Antihypertensive effects of selective prostaglandin E₂ receptor subtype 1 targeting

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Clinical use of prostaglandin synthase–inhibiting NSAIDs is associated with the development of hypertension; however, the cardiovascular effects of antagonists for individual prostaglandin receptors remain uncharacterized. The present studies were aimed at elucidating the role of prostaglandin E₂ (PGE₂) E-prostanoid receptor subtype 1 (EP1) in regulating blood pressure. Oral administration of the EP1 receptor antagonist SC51322 reduced blood pressure in spontaneously hypertensive rats. To define whether this antihypertensive effect was caused by EP1 receptor inhibition, an EP1-null mouse was generated using a “hit-and-run” strategy that disrupted the gene encoding EP1 but spared expression of protein kinase N (PKN) encoded at the EP1 locus on the antiparallel DNA strand. Selective genetic disruption of the EP1 receptor blunted the acute pressor response to Ang II and reduced chronic Ang II–driven hypertension. SC51322 blunted the constricting effect of Ang II on in vitro–perfused preglomerular renal arterioles and mesenteric arteriolar rings. Similarly, thepressor response to EP1-selective agonists sulprostone and 17-phenyltrinor PGE₂ were blunted by SC51322 and in EP1-null mice. These data support the possibility of targeting the EP1 receptor for antihypertensive therapy.

Introduction
Prostaglandins are endogenous oxygenated fatty acid metabolites and play important roles in inflammation as well as in modulating arterial blood pressure and renal salt excretion (1–3). The predominant effects of endogenous prostanoids are generally poised to reduce blood pressure, consistent with clinical observations that NSAIDs that inhibit prostaglandin synthase (cyclooxygenase; COX), such as COX2 inhibitors, cause hypertension (4–7). More recently, divergent cardiovascular effects of COX1 versus COX2 inhibition have been observed in mouse models of atherosclerosis and hypertension. Instead of exacerbating hypertension, chronic treatment with a selective COX1 inhibitor or COX1 gene knock-out reduced the pressor effect of Ang II (8). Conversely, pretreatment with an oral COX2 inhibitor enhanced the pressor effect of Ang II. These findings support the possibility that COX2-derived prostaglandins reduce blood pressure, while COX1-derived prostanoids increase blood pressure. The specific prostanoids that are responsible for these divergent effects of COX products on Ang II action remain poorly defined.

Endogenous prostanoids include 5 major bioactive compounds: prostaglandin E₂ (PGE₂), prostaglandin F₂α (PGF₂α), prostaglandin D₂ (PGD₂), prostacyclin (PGI₂), and thromboxane A₂ (TXA₂) (9). Each prostanoid exerts local biological effects via specific G protein–coupled receptors expressed on nearby target cells. PGE₂ is a major prostanoid synthesized in mouse kidney and vasculature (10). It is unique among the prostanoids in that it can activate 4 distinct G protein–coupled membrane receptors—designated E-prostanoid receptor 1 (EP1), EP2, EP3, and EP4—that are encoded by different genes and exhibit different tissue distribution (1, 9). These EP receptors can be selectively activated by different structural PGE₂-agonists, and each receptor activates different cell signaling pathways (11, 12). Infusion of PGE₂ i.v. in mice causes a transient fall in blood pressure, while disruption of the EP2 receptor unmasks a pressor response to PGE₂ (13), consistent with a depressor role for the EP2 receptor. In the absence of EP2, the pressor effects of PGE₂ are presumably mediated by the remaining receptors, possibly EP1 or EP3. Because Ang II stimulates PGE₂ synthesis (10, 14, 15), endogenous PGE₂ could modulate Ang II action in either a pressor or a depressor fashion.

The contribution of endogenous EP receptors to hypertension remains incompletely characterized. Both EP1 receptor antagonists (16–18) and EP1 knockout mice exist, but have not been characterized in models of hypertension. While conventional targeting of the gene encoding EP1 in mice suggests a role for EP1 in maintaining basal blood pressure (19), interpretation of these data is complicated by the circumstance that the EP1 locus encompasses a second gene on the antiparallel strand, the serine/threonine protein kinase N (PKN) (20). PKN is homologous to PKC and activated by rho GTPase and by fatty acids including arachidonate, and so might contribute to the regulation of vascular tone independent of EP1 (21). The aim of the present studies was to elucidate the role of the EP1 receptor in hypertension using an EP1 receptor–specific antagonist and an EP1 knockout mouse (Ptgcr1−/−, referred to herein as EP1−/−) that disrupts EP1 while sparing the PKN locus. Our findings demonstrate that either an EP1 receptor antagonist or EP1 receptor disruption ameliorate hypertension, supporting the feasibility of targeting the EP1 receptor for antihypertensive therapy.
Results

Pharmacologic blockade of the EP1 receptor reduces blood pressure in the spontaneously hypertensive rat. The spontaneously hypertensive rat (SHR) is a well established model of hypertension (22). SC51322 is a selective and potent EP1 receptor antagonist, and was originally developed as an analog of SC51322 on blood pressure in SHRs was assessed using daily radiotelemetric recordings of mean arterial blood pressure (MAP). Treatment with SC51322 (10 mg/kg/d orally) significantly reduced MAP over 3 days of treatment without affecting heart rate. Following discontinuation of SC51322 treatment, MAP returned to elevated levels (Figure 1). These results suggest that EP1 receptor activation contributes to hypertension in the SHR model.

Generation of EP1+/− mice. To confirm a role for the EP1 receptor in promoting hypertension, we generated what we believe to be a novel EP1 knockout mouse. Because the EP1 locus overlaps the PKN locus on the antiparallel strand (20), we selectively targeted the EP1 locus using a “hit-and-run” targeting strategy (25), where- in a premature stop codon (R242X) was created into fragments of 670 bp and resistant to the EcoRI digestion, while PCR fragments from the EP1+/− allele were cleaved into fragments of 670 bp and 110 bp. This demonstrated the presence of the EcoRI in mutant EP1 mRNA (Figure 2D), as was additionally confirmed by sequencing the PCR fragments (data not shown).

Disrupted signaling by EP1 receptor mutant. We confirmed that PGE2 failed to elicit the expected signaling events in HEK293 cells stably transfected with the mutant EP1 expression vector. The EP1 receptor signals via increased intracellular Ca2+ ([Ca2+]i) (26–28), as seen in fura-2–loaded HEK293 cells transfected with EP1+/− vector and treated with PGE2 (Figure 2E). In contrast, in untransfected HEK293 cells or cells expressing the mutant EP1 receptor, PGE2 failed to increase [Ca2+]i (P < 0.005).

PKN expression is unaffected in EP1-targeted mice. In situ hybridization detected renal expression of both PKN and EP1 in a nonoverlapping distribution, with PKN expression predominating in the renal cortex and EP1 predominating in the renal medulla (Figure 2F). Identical intrarenal localization of PKN was seen in EP1+/+ and EP1−/− mice (Figure 2G). PKN expression was also studied in immunoprecipitates from kidneys and whole brains of EP1+/+ and EP1−/− mice, and PKN protein levels were comparable between groups. These results confirm that the 3-bp change in the 3′ UTR of the long transcript did not affect PKN expression.

Role of the EP1 receptor in the pressor response to Ang II. Having confirmed that the truncation mutation disrupted EP1 receptor function, we examined whether EP1 receptor disruption affects the acute pressor response to i.v. Ang II infusion (75 pmol/kg/min) by using indwelling carotid catheter in anesthetized male 129S6/SvEvTac mice. MAP was significantly reduced in EP1−/− versus EP1+/+ mice (Figure 3A), with a peak increase in MAP of 20.9 ± 4.7 mmHg in EP1−/− mice after Ang II versus 26.2 ± 2.7 mmHg in EP1+/+ mice (P < 0.005). This reduction in the hypertensive response to Ang II was sustained for the duration of its infusion (P < 0.01; Figure 3B).

To further explore the role of EP1 receptor deletion on blood pressure regulation, a model of Ang II–dependent hypertension was established in wild-type male N10 129S6/SvEvTac EP1+/+ and EP1−/− littersmates using chronic Ang II infusion (1,000 ng/kg/min i.p.; n = 5 per genotype). Systolic arterial blood pressure, measured both by tail-cuff method in conscious mice and by preterminal carotid catherization in the same mice following anesthesia (13, 29), was significantly greater in EP1+/+ mice than in EP1−/− mice (P < 0.0001; Figure 3C). Baseline tail-cuff systolic blood pressure in male 129S6/SvEvTac mice was slightly, but significantly, lower in EP1+/− than in EP1−/− mice (113.20 ± 2.97 versus 119.84 ± 2.18 mmHg; n = 5; P < 0.001; Figure 3C). While Ang II significantly increased systolic blood pressure in both genotypes (Figure 3C), the increase in systolic blood pressure was significantly greater in EP1−/− mice than EP1+/− mice (26 ± 1.1 versus 18 ± 1.1 mmHg; P < 0.001; Figure 3D). Over the 4-week period of Ang II infusion, systolic blood pressure averaged approximately 16 mmHg lower in EP1−/− mice compared with EP1+/+ mice (P < 0.001), and this significant difference in MAP was confirmed at the end of the 4-week Ang II period by direct intracarotid measurements (Figure 3E). Furthermore, Ang II–associated cardiac hypertrophy was significantly reduced in EP1−/− mice compared with EP1+/+ mice (164 ± 8 versus 186 ± 11 mg; n = 5 and 4, respectively; P = 0.045). This difference in cardiac weight was maintained when corrected for body weight (6.0 ± 0.18 versus 7.1 ± 0.48 mg heart wt/g body wt; P = 0.05).

Arteriolar response to angiotensin following EP1 blockade. The effect of EP1 receptor blockade on the renal afferent arteriolar response to angiotensin was determined. Afferent arteriolar diameter averaged 17.4 ± 0.6 μm (n = 14). Angiotensin dose-dependently decreased afferent arteriolar diameter, and this vascular response was significantly attenuated in the presence of the EP1 receptor antagonist.
The vascular response to the EP1 and EP3 receptor agonist 17-phenyltrinor PGE\textsubscript{2} was also determined. Afferent arteriolar diameter decreased by 19% ± 4% (n = 7) in response to 1 \textmu M 17-phenyltrinor PGE\textsubscript{2}.

Mesenteric arteriolar tension. Expression of EP1 receptor was demonstrated by RT-PCR using mRNA obtained from mesenteric arteries, a systemic resistance vessel. The specificity of the PCR product was confirmed by sequencing and absence of a PCR product.

Figure 2
Generation and characterization of a hit-and-run–targeted EP1\textsuperscript{−/−} mouse. (A) Genomic organization of mouse EP1 and PKN genes. The EP1 gene consists of 3 exons (black boxes) spanning approximately 7.2 kb. Shown are the translational ATG start site (nucleotides 2,700–2,702) and the premature stop codon and EcoRI site introduced in the mutant. PKN is antiparallel with EP1, and 2 alternative 3′ splice variants (arrows) are characterized by differences in the 3′ UTR. (B) Southern blot of EcoRI digest demonstrated successful incorporation of the EcoRI site into the EP1\textsuperscript{−/−} mouse. The radiolabeled EP1 probe recognized a 10-kb EcoRI fragment derived from the wild-type allele and a 5.8-kb fragment from the mutant allele. (C) Design of EP1\textsuperscript{+/+} and EP1\textsuperscript{−/−} cDNA. The C795T mutation in knockout cDNA caused an in-frame R242X stop codon (CGA to TGA). Additionally, mutations C798G and G799A created a new EcoRI restriction site. (D) Secondary structure of EP1 receptor showing the site of truncation in the mutant receptor following the putative fifth membrane–spanning \( \alpha \)-helix. (E) \([\text{Ca}^{2+}]\)\textsubscript{i} signaling in fura-2–loaded HEK293 cells transfected with EP1\textsuperscript{+/+} and EP1\textsuperscript{−/−} cDNA was determined using the 340/380 nm emission intensity ratio. PGE\textsubscript{2} (1 \textmu M) significantly increased the ratio above basal values in cells transfected with EP1\textsuperscript{+/+} and EP1\textsuperscript{−/−} cDNA but not in HEK293 cells transfected with EP1\textsuperscript{+/−}. (F) Autoradiogram in situ hybridization showed distribution of 35S-labeled riboprobes for EP1 antisense and PKN (EP1 sense) probes demonstrating nonoverlapping and inverse distribution patterns in mouse kidney. (G) Immunoprecipitation of EP1\textsuperscript{+/+} and EP1\textsuperscript{−/−} mouse kidney and brain lysates showed PKN expression was not altered in EP1\textsuperscript{−/−} mice.
using mRNA as template without reverse transcription. Both dissected mouse mesenteric arteries and aortas were found to express EP1 receptor mRNA (Figure 3F). These findings support a model whereby the EP1 receptor directly exerts its systemic pressor activity on arterial resistance vessels.

Ang II induced concentration-dependent contraction of isolated mouse mesenteric arterioles. The maximal contraction was obtained using $10^{-7}$ M Ang II. While the EP1 receptor blocker SC51322 ($10^{-6}$ M; ref. 17) had no effect on the basal tension ($n = 7$; $P > 0.05$; data not shown), it almost completely eliminated Ang II-evoked contraction (Figure 3G).

**Effect of EP1 receptor agonists on MAP.** To examine whether EP1 receptor disruption alters regulation of blood pressure by infusion of PGE$_2$, PGE$_2$- and EP-specific analogs were performed. The pressure effect of the EP1/EP3 receptor–selective agonist 17-phenylltroper PGE$_2$ was markedly reduced in male EP1$^{-/-}$ mice, increasing MAP by
9.7 ± 0.6 versus 20.6 ± 0.7 mmHg in EP1+/− littermates (P < 0.0001; Figure 4, A and B). The blunted vasopressor effect of 17-phenyltrinor PGE2 is consistent with a systemic vasopressor role for the EP1 receptor. Because 17-phenyltrinor PGE2 also has activity at the EP3 receptor, we also tested the effect of a pure EP3 agonist, MB28767, on blood pressure in EP1+/− and EP1−/− mice. MB28767 produced a maximal pressor effect in EP1−/− mice identical to that observed in EP1−/− littermates (Figure 4C), suggesting the diminished pressor activity of the 17-phenyltrinor PGE2 was due to lack of a pressor EP1 receptor activity, rather than an effect on EP3 receptor.

Sulprostone, another EP1/EP3 agonist, also exhibited blunted pressor activity in EP1−/− mice (Figure 4D). Similarly, pretreatment of EP1+/− mice with selective EP1 receptor antagonist SC51322 significantly blunted the pressor effect of sulprostone (Figure 4E). In contrast, administration of EP1 receptor antagonist SC51322 had no effect on the pressor activity of sulprostone in EP1+/− mice (data not shown). The residual pressor effect of sulprostone in EP1+/− mice may be attributable to a separate pressor action of this agonist via the EP3 receptor.

Previous studies using a standard EP1 knockout generated in DBA1 mice suggested that deletion of the EP1 receptor reduced the vasodepressor response to the endogenous EP ligand, PGE2 (30). The present studies also found a modest reduction in the vasodepressor response to PGE2 (100 μg/kg i.v.) in male 129S6/SvEv EP1+/− mice (n = 8 per group; P < 0.005; Figure 5, A and B). To further characterize the mechanism underlying this difference, we tested the vascular effects of i.v. infusion of the vasodepressor EP2 and EP4 agonists (31) in EP1+/− mice. Although the vasodepressor effect of the EP2 agonist butaprost was similar in EP1+/− and EP1−/− mice (Figure 5C), the vasodepressor effect of the preferential EP4 agonist PGF2α-OH was significantly blunted in EP1+/− mice (Figure 5D), consistent with a functional interaction between vascular EP1 and EP4 receptors.

Effect of the EP1 receptor on renal salt and urine excretion. Acute infusion of PGE2 did not affect urine volume or sodium excretion in either EP1+/− or EP1−/− mice (data not shown). Furthermore, no differences in plasma aldosterone in EP1+/− versus EP1−/− mice on standard (0.25% w/w) NaCl diet were observed (835 ± 105 versus 695.9 ± 45 pg/ml; P = NS). To further examine whether the EP1 receptor affects renal salt or water excretion, we measured urine volume and sodium and potassium excretion in EP1+/− and EP1−/− mice sequentially placed on diets containing 0.25%, 0.02%, and 8% (w/w) NaCl. Urine volume and sodium excretion were significantly greater in EP1−/− than in EP1+/− mice following the switch from low- to high-sodium diet; however, this was accompanied by greater water and food intake in EP1−/− mice (Figure 6). Despite these differences in dietary sodium and water intake and excretion, systolic blood pressure remained unchanged in EP1+/− mice and significantly lower than in EP1−/− mice (Figure 6).

Discussion

The present studies demonstrate that endogenous EP1 receptor activation contributes to hypertension in 2 well-established rodent models: the SHR and chronic Ang II infusion. This conclusion is independently supported by results using what we believe to be a novel EP1−/− mouse and an EP1-selective receptor antagonist SC51322 (32). The latter not only reduced blood pressure in SHRs but also blocked the acute pressor effect of the EP1/EP3 agonist sulprostone in mice. Whether prolonged treatment with SC51322 results in a persistent reduction in blood pressure remains to be determined; however, a role for EP1 receptor antagonism is further
supported by findings that genetic disruption of the EP1 receptor in mice was associated with both sustained reduction in blood pressure and reduced cardiac weight following 4 weeks of Ang II infusion. Our results add to previous reports that endogenous COX products contribute to the vasoconstrictor activity of Ang II (10, 33) and are consistent with the prior finding that altered PGE2 action is involved in the pathogenesis of hypertension in SHRs (34). Notably, in contrast to the effects of prostaglandin synthase inhibition by NSAIDs, selective EP1 receptor inhibition reduced blood pressure, rather than increasing it.

The mechanism by which EP1 receptor disruption ameliorates hypertension is consistent with an altered vascular response to Ang II, as supported by the observation that EP1+/− mice exhibited a reduced acute vasopressor effect of Ang II infusion. This is further supported by the reduced pressor effect caused by the EP1 agonists sulprostone and 17-phenyltrinor PGE2 in EP1+/− mice, or following pretreatment with the EP1 antagonist SC532. Expression of EP1 receptor mRNA was demonstrated in peripheral resistance vessels and aortas, demonstrating vascular expression of the EP1 receptor. Direct functional interaction between angiotensin and EP1 receptor activity was demonstrated in in vitro mesenteric arterioles and preglomerular resistance arterioles, in which constrictor effects of Ang II were dramatically attenuated by the EP1 receptor antagonism, consistent with dependence of Ang II vasoconstriction on the EP1 receptor.

Gene targeting has proven a valuable tool for examining the biological roles of prostaglandin receptors (35). However, interpretation of the cardiovascular phenotypes in EP1+/− animals is complicated by the fact that the EP1 locus encodes both the EP1 receptor and PKN on the antiparallel strand (20). PKN is a serine/threonine protein kinase involved in PKC regulation and Rho function (21). Conventional gene targeting strategy uses the placement of an approximately 2-kb neomycin resistance cassette within the EP1 locus, and in this case could alter both the EP1 receptor and PKN transcripts. Although previous studies did not describe differences in PKN expression, immunoblots presented in those studies do not exclude the possibility of reduced PKN protein expression in EP1+/− mice (19). To circumvent alterations in PKN expression, we used a hit-and-run strategy whereby a premature stop codon was introduced in exon 2 of the EP1 locus and only 3 nucleotides were changed in the 3’ UTR of the PKN gene. As expected, altered PKN protein expression was not observed in EP1+/− mice generated in the present studies.

Despite concerns regarding PKN effects, many of the present findings are consistent with previous studies of standard DBA/1Jc EP1+/− mice (19). Both EP1+/− strains exhibited reduced basal blood pressure compared with EP1+/+ mice. In addition to its presence in arterial vasculature, the EP1 receptor is abundantly expressed in the renal collecting duct (36). The authors of the previous study speculated that the lower baseline blood pressure in EP1+/− mice was the result of renal sodium wasting, but did not perform studies directly testing this hypothesis (19). From our analysis of urinary sodium and water excretion, we failed to find any evidence for sodium wasting: during sodium restriction, there was no difference in sodium retention or in serum aldosterone levels between EP1+/− and EP1+/+ mice. Nor did we observe an acute drop in blood pressure over 3 days of salt restriction.

High salt intake also failed to normalize systolic blood pressure in EP1+/− mice. Previous studies of mice with collecting duct–specific modulation of endothelin-1 show the increase in blood pressure occurs within 1–2 days of instituting a high sodium diet (37), so the time frame of the present studies should have been adequate to discriminate a hypotensive phenotype due to EP1 action in the collecting duct. In contrast, on a high-salt diet, EP1+/− mice exhibited reduced rather than enhanced natriuresis and diuresis compared with EP1+/+ mice. While this is consistent with a role for the EP1 receptor in modulating either renal salt and water excretion (or dietary sodium and water intake), reduced sodium excretion on a high-salt diet is not consistent with sodium wasting being the primary cause of lower blood pressure in EP1+/− mice. Conversely, reduced sodium excretion on a high-salt...
butaprost reduced blood pressure to a similar absolute value in EP1+/− mice and EP1+/+ mice. These findings are consistent with a functional interaction between the EP1 and EP4 receptors. Although the precise mechanism(s) involved in this unexpected phenomenon remain speculative, one possibility is that EP1 receptor activity in endothelium contributes to EP4 receptor–dependent generation of vasodilators (e.g., nitric oxide), because both of these receptors have been identified in endothelial cells (43) and heterodimerization and crosstalk between prostanoid receptors has been suggested (44, 45).

PGE2 is a major COX product of vascular smooth muscle, and Ang II stimulates its synthesis in this tissue (46–48). Recent studies implicate COX1 activity in promoting PGE2 synthesis (10) and as critical for the pressor response to Ang II in both acute and chronic Ang II–dependent models (33, 49). The present data are consistent with a model whereby Ang II–stimulated synthesis of COX1-derived PGE2 activates the EP1 receptor to promote Ang II–dependent hypertension. The vasodepressor effect of the EP1 antagonist in the SHR is also consistent with the possibility that the EP1 receptor may act as a tonic vasoconstrictor contributing to hypertension. Recent studies of Ang II–induced hypertension used kidney crosstransplantation in AT1a knockout mice to and from wild-type mice to define nonredundant pressor roles for intrarenal and extrarenal AT1a receptors through which Ang II increases blood pressure (50). The present findings support hemodynamic effects of EP1 receptors promoting Ang II–dependent hypertension. Thus, as opposed to classic NSAIAD action, which enhances vasomotor tone and increases blood pressure (6, 51), EP1 receptor antagonism reduces blood pressure. The EP1 receptor may provide a novel target for the treatment of hypertension.

**Methods**

Chemical and molecular reagents. PGE2 and PGE2-OH were purchased from Cayman Chemical and BioMol Research Laboratories. Sulprostone was provided by Berlex Laboratories. MR28767 was generously provided by M. Caton (Rhone-Poulenc Rorer, Dagenham, United Kingdom). Butaprost and 17-phenyltrinor PGE2 were purchased from Cayman Chemical. SC51322 was purchased from BioMol Research Laboratories.

Construction of EP1 targeting vector and generation of EP1−/− mice using the hit-and-run strategy. All studies performed on rodents were reviewed and approved by the Institutional Animal Care and Use Committees of the Medical College of Georgia and Vanderbilt University. Mouse genomic clones containing the EP1 locus were obtained by screening a 129SVJ/ola genomic P1 library (Genome Systems) with a 233-bp murine EP1 cDNA fragment from the coding region of exon 2. A positive P1 clone was isolated and was sequenced. A 5,067-bp fragment containing all 3 exons of the EP1 gene was subcloned into pBluescript (Stratagene) by digesting with HindIII and XbaI. An 895-bp fragment was then amplified from this clone, and its sequence was altered using PCR-based mutagenesis. The mutations were introduced in the 3′UTR of the EP1 coding region to introduce a stop codon in the coding region of the EP1 receptor and a frame shift in the coding region of the EP1 receptor (30). The present findings using EP-selective agonists suggest a more complex mechanism, because the EP4-selective agonist PGE2-OH, which has an exceedingly low affinity for the EP1 receptor (41, 42), also exhibited reduced vasodilator activity in EP1−/− mice. In contrast, the EP2-selective agonist
cytoplasmic loop of the EP1 receptor, resulting in a nonfunctional receptor. The normal sequence within the 5,067 bp of HindIII-XbaI fragment was replaced with mutant fragment, reconstituting EP1 with a stop at codon 242. The 5,067-kb mutant EP1 fragment was then subcloned into the plasmid pKOSScrambler NTKV (Lexigen Inc.) that contains neo and TK selection cassettes.

Targeting the murine EP1 gene was accomplished by 2 steps using the hit-and-run strategy (25, 52). First, EP1 gene targeting vector was linearized by BstEII at a unique site between exons 1 and 2, prior to electro- porteration of TLI-1 ES cells. The linearized mutant gene inserts along with the neo and TK gene were introduced into ES cells, allowing homologous recombination via a single crossover event that results in a direct repeat tandem duplication of the EP1 gene. Cells were grown for 5 days, insertion events were selected by G418 resistance, and resistant clones were isolated and confirmed by Southern blot using 3 DNA probes (probes A, B, and C) shown in Suplemental Figure 1. The detection of a 5.7-kb EcoRI fragment hybridizing probe A indicates targeting of the vector to the EP1 locus, while the wild-type locus, lacking the mutant EcoRI site in exon 2, yielded a 10-kb fragment (Figure 2). Selecting EcoRI digests with probe C, composed of genomic sequence flanking the 3′ end of the EP1 gene, yielded an 11.5-kb fragment for events with the wild-type allele in the 3′ duplicate, documenting the insertion of an EcoRI site between neo and TK in the inserted vector (Supplemental Figure 1). The presence of the mutation in the 3′ duplicate resulted in a 4.3-kb fragment. In contrast, nontargeted EP1 loci yielded a 10-kb EcoRI fragment with all 3 probes. Second, because the direct repeat of the EP1 gene is unstable, spontaneous deletion of 1 allele arising via single, reciprocal intrachromosomal recombination occurred between the direct repeats, leading to deletion of the selectable cassettes, the plasmid backbone, and 1 repeat of the targeted loci. Targeted ES cells bearing the mutant allele were injected into blastocysts and introduced into a foster mother according to standard procedure carried out at Vanderbilt Transgenic Core.

**Analysis of wild-type and targeted EP1 alleles.** Both Southern blot- and PCR-based strategies were used for genotyping wild-type and EP1 mutant alleles. For Southern blot, mouse tail DNA digested with EcoRI was probed with a genomic DNA fragment (probe B; Supplemental Figure 1). The detection of a 10-kb EcoRI fragment with all 3 probes. For PCR analysis, a pair of primers was used to amplify a 1.066-bp genomic DNA fragment sparing the mutant site using the Advantage-GC Genomic PCR kit (BD Biosciences — Clontech). The sequences of the primers were as follows: sense, 5′-ACCCAGGCT-GCCATCTCTTTCTCCAT-3′; antisense, 5′-GTGAACAGCTGGGAAC-GAAAGGCTATGGA-3′. PCR reactions were carried out at 94°C for 30 seconds and 68°C for 3 minutes for 35 cycles. PCR products were then cut with EcoRI and separated on 1% agarose gel.

**RNA expression analysis.** Kidneys were harvested from EP1+/+ and EP1−/− mice. Total RNA was extracted using TRIzol Reagent (Invitrogen), treated with RNase-free DNase I (Promega), and reverse-transcribed to single-stranded cDNA using Moloney murine leukemia virus reverse transcriptase and 2.5 μM of random hexamers according to the manufacturer’s protocol (GeneAmp RNA PCR kit; PerkinElmer Cetus). The synthesized cDNA was then used to amplify a portion of EP1 cDNA sparing the mutation site. The upstream sense primer was 5′-CACCCTGGTGTATTATGCT-3′, and the downstream antisense primer was 5′-TAGCCTCAGCCATCATCAGC-3′. PCR reactions were carried out at 94°C for 30 seconds and 60°C for 60 seconds for 35 cycles in a PerkinElmer Cetus 2400 thermal cycler using the Advantage-GC DNA PCR kit (BD Biosciences — Clontech). The predicted 780-bp fragment was sequenced and subjected to EcoRI digestion (Supplemental Figure 1B).

**RT-PCR analysis of vascular expression of the EP1 mRNA.** Total RNA was extracted from aortas and mesenteric arteries of EP1+/+ mice and reverse-transcribed into cDNAs. The following set of primers specific for EP1 cDNA was used to examine EP1 mRNA expression: sense, 5′-TAACGATGG- GTCCCCAGGTG-3′; antisense, 5′-ATCCAGTGGGCGGTTAGGG-3′. The PCR conditions were the same as described in RNA expression analysis. Expressions of PKN in EP1−/− animals. Brain and kidney lysates (50 μg) prepared from EP1−/− mice and EP1+/+ controls were resuspended in RIPA buffer containing proteases inhibitors and subjected to immunoprecipitation adding 2 μg of PKN C-19 goat polyclonal antibody (Santa Cruz Biotechnology Inc.). Samples were incubated overnight at 4°C on a rocking platform. Hydrated protein G CL4B (20 μl) was added to each sample and incubated for 1 hour at 4°C. Immunocomplexes were pelleted by a brief centrifugation at 13,000 g, and the supernatant was removed. The pellet was washed 3 times with RIPA buffer containing protease inhibitors, resuspended in a volume of Laemmli buffer, and boiled for 10 minutes. Solubilized immunocomplexes were resolved by 4%–20% gradient SDS-PAGE and transferred on PVDF membrane. Western blot analysis was performed using PKN C-19 goat polyclonal antibody (1:200 dilution) followed by a rabbit anti-goat horseradish peroxidase–conjugated secondary antibody (1:2,000 dilution).

**Blood pressure measurement using tail-cuff and carotid catheterization in mice.** Male congenic 129S6/SvEVTac or C57BL/6 mice aged 12–16 weeks and with 20–25 g body weight were used. Following a 2-week training period, basal blood pressure was measured in conscious EP1+/+ and EP1−/− mice using the tail-cuff method as previously reported (29). These mice were then provided with normal-sodium diet for 2 days (0.25% w/w), followed by 4 days of low-salt diet (0.025% w/w) and 4 days of high-salt diet (8% w/w). Urine was collected over 24-hour periods by housing mice in metabolic cages.

For infusion study, mice were anesthetized with 80 mg/kg ketamine (Fort Dodge Laboratories) and 8 mg/kg inactin (BYK) by i.p. administration. Mice were placed on a temperature-controlled pad. After tracheostomy, PE-10 tubing was inserted into the right carotid artery, a jugular vein catheter was placed for infusion, and a urinary bladder catheter was inserted for urine collection. Blood pressure was measured with a Cobe CDX II transducer connected to a blood pressure analyzer (BPA 400; Micromed) as previously reported (13, 29). The readings of blood pressure and heart rate were equilibrated for 30–60 minutes until stable values were obtained. The test agents (PGE2, salpstone, SCS1322, 17-phenyltrinor PGE2, and PGE2OH dissolved in absolute ethanol) were mixed with 25 μl of saline and injected as a bolus via the jugular vein. To examine the hypertensive response to Ang II in EP1−/− and EP1+/+ mice, Ang II (Sigma-Aldrich) was dissolved in normal saline. Mice were continuously infused via carotid vein with Ang II at a rate of 75 pmol/kg/min for 30–45 minutes. Blood pressure, including systolic and diastolic pressure and MAP, and heart rates were recorded continuously on a thermal printer or computerized data record (13, 29).

**Telemetric recording of blood pressure in SHRs.** Male SHRs (n = 5) weighing approximately 250 g were used. Animals were anesthetized with phenobarbital (50 mg/kg i.p.), a midline abdominal incision was made, the intestines were displaced cranially, and the lower abdominal aorta was exposed. Vaso- spasm was reduced by the application of lidocaine 2% along the aorta. A small puncture was made in the aorta proximal to the femoral bifurcation, and the catheter of the transmitter was inserted and sealed in place with tissue adhesive (Verbond; 3M Animal Care Products) and a cellulose patch (53). All procedures were performed aseptically. Following implantation, rats were returned to their home cages for monitoring and allowed to recover for 3 days prior to data collection. Continuous ambulatory systolic and diastolic blood pressure recordings were made via a radiofrequency receiver in conscious, unrestrained animals at a sampling rate of 64 Hz for 10 seconds, performed every 3 minutes for 1 hour (20 measurements), using the TAI11PA-C20 pressure transmitter (Data Sciences International). Following surgery, the telemetry failed in 1 rat, which died; in another rat, the MAP was less than 110 mmHg, and so it was excluded from further experimentation. The remaining 3 animals were housed individually, and baseline heart
rate and MAP were determined for 6 days followed by a 3-day baseline blood pressure determination prior to treatment with the EPI receptor antagonist for 3 days. MAP was monitored for 3 days during daily oral treatment with SC51322 (10 mg/kg) and for 2 days after discontinuation of treatment.

Establishment of Ang II–dependent hypertension. Ang II–dependent hypertension was induced in EPI+/− and EPI−/− mice (n = 5 per group) as previously reported (49). Briefly, Ang II (1000 ng/kg/min; Sigma-Aldrich) dissolved in sterile saline or sterile saline alone for control animals was infused using an osmotic minipump (Alzet model 2004; Alza Corp.) that was inserted subcutaneously during an isoflurane anesthesia (3%). After 1 week of full recovery from the surgery, systolic blood pressure was recorded once a week for 1 month via computerized tail-cuff method. At the end of study, mice were anesthetized and catheters were inserted in carotid arteries and jugular veins. After 10-minute equilibration, MAP and heart rate were determined using a blood pressure analyzer (BPA-400; Micromed) as described above.

Renal microvascular responses. The perfused juxtamedullary nephron preparation was used to assess renal microvascular reactivity as described previously (54). Male C57BL/6 mice weighing 20–25 g were used. Mice were anesthetized with thiobutabarbital (inactin; 100 mg/kg i.p.). The kidney was removed and immediately isolated and perfused in vitro with a physiologic salt solution and mixture of l-amino acids (54). The juxtamedullary vasculature was isolated for study, and an afferent arteriole was monitored continuously by videomicroscopy. After control diameter measurements, angiotensin (0.1–100 nM) or 17-phenyltrinor PGE2 (1 μM) was delivered by superfusion, and the afferent arteriolar diameter response was determined.

In a separate set of experiments, the response to angiotensin was determined in the presence of the EPI antagonist SC51322 (1 μM).

Mesenteric arterial vascular tension. Mesenteric arteries (outer diameter, 0.4–0.7 mm) were dissected and cut into rings 5 mm in length in ice-cold modified Krebs-Ringer bicarbonate buffer (118.3 mM NaCl; 4.7 mM KCl; 2.5 mM CaCl2; 1.2 mM MgSO4; 1.2 mM KH2PO4; 25.0 mM NaHCO3; and 11.1 mM glucose). Rings of mesenteric arteries were suspended in organ chambers filled with 15 ml of the modified Krebs-Ringer bicarbonate solution maintained at 37 ± 0.5°C and aerated with 95% O2 and 5% CO2 (pH 7.4). Two stirrups passed through the lumen suspended each ring. One stirrup was anchored to the bottom of the organ chamber and the other connected to a strain gauge, and the isometric force was measured with a ML785 PowerLab/8sp recording and Analysis System (ADInstruments) (55).

At the beginning of the experiment, each vessel ring was stretched to its optimal resting tension by step-wise stretching until the active contraction of the vessel ring to 100 mM KCl reached a plateau. The optimal resting tension of mouse mesenteric arteries was approximately 0.6 g. After the vessels were brought to their optimal resting tensions, 1 hour of equilibration was allowed. Concentration-response curves to Ang II were constructed in a cumulative fashion in vessels. In some experiments, SC51322, an EPI receptor blocker, was administered to vessels at least 20 minutes prior to testing its effect contraction of the vessels evoked by Ang II.

Urinary sodium and potassium measurement. Urine volumes were measured during perfusion study, and urinary sodium and potassium concentrations were analyzed using an IL943 Flame Photometer (Instrumentation Laboratory).

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