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

Christophe Guilluy1,2,8, Jérémy Brégeon1,2,8, Gilles Toumaniantz1,2, Malvyne Rolli-Derkinderen1,2, Kevin Retailleau3, Laurent Loufrani3, Daniel Henrion3, Elizabeth Scalbert6, Antoine Bril5, Raul M Torres5, Stephan Offermanns8, Pierre Pacaud1,2 & Gervaise Loirand1,2,7

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 II2,3.

Increasing evidence implicates overactivation of the monomeric G protein RhoA as a critical component of the pathogenesis of hypertension in several experimental models4–7 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 hypertension2,11. Inhibition of the AT1 receptor reduces the upregulation of RhoA and Rho kinase activity in hypertensive rats2,11, and in vivo long-term infusion of Ang II increases the activity of RhoA and Rho kinase in arteries12, 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 GTP13,14 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 GEFs15, to identify interacting proteins in VSMCs with and without Ang II treatment. After incubating nucleotide-free
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 with Ang II (0.1 μM, 40 min) (+), precipitated with the G17A-RhoA mutant, which preferentially binds to active RhoA GEFs. 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 ± 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 μM, 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 (see Supplementary Table 1). In vitro phosphorylation of the Rho kinase target MYPT is mediated by Tyr738 phosphorylation of Arhgef11. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and Arhgef1. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and Arhgef1. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and Arhgef1. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and Arhgef1. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and Arhgef1. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and Arhgef1. The precipitated proteins (top blot) and lysates (bottom blot) were separated by SDS-PAGE and were blotted with antibodies to AU1 and Arhgef1.
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 Gβ13 in Ang II–induced Arhgef1 activation because we did not detect any interaction between Arhgef1 and Gβ13 after Ang II stimulation, and overexpression of the RGS domain of Arhgef1, the region of the protein that specifically binds Gβ13, 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 phosphorylated. 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 DbI 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 DbI 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-Y487F-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...
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 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 Gαq-dependent Ca2+ signaling by siRNA targeting Gαq, or using pharmacological inhibitors of Ca2+-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 Gαq and Ca2+ 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 vasomotor 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 (Arhgef1lox/lox) mice19 to mice expressing a fusion protein of the Cre recombinase with a modified estrogen receptor–binding domain (CreERT2) under the control of the smooth muscle myosin heavy chain (SMMHC, also known as Myh11) promoter (SMMHC-CreERT2 mice)20 to produce SMMHC-CreERT2;Arhgef1lox/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 ± 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 ± 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 ± 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 (p-MYPT) in blots, relative to MYPT expression (mean ± 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). Presor 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 floxed 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 Nω-nitro-l-arginine (L-NAME)
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 Arhgef1lox/lox mice (Fig. 6b). Moreover, this treatment lowered the levels of phosphorylated MYPT, Jak2 and Arhgef1 in arteries from Ang II–treated Arhgef1lox/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 genome. Among them, the small subfamily of RGS-containing Rho GEFs are the only ones subject to regulation by the Gα11 family of heterotrimeric G protein α subunits (Gα11 and Gαq)21–25. However, our results showed that Ang II–induced Arhgef1 activation was independent of the RGS domain and regulation by Gα11 and Gαq, 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 Gαq and Ca2+ signaling, in agreement with previous observations describing the Ca2+ dependence of Ang II–induced Jak2 activation26,27, as well as with previous work identifying Gαq or Gα11-dependent pathways as a main signaling mechanism for Ang II28,29. Thus, the RhoA/Rho kinase and Ca2+ signaling pathways are not completely independent, parallel processes controlling Ang II–mediated VSMC contraction, but are connected through Jak2. The rise in intracellular Ca2+ concentration induced by Ang II in VSMCs seems to be essential for inducing Arhgef1/RhoA signaling and Ca2+ sensitization; both this rise in Ca2+ concentration and RhoA-mediated Ca2+ 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 Ca2+ levels induced by Ang II is not by itself sufficient to lead to sustained vasoconstriction. The initial transient rise in the intracellular Ca2+ concentration needs to be relayed by a RhoA/Rho kinase–dependent Ca2+ sensitization mechanism to allow continued contraction.

Although Jak2 has been shown to be involved in Ang II–induced VSMC proliferation30,31, 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 Ca2+ 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.\textsuperscript{15,33} Accordingly, depending on the upstream signal, different Rho A 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 \( \gamma \)G12-\( \alpha \)G12-activated Arhgef2 signaling downstream of the endothelin-1 and thromboxane A2 receptors.\textsuperscript{39} Our present study, together with that previous study\textsuperscript{20}, supports the concept that RhoA GEFs discriminate upstream signals: whereas the \( \gamma \)G12-\( \alpha \)G12/Arhgef12 pathway is crucial in salt-induced hypertension, Arhgef1 signaling, downstream of \( \gamma \)Gq and Ca\textsuperscript{2+}, is essential for Ang II-induced vasoconstriction and hypertension.

Ang II–induced vasoconstriction of arterial rings \textit{in vivo} was virtually abolished in arteries from SM-Arhgef1-KO mice; however, small, transient pressor responses to acute Ang II injections \textit{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.\textsuperscript{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 \textit{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.\textsuperscript{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,\textsuperscript{41,42} and it has recently been shown that aldosterone and Ang II synergistically activate RhoA.\textsuperscript{43} 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\textsuperscript{44,45} or indirect\textsuperscript{46} 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, proteinomics and pulldown analyses. K.R., L.L. and D.H. collaborated on ex vivo contracture measurements. E.S. and A.B. helped with the organization of the study. R.M.T. and S.O. generated \textit{Arhgef1lox/lox} mice and SMMHC-CreER\textsuperscript{ERT2} 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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ARTICLES

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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 (ΔN-Arhgef1, amino acids 351–912, and DPHH-Arhgef1, amino acids 357–800). We created phosphoryrestain mutants by substituting Tyr487 and/or Tyr738 with a phenylalanine residue (Y487F-Arhgef1, Y738F-Arhgef1 and DPHH-YY/FF-Arhgef1), which corresponds to DPHH-Arhgef1 carrying the Y487F and Y738F mutations). The phosphomimetic Y738F-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′-GUAGUUGCCAGAAGAAUUDTdT-3′ and the scrambled siRNA sequence was 5′-CGACUUCUCCUACCUAAUCGdTdT-3′. Cells were transfected with Ang II (0.1 μM, Sigma) in the presence of the Ang II type 2 receptor antagonist PD 123319 (1 μM, Sigma). The deletion of Arhgef1 phosphorylation by autoradiography, cells were incubated in the presence of [3P] (0.6 μCi) 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 phosphorytrosine 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 nonphospho-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 μM Y-27632 (Sigma) or 10 μM fasudil (LC laboratories); data not shown). Blots were quantitated 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 elsewhere47. 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 the gel was stained with Coomassie blue, spots of interest analyzed by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by MALDI-TOF mass spectrometry (Micromass-Waters), and selected tryptic peptides sequenced by 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The Rho exchange factor Arhgef1 mediates Angiotensin II effects on vascular tone and blood pressure

Christophe Guilluy, Jérémy Brégeon, Gilles Toumaniantz, Malvyne Rolli-Derkinderen, Kevin Retailleau, Laurent Loufrani, Daniel Henrion, Elizabeth Scalbert, Antoine Bril, Raul Torres, Stephan Offermanns, Pierre Pacaud, Gervaise Loirand
Supplementary Table 1 Arhgef1 identification by mass spectrometry. Name and accession numbers for the protein showing increased association to the active GEF trap (G17A-RhoA) identified by mass spectrometry.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Molecular weight</th>
<th>pI</th>
<th>sequence coverage (%)</th>
<th>number of peptides</th>
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<td>Arhgef1: Rho guanine exchange factor 1</td>
<td>Q9Z116</td>
<td>103055.61</td>
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Supplementary Figure 1 Arghef1 knock-down in rat aortic smooth muscle cells prevents RhoA/Rho kinase activation by Ang II but not by other vasoconstrictors. (a,b) Analysis of the effect of siRNA-mediated Arhgef1 silencing on Ang II-induced RhoA/Rho kinase activation. The effect of siRNA-mediated silencing of Arhgef11, Arhgef12, vav2 and Vav3, suggested to be involved in Ang II signaling1, was also analyzed. Sequences of siRNA and PCR-primers used are given in the Supplementary Table 2 below. Selected siRNAs decreased the expression of their target mRNA, measured by quantitative RT-PCR, by at least 60%. Results are considered significant if the 3 siRNAs gave qualitatively similar results. Results shown were obtained with the siRNA indicated in bold (Supplementary Table 2). Silencing of Arhgef1 inhibited phosphorylation of the Rho kinase target MYPT (a) and RhoA (b) activation induced by Ang II (0.1 µM) while siRNA-mediated silencing of Arhgef11, Arhgef12,
vav2 and vav3 had no effect. The level of MYPT phosphorylation was expressed relative to the MYPT phosphorylation induced by Ang II (5 and 60 min) in scrambled siRNA-treated cells. The level of GTP-bound RhoA was expressed relative to its basal level in the absence of Ang II. *P<0.01. (c) In addition to the quantitative RT-PCR validation, the efficiency of siRNA was also checked at the level of the protein expression by western blot. (d) Measurement of RhoA activation (left) and MYPT phosphorylation (right) in smooth muscle cells treated with scramble or Arhgef1 siRNA, in control condition and after stimulation with U46619 (1 µM) endothelin-1 (0.1 µM) or noradrenaline (1 µM) (results displayed are parts of the same blot). Arhgef1 silencing does not modify the activation of RhoA/Rho kinase induced by these vasoconstrictors.

**Supplementary Table 2 : Quantitative RT-PCR primers and siRNA sequences (rat sequences).**

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<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>5'-3' primer sequence</th>
<th>Base pair</th>
<th>siRNA sequence (sense)</th>
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<tr>
<td>GAPDH</td>
<td></td>
<td>FW CCATGCCATCAGCCTGACT</td>
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<td></td>
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<td>RW GTCATCATACTGGCAGGTTTC</td>
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<td></td>
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<td>5'-GAA-GAC-UUA-UGA-GUG-AAG-A-dTdT-3'</td>
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<tr>
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<td>5'-GCA-AAU-UUA-UGG-AAG-AGG-dTdT-3'</td>
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<td></td>
<td>5'-UGA-AGG-AGA-UUA-UUUA-A-dTdT-3'</td>
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<td>VAV3</td>
<td>XM_227600.4</td>
<td>FW F:CGGAAGAAAGTGTAAATGATGAAGA</td>
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<td>5'-GGA-GGA-GAU-UCA-UCA-GAU-C-dTdT-3'</td>
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<td></td>
<td></td>
<td></td>
<td>5'-UUC-UCG-UCU-ACC-UCU-CUG-A-dTdT-3'</td>
</tr>
<tr>
<td>Scramble</td>
<td></td>
<td></td>
<td></td>
<td>5'-CGA-CUU-CCU-UAC-UUA-A-dTdT-3'</td>
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Supplementary Figure 2 Arhgef1-induced RhoA signaling activation does not depend on RGS/Gα13 signaling but depend on Gαq and Ca²⁺ signaling. (a) Western blot with anti-Gα13 antibody of immunoprecipitated Arhgef1 from control or Ang II (0.1 µM)-stimulated aortic smooth muscle cells indicates that the two proteins does not interact. This interaction between Arhgef1 and Gα13 is observed in leukocytes stimulated by U46619 used as positive control. Blots were also performed in total lysates. (b) Rho kinase activity monitored by the phosphorylation of MYPT in smooth muscle cells expressing AU1-tagged WT-Arhgef1, ΔN-Arhgef1 or DHPH-Arhgef1 under control condition or stimulated by Ang II (0.1 µM). The ΔN-Arhgef1, that lacks the RGS domain, did not modify Ang II-induced Rho kinase activation. (c,
d) Expression of the RGS domain of Arhgef1 (myc-tagged), characterized as a G\(\alpha_{13}\) signaling inhibitor did not modify Ang II (0.1 µM)-induced RhoA activation (c), Rho kinase and Arhgef 1 activation (d). (e) Analysis of the effect of siRNA targeting G\(\alpha_q\), G\(\alpha_{12}\) and G\(\alpha_{13}\) on the phosphorylation of the Rho kinase target MYPT induced by 0.1 µM Ang II in rat aortic smooth muscle cells. Results shown were obtained with the following siRNA sequences (Eurogentec) (sense): G\(\alpha_q\): 5’-AGA-ACU-UGA-UCG-UAU-UCG-C-dTdT-3’; G\(\alpha_{12}\): 5’-UAA-GUU-AUC-CAG-GAA-GUA-C-dTdT-3’; G\(\alpha_{13}\): 5’-AAG-AGU-AUU-GAU-GUC-ACA-C-dTdT-3’. Efficiency of target silencing was checked by quantitative RT-PCR and was more than 60% for each target at 48 h post-transfection. Only siRNA-mediated G\(\alpha_q\) silencing inhibited the effect of Ang II. (f) Analysis of the effect of Ca\(^{2+}\) modulating agents on the phosphorylation of the Rho kinase target MYPT induced by 0.1 µM Ang II (60 min) in rat aortic smooth muscle cells. Ca\(^{2+}\)-free medium, BAPTA-AM (30 µM), thapsigargin (2 µM) or nifedipine (5 µM) were applied to the cells 1 h before Ang II stimulation. The effect of Ang II on MYPT, Arhgef1 and Jak2 phosphorylation in each condition was quantified by densitometric analysis. The level of MYPT, Arhgef1 and Jak2 phosphorylation was expressed relative to its level under control condition. Results shown are mean of 4 experiments, *P<0.001. These data indicate that Ang II-induced activation of the jak2/Arhgef1 pathway is G\(\alpha_q\)- and Ca\(^{2+}\)-dependent.
Supplementary Figure 3 Endogenous Jak2 mediated Ang II-induced Arhgef1 phosphorylation and activation in vascular smooth muscle cells. (a) Phosphorylation of Arhgef1 by Ang II, measured by blotting immunoprecipitated phospho-tyrosine proteins with anti-Arhgef1 antibody, was inhibited by the Jak2 inhibitor AG490 (20 µM) whereas the non-selective tyrosine kinase inhibitor genistein (10 µM), the Src family inhibitor SU6656 (5 µM) and the epidermal growth factor receptor kinase blocker AG1478 (5 µM) had no effect. (b) AG490 also inhibited Ang II-induced Rho kinase activation, monitored by the level of P-MYPT, and Jak2 activation, monitored by the level of P-Jak2.
Supplementary Figure 4 Western blot analysis of Arhgef1 expression in lysates from mesenteric arteries of SM-Arhgef1-floxed and SM-Arhgef1-KO mice (typical results, 2 mice in each group). Cre-mediated recombination in mesenteric artery led to a decrease in Arhgef1 expression of 80-90% in SM-Arhgef1-KO mice compared to control SM-Arhgef1-floxed.
**Supplementary Figure 5** Profiles over 24 h in blood pressure pressure in SM-Arhgef1-KO and Arhgef1^lox/lox^ mice. Measurements were performed under a 12:12-hour light/dark schedule. Both group of mice (n=5 in each group) have been treated with tamoxifen one week before being instrumented with a telemetric system. (a) Profiles over 24 h in systolic and diastolic blood pressure. (b) Boxes show blood pressure amplitudes, with upper line representing systolic pressure and lower line, diastolic pressure. Blood pressures were similar in the two groups of mice.
Supplementary Figure 6 AT1 expression and plasma Ang II concentration were not modified in SM-Arhgef1-KO mice. (a) Western blot analysis of AT1 receptor expression in aortas of SM-Arhgef1-floxed and SM-Arhgef1-KO mice (2 mice in each group). (b) Measurement of plasma Ang II concentration (Elisa kit, Phoenix Pharmaceuticals) in SM-Arhgef1-floxed and SM-Arhgef1-KO mice fitted with osmotic minipump releasing saline (−) or Ang II (1 µg⁻¹ kg⁻¹ min⁻¹) (+). Results are expressed relative to the values obtained in control condition in SM-Arhgef1lox/lox mice.
Supplementary Figure 7 Role of Arhgef1 in L-NAME and DOCA-salt induced hypertension. Systolic blood pressure in Arhgef1lox/lox and SM-Arhgef1-KO mice chronically treated with L-NAME (a) or DOCA-Salt (b). Both groups of mice have been treated with tamoxifen one week before the beginning of the experiments. (a) Arhgef1lox/lox and SM-Arhgef1-KO mice have been treated with L-NAME (3 g l−1 in drinking water). This treatment induced rise in arterial blood pressure in Arhgef1lox/lox mice but not in SM-Arhgef1-KO mice. Additional treatment with the AT1 receptor antagonist losartan (16 mg kg−1 d−1) restored normal blood pressure value in Arhgef1lox/lox mice, thus attesting the involvement of AT1 receptor in L-NAME-induced hypertension. Losartan had no significant effect in SM-Arhgef1-KO mice. (mean of 6 mice in each group) (b) Arhgef1lox/lox and SM-Arhgef1-KO mice were unilaterally nephrectomized, subcutaneously implanted with a DOCA pellet (50 mg DOCA, 60-d release time, Innovative research of America) and received 1% NaCl in drinking water. This treatment increased blood pressure in both groups, but the rise in blood pressure induced in SM-Arhgef1-KO mice was significantly lower than in Arhgef1lox/lox (P<0.001, n=6). Additional treatment with losartan (16 mg kg−1 d−1) partially reversed DOCA-salt-induced hypertension in Arhgef1lox/lox but had no effect in SM-Arhgef1-KO mice. These results are in agreement with previous data showing that DOCA-salt-induced hypertension was not or only partially inhibited by RAS inhibitors, but was blocked by endothelin and thromboxane A2 antagonist2−4. Taken together, these results thus suggest that Arhgef1 is selectively involved in Ang-II dependent control of blood pressure.