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Regulation of Arterial Reactivity by Concurrent Signaling through the E-Prostanoid Receptor 3 and Angiotensin Receptor 1

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Abstract

Prostaglandin E_2 (PGE_2), a cyclooxygenase metabolite that generally acts as a systemic vasodepressor, has been shown to have vasopressor effects under certain physiologic conditions. Previous studies have demonstrated that PGE_2 receptor signaling modulates angiotensin II (Ang II)-induced hypertension, but the interaction of these two systems in the regulation of vascular reactivity is incompletely characterized. We hypothesized that Ang II, a principal effector of the renin-angiotensin-aldosterone system, potentiates PGE_2-mediated vasoconstriction. Here we demonstrate that pre-treatment of arterial rings with 1 nM Ang II potentiated PGE_2-evoked constriction in a concentration dependent manner ($AUC_{-Ang\ II}$ 2.778 ± 2.091, $AUC_{+Ang\ II}$ 22.830 ± 8.560, ***P<0.001). Using genetic deletion models and pharmacological antagonists, we demonstrate that this potentiation effect is mediated via concurrent signaling between the angiotensin II receptor 1 (AT1) and the PGE_2 E-prostanoid receptor 3 (EP3) in the mouse femoral artery. EP3 receptor-mediated vasoconstriction is shown to be dependent on extracellular calcium in combination with proline-rich tyrosine kinase 2 (Pyk2) and Rho-kinase. Thus, our findings reveal a novel mechanism through which Ang II and PGE_2 regulate peripheral vascular reactivity.

Graphical Abstract

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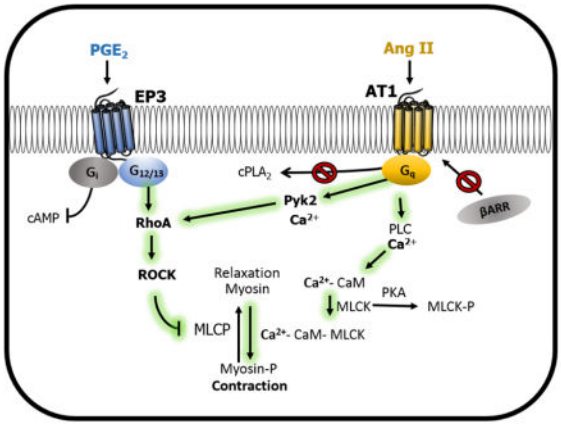

Keywords

prostaglandin E_2; angiotensin II; EP3 receptor; vascular reactivity; Pyk2; AT1 receptor

1. Introduction

High blood pressure is a major risk factor for cardiovascular diseases, including myocardial infarction, stroke and renal failure [1, 2]. The renin-angiotensin-aldosterone-system (RAAS) has long been regarded as a major drug target for treatment of hypertension due to its critical role in maintaining blood pressure through regulation of the vascular, renal, endocrine, and neural systems [3, 4]. Angiotensin II (Ang II), a principal effector of the RAAS system, signals via the G-protein coupled receptors AT1 and AT2. AT1 receptors mediate classical Ang II pressor effects including smooth muscle contraction, increased sympathetic nerve activity, and renal tubular reabsorption of Na^+ [5]. The AT2 receptor, although not fully characterized, has been shown to mediate opposing responses [6, 7]. The AT1 receptor induces vasoconstriction by coupling to G_q, thereby leading to activation of phospholipase C and increasing intracellular calcium [7, 8]. Such rises in calcium can then activate kinases that promote an increase in myosin light chain phosphorylation and ultimately to smooth muscle contraction. The AT1 receptor is also known to increase phospholipase A_2 activation, resulting in liberation of arachidonic acid and synthesis of prostaglandins [9].

Prostaglandin E2 (PGE_2) is an oxygenated metabolite of arachidonic acid, which among many functions modulates blood pressure [10, 11]. PGE_2 acts primarily as a vasodilator and vasodepressor; however, under certain circumstances PGE_2 can have vasopressor effects [12–17]. These divergent functions are due in part to the ability of PGE_2 to activate four distinct E-prostanoid receptors, EP1 – EP4 [10, 18]. EP1 and EP3 receptors facilitate vasoconstriction while the EP2 and EP4 receptors mediate vasodilation [13, 17]. As with the AT1 receptor, activation of the EP1 receptor leads to a rise in intracellular calcium via G_q signaling [19]. The EP3 receptor couples to G_i and $G_{12/13}$, which inhibits cAMP production, activates Rho GTPase, and elevates intracellular calcium levels through its $G_{\beta\gamma}$ subunit [18]. EP2 and EP4 receptors couple to G_s and upon activation increase cAMP.

We have previously demonstrated that blockade of the EP1 or EP3 receptor has salutary effects in mouse models of Ang II-mediated hypertension. EP1 receptor knockout mice display a reduced rise in mean arterial blood pressure in response to Ang II infusion and are protected against end-organ damage in hypertensive mouse models [20, 21]. Similarly, EP3 receptor knockout mice have a blunted blood pressure response to acute administration of Ang II [22]. Although these results indicate an interaction between Ang II and PGE_2 signaling, the molecular mechanisms through which these systems synergize to regulate vascular tone is incompletely characterized.

Previous studies have indicated a unique characteristic of PGE_2-mediated vessel reactivity, where pre-stimulation of rat arterial tissue with a contractile agent followed by PGE_2 unmasks a significant vasoconstrictive response to PGE_2 [23]. Thus, we hypothesized that Ang II, a potent vasoconstrictor, could potentiate PGE_2-mediated vasoconstriction. Additionally, we predicted that this enhanced vasoconstriction would be mediated by potentiation of EP3 or EP1 receptor signaling rather than through desensitization of the vasodilator EP receptors. In the present study, we investigate the effects of low dose Ang II "priming" on PGE_2-incited contraction in isolated blood vessels to determine whether Ang II can alter PGE_2 vessel responses. We demonstrate that Ang II primes vessels to exhibit potentiated vasoconstriction in response to PGE_2. This effect is mediated by the AT1 and EP3 receptors and requires calcium-dependent mechanisms involving Rho-kinase (ROCK) and the proline-rich tyrosine kinase 2 (Pyk2).

2. Materials and methods

2.1 Animals

All studies were performed in accordance with the National Institutes of Health animal care standards and approved by the Vanderbilt University Institutional Animal Care and Use Committee. Male mice 9 to 15 weeks old on a C57BL/6 background were used for all studies unless noted otherwise. Generation of EP1 (*ptger1*), EP2 (*ptger2*), and EP3 (*ptger3*) receptor knockout mice has been previously described [16, 20, 24]. Mice deficient for the EP4 receptor (*ptger4*) have a lethal newborn phenotype when maintained on a C57BL/6 genetic background [25]. We bred C57BL/6 $EP4^{+/-}$ male mice to an outbred strain (CD-1, Charles River laboratories; 10-generation outcross) and observed $EP4^{-/-}$ mice viability into adulthood; thus, these mice were used for the EP4 studies [26, 27].

2.2 Isolated femoral arteries

Following euthanasia, femoral arteries were excised and placed into ice-cold Modified Krebs-Henseleit solution ($NaHCO_2$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, KCl 4.7, NaCl 118, $CaCl_2$ 2.5, Dextrose 10 mM). Vessel rings (~2 mm long) were cut and suspended between 40 μm stainless steel wire in a Danish Myo Technology Model 620M myograph system to record tension. Tissue baths contained 5.0 mL of Krebs-Henseleit at 37 °C aerated with 95% O_2, 5% CO_2 gas. Vascular preparations were allowed to stabilize for 45 minutes at a resting tension of 2.5 mN, which had been determined to give about 90% of maximal response to 50 mM K^+ in mouse femoral arteries. Each preparation was initially challenged with 50 mM K^+, followed by 3 washes of Krebs-Henseleit solution before testing experimental reagents.

Separate rings were used for each data point to avoid the rapid tachyphylaxis of the AT1 receptors. For Ang II priming, Ang II was added to vessel ring baths for 10 minutes followed by addition of PGE_2. At the end of each experiment, endothelium integrity was confirmed by reversal of 1 μM phenylephrine-evoked constriction with 10 μM acetylcholine. Rings were excluded that did not dilate at least 70% of the phenylephrine contraction (tension reduced to < 30% of the phenylephrine response). Finally, rings were washed and retested with 50 mM K^+ to assure maintained responsiveness throughout the entire experiment. All drugs were dissolved in water, ethanol (PGE_2, nifedipine, ibuprofen, SC-236), or DMSO (DG-041, PF-431396) and then further diluted to ≥ 1:1000 or more in Krebs buffer before being added to the vessel bath. Data were collected and stored using ADInstuments LabChart 7 software.

2.3 Chemicals

Angiotensin II (human), acetylcholine, losartan, ibuprofen, nifedipine, PF-431396 hydrate, salicylic acid, and phenylephrine were purchased from Sigma (St. Louis, MO). Prostaglandin E_2 and SC-236 were purchased from Cayman Chemical (Ann Arbor, MI). PD123319 was purchased from Tocris (Bristol, UK). Y-27632 was purchased from Abcam Biochemicals (Cambridge, MA). EGTA was purchased from Thermo Fisher Scientific (Waltham, MA). FAK inhibitor 14 was purchased from Santa Cruz Biotechnology (Dallas, TX). (Sar^1,$Ile^{4,8}$)-Angiotensin II Trifluoroacetate salt was purchased from Bachem (Bubendorf, Switzerland). The Vanderbilt Chemical Synthesis Core synthesized DG-041. Salicylamine was a gift from L. Jackson Roberts (Vanderbilt University).

2.4 Competitive binding of [^{3}H]PGE_2

Total kidney membranes (100 μg) from wildtype C57BL/6 mice were incubated with [^{3}H]PGE_2 (2 nM) in binding buffer (25mM potassium phosphate, 1mM EDTA, and 10mM MgCl, pH 6.2) at a total volume of 200 μl for 1 hr at 30 °C. Nonspecific binding was determined in the presence of excess unlabeled PGE_2 (1 μM). EP1/3 selective agonist sulprostone (1 μM) was used as a positive control. Competitive binding experiments were performed in the presence of 2 nM [^{3}H]PGE_2 and 1 μM of competing ligands, either Losartan or PD123319. Binding reactions were terminated by vacuum filtration and radioactivity was quantified [28, 29].

2.5 Data analysis

Vascular responses were quantified as a change in force (maximal contraction-baseline tension) normalized to the first 50 mM K^+ response (K-standard) of each ring. All data were graphed as means ± SEM using GraphPad Prism 5 software. Data were fitted with variable slope sigmoidal curves using GraphPad Prism. Area under the curve (AUC) (% contraction/Log[treatment]) values were used to statistically compare concentration response curves (CRC) of PGE_2 with or without priming agents. Student's t test was used to determine significance between AUCs as well as for the [H^3]PGE_2 binding data. Ang II CRCs were fitted to a variable-slope sigmoidal curve. Comparison of single dose PGE_2 to various conditions was performed by one-way ANOVA with Bonferroni post-hoc test. For all studies, $P \leq 0.05$ was considered statistically significant. Significance was represented throughout each figure as the following: *= $P \leq 0.05$, **= $P \leq 0.01$, and ***= $P \leq 0.001$.

3. Results

3.1 Enhanced prostaglandin E_2-mediated contraction after priming with low-dose Ang II

To investigate the effect of Ang II on PGE_2-mediated vasoconstriction, mouse femoral arteries (I.D. ~0.25–0.3 mm) were studied *ex vivo* using a wire myograph. Femoral arteries were selected for our studies as a model of peripheral vasculature in order to compare to previous studies conducted using rat femoral arteries. Arterial rings were assayed for responsiveness to a range of single Ang II concentrations to determine the concentration response curve (CRC) (Fig. 1A). Arterial rings were then primed with a sub-threshold constrictor concentration of Ang II (1 nM, ~ EC_{15}) followed by a single concentration of PGE_2 at varying doses to construct a PGE_2 CRC. Exposure of arterial rings to PGE_2 alone, even at a high concentration of 1 μM (3.326 ± 0.647 % contraction of K-standard, N = 5) did not cause significant vasoconstriction. However, priming of arterial rings with a submaximal concentration of Ang II (1 nM) resulted in robust PGE_2-mediated vessel contraction (Fig. 1B, C). Ang II priming facilitated arterial contraction following exposure to doses of 2 nM PGE_2 or greater ($AUC_{untreated}$ 2.778 ± 2.091, $AUC_{Ang\ II\ primed}$ 22.830 ± 8.560, t test ***P<0.001) (Fig. 1C).

3.2 EP3 receptor signaling primed by AT1 receptor activation

To determine which prostaglandin receptor mediates the PGE_2-induced vasoconstriction of Ang II-primed vessels, wire myography studies were performed utilizing a combination of pharmacological antagonists and genetic deletion models. Femoral arteries isolated from $EP3^{-/-}$ mice displayed a lack of PGE_2-mediated vasoconstriction even with Ang II priming (Fig. 2A, B). PGE_2-mediated contraction was then assessed using wildtype femoral arteries treated with the EP3 receptor antagonist, DG-041. Pre-treatment with 1 μM DG-041 had no effect on resting tone or Ang II-mediated constriction (data not shown); however, Ang II failed to potentiate PGE_2-mediated vasoconstriction in DG-041 pretreated vessels (Fig. 2B). The apparent PGE_2-induced vasodilation of vessels primed with Ang II in the presence of DG-041 (Fig. 2B, far right bar) was not statistically significant compared to zero priming. By contrast, in femoral arteries isolated from $EP1^{-/-}$ mice, Ang II was able to potentiate PGE_2-mediated vasoconstriction to a similar level as observed with wildtype mice (Fig. 2B). Although these data indicated that upon Ang II priming the subsequent PGE_2-induced contraction was EP3 receptor-mediated, Ang II promoting desensitization of either vasodilator receptor, EP2 or EP4, could also explain the PGE_2-induced contraction. Thus, to test the contribution of the EP2 or EP4 receptor, we assessed the effect of PGE_2 on $EP2^{-/-}$ and $EP4^{-/-}$ femoral arteries. In the absence of Ang II priming, deletion of either EP2 or EP4 was insufficient to facilitate comparable PGE_2-induced vasoconstriction to that observed in wildtype vessels primed with Ang II (Fig. 2B, $EP2^{-/-}$ & $EP4^{-/-}$ white bars). PGE_2-mediated contraction of Ang II-primed vessels was notably greater in $EP2^{-/-}$ vessels, presumably from loss of the EP2 receptor vasodilator function (Fig. 2B, $EP2^{-/-}$ black bar, $P \leq 0.01$). Arterial rings isolated from $EP4^{-/-}$ mice exhibited responses that were indistinguishable from the responses observed in wildtype vessels.

To investigate whether $EP3^{-/-}$ mice have altered contractile responses to Ang II, concentration-response relationships for wildtype or $EP3^{-/-}$ vessels were compared. The

EC_{50} and E_{max} for Ang II-induced vasoconstriction were not different between wildtype and $EP3^{-/-}$ mice (Fig. 2C). Interestingly, the CRC to Ang II exhibited a steeper slope in wildtype vessels (Hill coefficient of 2.581) as compared to that observed in vessels isolated from $EP3^{-/-}$ mice (Hill coefficient of 1.198). PGE_2 alone did not cause significant vasoconstriction in any of the mice (wildtype, $EP1^{-/-}$, $EP2^{-/-}$, $EP3^{-/-}$, $EP4^{-/-}$). Taken together, these results indicate the EP3 receptor is necessary for PGE_2-induced vasoconstriction potentiated by Ang II.

To identify the Ang II receptor responsible for potentiation of PGE_2-mediated vasoconstriction by Ang II, vessel contraction was measured in arterial rings pre-treated (30 minutes) with the AT1 antagonist losartan or the AT2 antagonist PD123319 prior to Ang II priming and PGE_2 addition. Losartan blocked the ability of Ang II to potentiate PGE_2-induced vasoconstriction, while PD123319 had no significant effect. (Fig. 3A, B, C) To verify that the effect of losartan was not due to off target competitive inhibition of the prostanoid receptors, the ability of either losartan or PD123319 to compete with $[^3H]PGE_2$ binding in mouse kidney membrane preparations was assessed. In contrast to the EP1/3 selective agonist, sulprostone, neither losartan nor PD123319 competed with $[^3H]PGE_2$ binding (Fig. 3D). Together, these results indicate that the AT1 receptor facilitates the Ang II priming effect on EP3 receptor-mediated contraction.

3.3 AT1-mediated β-arrestin pathways are not sufficient to potentiate PGE_2-mediated contraction

AT1 has been identified to mediate several physiological responses mainly through activation of G_q; however, some AT1-mediated effects can be attributed to activation of G-protein independent pathways via GRK/β-arrestin/MAPK signaling. To determine whether Ang II-induced potentiation of PGE_2-mediated vasoconstriction involved activation of β-arrestin pathways, 30 μM of the biased agonist $Sar^1,Ile^{4,8}$-Angiotensin II (SII) (AT1a K_D = 300 nM, [30]) was used in wire myography studies. SII has been previously shown to activate β-arrestin signaling pathways via AT1 while not stimulating the G_q-associated calcium cascade [31]. SII did not evoke vasoconstriction at doses up to 30 μM. SII also did not exhibit a potentiation effect on PGE_2-mediated constriction of femoral arteries (Fig. 4A, C). Furthermore, SII blocked Ang II-induced contraction as well as Ang II priming of PGE_2-mediated contraction, presumably by acting as a competitive antagonist for Ang II-AT1 priming (Fig. 4B, C).

3.4 Extracellular calcium stores and Rho-kinase are required for contraction

Activation of AT1 by Ang II but not SII leads to increased intracellular calcium levels $[Ca^{2+}]_i$ in smooth muscle cells to stimulate contraction [31]. The contribution of extracellular stores of calcium to Ang II-induced potentiation of PGE_2-mediated vasoconstriction was assessed by analyzing vessels in Ca^{2+} free buffer. Arterial rings were incubated in calcium free Krebs buffer containing 1 mM EGTA. EGTA was subsequently removed from the bath, and the contractile response of Ang II-primed vessels to PGE_2 was tested. In control vessels without Ang II pre-treatment, 100 nM PGE_2 did not cause significant contraction in normal Krebs or upon addition of higher calcium concentrations to the bath (3.5 or 4.5 mM), demonstrating that increasing extracellular calcium concentration

is not sufficient to evoke potentiated PGE_2-mediated contraction (Fig. 5A). In the absence of extracellular Ca^{2+}, femoral arteries exhibited minimal responsiveness to Ang II. Furthermore, Ang II did not potentiate PGE_2-induced contraction in Ca^{2+} free media. However, re-addition of Ca^{2+} into the bath restored PGE_2-mediated contraction (Fig. 5B, C). Pre-treatment (30 minutes) of arteries with the L-type calcium channel blocker, Nifedipine, inhibited the PGE_2-mediated contraction (Fig. 5C, far right bar). Taken together, these studies indicate a significant contribution of extracellular sources of calcium, particularly from calcium influx by L-type channels, in mediating PGE_2-induced contraction after priming with Ang II. Due to its known role in sensitizing smooth muscle cells to calcium-induced contraction, ROCK was evaluated for its effect on PGE_2-induced vasoconstriction of Ang II-primed vessels. Addition of the ROCK inhibitor Y-27638 to PGE_2-induced vasoconstriction returned vascular tone to baseline (Fig. 5D & E). These data indicate that ROCK is required for EP3-induced arterial contraction.

3.5 Salicylate diminishes Ang II potentiation of PGE_2-mediated contraction

To determine whether Ang II potentiates PGE_2-mediated vasoconstriction through its action on $cPLA_2$, resulting in increased prostaglandin biosynthesis, femoral arterial rings were pre-incubated with nonsteroidal anti-inflammatory drugs (NSAID). 100 μM ibuprofen, a potent COX inhibitor (COX-1 IC_{50} = 7.6 μM, COX-2 IC_{50} = 7.2 μM) [32], did not affect PGE_2-induced constriction of Ang II-primed vessels (Fig. 6A, $P>0.05$), nor did 100 nM SC-236 (COX-1 IC_{50} = 17.8 μM, COX-2 IC_{50} = 10 nM) [33], a COX-2 specific inhibitor. However, 10 mM salicylate, an active metabolite of aspirin and poor COX enzyme inhibitor (COX-1 IC_{50} = 5 mM, COX-2 IC_{50} = 0.5–34 mM) [32], significantly attenuated PGE_2-mediated vasoconstriction of Ang II-primed vessels (Fig. 6A, $P<0.01$). Pretreatment with 100 μM salicylamine, a structurally similar compound to salicylate and identified isoketal scavenger, did not inhibit PGE_2-mediated vasoconstriction (Fig. 6A, $P>0.05$) [34]. Inhibition of contraction by salicylate but not ibuprofen or SC-236, suggests that the salicylate effect was a result of a non-COX mechanism.

3.6 Effects of proline-rich tyrosine kinase 2 inhibitors

Salicylate has been shown to inhibit the non-receptor tyrosine kinase Pyk2 [35, 36]. To test whether salicylate exerted its effect by inhibition of Pyk2, the Pyk2 inhibitor PF-431396 was tested. PF-431396 abolished PGE_2-induced contraction of Ang II-primed vessels (Fig 6B). Since Pyk2 is structurally similar to focal adhesion kinase (FAK), arteries were pre-treated with a FAK specific inhibitor, 10 μM FAK inhibitor 14, to test the contribution of FAK (Fig 6B). The FAK inhibitor did not affect Ang II priming of PGE_2, indicating that Pyk2 underlies PGE_2-mediated contraction. Collectively, these findings demonstrate that Ang II potentiates vasoconstriction induced by PGE_2 via convergent signaling between the AT1 and EP3 receptors. These convergent events occur through a Ca^{2+}-Pyk2-ROCK mechanism and are independent of β-arrestin signaling and AT1-mediated activation of PLA_2 (Fig 6, C).

4. Discussion

In the present study, we demonstrate that PGE_2 functions as a vasoconstrictor in femoral arteries pretreated with low-dose Ang II. In contrast, PGE_2 has no detectable effect on

vascular tone in the absence of priming by Ang II. This finding reveals a novel interaction between Ang II and PGE_2 in regulating peripheral vascular reactivity. Although other constrictors may be able to potentiate EP3 receptor-induced contraction, indicated by published results with rat femoral arteries primed with thromboxane or phenylephrine [23], Ang II is of particular interest due to its central role in the development of cardiovascular disease. One contributing factor of high blood pressure is increased peripheral vascular resistance [37]. Increased formation of vasoconstrictors and altered effectiveness of vasodilators may underlie such impairments. PGE_2 has been demonstrated to function primarily as a vasodilator and vasodepressor when intravenously infused into many species, including humans, dogs, rabbits, mice and rats; however, in certain physiologic settings PGE_2 acts as a vasoconstrictor [12, 16, 17, 38–43]. Our studies indicate that in the presence of Ang II, PGE_2 exhibits vasoconstrictor effects in mouse femoral arteries. Because Ang II and PGE_2 are well known to regulate peripheral vasculature, contractile responses mediated by concurrent signaling by Ang II and PGE_2 may contribute to vascular dysfunction associated with hypertension.

The Ang II concentration of 1 nM used for priming femoral arteries in our studies is greater than typical reported plasma concentrations of Ang II, which have been measured to be in the pM range [44, 45]. However, evidence indicates that tissue sources of Ang II may contribute more to vascular contractions than circulating Ang II, and interstitial levels of Ang II have been detected in the nM range in femoral arteries and kidneys [46, 47]. Therefore, our priming concentration of Ang II, which produced only a modest contractile response prior to PGE_2 administration (Figure 1), may approximate levels observed by AT1 receptors *in vivo* and could thus be relevant to vascular responsiveness.

Studies presented here demonstrate that the EP3 receptor is required for PGE_2-mediated vasoconstriction. We found that PGE_2-induced vasoconstriction following Ang II priming was lost upon knock-out of the EP3 receptor. Previous studies indicate that $EP3^{-/-}$ mice have lower mean arterial pressure (MAP) in response to acute or chronic Ang II infusions [22]. The observed changes in vascular reactivity described in the present studies may contribute to the reduction in MAP in response to Ang II observed in $EP3^{-/-}$ mice [22]. Of note, femoral arteries from $EP3^{-/-}$ mice exhibited Ang II concentration response curves similar to wildtype femoral arteries, suggesting the alterations in vascular reactivity to PGE_2 observed in $EP3^{-/-}$ femoral vessels are not due to a loss of Ang II responsiveness. In contrast, a previously reported study investigating the effect of EP3 receptor signaling on mouse mesenteric vasculature indicated that Ang II-mediated contraction was altered by knockout of the EP3 receptor [22]. Considering these findings, our data suggest differences in the interactions between Ang II and PGE_2 signaling that are specific to particular vascular beds.

Our findings support a model, diagrammed in Figure 6C, in which activation of the AT1 receptor by Ang II sensitizes smooth muscles cells to calcium, thereby facilitating a contractile response to PGE_2. This vasoconstriction does not require AT1-mediated production of endogenous prostaglandins or β-arrestin scaffolding. Importantly, our studies identified Pyk2 as a tyrosine kinase crucial for PGE_2-induced contraction. This finding aligns well with previous investigations of Pyk2 function. Pyk2 is a calcium-dependent

tyrosine kinase that acts upstream of the RhoA/ROCK pathway [35, 36]. Pyk2 can be activated by Ang II and plays a role in the ability of Ang II to mediate many cellular functions, including vasoconstriction [35, 48]. The EP3 receptor has been identified to mediate contractile responses through activation of ROCK [22, 23, 49]. Therefore, Ang II signaling may activate Pyk2, thereby sensitizing the ROCK pathway to EP3 signaling (depicted in Figure 6C); alternatively, EP3 and AT1 signaling may concurrently converge through a Pyk2-RhoA-ROCK pathway. Activation of ROCK would ultimately lead to inhibition of myosin phosphatase and enhance Ca^{2+} sensitivity of myosin filaments. The inhibition of Ang II priming of PGE_2-mediated contraction observed with salicylate and the specific Pyk2 inhibitor (PF-431396) may also explain some of the beneficial cardiovascular effects of salicylate.

Although the present studies focused on peripheral vascular reactivity, the observed interaction between the EP3 and AT1 receptors may be important in other tissue types and physiologic events associated with hypertension. For example, studies using rats have indicated that intracerebroventricular infusion of PGE_2 results in a rise in blood pressure and heart rate [50]. Interestingly, these responses were exacerbated in spontaneously hypertensive rats (SHRs), a hypertension model known to have elevated brain levels of Ang II [51]. These findings suggest a potential interaction of Ang II and PGE_2 in the central nervous system that might affect blood pressure. Future studies investigating additional mechanisms through which the EP3 and AT1 receptors synergize and how this interaction relates to the pathology of hypertension may reveal novel therapeutic strategies.

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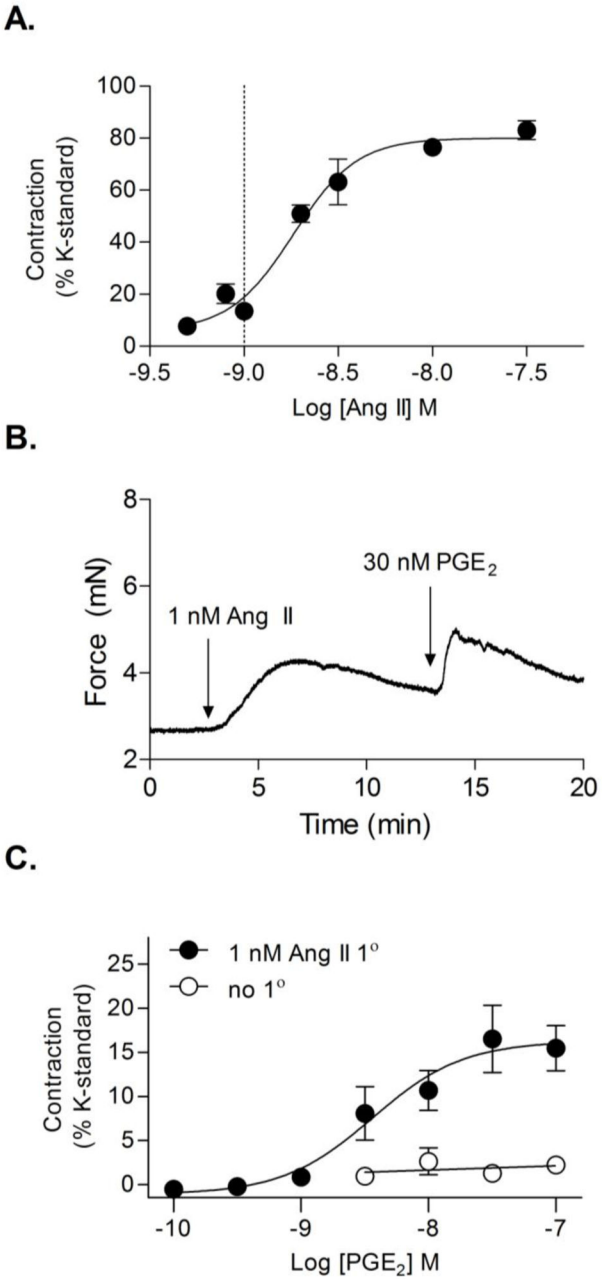

Figure 1. Ang II priming enhances PGE_2-mediated vasoconstriction

(A) Ang II CRC in wildtype mice. (N = 5–7) Dotted line indicates concentration of Ang II used to prime vessels in B and C. (B) Representative trace of 30 nM PGE_2-induced contraction of vessels primed with 1 nM Ang II. (C) Comparison of PGE_2 concentration-response curve with (filled circles, N = 5–6) or without (open circles, N = 5) 1 nM Ang II priming. Data for each concentration was collected on separate rings. Results were normalized to each ring's response to 50 mM KCl. Vertical bars indicate SEM.

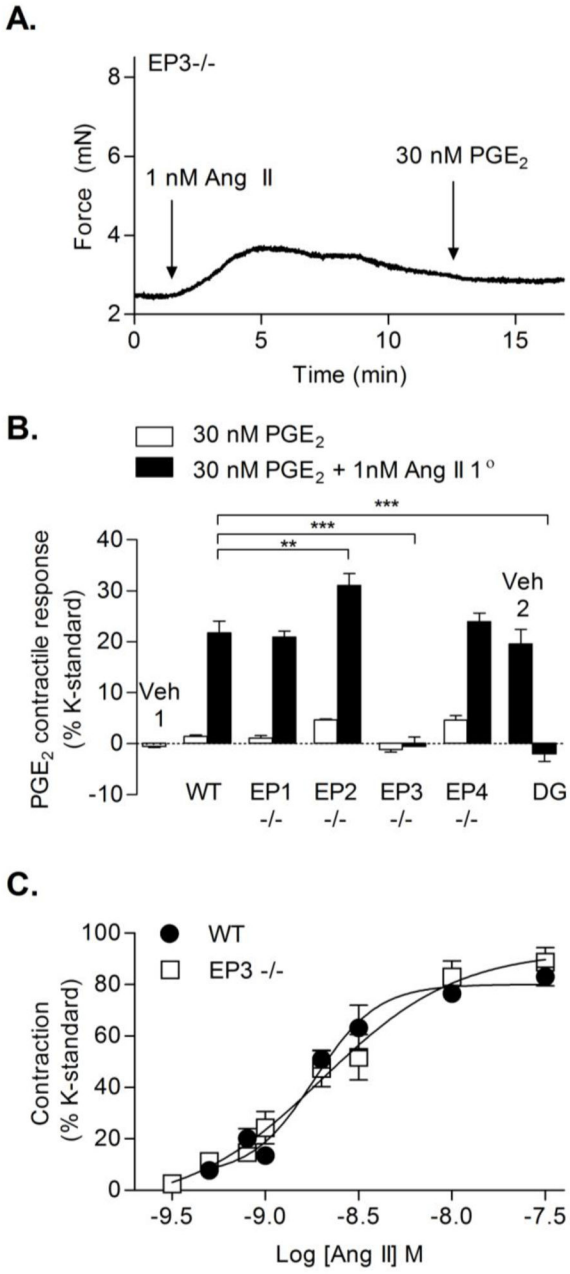

Figure 2. Effects of EP-receptors on PGE_2-induced contraction of Ang II-primed arteries

(A) Representative wire myography trace of an $EP3^{-/-}$ femoral artery that shows the lack of PGE_2 response when primed with Ang II. (B) Comparison of % contraction during 30 nM PGE_2-induced vasoconstriction with or without 1 nM Ang II priming in $EP1^{-/-}$, $EP2^{-/-}$, $EP3^{-/-}$, or $EP4^{-/-}$ vessels or in wildtype vessels treated with or without the EP3 antagonist DG-041 (DG) (N = 4–5 for $EP1^{-/-}$, all other bars N = 5; two $EP2^{-/-}$ mice were one year old). PGE_2 alone did not cause significant vasoconstriction in any of the mouse strains (wildtype, $EP1^{-/-}$, $EP2^{-/-}$, $EP3^{-/-}$, $EP4^{-/-}$). Veh1 indicates Ang II priming of PGE_2 solvent (ethanol). Veh2 indicates pre-treatment with DG-041 solvent (DMSO). Wildtype data with

or without Ang II are taken from Fig 1C. (C) Ang II concentration-response curves comparing contraction of wildtype (taken from Fig 1A, N = 5–7) and $EP3^{-/-}$ vessels (N = 5–6). EC_{50} for each was −8.74 and −8.70. Vertical bars indicate SEM.

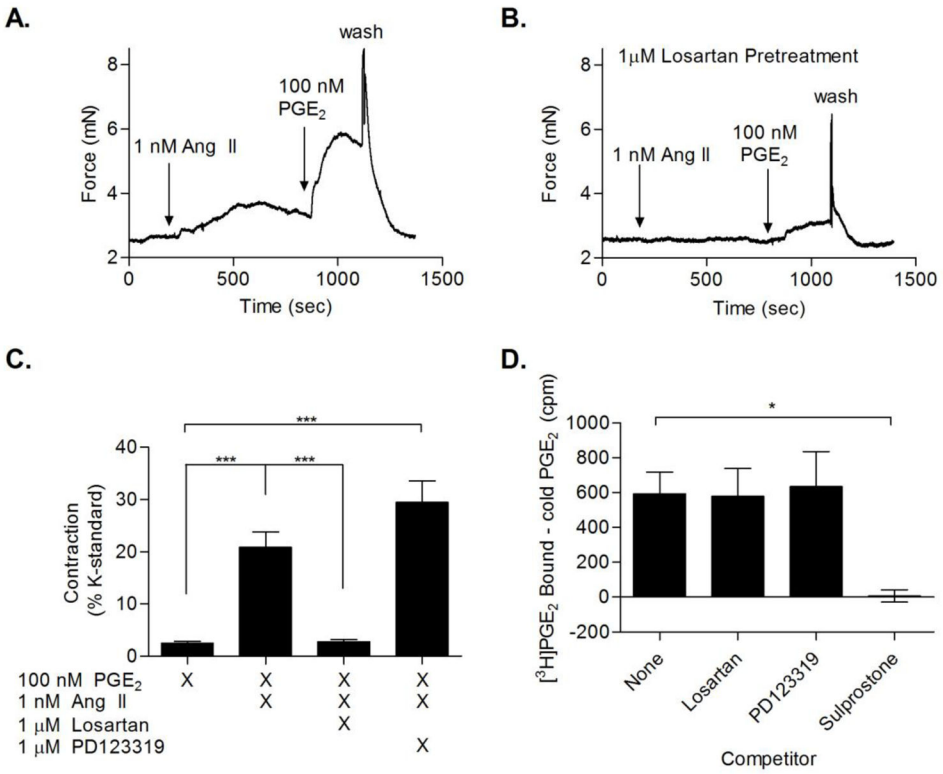

Figure 3. AT1 antagonist inhibits Ang II priming of PGE_2

(A) Representative wire myography trace of femoral arterial rings primed with 1 nM Ang II, followed by addition of 100 nM PGE_2. (B) Representative trace of femoral artery with 30 minutes pretreatment with 1 μM losartan followed by addition of 1 nM Ang II priming of 100 nM PGE_2. (C) Comparison between femoral arterial ring responses to 100 nM PGE_2 with and without 1 nM Ang II priming, as well as with priming in the presence of an AT1 (losartan) or AT2 antagonist (PD123319) (N = 5). (D) Competition binding in wildtype mouse kidney membranes; displacement of $[^3H]PGE_2$ by the EP3 agonist sulprostone, losartan, and PD123319. Graph is normalized to competition binding with cold PGE_2. (N = 3). * = $P < 0.05$

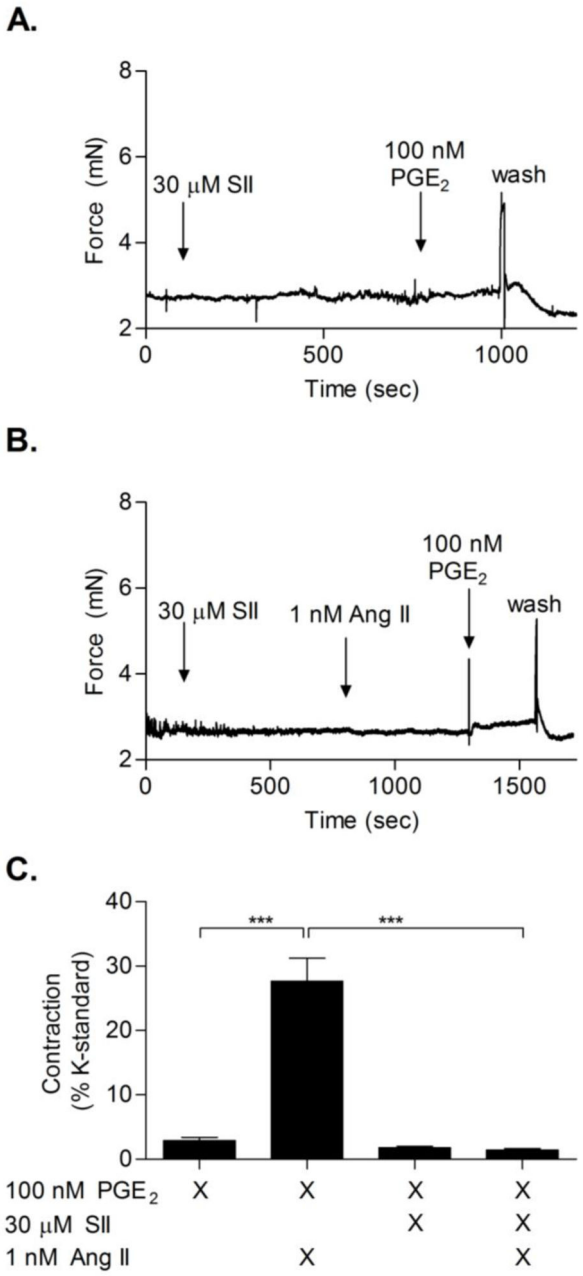

Figure 4. SII does not potentiate PGE_2-mediated contraction

(A) Representative wire myography trace showing that 10 minute pre-treatment with 30 μM SII did not potentiate 100 nM PGE_2-mediated contraction. (B) Representative trace of arterial rings preincubated with 30 μM SII for 10 minutes, followed by priming with 1 nM Ang II and addition of 100 nM PGE_2. (C) Quantification of SII results, showing a lack of potentiation by SII on PGE_2-induced contraction (3rd bar from left), and SII blockade of PGE_2-mediated contraction of Ang II-primed vessels (4th bar from left) (N = 5).

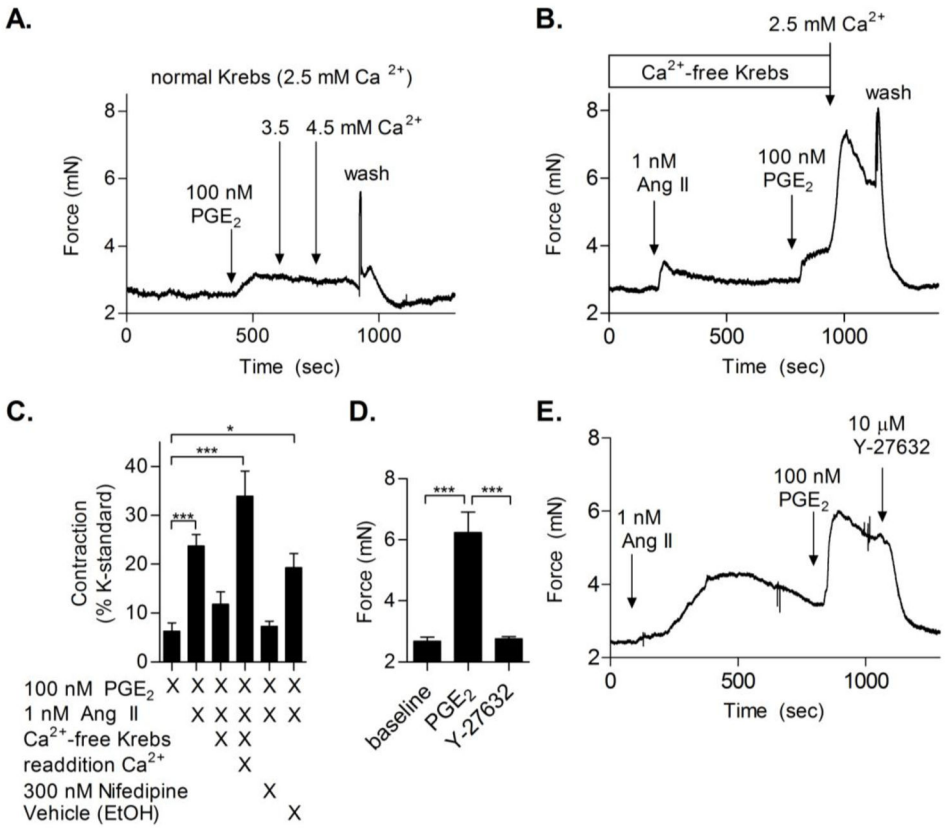

Figure 5. Contribution of extracellular calcium and Rho-kinase to PGE_2 primed vasoconstriction

(A) Representative wire myography trace (N = 5) of PGE_2-mediated contraction with additional calcium added (normal Krebs 2.5 mM, additional calcium 3.5 or 4.5 mM). (B) Example trace of femoral arterial ring maintained in calcium-free Krebs. Vessels were briefly treated with EGTA, washed, and subsequently administered Ang II, followed by PGE_2. Calcium was then added back to the bath to restore contraction. (C) Quantification of PGE_2 response with and without Ang II priming, in the presence or absence of calcium, compared to vessels pre-incubated with 300 nM Nifedipine or vehicle control (ethanol) (N = 5–8). (D) Quantification of the effects of the ROCK inhibitor Y-27632 on PGE_2-mediated contraction (N = 5). (E) Example trace of Ang II priming of PGE_2-induced contraction with ROCK inhibitor, Y-27632, addition during PGE_2 response.

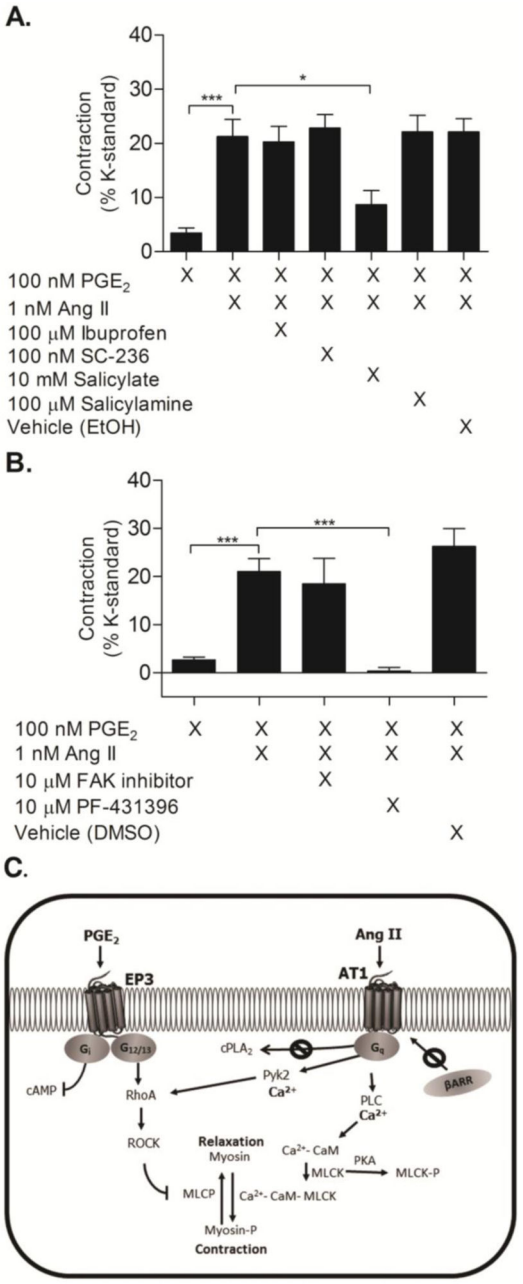

Figure 6. Pyk2 is necessary for PGE_2-mediated contraction of Ang II-primed vessels

(A) PGE_2-induced contraction of femoral arterial rings pretreated for 30 minutes with the indicated concentrations of ibuprofen, SC-236, salicylate, salicylamine, or control solvent for ibuprofen/SC-236 (EtOH) followed by priming with Ang II and subsequent addition of PGE_2 (N = 7–10). Salicylate attenuated PGE_2-induced constriction of vessels primed with Ang II ($P<0.05$ 1way ANOVA). (B) Preincubation with the Pyk2 inhibitor PF-431396 blocked PGE_2-induced contraction of Ang II-primed vessels, while inhibiting FAK with FAK inhibitor 14 did not affect contraction (N = 5). Vehicle represents control solvent for PF-431396 (N = 5). Vertical bars indicate SEM. (C) Schematic of key proteins involved in

PGE_2-induced contractile responses. Prior activation of the AT1a receptor by Ang II facilitates vasoconstriction via a PGE_2 – EP3 receptor pathway. PGE_2-induced vasoconstriction is dependent upon signaling through a Ca^{2+}-Pyk2-ROCK cascade, as opposed to other defined pathways such as activation of phospholipase A_2 or signaling via β-arrestin. EP3 and AT1 signaling converges through Pyk2-ROCK to sensitize smooth muscle cells to calcium and thus trigger a significant contractile response.