

G₁₂-G₁₃-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension

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The tone of vascular smooth muscle cells is a primary determinant of the total peripheral vascular resistance and hence the arterial blood pressure. Most forms of hypertension ultimately result from an increased vascular tone that leads to an elevated total peripheral resistance¹⁻³. Regulation of vascular resistance under normotensive and hypertensive conditions involves multiple mediators, many of which act through G protein-coupled receptors on vascular smooth muscle cells⁴. Receptors that mediate vasoconstriction couple with the G-proteins G_q-G₁₁ and G₁₂-G₁₃ to stimulate phosphorylation of myosin light chain (MLC) via the Ca²⁺/MLC kinase- and Rho/Rho kinase-mediated signaling pathways, respectively⁴⁻⁶. Using genetically altered mouse models that allow for the acute abrogation of both signaling pathways by inducible *Cre/loxP*-mediated mutagenesis in smooth muscle cells, we show that G_q-G₁₁-mediated signaling in smooth muscle cells is required for maintenance of basal blood pressure and for the development of salt-induced hypertension. In contrast, lack of G₁₂-G₁₃, as well as of their major effector, the leukemia-associated Rho guanine nucleotide exchange factor (LARG), did not alter normal blood pressure regulation but did block the development of salt-induced hypertension. This identifies the G₁₂-G₁₃-LARG-mediated signaling pathway as a new target for antihypertensive therapies that would be expected to leave normal blood pressure regulation unaffected.

Arterial hypertension is a common health problem that is a major risk factor for a variety of diseases, such as myocardial infarction and stroke^{7,8}. However, the pathogenesis of hypertension, as well as the basic mechanisms of blood pressure control, are still insufficiently understood. It is well established that dietary salt intake and a tendency toward salt retention are important in the pathogenesis of hypertension^{9,10}. The prevailing hypothesis suggests that increased sodium retention leads to an elevation of the extracellular fluid

volume and an increased cardiac output. The resulting tissue over-perfusion is normalized by autoregulatory processes that lead to an increased tone of resistance blood vessels and an elevated total peripheral vascular resistance, a hallmark of hypertension². More recently, evidence has been provided that the increase in vascular tone after salt and water retention involves endogenous factors that promote increased Ca²⁺ entry via the Na⁺-Ca²⁺ exchanger¹¹⁻¹³. However, the cellular mechanisms underlying the local autoregulatory increase in vascular tone have been elusive.

The vascular tone is under the control of various neural, humoral and rheological stimuli that regulate the phosphorylation state of the MLC. Phosphorylation of the MLC allows myosin to interact with actin and generate contractile forces. MLC phosphorylation can be increased by activation of the MLC kinase (MLCK) through a Ca²⁺- and calmodulin-dependent mechanism or by inhibition of myosin phosphatase via the Rho/Rho kinase pathway^{14,15}. Many contractile stimuli, acting through G protein-coupled receptors, induce MLC phosphorylation via activation of the G proteins G_q and G₁₁ and the subsequent stimulation of phospholipase C-β, resulting in the Ca²⁺ and calmodulin-dependent activation of MLCK (ref. 6). The receptors of many vasoconstrictors also couple to the G proteins G₁₂ and G₁₃ to activate the Rho/Rho kinase pathway, resulting in the inhibition of myosin phosphatase⁴⁻⁶. Activation of RhoA through G₁₂ and G₁₃ is mediated by a subgroup of Rho guanine nucleotide exchange factors (RhoGEFs), which are activated through the direct interaction with Gα₁₂ and Gα₁₃ (ref. 16). Thus, the dual regulation of MLC phosphorylation through Ca²⁺-dependent MLCK activation and Rho/Rho kinase-mediated myosin phosphatase inhibition is initiated by the dual coupling of receptors to G_q-G₁₁ and G₁₂-G₁₃, respectively^{5,6}.

To understand the role of the G_q-G₁₁- and G₁₂-G₁₃-mediated signaling pathways in the regulation of vascular tone under normal and hypertensive conditions, we generated mice lacking the α-subunits of G_q-G₁₁ or G₁₂-G₁₃ specifically in smooth muscle cells. We have generated floxed alleles of the genes coding for Gα_q (*Gnaq*) and

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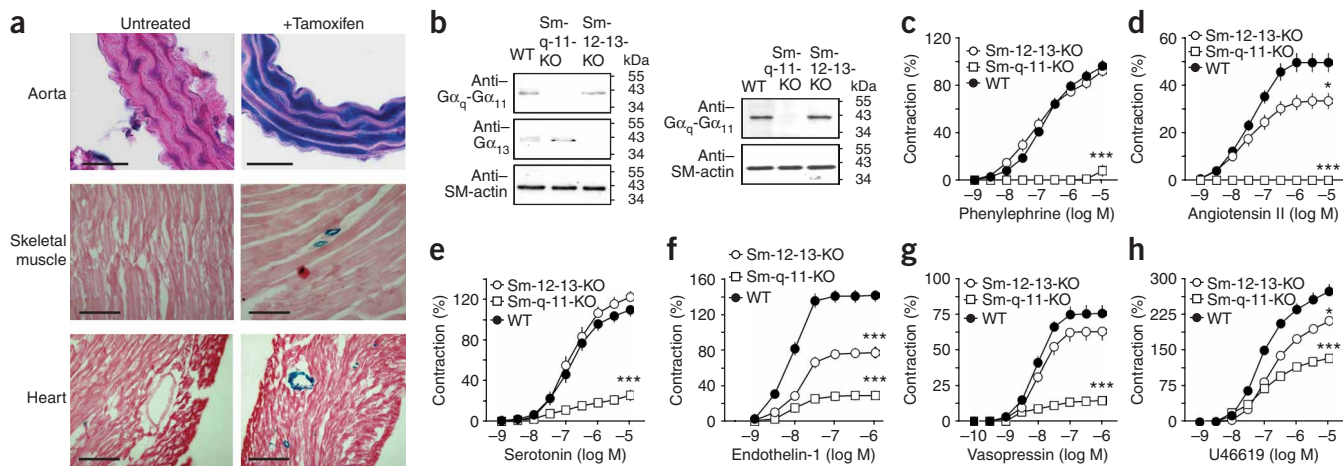


Figure 1 Generation of mice with smooth muscle-specific $G\alpha_q$ - $G\alpha_{11}$ and $G\alpha_{12}$ - $G\alpha_{13}$ deficiency and *in vitro* analysis. **(a)** Gt(ROSA)26SorCre reporter mice carrying the SMMHC-*CreERT*² transgene were treated with vehicle alone or with tamoxifen and then killed. The indicated organs were sectioned and stained for β -galactosidase activity. Scale bars, 20 μ m (aorta) and 100 μ m (skeletal muscle and heart). **(b)** Aorta lysates (left) and vascular smooth muscle cells (right) prepared from wild-type (WT), Sm- $G\alpha_q$ - $G\alpha_{11}$ -KO (Sm-q-11-KO) and Sm- $G\alpha_{12}$ - $G\alpha_{13}$ -KO (Sm-12-13-KO) mice were analyzed by western blotting with antibodies to $G\alpha_q$ - $G\alpha_{11}$, $G\alpha_{13}$ or SM-actin. **(c–h)** Dose-response curves of different vasoconstrictor agents in aortic segments of WT, Sm-q-11-KO and Sm-12-13-KO mice. All values are expressed as percentages of the 124-mM K^+ -induced reference contraction. Shown are mean values \pm s.e.m. * P < 0.05 and *** P < 0.001 versus WT.

$G\alpha_{13}$ (*Gna13*), which allow us to conditionally inactivate these genes alone or in a $G\alpha_{11}$ - or $G\alpha_{12}$ -deficient background^{17,18}. To restrict conditional $G\alpha_q$ - $G\alpha_{11}$ and $G\alpha_{12}$ - $G\alpha_{13}$ double deficiencies to smooth muscle cells, we developed a transgenic mouse line expressing a fusion protein of the Cre recombinase with the modified estrogen receptor binding domain (*CreERT*²)¹⁹ under the control of the smooth muscle myosin heavy chain (SMMHC) promoter. After treating the mice with tamoxifen, we observed Cre-mediated recombination exclusively in smooth muscle cells, whereas we did not observe recombination in other cell types, including heart and skeletal muscle cells (**Fig. 1a** and **Supplementary Fig. 1** online). $G\alpha_q$ - $G\alpha_{11}$ and $G\alpha_{12}$ - $G\alpha_{13}$ double deficiency in tamoxifen-treated SMMHC-*CreERT*²;*Gnaq*^{flx/flx}, *Gna11*^{-/-} (Sm- $G\alpha_q$ - $G\alpha_{11}$ -KO) and SMMHC-*CreERT*²;*Gna12*^{-/-}; *Gna13*^{flx/flx} (Sm- $G\alpha_{12}$ - $G\alpha_{13}$ -KO) mice could be verified by western blotting of vascular wall lysates and isolated vascular smooth muscle cells from the respective mice (**Fig. 1b**).

We then studied the effect of various vasoconstrictors on vascular segments from Sm- $G\alpha_q$ - $G\alpha_{11}$ -KO and Sm- $G\alpha_{12}$ - $G\alpha_{13}$ -KO mice. In aortic segments prepared from tamoxifen-treated SMMHC-*Cre*; $G\alpha_q$ ^{flx/flx}; $G\alpha_{11}$ ^{-/-} mice, phenylephrine had no contractile effect (**Fig. 1c**), whereas its ability to induce contraction was unaffected by $G\alpha_{12}$ - $G\alpha_{13}$ deficiency. Serotonin, vasopressin, U46619 and endothelin-1, but not angiotensin II, were still able to induce some contraction of $G\alpha_q$ - $G\alpha_{11}$ -deficient vessels (**Fig. 1d–h**); however, their potency, as well as efficacy, was severely reduced. The potency and efficacy of angiotensin II, U46619 and endothelin-1 was also reduced in $G\alpha_{12}$ - $G\alpha_{13}$ -deficient vessels compared to wild-type vessels (**Fig. 1d,f,h**).

The effects of the various pressor substances on isolated aortic segments were comparable to their acute pressor effects *in vivo* (**Fig. 2a**). Pressor responses to phenylephrine in Sm- $G\alpha_q$ - $G\alpha_{11}$ -KO mice were completely abrogated (**Fig. 2a**), whereas the effects of angiotensin II, vasopressin and endothelin were reduced. Pressor

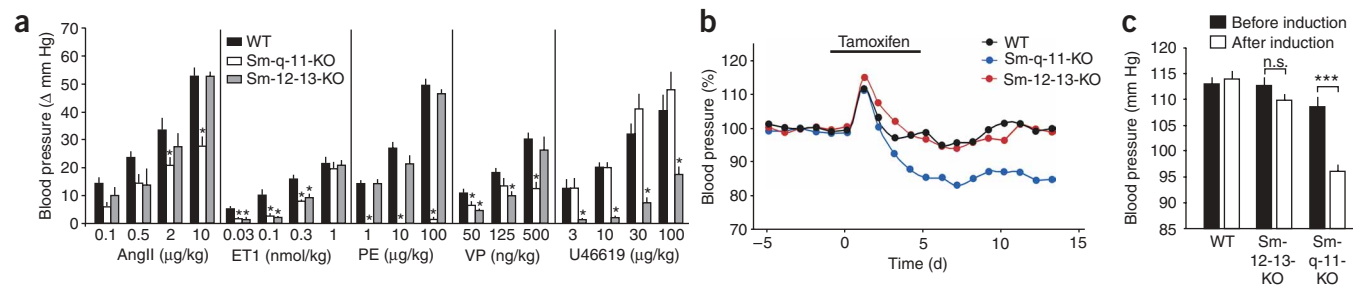
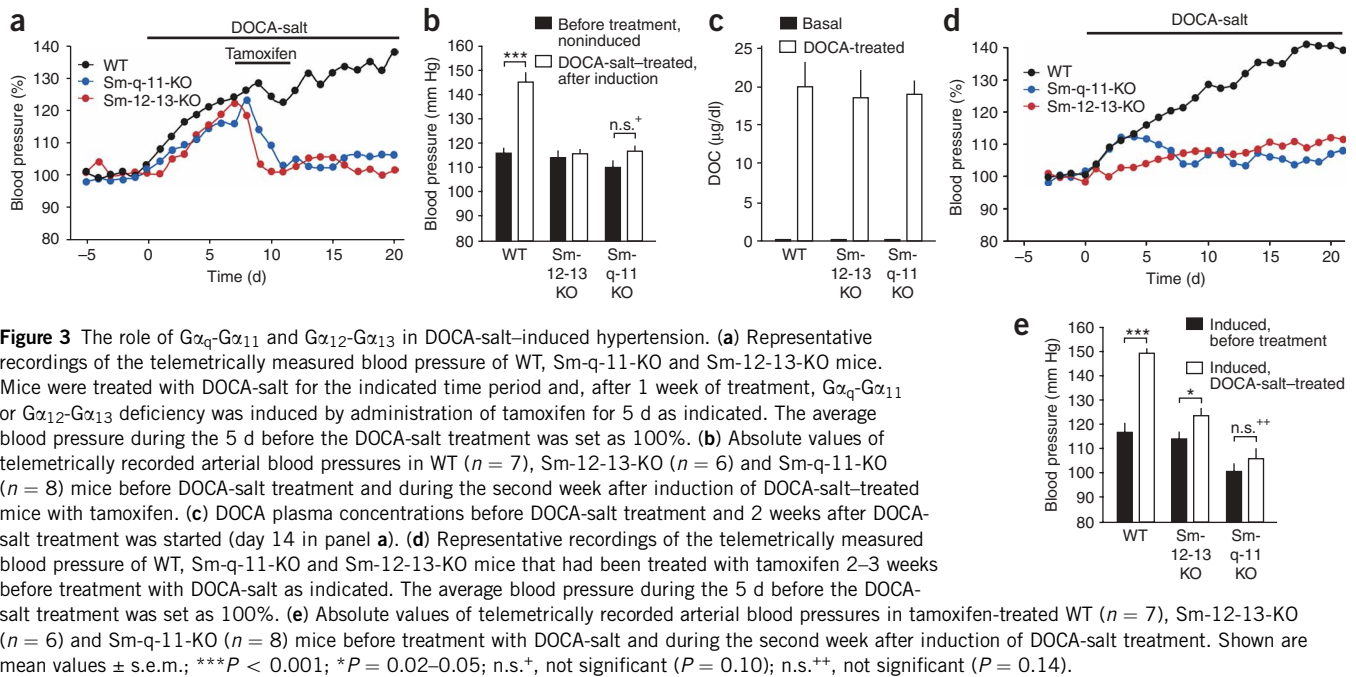


Figure 2 Basal blood pressure and pressor responses of smooth muscle-specific $G\alpha_q$ - $G\alpha_{11}$ - and $G\alpha_{12}$ - $G\alpha_{13}$ -deficient mice. **(a)** Arterial blood pressure was monitored telemetrically in anesthetized mice before and after the intravenous injection of the indicated doses of phenylephrine (PE), angiotensin II (AngII), endothelin-1 (ET1), U46619 and vasopressin (VP). Shown is the maximal blood pressure change, in mm Hg, after injection of the stimulus. Values are means \pm s.e.m.; * P < 0.05 versus WT. **(b)** Representative recordings of the telemetrically measured blood pressure of WT, Sm-q-11-KO and Sm-12-13-KO mice. $G\alpha_q$ - $G\alpha_{11}$ and $G\alpha_{12}$ - $G\alpha_{13}$ deficiency was induced by administration of tamoxifen on five consecutive days as indicated. The average blood pressure during the 5 d before injection was set as 100%. **(c)** Absolute values of telemetrically recorded arterial blood pressure in wild WT (n = 8), Sm-12-13-KO (n = 6) and Sm-q-11-KO (n = 6) mice before and after induction with tamoxifen. Blood pressure values were averaged for 1 week before administration of tamoxifen (before induction) and during the second week after induction with tamoxifen (days 8–14; after induction). Shown are mean values \pm s.e.m.; *** P < 0.001; n.s., not significant.



responses to endothelin-1, U46619 and vasopressin were affected by $G\alpha_{12}$ - $G\alpha_{13}$ deficiency (Fig. 2a).

Non-induced SMMHC-CreER^{T2};*Gnaq*^{fllox/fllox};*Gna11*^{-/-} mice, which lack only $G\alpha_{11}$, had slightly reduced mean arterial blood pressure values compared to wild-type mice and noninduced SMMHC-CreER^{T2};*Gna12*^{-/-};*Gna13*^{fllox/fllox} mice, which lack only $G\alpha_{12}$ (Fig. 2). During the tamoxifen treatment period, there was a transient increase in mean arterial blood pressure in mice of all genotypes (Fig. 2b). Three days after induction of Cre activity, the mean arterial blood pressure of Sm- $G\alpha_q$ - $G\alpha_{11}$ -KO mice dropped by about 10–15 mm Hg, whereas the blood pressure of wild-type and Sm- $G\alpha_{12}$ - $G\alpha_{13}$ -KO mice remained at normal levels (Fig. 2b,c). Thus, regulation of basal blood pressure requires the G_q - G_{11} -mediated but not the G_{12} - G_{13} -mediated signaling pathway in vascular smooth muscle cells.

To determine the role of G_q - G_{11} and G_{12} - G_{13} in salt-sensitive hypertension, we treated mice with deoxycorticosterone acetate and NaCl (DOCA-salt). In wild-type and noninduced SMMHC-CreER^{T2};*Gnaq*^{fllox/fllox};*Gna11*^{-/-} or SMMHC-CreER^{T2};*Gna12*^{-/-};*Gna13*^{fllox/fllox} mice, DOCA-salt treatment produced a strong increase in blood pressure within a few days (Fig. 3), which, in wild-type mice, was not affected by tamoxifen treatment. However, induction of smooth muscle-specific $G\alpha_q$ - $G\alpha_{11}$ or $G\alpha_{12}$ - $G\alpha_{13}$ deficiency resulted in a rapid decrease in blood pressure to normotensive levels (Fig. 3a,b). Also, in mice in which $G\alpha_q$ - $G\alpha_{11}$ and $G\alpha_{12}$ - $G\alpha_{13}$ deficiency was induced before DOCA-salt treatment, the development of DOCA-salt-dependent hypertension was severely impaired (Fig. 3d,e). In mice lacking only $G\alpha_{11}$ or $G\alpha_{13}$, DOCA-salt treatment resulted in 16% and 17% increases in blood pressure, respectively, during the second week of treatment (data not shown), which was less than in wild-type mice (28%) but more than in Sm- $G\alpha_q$ - $G\alpha_{11}$ -KO and Sm- $G\alpha_{12}$ - $G\alpha_{13}$ -KO mice (5% and 7%, respectively), indicating that both G protein α -subunits are required. Efficient DOCA-salt treatment was verified by determination of DOCA plasma levels (Fig. 3c and data not shown). Thus, both G_q - G_{11} - and G_{12} - G_{13} -mediated signaling are crucially involved in the elevation of blood pressure in DOCA-salt-dependent hypertension.

The G-proteins G_{12} - G_{13} activate the Rho/Rho kinase-mediated signaling pathway by direct interaction of their α -subunits with the RhoGEF proteins p115-RhoGEF, PDZ-RhoGEF and LARG¹⁶. Of the three RhoGEF proteins, LARG has been shown to be expressed in blood vessels^{16,20,21} and was the predominant RhoGEF effector of G_{12} - G_{13} in the media of the mouse aorta (Fig. 4a). We found that aortic segments from LARG-deficient mice showed similar defects in angiotensin II- and endothelin-1-induced contraction as were seen in vessels from Sm- $G\alpha_{12}$ - $G\alpha_{13}$ -KO mice, whereas the effects of phenylephrine and serotonin were not affected (Fig. 4b and Fig. 1c–f). Telemetric analysis of basal blood pressure in LARG-deficient mice showed no abnormalities (Fig. 4c,d), indicating that LARG function is not required to maintain basal blood pressure. However, when LARG-deficient mice were treated with DOCA-salt, they did not develop hypertension (Fig. 4c,d). This further indicates the crucial role for the G_{12} - G_{13} -RhoGEF-mediated signaling pathway in the regulation of vascular smooth muscle tone during the development of salt-induced hypertension.

Although both the G_q - G_{11} - and the G_{12} - G_{13} -LARG-mediated signaling pathways in smooth muscle cells are required for the increased vasoconstriction in the context of salt-induced hypertension, only G_q - G_{11} are necessary to maintain basal blood pressure (Fig. 4e). A central role of G_q - G_{11} -mediated signaling in the regulation of the basal blood pressure has been suggested by the phenotype of mice deficient in regulator of G protein signaling-2, which have increased $G\alpha_q$ - $G\alpha_{11}$ activity as well as elevated basal blood pressure^{22,23}. The different roles of both pathways in the regulation of normal and elevated blood pressure may be due to the involvement of a different set of vasoconstrictors. On the basis of our data, α_1 -adrenergic effects on smooth muscle cells are exclusively mediated by G_q - G_{11} , whereas the effects of other vasoconstrictors such as angiotensin II, endothelin-1 or thromboxane A₂ also involve G_{12} - G_{13} . A role of endothelin-1 and thromboxane A₂ in salt-induced hypertension is suggested by the relatively strong antihypertensive effects of endothelin and thromboxane A₂ antagonists in DOCA-salt-hypertensive mice compared to normotensive mice (Supplementary Fig. 2 online). Hence, several

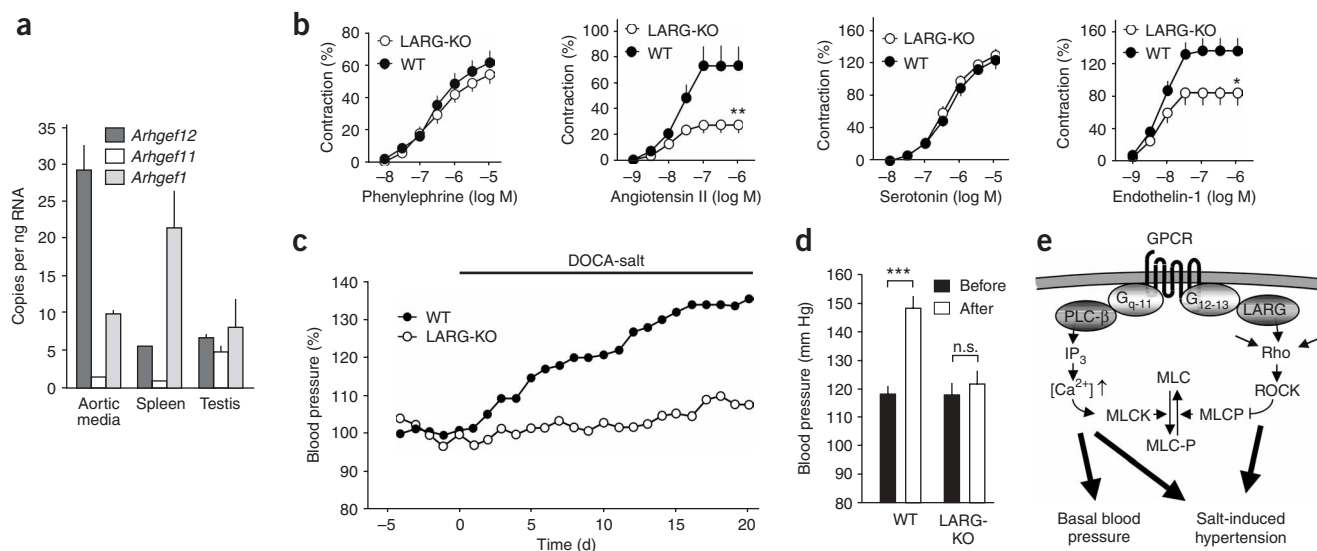


Figure 4 Role of LARG in DOCA-salt-induced hypertension. **(a)** Quantitative RT-PCR analysis of mRNAs for LARG (*Arhgef12*), PDZ-RhoGEF (*Arhgef11*) and p115-RhoGEF (*Arhgef1*) in the indicated tissues. Shown are mean values \pm s.e.m. ($n = 5$). **(b)** Dose-response curves of different vasoconstrictor agents in aortic segments of WT and LARG-deficient (LARG-KO) mice. All values are expressed as percentages of the contraction induced by 124 mM K^+ . Shown are mean values \pm s.e.m.; * $P < 0.05$ and ** $P < 0.01$ versus WT. **(c)** Representative recordings of the telemetrically measured blood pressure of WT and LARG-deficient mice. Mice were treated with DOCA-salt for the indicated time period. The average blood pressure during the 5 d before the DOCA-salt treatment was set as 100%. **(d)** Absolute values of telemetrically recorded arterial blood pressures in WT ($n = 6$) and LARG-KO ($n = 6$) mice before DOCA-salt treatment and during the second week after induction. Shown are mean values \pm s.e.m.; *** $P < 0.001$. **(e)** Proposed roles for G_q - G_{11} - and G_{12} - G_{13} -mediated signaling in blood pressure regulation. Activated G protein-coupled receptors (GPCRs) can increase vascular smooth muscle tone via G_q - G_{11} -mediated and Ca^{2+} -dependent activation of MLCK and via G_{12} - G_{13} -mediated inhibition of myosin phosphatase (MLCP) through the Rho/Rho kinase pathway. Although both pathways are crucially involved in the induction of hypertension by DOCA-salt, regulation of basal blood pressure requires only G_q - G_{11} -mediated signaling. PLC, phospholipase C- β ; IP₃, inositol triphosphate; ROCK, Rho kinase.

G protein-coupled receptor systems seem to mediate the increased vascular tone in salt-induced hypertension. We therefore think that the characterization of common downstream signaling steps represents a promising strategy for identifying new targets that will interfere with the increased vascular tone during salt-induced hypertension, independently of the G protein-coupled receptor systems involved.

The inhibition of Rho kinase, the final step in the RhoA-mediated regulation of vascular smooth muscle tone, has been shown to decrease blood pressure in various models of hypertension^{21,24–26}. The Rho/Rho kinase pathway can be activated by several mechanisms involving integrins, receptor tyrosine kinases or G protein-coupled receptors^{27,28}. Our data show that the G_{12} - G_{13} -mediated activation of Rho/Rho kinase via the RhoGEF protein LARG is a central mechanism of vascular smooth muscle tone regulation in salt-dependent hypertension.

Multiple pieces of evidence indicate that several mechanisms link dietary salt and hypertension, suggesting that hypertension is a multifactorial disorder^{3,29}. Our data here suggest that salt-induced increase in total peripheral resistance involves mediators acting through smooth muscle cell receptors, which are coupled to G_q - G_{11} and G_{12} - G_{13} . Although both G protein-mediated signaling pathways in vascular smooth muscle cells are crucial in salt-induced hypertension, maintenance of basal blood pressure depends only on G_q - G_{11} . This identifies the G_{12} - G_{13} -LARG-mediated signaling pathway as a new target for therapeutic approaches to reducing elevated vascular tone under hypertensive conditions without affecting normal blood pressure regulation.

METHODS

Chemicals and antibodies. Phenylephrine, angiotensin II, vasopressin, [deamino-Pen¹-O-Me-Tyr²,Arg⁸]-vasopressin and prazosin were purchased

from Sigma-Aldrich; endothelin-1 was from Calbiochem. U-46619 and SQ29,548 were from Cayman Europe, losartan was from Merck and darusentan was from Abbott. Antibodies to G_{α_q} - $G_{\alpha_{11}}$ were from Santa Cruz Biotechnology, antibodies to smooth muscle actin were from Sigma-Aldrich and the antibody to $G_{\alpha_{13}}$ has been described¹⁸.

Genetic mouse models. All animal care and use procedures in this study were approved by the local authorities (Regierungspräsidium Karlsruhe). The generation of floxed alleles of the genes encoding G_{α_q} (*Gnaq*) and $G_{\alpha_{13}}$ (*Gna13*), as well as of null alleles of the genes encoding $G_{\alpha_{11}}$ (*Gna11*) and $G_{\alpha_{12}}$ (*Gna12*), has been described previously^{17,18}.

The generation of an inducible, smooth muscle-specific Cre transgenic mouse line, as well as of mice lacking the RhoGEF protein LARG, is described in the **Supplementary Methods** and **Supplementary Figures 1** and **3** online. To verify the inducibility and activity of the Cre fusion protein, we mated SMMHC-CreER^{T2} mice with mice of the Cre-reporter transgenic line Gt(ROSA)26Sortm1sor (ROSA26-LacZ) (obtained from the Jackson Laboratory). We treated cotransgenic progeny from SMMHC-CreER^{T2} and ROSA26-LacZ crosses with tamoxifen (1-mg intraperitoneal injections per day for five consecutive days) or vehicle alone and killed them 14 d after induction. For histological analysis of β -galactosidase activity, we performed a staining reaction on 10–12- μ m cryosections and then counterstained with eosin.

Myography. For isometric tension recording in the isolated mouse aorta, we prepared 3-mm-long aortic segments, mounted them on a conventional myograph setup (610-M, Danish Myo Technology) and kept them in Krebs solution (119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 1.17 mM $MgSO_4 \cdot 7H_2O$, 20 mM $NaHCO_3$, 1.18 mM KH_2PO_4 , 0.027 mM EDTA, 11 mM glucose) aerated with carbogen. We adjusted the resting tension to 10–15 mN after 30 min at 37 °C, and exposed the segments to 124 mM K^+ Krebs solution in order to elicit a reference contraction. After a 30-min recovery period, we determined contractile responses by cumulative administration of one of the tested agonists. We performed experiments at least

eight times, and we performed statistical evaluation with one-way ANOVA and Tukey *post hoc* tests.

Telemetric blood pressure measurements. We used a radiotelemetry system (PA-C10, Data Sciences International) to monitor blood pressure in conscious, unrestrained mice, as described previously³⁰. We implanted the pressure-sensing catheter into the left carotid artery, and we inserted the transducer unit into a subcutaneous pouch along the right flank. After a recovery period of at least 1 week, we collected, stored and analyzed arterial pressure recordings with Dataquest A.R.T. software 4.0. We collected data for basal blood pressure measurements from DOCA-salt hypertensive mice with a 10-s scheduled sampling every 5 min and used the 24-h mean values for analysis. For analyzing the acute effects of agonists and antagonists, we collected data continuously in 5-s intervals for a total period of 30 min, and we averaged blood pressure values over an interval of 1 min. We performed the experiments in mice anesthetized with medetomidine (150 ng/g), midazolam (2 µg/g) and fentanyl (5 ng/g).

DOCA-salt hypertension. To produce DOCA-salt hypertensive mice, we unilaterally nephrectomized and implanted mice with the telemetric catheter. After 1 week, we implanted a DOCA pellet (50 mg DOCA, 21-d release time, Innovative Research of America) subcutaneously, and gave the mice drinking water containing 1% (w/v) NaCl. In all experiments, we compared wild-type, Sm-Gα_q-Gα₁₁-KO and Sm-Gα₁₂-Gα₁₃-KO mice or LARG-deficient mice subjected to the same treatment.

Quantitative reverse transcription-PCR. We used DNase-treated RNA isolated from the indicated tissues using the RNeasy Micro/Mini Kit (Qiagen) to prepare random-primed cDNA. We conducted quantitative PCR in reactions containing cDNA from 320 ng tissue RNA and SYBR Green indicator. We performed RT-PCR in a Chromo4 Real-Time Thermal Cycler using Opticon Monitor 3 software (Bio-Rad) for fluorescence detection and data evaluation. We generated standard curves from serially diluted standardized tissue genomic DNA. For oligonucleotide primer sequences, see **Supplementary Methods**.

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

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AUTHOR CONTRIBUTIONS

A.W. planned and performed most *in vivo* experiments, analyzed SMMHC-CreER^{T2} mice and was involved in writing the manuscript, Z.B. and M.L. planned and performed most *in vitro* experiments, B.L. helped generate and analyze SMMHC-CreER^{T2} mice, N.W. and S.G. helped perform *in vivo* experiments, P.Ö. and B.H. helped perform *in vitro* experiments, C.M.-G. performed DOCA level determinations, E.G. helped generate SMMHC-CreER^{T2} mice, B.L. helped perform *in vivo* experiments, G.S. helped generate SMMHC-CreER^{T2} mice, S.G. generated LARG-deficient mice, and S.O. planned and supervised the project and wrote the manuscript.

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