ORIGINAL RESEARCH

American Heart Association.

Inositol 1,4,5-Trisphosphate Receptors in Endothelial Cells Play an Essential Role in Vasodilation and Blood Pressure Regulation

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***Background*—**Endothelial NO synthase plays a central role in regulating vasodilation and blood pressure. Intracellular Ca^{2+} mobilization is a critical modulator of endothelial NO synthase function, and increased cytosolic Ca^{2+} concentration in endothelial cells is able to induce endothelial NO synthase phosphorylation. Ca^{2+} release mediated by 3 subtypes of inositol 1,4,5-trisphosphate receptors (IP_3Rs) from the endoplasmic reticulum and subsequent Ca^{2+} entry after endoplasmic reticulum Ca^{2+} store depletion has been proposed to be the major pathway to mobilize Ca^{2+} in endothelial cells. However, the physiological role of IP_3Rs in regulating blood pressure remains largely unclear.

***Methods and Results*—**To investigate the role of endothelial IP_3Rs in blood pressure regulation, we first generated an inducible endothelial cell–specific IP_3R1 knockout mouse model and found that deletion of IP_3R1 in adult endothelial cells did not affect vasodilation and blood pressure. Considering all 3 subtypes of IP_3Rs are expressed in mouse endothelial cells, we further generated inducible endothelial cell–specific IP_3R triple knockout mice and found that deletion of all 3 IP_3R subtypes decreased plasma NO concentration and increased basal blood pressure. Furthermore, IP_3R deficiency reduced acetylcholine-induced vasodilation and endothelial NO synthase phosphorylation at Ser1177.

***Conclusions*—**Our results reveal that IP_3R-mediated Ca^{2+} release in vascular endothelial cells plays an important role in regulating vasodilation and physiological blood pressure. **(*J Am Heart Assoc*. 2019;8:e011704. DOI: 10.1161/JAHA.118.011704.)**

Key Words: blood pressure • calcium • calcium signaling • endothelial cell • hypertension

Hypertension or high blood pressure is characterized as a sustained elevated level of systolic and/or diastolic blood pressure and is a major risk factor for cardiovascular morbidity and mortality.[1] Accumulating evidence suggests that hypertension could also be linked to noncardiovascular diseases, including dementia, cancer, oral health disorders, and osteoporosis.[2] Affecting ≈1 in 4 adults, hypertension is one of the most important preventable causes of premature death worldwide. However, despite the availability of various types of treatments, optimal blood pressure control in patients with hypertension is hard to achieve. One reason is that the pathogenesis of essential or primary hypertension is multifactorial and extremely complex. In fact, the origin of essential hypertension is generally unclear, and the pathogenesis of hypertension, as well as the molecular mechanism of blood pressure control, is still not well understood.

The endothelium is located at the interface between the vessel wall and lumen and has been recognized for decades

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Accompanying Table S1, Figures S1 through S3 are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.118.011704

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Received December 7, 2018; accepted January 17, 2019.

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Clinical Perspective

What Is New?

- We described the role of inositol 1,4,5-trisphosphate receptors, a family of intracellular Ca^{2+} release channels localized in the membrane of the endoplasmic reticulum, in regulating vasodilation and blood pressure using genetically engineered mouse models.
- We show that deletion of all 3 inositol 1,4,5-trisphosphate receptor subtypes in mouse endothelial cells leads to hypertension, with reductions in both plasma NO concentration and acetylcholine-induced vasodilation.

What Are the Clinical Implications?

- Given that genome-wide association studies suggested that variants in inositol 1,4,5-trisphosphate receptors are associated with hypertension in humans, inositol 1,4,5-trisphosphate receptor deficiency in endothelial cells may exacerbate human hypertension through reducing NO production and vasodilation.

as a critical mediator in controlling blood pressure, primarily via producing a variety of vasoactive substances, such as NO, thromboxane, and endothelin-1.[3] NO is one of the most well-known vessel-relaxing factors and is produced by a NO synthase (NOS) from L-arginine in the presence of oxygen and the cofactors Ca^{2+}, calmodulin, reduced nicotinamide adenine dinucleotide phosphate, and tetrahydrobiopterin.[4] Intracellular Ca^{2+} has long been proposed as a critical factor regulating the activity of endothelial NOS (eNOS). Numerous stimuli, including hormonal and chemical signals, but also mechanical changes such as shear stress, can elicit rapid increases in intracellular Ca^{2+} in endothelial cells, resulting in Ca^{2+}-dependent activation of eNOS and subsequent NO release.[5–9] Genetically deficient eNOS mice are hypertensive, with lower circulating nitrite levels, indicating the importance of eNOS and NO in blood pressure regulation.[10,11] However, how endothelial Ca^{2+} mobilization via plasma membrane Ca^{2+} entry and endoplasmic reticulum (ER) Ca^{2+} release regulates eNOS in vivo is not clear.

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are a family of intracellular Ca^{2+} release channels located on the ER membrane, which in mammals consists of 3 different subtypes (IP_3R1, IP_3R2, and IP_3R3) encoded by 3 genes, *Itpr1*, *Itpr2*, and *Itpr3*, respectively.[12] All 3 subtypes of IP_3Rs are expressed in endothelial cells.[13,14] However, it remains unclear whether the different subtypes of IP_3Rs in endothelial cells have distinct or redundant functions. A recent study found that IP_3R1 deletion by the endothelial-specific receptor tyrosine kinase-Cre (Tie2-Cre) in mice reduced acetylcholine-induced vasodilation and increased basal blood pressure.[15] However, Tie2-Cre is expressed early during embryonic development. Furthermore, Tie2-Cre is expressed not only in endothelial cells but also in blood cell lineages.[16] Therefore, Tie2-Cre is not the ideal tool to use when generating endothelial cell–specific gene deletion mouse models, especially for physiological studies. To investigate the specific roles of IP_3Rs in adult endothelial cells, we used an inducible endothelial cell–specific platelet-derived growth factor receptor-β–Cre ($iCre^+$) to delete IP_3R1 alone as well as all 3 IP_3R subtypes together in mouse endothelial cells. Our results demonstrate that deletion of IP_3R1 in adult endothelial cells does not affect vascular reactivity or blood pressure. In fact, the different IP_3R subtypes in adult endothelial cells function redundantly because deletion of all 3 IP_3R subtypes reduced plasma NO concentration and acetylcholine-induced eNOS phosphorylation, resulting in less vasodilation and hypertension. Our study reveals an essential role of IP_3R-mediated Ca^{2+} signaling in regulating vascular function and blood pressure with a mechanism of functional redundancy among the different IP_3R subtypes.

Methods

The data, analytical methods, and study materials are available to other researchers for purposes of reproducing results or replicating procedures, as described in this article or by contacting corresponding authors.

Mice

The generation of floxed mice for each IP_3R subtype has been described previously.[17,18] The $iCre^+$ mice expressing an inducible Cre recombinase under the control of the platelet-derived growth factor receptor-β promoter has also been described previously.[19] All IP_3R floxed mice and the $iCre^+$ mice were backcrossed with C57BL/6 mice for >8 generations. The B6.Cg-Tg (Tek-cre)1Ywa/J ($Tie2\text{-}Cre^+$) mice and the $Gt(ROSA)26Sor^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}/J$ ($ROSA^{mT/mG}$) reporter mice carrying membrane-targeted, 2-color fluorescent Cre-reporter allele were purchased from the Jackson Laboratory.

To generate endothelial cell–specific single IP_3R1 knockout and triple IP_3R knockout mice, the 7- to 8-week-old $iCre^+$ IP_3R1 floxed ($IP_3R1^{f/f}$) and $iCre^+IP_3R1^{f/f}IP_3R2^{f/f}IP_3R3^{f/f}$ mice were intraperitoneally injected with tamoxifen (50 mg/kg per day; Sigma-Aldrich) for 5 consecutive days, and they were considered as endothelial cell–specific IP_3R1 knockout (ECR1KO) and endothelial cell–specific IP_3R triple knockout (ECTKO) mice, respectively. The littermate $iCre^-IP_3R1^{f/f}$ and $iCre^-IP_3R1^{f/f}IP_3R2^{f/f}IP_3R3^{f/f}$ mice also treated with tamoxifen using the same protocol were considered as control mice. Only male mice were used in all experiments. All mice were housed under a 12-hour day/night cycle at a temperature of 25.0°C, with free access to a standard diet and clean water.

Endothelial Cell Isolation

Endothelial cells were freshly isolated by enzymatic digestion. After euthanasia, the lungs were perfused with ice-cold Hank's balanced salt solution (8 g/L NaCl, 0.4 g/L KCl, 1 g/L glucose, 60 mg/L KH_2PO4, and 47.5 mg/L Na_2HPO4, pH 7.4) via the right ventricle to remove blood cells from inside the tissue. The lung was then cut into small pieces and digested in serum-free M199 medium containing 1.5 mg/mL collagenase II (Worthington) and 0.5607 mg/mL dispase II (Roche) for 1 hour at 37°C. After digestion, the cells were filtrated through a 70-μm cell strainer and precipitated by centrifugation at 225 g for 10 minutes. The cells were resuspended and incubated with magnetic beads (sheep anti-rat; Invitrogen) linked to anti-CD31 antibody (rat anti-mouse; BD Pharmingen) for 30 minutes at 4°C, and endothelial cells were harvested by magnetic separation.

Ca^{2+} Imaging

Ca^{2+} imaging was performed and analyzed, as previously described.[20] Briefly, isolated endothelial cells from individual mice (3 mice per group) were washed with physiological saline solution (in mmol/L: NaCl 137, KCl 5.4, $MgSO_4$ 1.0, glucose 10, $CaCl_2$ 1.8, and HEPES 10, pH 7.4) and then incubated with 5 μmol/L Fluo-4-AM (Invitrogen) for 30 minutes at 37°C. After that, cells were washed with physiological saline solution 3 times and imaged with a Zeiss 880 inverted confocal microscope. Acetylcholine (10 μmol/L) was then applied to elicit intracellular Ca^{2+} mobilization in endothelial cells. The fluorescence was excited with 488-nm light, and emitted light >510 nm was collected.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Quantitative real-time polymerase chain reaction (PCR) was performed and analyzed, as previously described.[21] Briefly, total RNA was extracted from freshly isolated endothelial cells using the RNeasy MiniKit (Qiagen), and cDNA was synthesized using the *TransScript* One-Step gDNA removal and cDNA Synthesis SuperMix Kit (TransGen Biotech). Quantitative real-time PCR was performed using the *TransStart* Tip Green qPCR SuperMix (TransGen Biotech), according to the manufacturer's instructions. The sequences for primers of *Itpr1-3* and *Gapdh* have been previously described.[22] The primer sequences for quantitative PCR are listed in Table S1. Endothelial cells isolated from 3 mice were pooled as 1 sample. At least 3 samples were prepared for quantitative real-time PCR per group. Each sample was assayed in triplicate, and the transcript level of each target gene was normalized to *Gapdh*.

Immunoblotting

Thoracic aortas were isolated from individual mice (4 mice per group), cleaned from connective tissues and adipose, and equilibrated in Krebs solution (in mmol/L: NaCl 118, KCl 4.6, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, and glucose 12, pH 7.4) at 37°C for 1 hour. After equilibration, the arteries were stimulated with vehicle or acetylcholine (10 μmol/L; 10 minutes) and then were quickly frozen in liquid nitrogen and homogenized with protein lysis buffer consisting of 8 mol/L urea, 2 mol/L thiourea, 3% SDS, 75 mmol/L dithiothreitol, 0.05 mol/L Tris-HCl (pH 6.8), and 0.03% Bromophenol Blue. Western blotting was then performed as previously described.[23] The primary antibodies against mouse eNOS (Millipore, catalog No. 07–520, at 1:1000 dilution), phosphorylated eNOS at Ser1177 (Abcam, catalog No. ab195944, at 1:1000 dilution), phosphorylated eNOS at Thr495 (Cell Signaling Technology, catalog No. 9574s, at 1:1000 dilution), and GAPDH (Cell Signaling Technology, catalog No. 97166, at 1:1000 dilution) were commercially purchased.

Myography

To assess vascular reactivity, thoracic aortas and the second order of superior mesenteric arteries were isolated from individual mice (at least 6 mice per group) and quickly immersed in ice-cold Krebs solution. After the connective tissues were carefully removed, the vessel rings were prepared and mounted in a myograph chamber (620 mol/L; Danish Myo Technology, Denmark), as previously described.[22] Each chamber was filled with 5 mL Krebs solution aerated with 95% O_2 to 5% CO_2 and maintained at 37°C. Each vessel ring was stretched in a stepwise manner to the optimal resting tension (thoracic aortas to ≈9 mN; mesenteric arteries to ≈1.5 mN), equilibrated for 30 minutes, and then exposed to 100 mmol/L K^+ Krebs solution to induce a reference contraction. Thereafter, after the application of phenylephrine (10 μmol/L; Sigma-Aldrich) to elicit a preconstriction, cumulative doses of acetylcholine (1 nmol/L to 10 μmol/L) and sodium nitroprusside (1 nmol/L to 10 μmol/L; Sigma-Aldrich) were then applied to elicit vasodilation. The vasodilation in response to the cumulatively administrated acetylcholine or sodium nitroprusside was expressed as decreases in force and as percentages of the peak of phenylephrine-induced contraction.

Measurement of Blood Pressure by a Tail-Cuff System

Systolic blood pressure was measured by a noninvasive tail-cuff system (NIBP system, IN125/m; AD Instruments), as

previously described.[22] Briefly, the mice were placed in a restrainer with the caudal artery positioned right above the sensor. All the mice (11 control and 12 mutant mice for the IP_3R1 single knockout studies; 13 control and 17 mutant mice for the IP_3R triple knockout studies) were given at least 1 week to adapt to the system before blood pressure measurement. Systolic blood pressure was continuously measured at least 6 times and averaged for each mouse.

Telemetry Measurement

A total of 14 male mice at the age of 6 months (4 months after tamoxifen injection) were anesthetized with IP injection of a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg). Each mouse was implanted with a radiotelemetric transmitter (PA-C10; Data Sciences International) and housed in a separated cage under a 12-hour day/night cycle. Animals undergoing survival surgery were treated with carprofen for 2 to 5 days after operation to reduce discomfort. After a 1-week recovery period from surgery, blood pressures and heart rates were recorded every 5 minutes for 3 consecutive days in conscious, freely moving mice, as previously described.[22] After data acquisition, the mice will be euthanized by IP injection of nembutal (150 mg/kg), followed by cervical dislocation.

Histology

The second order of superior mesenteric arteries was isolated from 5 control and 6 ECTKO mice, fixed in 4% paraformaldehyde at 4°C overnight, and thereafter processed for frozen sections. Hematoxylin-eosin staining was performed on 8-μm sections, and images were acquired with light microscopy, as previously described.[24] The wall thickness and the media/lumen ratio were quantified using Image J software.

Measurement of Serum Nitrite and Nitrate

NO in solution is rapidly oxidized into nitrite and nitrate, which can be used to quantitate NO production. Serum was collected from 6 control and 6 ECTKO mice 4 months after tamoxifen induction, and NO production was assessed using the Nitric Oxide Assay Kit (Nanjing Jiancheng Bioengineering Institute), which is designed to accurately measure NO production after reduction of nitrate to nitrite using the improved Griess method.

Statistical Analysis

Scatter diagram and box-and-whisker plots were drawn using GraphPad Prism 5. Statistical analysis was performed using 2-tailed, unpaired, Student *t* test or 2-way ANOVA with Bonferroni post hoc test for multiple comparisons. All data were presented as mean±SEM (error bars). $P<0.05$ was considered statistically significant ($P<0.05$ and $P<0.01$ versus control).

Study Approval

All animal care and use procedures in this study were approved by the Institutional Animal Care and Use Committee at UC San Diego (San Diego, CA; protocol reference No. S01049) and at Peking University Shenzhen Graduate School (Shenzhen, China; protocol reference No. AP0017).

Results

Single Deletion of IP_3R1 in Endothelial Cells Does Not Affect Vascular Function and Blood Pressure

To investigate the specific role of IP_3R1-mediated Ca^{2+} release in endothelial cells of adult mice, we crossed $IP_3R1^{f/f}$ mice with mice that express a tamoxifen-inducible form of Cre recombinase in vascular endothelial cells using a phage artificial chromosome containing the platelet-derived growth factor receptor-β (*Pdgfb)* gene ($iCre^+$).[19] To confirm the efficiency and cell specificity of gene deletion by $iCre^+$, we also crossed $iCre^+$ mice with $ROSA^{mT/mG}$ reporter mice that express a cell membrane–targeted, 2-color fluorescent Cre reporter allele.[25]

Administration of tamoxifen in adult $iCre^+ROSA^{mT/mG}$ mice led to expression of cell membrane–localized enhanced green fluorescent protein in almost all endothelial cells of the mesenteric artery, whereas nonendothelial cells expressed cell membrane–localized tdTomato (Figure S1), suggesting that the Cre recombinase activity of $iCre^+$ was specifically induced in endothelial cells. Furthermore, we found that *Itpr1* mRNA levels were dramatically reduced in endothelial cells isolated from $iCre^+IP_3R1^{f/f}$ (ECR1KO) mice 4 months after tamoxifen injection compared with control cells, whereas *Itpr2* and *Itpr3* mRNA levels were comparable between control and ECR1KO cells (Figure 1A). However, single deletion of IP_3R1 was not sufficient to block Ca^{2+} mobilization induced by the endothelium-dependent vasodilator acetylcholine in endothelial cells (Figure 1B). In fact, the amplitude of Ca^{2+} transient induced by 10 μmol/L acetylcholine in ECR1KO cells was >80% of that in control cells (Figure 1C), implicating that the other 2 IP_3R subtypes can compensate for the loss of IP_3R1 in endothelial cells. Accordingly, systolic blood pressure measured by the tail-cuff system was comparable between control and ECR1KO mice at 2, 3, and 4 months after tamoxifen injection (Figure 1D). In addition, vascular reactivity in response to either acetylcholine or the endothelium-independent vasodilator sodium nitroprusside (a NO donor) in the thoracic aorta and mesenteric artery was not significantly

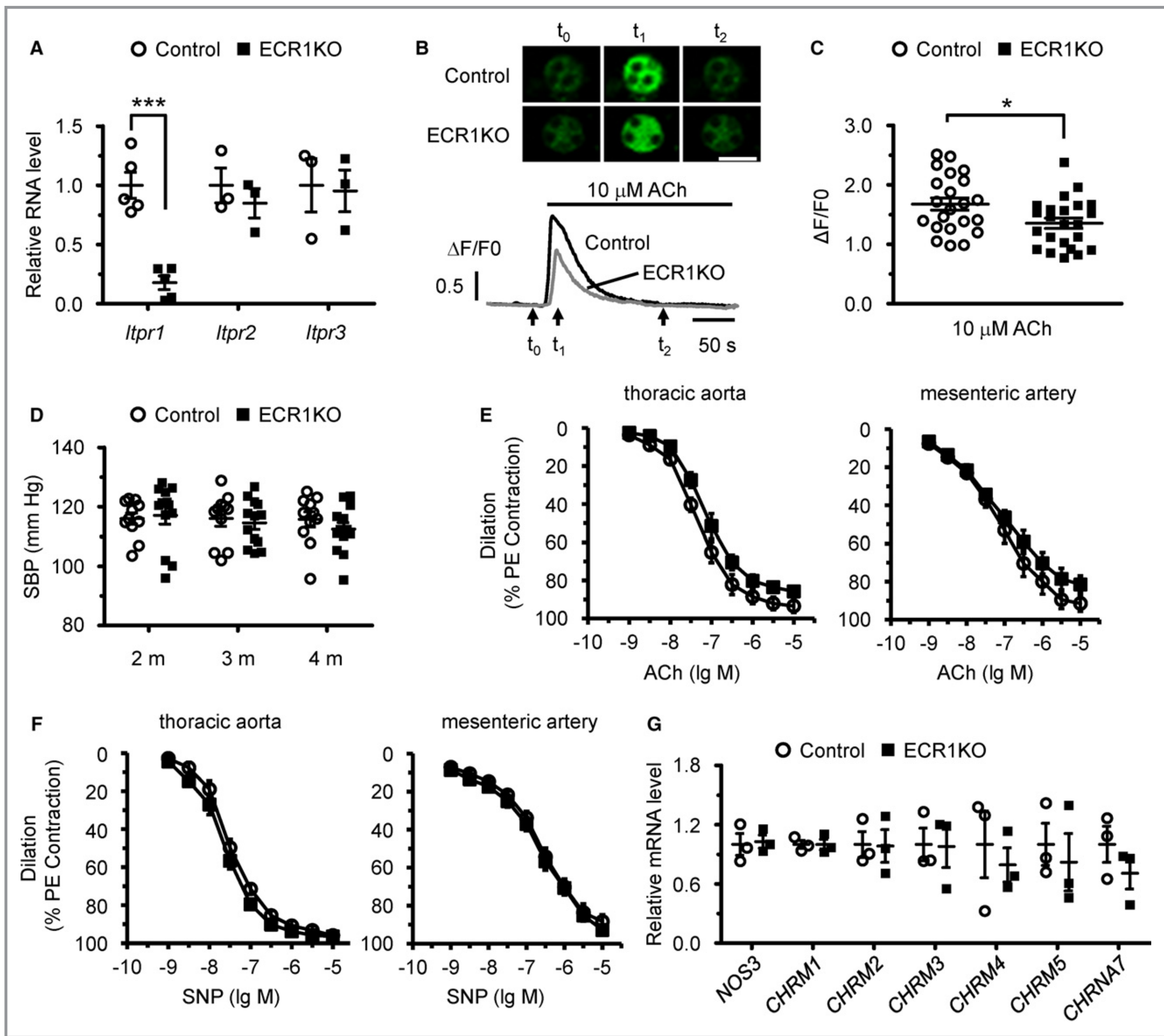

Figure 1. Endothelial cell–specific deletion of inositol 1,4,5-trisphosphate receptor 1 (IP$_3$R1) in adult mice had no major effects on blood pressure and vasodilation. **A**, mRNA levels of IP$_3$Rs in isolated endothelial cells from control and endothelial cell–specific IP$_3$R1 knockout (ECR1KO) mice. n=3 to 5 (with endothelial cells from 3 mice pooled as 1 sample) per group. **B**, Confocal Ca^{2+} imaging of isolated endothelial cells using Fluo-4-AM. Intracellular Ca^{2+} mobilization was elicited by 10 μmol/L acetylcholine. **Top**, Sequential confocal images of endothelial cells at the time points of time 0, time 1, and time 2, as indicated by the arrows at the **bottom**. Bar=20 μm. **Bottom**, Representative traces of Ca^{2+} signals in control (black) and ECR1KO (gray) endothelial cells. F0, the Fluo-4 fluorescence at rest. F/F0, normalized fluorescence. **C**, The amplitude of Ca^{2+} signals induced by 10 μmol/L acetylcholine. n=20 to 30 cells from 3 independent experiments per group. **D**, Systolic blood pressure (SBP) measured in control and ECR1KO mice at 2, 3, and 4 months after tamoxifen injection using the tail-cuff system. n=11 to 12 mice per group. **E**, Vascular reactivity in response to endothelium-dependent agonist acetylcholine in ECR1 aortas and mesenteric arteries showed a slight trend toward reduced vasodilation that was not statistically significant. **F**, Vascular reactivity in response to endothelium-independent agonist sodium nitroprusside (SNP) in aortas and mesenteric arteries. The vessels were preconstricted by 10 μmol/L phenylephrine, and the vasorelaxing effects of acetylcholine and sodium nitroprusside (SNP) were presented as a percentage of phenylephrine-induced contraction. n=6 mice per group. **G**, Quantitative real-time polymerase chain reaction analysis of the expression of *NOS3* and major acetylcholine receptors, including *CHRM1*, *CHRM2*, *CHRM3*, *CHRM4*, *CHRM5*, and *CHRNA7*, in control and ECR1KO endothelial cells. n=3 (with endothelial cells from 3 mice pooled as 1 sample) per group. Significance was determined using a 2-tailed, unpaired, Student *t* test or 2-way ANOVA analysis with Bonferroni post hoc test. Error bars represent mean±SEM. **P*<0.05, ****P*<0.001 vs control.

altered in ECR1KO mice compared with control mice 4 months after tamoxifen injection (Figure 1E and 1F). Furthermore, the mRNA levels of muscarinic acetylcholine receptor M1 (*CHRM1*), M2 (*CHRM2*), M3 (*CHRM3*), M4 (*CHRM4*), and M5 (*CHRM5*), nicotinic acetylcholine receptor subunit α7 (*CHRNA7*), and eNOS (*NOS3*) were also

comparable between control and ECR1KO mice (Figure 1G). Taken together, these results demonstrated that single deletion of IP_3R1 in adult endothelial cells was not sufficient to alter vasodilation and cause hypertension, as reported in a recent study that found that IP_3R1 deletion by Tie2-Cre in mice reduced acetylcholine-induced vasodilation and increased basal blood pressure.[15]

The difference between our ECR1KO mice and the recently published Tie2-Cre-IP_3R1 knockout mice could be explained by the fact that Tie2-Cre is expressed at early embryonic stages not only in endothelial cells but also in multiple blood cell lineages.[16] To further confirm this possibility, we then generated the same Tie2-$Cre^+IP_3R1^{f/f}$ mice (Figure S2A). However, no significant changes in systolic blood pressure were found between Tie2-$Cre^-IP_3R1^{f/f}$ and Tie2-$Cre^+IP_3R1^{f/f}$ mice at the ages of 3 and 6 months, respectively (Figure S2B). Acetylcholine-induced vasodilation in the thoracic aorta and mesenteric artery was also comparable between Tie2-$Cre^-IP_3R1^{f/f}$ and Tie2-$Cre^+IP_3R1^{f/f}$ mice at the age of 6 months (Figure S2C). In addition, the ratio of heart weight/body weight was not significantly increased in Tie2-$Cre^+IP_3R1^{f/f}$ mice at the age of 6 months when compared with Tie2-$Cre^-IP_3R1^{f/f}$ mice (Figure S2D). Thus, our data also do not support the published results that IP_3R1 deletion by Tie2-Cre in mice reduced acetylcholine-induced vasodilation and increased basal blood pressure.[15]

Deletion of All 3 IP_3R Subtypes in Adult Endothelial Cells Results in Hypertension

Because we did not observe any obvious functional deficits in the IP_3R1 single knockout animals, we considered that the other 2 IP_3R subtypes might play a compensatory function in the absence of IP_3R1 because both our data (Figure 1A) and others showed that all 3 IP_3R subtypes are expressed in endothelial cells.[13,14] Therefore, we generated $iCre^+IP_3R1^{f/f}IP_3R2^{f/f}IP_3R3^{f/f}$ (ECTKO) mice (Figure S3A). In isolated endothelial cells from control and ECTKO mice 4 months after tamoxifen injection, mRNA levels of all 3 IP_3R subtypes were dramatically reduced in ECTKO cells compared with control cells (Figure 2A), suggesting that all IP_3Rs were efficiently deleted by tamoxifen injection. Furthermore, Ca^{2+} transients induced by 10 μmol/L acetylcholine were almost abolished in ECTKO endothelial cells (Figure 2B and 2C). Considering the effect of single deletion of IP_3R1 on acetylcholine-induced Ca^{2+} transient in endothelial cells (Figure 1B and 1C), our data demonstrate that the different IP_3R subtypes in mouse endothelial cells have redundant roles in acetylcholine-induced Ca^{2+} mobilization.

We next investigated whether deletion of all 3 subtypes of IP_3Rs in endothelial cells affects blood pressure regulation. We found that systolic blood pressure, measured by the tail-cuff system, was significantly increased in ECTKO mice compared with control mice at 2, 3, and 4 months after tamoxifen injection (Figure 2D). Consistently, we also found that the wall thickness and the media/lumen ratio of mesenteric arteries were both significantly increased in ECTKO mice 4 months after tamoxifen injection when compared with control mice (Figure 2E and 2F). The importance of the vasodilator, NO, in regulating blood pressure has been well recognized, and lower circulating NO levels have been highlighted in numerous animal models as well as in patients with hypertension.[10,11,26] Therefore, we investigated whether loss of endothelial IP_3Rs in mice affects NO production by measuring the concentration of nitrite and nitrate (NOx) in the plasma. At 4 months after tamoxifen injection, the concentration of NOx in the plasma of ECTKO mice at baseline was significantly reduced compared with control mice (Figure 2G), indicating that deletion of IP_3Rs in adult mouse endothelial cells decreases NO production.

We further used a telemetry system to analyze the hemodynamics of control and ECTKO mice under conscious and unrestrained conditions. The averaged results from data collected continuously over 24 hours showed that all control and ECTKO mice 4 months after tamoxifen injection displayed characteristic diurnal variations in blood pressure (Figure 3A). Systolic blood pressure, diastolic blood pressure, and mean blood pressure measured at every time point, or averaged days or nights, are all significantly increased in ECTKO mice compared with control mice (Figure 3A and 3B), consistent with what was observed by the tail-cuff system. Taken together, all these data strongly suggest that IP_3R-mediated Ca^{2+} release in mouse endothelial cells is required for normal blood pressure regulation, and IP_3R deficiency in endothelial cells is sufficient to cause hypertension.

IP_3R Deletion in Endothelial Cells Caused Vascular Dysfunction and Defective eNOS Activity

Next, we investigated whether IP_3R deficiency influences vascular reactivity in response to vasoconstrictor and vasodilator treatment. Using wire myography, we measured vascular contractility and dilation in control and ECTKO mice 4 months after tamoxifen injection. Both the contraction induced by 100 mmol/L potassium and the dose-dependent response to phenylephrine in the aorta were not significantly altered in ECTKO mice relative to their controls (Figure S3B). The contraction of the mesenteric artery induced by 100 mmol/L potassium was also not significantly changed in ECTKO mice (Figure S3C). However, the dose-dependent response to phenylephrine in the ECTKO mesenteric arteries displayed a small leftward shift compared with control samples (Figure S3C), implicating that endothelial dysfunction resulting from IP_3R deficiency might also affect the basal

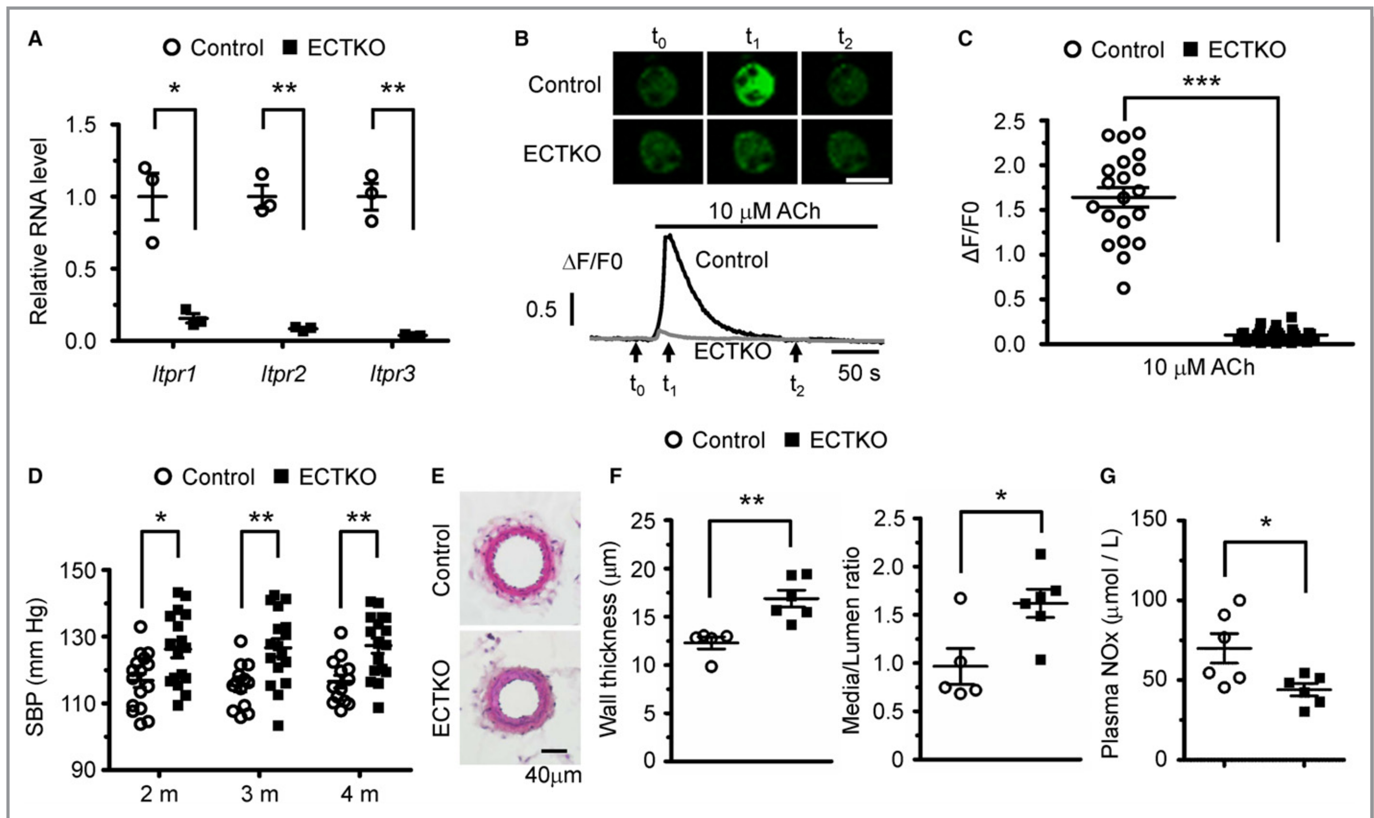

Figure 2. Endothelial cell–specific deletion of all 3 subtypes of inositol 1,4,5-trisphosphate receptors (IP_3Rs) in adult mice increased systolic blood pressure (SBP). **A**, Quantitative real-time polymerase chain reaction analysis of the 3 IP_3R subtypes in isolated endothelial cells from control and endothelial cell–specific IP_3R triple knockout (ECTKO) mice. n=3 (with endothelial cells from 3 mice pooled as 1 sample) per group. **B**, Confocal Ca^{2+} imaging of endothelial cells isolated from control and ECTKO mice. **Top**, Sequential confocal images of endothelial cells at the time point of time 0, time 1, and time 2, as indicated by the arrows at the **bottom**. Bar=20 μm. **Bottom**, Representative traces of Ca^{2+} signals in control (black) and ECTKO (gray) endothelial cells. F0, the Fluo-4 fluorescence at rest. F/F0, normalized fluorescence. **C**, The amplitude of Ca^{2+} signals induced by 10 μmol/L acetylcholine in control and ECTKO endothelial cells. n=20 to 30 cells from 3 independent experiments per group. **D**, SBP measured in control and ECTKO mice at 2, 3, and 4 months after tamoxifen administration using the tail-cuff system. n=13 to 17 mice per group. **E**, Representative hematoxylin and eosin–stained sections of the second-order mesenteric arteries isolated from control and ECTKO mice 4 months after tamoxifen administration. Bar=40 μm. **F**, The wall thickness and the ratio of medial/luminal area were calculated in control and ECTKO mice. n=5 to 6 mice per group. **G**, The concentration of nitrite and nitrate (NOx) in the serum measured in control and ECTKO mice 4 months after tamoxifen administration. n=6 mice per group. Significance was determined using a 2-tailed, unpaired, Student *t* test. Error bars represent mean±SEM. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control.

vascular tone toward increased constriction. Consistently, vasodilation induced by acetylcholine in both the aorta and mesenteric artery was markedly reduced in ECTKO mice compared with control mice (Figure 4A). In contrast, vasodilation induced by the endothelium-independent vasodilator sodium nitroprusside (a NO donor) in both the aorta and the mesenteric artery was not significantly changed between control and ECTKO mice (Figure 4B). Considering that the plasma NOx concentration at baseline was reduced in ECTKO mice (Figure 2G), we next investigated whether the observed blunted vasodilation was attributable to the defective response of eNOS to acetylcholine because eNOS is the primary enzyme to produce NO in endothelial cells. Therefore, we isolated aortas from control and ECTKO mice and measured the protein and phosphorylation levels of eNOS in response to acetylcholine stimulation. We found that the protein level of eNOS was not significantly altered in ECTKO aortas (Figure 4C and 4D). However, the increase in the phosphorylation level of eNOS at Ser1177 in response to acetylcholine stimulation was blunted in ECTKO aortas compared with control aortas (Figure 4C and 4D), which is consistent with the blunted vasodilation observed in ECTKO arteries. On the other hand, reduction of eNOS phosphorylation at Thr495 in response to acetylcholine stimulation was comparable between control and ECTKO aortas (Figure 4C and 4D). Taken together, our data strongly suggest that IP_3R-mediated Ca^{2+} release plays an essential role in regulating vasodilation via modulating eNOS phosphorylation.

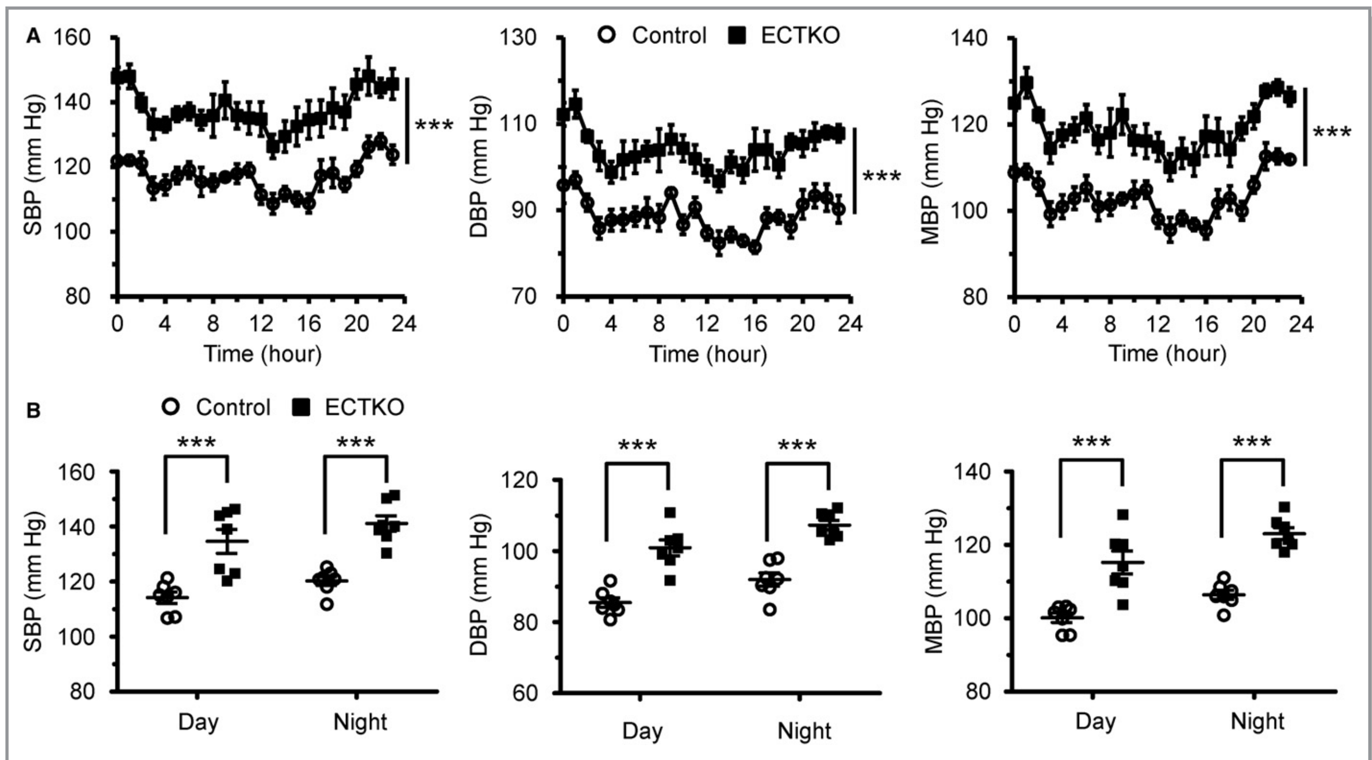

Figure 3. Basal blood pressure was significantly elevated in endothelial cell–specific inositol 1,4,5-trisphosphate receptor triple knockout (ECTKO) mice. Blood pressures were measured in unstrained mice using an implantable telemetry system. **A**, Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean blood pressure (MBP) in control and ECTKO mice. The data were presented at times indicated on a 24-hour scale, and data shown at each point represent 1-hour rolling averages of data sampled each minute in 3 consecutive days. n=7 mice per group. **B**, Mean values for SBP, DBP, and MBP were calculated during the day (8 AM–8 PM) and night (8 PM–8 AM). n=7 mice per group. Significance was determined using a 2-tailed, unpaired, Student *t* test or 2-way ANOVA analysis with Bonferroni post hoc test. Error bars represent mean±SEM. ***P<0.001 vs control.

Discussion

In this study, we demonstrated that IP$_3$Rs play an essential role in regulating vasodilation and blood pressure, with the different IP$_3$R subtypes displaying functional redundancy. The IP$_3$R is a ubiquitously expressed ER Ca^{2+} release channel, which has 3 different subtypes in mammals encoded by 3 distinct genes. The 3 full-length amino acid sequences are 60% to 80% homologous overall, with regions, including the ligand-binding and pore domains, having much higher homology.[27,28] Furthermore, multiple IP$_3$R subtypes are coexpressed in most mammalian cell types outside the central nervous system,[12] suggesting a functional redundancy between different subtypes. Indeed, various studies using genetically engineered mouse models have demonstrated that there is functional redundancy among the IP$_3$R subtypes in cells that express >1. Deletion of both IP$_3$R2 and IP$_3$R3 was required to create a pancreatic acinar cell secretion phenotype.[29] In T lymphocytes, deletion of all 3 IP$_3$R subtypes was required to induce a developmental defect in double-negative to double-positive transition and T-cell acute lymphoblastic leukemia.[17] In B cells, different IP$_3$R subtypes also play a redundant role in regulating both B-cell receptor–mediated Ca^{2+} release and B-cell development.[20]

In contrast to our current experiments, a recent publication performed concomitantly to our study reported that single deletion of IP$_3$R1 by Tie2-Cre in mice impaired endothelium-dependent vasodilation, elevated the basal blood pressure, and eventually increased the ratio of heart weight/body weight,[15] suggesting that IP$_3$R1-mediated Ca^{2+} release plays a dominant role in regulating endothelial cell function. In contrast with these findings, we found that single deletion of IP$_3$R1 in endothelial cells by the same Tie2-Cre was not able to cause the same phenotype in mice. The parameters, including blood pressure, vascular reactivity in response to acetylcholine, and the ratio of heart weight/body weight in our Tie2-Cre$^+$R1$^{f/f}$ mice, are all comparable with control mice. The difference between our current study and the previous report may result from different gene-targeting strategies and/or mouse genetic backgrounds. In our IP$_3$R1 floxed mouse model, the exon 5 was flanked, whereas the exon 4 was targeted in the mouse model of Yuan et al.[15] In addition, the mice reported by Yuan et al[15] were backcrossed with C57BL/6 2 more generations than ours. It is possible that

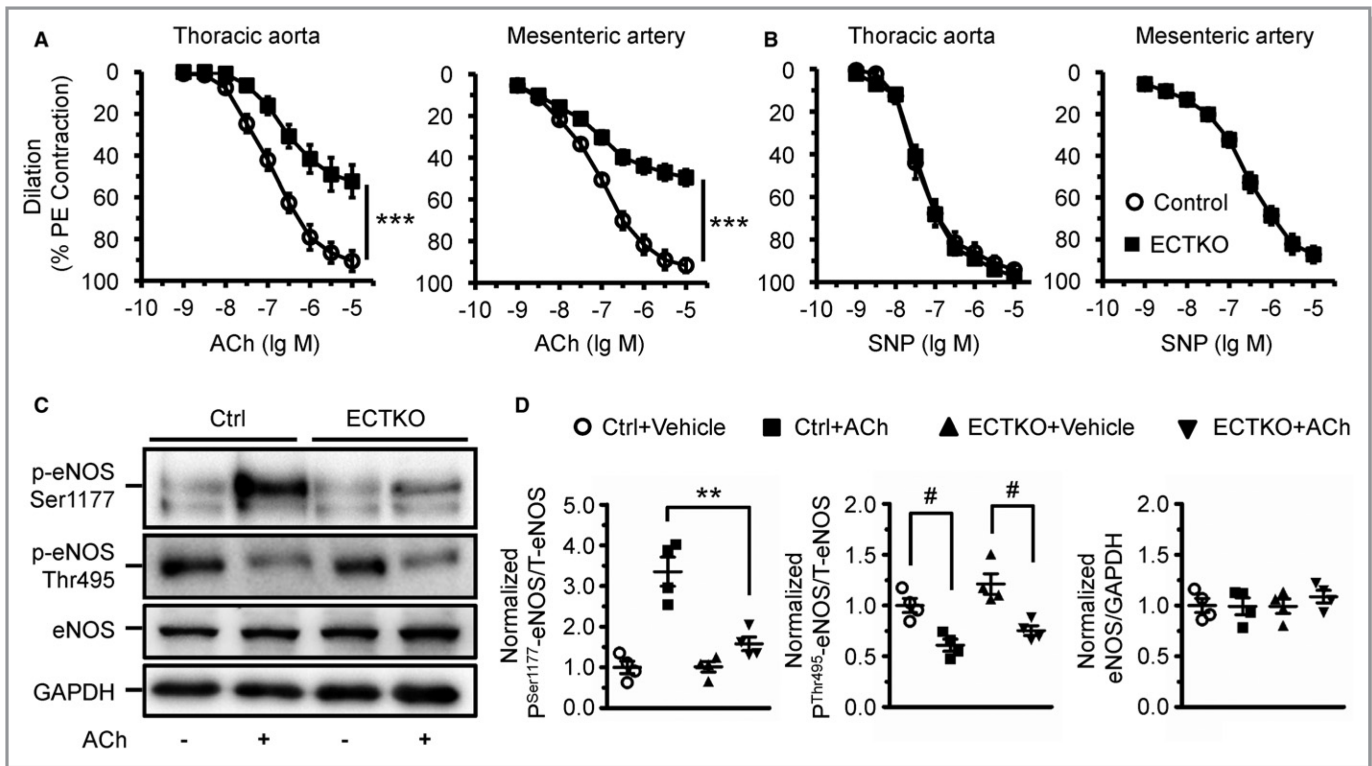

Figure 4. Deficiency of inositol 1,4,5-trisphosphate receptors (IP$_3$Rs) in endothelial cells affected acetylcholine-induced vasodilation and endothelial NO synthase (eNOS) phosphorylation. **A** and **B**, Vascular reactivity in response to endothelium-dependent agonist acetylcholine (**A**) and endothelium-independent agonist sodium nitroprusside (SNP) (**B**) in aortas and mesenteric arteries. The vessels were preconstricted by 10 μmol/L phenylephrine, and the vasorelaxing effects of acetylcholine and SNP were presented as a percentage of phenylephrine-induced contraction. n=6 to 12 mice per group. **C**, Expression and phosphorylation of eNOS measured by Western blot. Thoracic aortas isolated from control and endothelial cell–specific IP$_3$R triple knockout (ECTKO) mice were treated with or without 10 μmol/L acetylcholine for 10 minutes. **D**, The levels of phosphorylated eNOS at Ser1177 and Thr495 were normalized to total eNOS. The levels of total eNOS were normalized to GAPDH. n=4 mice per group. Significance was determined using a 2-tailed, unpaired, Student *t* test or 2-way ANOVA analysis with Bonferroni post hoc test. Error bars represent mean±SEM. $^{**}P<0.01$, $^{***}P<0.001$ vs control; $^{\#}P<0.05$ vs vehicle.

these differences could contribute, in part, to the discrepancy in the phenotypes observed in our and their mouse models. However, our results showed that deletion of IP$_3$R1 alone has minimal effects on the amplitudes of acetylcholine-induced Ca^{2+} signals in isolated endothelial cells. Furthermore, the 3 IP$_3$R subtypes are all expressed in endothelial cells, as confirmed by quantitative real-time PCR in this study and also reported by others.[13,14] In addition, our previous work demonstrated that deletion of all 3 IP$_3$R subtypes by Tie2-Cre resulted in T-cell acute lymphoblastic leukemia and mouse lethality from 2 months of age.[17] Tie2-Cre is not an ideal tool to study the role of endothelial cells in vascular biology because Tie2-Cre is also expressed in hematopoietic cells.[16,30] Another constitutively active panendothelial Cre, VE-cadherin-Cre, which has also been widely used for endothelium-specific gene deletion, is also expressed in hematopoietic cells.[30,31] Therefore, we used the iCre$^+$, an inducible panendothelial Cre, to target IP$_3$R genes in adult vascular endothelial cells, and we found that deletion of all IP$_3$R genes in endothelial cells was efficient after tamoxifen administration. Accordingly, deletion of all 3 IP$_3$R subtypes was able to block acetylcholine-induced Ca^{2+} signals in isolated endothelial cells. Consistently, deletion of all 3 IP$_3$R subtypes in endothelial cells by iCre$^+$ reduced acetylcholine-induced vasodilation and increased the basal blood pressure. Therefore, our study strongly suggests there is functional redundancy between the different IP$_3$R subtypes in endothelial cells.

In contrast to the vasodilatory effects of IP$_3$R-mediated Ca^{2+} release in endothelial cells, it has been well recognized that Ca^{2+} release from ER via IP$_3$Rs in smooth muscle cells leads to vasoconstriction via activation of myosin light chain kinase that subsequently phosphorylates myosin light chain, enabling molecular cross-bridge formation between myosin and actin filaments.[32,33] Deletion of all IP$_3$R subtypes in smooth muscle cells has been shown to reduce vascular contraction and myosin light chain 20 phosphorylation in response to vasoconstrictor, and it alleviated angiotensin II–

induced hypertension.[22] Therefore, IP_3R-mediated Ca^{2+} mobilization in vascular endothelial cells and smooth muscle cells plays an opposing role in regulating vascular contractility, which might provide the basis for paradoxical vasoconstriction induced by acetylcholine in atherosclerotic coronary arteries.[34,35] On the other hand, endothelial cells from different tissues or organs have distinct morphological, structural, and gene-expression profiles.[36–38] In additional to vascular tone, endothelial cells may also play essential roles in regulating vascular permeability, angiogenesis, inflammation, and vascular remodeling.[26,37] Thus, it will be worthy to examine diverse tissues or organs to determine whether and how IP_3R-mediated intracellular Ca^{2+} mobilization could mediate different functions.

Among the various vasoactive factors released by vascular endothelial cells, NO is definitely one of the most well-characterized molecules. NO regulates vascular tone and physiological blood pressure, and it participates in the pathogenesis of many cardiovascular diseases, including atherosclerosis, myocardial infarction, diabetes mellitus, and hypertension.[39–43] In endothelial cells, the production of NO is controlled by eNOS, an enzyme highly dependent on Ca^{2+}/calmodulin-mediated activation. Active calmodulin binds to eNOS, leading to caveolin detaching from eNOS, and transforms eNOS into its active form.[44,45] Previous studies have shown that removal of extracellular Ca^{2+}, treatment with calmodulin antagonist, or addition of IP_3R blockers, such as heparin, dramatically reduces NO production and endothelium-dependent artery dilation.[45–47] In addition, genetic depletion of stromal interaction molecule 1 from endothelial cells also significantly impairs the endothelium-dependent vasorelaxation.[48] Consistent with these findings, our data also demonstrated that deficiency of IP_3R-mediated Ca^{2+} release in endothelial cells reduces endothelium-dependent vasodilation and plasma NOx levels. Considering that shear stress has been proposed to play a critical role in regulating NO production and intracellular Ca^{2+} homeostasis in endothelial cells,[44,49–51] it remains to be determined whether loss of IP_3R-mediated Ca^{2+} release could also affect NO production and Ca^{2+} signals induced by shear stress.

In addition to the classic Ca^{2+}/calmodulin-dependent activation, eNOS can also be activated via phosphorylation at multiple sites.[52,53] The most extensively studied eNOS phosphorylation site is Ser1177, which is located in the reductase domain close to the carboxy-terminus. Phosphorylation of this site has been associated with eNOS activation in response to numerous stimuli, including mechanical factors, such as shear stress,[54] and humoral factors, such as bradykinin[55] and insulin.[56] Up to now, it has been proposed that kinases, such as 5' AMP-activated protein kinase, protein kinase B, protein kinase A, calmodulin-dependent protein kinase II, and protein kinase G, as well as phosphatase protein phosphatase 2A were able to regulate the phosphorylation of eNOS at Ser1177.[52] Overall, Ser1177 has been generally considered the most important of the regulatory eNOS phosphorylation sites. Phosphorylation of eNOS at Ser1177 increases eNOS activation mediated by Ca^{2+}/calmodulin binding, and it can also lead to activation of eNOS at resting levels of [Ca^{2+}]. Consistently, deficiency of IP_3R-mediated Ca^{2+} release in endothelial cells dramatically reduced acetylcholine-induced phosphorylation of eNOS at Ser1177. We assume that this could be, at least in part, a consequence of insufficient activation of Ca^{2+}/calmodulin-dependent protein kinase II because intracellular Ca^{2+} signals were almost eliminated in IP_3R-deficient endothelial cells. On the other hand, activity of eNOS is reduced by phosphorylation of eNOS at Thr495 in the Ca^{2+}/calmodulin binding domain.[57,58] Protein kinase C has been suggested to phosphorylate eNOS at Thr495,[59] whereas protein phosphatase 1, protein phosphatase 2A, and calcineurin dephosphorylate eNOS at Thr495.[52,53] However, dephosphorylation of eNOS at Thr495 induced by acetylcholine was not significantly altered in ECTKO arteries. Furthermore, in contrast to results reported by Yuan et al,[15] we found that protein levels of aortic eNOS were not significantly changed between control and ECTKO mice, suggesting that IP_3R-mediated Ca^{2+} release is not required for maintaining eNOS expression in endothelial cells.

Taken together, we demonstrated that IP_3R-mediated Ca^{2+} mobilization plays an essential role in regulating vascular dilation and basal blood pressure. In endothelial cells, all 3 IP_3R subtypes exist and may function redundantly. Deletion of all 3 IP_3R subtypes in mouse endothelial cells impaired acetylcholine-induced Ca^{2+} signals, eNOS phosphorylation at Ser1177, and vasodilation. Deficiency of all IP_3Rs also reduced plasma NOx levels and increased basal blood pressure. Considering that IP_3Rs have been strongly implicated in hypertension in humans, as revealed by genome-wide associated studies, our study provides mechanistic insight into the relationship between IP_3R function and human hypertension.

Acknowledgments

The authors would like to thank Marcus Fruttiger at UCL Institute of Ophthalmology (London, UK) for providing us the inducible platelet-derived growth factor receptor-β–Cre mice.

Author Contributions

Lin, Zhao, Jing, Li, Tang, Huang, Zhang, and Wang performed research; Ouyang, Fang, Liu, Jia, and Chen designed the research; Ouyang, Fang, Trexler, and Chen wrote the manuscript. Liu, and Jia provided materials.

Sources of Funding

The work was supported by the National Science Foundation of China (31370823, 91439130, 81700289, 31800767), the Guangdong Province Basic Research Foundation (2018A030310012), the Shenzhen Basic Research Foundation (KCYJ20160428154108239, KQJSCX20170330155020267, KCYJ20170818090044949, KQTD2015032709315529), and the National Institutes of Health (NIH) (Chen). Chen is the American Heart Association Endowed Chair in Research. Fang is also supported by NIH grant K99HL143210.

Disclosures

None.

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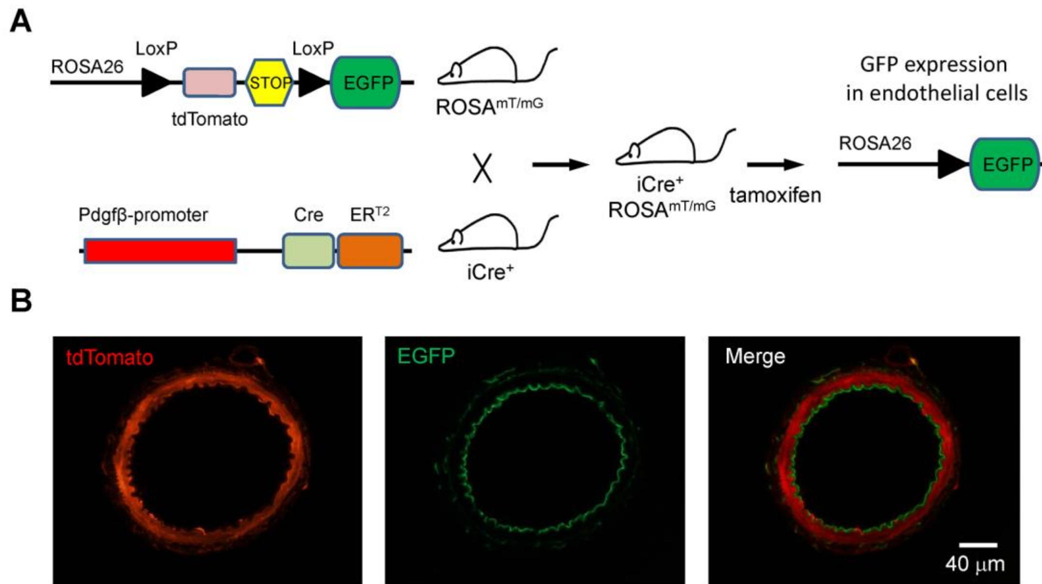
SUPPLEMENTAL MATERIAL

Table S1. primers sequences for RT-PCR.

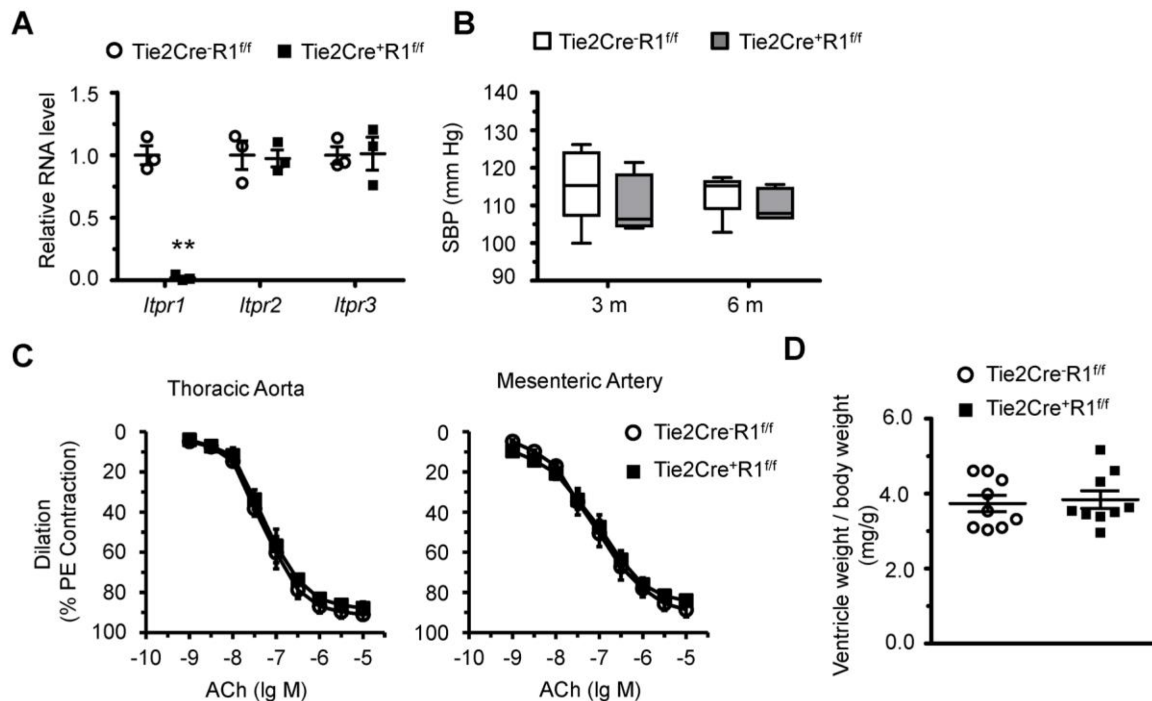
Gene	Forward primer（5'-3')	Reverse primer（5'-3')
Itpr1	CTCTGTATGCGGAGGGATCTAC	GCGGAGTATCGATTCATAGGAC
Itpr2	CTTCCTCTACATTGGGGACATC	GGCAGAGTATCGATTCATAGGG
Itpr3	AGCCAAGCAGACTAAACAGGAC	GCCGCTTGTTCACAGTTAAGTA
NOS3	TACGCACCCAGAGCTTTTCT	CTTGGTCAACCGAACGAAGT
CHRM1	TGACAGGCAACCTGCTGGTGCT	AATCATCAGAGCTGCCCTGCGG
CHRM2	CGGACCACAAAAATGGCAGGCAT	CCATCACCACCAGGCATGTTGTTGT
CHRM3	CCTCTTGAAGTGCTGCGTTCTGACC	TGCCAGGAAGCCAGTCAAGAATGC
CHRM4	TGTGGTGAGCAATGCCTCTGTCATG	GGCTTCATCAGAGGGCTCTTGAGGA
CHRM5	ACCACTGACATACCGAGCCAAGCG	TTCCCGTTGTTGAGGTGCTTCTACG
CHRNA7	GTAACCATGCGCCGTAGG	CCGAGGCTTGTGCTGAC
Gapdh	TGGCCTTCCGTGTTCCTAC	GAGTTGCTGTTGAAGTCGCA

Figure S1. Endothelial cell-specific gene deletion in adult mice by Pdgfb-iCreER.



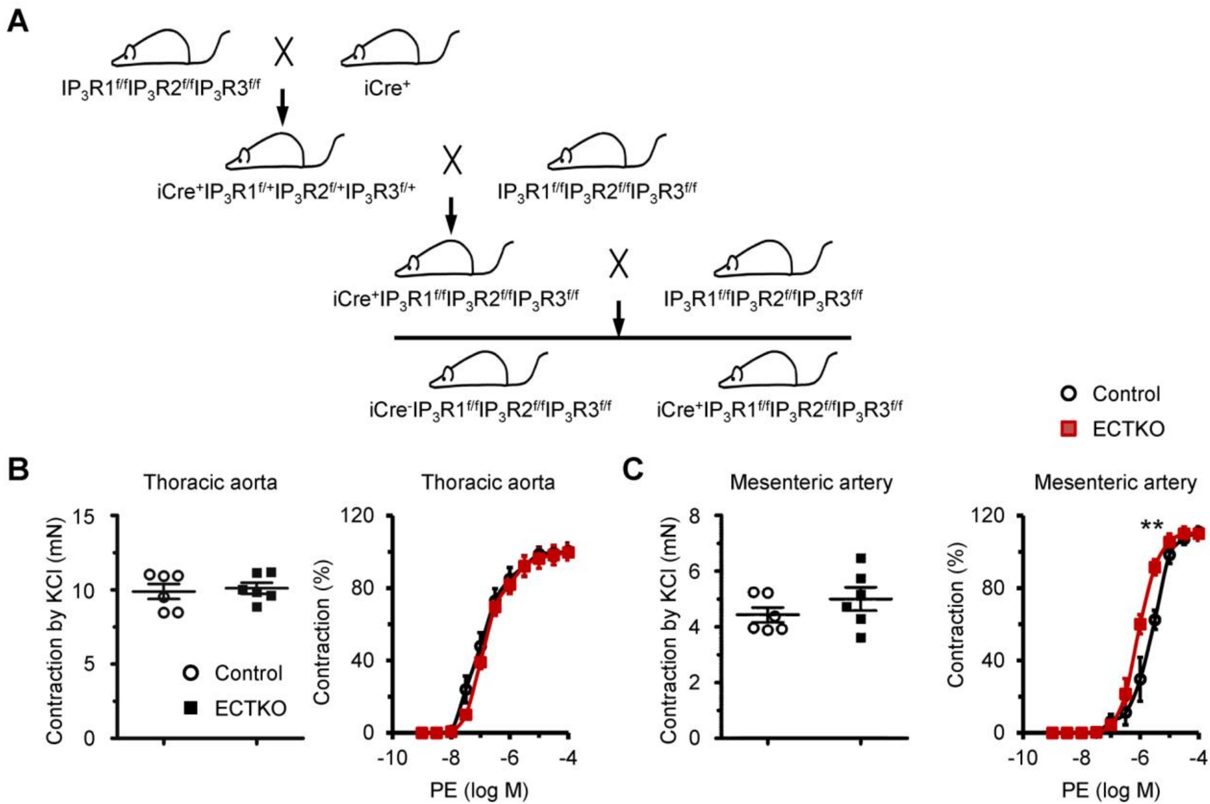
The Pdgfb-iCreER (iCre^{+}) mice were crossed with the ROSA$^{mT/mG}$ reporter mice to generate the iCre^{+}ROSA$^{mT/mG}$ mice. Administration of tamoxifen in adult iCre^{+}ROSA$^{mT/mG}$ mice led to expression of cell membrane-localized EGFP in endothelial cells, while non-endothelial cells expressed cell membrane-localized tdTomato. (**A**) Schematic diagram demonstrating the crossing strategy and the induction of EGFP expression. (**B**) Confocal fluorescent imaging showing the localization of EGFP and tdTomato in the cross-section of the mesenteric artery isolated from iCre^{+}ROSA$^{mT/mG}$ mice after tamoxifen administration. Scale bar, 40 μm.

Figure S2. Deletion of IP3R1 by Tie2-Cre in mice did not alter blood pressure and vasodilation.



The constitutively active Tie2-Cre instead of the inducible Pdgfb-iCreER was used to delete IP_3R1 in endothelial cells from as early as the embryonic stage. (**A**) Quantitative RT-PCR analysis of the expression of 3 IP_3R subtypes in isolated endothelial cells from Tie2-Cre$^-$R1$^{f/f}$ and Tie2-Cre$^+$R1$^{f/f}$ mice. n = 3 (with endothelial cells from 3 mice pooled as one sample) per group. Significance was determined by the 2-tailed, unpaired Student's t test. ***P* < 0.01 versus Tie2-Cre$^-$R1$^{f/f}$ mice. Data are presented as mean ± SEM. (**B**) Systolic blood pressure (SBP) were measured at the ages of 3 months and 6 months using the tail cuff system, respectively. n = 5-8 mice per group. Significance was determined by 2-way ANOVA analysis with Bonferroni post-hoc test. (**C**) Vascular reactivity in response to ACh in aortas and mesenteric arteries. The vessels were pre-constricted by 10 μM phenylephrine (PE) and the vasorelaxing effects of ACh were presented as a percentage of PE-induced contraction. n = 6 mice per group. Significance was determined by 2-way ANOVA analysis with Bonferroni post-hoc test. (**D**) The ratio of ventricle weight to body weight was comparable between Tie2-Cre$^-$R1$^{f/f}$ and Tie2-Cre$^+$R1$^{f/f}$ mice at the age of 6 months. n = 9 mice per group. Significance was determined by 2-tailed, unpaired Student's t test.

Figure S3. Mouse breeding strategy and measurement of vascular contractility in control and ECTKO mice.



(**A**) Schematic diagram showing the mouse breeding strategy to generate $iCre^+IP_3R1^{f/f}$ $IP_3R2^{f/f}$ $IP_3R3^{f/f}$ mice. (**B**) Reference contraction induced by high potassium (100mM) and the dose-dependent contractile response to phenylephrine (PE) in control and ECTKO aortas. n = 6 per group. (**C**) Reference contraction induced by high potassium (100mM) and the dose-dependent contractile response to phenylephrine (PE) in control and ECTKO mesenteric arteries. n = 6 per group. For all dose-response curves, data were expressed as a percentage of the peak of K^+-induced contraction, and significance was determined by 2-tailed, unpaired Student's t test or 2-way ANOVA analysis with Bonferroni post-hoc test. **P < 0.01 versus control. Error bars represent mean ± SEM.