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# Maintenance of normal blood pressure is dependent on IP3R1-mediated regulation of eNOS

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Endothelial cells (ECs) are critical mediators of blood pressure (BP) regulation, primarily via the generation and release of vasorelaxants, including nitric oxide (NO). NO is produced in ECs by endothelial NO synthase (eNOS), which is activated by both calcium ( $\text{Ca}^{2+}$ )-dependent and independent pathways. Here, we report that intracellular  $\text{Ca}^{2+}$  release from the endoplasmic reticulum (ER) via inositol 1,4,5-trisphosphate receptor (IP3R) is required for  $\text{Ca}^{2+}$ -dependent eNOS activation. EC-specific type 1 1,4,5-trisphosphate receptor knockout (IP3R1<sup>-/-</sup>) mice are hypertensive and display blunted vasodilation in response to acetylcholine (ACh). Moreover, eNOS activity is reduced in both isolated IP3R1-deficient murine ECs and human ECs following IP3R1 knockdown. IP3R1 is upstream of calcineurin, a  $\text{Ca}^{2+}$ /calmodulin-activated serine/threonine protein phosphatase. We show here that the calcineurin/nuclear factor of activated T cells (NFAT) pathway is less active and eNOS levels are decreased in IP3R1-deficient ECs. Furthermore, the calcineurin inhibitor cyclosporin A, whose use has been associated with the development of hypertension, reduces eNOS activity and vasodilation following ACh stimulation. Our results demonstrate that IP3R1 plays a crucial role in the EC-mediated vasorelaxation and the maintenance of normal BP.

hypertension | calcium | endothelium | IP3 receptor | eNOS

Hypertension (HTN) is a major cause of morbidity and mortality affecting millions of adults worldwide (1, 2). Globally, among the ~17 million deaths caused by cardiovascular diseases, nearly half can be attributed to complications of HTN (3–9). In addition, vasodilator drugs that activate nitric oxide (NO) production have been used to treat HTN for decades (4, 10). Calcineurin (also known as protein phosphatase 2B), is a  $\text{Ca}^{2+}$ /calmodulin-activated serine/threonine protein phosphatase. Calcineurin inhibitors are first-line immunosuppressants used in organ transplantation (11, 12). However, calcineurin inhibition causes HTN in up to 70% of patients (13), and the exact underlying mechanisms are not fully understood.

Vascular endothelial cells (ECs), located at the interface between the vessel wall and blood, release vasorelaxants that influence vascular smooth muscle tone in response to mechanical (e.g., shear stress) and biochemical stimuli (14, 15). These stimuli induce a rapid increase in intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) in ECs activating  $\text{Ca}^{2+}$ -dependent signaling pathways, resulting in release of endothelium-derived relaxing factors, including NO (14, 16–19). Mice lacking endothelial nitric oxide synthase (eNOS) develop severe HTN (20). However, how eNOS is regulated in vivo remains essentially unclear. The modulation of both plasma membrane  $\text{Ca}^{2+}$  entry and endoplasmic reticulum (ER)  $\text{Ca}^{2+}$  release is critical in EC function (21, 22).

Moreover, recent meta-analysis and genome-wide association studies in hypertensive individuals have linked type 1 1,4,5-trisphosphate receptor (IP3R1), a major  $[\text{Ca}^{2+}]_i$  release channel (23, 24), to high blood pressure (BP) (25, 26). On these grounds, we sought to investigate in detail the functional role of the IP3R1/ $\text{Ca}^{2+}$ /calcineurin/nuclear factor of activated T cells (NFAT)/eNOS pathway in BP regulation.

## Results and Discussion

**Generation and Characterization of Endothelium-Specific IP3R1 Knockout Mice.** Previous studies have reported embryonic lethality in calcineurin B mutant and NFATc3 and NFATc4 double-knockout mice models (27). Furthermore, there are three isoforms of calcineurin A and one of calcineurin B in ECs (28) plus four isoforms of NFAT (29). Thus, genetic approaches to elucidate the functions of these molecules are problematic. Therefore, to dissect the IP3R1/ $\text{Ca}^{2+}$ /calcineurin/NFAT/eNOS pathway, and to understand better the molecular mechanisms underlying BP regulation, we examined the role of IP3R1 in the  $\text{Ca}^{2+}$ -dependent eNOS activation in ECs. We generated EC-specific IP3R1 knockout (EC IP3R1<sup>-/-</sup>) mice resulting from crosses of floxed IP3R1 mice with Tie2<sup>Cre</sup> mice (Fig. S1A and B). IP3R1 expression was significantly reduced in vascular ECs isolated from EC IP3R1<sup>-/-</sup> mice compared with mice harboring the IP3R1<sup>fllox/fllox</sup> allele (IP3R1<sup>fl/fl</sup>) (Fig. S1C). In contrast, levels of mRNA encoding the two other isoforms of IP3R, IP3R2 and IP3R3, did not exhibit compensatory up-regulation in vascular ECs from EC IP3R1<sup>-/-</sup> mice (Fig. S1C). Immunofluorescence analysis did not detect IP3R1 protein in vascular ECs from EC IP3R1<sup>-/-</sup> mice (Fig. S1D). Additionally, EC IP3R1<sup>-/-</sup> mice exhibited normal embryonic development (Fig. S2).

**Effect of Endothelial Disruption on BP.** Strikingly, mean arterial pressure (measured using radiotelemetry) was significantly elevated in freely ambulating EC IP3R1<sup>-/-</sup> mice by 3 mo of age (Fig. 1A). These findings were confirmed by measuring BP using a Millar catheter (Fig. S3A) or tail-cuff (Fig. S3B). At 6 mo of age, chronic HTN resulted in cardiac enlargement and overall reduced function in EC IP3R1<sup>-/-</sup> mice (Table S1). Mesenteric arteries exhibited markedly thickened tunica media (Fig. S4A and B) with an increased media/lumen ratio (Fig. S4C) in EC IP3R1<sup>-/-</sup> mice.

In this study, we demonstrate that type 1 inositol 1,4,5-trisphosphate receptor channels are critically involved in the activation of the calcineurin/nuclear factor of activated T cells (NFAT)/endothelial nitric oxide synthase signaling pathway in endothelial cells. This pathway plays an essential role in the maintenance of normal blood pressure, with important implications in the clinical scenario.

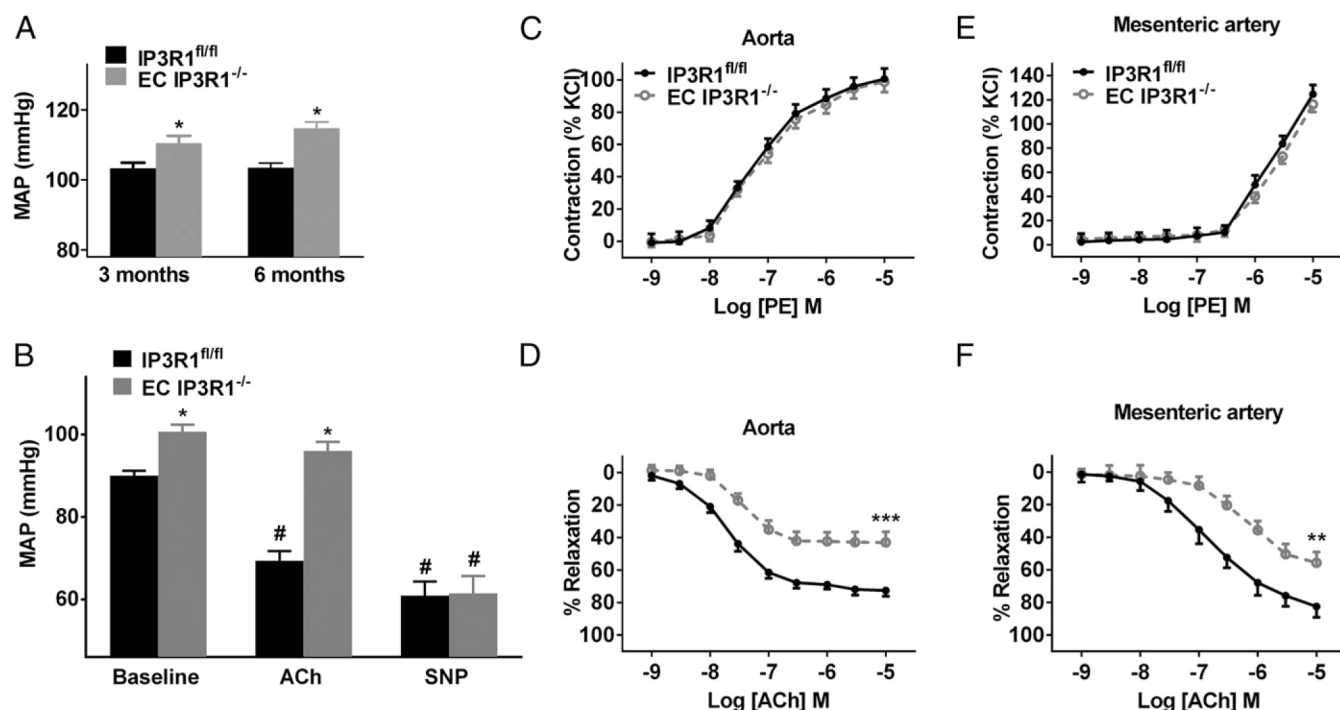
Author contributions: A.R.M. conceived of the study; G.S. and A.R.M. designed experiments; G.S. performed experiments; Q.Y. designed and performed ex vivo experiments; J.Y. performed in vitro experiments; S.R.R. and A.W. contributed new reagents/analytic tools; G.S., M.M.K., B.W.O., A.L., Y.Y., and A.R.M. analyzed data; and Q.Y., J.Y., G.S., and A.R.M. wrote the paper.

The authors declare no conflict of interest.

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**Fig. 1.** Effect of endothelial disruption of IP3R1 in ECs on BP and vascular reactivity. (A) Mean arterial pressure (MAP) recorded by radiotelemetry in at least seven IP3R1<sup>fl/fl</sup> and EC IP3R1<sup>-/-</sup> mice. (B) MAP measured by an intra-arterial Millar catheter in IP3R1<sup>fl/fl</sup> and EC IP3R1<sup>-/-</sup> mice in response to ACh and SNP injection. \**P* < 0.05 vs. IP3R1<sup>fl/fl</sup> mice; #*P* < 0.05 vs. baseline (*n* ≥ 6 mice per each group) by Student's *t* test. (C) Vasoconstrictor reactivity to the  $\alpha_1$ -adrenoreceptor agonist phenylephrine (PE) in aorta arteries in IP3R1<sup>fl/fl</sup> and EC IP3R1<sup>-/-</sup> mice. (D) Endothelium-dependent relaxation to ACh in aorta arteries. There was a significant decrease in potency (rightward shift) of the concentration response curve to ACh in the aortic arteries of EC IP3R1<sup>-/-</sup> mice. \*\*\**P* < 0.001 vs. IP3R1<sup>fl/fl</sup> mice by Student's *t* test. (E) Vasoconstrictor reactivity to PE in mesenteric arteries. (F) Endothelium-dependent relaxation to ACh in mesenteric arteries. There was a significant decrease in potency (rightward shift) of the concentration response curve to ACh in the mesenteric arteries of EC IP3R1<sup>-/-</sup> mice. Data are presented as mean  $\pm$  SEM (*n* = 12–20 rings from six mice per group for aorta arteries, *n* = 8 for isolated mesenteric arteries). \*\**P* < 0.01 vs. IP3R1<sup>fl/fl</sup> mice by Student's *t* test.

In addition, both cardiac fibrosis and renal fibrosis were augmented in 6-mo-old EC IP3R1<sup>-/-</sup> mice (Fig. S4 D and E). Taken together, these data show that EC IP3R1<sup>-/-</sup> mice have a chronic hypertensive phenotype with typical target-organ damage, including the heart, kidney, and vasculature. Further studies are needed to investigate in detail the potential involvement of the regulation of sodium excretion in the kidney and other renal pathways (30, 31).

**Defective Vasodilation in EC IP3R1<sup>-/-</sup> Mice.** EC IP3R1<sup>-/-</sup> mice exhibited a blunted vasodilatory response to i.v. ACh (Fig. 1B). However, injection of the endothelium-independent vasodilator sodium nitroprusside (SNP; an NO donor) caused an equivalent BP reduction in both IP3R1<sup>fl/fl</sup> and EC IP3R1<sup>-/-</sup> mice (Fig. 1B), indicating that the vasodilatory response to NO was intact.

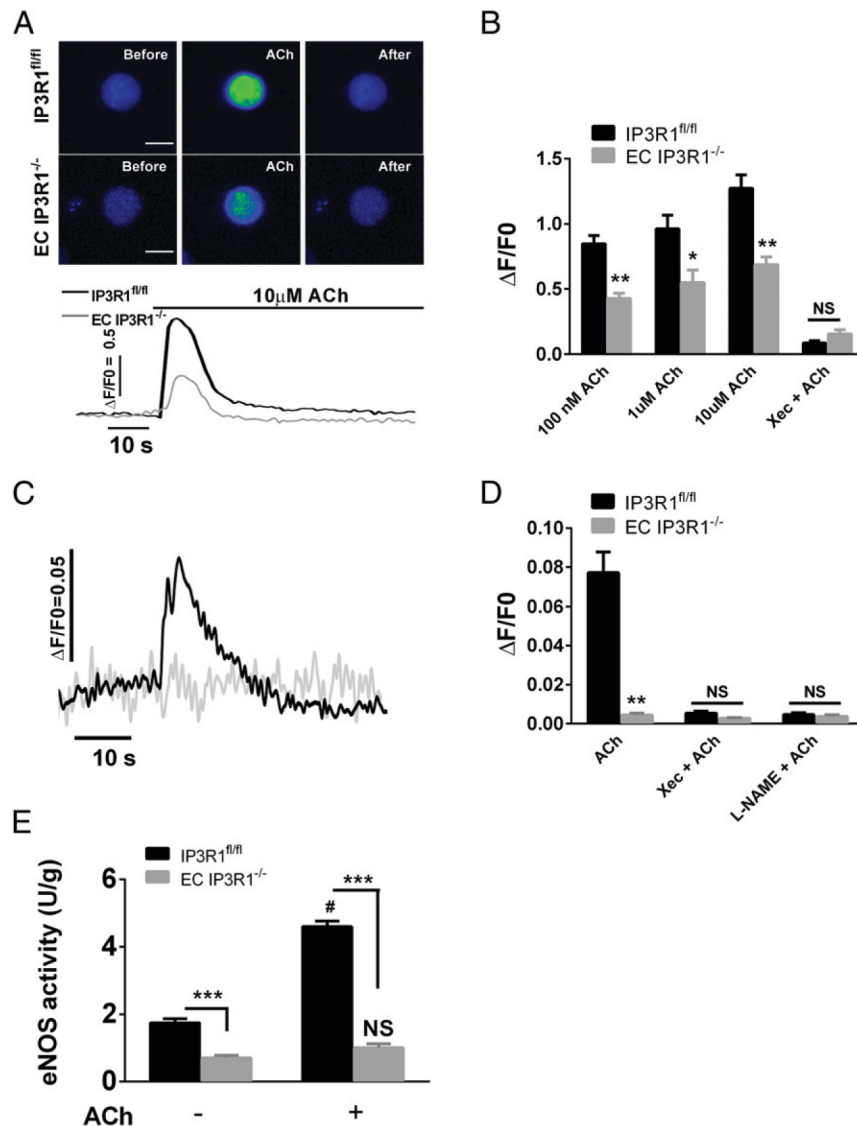
Responses to the vasoconstrictor phenylephrine (an  $\alpha_1$ -adrenoreceptor agonist) were intact in aortas and mesenteric arteries from both IP3R1<sup>fl/fl</sup> and EC IP3R1<sup>-/-</sup> mice (Fig. 1C and E). However, the ACh-induced aortic relaxation was markedly blunted (by ~41%) in EC IP3R1<sup>-/-</sup> mice compared with IP3R1<sup>fl/fl</sup> mice (Fig. 1D). Responses to the Ca<sup>2+</sup> ionophore A23187 were also markedly impaired (by ~23%) in EC IP3R1<sup>-/-</sup> mice (Fig. S5). Similarly, there was a significant reduction in the response to ACh (Fig. 1F) in EC IP3R1<sup>-/-</sup> mesenteric arteries. Importantly, responses to SNP were comparable between IP3R1<sup>fl/fl</sup> and EC IP3R1<sup>-/-</sup> mice in both vascular districts (Fig. S6 A and B).

**Diminished ACh-Induced Ca<sup>2+</sup>-Dependent NO Production in EC IP3R1<sup>-/-</sup> Mice.** To confirm that the observed blunted vasodilation was due to deficient IP3R1-mediated ER Ca<sup>2+</sup> release, we examined the ACh-induced Ca<sup>2+</sup> response in isolated aortic ECs. Addition of ACh elicited a rapid dose-dependent increase in

[Ca<sup>2+</sup>]<sub>i</sub> in IP3R1<sup>fl/fl</sup> ECs that was significantly reduced in IP3R1<sup>-/-</sup> ECs. Incubation of ECs with Xestospongin C (Xec; an IP3R inhibitor) also inhibited the rise in [Ca<sup>2+</sup>]<sub>i</sub> in response to ACh in both groups (Fig. 2B). Similar results were obtained using a second IP3R inhibitor (2-aminoethoxydiphenyl borate; Fig. S7). These genetic and pharmacological data indicate that IP3R1 activation contributes to the ACh-induced [Ca<sup>2+</sup>]<sub>i</sub> increase in vascular ECs. The increase in ACh-induced EC NO was blunted in EC IP3R1<sup>-/-</sup> mice (Fig. 2C), and NO production in control mice was reduced by Xec or L-NG-nitroarginine methyl ester (L-NAME) (Fig. 2D), indicating that IP3R1-mediated ER Ca<sup>2+</sup> plays a pivotal role in EC NO generation.

EC NO is produced primarily by eNOS following agonist stimulation, and eNOS activity is dependent on Ca<sup>2+</sup>-calmodulin (17, 32). Under basal conditions, eNOS activity in ECs isolated from EC IP3R1<sup>-/-</sup> mice was reduced compared with eNOS activity in ECs from IP3R1<sup>fl/fl</sup> mice (Fig. 2E). ACh (10  $\mu$ M) increased eNOS activity by 2.6-fold in IP3R1<sup>fl/fl</sup> mice, whereas such an increase was not observed in EC IP3R1<sup>-/-</sup> mice (Fig. 2E).

**Defective eNOS Activity Due to Decreased Expression and Phosphorylation.** eNOS activity is determined both by basal protein level and by enzymatic activity, which is regulated by phosphorylation (33). HTN was associated with reduced eNOS protein in EC IP3R1<sup>-/-</sup> mesenteric arteries (Fig. 3A), as well as attenuated eNOS phosphorylation at Ser<sup>1176</sup> in response to ACh stimulation (Fig. 3A). Moreover, phosphorylation of eNOS at Thr<sup>494</sup>, which is an inhibitory site (34), was increased in EC IP3R1<sup>-/-</sup> mesenteric arteries (Fig. 3B). IP3R1-deficient human ECs, generated by transfecting IP3R1 siRNAs into human umbilical vein endothelial



**Fig. 2.** ACh-induced  $\text{Ca}^{2+}$  increase and NO production in isolated aortic ECs. (A) Representative images (Upper) and traces (Lower) of endothelial  $\text{Ca}^{2+}$  responses to ACh measured with the fluorescence  $\text{Ca}^{2+}$  indicator Fluo-4. (Scale bars: 20  $\mu\text{m}$ .) (B) Summarized data regarding endothelial  $\text{Ca}^{2+}$  increase. Data are presented as mean  $\pm$  SEM ( $n = 30$ –40 cells from six mice per each group). \* $P < 0.05$ ; \*\* $P < 0.01$ . NS, not significant. (C) Representative traces of endothelial NO increase in response to ACh. (D) Summarized data of maximal endothelial NO fluorescence. Data are presented as mean  $\pm$  SEM ( $n = 20$ –30 cells from six mice per group). \*\* $P < 0.01$ . (E) NOS activity measured in isolated aortic ECs with (+) or without (–) 10  $\mu\text{M}$  ACh treatment at 37  $^{\circ}\text{C}$  for 10 min. Data are shown as mean  $\pm$  SEM of at least three independent experiments. \*\*\* $P < 0.001$  vs. IP3R1<sup>fl/fl</sup> ECs within the same group; # $P < 0.001$  vs. untreated IP3R1<sup>fl/fl</sup> ECs; NS, not significant vs. untreated EC IP3R1<sup>-/-</sup> ECs (all by Student's  $t$  test).

cells (HUVECs), exhibited an  $\sim 50\%$  decrease in eNOS protein (Fig. 3C) and mRNA (Fig. 3D).

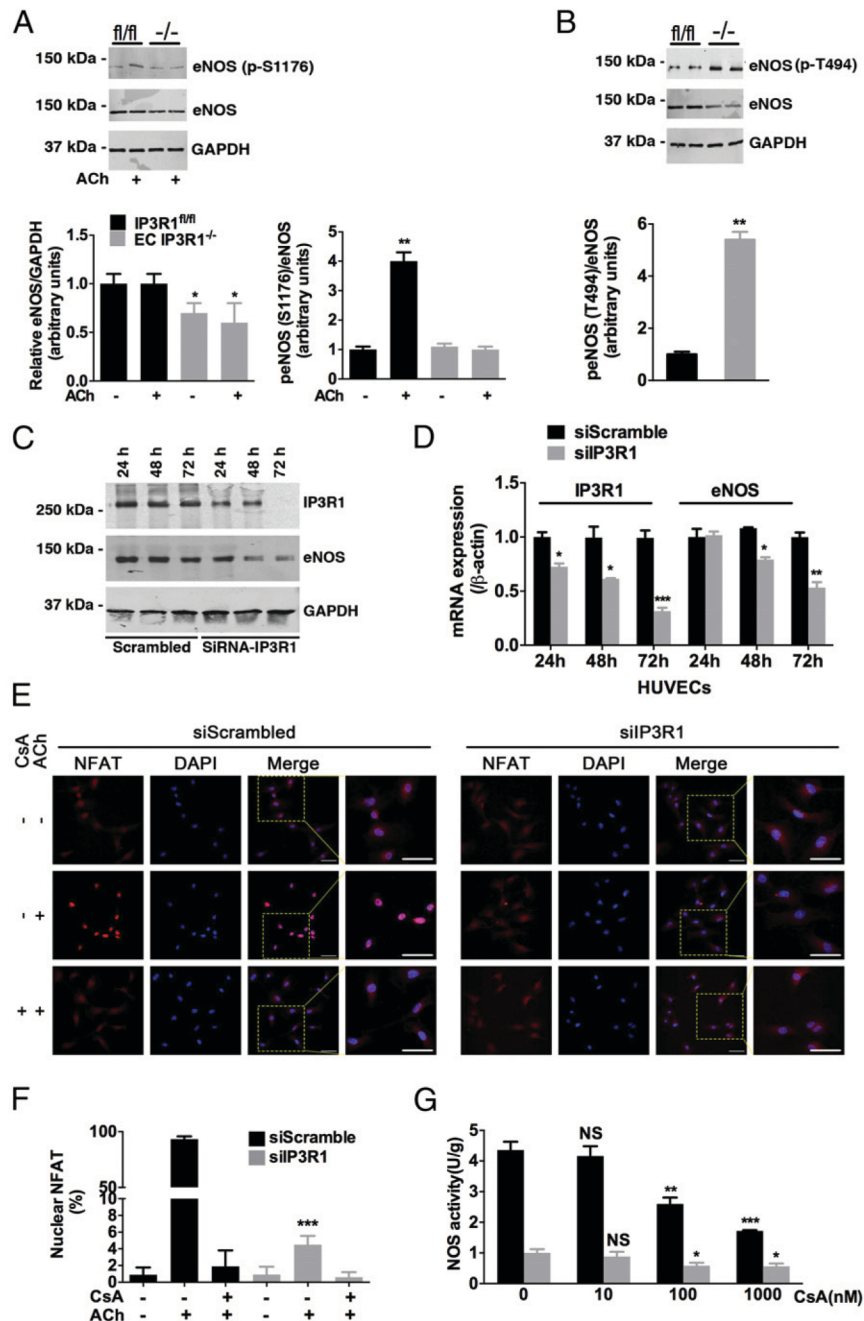
#### Effects of IP3R1 Deletion on the Calcineurin/NFAT Pathway in ECs.

The calcineurin/NFAT pathway has been identified as being located downstream of the angiotensin II type 2 receptor to promote eNOS expression in the myocardium, where it is believed to protect against cardiac hypertrophy (35). We examined NFAT translocation to the nucleus to determine whether NFAT might be a regulator of eNOS expression in ECs. Indeed, nuclear translocation of NFAT was reduced in response to ACh stimulation in both IP3R1 knockdown HUVECs (Fig. 3E and F) and isolated IP3R1-deficient ECs (Fig. S8). Taken together, these data provide a mechanism for the reduced eNOS levels observed in IP3R1-deficient ECs whereby decreased ER  $\text{Ca}^{2+}$  release impairs activation of downstream calcineurin/NFAT signaling.

The calcineurin inhibitor cyclosporin A (CsA) significantly reduced ACh-induced vasodilation, but did not reduce SNP-mediated vasodilation in IP3R1<sup>fl/fl</sup> mice (Fig. S9). Preincubation of HUVECs with CsA for 1 h blocked ACh-induced NFAT translocation (Fig. 3E and F). eNOS activation by ACh was also inhibited by CsA in a dose-dependent manner (Fig. 3G). These data suggest that the IP3R1/calcineurin/NFAT signaling pathway plays a key role in ACh-induced eNOS activity in vascular ECs.

Stimulation of muscarinic  $\text{M}_1$  receptors in ECs by ACh activates phospholipase C- $\gamma_1$ , resulting in IP3 production and IP3-mediated  $\text{Ca}^{2+}$  release from the ER (36). Our data show that increased  $[\text{Ca}^{2+}]_i$  in turn, activates the calcineurin/NFAT pathway, resulting in increased eNOS expression and activity in vascular ECs (Fig. 4). Although  $\text{Ca}^{2+}$ -induced eNOS activation appears to be an important pathway for vasodilation, we cannot rule out the involvement of other vasodilators [e.g., prostaglandin



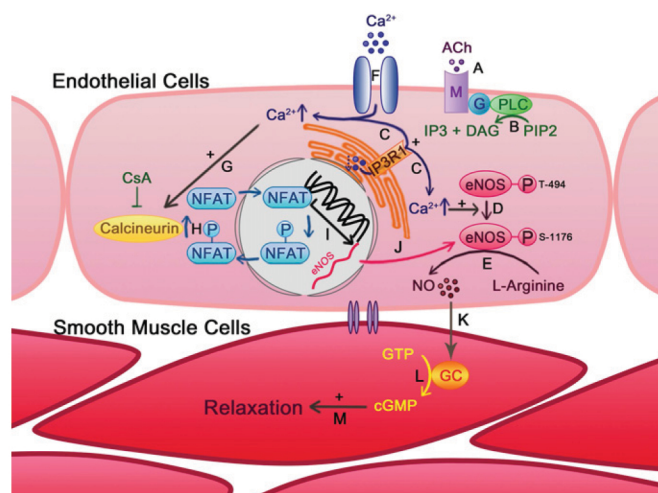


**Fig. 3.** Calcineurin in eNOS regulation. (A) Expression of eNOS and phosphorylation of eNOS at Ser1176 (p-S1176) in lysates from mesenteric arteries prepared under basal conditions or after 30-min ACh incubation in *IP3R1<sup>fl/fl</sup>* and EC *IP3R1<sup>-/-</sup>* mice. The graph shows the densitometric evaluation ( $n = 3$ ).  $*P < 0.05$ ;  $**P < 0.01$ . (B) Phosphorylation of eNOS at Thr494 (p-T494) in lysates from mesenteric arteries in *IP3R1<sup>fl/fl</sup>* and EC *IP3R1<sup>-/-</sup>* mice. Data are presented as mean  $\pm$  SEM of three independent experiments.  $**P < 0.01$ . (C) Immunoblots conducted in HUVECs transfected with scrambled or IP3R1-specific siRNAs. Cells were harvested 24 h, 48 h, and 72 h after transfection. (D) Relative IP3R1 and eNOS mRNA levels were measured by real-time RT-quantitative PCR in HUVECs after indicated times of transfection.  $\beta$ -Actin was used as an internal control. (A–D)  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$  vs. scrambled by Student's  $t$  test. (E) Representative immunofluorescence images of NFAT intracellular localization in transfected HUVECs, with or without CsA (1  $\mu$ M, 1 h) and ACh (10  $\mu$ M, 10 min) treatment. NFAT, red; DAPI, blue. (Magnification: 20 $\times$ .) (Scale bars: 20  $\mu$ m.) (F) Quantification of cells with nuclear-translocated NFAT. More than 30 cells were calculated for each condition, and data are presented as mean  $\pm$  SEM of three independent experiments.  $***P < 0.001$  vs. siScrambled treated with ACh only, analyzed by Student's  $t$  test. (G) Isolated aortic ECs were pretreated with gradient concentrations (0, 10, 100, and 1,000 nM) of CsA at 37  $^{\circ}$ C for 1 h, followed by ACh stimulation and NOS activity measurement as described in Fig. 2E.  $*P < 0.05$ ;  $**P < 0.01$ ;  $***P < 0.001$ ; NS, not significant vs. 0 nM CsA-treated cells of respective group, analyzed by Student's  $t$  test.

I2, endothelium-derived hyperpolarizing factor (EDHF)], particularly in mesenteric arteries, where EDHF is a major vasodilator downstream of increased  $[Ca^{2+}]_i$  (18). In addition, eNOS posttranslational modification at other sites upon ACh

stimulation may contribute to the low activity in EC *IP3R1<sup>-/-</sup>* mice (Fig. 3A and B). Phosphorylation of eNOS at Ser<sup>1176</sup> is a positive regulator of eNOS activity and is mediated by  $Ca^{2+}$ /calmodulin/calmodulin-dependent protein kinase II (CaMKII) cascade,





**Fig. 4.** Role of IP3R1 in ACh-induced vasodilation. ACh binds and activates muscarinic receptor (M) on the EC membrane (A), which leads to the production of diacylglycerol (DAG) and IP3 (B). (C) IP3 activates IP3R1 on the ER membrane to induce  $\text{Ca}^{2+}$  release from the ER store, resulting in the elevation of  $[\text{Ca}^{2+}]_i$ . Increased  $[\text{Ca}^{2+}]_i$  up-regulates eNOS activity by modifying the eNOS protein phosphorylation status (D) and promotes NO production from L-arginine (E). (F)  $\text{Ca}^{2+}$  reduction in the ER store induces extracellular  $\text{Ca}^{2+}$  entry through calcium channels.  $[\text{Ca}^{2+}]_i$  increase can also activate calcineurin (G), which dephosphorylates NFAT and promotes NFAT nuclear translocation (H). (I) NFAT induces eNOS gene transcription and translocates back into the cytoplasm. (J) eNOS protein level is transcriptionally up-regulated by an IP3R1-mediated  $\text{Ca}^{2+}$ -release-dependent calcineurin/NFAT mechanism. EC-generated NO diffuses into adjacent smooth muscle cells (K) and activates guanylyl cyclase (GC) to generate cGMP from GTP (L), leading to smooth muscle relaxation (M).

whereas the phosphorylation of Thr<sup>494</sup> inhibits eNOS catalytic activity (33). Calcineurin has been reported to dephosphorylate eNOS at Ser<sup>116</sup> in human and bovine ECs (37); however, this site

shows very low homology in murine EC eNOS. Further investigations are warranted to explore in detail the role of IP3R1 in the modulation of EC bioenergetics (38), which might contribute to the observed hypertensive phenotype.

Taken together, our results demonstrate that EC IP3R1 channels are crucial for the activation of the calcineurin/NFAT/eNOS signaling pathway, which, in turn, plays a fundamental role in the maintenance of normal BP.

## Materials and Methods

**Animal Studies.** Exon 4 of the gene encoding for IP3R1 was targeted by flanking it with loxP sites (Fig. S1A). Methodologies for the generation of embryonic stem cell-derived embryos have been described elsewhere (39). IP3R1<sup>fl/fl</sup> mice were bred with Tie2-Cre transgenic mice to obtain an EC-specific ablation of IP3R1 (EC IP3R1 KO). Genotypes were verified by PCR (Fig. S1B and Table S2). All mice were backcrossed into the C57BL/6 background for >10 generations. BP was recorded via radiotelemetry, Millar catheter, and tail-cuff, as previously described (39). Vascular reactivity of mouse thoracic aortic and mesenteric arterial ring preparations was determined following established protocols (40).

A detailed description of materials and methods can be found in *SI Materials and Methods*.

**Ethical Approval.** All studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Columbia University and according to NIH guidelines. All experiments were conducted by operators who were blinded to the genotypes of the animals. No samples, mice, or data points were excluded from the reported analyses.

**Statistics.** All results are presented as mean  $\pm$  SEM. Statistical analysis was performed using an unpaired two-tailed Student's *t* test unless otherwise indicated. *P* values <0.05 were considered significant.

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