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Supplementary Information for

# VSMC-specific EP4 deletion exacerbates angiotensin II-induced aortic dissection by increasing vascular inflammation and blood pressure

Hu Xu[1*], Shengnan Du[2*], Bingying Fang[1*], Chaojie Li[1*], Xiao Jia[3], Senfeng Zheng[1], Sailun Wang[1], Qingwei Li[1], Wen Su[4,5], Nanping Wang[1], Feng Zheng[1], Lihong Chen[1], Xiaoyan Zhang[1#], Jan-Ake Gustafsson[5, 6, #] and Youfei Guan[1#]

[1]Advanced Institute for Medical Sciences, Dalian Medical University, Dalian 11644, China.
[2]Department of Pharmacology, School of Basic Medical Sciences, Zhengzhou University, 100 Science Avenue, Zhengzhou 450001, China.
[3]Biobank, Peking University Third Hospital，Beijing 100191, China.
[4]Shenzhen University Medical Center, Department of Pathology, Shenzhen 518060, China.
[5]Center for Nuclear Receptors and Cell Signaling, University of Houston, 3013 Cullen Blv, 77204 Houston, Texas, USA.
[6]Center for Innovative Medicine, Department of Biochemistry and Nutrition, Karolinska Institutet, Huddinge, Sweden

* These authors contributed equally to this work.
# Corresponding Authors: Prof. Xiaoyan Zhang (wserien@163.com), Jan-Ake Gustafsson (jgustafs@central.uh.edu), or Youfei Guan (guanyf@dmu.edu.cn)

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Supplementary text;
Figs. S1 to S10;
Tables S1;
References for SI reference citations



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## Supplemental Materials and Methods

### AngII-infused hypertensive model and ultrasound imaging

Mice were treated with AngII dissolved in sterile saline at 1 μg per kg body weight (μg/kg) per minute using subcutaneous osmotic minipumps (Alzet Corp). After surgery, systolic blood pressure was recorded via the tail-cuff method as previously described (1), and sacrificed on the 28$^{th}$ day after surgery for further experiments.

For in vivo ultrasound images, the aortic diameter was monitored in isoflurane-anesthetized mice (2% isoflurane) by high-frequency ultrasound with a VEVO 3100 echography device (VisualSonics, Toronto, Canada) with 30-μm resolution. Maximal internal diameters of aortic images were measured using VEVO 3100 software (version 1.5.0). All recordings were made by a cardiologist and a technician who were blinded to animal genotype and treatment.

### Chemicals and Reagents

Primary antibodies against EP4 and EIf5 in the present study were purchased from Santa Cruz Biotechnology. MMP2, MMP9 and NOX1 antibodies were obtained from Abcam. α-SMA, SM22α, SMMHC antibodies were from Proteintech. The EP4 agonists prostaglandin E1 alcohol (PGE1-OH) and CAY10580 and antagonists L161,982 and MF498 were purchased from Cayman.

### Histology

After euthanization by CO2 inhalation, mouse aortas were perfused with saline. Aortas were then isolated and fixed in 4% formalin overnight at 4 °C. Paraffin cross-sections (4μm) from organs were stained with hematoxylin and eosin, EVG staining, Masson staining, and Sirius Red staining. Sections were also used for immunohistochemistry or immunofluorescence. Briefly, deparaffinized sections were rehydrated, boiled to retrieve antigens (10 mM citrate buffer, pH 6) and blocked for 45 min with 5% BSA in PBS. Samples were incubated with the following antibodies and reagents for immunohistochemistry or immunofluorescence: mouse anti-α-SMA, mouse anti- SM22α,

mouse-anti-SMMHC, rabbit anti-EP4, rabbit anti-F4/80 and dihydroethidium (DHE). Specificity was determined by substituting primary antibody with unrelated IgG (Santa Cruz). For immunohistochemistry, color was developed with DAB, and sections were stained with hematoxylin. For immunofluorescence, secondary antibodies were Alexa-Fluor-633-conjugated goat anti-rabbit antibodies. Sections were mounted with DAPI.

**Primary culture of VSMCs**

Mouse VSMCs were isolated from 8-12 weeks old EP4 flox/flox mice as described (2), and the cells at passages 3 to 7 were used in experiments. Rat VSMCs were isolated from the thoracic aortic arteries of Sprague-Dawley rats (150-180 g) as described previously (3). The purity of VSMCs was verified by anti-smooth muscle-actin antibody staining, and cells at passages 1 to 6 were used in all experiments.

**Western blot and real-time PCR**

Western blot was performed as described previously (4). Briefly, proteins from tissues or cells were extracted and quantified. Aliquots of 50 μg total protein or 10 μg membrane protein of each sample were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was blocked at room temperature for 1 h in TBS-T (Tris-buffered saline containing 0.1% Tween 20) containing 5% skimmed milk and was then incubated with primary antibodies at 4°C overnight. The membrane was washed for 5 min with TBS-T buffer for 3-5 times and then incubated with a horseradish peroxide-conjugated secondary antibody at a dilution 1:5,000 at room temperature for 1 h. After washing, the membrane was developed with ECL Reagent (Vigorous Biotechnology, Beijing) and exposed to Kodak XBT-1 film. The density of the signals was quantified with Image J (NIH) software.

Real-time PCR was performed as described previously (4). Briefly, total RNA was extracted, quantified, and reverse transcribed to cDNA. SYBR Green (Invitrogen, CA) was used as fluorochrome according to the manufacturer's instructions. Primers were designed from the known sequences of mouse genes listed in SI Appendix, Table S1. β-actin was used as an internal standard. The PCR reactions were 94°C for 5 min, and then 35 cycles

of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s, followed by extension at 72°C for 5 min. Target gene mRNA level was normalized to that of β-actin in the same sample using the 2-ΔCt method. Each sample was measured in triplicate in each experiment. Moreover, melting curves for each PCR product were analyzed to ensure the specificity of the amplification product.

**Blood pressure Measurement**

Male mice aged 6-8 months and with 28-33g body weight were used. Following a 2-week training period, blood pressure of mice at the basal level or fed with 7-day high salt diet was measured in conscious $EP4^{f/f}$ and VSMC-$EP4^{-/-}$ mice using the tail-cuff method as previously reported (1) . $EP4^{f/f}$ and VSMC-$EP4^{-/-}$ mice were also used in chronic AngII infusion experiments. For acute infusion studies, mice were anesthetized with 80mg/kg ketamine and 8mg/kg inactin i.p. and placed on a temperature controlled pad. After tracheostomy, PE-10 tubing was inserted into the right carotid artery, and blood pressure was measured with a Cobe CDX II transducer connected to a blood pressure analyzer (BPA 400; Micromed, Louisville, KY) as previously reported (5).

**Vascular Tension Assay of Mesenteric Arteries**

Mesenteric arteries were isolated from male $EP4^{f/f}$ and VSMC-$EP4^{-/-}$ mice after anesthetized with chloral hydrate (10%) i.p. and cut into rings of 1.0-1.3 mm long in ice-cold modified Krebs-Ringer bicarbonate buffer as previously reported (6). Each segment was suspended between two tungsten wires in chambers of a Multi Myograph System (610M, Danish Myo Technology A/S, Aarhus N, Denmark) for the measurement of isometric force. Each organ chamber was filled with 5ml of the modified Krebs-Ringer bicarbonate solution maintained at 37±0.5 ℃ and aerated with 95% O2-5% CO2 (pH = 7.4). At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension of 1mN for 30 minutes by step-wise stretching and contracted with 60mM KCl to test its contractility. Vessels were brought to their optimal resting tension, equilibrated for 30 minutes and a dose-response to AngII in vessels was determined after pretreating with L-NAME (0.1mM). A dose-response to phenylephrine in vessels was also determined after pretreating with/without L-NAME (0.1mM). To eliminate the

vasodilation responses of mesenteric resistance arteries, a dose-response to acetycholine was determined.

**$Ca^{2+}$ Imaging in Cultured VSMCs**

Rat and mouse VSMCs were loaded with the $Ca^{2+}$ indicator fluo-4 AM (2.5μmol/L, Molecular Probes) in Tyrode's solution for 10 minutes in the dark at 37°C. After washed to remove excess indicator, cells were imaged in $Ca^{2+}$ free Tyrode's solution at 23°C using a Leica SP8 Live inverted confocal microscope (Leica, Germany) equipped with a 40x oil immersion objective, at 90 frames/min, with the excitation laser at 488 nm and 505-530 nm emission as previously reported (6) .

**Statistical analysis.**

Data are presented as mean±SEM. Statistical analyses were performed using GraphPad Prism 5 software. Comparisons between 2 groups were analyzed by Student's t test except the time-course studies in the two genotypes. Comparison among groups in the time-course studies was made by repeated-measures ANOVA using SAS. $p<0.05$ was considered statistically significant.

# Supplemental Figures and tables

**Figure S1**

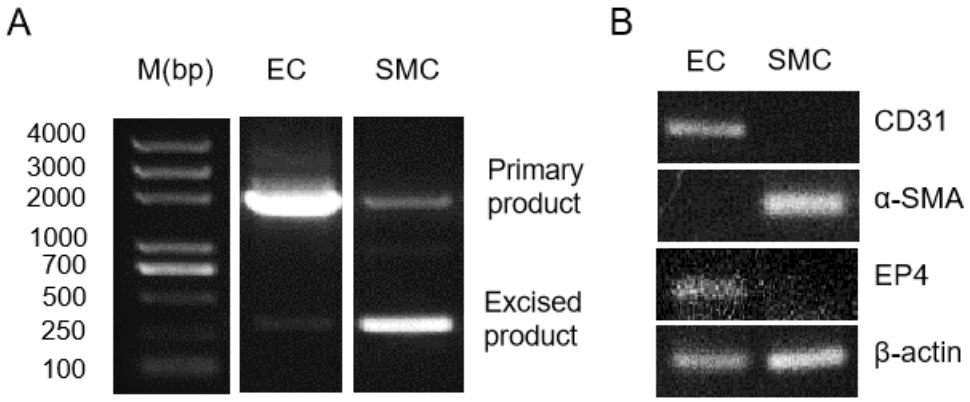


**Figure S1. Validation of EP4 deficiency in VSMCs.**

**A.** PCR analysis showing sufficient deletion of the exon 2 of EP4 gene in the aortic smooth muscle layer in VSMC-EP4-/- mouse. Little deletion of EP4 gene was evident in aortic endothelium layer. EC: endothelial cell; SMC: smooth muscle cell.

**B.** RT-PCR assay demonstrating the absence of EP4 mRNA in the aortic smooth muscle layer of VSMC-EP4-/- mouse.

**Figure S2**

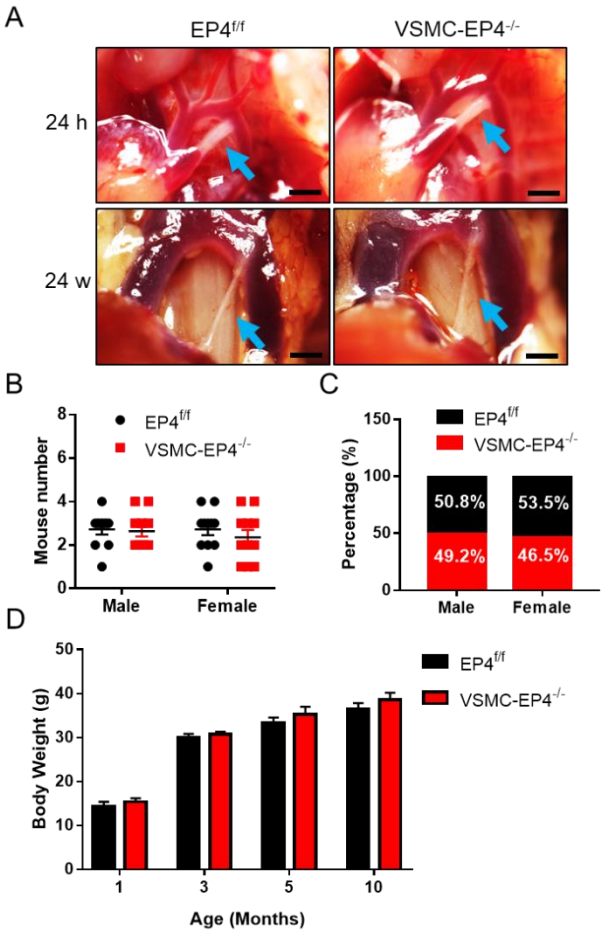


**Figure S2. VSMC-specific deletion of EP4 does not affect the closure of the ductus arteriosus, the survival and growth of mice.**

**A.** Photographic figures showing the ductus arteriosus of neonatal (24 hours after birth) and adult (24 weeks old) $EP4^{f/f}$ and $VSMC\text{-}EP4^{-/-}$ mice.

**B.** Gender difference in mice with $EP4^{f/f}$ and $VSMC\text{-}EP4^{-/-}$ genotypes. $EP4^{f/f}$ mice were crossed with $SMMHC\_Cre^{+}$ mice. Male and female offsprings with different genotypes were counted.

**C.** Genotypic difference in both male and female mice. The percentage of cre-positive ($VSMC\text{-}EP4^{-/-}$) and cre-negative ($EP4^{f/f}$) mice were calculated.

**D.** Body weight of male $EP4^{f/f}$ and $VSMC\text{-}EP4^{-/-}$ mice with different ages.

**Figure S3**

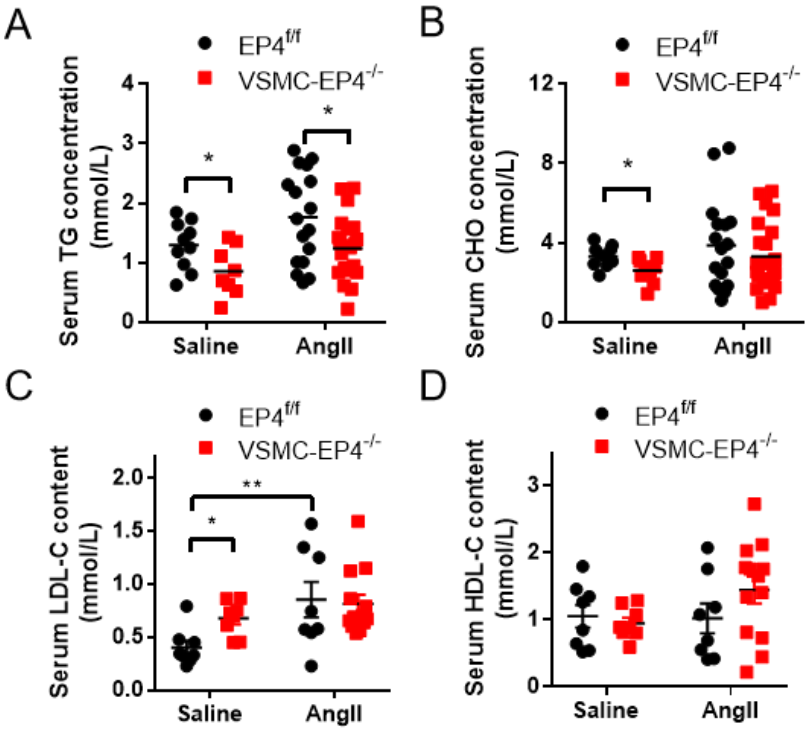


**Figure S3. Effect of AngII infusion on serum lipid profile in WT and VSMC-EP4$^{-/-}$ mice.**

**A&B**. Total serum triglycerides (TG) and cholesterol (Ch) concentrations in WT (EP4$^{f/f}$) and VSMC-EP4$^{-/-}$ mice following saline or AngII infusion for 28 days. n=8-22, *p<0.05.

**C&D**. Serum LDL-Ch and HDL-Ch levels of saline- or AngII-infused EP4$^{f/f}$ and VSMC-EP4$^{-/-}$ mice. n=8-13, *p<0.05, **p<0.01.

**Figure S4**

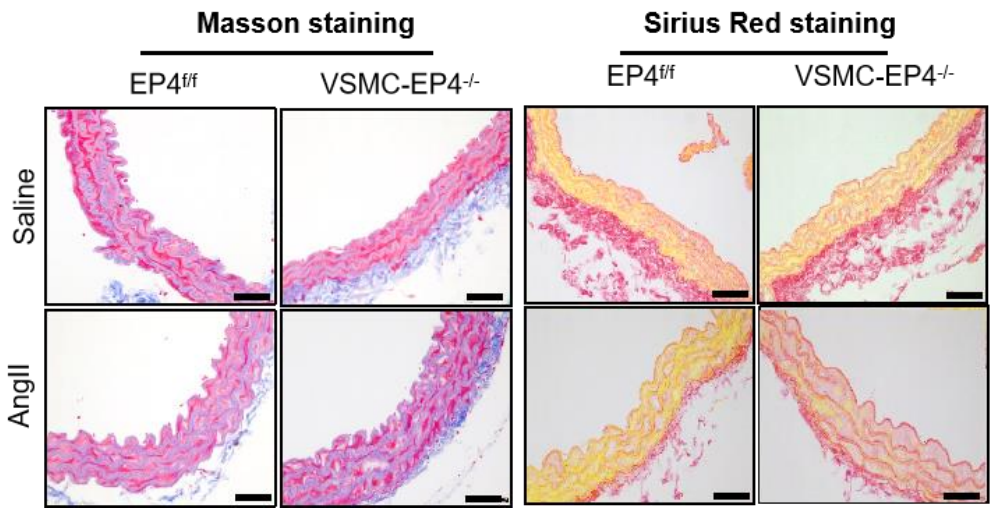


**Figure S4. No difference in aortic collagen content between EP4$^{f/f}$ and VSMC-EP4$^{-/-}$ mice.**

Representative staining of collagen in saline- and AngII-treated mice as assessed by the Masson and Sirius Red stains. Scale bars: 100μm.

**Figure S5**

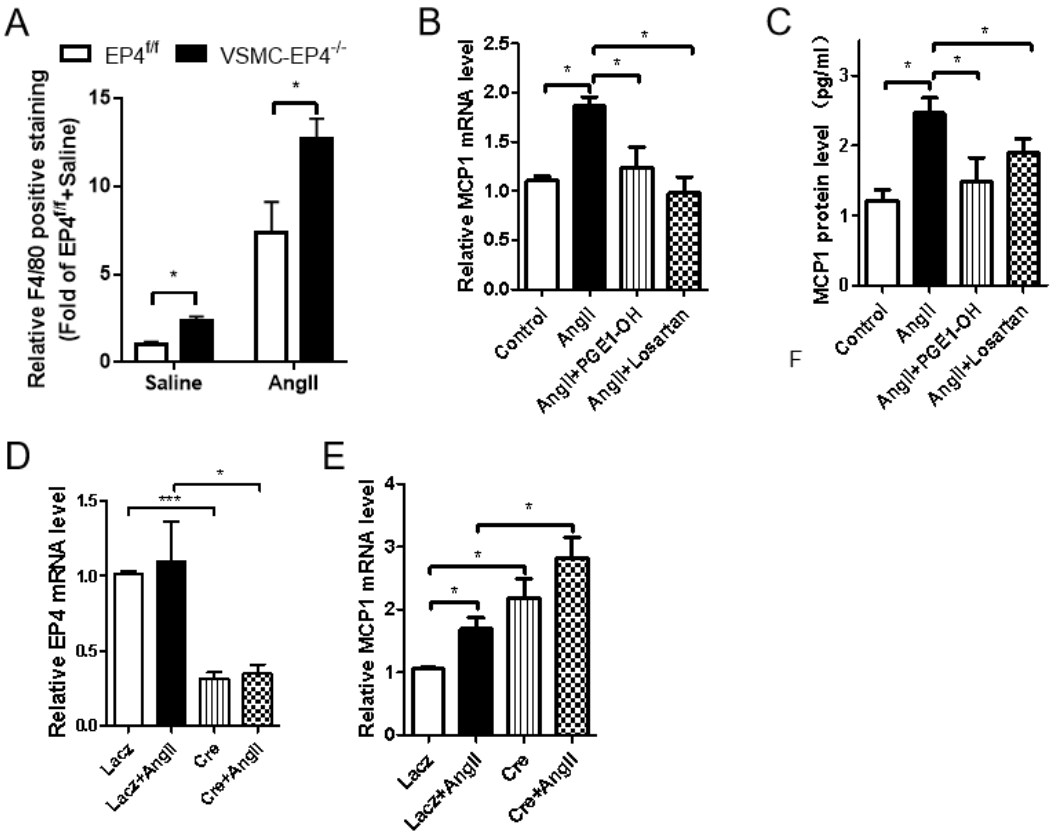


**Figure S5. EP4 suppresses MCP-1 expression in cultured VSMCs.**

**A.** Semi-quantitative analysis of aortic F4/80 expression in Figure 4B. n=3-4, *p<0.05.

**B&C.** Real-time PCR analysis (A) and enzyme-linked immunosorbent assay (ELISA) (B) of MCP-1 mRNA expression and protein secretion. Cultured wild-type VSMCs were pretreated with the EP4 agonist PGE1-OH (100 nM) or the AT1 antagonist losartan (1μM) for 2 hours, followed by AngII (100 nM) treatment for 24 hours. n=3, *p<0.05.

**D&E.** EP4 mRNA expression (C) and MCP-1 mRNA expression (D) in $EP4^{f/f}$ VSMCs infected with Cre adenovirus or Lacz adenovirus for 48 hours. n=3, *p<0.05.

**Figure S6**

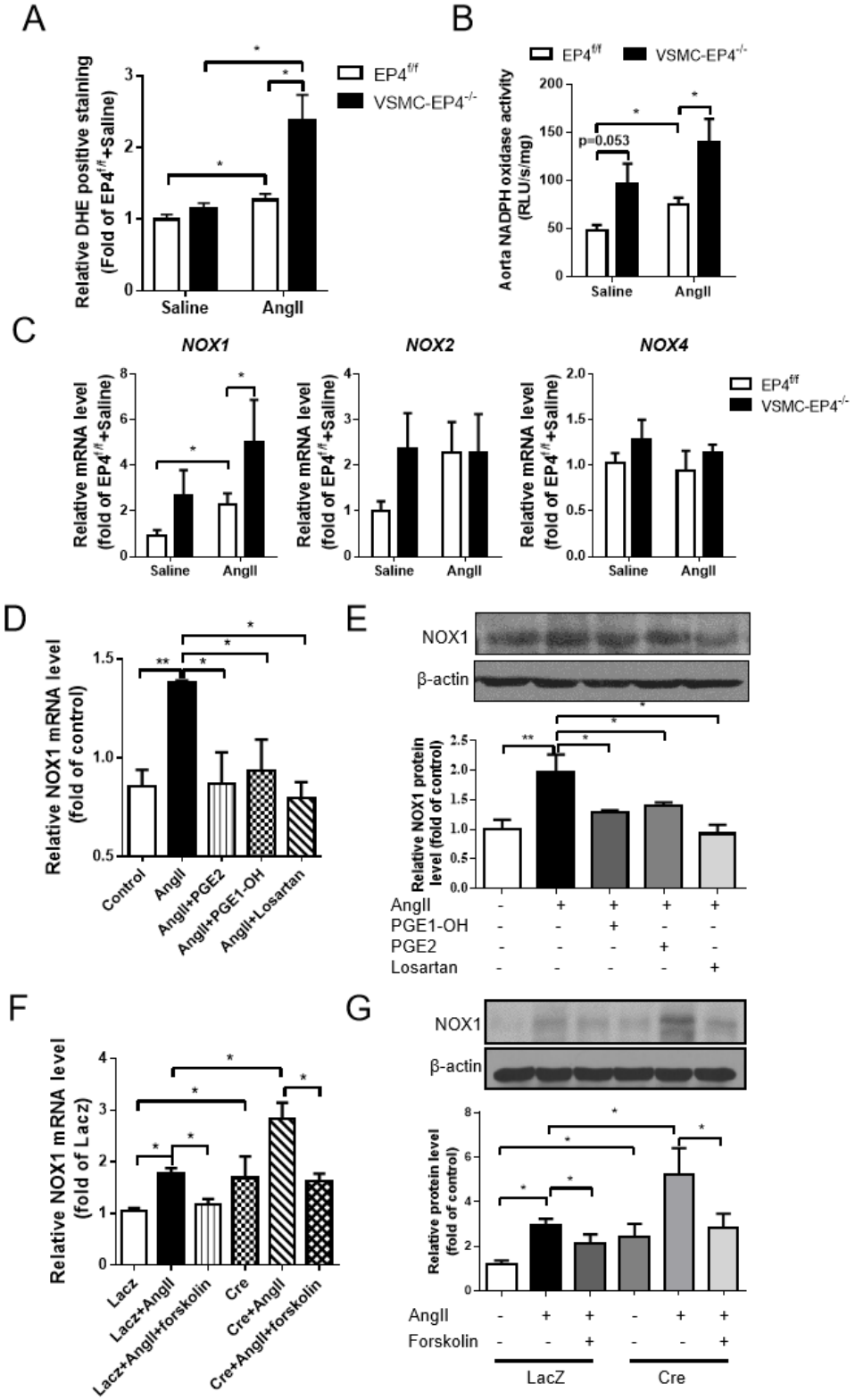
A
Relative DHE positive staining (Fold of EP4f/f+Saline)
EP4f/f
VSMC-EP4-/-
Saline
AngII
B
Aorta NADPH oxidase activity (RLU/s/mg)
p=0.053
C
NOX1
NOX2
NOX4
Relative mRNA level (fold of EP4f/f+Saline)
D
Relative NOX1 mRNA level (fold of control)
Control
AngII
AngII+PGE2
AngII+PGE1-OH
AngII+Losartan
E
NOX1
β-actin
Relative NOX1 protein level (fold of control)
AngII
PGE1-OH
PGE2
Losartan
F
Relative NOX1 mRNA level (fold of Lacz)
Lacz
Lacz+AngII
Lacz+AngII+forskolin
Cre
Cre+AngII
Cre+AngII+forskolin
G
NOX1
β-actin
Relative protein level (fold of control)
AngII
Forskolin
LacZ
Cre

**Figure S6. EP4 suppresses NOX activity in mice and NOX1 expression in cultured VSMCs.**

**A.** Semi-quantitative analysis of aortic superoxide anion levels in Figure 4C. n=3-4, *p<0.05.

**B.** Lucigenin chemiluminescence analysis for aortic NADPH oxidase activity in each treatment group. n=3, *p<0.05.

**C.** Real-time PCR analysis of mRNA levels of NOX1, NOX2 and NOX4 in the aortas of AngII-infused mice. n=7-9, *p<0.05.

**D&E.** Quantitative PCR (G) and Western blot (H) analysis of NOX1 expression in VSMCs treated with AngII (100nM) with or without PGE2 (1 μM), PGE1-OH (100 nM) and losartan (1 μM). n=3, *p<0.05, **p<0.01.

**F&G.** mRNA (I) and protein (J) expression of NOX1 in $EP4^{f/f}$ VSMCs infected with Cre adenovirus or LacZ adenovirus in the presence of AngII (100 nM) with or without forskolin (1 μM). n=4, *p<0.05.

**Figure S7**

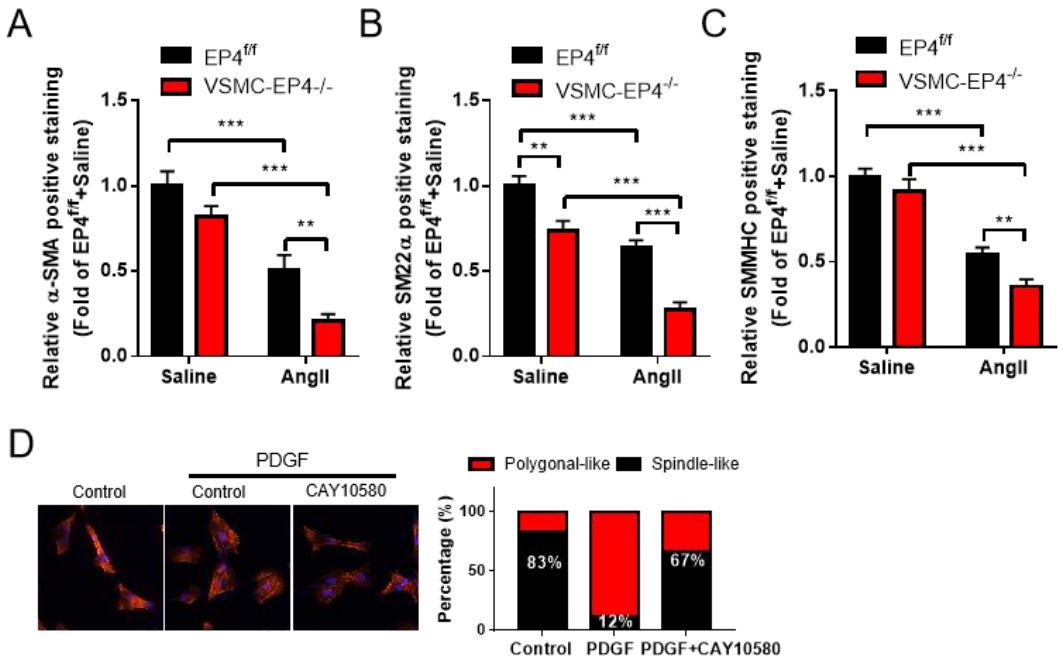


**Figure S7. EP4 is essential for maintaining VSMC contractile phenotype.**

**A-C.** Semi-quantitative analysis of α-SMA (A), SM22α (B) and SMMHC (C) in Figure 5A-C. n=7-14, **p<0.01, ***p<0.001.

**D.** Representative images of F-actin (red) distribution of the EP4 agonist CAY10580 (100 nM)-treated aortic VSMCs in the presence of PDGF-BB (25 ng/ml). DAPI staining was used to stain the nuclei (blue). The values were expressed as a percentage of spindle-like or polygonal-like cells.

**Figure S8**

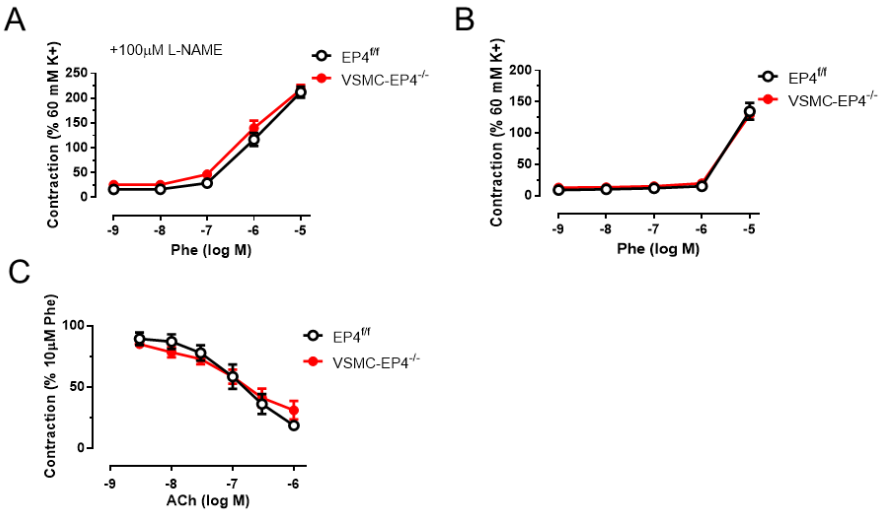


**Figure S8. No difference in phenylephrine (Phe)-evoked vasoconstriction and acetylcholine (Ach)-induced vasodilatation between EP4$^{f/f}$ and VSMC-EP4$^{-/-}$ mice.**

**A&B.** Phenylephrine (Phe)-evoked vasoconstriction of mesenteric arterial rings from EP4$^{f/f}$ and VSMC-EP$^{4-/-}$ mice. The rings were pretreated with or without L-NAME (100 μM) and then challenged with various doses of Phe. No difference was found between two genotypes, n=5-10.

**C.** Acetylcholine (Ach)-induced vasodilation responses of mesenteric arteries from EP4$^{f/f}$ and VSMC-EP4$^{-/-}$ mice. No difference was found between two genotypes, n=6.

**Figure S9**

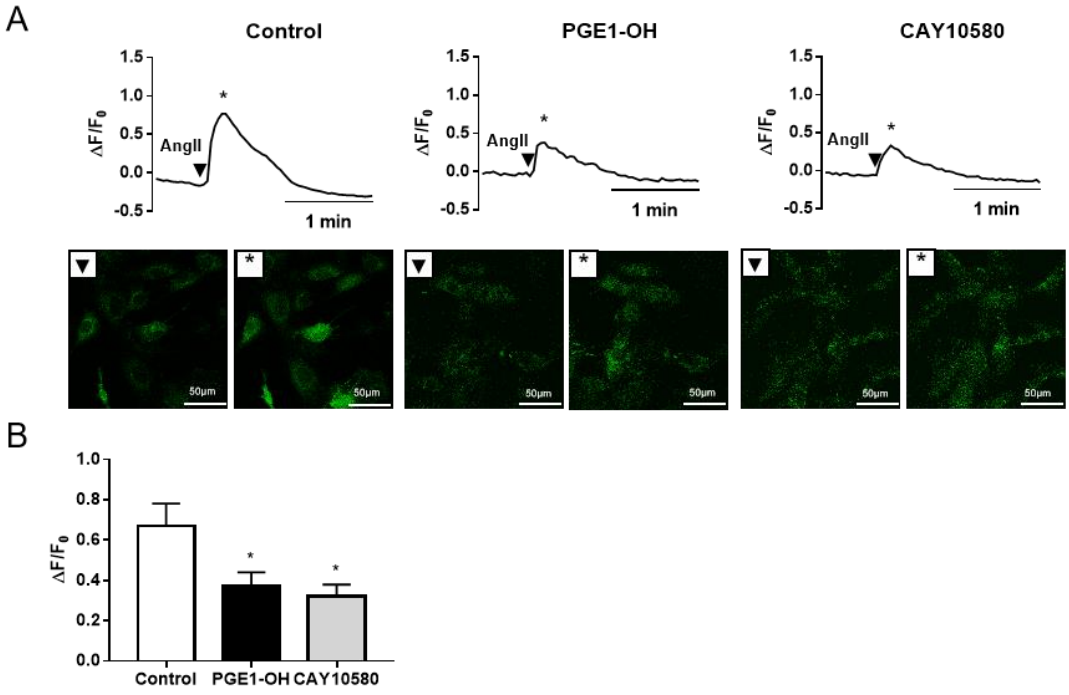


**Figure S9. Activation of EP4 suppresses AngII-induced increase in intracellular $Ca^{2+}$ levels.**

**A.** Representative images of AngII-induced intracellular $Ca^{2+}$ spark in VSMCs by the EP4 agonists. VSMCs were pre-incubated with the EP4 agonist PGE1-OH or CAY10580 for 30 minutes. ▼ represents $Ca^{2+}$ fluorescence images at baseline. * indicates images at the peak of induction.

**B.** Ratio of peak-to-basal change (ΔF/F0) in Fluo-4 AM fluorescence intensity in VSMCs treated with PGE1-OH or CAY10580. n=5-8, *p<0.05 *vs.* control.

**Figure S10**

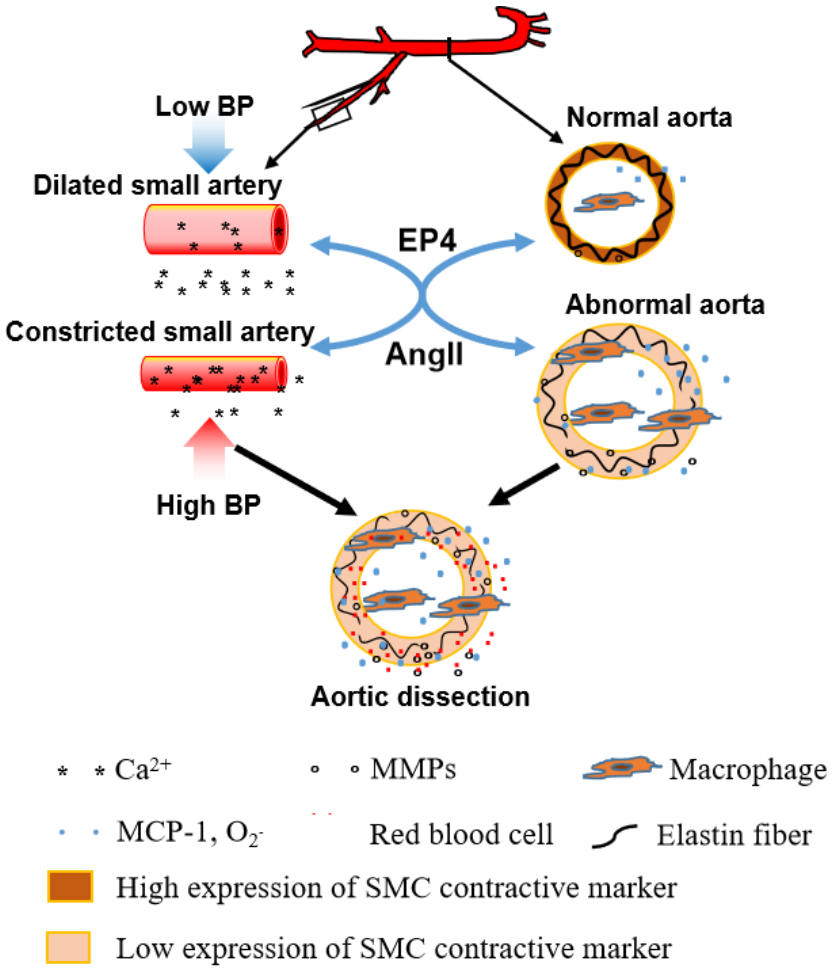


**Figure S10. Graphic illustration of the effect of VSMC-specific EP4 deficiency on aortic dissection.**

EP4 attenuates AngII-induced small artery vasocontraction and hypertension via reducing intracellular $Ca^{2+}$ concentration in VSMCs. VSMC-specific EP4 deletion sensitizes mice to AngII-induced hypertension and enhances AngII-elicited vascular inflammation, smooth muscle cell dedifferentiation, oxidative stress and MMP2/9 activity, leading to the occurrence of aortic dissection.

**Table S1.** Primer pairs used for amplifying mouse genes.

| Gene Name | Forward primer sequence (5′-3′) | Reverse primer sequence (5′-3′) |
|---|---|---|
| EP4 | ATGGTCATCTTACTCATCGCCAC | CCTTCACCACGTTTGGCTGAT |
| NOX1 | ACAACAGCACTCACCAATGC | TTTTGAGCTTGTTGTCGGCA |
| NOX2 | TTTGTCAAGTGCCCCAAGGT | GGCATCTTGGAACTCCTGCT |
| NOX4 | TGTGCCTTTATTGTGCGGAG | GCTGATACACTGGGGCAATG |
| MCP1 | AATGAGTAGCAGCAGGTGAGTG | GAAGCCAGCTCTCTCTTCCTC |
| MMP2 | GCACATCCTATGACAGCTGC | TTTGTTGCCCAGGAAAGTGA |
| MMP9 | GAGACTCTACACCCAGGACG | GAAAGTGAAGGGGAAGACGC |
| EP1 | TAACGATGGTCACGCGATGG | ATGCAGTAGTGGGCTTAGGG |
| EP2 | ATGCTCCTGCTGCTTATCGT | AGGGCCTCTTAGGCTACTGC |
| EP3 | GGATCATGTGTGTGCTGTCC | GCAGAACTTCCGAAGAAGGA |
| β-actin | CCCTGGAGAAGAGCTACGAG | CGTACAGGTCTTTGCGGATG |

Reaction temperature was 59°C for all genes.

## Supplementary References


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