assessed by its binding to G17A-RhoA (**Fig. 3d**). Similar results were obtained in cells expressing a catalytically inactive, dominant negative Jak2 mutant (KE-Jak2) (**Fig. 3e**). Inhibition of Ang-II induced $G\alpha_q$ -dependent Ca^{2+} signaling by siRNA targeting $G\alpha_q$ or using pharmacological inhibitors of Ca^{2+} -increase mechanisms prevented Ang II-induced phosphorylation of Jak2 and *Arhgef1* as well as activation of RhoA signaling, suggesting that activation of Jak2 depends on Ang-II induced $G\alpha_q$ and Ca^{2+} signaling

activation (**Supplementary Fig. 2**). These results indicate that upon Ang II stimulation, Jak2 phosphorylates and activates *Arhgef1*, which in turn activates RhoA signaling.

According to this model, we hypothesized that by preventing *Arhgef1* activation, the Jak2 inhibitor AG490 should affect the contractile properties of arterial rings. AG490 did not modify contraction-response curves to phenylephrine, U46619 and endothelin-1, but did selectively inhibit Ang II-induced contraction, thus confirming the essential role of the Jak2-*Arhgef1* pathway in the vasoconstrictor effect of Ang II (**Fig. 3f–i**).

Arhgef1 deletion prevents RhoA activation by Ang II

To test the role of *Arhgef1* in Ang II-dependent regulation of vascular tone and hypertension *in vivo*, we generated mice lacking *Arhgef1* specifically in smooth muscle cells (SM-*Arhgef1*-KO mice). We mated floxed *Arhgef1* (*Arhgef1*^{lox/lox}) mice¹⁹ to mice expressing a fusion protein of the Cre recombinase with a modified estrogen receptor-binding domain (CreER^{T2}) under the control of the smooth muscle myosin heavy chain (*SMMHC*, also known as *Myh11*) promoter (*SMMHC*-CreER^{T2} mice)²⁰ to produce *SMMHC*-CreER^{T2};*Arhgef1*^{lox/lox} mice (SM-*Arhgef1*-floxed mice). SM-*Arhgef1*-KO mice were then obtained

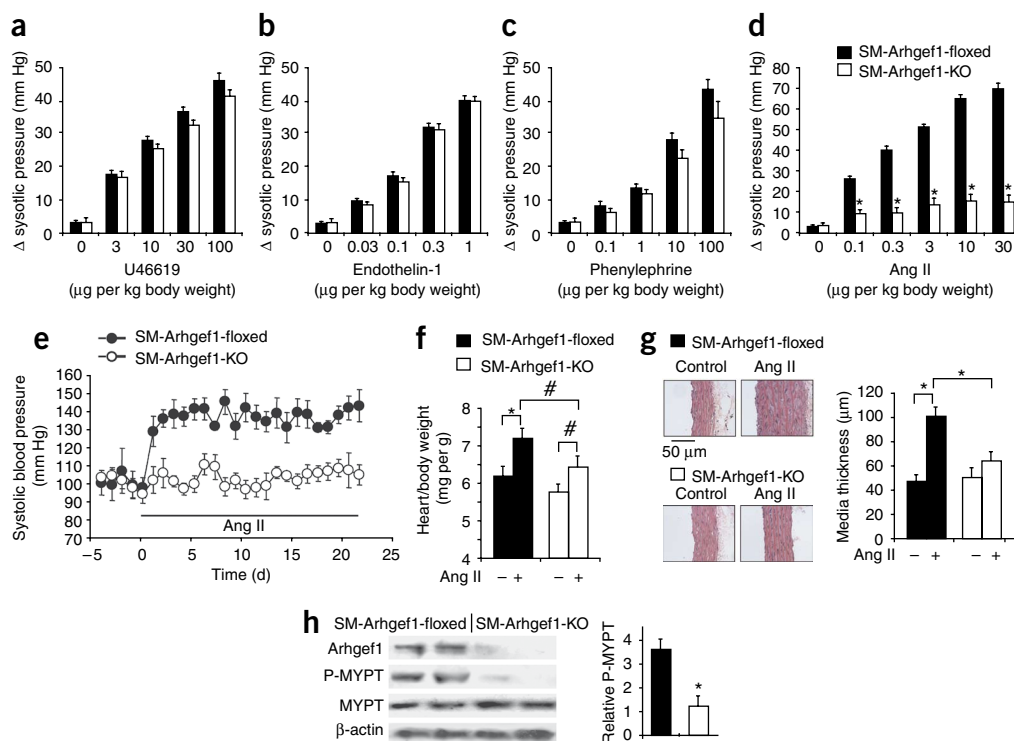


Figure 5 Smooth muscle cell–specific *Arhgef1* deficiency prevents Ang II–induced acute rise in blood pressure and Ang II–dependent hypertension. (a–d) Dose-dependent transient rise in systolic arterial pressure induced by intravenous injection of indicated vasoconstrictors in SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice. Data shown are the means \pm s.e.m. from five mice in each group; * P < 0.001. (e) Systolic blood pressure in SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice chronically treated with Ang II. Data shown are the means \pm s.e.m. from six mice in each group. (f–g) Assessment of cardiac hypertrophy (heart-to-body weight, f) and arterial wall thickening (aortic media thickness, g) in SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice infused for 21 d with saline (control) or Ang II. Results shown are means \pm s.e.m. from 6–11 mice in each group; # P < 0.01; * P < 0.001. Typical H&E-stained aortic cross-sections are shown in g. (h) Immunoblot analysis (two representative mice in each group) of *Arhgef1* expression and MYPT phosphorylation in the aortas of SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice treated with Ang II for 21 d. Graph shows densitometric analysis of phospho-MYPT in blots, relative to MYPT expression (mean \pm s.e.m. from six mice in each group; * P < 0.001).

by treating SM-*Arhgef1*-floxed mice with tamoxifen; loss of *Arhgef1* expression was verified by PCR and immunoblotting of lysates from vascular wall (aorta or mesenteric arteries) or isolated VSMCs (Fig. 4a,b and Supplementary Fig. 4). In VSMCs from SM-*Arhgef1*-KO mice, stimulation with Ang II did not induce MYPT phosphorylation (Fig. 4c), thus demonstrating functional loss of *Arhgef1* activity in smooth muscle cells of these mice.

We next studied the contractile properties of aortic rings *ex vivo* from SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice. In aortic segments from SM-*Arhgef1*-KO mice, KCl-, phenylephrine-, U46619- and endothelin-induced contractions were similar to those measured in aortic rings from SM-*Arhgef1*-floxed mice (Fig. 4d–g). In contrast, the amplitude of Ang II–induced contractions was much lower in aortic rings from SM-*Arhgef1*-KO mice than in rings from SM-*Arhgef1*-floxed mice (Fig. 4h), indicating that the loss of *Arhgef1* specifically inhibits the vasoconstrictor effect of Ang II.

SM-*Arhgef1*-KO mice resist to Ang II–induced hypertension

To assess the consequences of smooth muscle–specific *Arhgef1* deletion *in vivo*, we first measured the acute pressor effect of vasoactive agents (Fig. 5a–d). Pressor responses to U46619, endothelin-1 and phenylephrine were similar in SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice (Fig. 5a–c), but responses to Ang II were reduced in SM-*Arhgef1*-KO mice (Fig. 5d).

We next determined the role of *Arhgef1* in Ang II–induced hypertension. We placed minipumps chronically releasing Ang II into

SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice. Basal blood pressures were similar in SM-*Arhgef1*-floxed, SM-*Arhgef1*-KO mice and tamoxifen-treated *Arhgef1*^{lox/lox} mice (Figs. 5 and 6a,b and Supplementary Fig. 5). Ang II treatment increased blood pressure in SM-*Arhgef1*-floxed but not in SM-*Arhgef1*-KO mice (Fig. 5e). Both plasma Ang II concentration and AT1 receptor expression were similar between control and SM-*Arhgef1*-KO mice whether or not the mice were treated with Ang II (Supplementary Fig. 6). Although not abolished, cardiac hypertrophy, as assessed by the heart to body weight ratio, was significantly reduced in Ang II–treated SM-*Arhgef1*-KO mice (Fig. 5f). Ang II–induced hypertension in SM-*Arhgef1*-floxed mice was associated with a marked arterial wall remodeling, which was absent in SM-*Arhgef1*-KO mice (Fig. 5g). In addition, RhoA signaling pathway activity, as monitored by MYPT phosphorylation, was much lower in the arteries of Ang II–treated SM-*Arhgef1*-KO mice compared to Ang II–treated SM-*Arhgef1*-floxed mice (Fig. 5h). Moreover, tamoxifen treatment of Ang II–treated SM-*Arhgef1*-floxed mice, starting 10 d after the beginning of Ang II treatment, was able to reverse preexisting hypertension (Fig. 6a). The effect of *Arhgef1* deletion on blood pressure was similar to that of losartan (Fig. 6a). These results demonstrate that *Arhgef1* deletion in smooth muscle prevents the hypertensive effect of Ang II and suggest a crucial role for *Arhgef1*-mediated RhoA activation in arterial smooth muscle in controlling blood pressure.

We investigated the specificity of *Arhgef1* in Ang II/AT1 receptor–dependent regulation of blood pressure by testing the response of SM-*Arhgef1*-KO mice in the *N*^ω-nitro-L-arginine (L-NAME)

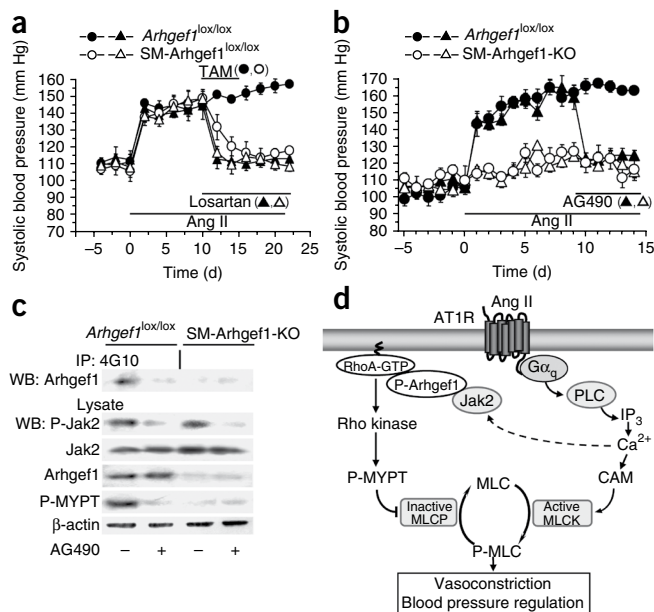


Figure 6 Arhgef1 deletion in smooth muscle or Jak2 inhibition reverses Ang II-induced hypertension. **(a)** Systolic blood pressure in *Arhgef1*^{lox/lox} and SM-*Arhgef1*-floxed mice chronically treated with Ang II and then with tamoxifen (TAM) or losartan. Data shown are the means \pm s.e.m. from six mice in each group. **(b)** Systolic blood pressure in *Arhgef1*^{lox/lox} and SM-*Arhgef1*-KO mice chronically treated with Ang II then with AG490. Data shown are means \pm s.e.m. from five mice in each group. **(c)** Immunoblot (WB) analysis of the aortas of *Arhgef1*^{lox/lox} and SM-*Arhgef1*-KO mice chronically treated with Ang II for 21 d with or without AG490 administration for the last 6 d. Results shown are representative of those of six mice in each condition. Immunoblotting was performed on tyrosine-phosphorylated proteins immunoprecipitated (IP) with 4G10 antibody and on total lysate. **(d)** Proposed role for Arhgef1 in vasoconstriction and blood pressure regulation mediated by the AT1 receptor in VSMCs. AT1 receptor (AT1R) stimulation by Ang II mediates vasoconstriction and controls blood pressure through $G\alpha_q$ -mediated Ca^{2+} - and calmodulin (CAM)-dependent activation of myosin light chain kinase (MLCK) (Ca^{2+} signaling) and through Arhgef1/RhoA/Rho kinase-mediated phosphorylation of MYPT and inhibition of myosin light chain phosphatase (MLCP) activity (Ca^{2+} sensitization) through $G\alpha_q$ and Ca^{2+} -dependent Jak2 activation. PLC, phospholipase C; IP₃, inositol-1,4,5-trisphosphate.

and deoxycorticosterone acetate plus NaCl (DOCA-salt) models of hypertension (**Supplementary Fig. 7**). L-NAME treatment of control mice induced hypertension that was completely reversed by treatment with an AT1 receptor antagonist; however, L-NAME treatment did not induce hypertension in SM-*Arhgef1*-KO mice, consistent with the idea that Arhgef1 acts downstream of the AT1 receptor. In contrast, DOCA-salt treatment of control mice induced hypertension that was only partially inhibited by treatment with an AT1 receptor antagonist, indicating that hypertension in this model does not depend entirely on signaling through this receptor. Accordingly, we found that SM-*Arhgef1*-KO mice were not completely protected from DOCA-salt-induced hypertension and were resistant to the blood pressure-lowering effects of the AT1 receptor antagonist.

Finally, to confirm the involvement of Jak2 in Ang II-mediated Arhgef1 activation *in vivo*, we treated SM-*Arhgef1*-floxed and SM-*Arhgef1*-KO mice with Ang II for 10 d, followed by treatment with AG490. AG490 treatment restored normal blood pressure in *Arhgef1*^{lox/lox} mice (**Fig. 6b**). Moreover, this treatment lowered the levels of phosphorylated MYPT, Jak2 and Arhgef1 in arteries from Ang II-treated *Arhgef1*^{lox/lox} mice (**Fig. 6c**).

DISCUSSION

Our study unveils a new Ang II signaling pathway, operating in VSMCs, which is essential for the control of vascular tone and blood pressure. First, we demonstrate that the RhoA GEF Arhgef1 is specifically responsible for AT1 receptor-mediated RhoA activation through a mechanism involving the tyrosine kinase Jak2. To our knowledge, activation of Arhgef1 by tyrosine phosphorylation has not been previously described, and Jak2 has not been previously shown to activate Arhgef1 and RhoA. Second, we show that ablation of the *Arhgef1* gene in VSMCs protects against Ang II-dependent hypertension without affecting blood pressure at baseline or the response to other vasoactive factors, supporting the hypothesis that Arhgef1 activation and subsequent RhoA signaling is causally involved in the development of Ang II-dependent hypertension. Most importantly, Arhgef1, as the molecular link connecting Ang II to RhoA activation and vasoconstriction, represents a new target for reducing elevated vascular

tone under hypertensive conditions without affecting normal blood pressure regulation by other vasoactive factors.

Rho GEFs form a large family, comprising ~ 70 members in the human genome¹⁵. Among them, the small subfamily of RGS-containing Rho GEFs are the only ones subject to regulation by the $G\alpha_{12}$ family of heterotrimeric G protein α subunits ($G\alpha_{12}$ and $G\alpha_{13}$)^{21–25}. However, our results showed that Ang II-induced Arhgef1 activation was independent of the RGS domain and regulation by $G\alpha_{12}$ and $G\alpha_{13}$, and instead was due to Jak2-dependent phosphorylation of Tyr738 of Arhgef1, which resulted in a stimulation of its exchange activity. Indeed, Ang II induction of Jak2-dependent activation of Arhgef1 and RhoA depended on $G\alpha_q$ and Ca^{2+} signaling, in agreement with previous observations describing the Ca^{2+} dependence of Ang II-induced Jak2 activation^{26,27}, as well as with previous work identifying $G\alpha_q$ - or $G\alpha_{11}$ -dependent pathways as a main signaling mechanism for Ang II^{28,29}. Thus, the RhoA/Rho kinase and Ca^{2+} signaling pathways are not completely independent, parallel processes controlling Ang II-mediated VSMC contraction, but are connected through Jak2. The rise in intracellular Ca^{2+} concentration induced by Ang II in VSMCs seems to be essential for inducing Arhgef1/RhoA signaling and Ca^{2+} sensitization; both this rise in Ca^{2+} concentration and RhoA-mediated Ca^{2+} sensitization of contractile proteins are necessary for Ang II-induced contraction and control of vascular tone (**Fig. 6d**). The suppressive effects of a Jak2 inhibitor on Ang II-induced VSMC contraction and hypertension suggest that the rise in intracellular Ca^{2+} levels induced by Ang II is not by itself sufficient to lead to sustained vasoconstriction. The initial transient rise in the intracellular Ca^{2+} concentration needs to be relayed by a RhoA/Rho kinase-dependent Ca^{2+} sensitization mechanism to allow continued contraction.

Although Jak2 has been shown to be involved in Ang II-induced VSMC proliferation^{30,31}, its role in Ang II-induced regulation of vascular tone has not previously been directly addressed. However, diabetes-associated hypertension, which depends on Ang II, is prevented by Jak2 inhibition³², suggesting a crucial function for Jak2 in Ang II-dependent blood pressure regulation. Our results demonstrate the key role of Jak2 in linking Ang II-induced Ca^{2+} signaling to RhoA activation. Based on these findings, we suggest that, in other contexts, other Jak kinase family members (Jak1, Jak3, Tyk2) might play a similar role in regulating Arhgef1 and consequently RhoA activity.

Because of the multiplicity of Rho GEFs, compared to only 20 Rho proteins, it has been suggested that different upstream signals use different Rho GEFs to activate Rho proteins^{15,33}. Accordingly, depending on the upstream signal, different RhoA GEFs might be used to similarly activate RhoA in VSMCs. Although RhoA activation is a common feature of hypertension, the stimulus and the Rho GEF(s) involved in its activation are probably different according to the pathological context. Here, we demonstrate that Arhgef1 specifically mediates RhoA activation by Ang II but not by other vasoconstrictors. We cannot exclude the possibility that Ang II-mediated Arhgef1 activation could also lead to activation of the close RhoA relatives RhoB and RhoC, the role of which in vascular tone regulation is unknown. It has recently been shown that DOCA-salt-induced hypertension depends on $G\alpha_{12}$ - $G\alpha_{13}$ -activated Arhgef12 signaling downstream of the endothelin-1 and thromboxane A2 receptors²⁰. Our present study, together with that previous study²⁰, supports the concept that RhoA GEFs discriminate upstream signals: whereas the $G\alpha_{12}$ - $G\alpha_{13}$ /Arhgef12 pathway is crucial in salt-induced hypertension, Arhgef1 signaling, downstream of $G\alpha_q$ and Ca^{2+} , is essential for Ang II-induced vasoconstriction and hypertension.

Ang II-induced vasoconstriction of arterial rings *ex vivo* was virtually abolished in arteries from SM-Arhgef1-KO mice; however, small, transient pressor responses to acute Ang II injections *in vivo* that were not concentration dependent remained in these mice. This remaining response likely results from indirect effects of Ang II, as acute pressor responses to Ang II have been shown to be mediated by both direct vasoconstrictive effects on blood vessels as well as indirect vasoconstrictive effects through the sympathetic nervous system^{34–38}. As SM-Arhgef1-KO mice were resistant to Ang II-induced hypertension, this residual transient response to acute Ang II administration does not seem to contribute to long-term Ang II-mediated blood pressure regulation.

The pronounced effects of smooth muscle-specific *Arhgef1* gene deletion, which both prevents and reverses Ang-II induced hypertension, provides definitive evidence for a causal role of a Arhgef1-RhoA signaling pathway in the development of hypertension. Although several studies have suggested that effects of renal Ang II on sodium handling contribute to hypertension, our findings suggest that the renal effects of Ang II are due to AT1-mediated renal vasoconstriction^{39,40}. The absence of vascular remodeling in Ang II-treated SM-Arhgef1-KO mice suggests that either elevated blood pressure is required for this remodeling or that Arhgef1 in VSMCs is involved in the signaling process leading to vascular hypertrophy. Synergistic interactions between aldosterone and Ang II have been implicated in cardiovascular remodeling^{41,42}, and it has recently been shown that aldosterone and Ang II synergistically activate RhoA⁴³. Arhgef1 may thus be a key molecule in the signaling pathway responsible for the synergistic action of aldosterone and Ang II on RhoA in VSMCs. The observation that cardiac hypertrophy was only partially blocked in Ang II-treated SM-Arhgef1-KO mice suggests that Ang II-mediated cardiac remodeling is partially independent of pressure overload; this pressure overload-independent pathway could involve either direct^{44,45} or indirect⁴⁶ Ang II effects on aldosterone-dependent processes.

In conclusion, we suggest that Arhgef1 may constitute a new target for antihypertensive therapies. Because of the strategic position of RhoA GEFs in the regulation of RhoA activity, inhibitors of these GEFs could be used to selectively reverse the hyperactivity of RhoA occurring in different forms of hypertension, allowing targeted antihypertensive treatments.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

C.G. contributed to study design and performed all experiments with J.B. G.T. and M.R.-D. collaborated on *in vitro* experiments, proteomics and pulldown analyses. K.R., L.L. and D.H. collaborated on *ex vivo* contraction measurements. E.S. and A.B. helped with the organization of the study. R.M.T. and S.O. generated *Arhgef1*^{lox/lox} mice and SMMHC-CreER^{T2} mice, respectively. P.P. and G.L. planned and directed the study and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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1. Staessen, J.A., Wang, J., Bianchi, G. & Birkenhager, W.H. Essential hypertension. *Lancet* **361**, 1629–1641 (2003).
2. de Gasparo, M., Catt, K.J., Inagami, T., Wright, J.W. & Unger, T. International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacol. Rev.* **52**, 415–472 (2000).
3. Touyz, R.M. & Berry, C. Recent advances in angiotensin II signaling. *Braz. J. Med. Biol. Res.* **35**, 1001–1015 (2002).
4. Christobolis, S. & Sobey, C.G. Evidence that Rho-kinase activity contributes to cerebral vascular tone *in vivo* and is enhanced during chronic hypertension: comparison with protein kinase C. *Circ. Res.* **88**, 774–779 (2001).
5. Mukai, Y. *et al.* Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. *FASEB J.* **15**, 1062–1064 (2001).
6. Seko, T. *et al.* Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ. Res.* **92**, 411–418 (2003).
7. Uehata, M. *et al.* Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature* **389**, 990–994 (1997).
8. Masumoto, A. *et al.* Possible involvement of Rho-kinase in the pathogenesis of hypertension in humans. *Hypertension* **38**, 1307–1310 (2001).
9. Jaffe, A.B. & Hall, A. Rho GTPases: biochemistry and biology. *Annu. Rev. Cell Dev. Biol.* **21**, 247–269 (2005).
10. Somlyo, A.P. & Somlyo, A.V. Ca²⁺ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.* **83**, 1325–1358 (2003).
11. Loirand, G., Guerin, P. & Pacaud, P. Rho kinases in cardiovascular physiology and pathophysiology. *Circ. Res.* **98**, 322–334 (2006).
12. Kataoka, C. *et al.* Important role of Rho-kinase in the pathogenesis of cardiovascular inflammation and remodeling induced by long-term blockade of nitric oxide synthesis in rats. *Hypertension* **39**, 245–250 (2002).
13. Moriki, N. *et al.* RhoA activation in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats. *Hypertens. Res.* **27**, 263–270 (2004).
14. Higashi, M. *et al.* Long-term inhibition of Rho-kinase suppresses angiotensin II-induced cardiovascular hypertrophy in rats *in vivo*: effect on endothelial NAD(P)H oxidase system. *Circ. Res.* **93**, 767–775 (2003).
15. Rossman, K.L., Der, C.J. & Sondek, J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. *Nat. Rev. Mol. Cell Biol.* **6**, 167–180 (2005).
16. Bos, J.L., Rehmann, H. & Wittinghofer, A. GEFs and GAPs: critical elements in the control of small G proteins. *Cell* **129**, 865–877 (2007).
17. Garcia-Mata, R. *et al.* Analysis of activated GAPs and GEFs in cell lysates. *Methods Enzymol.* **406**, 425–437 (2006).

18. Mehta, P.K. & Griendling, K.K. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am. J. Physiol. Cell Physiol.* **292**, C82–C97 (2007).
19. Rubtsov, A. *et al.* Lsc regulates marginal-zone B cell migration and adhesion and is required for the IgM T-dependent antibody response. *Immunity* **23**, 527–538 (2005).
20. Wirth, A. *et al.* G12–G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension. *Nat. Med.* **14**, 64–68 (2008).
21. Fukuhara, S., Murga, C., Zohar, M., Igishi, T. & Gutkind, J.S. A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. *J. Biol. Chem.* **274**, 5868–5879 (1999).
22. Kozasa, T. *et al.* p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. *Science* **280**, 2109–2111 (1998).
23. Suzuki, N., Nakamura, S., Mano, H. & Kozasa, T. Galpha 12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF. *Proc. Natl. Acad. Sci. USA* **100**, 733–738 (2003).
24. Fukuhara, S., Chikumi, H. & Gutkind, J.S. Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho. *FEBS Lett.* **485**, 183–188 (2000).
25. Wells, C.D. *et al.* Mechanisms for reversible regulation between G13 and Rho exchange factors. *J. Biol. Chem.* **277**, 1174–1181 (2002).
26. Frank, G.D. *et al.* Requirement of Ca(2+) and PKCdelta for Janus kinase 2 activation by angiotensin II: involvement of PYK2. *Mol. Endocrinol.* **16**, 367–377 (2002).
27. Marrero, M.B. *et al.* Direct stimulation of Jak/STAT pathway by the angiotensin II AT1 receptor. *Nature* **375**, 247–250 (1995).
28. Ohtsu, H. *et al.* Central role of Gq in the hypertrophic signal transduction of angiotensin II in vascular smooth muscle cells. *Endocrinology* **149**, 3569–3575 (2008).
29. Keys, J.R., Greene, E.A., Koch, W.J. & Eckhart, A.D. Gq-coupled receptor agonists mediate cardiac hypertrophy via the vasculature. *Hypertension* **40**, 660–666 (2002).
30. Marrero, M.B. *et al.* Role of Janus kinase/signal transducer and activator of transcription and mitogen-activated protein kinase cascades in angiotensin II- and platelet-derived growth factor-induced vascular smooth muscle cell proliferation. *J. Biol. Chem.* **272**, 24684–24690 (1997).
31. Shaw, S. *et al.* High glucose augments the angiotensin II-induced activation of JAK2 in vascular smooth muscle cells via the polyol pathway. *J. Biol. Chem.* **278**, 30634–30641 (2003).
32. Baner-Berceli, A.K. *et al.* Angiotensin II and endothelin-1 augment the vascular complications of diabetes via JAK2 activation. *Am. J. Physiol. Heart Circ. Physiol.* **293**, H1291–H1299 (2007).
33. Garcia-Mata, R. & Burrige, K. Catching a GEF by its tail. *Trends Cell Biol.* **17**, 36–43 (2007).
34. Fujii, A.M. & Vatner, S.F. Direct versus indirect pressor and vasoconstrictor actions of angiotensin in conscious dogs. *Hypertension* **7**, 253–261 (1985).
35. Rowe, B.P., Noble, A.R. & Munday, K.A. Blockade of pressor responses to angiotensins I and II and noradrenaline using phentolamine, propranolol and hexamethonium in conscious rabbits. *Pflugers Arch.* **382**, 269–274 (1979).
36. Cline, W.H. Jr. Role of released catecholamines in the vascular response to injected angiotensin II in the dog. *J. Pharmacol. Exp. Ther.* **216**, 104–110 (1981).
37. Ferrario, C.M., Barnes, K.L., Szilagyi, J.E. & Brosnihan, K.B. Physiological and pharmacological characterization of the area postrema pressor pathways in the normal dog. *Hypertension* **1**, 235–245 (1979).
38. Falcon, J.C. II, Phillips, M.I., Hoffman, W.E. & Brody, M.J. Effects of intraventricular angiotensin II mediated by the sympathetic nervous system. *Am. J. Physiol.* **235**, H392–H399 (1978).
39. Crowley, S.D. *et al.* Angiotensin II causes hypertension and cardiac hypertrophy through its receptors in the kidney. *Proc. Natl. Acad. Sci. USA* **103**, 17985–17990 (2006).
40. Ruilope, L.M., Lahera, V., Rodicio, J.L. & Carlos Romero, J. Are renal hemodynamics a key factor in the development and maintenance of arterial hypertension in humans? *Hypertension* **23**, 3–9 (1994).
41. Schiffrin, E.L. Effects of aldosterone on the vasculature. *Hypertension* **47**, 312–318 (2006).
42. Lemarie, C.A., Paradis, P. & Schiffrin, E.L. New insights on signaling cascades induced by cross-talk between angiotensin II and aldosterone. *J. Mol. Med.* **86**, 673–678 (2008).
43. Montezano, A.C. *et al.* Aldosterone and angiotensin II synergistically stimulate migration in vascular smooth muscle cells through c-Src-regulated redox-sensitive RhoA pathways. *Arterioscler. Thromb. Vasc. Biol.* **28**, 1511–1518 (2008).
44. Hein, L. *et al.* Overexpression of angiotensin AT1 receptor transgene in the mouse myocardium produces a lethal phenotype associated with myocyte hyperplasia and heart block. *Proc. Natl. Acad. Sci. USA* **94**, 6391–6396 (1997).
45. Paradis, P., Dali-Youcef, N., Paradis, F.W., Thibault, G. & Nemer, M. Overexpression of angiotensin II type I receptor in cardiomyocytes induces cardiac hypertrophy and remodeling. *Proc. Natl. Acad. Sci. USA* **97**, 931–936 (2000).
46. Struthers, A.D. & MacDonald, T.M. Review of aldosterone- and angiotensin II-induced target organ damage and prevention. *Cardiovasc. Res.* **61**, 663–670 (2004).

ONLINE METHODS

Cell culture and transfection. We isolated rat aortic smooth muscle cells by enzymatic dissociation and cultured them in DMEM (Gibco). We transfected plasmids and siRNA into VSMCs by electroporation (Nucleofector, Amaxa) with a transfection efficiency of 60%–80%. We used the human *ARHGEF1* DNA constructs pCEF-AU1-Arhgef1 (WT-Arhgef1, amino acids 1–912) and two truncated forms (Δ N-Arhgef1, amino acids 351–912, and DHPH-Arhgef1, amino acids 357–800). We created phosphoresistant mutants by substituting Tyr487 and/or Tyr738 with a phenylalanine residue (Y487F-Arhgef1, Y738F-Arhgef1 and DHPH-YY/FF-Arhgef1, which corresponds to DHPH-Arhgef1 carrying the Y487F and Y738F mutations). The phosphomimetic Y738E-Arhgef1 mutant was generated by replacing Tyr738 with a glutamate. For studies in which we transfected the RGS domain of Arhgef1 into cells, we used the pEF4-Myc-RGS-Arhgef1 construct, encoding amino acids 61–266 of Arhgef1 (RGS-Arhgef1). For studies in which we transfected Jak2 into cells, we used the pRK5 expression plasmid encoding hemagglutinin-tagged full-length wild-type mouse Jak2 (WT-Jak2) or a kinase-dead mutant (K882E-Jak2). The sense strand of the siRNA (Eurogentec) used for Jak2 silencing was 5'-GUAUGUUGCCGAAGAAAUdTdT-3' and the scrambled siRNA sequence was 5'-CGACUUCUUACUCUAUACdTsT-3'. Cells were stimulated with Ang II (0.1 μ M, Sigma) in the presence of the Ang II type 2 receptor antagonist PD 123319 (1 μ M, Sigma). For the detection of Arhgef1 phosphorylation by autoradiography, cells were incubated in the presence of 32 P (0.6 mCi) for 4 h, and then stimulated by Ang II.

Immunoblot analysis. Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes and then incubated with specific antibodies. Equal loading was checked by reprobing the membrane with monoclonal antibody to β -actin (A-5316, Sigma). Immunoprecipitation of phosphotyrosine was performed using the antibody 4G10 (Upstate Biotechnology), and immunoprecipitation of transfected AU1-tagged Arhgef1 was done using an antibody (MMS.130R, Covance) that recognizes the AU1 tag. Phosphorylation of the Rho kinase target protein myosin phosphatase target subunit 1 (phospho-MYPT) on Thr-696 (ref. 47), as assessed using a rabbit polyclonal antibody to phospho-MYPT (Thr696) (sc17556-R, Santa Cruz), was used to monitor Rho kinase activity. Antibody to MYPT was used to analyze total MYPT expression (sc25618, Santa Cruz). The dependence of phospho-MYPT generation on Rho kinase activity was confirmed by showing that the Ang II-induced increase in MYPT phosphorylation was abolished by Rho kinase inhibitors (10 μ M Y-27632 (Sigma) or 10 μ M fasudil (LC laboratories); data not shown). Blots were quantified by densitometric analysis using QuantityOne (Biorad).

Pulldown experiments. We assessed RhoA activity by pulldown assay using the Rho-binding domain of the Rho effector Rhotekin, produced as described previously⁴⁸. Affinity precipitation of active RhoA-GEFs with the nucleotide-free RhoA mutant (G17A) has been described in detail elsewhere¹⁷. For mass spectrometric analysis, the gel was stained with Coomassie blue, spots of interest analyzed by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by LC-MS/MS (Micromass-Waters) at the INRA Proteomics Facility (Nantes).

In vitro exchange activity assay. We determined Arhgef1 exchange activity on RhoA using a fluorophore-based RhoGEF exchange activity assay kit according to the manufacturer's instructions (Cytoskeleton).

In vitro phosphorylation. We performed *in vitro* phosphorylation assays using WT-Arhgef1 and Arhgef1 mutants that had been immunoprecipitated from transfected cells. The reaction was carried out with 100 ng of recombinant Jak2 (Cell Signaling) in a phosphorylation buffer for 30 min at 30 °C and stopped by addition of cold phosphorylation buffer. Proteins were separated by SDS-PAGE and phosphorylation was visualized by blotting with antibody to phosphotyrosine (4G10).

Contraction measurements. We performed contraction measurements in rat aortic rings mounted in organ baths and connected to force transducers for isometric tension recordings (Pioden Controls Ltd) and in mouse aortic rings mounted on a multichannel isometric myograph (Danish Myo Technology). We constructed cumulative concentration-response curves in response to KCl, ET-1, noradrenaline, phenylephrine and the thromboxane A2 receptor agonist U46619 (all from Sigma).

Generation of knockout mice. *Arhgef1*^{lox/lox} and SMMHC-CreER^{T2} mice have been previously described^{19,20,47}. They were generated in, and bred on, a C57BL/6 genetic background. We mated *Arhgef1*^{lox/lox} mice to SMMHC-CreER^{T2} transgenic mice to generate mice lacking the SMMHC-CreER^{T2} allele but containing *Arhgef1* floxed alleles (*Arhgef1*^{lox/lox} mice) and mice containing both the CreER^{T2} allele and *Arhgef1* floxed alleles (SMMHC-CreER^{T2};*Arhgef1*^{lox/lox} mice: SM-*Arhgef1*-floxed). Cre-mediated deletion in smooth muscle (SM-*Arhgef1*-KO mice) was induced by tamoxifen treatment (intraperitoneal injection of 1 mg per day, for 5 consecutive days). Tamoxifen-treated *Arhgef1*^{lox/lox} and untreated SM-*Arhgef1*-floxed mice were used as controls. All animal care and use procedures of the present study were performed in accordance with the European Community Standards on the Care and Use of Laboratory Animals and were approved by the local ethics committee (Comité d'Éthique en Expérimentation Animale des Pays de Loire). Real-time reverse transcription-PCR analysis of *Arhgef1* transcripts was performed to assess the extent of the deletion. Only mice showing >80% reduction of *Arhgef1* transcript abundance in the aorta, with respect to control levels, were used in this study. Immunoblot analysis of aorta, carotid, caudal and mesenteric artery protein extracts was used to confirm a corresponding (80%–90%) reduction in the abundance of Arhgef1 protein.

Ang II-induced hypertension. Osmotic minipumps (Alzet, model 2004) filled with saline solution set to deliver Ang II (Sigma) at 1 mg d⁻¹ per kilogram body weight were placed subcutaneously. Losartan treatment (Sigma, 16 mg kg⁻¹ d⁻¹) was administered in the drinking water and AG490 (Sigma, 10 mg kg⁻¹ d⁻¹) by daily intraperitoneal injections. At the end of the treatment regimen, aortas were removed and prepared for protein extraction or fixed for histological analysis. We fixed aortas were fixed in 4% paraformaldehyde in PBS and paraffin-embedded them. We then stained transverse sections (2–3 μ m) with hematoxylin and eosin (Sigma) and quantified arterial wall area in a blinded manner using Metamorph-Metaview software (Universal Imaging).

Arterial pressure measurements. Blood pressure was measured in conscious, unrestrained mice using a radiotelemetry system as described previously⁴⁹ (PA-C10 and Dataquest software, Data Sciences International; **Figs. 5 and 6** and **Supplementary Fig. 5**) or by computerized tail cuff plethysmography in conscious mice⁵⁰ using the BP 2000 analysis system (Visitech Systems; **Supplementary Fig. 7**). For analyzing the acute effects of agonists (**Fig. 5a–d**), mice implanted with telemetry transmitters were anesthetized with ketamine (0.1 mg g⁻¹) and xylazine (2.5 μ g g⁻¹), vasoconstrictors were injected through a catheter introduced into the jugular vein and blood pressure was measured continuously. After a 30-min stabilizing period, bolus injections of increasing doses of vasoconstrictors were administered at 10-min intervals. Dose-response curves were established for the peak of the systolic pressure responses.

Chemicals and antibodies. Antibodies to RhoA (sc418), Arhgef1 (sc20804), Arhgef12 (sc15439), Myc (sc40), Vav2 (sc20803), Vav3 (sc28474) and α_{13} (sc410) were purchased from Santa Cruz Biotechnology. Antibody to AU1 was obtained from Covance (MMS.130R) and antibody to Arhgef1 I was from Alpha Diagnostic (GTRAP48-A). Jak2-specific antibody was purchased from Cell Signaling (33302) and phospho-Jak2-specific antibody was from Millipore (09-275). AG490 and genistein were obtained from Calbiochem. All other reagents were purchased from Sigma.

Statistics. All results are expressed as the means \pm s.e.m. of sample size *n*. Significance was tested by analysis of variance or Student's *t*-test. Probabilities less than 5% (*P* < 0.05) were considered to be significant.

47. Feng, J. *et al.* Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. *J. Biol. Chem.* **274**, 37385–37390 (1999).

48. Ren, X.D., Kiosses, W.B. & Schwartz, M.A. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J.* **18**, 578–585 (1999).

49. Mills, P.A. *et al.* A new method for measurement of blood pressure, heart rate, and activity in the mouse by radiotelemetry. *J. Appl. Physiol.* **88**, 1537–1544 (2000).

50. Krege, J.H., Hodgin, J.B., Hagaman, J.R. & Smithies, O. A noninvasive computerized tail-cuff system for measuring blood pressure in mice. *Hypertension* **25**, 1111–1115 (1995).