ORIGINAL RESEARCH

Stretch-activated Piezo1 Channel in Endothelial Cells Relaxes Mouse Intrapulmonary Arteries

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Abstract

In intrapulmonary arteries (IPA), endothelial cells (EC) respond to mechanical stimuli by releasing vasoactive factors to set the vascular tone. Piezo1, a stretch-activated, calcium-permeable channel, is a sensor of mechanical stress in EC. The present study was undertaken to investigate the implication of Piezo1 in the endothelium-dependent regulation of IPA tone and potential involvement of Piezo1 in pulmonary hypertension, the main disease of this circulation. IPA tone was quantified by means of a myograph in control Piezo1$^{+/+}$ mice and in mice lacking endothelial Piezo1 (EC-Piezo1$^{-/-}$). Endothelial intracellular calcium concentration ($[Ca^{2+}]_i$) and nitric oxide (NO) production were measured, in mouse or human EC, with Fluo-4 or DAF-FM probe, respectively. Immunofluorescent labeling and patch-clamp experiments revealed the presence of Piezo1 channels in EC. Yoda1, a Piezo1 agonist, induced an endothelium-dependent relaxation that was significantly reduced in pulmonary arteries in EC-Piezo1$^{-/-}$ compared with Piezo1$^{+/+}$ mice. Yoda1 as well as mechanical stimulation (by osmotic stress) increased $[Ca^{2+}]_i$ in mouse or human EC. Consequently, both stimuli increased the production of NO. NO and $[Ca^{2+}]_i$ increases were reduced in EC from Piezo1$^{-/-}$ mice or in the presence of Piezo1 inhibitors. Furthermore, deletion of Piezo1 increased α-adrenergic agonist–mediated contraction. Finally, in chronically hypoxic mice, a model of pulmonary hypertension, Piezo1 still mediated arterial relaxation, and deletion of this channel did not impair the development of the disease. The present study thus demonstrates that endothelial Piezo1 contributes to intrapulmonary vascular relaxation by controlling endothelial $[Ca^{2+}]_i$ and NO production and that this effect is still present in pulmonary hypertension.

Keywords: Piezo1; calcium; pulmonary artery; relaxation

Although vascular tone regulation by hormonal stimuli is well described, much less is known regarding signaling pathways triggered by mechanical forces. Endothelial cells (EC) are sensitive to different types of mechanical stimuli generated by the blood flow, including pulsatile stretch and shear stress. These stimuli trigger a physiological response via EC release of vasodilatory factors such as nitric oxide (NO) or, conversely, constrictive factors such as endothelin 1 (1). A tight regulation of these factors precisely controls the arterial tone.

Important sensors of mechanical pressure in EC are mechanosensitive channels or stretch-activated channels (SAC) (2). SAC belong to a channel family among which are Piezo1 channels, which are nonselective cation channels that allow calcium influx into the cell (3). The three-dimensional structure of Piezo1 shows that it forms a three-bladed, propeller-shaped homotrimeric channel complex. Drugs such as Yoda1 can also directly activate Piezo1 (4). Piezo1 is very important for touch sensitivity as well as for processing of vascular architecture during embryonic development (5, 6). In systemic mesenteric arteries, Piezo1, located in EC, is involved in vasoconstriction when activated by blood flow modulation during physical exercise (7). In these EC, sodium influx through Piezo1 induces a depolarization that

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spreads to the underlying smooth muscle cells, thus inducing vasoconstriction. By contrast, EC-Piezo1 relaxes vessels through the release of ATP by EC in mesenteric arteries (8) or NO in aorta (9).

Pulmonary circulation regulation is very specific. For example, hypoxia induces vasoconstriction rather than vasodilation, as for systemic circulation. Because there is little data on Piezo in pulmonary circulation, the first aim of the present study was to determine the effect of endothelial Piezo1 channel activation on pulmonary vascular tone. We focused on calcium influx through Piezo1 (3) that could stimulate endothelial nitric oxide synthase (eNOS) (1, 10, 11). Therefore, production of NO linked to a putative Piezo1 activation may counterbalance the depolarizing effect of sodium influx, thus producing vasodilation in arteries.

Pulmonary hypertension (PH) is one of the main pulmonary circulation diseases. PH is characterized by an increase in pressure (>25 mm Hg) and mechanical forces inside the intrapulmonary arteries (IPA) (12, 13). PH is associated with *1*) vascular hyperreactivity to vasoconstrictive agents such as noradrenaline, *2*) pulmonary artery remodeling with cell proliferation, and *3*) endothelial dysfunction linked to intracellular calcium concentration ($[Ca^{2+}]_i$) alteration (14–19). Although Piezo1 has been clearly implicated in the development of systemic hypertension (20), its role in PH remains unknown.

The aims of the present study were thus to determine the role of endothelial Piezo1 channel in IPA vascular tone regulation and whether Piezo1 is involved in PH. To address these issues, we performed experiments in mice deleted for endothelial Piezo1 (EC-Piezo1$^{-/-}$) and used cultured murine and human pulmonary artery endothelial cells (HPAEC). In this article, we provide evidence that Ca^{2+} influx through Piezo1 channels directly participates in the NO-dependent relaxation in the pulmonary circulation.

Methods

Detailed descriptions of methods are provided in the data supplement.

Animal Models and Ethical Approval

Twelve-week-old (20–25 g) male C57BL/6 mice (Piezo1$^{+/+}$) (Cadherin5CreERT2^{-} Piezo1$^{-/flox}$ and Piezo1$^{flox/flox}$) or EC-Piezo1$^{-/-}$ (Cadherin5CreERT2^{+}Piezo1^{-}/flox and Piezo1$^{flox/flox}$) with deletion induced by injection of tamoxifen (150 mg/kg/d, diluted in corn oil) during 5 consecutive days were used at least 5 weeks after induction. Mice were bred in accordance with approval of the local animal care ethics committee ("Comité d'éthique de Bordeaux" no. 50, protocol no. APAFIS 9212-2017031018562273v5). The study was performed in accordance with the guidelines of Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and with NIH guidelines. EC-Piezo1$^{-/-}$ mice were produced by Prof. Ardem Patapoutian (Department of Neuroscience, ICND202, Scripps Research Institute/Howard Hughes Medical Institute, La Jolla, CA). For tissue and cellular experiments, mice were killed with an intraperitoneal injection of 190 mg of pentobarbital per kilogram of body mass (Exagon; Richter Pharma).

Chronic Hypoxia (Model of PH)

To develop PH, chronically hypoxic (CH) mice were housed during 4 weeks in a hypobaric chamber (50 kPa). PH was assessed by measuring the associated right ventricular hypertrophy through the ratio of right ventricle to left ventricle plus septum weight (Fulton's index). Remodeling of the arterial wall during PH was estimated by subsequent *ex vivo* histological studies.

Organ Bath IPA Contraction

IPA were mounted in a Mulvany myograph to record contraction. Values are expressed as wall tension (mN/mm^{-}) or as a percentage of the initial 80 mM KCl-induced contraction.

Immunostaining of EC

Immunostaining was performed on fixed whole IPA. The primary antibody used was against Piezo1 (15939-1-AP; Proteintech) or eNOS (N9532 dilution; Sigma-Aldrich).

EC

EC from mice were obtained by enzymatic dissociation of IPA as previously described (21). HPAEC were purchased (PromoCell) and cultured as recommended by the dealer. siRNA against Piezo1 (catalog no. sc93227; Santa Cruz Biotechnology) was used.

Ca^{2+} Fluorescence Confocal Imaging and NO Measurement

In real-time fluorescence imaging, the Fluo-4/AM probe was used for measuring variations in the $[Ca^{2+}]_i$. The fluorescent probe DAF-FM diacetate (2 μM) was used to measure NO production.

Electrophysiological Experiments

Patch-clamp experiments were performed as previously described (16, 22) with a cell-attached voltage-clamp configuration. Pclamp software (Molecular Devices) was used to record and analyze data. Mechanical stimulation was performed by applying negative pressure to the back end of the patch pipette using an HSPC-1 high-speed pressure-clamp device.

Data and Statistical Analysis

Results are expressed as a mean ± SEM. Statistical analysis was performed with nonparametric tests (Mann-Whitney *U* test or Kruskal-Wallis test with Dunn's posttest).

Drugs

Yoda1 was dissolved in DMSO to obtain a 2 mM stock solution. Other drugs were dissolved in saline solution.

Results

EC from Mouse IPA and HPAEC Express Piezo1 Channels

Immunostaining of Piezo1$^{+/+}$ mouse IPA indicated that the EC layer expressed Piezo1 (Figure 1A). In IPA from EC-Piezo1$^{-/-}$ mice, the red staining of Piezo1 in the endothelium was reduced (Figure 1B). In HPAEC that expressed Piezo1 (Figure 1C), the mean fluorescence intensity (Figures 1D and 1E) was decreased in the presence of siRNA directed against Piezo1. Then, to record the activity of Piezo1 in EC, the cell-attached patch-clamp configuration was used in HPAEC. A brief depression in the pipette stretched the cell membrane and activated SAC. As a consequence, a large inward current composed of several unitary inward currents was recorded (Figure 1F). The unitary current had a conductance of 24 ± 3 pS ($n = 10$ cells). In the presence of the Piezo1 agonist, Yoda1 (10 μM) in the pipette solution, basal unitary inward currents (23 ± 2.8 pS; $n = 10$ cells) could be recorded, and a brief depression induced a larger inward current (Figures 1F and

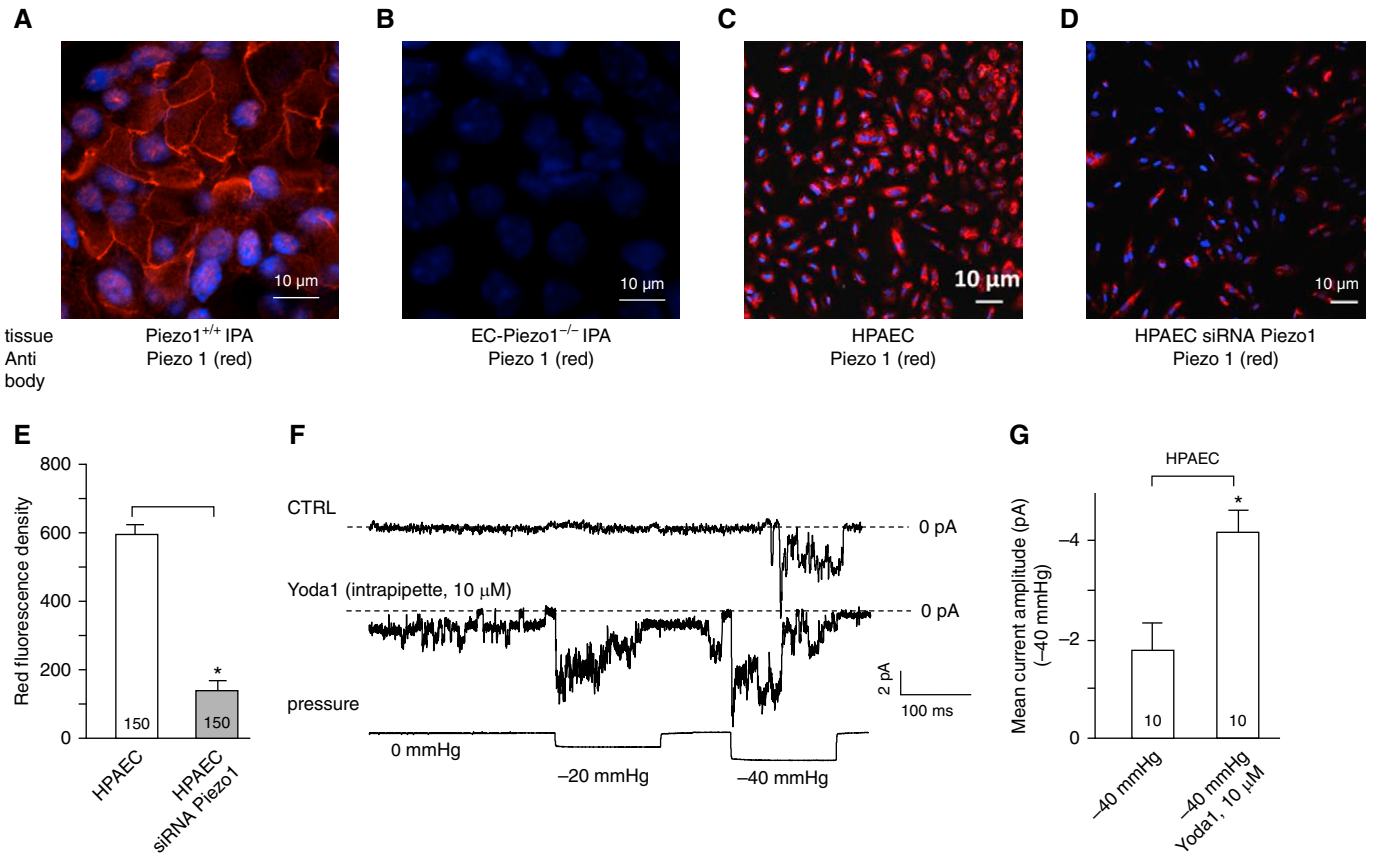

Figure 1. Mouse and human pulmonary endothelial cells (HPAEC) express Piezo1 channel. (*A* and *B*) The endothelial layer of intrapulmonary arteries (IPA) expressed Piezo1. IPA were observed in the *en face* configuration in (*A*) Piezo1$^{+/+}$ mice or (*B*) mice lacking endothelial Piezo1 (EC-Piezo1$^{-/-}$). Red = antibody against Piezo1; blue = nucleus. Scale bars: 10 μm. For both pictures, experiments were performed at the same time with the same condition of acquisition. (*C* and *D*) HPAEC expressed Piezo1. Immunostaining of HPAEC with antibody against Piezo1 (red) (*C*) in normal condition or (*D*) in the presence of siRNA against Piezo1. Scale bars: 10 μm. (*E*) Quantification of red fluorescence (antibody against Piezo1) in the presence or absence of siRNA against Piezo1 (10 different experiments). (*F*) Typical current traces of stretch-activated channel/Piezo1 current in HPAEC (cell-attached configuration) stimulated by different intrapipette depressions (pressure trace) in control conditions or in the presence of Yoda1 (10 μM) in the pipette solution (pipette potential, +60 mV; pipette resistance, 3 MΩ). (*G*) Quantification of the stretch-activated channel current amplitude in HPAEC with or without Yoda1; mean amplitude current was measured during the stretch at −40 mm Hg (10 different cells). *$P < 0.05$. The number inserted in the bar graph represents the number of cells analyzed. CTRL = control.

1G). Then, the current-clamp mode of the patch-clamp setup (whole-cell configuration) was applied to investigate whether this inward current modifies the transmembrane potential. Application of Yoda1 depolarized EC (+5 ± 4 mV from a mean resting potential of −41 ± 3 mV; n = 12; data not shown). Altogether, these experiments demonstrated that EC from human and mouse pulmonary vessels express functional Piezo1 channels.

Stimulation of Piezo1 Induces Relaxation

Involvement of Piezo1 in the regulation of IPA vascular tone was investigated in mouse IPA by means of its agonist Yoda1. Application of Yoda1 (20 μM) did not modify Piezo1$^{+/+}$ IPA basal tone (Figure 2A). However, addition of Yoda1 on IPA precontracted with phenylephrine (0.3 μM) concentration dependently relaxed the vessel (Figures 2B and 2C). In the absence of Yoda1, the precontraction remained stable for at least 20 minutes, and addition of the solvent DMSO (1:1,000) did not change the contraction (−2% ± 3%; n = 4; data not shown). Yoda1-induced relaxation was significantly reduced in IPA from EC-Piezo1$^{-/-}$ mice (Figure 2C), thus confirming that it is linked to endothelial Piezo1. Yoda1-induced relaxation did not depend on the agonist used to precontract the vessel, because it was observed in Piezo1$^{+/+}$ IPA precontracted with either KCl or prostaglandin $F_{2\alpha}$ (Figure 2D). The relaxation depended on an extracellular calcium influx because it was completely abrogated in the absence of extracellular calcium (prostaglandin $F_{2\alpha}$ precontraction) (Figure 2D). Finally, we investigated cross-talk between Yoda1 relaxation and the well-known vasodilatory factor acetylcholine. In the presence of Yoda1, acetylcholine still induced an additive relaxation (−17% ± 4%; n = 5) (Figure 2B), but of smaller amplitude than in the absence of Yoda1 (−48% ± 5%; n = 5).

Stimulation of Piezo1 Activates Calcium Influx

To decipher how Piezo1 relaxes vessels, we measured $[Ca^{2+}]_i$ and NO production in EC. Isolated EC from mouse Piezo1$^{+/+}$ IPA

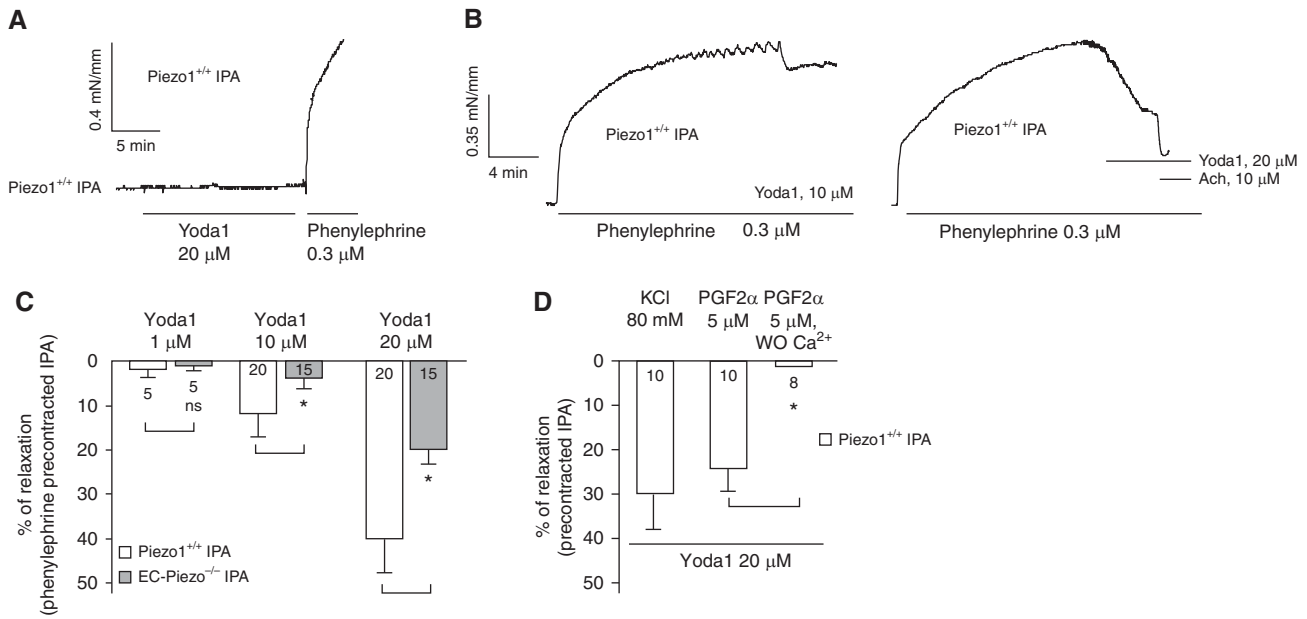

Figure 2. Endothelial Piezo1 relaxes IPA. (*A*) Typical trace of Piezo1$^{+/+}$ IPA tension recorded in the presence of Yoda1, which did not modify the wall tension, whereas phenylephrine (α_1-agonist) induced a large contraction. (*B*) Typical trace of Piezo1$^{+/+}$ IPA tension recorded in the presence of Yoda1 (10 or 20 μM) after a precontraction with phenylephrine (0.3 μM). Yoda1 relaxed IPA and acetylcholine (Ach; 10 μM) relaxed the vessels further. (*C*) Statistical analysis of the relaxation induced by Yoda1 (1–20 μM) in Piezo1$^{+/+}$ and EC-Piezo1$^{-/-}$ IPA (5–10 different mice). (*D*) Amplitude of the Yoda1-mediated relaxation in the presence of different contracting agents (KCl and prostaglandin $F_{2\alpha}$ [$PGF_{2\alpha}$]) and without extracellular calcium plus 0.5 mM EGTA (WO Ca^{2+}) (six different mice). *$P < 0.05$. The number inserted in the bar graph represents the number of arterial rings analyzed. ns = not significant.

express Piezo1 channels (Figure 3A). Yoda1 (20 μM) induced a rapid and sustained increase in $[Ca^{2+}]_i$ (Figure 3B), the amplitude of which was significantly smaller in EC from EC-Piezo1$^{-/-}$ mice (Figure 3C). By contrast, Yoda1 (20 μM) only induced a very small increase in $[Ca^{2+}]_i$ in isolated Piezo1$^{+/+}$ pulmonary arterial smooth muscle cells (F_{max}/F_0 = 1.3 ± 0.2; n = 50; data not shown) that was significantly smaller than in EC ($P < 0.05$). To better characterize Yoda1-induced $[Ca^{2+}]_i$ increase, we further investigated this response in HPAEC in which Yoda1 induced the same rapid and sustained increase in $[Ca^{2+}]_i$ (Figure 3D). Yoda1-induced $[Ca^{2+}]_i$ increase required extracellular calcium and was significantly reduced in the presence of several nonselective Piezo1 antagonists (100 μM Gd^{3+}, 5 μM GsMTx-4, 200 μM streptomycin) or the selective siRNA directed against Piezo1 (Figure 3E). As expected, siRNA directed against Piezo1 did not modify the calcium response induced by other agonists such as ATP (ATP alone, F_{max}/F_0 = 3.9 ± 0.4; n = 50; ATP in the presence of siRNA, F_{max}/F_0 = 3.7 ± 0.3; n = 50; data not shown). Then, we investigated whether this $[Ca^{2+}]_i$ increase required any other partner than Piezo1 (Figure 3F). In HPAEC, Yoda1-induced $[Ca^{2+}]_i$ increase was not reduced in the presence of either ryanodine (100 μM ryanodine receptor inhibitor) or thapsigargin (2 μM sarcoplasmic endoplasmic calcium ATPase pump inhibitor), two inhibitors of sarcoplasmic calcium release, thus confirming that it was exclusively due to an extracellular calcium influx. The calcium influx also was not inhibited by L-type calcium channel antagonist (1 μM nifedipine), T-type calcium channel antagonist (1 μM NNC 55-0396), or purinoceptor P2 (20 μM suramin), confirming that calcium flowed through Piezo1 channels.

In another set of experiments, the Piezo1 channel was stimulated by an osmotic shock at 144 mOsm that stretched membrane and activated SAC, thus reproducing mechanical stress effect. Osmotic shock increased $[Ca^{2+}]_i$ in HPAEC (Figure 3G). As for the Yoda1-induced $[Ca^{2+}]_i$ response, the osmotic shock-induced $[Ca^{2+}]_i$ response was significantly reduced in the presence of Piezo1 antagonists and siRNA directed against Piezo1 (Figure 3G).

Stimulation of Piezo1 Activates NO Production

To investigate whether Piezo1-induced $[Ca^{2+}]_i$ increase in EC could be involved in the production of NO, we used the fluorescent probe DAF-FM in HPAEC (Figure 4A). HPAEC were stimulated by either the Piezo1 agonist, Yoda1, or an osmotic shock and then in the additional presence of the eNOS inhibitor (N^{ω}-nitro-L-arginine methyl ester [L-NAME]), the antagonists of the mechanosensitive channels (GsMTx-4 and Gd^{3+}), or siRNA directed against Piezo1 or in the absence of extracellular calcium. Each of these four experimental conditions significantly reduced the observed Yoda1 calcium increase (Figures 4B–4D). Altogether, these data demonstrate that calcium influx through Piezo1 channel activates the eNOS leading to NO production, which accounts for IPA relaxation.

Piezo1 Interacts with Contractile Agonists

We investigated whether Piezo1 deletion impaired IPA contraction induced by constrictive factors. The depolarizing agent, 80 mM KCl, and the α_1-adrenergic

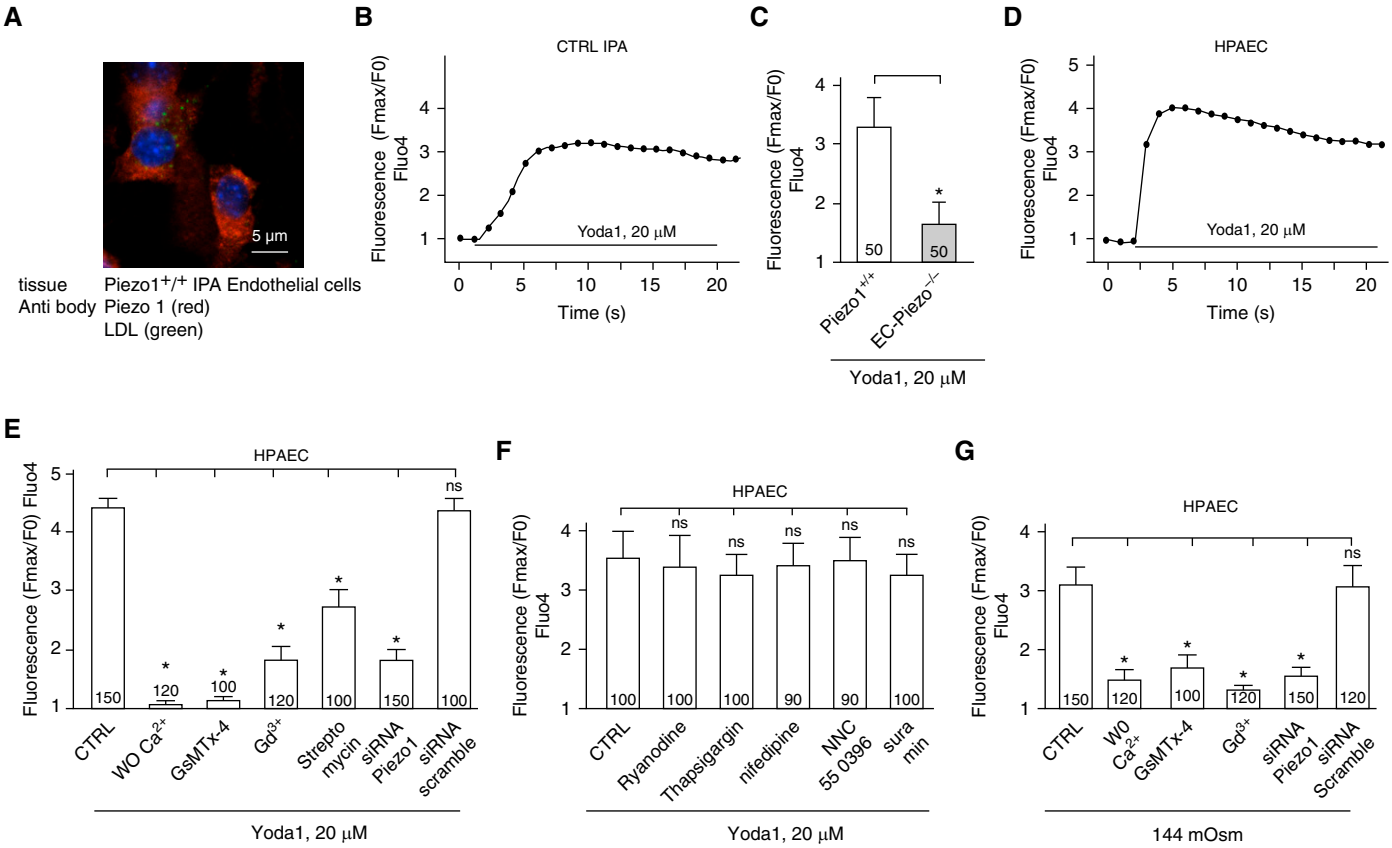

Figure 3. Piezo1 activation increases intracellular calcium concentration ($[Ca^{2+}]_i$). (*A*) Typical endothelial cells (EC) dissociated from Piezo1$^{+/+}$ IPA that have incorporated fluorescent low-density lipoprotein (LDL; green) and expressed Piezo1 channels (red). Nuclei were stained in blue. Scale bar: 5 μm. (*B*) Yoda1 (20 μM) induced a strong increase of Fluo-4 fluorescence in isolated EC from Piezo1$^{+/+}$ IPA. (*C*) Statistical analysis of the $[Ca^{2+}]_i$ increase induced by Yoda1 (20 μM) in ECs from Piezo1$^{+/+}$ and EC-Piezo1$^{-/-}$ IPA (five different mice). (*D*) Trace of Fluo-4 fluorescence of HPAEC in the presence of Yoda1 (20 μM). (*E*) In HPAEC, the effect of Yoda1 on the $[Ca^{2+}]_i$ increase in the absence of extracellular calcium (WO; solution without calcium and with 0.5 mM EGTA) or in the presence of stretch-activated channel inhibitors GSMTx4 (5 μM), $GdCl_3$ (100 μM), streptomycin (200 μM), or siRNA against Piezo1 (nine different experiments). (*F*) $[Ca^{2+}]_i$ increase was not reduced by thapsigargin (2 μM; calcium-ATPase inhibitor), ryanodine (100 μM; ryanodine receptor inhibitor), nifedipine (1 μM; L-type voltage-gated calcium channel inhibitor), NNC 55-0396 (1 μM; T-type voltage-gated calcium channel inhibitor), or suramin (20 μM; P2X inhibitor). (*G*) In HPAEC, the effect of a hypo-osmotic shock (144 mOsm) on the $[Ca^{2+}]_i$ increase in the absence of extracellular calcium (WO; solution without calcium and with 0.5 mM EGTA) or in the presence of GSMTx4 (5 μM), $GdCl_3$ (100 μM), or siRNA against Piezo1 (nine different experiments). *$P < 0.05$. The number inserted in the bar graph represents the number of cells analyzed.

agonist, phenylephrine (1 nM–3 μM), induced a contraction, the amplitude of which was significantly increased in IPA from EC-Piezo1$^{-/-}$ mice (Figures 5A and 5B). Likewise, phenylephrine-induced contraction in Piezo1$^{+/+}$ IPA was greater in the presence of the Piezo1 antagonist, streptomycin (200 μM) (Figure 5B), or the NO synthase inhibitor, L-NAME (100 μM) (Figure 5B). This greater contraction was not due to an alteration in Piezo1 activity in IPA smooth muscle cells. Indeed, Yoda1 induced a similar $[Ca^{2+}]_i$ increase in pulmonary arterial smooth muscle cells from either Piezo1$^{+/+}$ or EC-Piezo$^{-/-}$ mice ($F_{max}/F_0 = 1.3 \pm 0.2$, $n = 50$, or $F_{max}/F_0 = 1.4 \pm 0.2$, $n = 50$, respectively; data not shown). In the same way, endothelin 1 release by EC, which may contribute to the greater contraction, was not altered (Figure E1B in the data supplement).

Piezo1 Channel Is Active in CH Mouse

Because Piezo1 controls EC $[Ca^{2+}]_i$, we performed experiments in the CH mouse, a model of PH in which EC $[Ca^{2+}]_i$ is markedly altered. We first studied the typical parameters of the development of PH in the EC-Piezo1$^{-/-}$ mouse. CH similarly increased the Fulton's index ratio compared with normoxia (NX) in both Piezo1$^{+/+}$ mice and EC-Piezo$^{-/-}$ mice (Figure 6A). In this connection, remodeling of IPA wall induced by CH as well as cardiac pressure did not differ in both types of mice (Figures 6B and 6C). These results thus indicate that endothelial deletion of Piezo1 does not modify the development of PH in mice.

In a second set of experiments, we investigated whether Piezo1 remains present during PH. Immunostaining revealed that there was no difference in endothelial expression of Piezo1 between Piezo1$^{+/+}$ NX and Piezo1$^{+/+}$ CH IPA (Figure E1C). Contraction experiments indicated that Yoda1 relaxes CH IPA to an extent similar to that in NX IPA. As expected, this relaxation was reduced

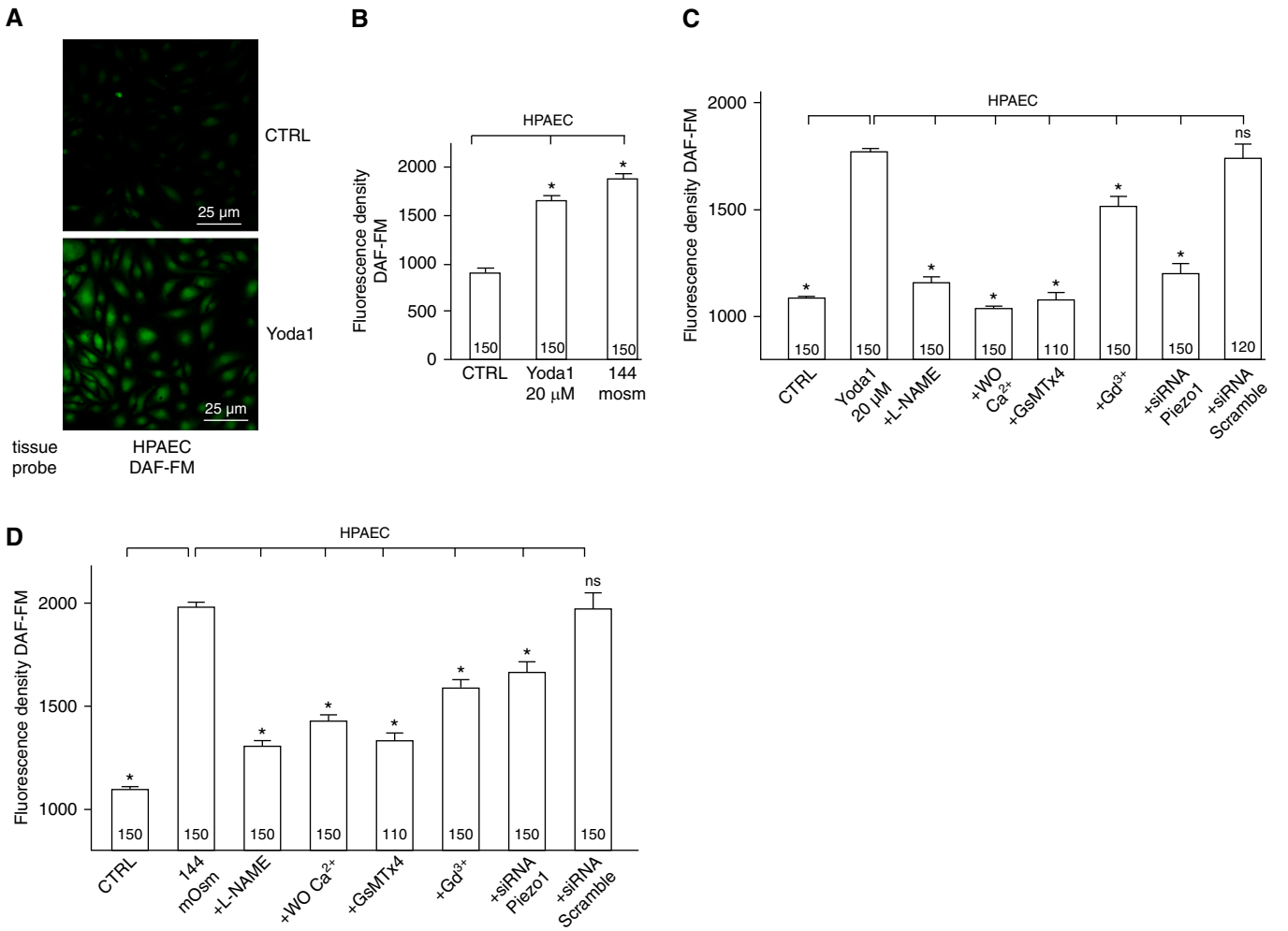

Figure 4. Piezo1 activation increases nitric oxide production. (*A*) Typical images of the green fluorescence of DAF-FM (4-amino-5-methylamino-2',7',-difluorofluorescein) recorded in HPAEC with or without Yoda1 (20 μM; image recorded at the end of 20 min of incubation). Scale bars: 25 μm. (*B*) Increase of DAF-FM fluorescence in the presence of Yoda1 (20 μM) or osmotic shock (144 mOsm) (nine different experiments). (*C* and *D*) Effects of nitric oxide synthase inhibitor (N^{ω}-nitro-L-arginine methyl ester [L-NAME] 100 μM), absence of extracellular calcium (WO Ca^{2+} solution without calcium and with 0.5 mM EGTA), Piezo1 inhibitor (GsMTx4 5 μM, $GdCl_3$ 100 μM), and siRNA against Piezo1 on the Yoda1- (*C*) or hypoosmotic shock–mediated DAF-FM fluorescence increase (nine different experiments) (*D*). *$P < 0.05$. The number inserted in the bar graph represents the number of cells analyzed.

in CH EC-Piezo$^{-/-}$ mice (Figure 6D). *In vivo* experiments were then performed to measure the effect of Yoda1 on right cardiac pressure. Injection of Yoda1 induced a similar diminution in the mean cardiac pressure in NX and CH Piezo1$^{+/+}$ mice: 1.67 ± 0.3 mm Hg ($n = 7$) and 1.96 ± 0.4 mm Hg ($n = 5$), respectively (Figure E2). As expected, Yoda1-induced relaxation was statistically smaller in Piezo1$^{-/-}$ mice (0.19 ± 0.1 mm Hg) ($n = 4$). These results suggest that Piezo1 activity is not altered during CH. This functional finding was confirmed by experiments performed in HPAEC. To mimic CH, HPAEC were cultured under hypoxic conditions (1% O_2) during 48 hours, a well-established technique that simulates *in vitro* CH (23). Under those conditions, the calcium response to either Yoda1 or osmotic stress remained unchanged (Figure 6E). In CH HPAEC, although, as expected, basal production of NO was reduced compared with NX conditions as a consequence of CH-induced endothelial dysfunction, both Yoda1 and osmotic stress were still able to increase NO production (Figure 6F). The percentage of increase in NO production (in the presence of Yoda1/control condition) was similar in NX and CH (Figure 6G). These data thus indicate that Piezo1 still mediates IPA relaxation in CH as in NX mouse.

Discussion

The present study demonstrates that Piezo1 channels are involved in endothelium-mediated relaxation of IPA in mice. Piezo1 channels trigger an influx of Ca^{2+} that subsequently activates NO release and, hence, IPA relaxation. In the CH mouse, a model of PH characterized by endothelial dysfunction, Piezo1 channels still contribute to relaxation of IPA. To our knowledge, this study is the first

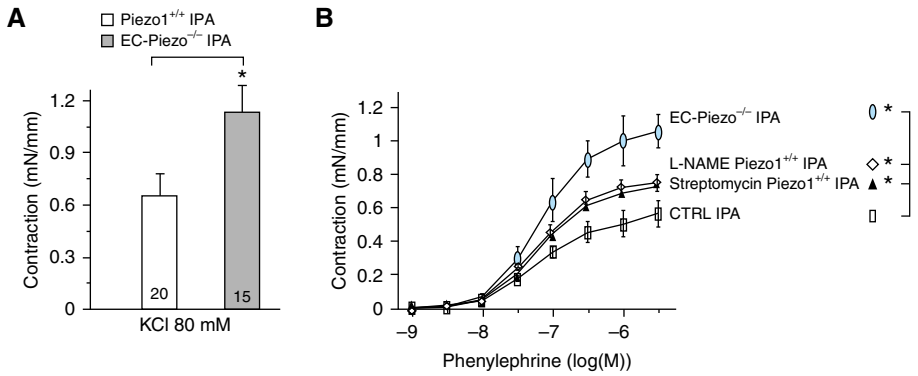

Figure 5. Piezo1 and vasoconstrictor factors. (*A*) The amplitude of the contraction induced by KCl (80 mM) was smaller in Piezo1$^{+/+}$ IPA than in EC-Piezo1$^{-/-}$ IPA (five or six different mice). (*B*) Amplitude of the contraction in Piezo1$^{+/+}$ IPA induced by phenylephrine (1 nM–3 μM; cumulative dose–response curve) with or without streptomycin (200 μM) or L-NAME (100 μM) (six to eight different arteries) and EC-Piezo1$^{-/-}$ IPA (six different arteries). *$P < 0.05$. The number inserted in the bar graph represents the number of arterial rings analyzed.

demonstration that EC-Piezo1 controls pulmonary circulation tone in health and disease.

On the basis of our use of mice deleted for both EC-Piezo1 channels and pharmacological SAC inhibitors, we report that Piezo1 activation relaxes IPA. Because the shear stress also activates Piezo1, these channels could participate in the relaxation induced by an increase in pulmonary blood flow (24). Piezo1 channels relax pulmonary vessels by increasing $[Ca^{2+}]_i$ and, accordingly, NO production in EC (Figures 3 and 4). If Yoda1 is known to selectively stimulate Piezo1, membrane stretch by the osmotic shock activates not only this channel but also all other mechanosensitive channels present on EC, such as transient receptor potential cation channel subfamily V member 4 channels (25). Because siRNA directed against Piezo1 as well as GsMTx4 also reduced the osmotic shock response, Piezo1 appears to be a major player in the stretch-induced pulmonary vascular response. The fact that Yoda1 still induced a small increase in calcium after Piezo1 silencing (Piezo$^{-/-}$ EC or with siRNA against Piezo1) (Figures 3C and 3E) deserves further discussion. This calcium response could be attributed to a lack of Piezo1 deletion in some cells or to some off-target effects of Yoda1. Furthermore, Piezo1 was first described in the reticulum (26), and therefore Yoda1 could also modify calcium flux at the site of the reticulum. In pulmonary EC, Yoda1 as well as osmotic shock increase $[Ca^{2+}]_i$ and, consequently, NO production via Piezo1 channel activation. This Piezo1-mediated calcium increase does not require intracellular reticular calcium stores (Figure 3F) or membrane L-type, T-type, or P2 channels. In pulmonary EC, Piezo1-activated signaling pathway is thus different from that in the mesenteric artery that implicates release of ATP and activation of $P2Y_2/G_q/G_{11}$ receptor coupled to the calcium release from reticular inositol trisphosphate–dependent store (8). In IPA, acetylcholine, which also relaxes vessels by activating NO synthase, induces an additional, although small, relaxation (Figure 2A) to that induced by Yoda1. Indeed, acetylcholine increases $[Ca^{2+}]_i$ by recruiting alternative signaling pathways such as inositol trisphosphate–dependent or voltage-gated T-type channels (25). Finally, NO synthase can also be activated by phosphorylation via AKT calcium-independent pathway (8). However, such pathway is not operative in HPAEC, because NO production is totally absent in the absence of extracellular calcium.

Regarding the resulting mechanical activity, in the mesenteric artery, Piezo1 activation induces a contraction due to sodium influx and EC depolarization (7). In contrast, in the pulmonary circulation, although Yoda1 depolarized HPAEC, the amplitude of the depolarization was much smaller than in the mesenteric circulation (5 and 20 mV, respectively). This discrepancy may be related to an increased presence of calcium-activated potassium channels in pulmonary EC (27), whose activation would have masked the depolarizing influx of sodium via Piezo1 channels. As a resulting effect, the small amplitude of Yoda1-induced depolarization does not counteract NO-dependent vasodilation.

Contractile agonists can also directly or indirectly activate endothelial Piezo1, thus inducing a negative feedback loop in IPA. Indeed, Piezo1 activation opposes the α_1-adrenergic agonist contracting effect because phenylephrine-induced contraction was greater in the absence of Piezo1. On one hand, phenylephrine-induced contraction of the vessel modifies its shape, which could lead to EC stretch, which in turn activates EC-Piezo1 channels. NO released by EC would then antagonize the contraction. On the other hand, phenylephrine could release factors derived from smooth muscle cells that may stimulate endothelial Piezo channels. In this connection, a cross-talk between smooth muscle cells and endothelium has been previously described (28) in the pulmonary artery, where serotonin stimulates both smooth muscle cell contraction and NO release (29).

Finally, we investigated the role of Piezo1 in a PH model. Two major factors contribute to the increase in pulmonary pressure during PH: enhanced vasoconstriction and/or reduced vasodilation combined with partial luminal obstruction due to complex remodeling of IPA (19). Taking into account that EC-Piezo1 favors relaxation, one could postulate that its deletion would favor the development of PH (30). The present study indicates that this is not the case, because CH induced a similar PH in EC-Piezo1$^{-/-}$ mice in terms of right ventricular hypertrophy or remodeling of the arterial wall or cardiac pressure compared with NX mice. This result highlights a specific role of EC-Piezo1 in pulmonary circulation compared with the systemic circulation, in which it has been implicated in systemic hypertension (20). PH characteristics were measured only at the end of 4 weeks of the CH condition. However, the time course of disease development in EC-Piezo1$^{-/-}$ mice could be different from that in Piezo1$^{+/+}$ mice. Piezo1-mediated relaxation could delay hypertension development, and further investigations are required to investigate this point. Endothelial dysfunction

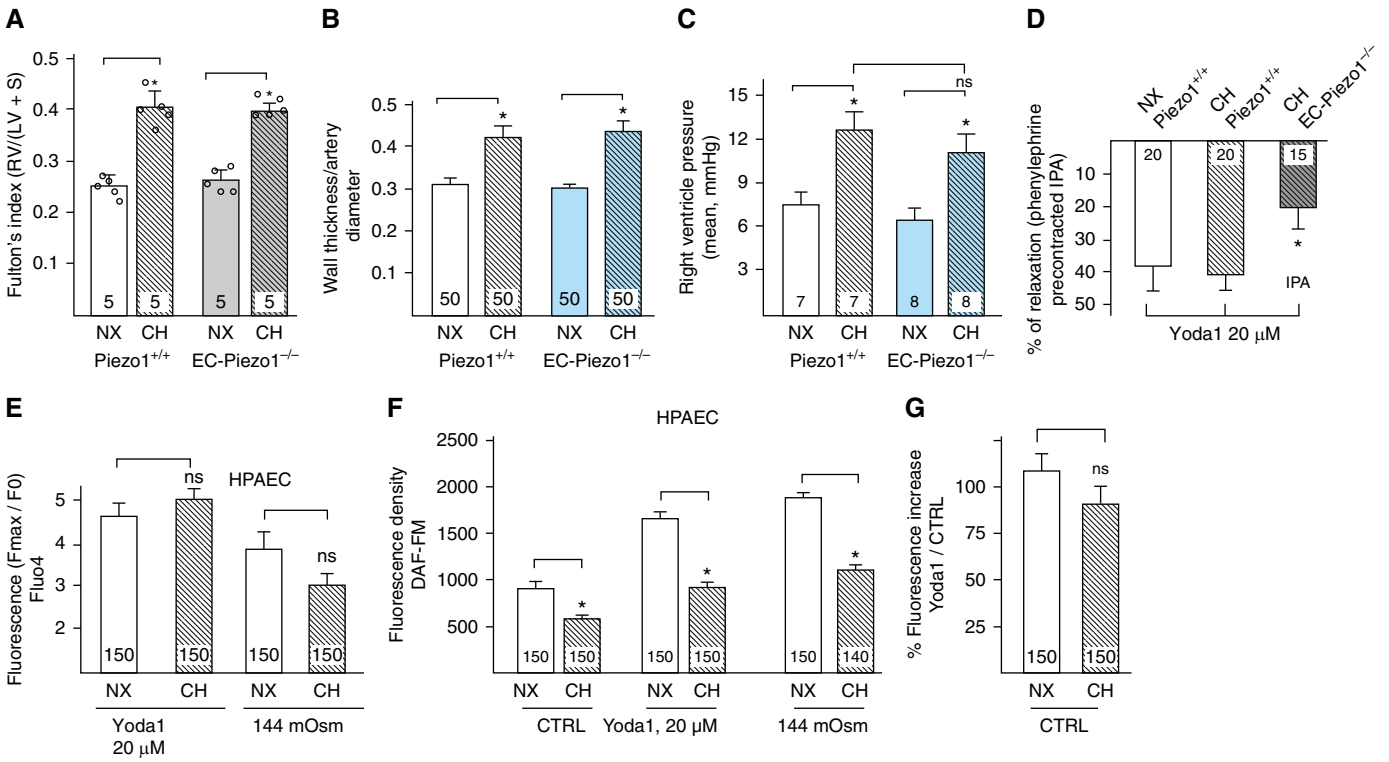

Figure 6. Piezo1 mediates relaxation in chronically hypoxic (CH) mice. (*A*) Fulton's index, a cardiac pulmonary hypertension marker in normoxic (NX) or in CH conditions (a model of pulmonary hypertension) of Piezo1$^{+/+}$ or EC-Piezo1$^{-/-}$ mice. Small circles represent individual values. (*B*) Wall thickness in NX or CH IPA from Piezo1$^{+/+}$ or EC-Piezo1$^{-/-}$ mice. Wall thickness increased in CH condition, regardless of mouse genotype. (*C*) Right ventricular (RV) mean pressure in NX or CH conditions in Piezo1$^{+/+}$ or EC-Piezo1$^{-/-}$ mice. Pressure increased in CH condition, regardless of mouse genotype. (*D*) Statistical analysis of the relaxation induced by Yoda1 (20 μM) in CH Piezo1$^{+/+}$ and EC-Piezo1$^{-/-}$ IPA (six or seven different mice). Yoda1 induced the same relaxation in CH IPA as in NX IPA. (*E*) Statistical analysis of the Yoda1 (20 μM) and osmotic stress–induced intracellular calcium concentration ($[Ca^{2+}]_i$) increase in NX or CH cultivated HPAEC (nine different experiments). (*F*) Statistical analysis of the Yoda1- (20 μM) and osmotic stress–induced NO production in NX or CH HPAEC (nine different experiments). (*G*) Percentage of NO production increase between none stimulated and Yoda1 stimulated HPAEC in NX or CH condition. Values were calculated from data shown in *D*. *$P < 0.05$. The number inserted in the bar graph represents the number of (*B*) arterial rings or (*C* and *D*) cells analyzed. LV = left ventricle; S = septum.

plays a very important role in IPA reactivity alteration (14, 31–33). For example, acetylcholine-mediated relaxation is considerably reduced in CH IPA (34). By contrast, in the present study, Piezo1-induced relaxation, which is also mediated via EC, was not altered. Likewise, Yoda1-mediated $[Ca^{2+}]_i$ increase was identical in normoxic and hypoxic HPAEC, even though alteration in calcium homeostasis in PH has been previously described (35–37). Nevertheless, the percentage increase in NO production upon stimulation by Yoda 1 or osmotic shock was the same, regardless of the oxygen culture conditions, thus explaining that Piezo1-mediated relaxation is preserved during PH. A similar result was previously obtained for the β_2-mediated relaxation that also involves NO synthase (32) and which is also preserved. In this connection, because endothelial Piezo1 is still active during PH, it could be a pharmacological target to relax IPA.

In conclusion, to our knowledge, this study is the first demonstration of the importance of Piezo1 channels in the control of endothelial Ca^{2+} homeostasis as well as NO synthesis leading to vasodilation in the pulmonary circulation in health and disease, illustrating the potential beneficial effect of targeting Piezo1 at the endothelial site on pulmonary vascular reactivity in PH. ■

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