Inactivation of the E-Prostanoid 3 Receptor Attenuates the Angiotensin II Pressor Response via Decreasing Arterial Contractility

Lihong Chen,* Yifei Miao,* Yahua Zhang,* Dou Dou, Limei Liu, Xiaoyu Tian, Guangrui Yang, Dan Pu, Xiaoyan Zhang, Jihong Kang, Yuansheng Gao, Shiqiang Wang, Matthew D. Breyer, Nanping Wang, Yi Zhu, Yu Huang, Richard M.Breyer, Youfei Guan

Objective—The present studies aimed at elucidating the role of prostaglandin E₂ receptor subtype 3 (E-prostanoid [EP] 3) in regulating blood pressure.

Methods and Results—Mice bearing a genetic disruption of the EP3 gene (EP₃−/−) exhibited reduced baseline mean arterial pressure monitored by both tail-cuff and carotid arterial catheterization. The pressor responses induced by EP3 agonists M&B28767 and sulprostone were markedly attenuated in EP₃−/− mice, whereas the reduction of blood pressure induced by prostaglandin E₂ was comparable in both genotypes. Vasopressor effect of acute or chronic infusion of angiotensin II (Ang II) was attenuated in EP₃−/− mice. Ang II-induced vasoconstriction in mesenteric arteries decreased in EP₃−/− group. In mesenteric arteries from wild-type mice, Ang II-induced vasoconstriction was inhibited by EP3 selective antagonist DG-041 or L798106. The expression of Arhgef-1 is attenuated in EP3 deficient mesenteric arteries. EP3 antagonist DG-041 diminished Ang II-induced phosphorylation of myosin light chain 20 and myosin phosphatase target subunit 1 in isolated mesenteric arteries. Furthermore, in vascular smooth muscle cells, Ang II–induced intracellular Ca²⁺ increase was potentiated by EP3 agonist sulprostone but inhibited by DG-041.

Conclusion—Activation of the EP3 receptor raises baseline blood pressure and contributes to Ang II–dependent hypertension at least partially via enhancing Ca²⁺ sensitivity and intracellular calcium concentration in vascular smooth muscle cells. Selective targeting of the EP3 receptor may represent a potential therapeutic target for the treatment of hypertension. (Arterioscler Thromb Vasc Biol. 2012;32:3024-3032.)

Key Words: angiotensin II □ calcium □ E-prostanoid 3 □ hypertension □ vasoconstriction

Hypertension is a major cardiovascular risk factor and a prominent worldwide health challenge. Prostaglandins are endogenous oxygenated fatty acid metabolites and play important roles in modulating arterial blood pressure (BP) and renal salt excretion.1-3 Prostaglandin E₂ (PGE₂) is a major prostanoïd synthesized in mouse kidney and vasculature4 and is unique among the prostanoïds in that it can activate 4 distinct G protein–coupled membrane receptors—designated E-prostanoid (EP) receptor 1 (EP1), EP2, EP3, and EP4. EP receptors exhibit characteristic tissue distribution1,4 and evoke receptor-specific signaling pathways.5 The EP1 and EP3 receptors were initially defined as involved in vasoconstriction, whereas the EP2 and EP4 receptors participate in the relaxation of vascular smooth muscle.7,9

At the cellular level, EP1 receptor activation leads to increased intracellular Ca²⁺,10,11 whereas EP3 receptor signals through a number of pathways, including activation of the inhibitory G protein, lead to a decrease in intracellular cAMP concentration as well as an increase in intracellular calcium.12-16 Moreover, EP3 receptor could activate G₁₃ protein, leading to the activation of the small GTPase Rho.17 The EP2 and EP4 receptors couple to the stimulatory G protein, leading to receptor-evoked increase of intracellular cAMP concentration.7,23 The multiple functionally antagonistic signaling pathways evoked by PGE₂ suggest that the 4 EP receptors may act in concert to maintain BP homeostasis.

An important role for EP1 receptors in modulating angiotensin II (Ang II) effects on systemic BP was revealed in our previous study, which showed that EP1 null mice exhibited reduced BP and a blunted vasopressor response to acute and chronic infusion of Ang II.18 Disruption of the EP2 receptor through a number of pathways, including activation of the inhibitory G protein, lead to a decrease in intracellular cAMP concentration as well as an increase in intracellular calcium.12-16 Moreover, EP3 receptor could activate G₁₃ protein, leading to the activation of the small GTPase Rho.17 The EP2 and EP4 receptors couple to the stimulatory G protein, leading to receptor-evoked increase of intracellular cAMP concentration.7,23 The multiple functionally antagonistic signaling pathways evoked by PGE₂ suggest that the 4 EP receptors may act in concert to maintain BP homeostasis.

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From the Department of Physiology and Pathophysiology, Peking University Health Science Center, Beijing, China (L.C., Y.M., D.D., L.L., G.Y., D.P., X.Z., J.K., Y.G., Y.G.); Key Laboratory of Cardiovascular Science of the Ministry of Education, Beijing, China (L.C., Y.M., G.Y., D.P., X.Z., J.K., N.W., Y.Z., Y.G.); Department of Medicine, Division of Nephrology and Hypertension, Vanderbilt University Medical Center, Nashville, TN (Y.Z., M.D.B., R.M.B.); Institute of Vascular Medicine, Li Ka Shing Institute of Health Sciences, School of Biomedical Sciences, Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China (X.T., Y.H.); and Institute of Life Science, Peking University, Beijing, China (S.W.).

*These authors contributed equally to this work.

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Correspondence to Youfei Guan, MD, PhD, Department of Physiology and Pathophysiology, Peking University Health Science Center, 38 Xueyuan Rd, Haidian District, Beijing, China 100191. E-mail youfeiguan@bjmu.edu.cn

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unmasked the pressor response to PGE₂ and was susceptible to salt-sensitive hypertension. In addition, our previous study demonstrated EP2 receptor played an important role in neointimal hyperplasia after arterial injury. PGE₂ may also induce vascular relaxation by EP4 receptor-mediated activation of endothelial NO synthase, consistent with the observed vasodilator effect of the EP4 agonist PGE₃-OH in wild-type mice. However, the role of EP3 receptor in regulating vascular tone and BP is only partially characterized. In the present studies, we used a mouse with targeted disruption of the EP3 gene to determine the role of the EP3 receptor in systemic hemodynamics and to examine the response to Ang II–induced hypertension. We found that EP3 gene disruption resulted in reduced basal BP and attenuated Ang II–evoked hypertension.

Methods

Experimental Animals and Reagents

EP3+/+ and EP3−/− mice were kindly gifted by Dr Richard M. Breyer. Male EP3−/− mice and sex- and age-matched EP3+/- littersates (12–16 weeks) on a pure C57BL/6 background were used in all studies. The detailed methods for genotyping those mice are shown in the online-only Data Supplement data (Figure I in the online-only Data Supplement). The study protocols and the use of the animals were reviewed and approved by the Animal Care and Use Review Committee of Peking University Health Science Center. The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health publication no. 85-23, revised 1996). All chemicals and reagents used are listed in the online-only Data Supplement data.

BP Measurement

Male congenic C57BL/6 mice aged 12 to 16 weeks and with 20 to 25 g body weight were used. After a 2-week training period, basal BP was measured in conscious EP3+/+ and EP3−/− mice using the tail-cuff method as previously reported. These mice were then used in chronic Ang II infusion experiments. For acute infusion studies, mice were anesthetized with 80 mg/kg ketamine and 8 mg/kg inactin intraperitoneally and placed on a temperature-controlled pad. After tracheostomy, PE-10 tubing was inserted into the right carotid artery, and BP was measured with a Cobe CDX II transducer connected to a computerized data recorder. Briefly, Ang II (1000 ng/kg per minute) was infused for 30 to 60 minutes until stable values were obtained, and then 100 μL of test agents (sulprostone, M&B28767, PGE₃, phenylephrine (PhE), or sodium nitroprusside) dissolved in saline were injected as a bolus via the jugular vein. For Ang II studies, Ang II dissolved in normal saline was continuously infused via the jugular vein at a rate of 75 pmol/kg per minute for 30 minutes. BP and heart rates were recorded continuously on a thermal printer or as a computerized data record.

Ang II-Dependent Chronic Hypertension

Chronic Ang II–dependent hypertension was induced in EP3+/+ and EP3−/− mice as previously described. Briefly, Ang II (1000 ng/kg per minute) dissolved in sterile saline was infused using an osmotic mini-pump (Alzet model 2004; Alza Corp) inserted subcutaneously during chloral hydrate anesthesia (10%). After 3 days of full recovery from the surgery, systolic BP (SBP) and heart rates were recorded every other day for 28 days via computerized tail-cuff method as previously reported. Mice were placed in individual mouse metabolic cages (Tecniplast, Agugliate, Italy) with free access to water and food, and at Day 0, 14, 28, urine output and drinking volume were collected and calculated.

Mesenteric Arterial Vascular Tension

Mesenteric arteries were dissected from male EP3+/+ and EP3−/− mice after anesthetized with chloral hydrate (10%) intraperitoneally and cut into rings of 1.0 to 1.3 mm long in ice-cold modified Krebs-Ringer bicarbonate buffer. Each segment was suspended between 2 tungsten wires in chambers of a Multi Myograph System (610M, Danish Myo Technology AS, Aarhus N, Denmark) for the measurement of isometric force. Each organ chamber was filled with 5 mL of the modified Krebs-Ringer bicarbonate solution maintained at 37±0.5°C 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 1 mN for 30 minutes by stepwise stretching and contracted with 60 mmol/L KCl to test its contractility. Vessels were brought to their optimal resting tension, equilibrated for 30 minutes, and a dose-response to Ang II in vessels was determined. To eliminate the possible involvement of endothelium-derived NO, nitro-L-arginine (100 μmol/L) was included in the medium and present throughout the experiments.

Ca²⁺ Imaging in Cultured Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMCs) were loaded with the Ca²⁺ indicator fluo-4 acetoxymethyl ester (2.5 μmol/L, Molecular Probes) in Tyrode’s solution for 5 minutes in the dark at 37°C. After being washed to remove excess indicator, cells were imaged in Tyrode’s solution at 23°C using a Zeiss LSM5 Live inverted confocal microscope (Carl Zeiss, Germany) equipped with a ×40 oil immersion objective, at 90 frames/min, with the excitation laser at 488 nm and >505 nm emission.

Myosin Light Chain 20 and Myosin Phosphatase Target Subunit 1 Phosphorylation

Mesenteric arteries were dissected from male mice and rats after being anesthetized with chloral hydrate (10%) intraperitoneally and cut into rings in ice-cold modified Krebs-Ringer bicarbonate buffer. Vessel rings were incubated for 30 minutes in the presence of solvent or DG-041 (1 μmol/L) after being equilibrated in serum-free DMEM for 30 minutes at 37°C. Nitro-L-arginine (100 μmol/L) was included in the medium. Then vessel rings were rapidly taken out and snappy frozen with liquid nitrogen immediately after stimulated by Ang II (1 μmol/L) for 5 minutes. Tissue lysates prepared from the frozen vessels, each containing 20 μg of protein, were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride membrane. Nonspecific binding of antibody was blocked by washing with Tris-buffered saline buffer containing 10% milk for 1 hour. The blot was then incubated with the first antibody of phospho-myosin light chain 20 and myosin phosphatase target subunit 1 (MYPT1; 1:1000 dilution), phospho-MYPT1 (Thr696; 1:500 dilution), Rho guanine nucleotide exchange factor (GEF) p115/Lac (also known as Arhgef-1; 1:500 dilution), respectively, overnight and the secondary antibody for 1 hour. It was developed using the chemiluminescent detection method (Pierce). The protein present on blots was quantified by densitometry using Image J (National Institutes of Health) and normalized to β-actin (1:4000).

Statistical Analysis

Prism (GraphPad, Software Inc, La Jolla, CA) was used for statistical analysis except in the case of chronic Ang II infusion (see below). The results were expressed as means±SE. Data were evaluated by 2-sided Student t test. A P<0.05 was required to reject the null hypothesis. The effect of chronic Ang II treatment on EP3+/+ and EP3−/− animals was assessed by comparing the change in SBP from baseline out to 28 days using general linear models with bootstrap covariance accounting for correlation among repeated measures within each mouse, in which the baseline SBP was adjusted as a covariate. Residuals were assessed graphically for normality. These analyses were performed with R-software version 2.9.1 (www.r-project.org), and a 2-sided P<0.05 was required to reject the null hypothesis.
Results

Disruption of the EP3 Receptor Gene Decreases Basal BP

Mean arterial pressure (MAP) was measured in anesthetized EP3+/+ and EP3−/− mice. As shown in Figure 1A, baseline MAP was significantly decreased in EP3−/− mice (EP3+/+ 83.7±1.7 mm Hg versus EP3−/− mice 94.0±2.1 mm Hg). Consistent result was observed in conscious state that EP3+/+ mice showed reduced SBP (101.1±1.1 mm Hg) compared with EP3+/+ mice (105.5±1.6 mm Hg; Figure 1B).

Deletion of the EP3 Receptor Blunts the Pressor Response to Ang II

Acute infusion of the pressor Ang II increased MAP in EP3+/+ mice, which was attenuated in EP3−/− mice. Peak increase in MAP was 90.4±3.0 mm Hg in EP3+/+ mice versus 131.3±3.9 mm Hg in EP3−/− mice (data not shown). The net change of MAP in EP3+/+ mice on Ang II infusion was also markedly reduced in EP3+/+ mice (10.5±2.0 mm Hg) versus EP3−/− mice (42.1±1.2 mm Hg; Figure 2A).

A similar effect was observed on chronic Ang II infusion in EP3−/− mice. As shown in Figure 2B, chronic Ang II infusion increased SBP in both genotypes, whereas compared with EP3+/+ mice, the EP3−/− mice had lower SBP without change in heart rate (Figure 2C) over time after treatment. Cardiac hypertrophy, as assessed by the heart to body weight ratio, was similar between EP3+/+ and EP3−/− mice (Figure 2D). Over the 28-day Ang II infusion, average SBP increased to 154.4±3.1 mm Hg in the EP3+/+ group but only to 138.9±2.2 mm Hg in the EP3−/− group (Figure 2B). The net increase was ≈14 mm Hg lower in EP3−/− mice (38.6±2.2 mm Hg) compared with EP3+/+ mice (52.3±3.1 mm Hg; data not shown). During the infusion of Ang II, the increase in SBP was associated with increased urine output and urinary Na+ excretion, but no difference was observed between the EP3+/+ and EP3−/− groups (Figure 2E and 2F).

EP3−/− Mice Display a Loss of Vascular Reactivity to EP3 Agonists

The effect of the EP3 gene disruption on vascular reactivity was assessed by infusion of the EP3 receptor–selective agonist M&B28767. The pressor response to M&B28767 was markedly reduced in EP3−/− mice, increasing MAP by 9.1±1.2 mm Hg versus 35.7±3.4 mm Hg in EP3+/+ littermates (Figure 3A). Similarly, infusion of the dual EP1/3 agonist sulprostone exhibited clear pressor activity in EP3+/+ mice (28.1±3.4 mm Hg), which was significantly diminished in EP3−/− mice (5.2±1.4 mm Hg; Figure 3B). Infusion of the endogenous ligand PGE2 resulted in an expected depressor effect, which was not different in magnitude between the EP3−/− and EP3+/+ groups; however, the EP3−/− mice appeared to have a more prolonged reduction in MAP as compared with EP3+/+ mice (Figure 3C).

EP3−/− Mice Exhibit Intact Hemodynamic Responsiveness to Other Vasoactive Agonists

The specificity of the alterations in vascular reactivity was assessed by administration of the α-adrenergic receptor agonist PhE. The peak pressor response to PhE was not significantly different in the EP3−/− as compared with the EP3+/+ mice (Figure 3D). Similarly, the vasodilator sodium nitroprusside exhibited clear depressor activity in both EP3+/+ and EP3−/− mice with no significant difference between the 2 genotypes (Figure 3E).

EP3−/− Mice Mesenteric Arteries Exhibit Reduced Ang II Responsiveness

Reverse transcription polymerase chain reaction analysis revealed that each of the 3 splice variants of mouse EP3 were constitutively expressed in mesenteric arteries and kidney of EP3+/+ mice (Figure IB in the online-only Data Supplement), whereas none of the splice variants was detected in the EP3−/− kidney (Figure IC in the online-only Data Supplement) and mesenteric arteries (data not shown). Ang II–induced contraction was reduced in the mesenteric arteries from EP3−/− mice compared with that from EP3+/+ mice (Figure 4A). However, no difference in PhE-evoked vasodilation was observed between EP3−/− and EP3+/+ mice (Figure 4B). In addition, the EP3 selective antagonist DG-041 (1 μmol/L) and L798106 (1 μmol/L) attenuated, whereas its agonist sulprostone (1 μmol/L) enhanced, the Ang II–induced contraction in mesenteric arteries from wild-type mice (Figure 4C). Neither the EP3 agonist nor antagonist had effect on PhE-evoked vasodilation (Figure 4D).

Role of EP3 in Ang II–Elicited Phosphorylation of MLC20 in Resistant Vessels

In addition to the Ca2+-dependent activation of MLC kinase, Ang II–induced contractility of VSMCs is attenuated by MLA phosphorylation of MLC20 in Resistant Vessels

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II–elicited vasoconstriction, we tested the effect of EP3 inactivation or blockage on the expression of RhoGEFs and the level of phosphorylated MYPT1. Among 3 VSMC-specific RhoGEFs, Arhgef-1 mRNA, but not Arhgef-11 and Arhgef-12, was significantly reduced in the mesenteric arteries from EP3 −/− mice (Figure 5A and 5B). In line with the decreased mRNA level, Arhgef-1 protein expression was also reduced in EP3-deficient mesenteric arteries (Figure IIA in the online-only Data Supplement). We noticed that Ang II stimulation significantly increased the phosphorylation of both MLC20 and MYPT1 in EP3+/+ arteries, whereas little change was observed in EP3−/− arteries (Figure IIB in the online-only Data Supplement). The Ang II–evoked phosphorylation of MLC20 and MYPT1 was similarly markedly inhibited by EP3 antagonist DG-041 in rat mesenteric arteries (Figure 5C and 5D). In addition, similar results were obtained using porcine coronary arteries, another type of resistant vessel rings (Figure IV in the online-only Data Supplement).

Role of the EP3 in Ang II–Elicited Increase in Intracellular Ca2+ Levels in VSMCs

On binding to its Gq-coupled receptor (Ang II type 1), Ang II stimulates phospholipase C activity and increases intracellular Ca2+ levels via inositol trisphosphate and protein kinase C–dependent mechanisms.24 Ca2+ binds to calmodulin and the Ca2+/calmodulin complex then activates MLC kinase to phosphorylate the MLC. To determine the effect of EP3 on Ang II–induced increase in intracellular Ca2+ levels, we examined the effect of sulprostone or DG-041 on Ang II–induced Ca2+...
increase. Incubation of VSMCs with sulprostone (10 μmol/L) for 30 minutes potentiated the Ang II–induced increase in intracellular Ca^{2+} levels (Figure 6A and 6B). Compared with Ang II, sulprostone at high dose (10 μmol/L) alone only slightly increased Ca^{2+} levels (Figure VA in the online-only Data Supplement). In contrast, the EP3 antagonist DG-041 pretreatment for 30 minutes concentration-dependently inhibited Ang II–evoked Ca^{2+} signal (Figure 6C and 6D), with little effect on calcium levels in PhE-treated cells (Figure VB in the online-only Data Supplement).

Discussion

PGE_{2} has been demonstrated to act as either a pressor or depressor depending on the EP receptor activated. The balance of pressor and depressor receptors activated by PGE_{2} plays an important role in the overall regulation of BP, and a functional imbalance of EP receptors may be an important mechanism in the development of essential hypertension. The current study demonstrated that targeted gene disruption of the EP3 receptor resulted in lower BP at baseline, in agreement with our earlier findings that EP3 was an important contributor to the pressor response to PGE_{2}.22 Our studies also found that the EP3 receptor sensitized the resistant vessels/VSMCs to the actions of Ang II, suggesting that the EP3 receptor may contribute to increased BP in Ang II–dependent hypertension.

The fall in baseline BP in the EP3−/− mice suggested that EP3 receptor activation played a tonic role in BP homeostasis. This was further supported by the findings that acute infusion of the EP3 agonists resulted in a lesser increase in BP in EP3−/− mice. However, on infusion of PGE_{2}, an endogenous ligand for all 4 EP receptors, no difference in the magnitude of the depressor response was observed in EP3−/− mice, suggesting that the vasodepressor response to PGE_{2}, which is
predominantly mediated by the activation of the stimulatory G protein–coupled EP2 and EP4, is intact.

In EP3−/− mice, there was also a striking loss of the acute pressor response specific to Ang II. Consistent with the blunted pressor effects of acute infusion of Ang II, chronic administration of Ang II also resulted in less of an increase of SBP in EP3−/− mice. Furthermore, the changes in renal function did not appear to contribute to the difference in BP between EP3+/+ and EP3−/− mice, because no significant alteration was observed in urine volume and sodium excretion between 2 genotypes. Together, these results suggest that the synergistic effect of EP3 on the Ang II pressor response is a direct effect in the vasculature.

Three additional independent lines of study support the hypothesis that the effect of EP3 on Ang II signaling is at the level of resistant vessels and is mediated at least in part by direct action on the contractility of vascular smooth muscle. First, the contraction of the mesenteric artery induced by Ang II was reduced in EP3−/− mice. In addition, the EP3 agonist potentiated, whereas its antagonist attenuated, Ang II–evoked mesenteric arterial contractility. Second, Ang II–induced phosphorylation of MLC20 and MYPT1 were inhibited by DG-041, and RhoGEF1 (Arhgef-1), an important GDP–GTP exchange factor specifically responsible for Ang II–induced RhoA activation, was significantly suppressed in EP3−/− mouse mesenteric arteries. Finally, the increase of Ang II–evoked intracellular calcium levels in VSMCs was attenuated by the EP3 antagonist DG-041 but enhanced by the EP3 agonist sulprostone.

Figure 4. Effect of E-prostanoid (EP) 3 disruption on angiotensin II (Ang II)–evoked vasoconstriction in mouse mesenteric arteries. A, Ang II–evoked vasoconstriction in EP3−/− mesenteric arterial rings ex vivo (n=7) was attenuated as compared with EP3+/+ (n=4). *P<0.05, **P<0.01 vs EP3+/+ group. B, No difference in phenylephrine-evoked vasoconstriction between EP3+/+ and EP3−/− mesenteric arterial rings (n=11). C, EP3 antagonist DG-041 and L798106 dose-dependently inhibited, whereas EP3 agonist sulprostone facilitated, Ang II–evoked vasoconstriction and Ang II–evoked mesenteric arterial rings ex vivo.**P<0.05, sulprostone vs control group; #P<0.05, DG-041 vs control group; $P<0.05, L798106 vs control group, n=5. D, EP3 agonist or antagonist failed to affect phenylephrine-evoked vasoconstriction in EP3−/− mesenteric arterial rings ex vivo (n=8).

Figure 5. Role of the E-prostanoid (EP) 3 receptor in the expression of Rho guanine nucleotide exchange factors (GEFs) and angiotensin II (Ang II)–induced phosphorylation of myosin light chain (MLC) 20 and myosin phosphatase target subunit 1 (MYPT1). A, Reverse transcription polymerase chain reaction (RT-PCR) analysis of the expression of Arhgef-1, Arhgef-11, and Arhgef-12 in mesenteric arteries of EP3+/+ and EP3−/− mice (n=3 in each group). B, Quantitative RT-PCR analysis of gene expression of RhoGEFs in mesenteric arteries of EP3+/+ and EP3−/− mice. The levels of Arhgef-1, but not Arhgef-11 and Arhgef-12, were significantly reduced in EP3−/− mice. **P<0.01, EP3−/− vs EP3+/+ group, n=3. C, Inhibitory effect of the EP3 antagonist DG-041 on Ang II–induced phosphorylation of MLC20 and MYPT1 in EP3−/− mesenteric arteries. The rings of mesenteric arteries were stimulated by Ang II (1 μmol/L) for 5 minutes after preincubation with the EP3 antagonist DG-041 (10 μmol/L) or a ROCK inhibitor Y27632 (20 μmol/L) for 30 minutes. Both DG-041 and Y27632 attenuated Ang II–induced phosphorylation of MYPT1 and

MLC20. D, Quantitative analysis of the levels of phospho-MLC20 and phospho-MYPT1. *P<0.05, ***P<0.001, Ang II+DG-041 vs Ang II alone; #P<0.001, Ang II+Y27632 vs Ang II alone. n=3 in each group.
It is well known that the contractility of vascular smooth muscle is potentiated by phosphorylation of MLC20 via Ca\(^{2+}\)/calmodulin-activated MLC kinase and reduced by dephosphorylation of MLC20 via MLC phosphatase. Ang II–induced activation of its Gq-coupled receptor causes a rise of Ca\(^{2+}\) levels to initiate Ca\(^{2+}\)/calmodulin/MLC kinase pathway, whereas activation of its G12/13-coupled receptor potentiates Ca\(^{2+}\) sensitivity by increasing activity of RhoGEF, activating Rho/Rho-kinase signaling and then inhibiting phosphorylation of MYPT1, the regulatory subunit of MLC phosphatase heterotrimer.\(^{33,34}\) Previous studies have demonstrated that in addition to inhibitory G protein signaling causing a decrease in cAMP levels, the EP3 receptor may also mobilize intracellular calcium.\(^{17,35,36}\) It is found that the EP3 agonist sulprostone-induced contraction of guinea-pig thoracic aorta may involve Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels.\(^{37}\) We showed that the EP3 receptor agonist sulprostone potentiated Ang II–evoked Ca\(^{2+}\) mobilization and the EP3 antagonist DG-041 attenuated Ang II–stimulated phosphorylation of MLC20 and MYPT1, which suggested that the EP3 receptor may synergize Ang II–induced vasoconstriction. Among 3 RGS-containing RhoGEFs (Arhgef-1, -11, and -12), important GDP–GTP exchange factors specifically responsible for Ang II–induced RhoA activation in VSMCs,\(^{38–40}\) only Arhgef-1 expression was significantly reduced in EP3−/− mesenteric arteries. The reason why the EP3 receptor selectively affects Arhgef-1 expression is currently unknown. However, given the critical role of RGS-containing RhoGEFs in linking Ang II type 1 to RhoA activation, it is expected that reduction of Arhgef-1 expression would result in diminished phosphorylation of MLC20 and MYPT1 in EP3−/− arteries exposed to chronic Ang II treatment.

PGE\(_2\) is a major cyclooxygenase product of vascular smooth muscle where Ang II stimulates its synthesis.\(^{41–43}\) Recent studies suggest that cyclooxygenase 1 activity is critical for the pressor response to Ang II in both acute and chronic Ang II–dependent models,\(^{44,45}\) and Ang II–stimulated synthesis of cyclooxygenase 1–derived PGE\(_2\) activates the EP1 receptor to promote Ang II–dependent hypertension.\(^{18}\) The present study further provides evidence that the EP3 receptor may also represent as a tonic vasoconstriction mediator, which acts synergistically with Ang II to increase BP. Because intrarenal and extrarenal Ang II type 1 receptors play nonredundant pressor roles in Ang II–induced hypertension,\(^{46}\) the present findings also support the possibility that the combination of Ang II receptor blockers and the EP3 antagonists may represent a novel therapeutic strategy for the treatment of hypertension (Figure VI in the online-only Data Supplement). Moreover, because both the EP1 and EP3 receptors are vasoconstrictive, it would be interesting to determine whether blockade of both receptors is additive in reducing BP at baseline or in Ang II–dependent hypertension. This important issue could be addressed in a mouse line deficient for both EP1 and EP3 genes, and it warrants further investigation.

One unexpected finding from our study is that although EP3−/− mice showed significantly attenuated Ang II–induced hypertension, no alleviated cardiac hypertrophy was observed. One possible explanation for this discrepancy is that in addition to a direct growth promoting effect on cardiac myocytes and fibroblasts, Ang II may synergize with the sympathetic nervous system, aldosterone, inflammation, and oxidative stress systemically or locally to drive cardiac hypertrophy and fibrosis in the face of high BP and increased heart afterload.\(^{47,48}\)

![Figure 6. Effect of E-prostanoid (EP) 3 agonist or antagonist on angiotensin II (Ang II)–elicited increase in intracellular Ca\(^{2+}\) in vascular smooth muscle cells (VSMCs).](image-url)
Because PGE₂ has been found to be involved in the regulation of inflammation, aldosterone release, sympathetic activation, and oxidative stress, 49, 50 EP3 gene deficiency may alter these biological processes and affect Ang II–elicited cardiac hypertrophy. However, given that multiple splicing variants of the EP3 receptor exist, 51 it is hard to predict the exact cardiac phenotypes in mice with all EP3 receptor isoform deleted. Therefore, the effect of the EP3 receptor on Ang II–dependent phenotypes in mice with all EP3 receptor isoform deleted requires further investigation.

In summary, the EP3 receptor is a vasconstrictor mediator and its inactivation attenuates Ang II–induced hypertension. It contributes to Ang II–dependent hypertension at least partially via enhancing intracellular calcium concentration and Ca²⁺ sensitivity. Selective targeting of the EP3 receptor may represent a potential therapeutic target for the treatment of hypertension.

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Disclosures
None.

References


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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°C for 30 seconds and 58°C for 30 seconds then 72°C 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°C 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°C 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’GCCUAGAAGUGGCUACAATT 3’, antisense, 5’UUGAUGACCACUUCUAGGCTT 3’; No. 1619: sense, 5’GCUCAGGCUCCAGAAGUTT 3’, antisense, 5’AUCUUCUGGAACCAUGAGCTT 3’; No. 704: sense, 5’CCACCUAGGAAAUGCAATT 3’, antisense, 5’UUGCAUUUCCUCUAGGGTT 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 5x10^5 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°C for 30 seconds, 59°C to 60°C for 30 seconds, and 72°C for 30 seconds, for 40 cycles, followed by a final extension at 72°C for 7 minutes. The primer sequences were listed in Supplemental Table S1. PCR products were validated by electrophoresis on 1.5% agarose 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:

Supplemental Figure Legends

Supplemental Figure I.  Genotyping and validation of EP3−/− mice.

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 5 minutes after preincubated in DG041 (10μmol/L) for 30 minutes. 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 DG041 treatment 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.

A

DNA Marker

1000bp
750bp
500bp
250bp
100bp

EP3+/+ EP3-/-

721bp
152bp

B

Kidney Mesenteric artery

EP3α
EP3β
EP3γ
β-actin

C

EP3+/+ EP3-/-

EP3 611bp
EP3α 241bp
EP3β 307bp
EP3γ 451bp

D

EP3+/+ EP3-/-

EP3
β-actin
Supplemental Figure II.

A

B

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Supplemental Figure IV.

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pMLC

anti-MLC20

pMYPT1

anti-MYPT1

β-actin

B

![Graph showing relative protein level (fold of control)](image)
Supplemental Figure V.

A

B

Phenylephrine

ΔF/F₀

ΔF/F₀
Supplemental Figure VI.
### Supplemental Table I. Sequence of primers for genotyping and RT-PCR analysis

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