Supplemental Material

Chemicals and Reagents

PGE2 and sulprostone were respectively purchased from Cayman Chemical (Ann Arbor, MI) and BioMol Research Laboratories (Plymouth Meeting, PA). M&B28767 was generously provided by M. Caton (Rhone-Poulenc Rorer, Dagenham, United Kingdom). The EP3 antagonist DG-041 was synthesized by the Vanderbilt Institute of Chemical Biology Chemical Synthesis Core. Cell culture media and all other related chemicals were purchased from Invitrogen (Carlsbad, CA). Ang II, phenylephrine (PE), sodium nitroprusside (SNP) and L798106 were obtained from Sigma-Aldrich (St. Louis, MO).

Analysis of Wild-type and Targeted EP3 Alleles

EP3+/+ and EP3-/- mice were kindly gifted by Dr. Richard M. Breyer. PCR-based strategies were used for genotyping wild-type and EP3 mutant alleles. Two pairs of primers were used to amplify a 152bp and 721bp genomic DNA fragment sparing the mutant site for wild type and EP3-/- mice, respectively (Fig. S1). The sequences of the primers were listed in table S1. PCR reactions were carried out at 94℃ for 30 seconds and 58℃ for 30 seconds then 72℃ for 30 seconds for 35 cycles for both wild-type and EP3 alleles. PCR products were separated on 1% agrose gel.

Primary Culture of VSMCs

VSMCs from Sprague-Dawley rats were cultured as previously reported 1. The thoracic aorta was dissected, vessels were cleared of fat in Dulbecco's modified Eagle's medium (DMEM), cut into 1- to 2-mm pieces and digested with collagenase in DMEM at 37℃ overnight. The cell suspension was centrifuged at 1000 xg for 10 min, and the pellet was resuspended in DMEM containing 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. The dispersed cells were incubated at 37℃ in a humidified 5% CO2/95% air atmosphere. Cells were subcultured in tissue culture dishes (100 mm diameter) containing 19 mm diameter type 0 glass coverslips, and the cells were grown to confluence. All studies were performed with cells between 6 and 12 passages. Six to twelve hours before the experiment, media was replaced with serum-free DMEM. On the day of the experiment, subconfluent cells were washed once with culture media and reincubated with fresh media containing sulprostone (1μmol/L, 10μmol/L) or DG-041 (0.1μmol/L, 1μmol/L, and 10μmol/L) for 30 mins.

Knockdown of Arhgef-1 expression in VSMCs

To knockdown rat VSMCs Arhgef-1 expression, three sets of siRNA against rat

Arhgef-1 cDNA coding sequence and a negative control (scramble) siRNA were synthesized by GenePharma Co., Ltd (No. 2723: sense, 5'GCCUAGAAGUGGUCAUCAATT 3', antisense, 5' UUGAUGACCACUUCUAGGCTT 3'; No. 1619: sense, 5' GCUCAUGGUUCCAGAAGAUTT 3', antisense, 5' AUCUUCUGGAACCAUGAGCTT 3'; No. 704: sense, 5' CCACCUAGAGGAAAUGCAATT 3', antisense, 5' UUGCAUUUCCUCUAGGUGGTT 3'). Transfections of siRNAs to VSMC were carried out with Lipofectamine 2000 as per manufacturer's instruction. In brief, cells were plated the day before transfection at a concentration of 5x105 cells per well in 6-well plates. The following day, cells were transfected with 40nM siRNA and incubated for additional 36h prior to harvesting. The mRNA and protein level of Arhgef-1 were monitored by Real-time PCR and Western blot, respectively.

RNA Extraction and Real-time PCR

Mesenteric artery segments and kidneys were quickly dissected and snap-frozen in liquid nitrogen. Vascular tissues were pooled from 3 animlas and total RNA was extracted by using Trizol reagent (Life Technologies). VSMCs were washed twice with PBS and 1ml Triol reagent were added into one well. Then the cells were scraped from well and pipetted into 1.5 ml EP tube. Total RNA was reverse-transcribed to cDNA by the use of RevertAidTM first strand cDNA synthesis kit (Fermentas) according to the manufacturer's protocol. Real-time PCR was performed with the use of iCycler with the SYBR Green I probe (Bio-rad, Hercules, CA). Each sample was analyzed in triplicate and normalized to the level of β-actin mRNA. The PCR protocol was 95℃ for 30 seconds, 59℃ to 60℃ for 30 seconds, and 72℃ for 30 seconds, for 40 cycles, followed by a final extension at 72℃ for 7 minutes. The primer sequences were listed in Supplemental Table S1. PCR products were validated by electrophoresis on 1.5% agrose gel.

MLC20 and MYPT1 Phosphorylation in Porcine Coronary Arteries

Left circumflex coronary arteries and left anterior descending coronary arteries of domestic pigs (4 months old, either sex) were obtained within 30 min of death from the local slaughterhouse in ice-cold oxygenated modified Krebs–Ringer bicarbonate solution. Arteries with external diameter ranging from 1.7 to 2.5 mm were dissected in ice-cold control buffer and used in the study. Vessel rings were incubated for 30 min in the presence of solvent or DG041 (1μmol/L) after equilibrated in serum-free Dulbecco's modified Eagle's medium for 30min at 37°C. Nitro-L-arginine (100μmol/L) was included in the medium. Then vessel rings were rapidly taken out and snappy frozen with liquid nitrogen immediately after stimulated by AngII

(1μmol/L) for 5min.

Tissue lysates prepared from the frozen vessels, each containing 20μg of protein, were subjected to SDS–PAGE and then transferred to PVDF membrane. Non-specific binding of antibody was blocked by washing with TBS buffer containing 10% milk for 1 h. The blots were then incubated with the primary antibody of phospho-MLC20 (1:1000 dilution), MLC20 (1:1000 dilution), MYPT1 (1:1000 dilution) or pMYPT1 (Thr696) (1:500 dilution) overnight and then the secondary antibody for 1h. The blots were visualized using the chemiluminescent detection method (Pierce). The levels of proteins present on the blots were quantified by densitometry using ImageJ (NIH) and normalized to the signals of β-actin (1:4000).

Supplemental Reference:

1. Gopalakrishnan V, Xu YJ, Sulakhe PV, Triggle CR, McNeill JR. Vasopressin (V1) receptor characteristics in rat aortic smooth muscle cells. *Am J Physiol.* 1991;261:H1927-1936.

Supplemental Figure Legends

Supplemental Figure I. Genotyping and validation of EP3-/- mice.
PCR genotyping of EP3+/+ and EP3-/- mice. Two pairs of primers were used for genotyping wild-type (152bp) and EP3-/- (721bp) alleles, respectively. B) RT-PCR analysis of mouse EP3α, EP3β, and EP3γ mRNA expression. Total RNA from kidneys and mesenteric arteries of wild type mice were used to detect EP3α, EP3β, and EP3γ variants and β-actin served as the loading control. C) RT-PCR analysis of EP3 mRNA and its subtype levels from kidneys of EP3+/+ and EP3-/- mice. D) Absence of the EP3 protein in the kidneys of EP3-/- mice, β-actin was used as the loading control.

Supplemental Figure II. Arhgef-1 protein expression and MLC20 and MYPT1 phosphorylation in EP3+/+ and EP3-/- mesenteric arteries.
Western blot analysis of Arhgef-1 protein expression in EP3+/+ and EP3-/- mesenteric arteries pooled from 3 animals in each group. EP3-/- mesenteric arteries showed markedly reduced Arhgef-1 protein expression. B) Western blot analysis of MLC and MYPT1 phosphorylation in EP3+/+ and EP3-/- mesenteric arteries pooled from 3 animals in each group with or without 5-min pretreatment of AngII (1μmol/L). AngII enhanced the phosphorylation of both MLC and MYPT1 in EP3+/+ mice, while a lack of EP3 diminished the effect. β-actin was used as the loading control.

Supplemental Figure III. Validation of Arhgef-1 antibody.
Rat VSMCs were transfected with three sets of Arhgef-1 siRNA (No. 2723, No. 1619 and No. 704) with the scramble siRNA as a control. Western blot (A) or Real-time PCR (B) was used to determine the protein and mRNA expression levels of Arhgef-1. A) Suppression of Arhgef-1 protein expression by three sets of Arhgef-1 siRNA. β-actin was used as the loading control. B) Arhgef-1 siRNAs reduced Arhgef-1 mRNA levels by 50%, with little effect on Arhgef-11 and Arhgef-12 expression. **P<0.01, siRNA vs. scramble. n=4 in each group.

Supplemental Figure IV. Effect of the EP3 receptor antagonist on AngII-induced MLC20 and MYPT1 phosphorylation in porcine coronary arteries.
The rings of porcine coronary arteries were stimulated by AngII (1μmol/L) for 5minutes after preincubated in DG041 (10μmol/L) for 30minutes. DG041 markedly inhibited AngII-induced phosphorylation of MLC20 and MYPT1. *P<0.05, **P<0.01, Control group vs. AngII alone; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001, AngII+DG041 vs. AngII alone. n=3 in each group.

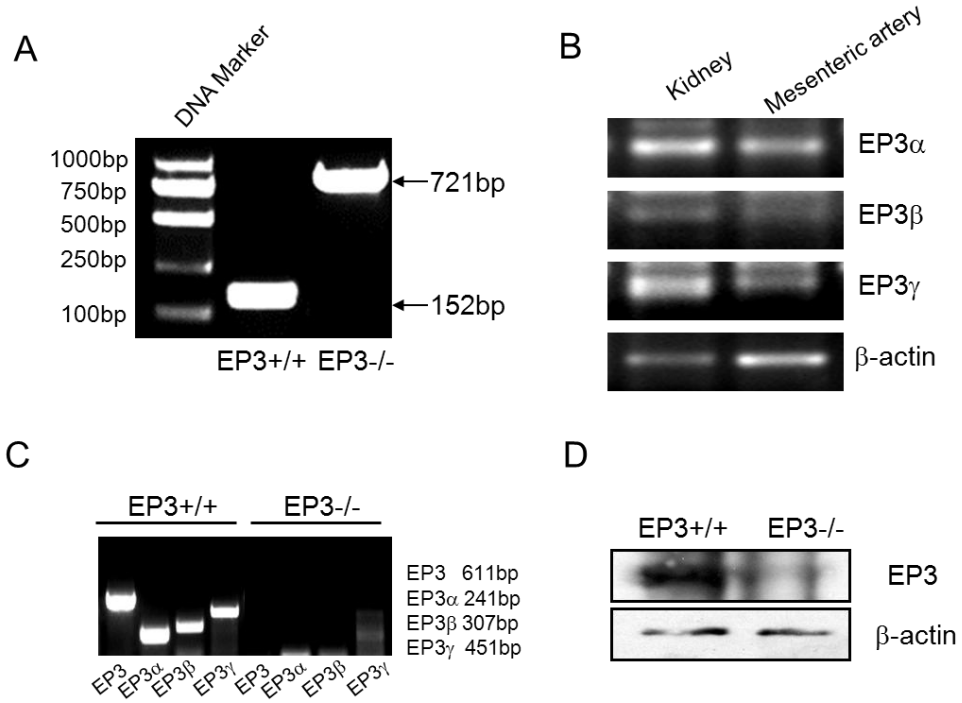
Supplemental Figure V. Effect of sulprostone and DG041treatment on intracellular Ca2+ levels in VSMCs.
A) Compared with AngII (1 mol/L), sulprostone alone only slightly increased intracellular Ca2+ levels. ***P<0.001, 10μmol/L sulprostone vs. 1μmol/L sulprostone.

n=10 in each group. B) Pretreatment of VSMCs with DG041 (10μmol/L) had little effect on phenylephrine (1 mol/L)-induced calcium signal. n=15 in each group.

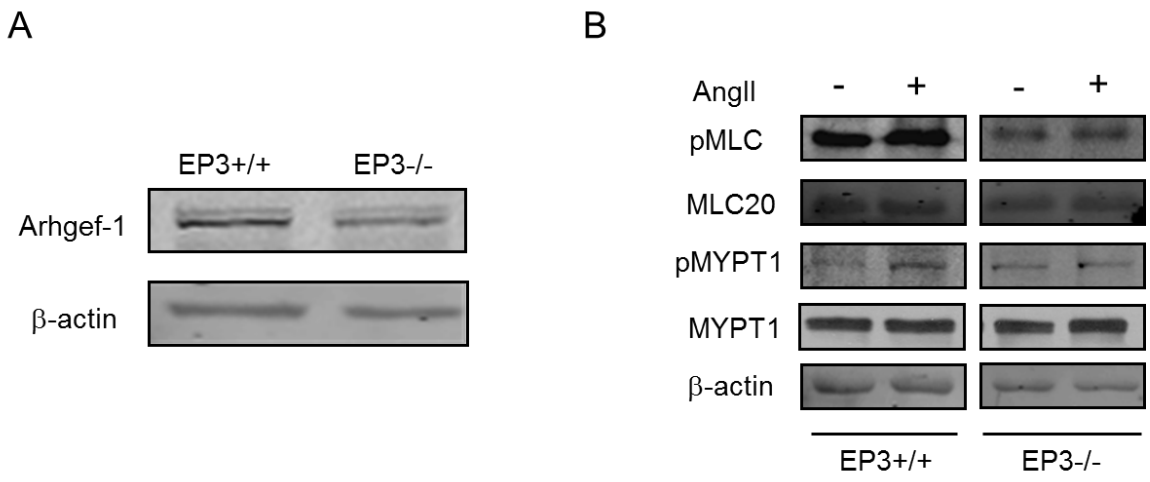
Supplemental Figure VI. The role of the EP3 receptor in AngII-induced vasoconstriction.

AngII evokes vasopressor response through both Gq/Ca2+/calmodulin-dependent activation of MLCK and G12/13/Arhger-1/RhoA/Rho Kinase-mediated phosphorylation of MYPT1. By modulating the levels of Ca2+, Arhgef-1 and phosphorylation of MYPT1/MLC, the EP3 receptor synergistically acts with AngII to contract VSMCs via Ca2+ signaling and Ca2+ sensitivity pathways .

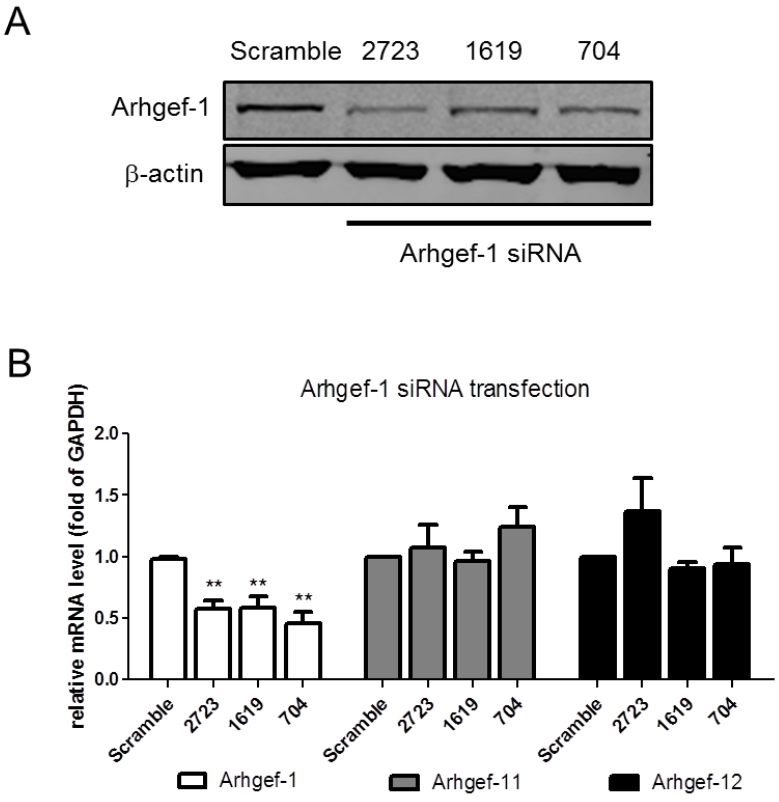
Supplemental Figure I.



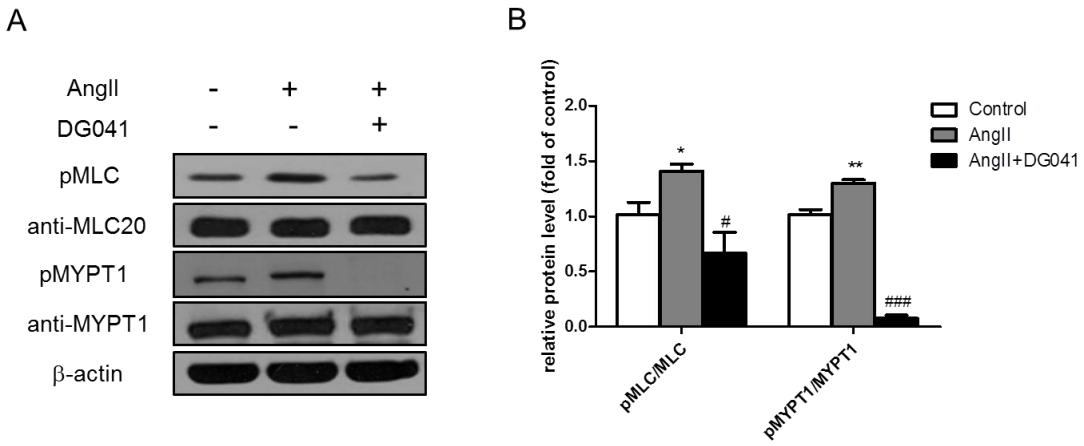
Supplemental Figure II.



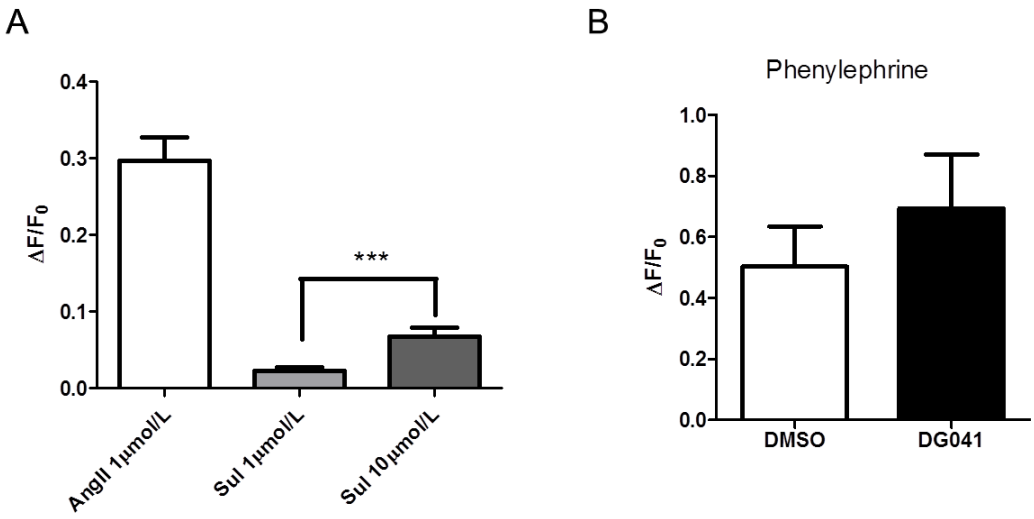
Supplemental Figure III.



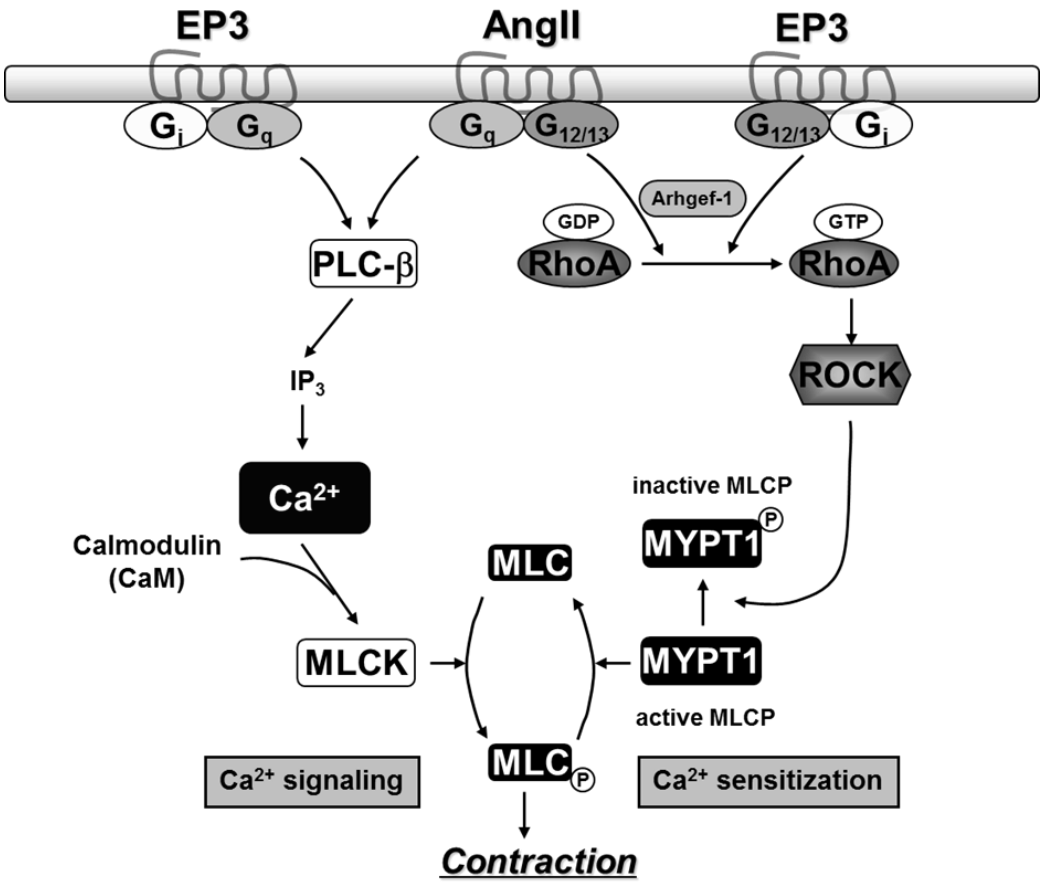
Supplemental Figure IV.



Supplemental Figure V.



Supplemental Figure VI.



Supplemental Table I. Sequence of primers for genotyping and RT-PCR analysis

For Genotyping

Name	Sequence	Product length(bp)
EP3+/+	Sense: 5’ GCTGTCTCCAGTTGTCTA 3’ Anti-sense: 5’ TGCCTCAGTCCATAAGGGTTAGGG 3’	152
EP3-/-	Sense: 5’ TGGCACAGAAAGGATTATCTA 3’ Anti-sense: 5’ AACGCTTGTCAAATGTTCAT 3’	721

For RT-PCR

Name NCBI No.	Sequence	Product length(bp)
EP3 NM_011196.2	Sense: 5’ ATCCTCGTGTACCTGTCACAGCGACGCTGG 3’ Anti-sense: 5’ TGCTCAACCG CATCTGATTGAAGATCATT 3’	611
EP3α D10204.1	Sense: 5’ GCTGTCCGTCTGTTGGTC 3’ Anti-sense: 5’ GGAGCTGGAAGCATAGTTG 3’	241
EP3β NM_011196.2	Sense: 5’ GGAAGTTCTGCCAGATGAT 3’ Anti-sense: 5’ GGTTCTGAGGCTGGAGATA 3’	307
EP3γ D17406.1	Sense: 5’ AACGCTGTCTCCAGTTGC 3’ Anti-sense: 5’ TGTGGCTTCATTCCTTGC 3’	451
Arhgef-1 NM_021694.3	Sense: 5’ GCCCCAAAGAAGCCAAGAA 3’ Anti-sense: 5’ ACCTCTTGTATGAACCGCCTCT 3’	163
Arhgef-11 NM_023982.1	Sense: 5’ GGGACCCTCTTCGAGAACGCCAAA 3’ Anti-sense: 5’ GGGCAGCCACTTGTCCTTGTCAGG 3’	209
Arhgef-12 NM_001013246.1	Sense: 5’ GCATGGAAGTATTTTGAACCGA 3’ Anti-sense: 5’ CCCGAATCCATTGTCATCTTT 3’	193
β-actin NM_007393	Sense: 5’ AGCCATGTACGTAGCCATCC 3’ Anti-sense: 5’ GCTGTGGTGGTGAAGCTGTA 3’	222