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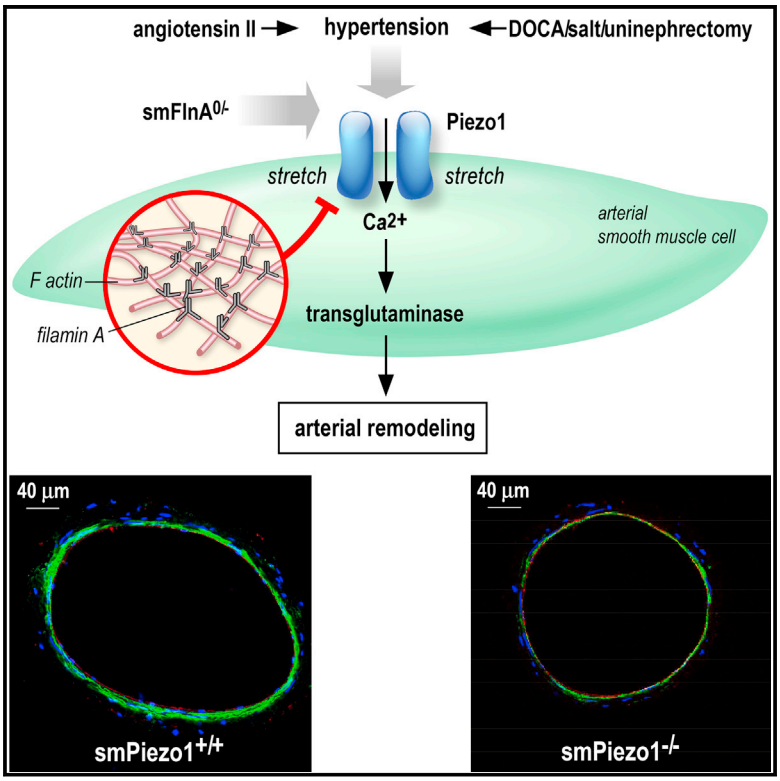
Piezo1 in Smooth Muscle Cells Is Involved in Hypertension-Dependent Arterial Remodeling

Graphical Abstract



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In Brief

Piezo1 is a stretch-activated ion channel expressed in the endothelium and its deletion alters vascular architecture. However, its role in arterial myocytes remains unknown. Retailleau et al. show that smooth muscle Piezo1 is dispensable for the arterial myogenic tone but involved in the remodeling of small arteries upon hypertension.

Highlights

- Smooth muscle Piezo1 is required for stretch-activated cationic channel activity
- Piezo1 is dispensable for the arterial myogenic tone
- Piezo1 opening has a trophic effect on the arterial wall of small arteries
- Piezo1 activation increases cytosolic calcium and transglutaminase activity

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Piezo1 in Smooth Muscle Cells Is Involved in Hypertension-Dependent Arterial Remodeling

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SUMMARY

The mechanically activated non-selective cation channel Piezo1 is a determinant of vascular architecture during early development. Piezo1-deficient embryos die at midgestation with disorganized blood vessels. However, the role of stretch-activated ion channels (SACs) in arterial smooth muscle cells in the adult remains unknown. Here, we show that Piezo1 is highly expressed in myocytes of small-diameter arteries and that smooth-muscle-specific Piezo1 deletion fully impairs SAC activity. While Piezo1 is dispensable for the arterial myogenic tone, it is involved in the structural remodeling of small arteries. Increased Piezo1 opening has a trophic effect on resistance arteries, influencing both diameter and wall thickness in hypertension. Piezo1 mediates a rise in cytosolic calcium and stimulates activity of transglutaminases, cross-linking enzymes required for the remodeling of small arteries. In conclusion, we have established the connection between an early mechanosensitive process, involving Piezo1 in smooth muscle cells, and a clinically relevant arterial remodeling.

INTRODUCTION

The molecular identity of non-selective stretch-activated ion channels (SACs) has long remained a mystery (Nilius, 2010; Pedersen and Nilius, 2007). Only recently, Piezo1 and Piezo2 were shown to be essential components of distinct SACs (Coste et al., 2010). Piezo1 is a pore-forming subunit, as demonstrated by functional reconstitution into artificial bilayers (Coste et al., 2012). Piezo1 exogenous depolarizing currents can be activated in the whole cell configuration upon pressure stimulation with a glass stylus, by fluid flow, by substrate deflection, or in the cell-attached patch configuration by applying a negative pressure (Bae et al., 2011; Coste et al., 2010; Gottlieb et al., 2012; Li et al., 2014; Peyronnet et al., 2013; Poole et al., 2014; Ranade et al., 2014). In terms of permeability, Na^+, K^+, Ca^{2+}, and Mg^{2+} all permeate the channel, with a slight preference for Ca^{2+} (Coste et al., 2010).

Global knockout of Piezo1 is embryonically lethal, thus indicating an important role for this mechanosensitive ion channel in early development (Li et al., 2014; Ranade et al., 2014). Piezo1 is expressed in the endothelium of developing blood vessels, and its genetic deletion profoundly alters vascular architecture (Li et al., 2014; Ranade et al., 2014). Piezo1 expression in the endothelium confers sensitivity to shear stress, resulting in a calcium influx in response to increased fluid flow (Li et al., 2014; Ranade et al., 2014). Loss of Piezo1 in endothelial cells leads to altered stress fiber organization and cell orientation in response to shear stress (Li et al., 2014; Ranade et al., 2014). These elegant findings indicate that shear stress activation of endothelial Piezo1 is required for the proper development of blood vessels (Li et al., 2014; Ranade et al., 2014).

The opening of SACs at the plasma membrane of smooth muscle cells has been proposed as a triggering mechanism for the myogenic response (Beech, 2005; Brayden et al., 2008; Davis and Hill, 1999; Folgering et al., 2008; Hill et al., 2006). The myogenic response is a tonic vasoconstriction of small-diameter arteries in response to an increase in intraluminal pressure and serves to maintain a constant blood flow within a wide range of blood pressures and is required for the establishment of a basal tone upon which vasorelaxing agents may act (Davis and Hill, 1999; Hill et al., 2006). While, various inhibitors of SACs have been shown to significantly reduce the amplitude of the myogenic response in different vascular beds (Drummond et al., 2004; Earley et al., 2004; Jernigan and Drummond, 2006; Takenaka et al., 1998a, 1998b; Welsh et al., 2002), non-specific effects of these pharmacological agents cannot be entirely ruled out. Moreover, the molecular identity of SACs in arterial myocytes remains obscure, with a possible involvement of epithelial Na^+ channel (ENaC) and/or transient receptor potential (TRP)

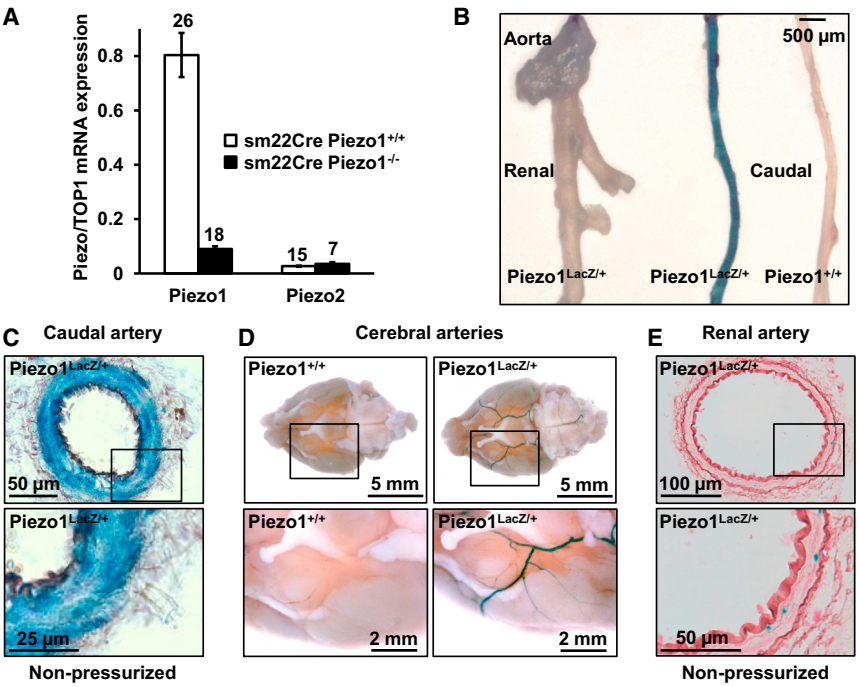

Figure 1. Piezo1 Expression in Arterial Smooth Muscle Cells

(A) qPCR data showing Piezo1 and Piezo2 mRNA expression (relative to the expression of topoisomerase 1; TOP1) in de-endothelized caudal arteries from sm22Cre Piezo1$^{+/+}$ mice (white bars) or sm22Cre Piezo1$^{-/-}$ mice (black bars). Number of mice is indicated. Data represent mean ± SEM.

(B) LacZ staining of aorta, renal artery, and caudal artery from Piezo1$^{LacZ/+}$ mice. Lack of staining is shown on the right side for a control Piezo1$^{+/+}$ caudal artery.

(C) LacZ staining of a caudal arterial section from a Piezo1$^{LacZ/+}$ mouse. The bottom panel shows a magnification of the boxed area.

(D) LacZ staining of cerebral arteries. The circle of Willis and cerebral arteries stained in blue are visible in the Piezo1$^{LacZ/+}$ mouse (right panel), compared to a control Piezo1$^{+/+}$ mouse brain (left panel). Bottom pictures illustrate a magnification of the medial cerebral artery, comparing Piezo1$^{+/+}$ (left) with Piezo1$^{LacZ/+}$ (right) mice (boxed in the upper pictures).

(E) Discrete LacZ staining in a renal artery section from a Piezo1$^{LacZ/+}$ mouse. The bottom panel shows a magnification of the boxed area.

See also Figure S1.

subunits (Drummond et al., 2004; Earley et al., 2004; Jernigan and Drummond, 2006; Lee et al., 2007; Park et al., 2003; Takenaka et al., 1998a, 1998b; Welsh et al., 2002).

In addition to an acute myogenic regulation of arterial diameter, resistance arteries also have the ability to regulate their caliber during chronic hypertension by a phenomenon called arterial remodeling, operating on a timescale of several days (Bakker et al., 2005, 2008; Martinez-Lemus et al., 2009; Mulvany, 2002). Arterial remodeling is a structural adaptation of the vessel wall to hemodynamic stimuli (Bakker et al., 2005, 2008; Martinez-Lemus et al., 2009; Mulvany, 2002). In chronic hypertension, conduit arteries undergo an hypertrophic remodeling, while resistance arteries show inward eutrophic remodeling, with a repositioning of smooth muscle cells around a smaller lumen diameter in the absence of hypertrophy (Mulvany, 2002). Those changes in wall thickness and diameter contribute, according to the law of Laplace ($\sigma = Pr_i/h$, where σ = wall stress, P = pressure, r_i = inner arterial radius, and h = wall thickness), to the arterial wall tensional homeostasis (Khavandi et al., 2009; Mulvany, 2002). Importantly, remodeling of small arteries has been linked to cardiovascular morbidity and mortality (Mathiassen et al., 2007; Rizzoni et al., 2003). Disorders in the structure and function of resistance arteries raise capillary pressure and may cause downstream organ damage, as occurring in diabetic nephropathy (Khavandi et al., 2009). The molecular mechanisms implicated in the remodeling of resistance arteries during hypertension are only starting to emerge (Bakker et al., 2005, 2008; Martinez-Lemus et al., 2009; Mulvany, 2002). Recent findings indicate that activation of the crosslinking enzyme transglutaminase 2 is involved in the remodeling of small arteries in hypertensive conditions (Bakker et al., 2005; Engholm et al., 2011; Huelsz-Prince et al., 2013).

Here, using a combination of smooth-muscle-specific knockout mouse models, we explored the functional role of Piezo1 in the adult circulation. We show that, while Piezo1 is dispensable for the arterial myogenic tone, it is centrally involved in the remodeling of small arteries.

RESULTS

Smooth-Muscle-Specific Piezo1 Knockout Mouse Model

qPCR analysis revealed that Piezo1, unlike Piezo2, is highly expressed in the cutaneous caudal artery (Figure 1A). We used the sm22Cre recombinase to specifically delete Piezo1 in smooth muscle cells of C57BL/6 male mice (sm22Cre Piezo1$^{-/-}$) (Figures S1A–S1F). We observed a robust knockdown of Piezo1 in the caudal artery without compensation by Piezo2 expression (Figure 1A). Mouse weight, tibial length, and viability were not affected by specific deletion of Piezo1 in smooth muscle cells (Figures S1G and S1H). Moreover, arterial pressure was not significantly modified in the absence of Piezo1, in awake or anesthetized mice (Figures S1K–S1M).

Taking advantage of a LacZ Piezo1 reporter mouse, we observed a remarkably strong expression of Piezo1 (shown in blue) in the media of small-diameter arteries, with the strongest signals seen in the cutaneous caudal artery and cerebral arteries (Figures 1B–1D). Indeed, isolated smooth muscle cells from the caudal resistance artery were strongly positive for Piezo1, as visualized by intense LacZ blue staining (Figures S1E and S1F). In contrast, a very low expression was detected in large-diameter conduit arteries, including the aorta or the main renal artery (Figures 1B and 1E).

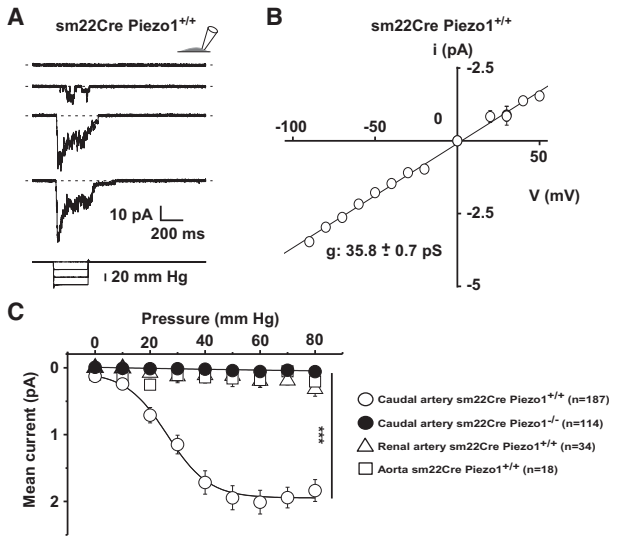

Figure 2. SAC Activity in Caudal Artery Myocytes Critically Requires Piezo1

(A) Non-selective SAC currents from a sm22Cre Piezo1$^{+/+}$ caudal artery myocyte were elicited in the cell-attached patch configuration by applying pulses of negative pressure in the patch pipette (bottom traces). Channel activity was recorded at a holding potential of −80 mV (top traces).

(B) Current-to-voltage relationship for single SACs in isolated myocytes from sm22Cre Piezo1$^{+/+}$ caudal artery myocytes (n = 5). i, single-channel current amplitude; V, voltage; g, single-channel conductance.

(C) Mean SAC current amplitude in sm22Cre Piezo1$^{+/+}$ (white circles) and sm22Cre Piezo1$^{-/-}$ (black circles) myocytes isolated from the caudal artery. For comparison, mean SAC current amplitude recorded in isolated myocytes from sm22Cre Piezo1$^{+/+}$ aorta (white squares) and sm22Cre Piezo1$^{+/+}$ renal artery (white triangles) is shown. Number of myocytes is indicated. SAC activity was measured in the cell-attached patch-clamp configuration in response to negative pressure pulses at a holding potential of −80 mV (as shown in A). The fitting parameters were $P_{0.5}$: −26.3 and k: 7.5 for caudal artery sm22Cre Piezo1$^{+/+}$.

Data represent mean ± SEM. ***p < 0.001.

See also Figure S2.

These findings indicate that Piezo1 is present at the adult stage in the smooth muscle of small arteries that participate actively in the regulation of peripheral resistance, as well as in the cerebral circulation. Moreover, Piezo1 in myocytes of small-diameter arteries is dramatically knocked down in the sm22Cre Piezo1$^{-/-}$ mouse model.

Piezo1 Is Critically Required for SAC Activity in Arterial Myocytes

We measured the activity of non-selective SACs in freshly isolated myocytes from the caudal artery using cell-attached patch clamp recordings (Figure 2A). SACs only partially inactivated during a maintained pressure pulse, and they reversed at about 0 mV, and the single-channel conductance was estimated to be about 35 pS (Figures 2A and 2B; Figure S2B). In line with the differential LacZ expression profile of Piezo1, SAC activity was significantly higher in smooth muscle cells derived from the caudal artery (128/187 active patches for sm22Cre Piezo1$^{+/+}$) compared to that elicited in myocytes from the aorta (1/18) or renal arteries (7/34) (Figure 2C). Most importantly, SAC activity in caudal artery myocytes was dramatically reduced upon homozygote deletion of Piezo1 (9/114 active patches for sm22Cre Piezo1$^{-/-}$; Figures 2C and S2A).

These findings demonstrate that Piezo1 is critically required for non-selective SAC activity in smooth muscle cells of the caudal artery.

Piezo1 Is Dispensable for the Caudal Artery Myogenic Tone

One possible mechanism for initiation of the myogenic tone is the opening of SACs in arterial smooth muscle cells in response to an increase in wall tension (for reviews, see Davis and Hill, 1999; Hill et al., 2006). Thus, we investigated whether Piezo1 knockout might influence the caudal artery myogenic tone. Pressure-dependent vasomotion was consistently present on top of the myogenic response in stop-flow experiments (Figure S3A). Myogenic response and vasomotion were also present when intraluminal flow was set at 15 μl/min, thus ruling out the possible contribution of an accumulating diffusible factor in the lumen (n = 3; data not shown). Moreover, myogenic response and vasomotion were observed in de-endothelized caudal arteries, while vasodilation induced by acetylcholine was lost (n = 3; not shown). The myogenic response reached a plateau at a pressure value of 50 mm Hg and further decreased in amplitude at pressure above 125 mm Hg (Figure S3B). The mean amplitude of vasomotion presented a maximum at 25 mm Hg and then gradually decreased at higher pressure values (Figure S3C). Remarkably, no significant change was observed upon Piezo1 knockout on either the myogenic response or pressure-dependent vasomotion (Figures S3B and S3C). Furthermore, the passive diameter measured in the absence of extracellular calcium and in the presence of vasorelaxants was unaffected in the absence of Piezo1 (Figure S3D). In addition, reactivity of the caudal artery to agonists—either vasoconstrictors or the vasorelaxant acetylcholine—as well as vasoconstriction induced by KCl (80 mM), was unaffected upon Piezo1 deletion (Figures S3E–S3I).

These results indicate that Piezo1-dependent SAC activity is not required for the myogenic tone and reactivity to agonists of the cutaneous caudal artery.

Piezo1 Is Dispensable for the Cerebral Artery Myogenic Tone

The lack of effect of Piezo1 knockout on the myogenic tone was confirmed with the rostral cerebellar artery, another vascular bed characterized by a high Piezo1 expression (Figure 1D). Again, SAC activity was absent (0/23 for sm22Cre Piezo1$^{-/-}$, as compared to 10/12 for sm22Cre Piezo1$^{+/+}$) in the isolated rostral cerebellar artery myocytes from sm22Cre Piezo1$^{-/-}$ mice, although myogenic response was unaffected (Figures S4A–S4C). Moreover, the passive arterial diameter of the rostral cerebellar artery was not modified in the absence of Piezo1 (Figure S4D).

Thus, Piezo1 is dispensable for the active myogenic tone of both caudal and cerebral arteries. Moreover, the lack of Piezo1 does not alter basal passive arterial diameter (i.e., in the absence of extracellular calcium). Next, we tested whether the influence of Piezo1 on arterial dimensions might be revealed at higher blood pressure.

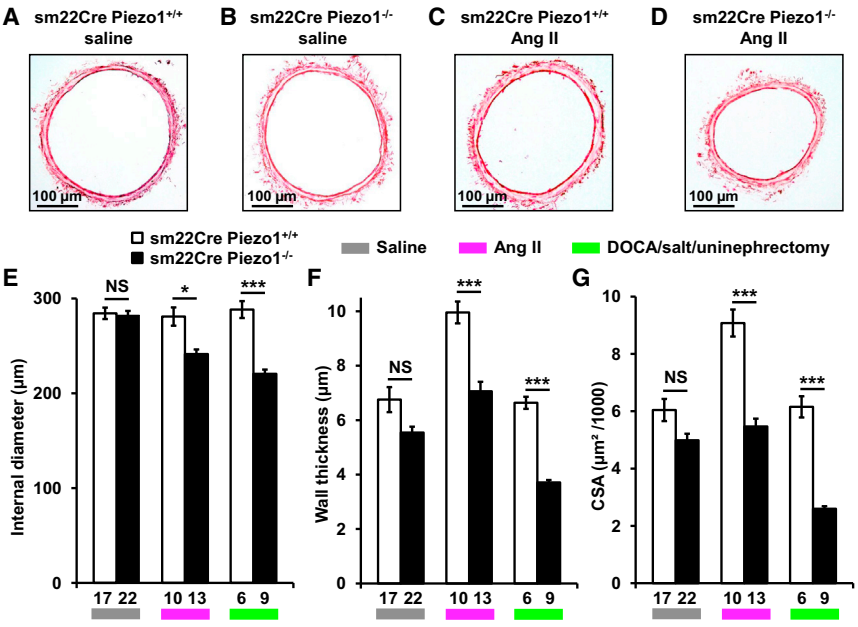

Figure 3. Caudal Artery Remodeling Is Influenced by Piezo1 in Hypertension

(A) Caudal artery sections are stained with orcein from a saline-infused normotensive sm22Cre Piezo1$^{+/+}$ mouse.

(B) Same as in (A) but from a saline-infused normotensive sm22Cre Piezo1$^{-/-}$ mouse.

(C) Same as in (A) but from an Ang-II-infused hypertensive sm22Cre Piezo1$^{+/+}$ mouse.

(D) Same as in (A) but from an Ang-II-infused hypertensive sm22Cre Piezo1$^{-/-}$ mouse.

(E) Internal diameter.

(F) Wall thickness.

(G) CSAs are illustrated for sm22Cre Piezo1$^{+/+}$ (white bars) and sm22Cre Piezo1$^{-/-}$ (black bars) arteries. Morphometric analysis has been performed on caudal artery sections stained with orcein. Five caudal artery sections were analyzed per mouse. Saline and hypertensive conditions (Ang II or DOCA/salt/uninephrectomy) are indicated by gray, magenta, and green horizontal bars, respectively. Number of mice is indicated.

Data represent mean ± SEM. *$p < 0.05$; ***$p < 0.001$. NS, not significant.

See also Figure S5.

Piezo1 Is Involved in Arterial Remodeling upon Hypertension

We investigated whether the opening of Piezo1 in smooth muscle cells might influence caudal artery remodeling upon hypertension. We used an in vivo experimental model of chronic hypertension involving angiotensin II (Ang II) infusion (Figures S1G and S1H; Figures S1L and S1M). Arterial segments in the absence of extracellular calcium and in the presence of the vasorelaxant agents sodium nitroprusside and papaverine (i.e., without active tone) were fixed at 75 mm Hg for morphological examination (Figure 3). In normotensive control conditions (saline), homozygote smooth muscle Piezo1 deletion did not significantly affect caudal artery morphology, in line with our previous measurements of passive diameter by arteriography (Figure 3; Figure S3D). However, Piezo1 knockout in Ang-II-infused hypertensive mice resulted in a significant reduction in arterial diameter; wall thickness; and, consequently, cross-sectional area (CSA) (Figure 3). We used a second in vivo model of hypertension, involving deoxycorticosterone acetate (DOCA)/salt/uninephrectomy (Figures S1G and S1H; Figures S1L and S1M). In the DOCA/salt/uninephrectomy hypertension model, arterial dimensions were unaffected in control animals, despite a strong hypertensive effect (Figures 3E–3G; Figures S1L and S1M). However, again, both diameter and wall thickness dramatically decreased, resulting in a significant drop in CSA during DOCA/salt/uninephrectomy hypertension in the absence of smooth muscle Piezo1 (Figures 3E–3G). By labeling smooth muscle cells with an antibody directed against sm22, we confirmed that media thickness was reduced upon smPiezo1 deletion in the DOCA/salt/uninephrectomy hypertension model, although the endothelial cell layer was not visibly altered (Figures S5A and S5B). Using Hoechst staining, we further estimated, by confocal microscopy in thin sections, the number of nuclei present in the media (Figures S5A and S5C). In the DOCA/salt/uninephrectomy smPiezo1$^{-/-}$ mice, the number of nuclei was significantly decreased (Figure S5C). However, TUNEL staining, as well as Ki67 immunolocalization were both negative after 3 weeks of DOCA/salt/uninephrectomy hypertension in both smPiezo1$^{+/+}$ and smPiezo1$^{-/-}$ mice (data not shown), suggesting that increased apoptosis and/or decreased proliferation upon Piezo1 deletion might have occurred at an earlier step during arterial remodeling or, alternatively, that an altered recruitment/differentiation from a non-smooth-muscle source might possibly be involved. In addition, we estimated collagen I and III expression in the arterial wall, taking advantage of Sirius red staining visualized under polarized light (Figures S5D–S5G). The expression of both collagen I and III isoforms was unchanged in the DOCA/salt/uninephrectomy smPiezo1$^{-/-}$ mice (Figures S5D–S5G). Notably, the effect of Piezo1 knockout on arterial dimensions tended to be correlated to blood pressure, with the strongest effect seen in the DOCA/salt/uninephrectomy condition (Figures 3E–3G; Figures S1L and S1M).

These findings demonstrate that, at high blood pressure, Piezo1 opening affects arterial structure (diameter and wall thickness), unlike at normal blood pressure. Next, we investigated whether an increase in the opening of Piezo1 in smooth muscle cells might be sufficient to induce arterial remodeling, independently of blood pressure. We took advantage of the potent mechanoprotection of SACs by the cytoskeletal element filamin A (FlnA) (Kainulainen et al., 2002; Sharif-Naeini et al., 2009) to generate a conditional mouse model in which Piezo1 opening can be selectively enhanced in smooth muscle cells but without the requirement of hypertension.

Smooth-Muscle-Specific and Conditional Deletion of FlnA

Our previous work and those of others have indicated an important role for the actin cytoskeleton in the down-modulation of

mechanosensitive ion channels, including non-selective cationic SACs and the K^+-selective TREK/TRAAK channels (Kainulainen et al., 2002; Lauritzen et al., 2005; Peyronnet et al., 2012; Sharif-Naeini et al., 2009). Notably, the actin crosslinking element FlnA exerts a tonic repression on the opening of SACs (Kainulainen et al., 2002; Peyronnet et al., 2013; Sharif-Naeini et al., 2009). We reasoned that selectively removing smooth muscle FlnA (smFlnA) might lead to an increase in the opening of Piezo1/SACs in arterial myocytes (i.e., loss of mechanoprotection), without the requirement of hypertension.

We used a tamoxifen (TAM)-inducible smMHCCre* system to specifically and conditionally delete FlnA (smMHCCre* $FlnA^{0/-}$, with or without Piezo1) in smooth muscle cells of adult C57BL/6 male mice, thus ruling out the developmental effects of the knockout (Feng et al., 2006; Wirth et al., 2008). qPCR experiments revealed that TAM induction of smMHCCre* activity resulted in a potent deletion of FlnA in caudal artery smooth muscle cells (Figure S6A). Of note, deletion of FlnA did not influence Piezo1 mRNA expression, and vice versa (Figures S6A and S6C). At the protein level, the turnover of this abundant cytoskeletal element is remarkably slow, and a period of 12 weeks post-TAM induction was required for complete removal of FlnA in myocytes of the caudal artery (Figure S6B). Mouse viability, weight, and size were not affected by specific deletion of FlnA in smooth muscle cells at the adult stage, at least 12 weeks after TAM induction (Figures S1I and S1J). Systolic arterial pressure was mildly lowered upon smFlnA deletion, unlike diastolic pressure (Figures S1N and S1O).

Thus, both FlnA and Piezo1 are highly expressed in the media of the caudal artery (Figures 1B and 1C; Figures S6B and S6D). Moreover, an efficient smooth-muscle-specific deletion of FlnA and/or Piezo1 can be achieved conditionally at the adult stage using the smMHCCre* line. Next, we examined the effect of smFlnA knockout on the Piezo1 currents.

Piezo1 Activity Is Enhanced in the Absence of smFlnA

The amplitude of SAC currents induced by step increases in pressure was significantly higher in myocytes from smMHCCre* $FlnA^{0/-}$ mice at 12 weeks post-TAM induction, unlike at 2 and 6 weeks (Figure 4, gray circles). Remarkably, SAC currents in caudal artery myocytes without FlnA were normalized when a single Piezo1 allele was deleted (smMHCCre* $FlnA^{0/-}$ $Piezo1^{+/-}$; Figure 4, magenta circles) but were fully suppressed upon homozygote deletion of Piezo1 (smMHCCre* $FlnA^{0/-}$ $Piezo1^{-/-}$; Figure 4E, red circles). Neither the single-channel current amplitude nor the current kinetics, nor the open channel probability were altered upon smFlnA deletion (Figures 4A and 4B; Figures S2D and S2E), indicating that the number of active Piezo1 channels is likely to be increased in the absence of smFlnA.

These findings demonstrate that Piezo1 activity in smooth muscle cells of the caudal artery is significantly enhanced upon FlnA deletion. Next, we examined whether this effect might be associated with a rise in cytosolic calcium since Piezo1 is a non-selective cationic channel permeable to calcium (Coste et al., 2010).

smFlnA Deletion Increases Cytosolic Calcium via Piezo1

Using ratiometric Fura-2 calcium imaging in isolated pressurized caudal arteries, we observed that smFlnA deletion induced a significant increase in cytosolic calcium (Figure 5, gray bar). However, Piezo1 deletion by itself (i.e., in the presence of smFlnA) did not significantly alter intracellular calcium concentration (Figure 5, black bar). Remarkably, when FlnA was deleted together with Piezo1 in smooth muscle cells, the rise in intracellular calcium was absent (Figure 5, red bar). In the presence of nifedipine, to avoid the possible contribution of L-type calcium channels, cytosolic calcium remained elevated when smFlnA was deleted alone (Figure 5, gray bar). However, without extracellular calcium, this increase in cytosolic calcium upon FlnA deletion was suppressed (Figure 5, gray bar).

These data indicate that deletion of smFlnA stimulates Piezo1 opening, which is associated with an increase in cytosolic calcium. Next, we examined whether caudal artery morphology might be altered in the absence of smFlnA, as anticipated if there is a link between arterial remodeling and Piezo1 opening.

Inward Eutrophic Remodeling of the Caudal Artery in the Absence of smFlnA

Mutations in the X-linked *FlnA* gene cause periventricular nodular heterotopia (PH), resulting from an abnormal neuronal migration from the periventricular region to the cortical area and associated with epileptic seizures (Fox et al., 1998; Poussaint et al., 2000). Moreover, PH patients have a propensity for aortic dilatation, aneurysms, abnormalities of the microcirculation, and premature stroke (de Wit et al., 2009; Hart et al., 2006; Parrini et al., 2006; Reinstein et al., 2013; Robertson, 2005; Sheen et al., 2005; Zhou et al., 2007). Thus, loss-of-function FlnA mutations also greatly affect the circulation.

Luminal diameter was not modified at 2 and 6 weeks after induction of the smFlnA knockout with TAM (Figures S6E and S6F). However, an inward remodeling was observed at 12 weeks post-TAM induction (Figures S6B and S6G). There was a striking parallel between the increase in SAC activity (Figures 4C–4E) and inward remodeling of the caudal artery, as a function of time after TAM induction (Figures S6E–S6G). Histological examination confirmed that internal diameter was reduced, while wall thickness was increased,a resulting in a higher media/lumen ratio without change in CSA (Figure 6, gray bars). Thus, smooth muscle specific deletion of FlnA results in an inward eutrophic remodeling of the caudal artery, occurring in the absence of hypertension. Next, we examined whether the increase in Piezo1 opening upon smFlnA deletion might influence this remodeling.

Role of Piezo1 in Caudal Artery Remodeling Induced by smFlnA Deletion

We studied the effect of a smooth-muscle-specific double FlnA/Piezo1 deletion (smMHCCre* $FlnA^{0/-}$ $Piezo1^{-/-}$; Figure 6, red bars) on caudal artery dimensions. By itself, smFlnA deletion already led to a major inward remodeling (Figures 6B and 6D). This decrease in arterial diameter in the absence of FlnA was not affected by a combined deletion with Piezo1, while upon Ang II or DOCA/salt/uninephrectomy hypertension (in the presence of smFlnA), the arterial diameter was preserved, and the effect of Piezo1 deletion on arterial diameter was evident (Figure 3E). However, the increase in wall thickness in the absence of smFlnA was fully reversed when either one or two alleles of Piezo1 were deleted together with FlnA (Figures 6C and 6E,

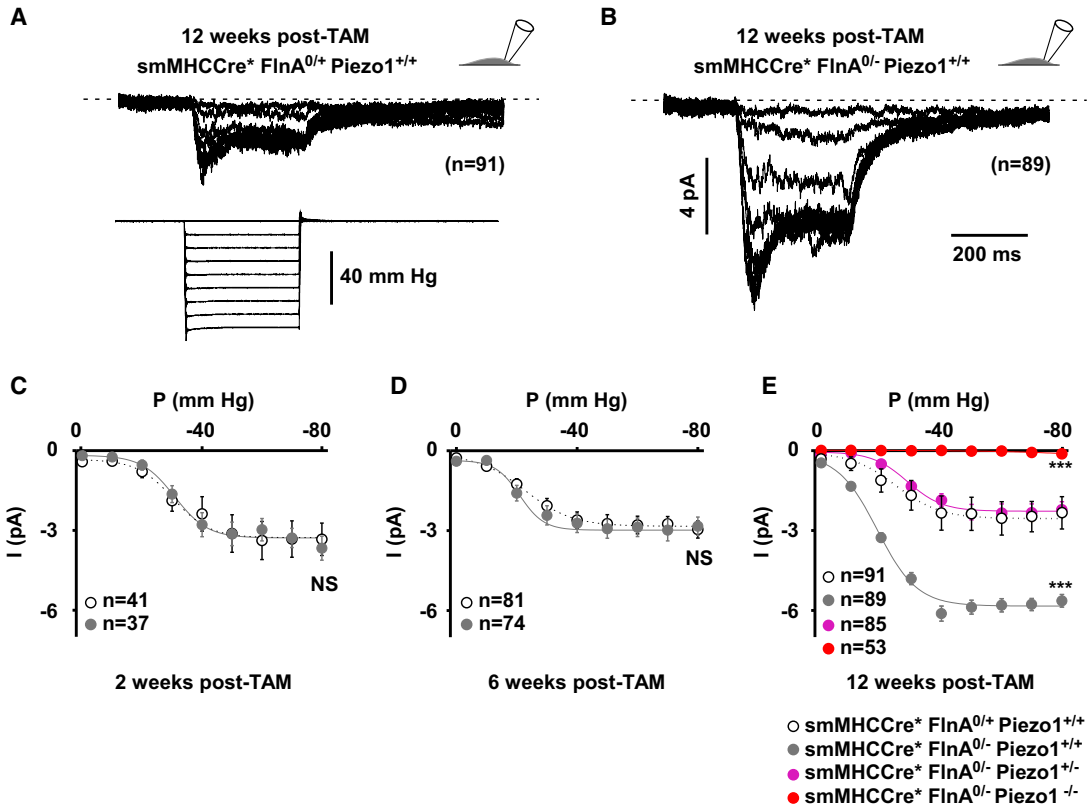

Figure 4. SAC Activity Is Enhanced in the Absence of smFlnA

(A) Mean SAC currents (at −80 mV) elicited in the cell-attached patch-clamp configuration in response to pressure pulses of increasing amplitude (shown in the bottom trace) at 12 weeks post-TAM induction for control smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ arterial myocytes.

(B) Same as in (A) but for smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ myocytes. Smooth muscle cells were isolated from caudal arteries of three different mice.

(C–E) Pressure-effect curves for SAC activity (holding potential: −80 mV) at (C) 2 weeks, (D) 6 weeks, and (E) 12 weeks post-TAM induction in arterial myocytes from smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ (white circles) and smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ (gray circles) caudal arteries. In (E), SAC activity for smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/-}$ (magenta dots) and smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$ (red dots) is also illustrated. Number of myocytes is indicated. The fitting parameters at 2 weeks post-TAM induction were $P_{0.5}$: −30.3 and k: 6.0 for smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$; and $P_{0.5}$: −30.8 and k: 5.4 for smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$. The fitting parameters at 6 weeks post-TAM induction were $P_{0.5}$: −22.6 and k: 7.8 for smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$; and $P_{0.5}$: −20.9 and k: 4.0 for smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$. The fitting parameters at 12 weeks post-TAM induction were $P_{0.5}$: −24.6 and k: 7.4 for smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$; $P_{0.5}$: −18.7 and k: 6.5 for smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$; and $P_{0.5}$: −28.6 and k: 5.8 for smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/-}$. I, mean current; P, pressure.

Data represent mean ± SEM. ***p < 0.001. NS, not significant.

See also Figure S2.

magenta and red bars). Accordingly, the remodeling index media/lumen ratio and the CSA were dramatically reduced when both Piezo1 and FlnA were inactivated at the same time in smooth muscle cells (Figures 6F and 6G, magenta and red bars). These findings were confirmed by labeling arterial myocytes with an antibody directed against sm22 and by counting the number of nuclei present in the media (Figures S5B and S5C, gray and red columns). However, as previously described, deletion of Piezo1 alone (i.e., in the presence of FlnA in smooth muscle cells) did not significantly affect arterial dimensions (Figures 3E–3G, black bars).

These findings indicate that the increase in Piezo1 opening in the absence of smFlnA (i.e., removal of mechanoprotection) influences arterial wall thickness, without a requirement for hypertension. Next, we examined whether transglutaminases (TG), calcium-dependent crosslinking enzymes previously reported to be involved in the hypertension-dependent remodeling of small arteries (Bakker et al., 2008; Engholm et al., 2011; Huelsz-Prince et al., 2013), might be involved downstream of Piezo1. Of note, TG requires the binding of up to six calcium ions to be catalytically active (Bakker et al., 2008; Engholm et al., 2011; Huelsz-Prince et al., 2013).

Stimulation of TG Activity in the Absence of smFlnA Requires Piezo1

TG activity was measured by incorporation of the fluorescent pseudo-substrate Alexa Fluor 647 cadaverine in whole caudal arteries (Figure 7; Figure S7A). As a positive control, TG was stimulated by the reducing agent DTT, and this activation was reversed by cystamine (Figure S7A) (Castorena-Gonzalez et al., 2014). TG activity in the smMHCCre* FlnA$^{0/+}$ Piezo1$^{-/-}$ arteries (Figure 7B, black bar) was comparable to that in the control

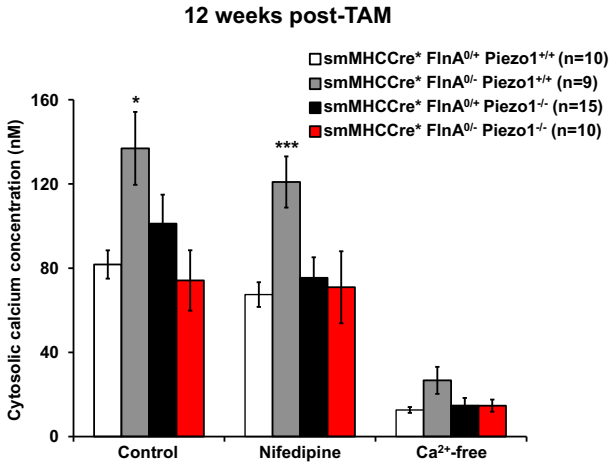

Figure 5. Piezo1-Dependent Increase in Cytosolic Calcium upon FlnA Deletion in Smooth Muscle Cells

Cytosolic calcium was monitored using Fura-2 ratiometric fluorescence imaging on isolated pressurized (50 mm Hg; intraluminal stop flow) caudal arteries at 12 weeks post-TAM induction. After a control period in the presence of extracellular calcium, arteries were bathed in the presence of 3 μM nifedipine to inhibit voltage-dependent L-type calcium channels. Subsequently, extracellular calcium was omitted, and 500 μM EGTA was added to the bath solution. smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ is indicated by white bars, smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ is indicated by gray bars, smMHCCre* FlnA$^{0/+}$ Piezo1$^{-/-}$ is indicated by black bars, and smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$ is indicated by red bars. Number of arteries is indicated. Data represent mean ± SEM. *$p < 0.05$; ***$p < 0.001$.

smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ condition (Figures 7A and 7B). By contrast, TG activity was significantly enhanced in caudal arteries from smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ mice (Figure 7A; Figure 7B, gray bar). However, qPCR and western blot analysis revealed that TG2 expression was not altered in the smFlnA$^{0/-}$ caudal artery (Figures S7B and S7C). Remarkably, this stimulation was fully reversed when Piezo1 was inactivated together with FlnA (smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$; Figure 7A; Figure 7B, red bar). We confirmed this decrease in TG activity by Piezo1 deletion in the caudal artery from smFlnA$^{0/-}$ mice using a quantitative gel assay (Figures S7D and S7E). Even when a single Piezo1 allele was deleted (smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/-}$), TG activity tended to be normalized in the absence of FlnA (Figure 7B, magenta bar).

These findings indicate that Piezo1 might be upstream of TG activation in smooth muscle cells. Previous studies have demonstrated that smooth muscle TG is potentially involved in hypertension-dependent remodeling of resistance arteries (Bakker et al., 2008; Huelsz-Prince et al., 2013). In addition, an important role for TG2 was demonstrated on arterial smooth muscle cell proliferation, with a key role in pulmonary artery hypertension (DiRaimondo et al., 2014; Penumatsa et al., 2014). Thus, we hypothesized that pharmacological inhibition of TG with cystamine might phenocopy the effect of Piezo1 deletion in smooth muscle cells. Next, we investigated whether in vivo chronic inhibition of TG by a cystamine treatment might affect the Piezo1-dependent arterial remodeling occurring in the absence of smFlnA.

The TG Inhibitor Cystamine Reverses Piezo1-Dependent Arterial Remodeling

Cystamine (225 mg/kg/day) was added to the drinking water of the mice for a period of 6 weeks. Cystamine treatment of both control (smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$; white bars) and FlnA knockout (smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$; gray bars) had no detectable effect on either diastolic or systolic blood pressure (Figures S7F and S7G). The decrease in arterial diameter in the absence of smFlnA was not altered by cystamine treatment (Figure 7C). However, the increase in wall thickness was completely reversed, with, accordingly, a significant decrease in the media/lumen ratio and CSA (Figures 7D–7F).

These findings suggest that TG contributes to the Piezo1-dependent arterial remodeling in the absence of smFlnA.

DISCUSSION

Altogether, our findings indicate that Piezo1 in arterial myocytes plays a role in the structural remodeling of small-diameter arteries. In the cutaneous resistance caudal artery, smooth muscle Piezo1 is associated with a trophic effect during hypertension (i.e., when myocytes are mechanically stressed) or when channel opening is enhanced at normal blood pressure in the absence of smFlnA (i.e., without mechanoprotection). A role for SACs in stressful conditions (i.e., hypertension) is reminiscent of the function of the mechanosensitive ion channels (MscS and MscL) in bacteria (Booth, 2014; Haswell et al., 2011; Kung, 2005). Indeed, the knockout of MscL and/or MscS does not affect basal bacterial growth. However, those channels become critically required for adaptation to an osmotic down-shock (i.e., environmental stress) and their opening allows the release of osmolytes, which prevents bacterial lysis (Booth, 2014; Haswell et al., 2011; Kung, 2005). Thus, both in arterial smooth muscle cells and bacteria, mechanosensitive ion channels appear to be required for adaptive responses to stress.

Remarkably, no SAC activity was left in the absence of smooth muscle Piezo1, demonstrating its central role in the mechanotransduction of arterial myocytes. Piezo1 opening is repressed by FlnA in smooth muscle cells. How might the cytoskeleton network affect Piezo1 gating? The cortical cytoskeleton divides the bilayer into submicrometer microdomains (the upholstery model) (Sharif-Naeini et al., 2009). Disruption or softening of the cytoskeleton, either chemically, mechanically, or genetically (in the present case, by deleting the crosslinking element FlnA), is likely to result in a larger radius of curvature of those microdomains, leading to an increase in membrane stress and, thus, sensitization of Piezo1 opening (Sharif-Naeini et al., 2009).

Upon smFlnA deletion, there is a striking temporal correlation between remodeling of the caudal artery and the increase in Piezo1 activity. Remarkably, normalization of SAC currents by removing a single Piezo1 allele was sufficient to reverse the increase in arterial wall thickness in the absence of smFlnA. Those findings suggest that it is the increase in Piezo1 opening (either induced by mechanical stress upon high blood pressure or by removing FlnA mechanoprotection) that is responsible for arterial remodeling, unlike the basal Piezo1 activity. The downstream events linking Piezo1 activity to arterial remodeling probably involve multiple effectors, which might possibly be activated by

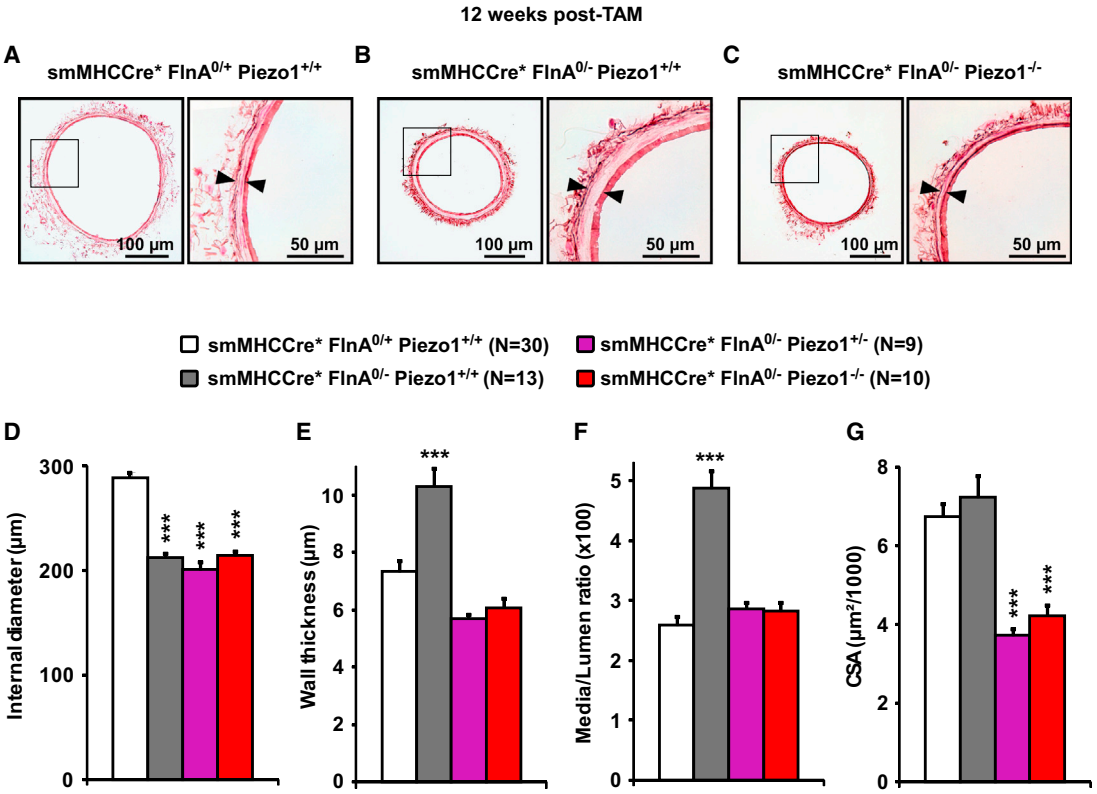

Figure 6. Deletion of Piezo1 in Smooth Muscle Cells Reduces Caudal Artery Wall Thickness in the Absence of FlnA

(A) Caudal artery section stained with orcein (left panel) and magnification of the wall (right panel) from a smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ mouse.

(B) Same as in (A) but from a smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ mouse.

(C) Same as in (A) but from a smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$ knockout mouse.

(D) Internal diameter.

(E) Wall thickness.

(F) Media/lumen ratio.

(G) CSAs are illustrated for control smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ (white bars), smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ (gray bars), smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/-}$ (magenta bars), and smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$ (red bars) arteries. Five caudal artery sections were analyzed per mouse at 12 weeks post-TAM induction, and the number of mice is indicated.

Data represent mean ± SEM. ***$p < 0.001$.

See also Figure S5.

intracellular calcium, as previously reported for the protease calpain-2 downstream of Piezo1 in the endothelium (Li et al., 2014). Interestingly, pharmacological inhibition or genetic deletion of the crosslinking enzyme TG2 has previously been shown to impair or delay arterial inward remodeling of small arteries in hypertensive conditions (Bakker et al., 2005, 2006; Engholm et al., 2011). How TG is activated upon hypertension, where it is active, and how it influences arterial remodeling are only starting to emerge (Huelsz-Prince et al., 2013; van den Akker et al., 2010). It has been recently hypothesized that mechanical force within smooth muscle cells could be involved in regulating the activity of TG during hypertension-dependent arterial remodeling (Huelsz-Prince et al., 2013). Notably, in vivo chronic treatment with the TG inhibitor cystamine, as well as Piezo1 deletion, reversed the increase in arterial wall thickness in the absence of smFlnA. Thus, our findings are supportive for a possible role of Piezo1 in calcium-dependent TG activation within smooth muscle cells.

Both constitutive (sm22Cre) and inducible (smMHCCre*) systems used to delete Piezo1 in smooth muscle cells have their own advantages and limitations (Frutkin et al., 2007; Moessler et al., 1996; Wirth et al., 2008). Thus, one might expect some common effects but also, eventually, some specific effects when comparing both models. One consistent finding in our study was an effect of Piezo1 on the arterial wall thickness, whatever the Cre driver or the experimental protocol used to induce arterial remodeling (hypertension or deletion of smFlnA).

In conclusion, our findings indicate that smooth muscle Piezo1 plays a key role in the structural remodeling of resistance arteries upon hypertension or when mechanoprotection by FlnA is removed. Recent elegant findings also demonstrate that Piezo1 fulfills an important function in the endothelium during early development, where it is activated by shear stress (Li et al., 2014; Ranade et al., 2014). Thus, Piezo1 is a key player in arterial mechanotransduction, responding to both flow and pressure. Here, we show that increased Piezo1 opening in smooth muscle

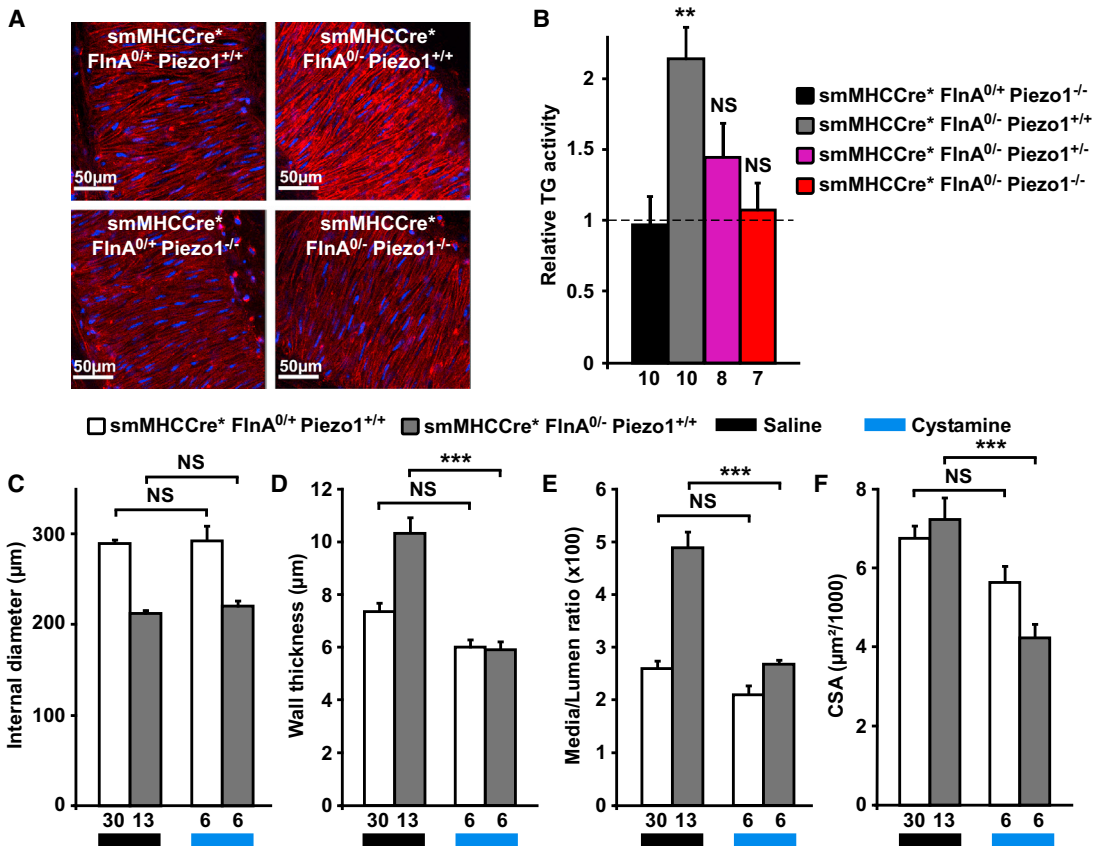

Figure 7. Increased Piezo1 Opening in the Absence of smFlnA Stimulates TG Activity

(A) The activity of TG in the caudal artery wall was reflected by the incorporation of the fluorescent pseudo-substrate cadaverine. smFlnA deletion at 12 weeks post-TAM (smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$) stimulates TG activity and this effect was reversed when Piezo1 was inactivated together with FlnA (smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$).

(B) TG activity was normalized to the control condition smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ in each experiment to avoid any possible variation in the labeling and/or imaging procedures. smMHCCre* FlnA$^{0/+}$ Piezo1$^{-/-}$ is indicated by black bars, smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ is indicated by gray bars, smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/-}$ is indicated by magenta bars, and smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$ in red bars.

(C) Internal diameter.

(D) Wall thickness.

(E) Media/lumen ratio.

(F) CSAs are illustrated for smMHCCre* FlnA$^{0/+}$ Piezo1$^{+/+}$ (white bars) and smMHCCre* FlnA$^{0/-}$ Piezo1$^{+/+}$ (gray bars) arteries in control conditions or chronically treated with cystamine (equivalent to 225 mg/kg/day) added to the drinking water for 6 weeks (starting at 6 weeks post-TAM induction). Morphometric analysis has been performed on caudal artery sections stained with orcein. Five caudal artery sections were analyzed per mouse. Control (water) and cystamine conditions are indicated by black and blue horizontal bars, respectively. Number of mice is indicated.

Data represent mean ± SEM. ***p < 0.001.

See also Figure S7.

cells conditions the structural remodeling of small-diameter arteries. Since arterial remodeling is now recognized as a major prognostic marker in patients at high cardiovascular risk, Piezo1 may represent an interesting pharmacological target for the treatment of hypertensive conditions (Mathiassen et al., 2007; Rizzoni et al., 2003).

EXPERIMENTAL PROCEDURES

Knockout Mice

sm22Cre Piezo1$^{-/-}$ Mice

For constitutive smooth-muscle-specific Piezo1 deletion, the sm22Cre line (Tg(Tagln-cre)1Her, Jackson Laboratories) was initially crossed with Piezo1$^{lox/lox}$ mice and further bred to yield the sm22Cre Piezo1$^{-/-}$ mice. Although recombination also occurs during embryogenesis in some cardiomyocytes, a major advantage for this constitutive sm22Cre driver is that there is no recombination in either visceral smooth muscle cells or veins (Moessler et al., 1996). Considering that Piezo1 mRNA expression is particularly low in the mouse heart and that the transient sm22 expression in the developing heart is spatially restricted to the presumptive right ventricle, the impact on the systemic circulation of an eventual Piezo1-dependent cardiac dysfunction is very unlikely (Coste et al., 2010; Moessler et al., 1996). In the control group, we included both Piezo1$^{lox/lox}$ and sm22Cre mice, and since no significant difference was observed between both genotypes, data were merged (controls indicated by sm22Cre Piezo1$^{+/+}$). The genetic background was C57BL/6, and 20-week-old adult male mice were used for this study.

smMHCCre Piezo1$^{-/-}$ Mice*

For conditional smooth-muscle-specific Piezo1 knockout, Piezo1$^{lox/lox}$ female mice were initially crossed with the TAM-inducible smMHCCre-ER(T2) male mice (inducible Cre* is inserted in the Y chromosome (Wirth et al., 2008)) and further bred to yield the smMHCCre* Piezo1$^{-/-}$ male mice. Twelve-week-old adult male mice were injected twice intraperitoneally with TAM (50 mg/kg/day dissolved in peanut oil, EtOH 10%). Of note, the smMHC-Cre* transgene is also expressed in visceral smooth muscle cells, although not in the heart (Frutkin et al., 2007; Wirth et al., 2008). In the control group, we included Piezo1$^{lox/lox}$ and smMHCCre* mice injected with TAM, and since no significant difference was observed between both genotypes, data were merged (controls indicated by smMHCCre* Piezo1$^{+/+}$). Mice were studied 12 weeks after TAM injection. TAM-injected heterozygote and homozygote smooth-muscle-specific Piezo1 knockout mice are indicated by smMHCCre* Piezo1$^{+/-}$ and smMHCCre* Piezo1$^{-/-}$, respectively.

smMHCCre FlnA$^{0/-}$ Mice*

TAM-inducible smMHCCre* male mice were crossed with female FlnA$^{lox/lox}$ mice to achieve a smooth-muscle-specific deletion of FlnA at the adult stage (smMHCCre* FlnA$^{0/-}$), thus avoiding developmental effects of the knockout (Feng et al., 2006). Of note, male mice are hemizygotes for FlnA, as the gene is on the X chromosome (FlnA$^{0/+}$). Mice were backcrossed at least 12 times onto a C57BL/6 background. Protocol for TAM injection was as described earlier for Piezo1 deletion. For generating double FlnA and Piezo1 KO mice, smMHCCre* FlnA$^{0/-}$ male mice were crossed with female Piezo1$^{lox/lox}$ mice and further bred to yield the smMHCCre* FlnA$^{0/-}$ Piezo1$^{-/-}$ male mice.

Statistics

Significance of the differences was tested with a permutation test (R Development Core Team: http://www.r-project.org/) ($n < 30$) or two-sample Student's t test ($n > 30$). One asterisk indicates $p < 0.05$; two asterisks indicate $p < 0.01$, and three asterisks indicate $p < 0.001$. Data represent mean ± SEM. N indicates the number of mice studied, and n indicates the number of cells or arteries.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.09.072.

AUTHOR CONTRIBUTIONS

K.R. performed the arteriography/myography experiments, analyzed the histological data, and generated the figures. F.D. performed the calcium imaging experiments and the DOCA/salt/uninephrectomy hypertension experiments and contributed to data analysis. M.A. performed the staining experiments and the TG experiments and was in charge of breeding the Piezo1 and FlnA mouse lines. S.S.R. generated the Piezo1$^{Laz/+}$ and the Piezo1$^{lox/lox}$ mice. R.P. and J.R.M. performed the patch-clamp recordings. M.J. isolated smooth muscle cells from caudal and cerebral arteries. C.M. did the histological sectioning and labeling experiments. S.O. provided the smMHCCre* line. Y.F. provided the FlnA$^{lox/lox}$ mice. S.D. performed the Ang II hypertension experiments. A.P. was involved in the molecular biology experiments, design of the experiments, and writing of the manuscript. E.H. performed some of the electrophyiological experiments, designed experiments, and wrote this manuscript.

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