Biphasic Actions of Prostaglandin E₂ on the Renal Afferent Arteriole
Role of EP₃ and EP₄ Receptors

Lilong Tang, Kathy Loutzenhiser, Rodger Loutzenhiser

Abstract—Prostaglandin (PG) E₂ is an important modulator of the actions of angiotensin (Ang) II. In the present study, we investigated the renal microvascular actions of PGE₂ and the EP receptor subtypes involved. Ibuprofen potentiated Ang II–induced vasoconstriction in in vitro perfused normal rat kidneys and augmented afferent arteriolar, but not efferent arteriolar, responses in the hydronephrotic rat kidney model. This preglobular effect of endogenous prostanoids was mimicked by exogenous PGE₂, which reversed Ang II–induced afferent arteriolar vasoconstriction at concentrations of 0.1 to 10 nmol/L without affecting the efferent arteriole. The PGE₂-induced vasodilation was potentiated by the phosphodiesterase inhibitor Ro 20-1724 and was mimicked by 11-deoxy-PGE₁ (0.01 to 1 nmol/L). Butaprost, which acts preferentially at EP₂ receptors, was relatively ineffective. Whereas 0.1 to 10 nmol/L PGE₂ elicited vasodilation, higher concentrations (1 to 10 μmol/L) restored Ang II–induced afferent arteriolar vasoconstriction. This response was blocked by pertussis toxin (200 μg/mL) and was mimicked by the EP₃/EP₄ agonist sulprostone (1 to 300 nmol/L). Reverse transcription–polymerase chain reaction of individually isolated afferent arterioles revealed the presence of message for EP₂ receptor subtypes.

Key Words: receptors ■ microcirculation ■ arterioles ■ cyclooxygenase ■ angiotensin II

Prostaglandin (PG) E₂ is the major prostanoid produced by the kidney and plays an important role in modulating the renal microvascular response to vasoconstrictors.¹ During conditions associated with low renal perfusion pressure, elevated renin activity, and increased renal sympathetic traffic, normal renal function may depend on the modulatory actions of PGE₂ on angiotensin (Ang) II– and catecholamine-induced renal vasoconstriction. In clinical settings such as congestive heart failure, interference with this compensatory mechanism by the administration of nonsteroidal anti-inflammatory agents can lead to a deterioration of renal function and renal insufficiency.² It has now been disclosed that the diverse biological actions of PGE₂ are mediated by specific members of a group of G protein–linked (EP) receptors (reviewed in Reference 3). Thus, future therapies may allow a more selective intervention based on the development of selective receptor antagonists capable of blocking undesirable effects of the prostanoids while preserving their renal protective actions. This approach will require information on the renal microvascular actions of PGE₂ and the identification of the specific EP receptor subtypes involved.

Although PGE₂ is recognized to play a critical role in modulating renal vasoconstriction, the actions of PGE₂ on the renal arterioles have not been thoroughly investigated. Only 2 studies to date have directly examined the renal microvascular actions of PGE₂. In a study of isolated rabbit arterioles, Edwards⁴ reported that PGE₂ elicited afferent arteriolar vasodilation but had no effect on the efferent arteriole. No constriction responses to PGE₂ were observed in this study. In contrast, Inscho et al.,⁵ using the blood-perfused juxtamedullary nephron preparation, found 1.0 μmol/L PGE₂ to evoke afferent arteriolar vasoconstriction in the rat. Only 1 concentration was used, and the effects of PGE₂ on the efferent arteriole were not examined. Neither study examined the EP receptor subtypes or signaling pathways involved.

At least 4 distinct genes encoding for 4 separate subtypes of PGE₂ receptors, EP₁ to EP₄ (reviewed in Reference 3), have been identified. The mRNA for all 4 EP receptor subtypes has been reported to be expressed in the kidney.⁶–⁹ EP₂ and EP₄ receptors are generally associated with smooth muscle relaxation, whereas EP₁ and EP₃ receptors are reported to elicit smooth muscle contractile responses. However, although the receptor subtypes involved in the tubular epithelial actions of PGE₂ have been partially characterized (reviewed in Reference 10), we know little of the EP receptor subtypes and...
signaling pathways involved in the renal microvascular actions of PGE2. In the present study, we used the in vitro perfused normal rat kidney and the in vitro perfused hydronephrotic rat kidney models to investigate the actions of PGE2 on the afferent and efferent arterioles. We used reverse transcription–polymerase chain reaction (RT-PCR) assays of microdissected vessels to determine EP receptor subtype expression in the renal afferent arteriole. Our findings indicate that PGE2 exerts a selective effect on the afferent arteriole, eliciting both vasodilation and vasoconstriction of this vessel. The vasodilation appears to be mediated by the EP3 receptor coupled to a cAMP-dependent mechanism. The vasoconstrictor actions of PGE2 are mediated by an EP3 receptor coupled to Gaii and appear to involve a functional antagonism of the EP2-induced vasodilation.

Materials and Methods

In Vitro Perfused Hydronephrotic Kidney

Male Sprague-Dawley rats were used in all experiments. The in vitro hydronephrotic rat kidney11 was used to examine the afferent and efferent arteriolar actions of PGE2. Unilateral hydronephrosis was induced by ligation of the left ureter. Kidneys were harvested after 6 weeks, when tubular atrophy allows a direct visualization of the renal microvasculature. The renal artery was cannulated, and the kidney was excised with continuous perfusion. Kidneys were perfused at a renal arterial pressure of 80 mm Hg, with a modified Dulbecco's medium containing 30 mmol/L bicarbonate, 5 mmol/L glucose, and 5 mmol/L HEPES and equilibrated with 95% air/5% CO2 (37°C, pH 7.4). Diameters were measured by online image processing. Afferent diameters were measured between the midpoint and origin. Efferent arteriolar diameters were measured within 50 μm of the glomerulus.

Initial studies were performed to determine the effects of basal prostaglandins. In all subsequent studies, kidneys were pretreated with 10 μmol/L ibuprofen to eliminate endogenous prostanoids. PGE2 was obtained from Calbiochem and prepared fresh daily. Ro 20-1724 was obtained from Research Biochemicals International and was administered at 0.5 μmol/L. Sulprostone and 11-deoxy-PGE1 were obtained from Cayman Chemical Co. Butaprost was provided by Dr Kluender of Bayer Pharmaceutical Division. SC-51322 was obtained from BIOMOL Research Laboratories.

In Vitro Perfused Normal Kidney

For the normal kidney studies, rats weighing from 220 to 275 g were anesthetized with methoxyflurane. The left renal artery was cannulated, and the kidney was excised with continuous perfusion. Kidneys were perfused in vitro at 80 mm Hg as described above. Perfusate flow was measured with a transonic flowmeter (model T106, Transonic Systems, Inc).

EP mRNA Expression in Isolated Afferent Arterioles

Normal kidneys were perfused with 2% agarose (37°C in DMEM) and chilled (4°C). Cortical slices (400 μm) were prepared and treated with collagenase IV, hyaluronidase IV, dispase II, and DNase I. Six to 8 arterioles were individually isolated and washed 3 times. RT-PCR was run on the final wash to test for contamination. Arteriolar total RNA was extracted, and cDNA was synthesized with random primers. To eliminate genomic contamination, a separate sample was treated identically, but without reverse transcriptase (Figure 8A and 8B). Primers for RT-PCR assays were designed from the reported EP receptor sequences12–17 (Table 1 online, see http://www.circresaha.org). We assayed for a sequence common to all EP receptors (EP,sub) and sequences specific for EP1, EP2, EP3, and each EP splice variant. A whole-kidney homogenate was used as a positive control, and primers for GAPDH18 were used for an internal control. After 35 cycles, PCR products were separated by electro-

Figure 1. A, Ibuprofen potentiated Ang II–induced afferent arteriolar vasoinhibition in in vitro perfused hydronephrotic rat kidney model (left) but had no effect on reactivity of the efferent arteriole (right, n=8, afferent, efferent). Basal diameters are indicated by squares. Open symbols represent control responses. Open symbols represent the responses after 10 μmol/L ibuprofen. *P<0.05. B, Ibuprofen potentiated the vasoconstrictor actions of Ang II in isolated perfused normal kidneys (n=5). Basal renal perfusate flows indicated by squares. Closed symbols represent control responses. Open symbols represent the responses after 10 μmol/L ibuprofen. *P<0.05.

phoresis (2% agarose gel) and stained with ethidium bromide. The identity of the products obtained with each set of primers was confirmed by DNA sequencing. When negative results were obtained, fresh polymerase was added after 35 cycles, and the PCR was continued to 70 cycles.

Analysis

Data are expressed as the mean±SEM. Differences were evaluated by ANOVA and Student’s t test (paired or unpaired). Values of P<0.05 were considered significant. For multiple comparisons, the Bonferroni correction was applied, and values of P<0.05/n were considered significant.

An expanded Materials and Methods section is available online at http://www.circresaha.org.

Results

Initial studies were conducted to determine the influence of endogenous prostaglandins on Ang II responses of the in vitro perfused hydronephrotic kidney preparation and of in vitro perfused normal rat kidneys. The effects of 10 μmol/L ibuprofen on the afferent and efferent arteriolar actions of Ang II are presented in Figure 1A. Basal diameters were not affected by ibuprofen in either the afferent (15.6±1.8 versus 15.4±1.6 μm, n=8, P=0.84) or efferent (11.2±1.2 versus 11.7±1.2 μm, n=8, P=0.68) arteriole. In the afferent arte-
perfusate flows were 13.4 ± 0.003). As presented in Figure 1B, ibuprofen significantly altered by ibuprofen, and 0.03 nmol/L Ang II elicited significant afferent arteriolar vasoconstriction in both settings.

Ibuprofen also potentiated Ang II–induced vasoconstriction in normal perfused rat kidneys (Figure 1B). In the control group, basal perfusate flows were 14.6 ± 0.9 mL·min⁻¹·g⁻¹ kidney wt⁻¹ (n=5), and Ang II elicited significant vasoconstriction at 0.1 nmol/L (9.5 ± 1.0 mL·min⁻¹·g⁻¹, P<0.0001). In kidneys pretreated with 10 µmol/L ibuprofen, basal perfusate flows were 13.4 ± 0.6 mL·min⁻¹·g⁻¹ (P=0.26 versus control, n=5) and Ang II elicited significant vasoconstriction at 0.03 nmol/L (11.8 ± 0.5 mL·min⁻¹·g⁻¹, P=0.0036). As presented in Figure 1B, ibuprofen significantly potentiated the vasoconstrictor response to Ang II at concentrations of 0.03 and 0.1 nmol/L (P<0.05). In contrast, the efferent arteriolar response to Ang II was not significantly altered by ibuprofen, and 0.03 nmol/L Ang II elicited significant efferent arteriolar vasoconstriction in both settings.

Because the major prostanoid produced by the kidney is PGE₂, we examined the effects of exogenous PGE₂ on the afferent and efferent arteriolar response to Ang II. In these studies and those that follow, kidneys were pretreated with 10 µmol/L ibuprofen to eliminate endogenous prostanoid formation. As shown in Figure 2, 0.1 nmol/L Ang II reduced afferent arteriolar diameter by 43±7% (n=8) and efferent arteriolar diameter by 23±5% (n=7, P<0.001). PGE₂ exerted a biphasic action on the afferent arteriole. Over concentrations ranging from 1 to 10 nmol/L, PGE₂ elicited afferent arteriolar vasodilation, completely reversing the Ang II–induced vasoconstriction at concentrations >100 nmol/L, PGE₂ elicited vasoconstriction. Thus, whereas PGE₂ completely reversed Ang II–induced vasoconstriction at a concentration of 10 nmol/L (14.2±0.6, 8.2±1.2,* and 13.4±0.7 µm for control, Ang II, and Ang II plus 10 nmol/L PGE₂, respectively; *P<0.05 versus control), increasing the PGE₂ concentration to 1.0 µmol/L fully restored the vasoconstriction (7.0±1.4 µm, P>0.05 versus Ang II alone). In the absence of Ang II (Figure 2, top), PGE₂ also elicited a modest vasodilation and constriction at concentrations of 10 nmol/L and 1 µmol/L (P<0.05). However, the constrictor actions of PGE₂ (1.0 µmol/L) were far greater in the presence of Ang II (51±8% decrease in diameter) than in its absence (11±3% decrease). Finally, in contrast to the afferent arteriole, the efferent arteriolar actions of Ang II were not significantly altered by PGE₂ over concentrations ranging from 1.0 nmol/L to 1.0 µmol/L (Figure 2, bottom).

To investigate the signaling pathway involved in the vasodilation, we determined the effects of the specific type IV phosphodiesterase inhibitor Ro 20-1724. A concentration of 0.5 µmol/L Ro 20-1724 was selected for these studies because we found that this concentration does not produce vasodilation by itself but rather potentiates the vasodilatory actions of adenosine (not shown). Two groups of hydronephrotic kidneys (controls and Ro 20-1724–treated, n=5 for each) were preconstricted with 0.1 nmol/L Ang II, and the afferent arteriolar vasodilatory actions of PGE₂ were assessed. The inhibition of the Ang II–induced vasoconstriction was calculated, and these data are summarized in Figure 3. Ro 20-1724 markedly potentiated the actions of PGE₂, shifting the ED₅₀ by ≈1 log unit. These findings are consistent with the currently held view that the vasodilatory actions of PGE₂ are mediated by cAMP.

Both EP₂ and EP₄ have been shown to be coupled to Gₐ and are thought to produce vasodilation by stimulating the
To characterize the receptor involved in the afferent arteriolar vasodilation, we compared the potency of 11-deoxy-PGE₁ and butaprost. These 2 agonists have similar affinities for the EP₂ receptor but markedly different affinities for the EP₄ receptor. Figure 4 summarizes the results of studies comparing the actions of these 2 agonists on afferent arterioles preconstricted with 0.1 nmol/L Ang II (n=5 for both groups). As depicted, 11-deoxy-PGE₁ fully reversed Ang II–induced afferent arteriolar vasoconstriction (ED₅₀ =0.1 nmol/L). In contrast, butaprost was a relatively weak vasodilator in this preparation. These results are consistent with an EP₄–mediated vasodilation.

The biphasic response of the afferent arteriole to PGE₂ indicates actions at multiple receptor subtypes, linked to both vasodilator and vasoconstrictor mechanisms. To characterize the constrictor response, we examined the effects of pretreatment with pertussis toxin (PTX). In these studies, kidneys were pretreated with 200 μg/mL PTX for 1 hour with a recirculating circuit. Control kidneys were treated in an identical manner with vehicle alone. The kidneys were then returned to the single-pass perfusion system and challenged with Ang II and PGE₂. As shown in Figure 5, PTX had no effect on the magnitude of the Ang II–induced vasoconstriction and did not alter the vasodilatory actions of 10 nmol/L PGE₂. However, PTX treatment completely abolished the vasoconstrictor actions of 1.0 μmol/L PGE₂.

Of the known PGE₂ receptors, the EP₃ receptor subtype has been shown to be coupled to the PTX-sensitive G protein Gₛ. We next examined the effects of the EP₁/EP₃ receptor agonist sulprostone. As shown in Figure 6A, sulprostone did not elicit afferent arteriolar vasodilation in kidneys preconstricted with Ang II and, in this setting, had no vasoconstrictor activity. However, as described above (Figure 2), the vasoconstrictor actions of PGE₂ were much more evident when administered to preconstricted vessels that were then dilated with low concentrations of PGE₂. We therefore designed studies to mimic this condition, first inducing vasoconstriction with Ang II, then eliciting full vasodilation with 10 nmol/L PGE₂, and then administering sulprostone (Figure 6B). Under these conditions, sulprostone caused a dose-dependent vasoconstriction, eliciting threshold contractions at concentrations of 1.0 to 10 nmol/L. Because sulprostone can activate both EP₁ and EP₃, we examined the effects of SC-51322, an EP₁-selective antagonist (pA₂ =8.1), on this response. As shown in Figure 1 online (see http://www.circresaha.org), SC-51322 at concentrations of 3 and 10 μmol/L had no effect on the sulprostone-induced vasoconstriction, suggesting that this response is mediated by EP₃ receptors.

The results of the above studies suggest that an EP₃ receptor coupled to a PTX-sensitive G protein mediates the afferent arteriolar vasoconstrictor effects of PGE₂ in the hydropnephrotic kidney model. To determine whether a similar response occurs in the nonhydronephrotic kidney, parallel studies were performed using isolated perfused normal kidneys. Kidneys were treated with 10 μmol/L ibuprofen and perfused at a constant arterial pressure of 80 mm Hg. Vaso-
constriction was established with 0.1 nmol/L Ang II, and then 10 nmol/L PGE$_2$ was administered. As depicted in Figure 7, PGE$_2$ partially reversed the Ang II–induced vasoconstriction, consistent with a selective afferent arteriolar vasodilation. In this setting, sulprostone elicited a similar vasoconstrictor response.

Finally, to determine the expression of EP receptors in the renal afferent arteriole, RT-PCR was used to assay EP receptor mRNA in individually isolated afferent arterioles. As depicted in Figure 8A, the presence of afferent arteriolar message for EP$_3$ (EP$_{3a}$) and EP$_4$ receptors, but not for EP$_1$ or EP$_2$ receptors, could be demonstrated with this approach. To further identify the EP$_1$, receptors present, individual primers for mRNA regions specific for the $\alpha$, $\beta$, and $\gamma$ splice variants were used. As shown in Figure 8B, we found the mRNA for all 3 splice variants to be expressed in the normal afferent arteriole.

**Discussion**

The diverse actions of PGE$_2$ on the kidney are mediated by a distinct group of G protein–coupled receptors. Four genes encoding for differing PGE$_2$ receptors have been cloned (EP$_1$ to EP$_4$), and message for all 4 receptor subtypes is found in the kidney. Previous studies have focused on the EP receptors mediating the renal tubular actions of PGE$_2$, but no information is currently available on the renal microvascular EP receptors. The present study indicates that the afferent arteriole of the rat expresses message for the EP$_3$ receptor and for 3 known splice variants of the EP$_1$ receptor ($\alpha$, $\beta$, and $\gamma$). We found PGE$_2$ to elicit vasodilation at low concentrations (ED$_{50} < 3$ nmol/L) by activating EP$_1$ receptors coupled to cAMP. This vasodilation is reversed at higher concentrations (>100 nmol/L). This vasococonstrictor response is mediated by an EP$_{3a}$ receptor coupled to $\gamma_{2}$ and reflects a functional antagonism of the EP$_{2a}$-mediated vasodilation.

The present study used the hydronephrotic rat kidney model to assess microvascular responses. Although this model is a widely used method of studying the renal microvasculature, it was important to characterize the role of endogenous prostanoids in this preparation. During the onset of hydronephrosis, infiltrating macrophages increase renal thromboxane production, eliciting vasoconstriction. For microvascular studies, a long-term preparation (6 to 8 weeks’ duration) is used. At this stage, tubular atrophy is complete, renal thromboxane production returns to normal levels, and SQ29548 has no effect on basal tone, indicating a lack of thromboxane-dependent vasoconstriction. The reactivity of our in vitro model to Ang II was similar to that of the normal kidney, because threshold vasoconstrictor responses were observed at concentrations of 0.03 to 0.1 nmol/L in both preparations. Moreover, the reactivity to Ang II was similarly potentiated by cyclooxygenase inhibition, suggesting a predominant influence of endogenous vasodilatory prostanoids in both models (Figure 1A and 1B).

Cyclooxygenase inhibition potentiated the afferent arteriolar actions of Ang II but had no effect on the efferent arteriole, indicating a selective preglomerular action of en-
dogenous prostanoids in our model. These findings are consistent with previous observations using in vivo preparations. For example, Heller and Horacek found that cyclooxygenase inhibition potentiates the ability of Ang II to reduce glomerular filtration rate in the dog. These authors suggested a protective role of vasodilatory prostanoids in suppressing Ang II–mediated afferent arteriolar vasoconstriction. Our results are in agreement with this interpretation and suggest that the prostanoid involved in this action is PGE₂, the major prostanoid produced by the normal kidney and by cortical interstitial cells of the hydropnephrotic kidney. Like the endogenous prostanoid, exogenous PGE₂ altered the afferent arteriolar effects of Ang II but had no effect on the efferent arteriole. This selective afferent arteriolar effect of PGE₂ observed in our rat kidney model agrees with previous findings by Edwards, who used isolated afferent and efferent arterioles from the rabbit. Edwards reported PGE₂ to block the afferent arteriolar response to norepinephrine but to have no effect on the efferent arteriolar actions of either norepinephrine or Ang II. Ang II does not constrict the afferent arteriole in Edwards’ preparation.

At concentrations >100 nmol/L, we found PGE₂ to promote afferent arteriolar vasconstriction. A number of previous studies have documented both vasodilator and vasoconstrictor actions of PGE₂ (reviewed in Reference 1). Edwards did not observe PGE₂-induced vasoconstriction in isolated rabbit arterioles. However, in a study using the blood-perfused juxtamedullary nephron preparation of the rat, Insho et al found PGE₂ to elicit afferent arteriolar vasoconstriction. In the Insho study, only a single concentration of PGE₂ (1.0 μmol/L) was used, and efferent arteriolar responses were not reported. Our observation that 1.0 μmol/L PGE₂ elicited afferent arteriolar vasoconstriction in the hydropnephrotic kidney model is consistent with these findings. The fact that PGE₂ elicits both constrictor and dilator responses clearly indicates an involvement of multiple EP receptor subtypes.

EP₂ and EP₄ receptors are both linked to vasodilation (reviewed in Reference 3). Our studies indicate that in the afferent arteriole, PGE₂ elicits vasodilation by activating EP₂ receptors. In this vessel, we found 11-deoxy-PGE₁ to be a potent vasodilator, whereas butaprost was relatively ineffective (Figure 4). Butaprost and 11-deoxy-PGE₂ are both EP₂ agonists and exhibit similar potencies at this receptor subtype. However, 11-deoxy-PGE₁ is also a potent EP₁ agonist, whereas butaprost has very weak activity at this receptor. Thus, the rank order of potencies we observed (11-deoxy-PGE₁ >> butaprost) suggests that the vasodilation involves the EP₁ receptor rather than the EP₂ receptor. This interpretation is consistent with the results of our RT-PCR studies, in that we found that message for EP₁ but not EP₂ was expressed in the afferent arteriole. We could not demonstrate the presence of EP₂ mRNA even under extreme conditions (70 cycles). Previous studies have reported low expression levels of EP₂ mRNA in the kidney and an absence of this signal in the renal cortex. We also found low levels of EP₂ mRNA in whole-kidney homogenates (Figure 8A). In contrast, EP₂ receptor mRNA is highly expressed in the kidney and has been localized to the glomerulus. Both EP₂ and EP₄ receptors have been shown to be coupled to Gα and stimulation of adenylyl cyclase (reviewed in Reference 3). The phosphodiesterase inhibitor Ro 20-1724 potentiated the vasodilatory response to PGE₂ in our study, a finding in agreement with previous observations suggesting that the renal microvascular actions of PGE₂ are mediated by an elevation in cAMP. Our observations are thus consistent with a signaling pathway involving PGE₂ stimulation of EP₄ receptors, leading to the activation of Gα, adenylyl cyclase, and vasodilation via cAMP-dependent mechanisms.

Our studies suggest that the vasconstrictor actions of PGE₂ involve an EP₃ receptor. Sulprostone caused vasoconstriction at concentrations of 10 to 100 nmol/L, whereas constrictor responses to PGE₂ occurred only at much higher concentrations (0.3 to 1.0 μmol/L). Sulprostone acts on both EP₁ and EP₃ receptors but is less potent than PGE₂ on EP₁ receptors and more potent on EP₂ receptors. The relative potency of sulprostone versus PGE₂ is thus consistent with an EP₁ response. This interpretation is supported by our observation that SC53122, an EP₁-selective antagonist, did not alter the vasconstrictor response to sulprostone. The RT-PCR studies are also consistent with an involvement of EP₁ rather than EP₂ receptors, because we found that the afferent arteriole expresses message for all 3 splice variants of the rat EP₁ receptor, but we could not demonstrate message for EP₂ in this vessel. Finally, the contractile response to PGE₂ was prevented by PTX, indicating a coupling to Gα or Gα. EP₁-mediated responses are insensitive to PTX and generally linked to Gα, whereas Gα coupling is a common characteristic of EP₃ receptors. In concert, these observations provide compelling evidence that the EP₁ receptor mediates PGE₂-induced afferent arteriolar vasconstriction.

Thus, our functional studies indicate that in the rat, the renal microvascular actions of PGE₂ are mediated by EP₁ and EP₂ receptors. We found no evidence for the expression of EP₂ or EP₃ receptors in the rat afferent arteriole. In contrast, Morath et al recently reported that in the human kidney, the afferent arteriole stains positively for all 4 EP receptor subtypes. One obvious explanation of this difference is the possibility of species-dependent variability in EP receptor expression. Another possibility is that EP₁ and EP₂ mRNAs are present, but at levels that were below the detection limits in our assay. However, we found the pharmacological attributes of the responses we observed to be consistent with the results of the RT-PCR assays in that each approach suggested a primary role of EP₁ and EP₂ receptors.

We found message for all 3 known splice variants of the rat EP₁ receptor in the rat afferent arteriole. The variable region of the EP₁ receptor subtypes is confined to the intracellular carboxylic tail and determines G protein coupling. Because the sequence is identical from the amino-terminus through the seventh transmembrane domain, including the external portion of the receptor, the EP₁ α, β, and γ isoforms are thought to have similar ligand-binding characteristics. All 3 rat EP₁ receptor isoforms can couple to Gα, EP₁γ can also couple to Gα, and can stimulate adenylyl cyclase. However, sulprostone had no vasodilatory effect on the afferent arteriole (Figure 6), which argues against a role of EP₁ receptors in the PGE₂-induced afferent arteriolar vasodilation. A fourth vari-
ant, EP3, is coupled to a PTX–insensitive G_i/o.3,34 However, to the best of our knowledge, this splice variant has not yet been found in the rat.

Although the role of the vasodepressant action of PGE2 is widely appreciated, the significance of the PGE2-induced vasocconstriction is not understood. In our study, PGE2 elicited a pronounced vasoconstriction in the presence of Ang II but only a modest response when administered alone (Figure 2). Similarly, full expression of the sulprostone-induced vasoconstriction required pretreatment with both Ang II and PGE2. We interpret these observations as suggesting that EP3 receptor activation alone is not sufficient to cause vasoconstriction but rather promotes vasoconstriction by counteracting EP4 signaling and reversing the PGE2-induced vasodilation. Thus, at low concentrations, PGE2 stimulates EP4 receptors, activates G_ia and adenyl cyclase, and elevates cAMP. At higher concentrations or under conditions in which the EP4 receptor expression is upregulated, PGE2 concurrently stimulates EP3 receptors. EP3 receptor activation inhibits adenyl cyclase and decreases cAMP via G_i/o, thereby attenuating the EP4-mediated vasodilation. Activation of EP3 receptors by PGE2 and sulprostone have been shown to reduce cAMP-dependent water flux in the cortical collecting duct by a similar mechanism.33 Thus, the EP3 receptor may provide a functional antagonism of the vasoconstrictor action of PGE2 to reduce cAMP-dependent water flux in the cortical collecting duct by a similar mechanism.33 Thus, the EP3 receptor may provide a functional antagonism of the vasoconstrictor action of PGE2.

In summary, the present study demonstrates that endogenous prostanooids and exogenous PGE2 preferentially modulate the reactivity of the afferent arteriole. In this vessel, PGE2 elicits a biphasic response, eliciting vasodilation at low concentrations (1 to 10 nmol/L) and reversing this effect at higher concentrations. The vasodilation is mediated by EP3 receptors and is dependent on cAMP. The vasoconstriction is mediated by an EP3 receptor and is blocked by PTX, implicating a mechanism dependent on G_i/o and reduced cAMP formation. These findings suggest a functional antagonism of these 2 receptor systems. We suggest that in the renal microvasculature, EP3 receptors may play a modulatory role on the vasodepressant and renal hemodynamic actions of PGE2.

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References


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Supplementary Methods, Table and Figure 1 Online

*In Vitro* Perfused Hydronephrotic Kidney Studies:

Male Sprague Dawley rats were used in all experiments. The *in vitro* perfused hydronephrotic rat kidney was used to examine the renal microvascular actions of PGE2. This model has been described in previous publications (1). In brief, unilateral hydronephrosis was induced by ligating the left ureter under halothane-induced anesthesia. Kidneys were harvested after 6 weeks, at which time the tubular atrophy has progressed to a stage that allows direct visualization of the renal microvasculature. The renal artery was cannulated and the hydronephrotic kidney was excised with continuous perfusion. The kidneys were perfused at a renal arterial pressure of 80 mmHg. The perfusate consisted of a modified Dulbecco's medium containing bicarbonate 30 mM, glucose 5 mM, and hepes 5 mM, and was equilibrated with 95% air / 5% CO2 (pO2 150 mmHg). The perfusate was not recirculated. Temperature and pH were maintained at 37°C and 7.4 respectively.

Initial studies were performed to determine the effects of basal prostaglandin production on angiotensin II-induced vasoconstriction. In all subsequent studies, kidneys were pre-treated with 10 μM ibuprofen to eliminate the influence of endogenous prostanoids. PGE2 (Calbiochem, La Jolla, CA) was prepared fresh from a 1.0 mM stock. Ro 20-1724 was obtained from Research Biochemicals International (Natick, MA) and was administered at a concentration of 0.5 μM. Sulprostone and 11-deoxy PGE1 were obtained from Cayman Chem. Co. (Ann Arbor, MI). Butaprost was generously provided by Dr. Kluender of Bayer Pharmaceutical Division (West Haven, CT). SC-51322 was
obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Pertussis toxin was obtained from Sigma (St Louis MO). In the studies examining the effects of pertussis toxin, kidneys were first equilibrated with single-pass perfusion for at least one hour, then switched to a recirculating system and perfused with sterile medium (100 ml, 100 U penicillin-streptomycin/ml, GIBCO-BRL) containing either pertussis toxin (200 μg/ml) or vehicle for one hour. The kidneys were then returned to the single pass perfusion system, allowed to equilibrate and the responses to angiotensin II and PGE2 were assessed.

Vessel diameters were measured by on-line image processing. Video images were digitized (model IVG-128, Datacube) and diameters were measured at each pixel point and averaged over a 10-20 μm segment of the vessel. Measurements were taken at a rate of approximately 2 Hz and steady-state responses were averaged over approximately 3-5 minutes. Afferent arteriolar diameters were measured between the mid-point and origin from the interlobular artery. Efferent arteriolar diameters were measured within 50 microns of the glomerulus. In the studies comparing afferent and efferent arteriolar responses, paired arterioles from the same glomerulus were used. Only one data set (afferent arteriole, or one pair of afferent and efferent arterioles) was obtained from each kidney preparation.

**In Vitro Perfused Normal Kidney Studies:**

For the normal perfused kidney studies, male Sprague Dawley rats, weighing from 220 to 275 g were anesthetized with methoxyflurane. The left renal artery was cannulated in situ and the kidney was excised with continuous perfusion. Kidneys were then perfused in vitro as described above at a constant renal arterial pressure of 80 mm Hg,
monitored at the level of the renal artery. Perfusate flow was measured using a transonic flow meter (model T106, Transonic Systems, Inc.).

**RT-PCR of EP mRNA Expression in Isolated Afferent Arterioles:**

Adult male Sprague Dawley rats were anesthetized with halothane and the left kidney was flushed in vivo with warm DMEM. The kidney was then perfused with 37°C seaprep/seaplaque (2% in DMEM), excised and chilled (4°C), to solidify the agarose. Cortical slices (400 μm) were prepared and treated with collagenase IV, hyaluronidase IV, dispase II, and DNase I to dissociate microvessels from tubules. Six to eight arterioles were individually transferred to fresh buffer, and this procedure was repeated 3 times. RT-PCR was run on the final wash as a control to test for possible contamination (Figure 1 Online).

Arteriolar total RNA was extracted using a method adapted to small samples. Vessels were placed in 250 μl of solution containing 0.14 M NaCl, 10 mM Tris (pH 8.6), 1.5 mM MgCl2, and 0.5% Nonidet 40. An equal volume of 0.2 M Tris (pH 8.0), 25 mM EDTA, 0.3 M NaCl, 2% SDS and proteinase K (200 μg/ml) were added. After incubating at 37°C for 30 minutes, proteins were removed by phenol-chloroform extraction and total nucleic acids precipitated with 2.5 volumes of 100% ethanol. To remove genomic DNA, the samples were incubated with DNase I (2 μg/ml, 37°C for 1 hour), then extracted with phenol-chloroform (to remove DNase). Total RNA was precipitated by adding 2 μl seeDNA (Amersham, England) and 2.5 volumes of 100% ethanol. The pellet was dissolved in 10 μl TE (10 mM Tris HCl, pH 8.0 and 1 mM EDTA). The following were then added: 2μl 10x RT buffer (100 mM TRIS pH 8.3, 500 mM KCl, 15 mM MgCl2), 1
μl deoxynucleotide mixture (10 mM), 1μl DTT (100 mM), 1 μl random primer mixture, 1 μl RNase inhibitor (GIBCO-BRL), 1 μl AMV reverse transcriptase (GIBCO-BRL), and 3 μl water. To rule out genomic contamination, a separate sample was treated as above, but without reverse transcriptase (Figure 1 Online). Reaction tubes were incubated at 42°C for 60 minutes and at 90°C for 3 minutes and then iced.

The primers for RT-PCR assays of the rat EP-receptor mRNAs were designed from the reported sequences (2-7) and are presented in Table 1. We assayed for a sequence common to all EP3 receptors (EP3αβγ), in addition to sequences specific for each EP3 splice-variant. The denaturing, elongating and annealing PCR conditions used for the EP1 and EP2 primers were 94, 55, and 72 °C and 1.0, 1.0, and 1.5 minutes, respectively (40 cycles). For the EP3αβγ primers, the conditions were 94, 55, and 72 °C for 1.0, 1.0 and 1.5 minutes (35 cycles). For EP3α, EP3β and EP3γ primers, the conditions were 94, 60, 72 °C for 1.0, 1.0, and 2.0 minutes, respectively (40 cycles). For the EP4 primers, the conditions were 94, 55, and 72 °C for 1.0, 1.0, and 1.5 minutes (35 cycles).

A whole-kidney homogenate was used as a positive control for the EP receptors. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control and for the negative controls (sense and antisense primers:

5'CGGAGTCAACGGATTTGG TCGTAT and 5'AGCCTTCTCCATGGTGCTGA

AGAC (8)). PCR products were separated by electrophoresis using 2% agarose gel and were stained with ethidium bromide. The identity of the PCR products obtained using each set of primers was confirmed by DNA sequencing. When negative PCR results were obtained (i.e., for EP1 and EP2 primers), fresh Taq DNA polymerase was added after 35
cycles and the PCR was then continued to 70 cycles.

**Analysis:** Data are expressed as the mean values followed by the standard error of the mean. Differences between means were evaluated by ANOVA followed by Student's t test (paired or unpaired). Values of P<0.05 were considered statistically significant. For multiple comparisons, the Bonferoni correction was applied and P values <0.05/n (where n= number of comparisons) were considered significant.

**REFERENCES:**


Figure 1 Online:

The EP1 receptor antagonist SC-51322 did not alter the vasoconstrictor response of the afferent arteriole to sulprostone (n=5, p>0.10 for sulprostone alone versus sulprostone + 10 μM SC51322).
Table 1  EP Receptor Primers

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Primer Sequence</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP1 sense</td>
<td>5'TTGGTGCCTCGCCTGTATACTG</td>
<td>285-306</td>
<td>(2)</td>
</tr>
<tr>
<td>antisense</td>
<td>5'CCAGTGATGCTCGATGTCATGG</td>
<td>534-555</td>
<td></td>
</tr>
<tr>
<td>EP2 sense</td>
<td>5'GACTACAGCACCTTCATCCTAC</td>
<td>277-298</td>
<td>(3)</td>
</tr>
<tr>
<td>antisense</td>
<td>5'CTTGTCACGTAGTGCTGTAG</td>
<td>378-399</td>
<td></td>
</tr>
<tr>
<td>EP3αβγ sense</td>
<td>5'TGCCAGCCACATGAAGACTCGC</td>
<td>426-447</td>
<td>(4)</td>
</tr>
<tr>
<td>antisense</td>
<td>5'ATAGCCGTCTCCGTGGTGATCC</td>
<td>754-775</td>
<td></td>
</tr>
<tr>
<td>EP3-sense</td>
<td>5'ATGGGGATCATGTGTGACTG</td>
<td>911-929</td>
<td>(3, 5,6)</td>
</tr>
<tr>
<td>α antisense</td>
<td>5'TGGAAGCATAGTTGGTGTTG</td>
<td>1013-1032</td>
<td></td>
</tr>
<tr>
<td>β antisense</td>
<td>5'AACTCCGCTTCAGGTTGTTC</td>
<td>1011-1031</td>
<td></td>
</tr>
<tr>
<td>γ antisense</td>
<td>5'TAGACAATGAGATGGCCTGC</td>
<td>1050-1069</td>
<td></td>
</tr>
<tr>
<td>EP4 sense</td>
<td>5'CCTTCTTCGGAAGACTGTGCTC</td>
<td>999-1020</td>
<td>(7)</td>
</tr>
<tr>
<td>antisense</td>
<td>5'CAGAAGATGCTCCTCCGACTCTC</td>
<td>1112-1133</td>
<td></td>
</tr>
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