Prostaglandin E₂ Mediates Connecting Tubule Glomerular Feedback

YiLin Ren, Martin A. D’Ambrosio, Jeffrey L. Garvin, Hong Wang, Oscar A. Carretero

Abstract—Connecting tubule glomerular feedback (CTGF) is a mechanism in which Na reabsorption in the connecting tubule (CNT) causes afferent arteriole (Af-Art) dilation. CTGF is mediated by eicosanoids, including prostaglandins and epoxyeicosatrienoic acids; however, their exact nature and source remain unknown. We hypothesized that during CTGF, the CNT releases prostaglandin E₂, which binds its type 4 receptor (EP4) and dilates the Af-Art. Rabbit Af-Arts with the adherent CNT intact were microdissected, perfused, and preconstricted with norepinephrine. CTGF was elicited by increasing luminal NaCl in the CNT from 10 to 80 mmol/L. We induced CTGF with or without the EP4 receptor blocker ONO-AE3-208 added to the bath in the presence of the epoxyeicosatrienoic acid synthesis inhibitor MS-PPOH. ONO-AE3-208 abolished CTGF (control, 9.4±0.5; MS-PPOH+ONO-AE3-208, −0.6±0.2 μm; P<0.001; n=6). To confirm these results, we used a different, specific EP4 blocker, L161982 (10⁻⁵ mol/L), that also abolished CTGF (control, 8.5±0.9; MS-PPOH+L161982, 0.8±0.4 μm; P<0.001; n=6). To confirm that the eicosanoids that mediate CTGF are released from the CNT rather than the Af-Art, we first disrupted the Af-Art endothelium with an antibody and complement. Endothelial disruption did not affect CTGF (7.9±0.9 versus 8.6±0.6 μm; P=NS; n=7). We then added arachidonic acid to the lumen of the CNT while maintaining zero NaCl in the perfusate. Arachidonic acid caused dose-dependent dilation of the attached Af-Art (from 8.6±1.2 to 15.3±0.7 μm; P<0.001; n=6), and this effect was blocked by ONO-AE3-208 (10⁻⁵ mol/L). We conclude that during CTGF, the CNT releases prostaglandin E₂, which acts on EP4 on the Af-Art inducing endothelium-independent dilation. (Hypertension. 2013;62:00-00.) • Online Data Supplement

Key Words: 11,12-epoxy-5,8,14-eicosatrienoic acid • arachidonic acid • arterioles • endothelium • microcirculation • prostaglandin E2 • receptors, prostaglandin E

In the kidney, the afferent arteriole (Af-Art) is a major contributor to vascular resistance, and thus it controls glomerular hemodynamics. At least 3 intrinsic mechanisms determine the tone of the Af-Art: the myogenic response, macula densa-mediated tubuloglomerular feedback, and connecting tubule-mediated glomerular feedback (CTGF). The mechanisms of myogenic response and tubuloglomerular feedback have been studied extensively; however, the newly discovered CTGF is not completely understood.

In rabbits, rats, mice, and humans, the CNT is in close contact with the Af-Art. We have reported that CTGF regulates Af-Art tone both in vitro and in vivo by causing dilation of the Af-Art in response to increases in NaCl in the lumen of the attached CNT. In vivo, CTGF antagonizes the constrictor effect of tubuloglomerular feedback on the Af-Art and partly mediates the resetting of tubuloglomerular feedback induced by volume expansion. In vivo, we studied the pathways that mediate CTGF; we found that Na entry into the CNT via the epithelial Na channel is required to induce CTGF, and that eicosanoids derived from arachidonic acid (AA), including epoxyeicosatrienoic acids (EETs) and prostaglandins, mediate CTGF. Possible sources for these eicosanoids include the CNT itself and the Af-Art endothelium.

Prostaglandins mediate about half of the CTGF response, whereas the other half is accounted for by EETs; however, the exact identity of the prostaglandin and prostaglandin receptor involved remains unknown. Prostaglandin E₂ (PGE₂) and PGI₂, the major renal metabolites of cyclooxygenase, are both synthesized by the vascular endothelium. In addition, PGE₂ is also synthesized in the nephron, with strong expression of microsomal PGE₂ synthase in the CNT. Both PGI₂ and PGE₂ are vasodilators, with PGE₂ being a more potent renal vasodilator compared with PGI₂. The actions of PGE₂ are attributable to its interaction with EP receptors (PGE₂ receptors). There are 4 EP receptors: EP1, EP2, EP3, and EP4; however, EP4 is the only vasodilator EP receptor expressed in the Af-Art.

We hypothesized that Na reabsorption by the CNT induces release of PGE₂, which binds EP4 receptors and dilates the Af-Art in an endothelium-independent manner. CTGF was studied using an in vitro preparation developed by us, in

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which an Af-Art and its adherent CNT are isolated and microperfused simultaneously. This approach avoids the confounding influence of the multiple systemic factors that regulate the renal microcirculation.

**Methods**

New Zealand White rabbits weighing 1.5 to 2 kg (Myrtle’s rabbirry, TN) were given standard chow (Ralston Purina, St. Louis, MO) and tap water ad libitum and anesthetized with ketamine (50 mg/kg IM), xylazine (10 mg/kg IM), and pentobarbital (25 mg/kg IV). All protocols were approved by Henry Ford Health System’s Institutional Animal Care and Use Committee and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. We used rabbits because their CNTs are well demarcated, and microdissection of the CNT and attached Af-Art is easier than in rats or mice. To isolate and microperfuse the Af-Art and CNT, we used methods similar to those described previously. The kidneys were sliced along the corticomедullary axis, and slices were placed in ice-cold minimum essential medium (Gibco Laboratories, Grand Island, NY) containing 5% bovine serum albumin (Sigma, St. Louis, MO). Using fine forceps, we dissected a single superficial Af-Art with its glomerulus intact together with the adherent CNT. Using a micropipette, we transferred the microdissected complex to a temperature-regulated perfusion chamber mounted on an inverted microscope with Hoffman modulation. Both the Af-Art and CNT were cannulated with an array of concentric glass pipettes as described previously. This system allows us to exchange the perfusion solution in a few seconds while keeping the holding and perfusion pipettes in place. The Af-Art was microperfused with minimum essential medium containing 5% bovine serum albumin gassed with room air. Intraluminal pressure was measured by Landis technique and maintained at 60 mm Hg. The CNT perfusion solution contained (in mmol/L): 4 KHCO₃, 10 HEPES, 0.5 Na acetate, 0.5 Na lactate, 0.5 K HPO₄, 1.2 MgSO₄, 1 CaCO₃, and 3.5 glucose, adding 1 mol/L NaCl to achieve the desired final NaCl concentration. Tubular perfusion was controlled by the use of a syringe microperfusion pump (Harvard Apparatus Inc, MA) set to 20 nL/min, which is within the range of physiological flow rates. The bath was superfused with minimum essential medium containing 0.15% bovine serum albumin at a rate of 1 mL/min.

Microdissection and cannulation of the Af-Art and CNT were completed within 90 minutes at 8°C, after which the temperature was gradually raised to 37°C. Once it was stable, a 30-minute equilibration period was allowed before taking any measurements. Because isolated arteries have little or no tone and our preliminary studies showed that increasing NaCl in the CNT perfusate caused only modest dilation of the Af-Art unless it was preconstricted, we performed all CTGF experiments with Af-Arts preconstricted with norepinephrine (NE; 2–5×10⁻⁷ mol/L) to about half of its baseline diameter. Using fine forceps, we dissected a single superficial Af-Art with its glomerulus intact together with the adherent CNT. Using a micropipette, we transferred the microdissected complex to a temperature-regulated perfusion chamber mounted on an inverted microscope with Hoffman modulation. Both the Af-Art and CNT were cannulated with an array of concentric glass pipettes as described previously. This system allows us to exchange the perfusion solution in a few seconds while keeping the holding and perfusion pipettes in place. The Af-Art was microperfused with minimum essential medium containing 5% bovine serum albumin gassed with room air. Intraluminal pressure was measured by Landis technique and maintained at 60 mm Hg. The CNT perfusion solution contained (in mmol/L): 4 KHCO₃, 10 HEPES, 0.5 Na acetate, 0.5 Na lactate, 0.5 K HPO₄, 1.2 MgSO₄, 1 CaCO₃, and 3.5 glucose, adding 1 mol/L NaCl to achieve the desired final NaCl concentration. Tubular perfusion was controlled by the use of a syringe microperfusion pump (Harvard Apparatus Inc, MA) set to 20 nL/min, which is within the range of physiological flow rates. The bath was superfused with minimum essential medium containing 0.15% bovine serum albumin at a rate of 1 mL/min.

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**Experimental Protocols**

1. Time control for experiments #2 and #3: 3 consecutive concentration–response curves were generated by increasing luminal NaCl in the CNT from 10 to 80 mmol/L.
2. Effect of the EP4 antagonist ONO-AE3-208 on CTGF: 3 consecutive concentration–response curves were generated by increasing luminal NaCl in the CNT from 10 to 80 mmol/L. The EET synthesis inhibitor MS-PPOH (10⁻⁶ mol/L) was added to the second and third curves and the EP4 antagonist ONO-AE3-208 (10⁻⁷ mol/L) to the third curve. This concentration of ONO-AE3-208 is 77x its Ki for the EP4 receptor and 2100x lower than its Ki for the EP2 receptor.
3. Effect of the EP4 antagonist L161982 on CTGF: this experiment was similar to #2, but the EP4 antagonist L161982 (10⁻⁶ mol/L) was used instead of ONO-AE3-208. This concentration of L161982 is 312x its Ki for the EP4 receptor and 6x lower than its Ki for the EP2 receptor.
4. Effect of endothelium disruption on CTGF: CTGF was induced by increasing NaCl in the CNT from 10 to 80 mmol/L. Then a goat antihuman antibody against von Willebrand factor (14.29 mg/mL diluted 1:1000) and 2% guinea pig complement were perfused into the lumen of the Af-Art for 10 minutes followed by a 20-minute wash-out period, and CTGF was induced again. To confirm complete functional removal of the endothelium, we added acetylcholine to the lumen of the Af-Art, 10⁻⁴ mol/L, a concentration we have repeatedly shown to be sufficient to dilate the Af-Art.
5. Effect of exogenous AA in the CNT: After the Af-Art was preconstricted with NE, AA was added to the lumen of the CNT at increasing concentrations from 10⁻⁷ to 10⁻⁵ mol/L in the absence of NaCl. At the end of the experiment, we removed AA and switched the CNT luminal perfusate to 80 mmol/L NaCl.
6. Effect of an EP4 antagonist on CTGF induced by exogenous AA: this experiment was similar to #5, except that MS-PPOH was added to the lumen of the CNT, and ONO-AE3-208 was added to the bath.

In all experiments, Af-Art diameter was measured in the region of maximal response to NE at 3 sites 3 to 5 µm apart and expressed as the average of these 3 measurements. Diameter was recorded at 5-second intervals with a video camera and measured with a computer equipped with Metavitue image analysis software (MDS Analytic Technologies, Toronto, Ontario, Canada).

**Chemicals**

ONO-AE3-208 was a gift from Ono Pharmaceutical Co (Osaka, Japan), and MS-PPOH was a gift from Dr J.R. Falck (University of Texas Southwestern Medical Center, Dallas, TX). L161982 was purchased from Tocris Bioscience (Ellisville, MO), NE and acetylcholine from Sigma-Aldrich (St Louis, MO), and AA from Cayman Chemical (Ann Arbor, MI).

**Statistics**

Values are expressed as mean±SEM. Paired t-tests were used to compare CTGF (ΔAf-Art diameter) between the control and experimental periods. Hochberg’s step-up procedure was used to adjust the P values for multiple comparisons so that the familywise type I error rate, predefined as 0.05, was controlled.

**Results**

To test whether CTGF is mediated by EETs and PGE₂ produced endogenously in response to increases in luminal NaCl in the CNT, we conducted a series of experiments involving 3 consecutive CTGF responses. First, we performed a control experiment by preconstricting the Af-Art with NE and repeatedly switching NaCl in the tubular perfusate from 10 to 80 mmol/L. Increasing NaCl in the CNT to 80 mmol/L dilated the attached Af-Art, and switching NaCl back to 10 mmol/L returned the Af-Art diameter to preconstricted levels. Furthermore, all 3 CTGF responses were similar, indicating that CTGF is reproducible and stable over time (Figure 1).

To test whether PGE₂ acting via EP4 receptors, mediates CTGF along with EETs, we again induced CTGF for 3 consecutive times, first with vehicle, then adding the EET synthesis inhibitor MS-PPOH, and finally adding both MS-PPOH and the EP4 antagonist ONO-AE3-208. CTGF was attenuated by MS-PPOH and abolished by the combination of MS-PPOH and ONO-AE3-208 (Figure 2). We confirmed these results with a second EP4 blocker, L161982. Again, CTGF was attenuated by inhibition of EET synthesis and abolished by addition of the EP4 blocker (Figure 3). Taken together, these data suggest that during CTGF, in addition to EETs, the CNT releases PGE₂, which act on EP4 receptors in the Af-Art to cause vasodilation.
To test whether the eicosanoids that mediate CTGF are released from the Af-Art endothelium or the CNT, we studied CTGF before and after Af-Art endothelium removal with an antibody directed to von Willebrand factor (an antigen present in the endothelium, also referred to as factor VIII–related antigen) and complement, perfused into the lumen of the Af-Art. Endothelium removal did not alter CTGF (Figure 4). At the end of the experiment, we confirmed that endothelium was removed functionally by adding the endothelium-dependent vasodilator, acetylcholine, into the lumen of the Af-Art, which failed to dilate the preconstricted Af-Arts. These data suggest that during CTGF, eicosanoids are released from the CNT, rather than from the Af-Art endothelium.

To confirm that the CNT produces the eicosanoids that mediate CTGF, we added AA, the substrate for eicosanoids synthesis, into the lumen of the CNT while perfusing the tubule with zero NaCl. The addition of AA to the CNT dose dependently dilated the attached preconstricted Af-Art, returning the Af-Art diameter to its baseline (ie, completely reversing NE-induced vasoconstriction). This is similar to what we observe when we induce CTGF by increasing lumenal NaCl in the CNT (Figure 5). Because we reported that EETs mediate about half of CTGF, and we hypothesized the other half is because of PGE2 acting on EP4 receptors, we repeated this experiment in the presence of both MS-PPOH and ONO-AE3-208. The addition of AA to the CNT lumen failed to elicit Af-Art dilation in the presence of MS-PPOH and ONO-AE3-208 (Figure 6). These data suggest that the CNT is capable of producing EETs and PGE2, which cause dilation of its attached Af-Art.

Discussion

We previously provided direct evidence of cross-talk between CNT and Af-Art. It is initiated by increasing NaCl concentration in the lumen of the CNT, which stimulates Na transport via epithelial Na channel in the CNT and dilates the Af-Art. We also found that 2 classes of AA metabolites, EETs and PGs, are involved. In this study, we demonstrated for the first time that the eicosanoids that mediate CTGF are produced in the CNT rather than in the Af-Art, that the prostaglandin that mediates CTGF is PGE2, and that the prostaglandin receptor involved is EP4.

We previously reported that addition of indomethacin to the CNT partially inhibits CTGF, suggesting that CTGF is mediated by a cyclooxygenase (COX)-derived metabolite, such as PGE2 and PGI2, both of which are synthesized in the kidney and can regulate renal hemodynamics. Here, we found that CTGF is mediated by PGE2. Our data are consistent with previous reports that PGE2 influences renal vascular resistance, causing an increase in renal vascular cAMP levels and inducing relaxation of preglomerular vessels, whereas PGI2 was a less likely candidate to mediate CTGF because it is less potent.
Likewise, Purdy et al. showed that EP4 is the major receptor for PGE2, whereas PGI2 synthase is not expressed in the connecting tubules. Studies have shown that connecting tubules express mPGES-1 (membrane-associated PGE synthase), the enzyme that synthesizes PGE2, whereas PG1 synthase is not expressed in the nephron. In addition, localization of mPGES by immunolabeling showed expression in the principal cells of the CNT (i.e., the cells that express epithelial Na channel and initiate CTGF), where it colocalizes with COX1, but not COX2.

PGE2 is a major renal cyclooxygenase metabolite of AA and interacts with G-protein–coupled receptors designated as EP1, EP2, EP3, and EP4. Acting through these receptors, PGE2 modulates renal hemodynamics, as well as salt and water excretion. Generally, activation of EP1 and EP3 results in vasoconstriction, whereas EP2 and EP4 receptors mediate vasodilation by activating Gs coupled to adenylate cyclase and elevating intracellular Ca2+, and could potentially mediate CTGF. Here, we found that CTGF is mediated by PGE2 because blockade of EP4 receptors with either of 2 different antagonists abolished CTGF. Our data are consistent with previous reports that butaprost, a PGE2 analog that preferentially activates EP2, is relatively ineffective as a vasodilator of the Af-Art, and that Af-Arts express EP4 but not EP2. Purdy et al. showed that EP4 is the major receptor in the preglomerular vasculature where it mediates the vasodilatory effects of PGE2. Furthermore, regulation of plasma renin activity and intrarenal renin mRNA is not different in wild-type and EP2-knockout mice, thus the EP2 receptor does not seem to mediate the effects of PGE2 in the Af-Art and was therefore an unlikely candidate to mediate CTGF. Taken together, these previous reports and our present data indicate that CTGF is mediated by PGE2 acting on EP4 receptors.

The exact mechanism by which Na entry into the principal cells of the CNT can induce PGE2 production is unknown; however, because Na entry via epithelial Na channel leads to increases in intracellular Ca2+, it is possible that Ca-dependent activation of phospholipases in the CNT cells initiates a cascade leading to PGE2 production.

The local concentration of PGE2 produced by the CNT during CTGF is not known; however, from a functional point of view, its effect is comparable with that of exogenous PGE2 when applied to isolated Af-Arts at a concentration of 10^-7 mol/L (see Figure S1 in the online-only Data Supplement). Similar to endogenous CTGF, this concentration of PGE2 dilates the Af-Art to ≈50% of its basal diameter from a preconstricted state. Likewise, the same concentration of the EP4 receptor antagonist, L161982, that completely blocks endogenous CTGF can also completely block the vasodilatory effect of PGE2, 10^-7 mol/L. These data suggest that the local concentration of PGE2 in the CNT during CTGF may be ≈10^-7 mol/L.

We have reported previously that CTGF is mediated by EETs and prostaglandins, with each of these classes of eicosanoids accounting for about half of the vasodilatory effect of CTGF. Here, we tested whether these eicosanoids were derived from CNT epithelial cells or from the Af-Art endothelium. For this, we took 2 approaches. First, we studied CTGF in the absence of a functional endothelium. We found that endothelial removal did not affect CTGF, indicating that CTGF is endothelium independent. Next, we tested the ability of the CNT to generate vasodilator eicosanoids when AA, the substrate for eicosanoid generation, was infused in the lumen of the tubule. AA diluted the attached Af-Art, indicating the CNT is able to produce dilator eicosanoids that can act on the attached Af-Art and cause dilation. Prostanoids are generally considered to be locally acting factors that modulate cellular function in the vicinity of their site of generation. This means that prostaglandins can act within the same compartment, such as endothelium-derived prostaglandins causing relaxation of vascular smooth muscle cells in a vessel. However, it is also possible for prostaglandins to be generated in one structure and function in another. For example, inhibition of prostaglandin synthesis with indomethacin increased the sensitivity of the efferent arteriole to vasoconstrictors when the arteriole was perfused orthogradely (through the glomerulus), but not when it was perfused retrogradely, suggesting that prostaglandins produced in the glomerulus can act on the efferent arteriole. Also, in the outer medulla, PGE2 is produced in collecting duct epithelial and interstitial cells and causes dilation of neighboring descending vasa recta vessels.

Of note, our finding that AA added to the lumen of the CNT dilates the Af-Art cannot be explained by diffusion of the AA to act directly on the Af-Art because AA, when added to isolated Af-Arts, is a vasoconstrictor, rather than a vasodilator, and thus the CNT is necessary to convert AA to vasodilator eicosanoids. In addition, the setting of our preparation makes direct diffusion from the tubular perfusate to the Af-Art...
unlikely because the effluent from the tubular perfusate is diluted 50000× in the bath before it can act on the Af-Art.

The interaction between prostaglandins and angiotensin II (AngII) plays a critical role in the modulation of the renal microcirculation. Specifically, AngII causes constriction of the renal vasculature and decreases renal blood flow. However, AngII also increases the production of prostaglandins by the nephron, as well as its modification by pharmacological agents. For example, nonsteroidal anti-inflammatory drugs are likely to be because of the fact that AngII induces the expression of vasodilatory prostaglandins, such as PGE2, which acts on EP4 receptors to renal microvascular reactivity in mice. AngII causes constriction of the renal vasculature and decreases renal blood flow. However, AngII also increases the production of prostaglandins by the kidney, and these prostaglandins partially buffer the decrease in renal blood flow induced by AngII. Microdissected superficial Af-Arts demonstrate an enhanced responsiveness to AngII during COX inhibition. This is likely to be because of the fact that AngII induces the expression of COX2 in some nephron segments, as the thin ascending limb and macula densa. However, it is also likely that it involves CTGF. We recently reported that AngII acting in the lumen CNT enhances CTGF. Taken together with our present findings that PGE2 mediates CTGF, it is likely that AngII, by enhancing CTGF, increases PGE2, and partially counters its direct constrictor effect on the Af-Art.

**Perspectives**

We report here for the first time that CTGF is an endothelium-independent vasodilator mechanism of the Af-Art, that the CNT is capable of producing and releasing vasodilator eicosanoids that act in a paracrine manner on the Af-Art, and that during CTGF, increases in NaCl in the lumen of the CNT trigger CTGF that is mediated by EETs and also by PGE2, which acts on EP4 receptors in the Af-Art to cause vasodilatation. Our findings shed light on the physiological regulation of renal hemodynamics by the nephron, as well as its modification by pharmacological agents. For example, nonsteroidal anti-inflammatory drugs are widely used medications with well-known adverse effects on the kidney function. Of relevance, they inhibit the production of vasodilatory prostaglandins, such as PGE2, thus decreasing renal blood flow.

Our studies suggest that part of this detrimental effect may be because of blockade of CTGF.

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**Disclosures**

None.

**References**

34. Tilley SL, Audoly LP, Hicks EH, Kim HS, Flannery PJ, Coffman TM, Kolter BH. Repressive failure and reduced blood pressure in mice.
What Is New?

• Connecting tubule glomerular feedback (CTGF) is a novel mechanism of regulation of the microcirculation in the kidney. This mechanism is activated by the presence of increased concentrations of sodium chloride (salt) in a segment of the kidney tubules known as the connecting tubule and causes the afferent arteriole (a vessel that is right next to the connecting tubule) to relax.

• Our data are the first to show that connecting tubule glomerular feedback is mediated by small lipid molecules known as eicosanoids, including epoxyeicosatrienoic acids and prostaglandin E2. We also show for the first time that said eicosanoids are produced by the connecting tubule.

What Is Relevant?

• The kidney plays a key role in high blood pressure. Kidney diseases cause hypertension, and hypertension can, in turn, damage the kidney.

What Is Relevant?

• Much of the renal function is controlled by the blood supply through the afferent arteriole, which, in turn, is in part controlled by connecting tubule glomerular feedback.

• Our studies will help to better understand the control of the renal microcirculation and function.

Summary

We found that during connecting tubule glomerular feedback, the connecting tubule produces eicosanoids, including epoxyeicosatrienoic acids and prostaglandin E2, that dilate the afferent arteriole. We also found that prostaglandin E2 does this by activating its type 4 receptor.
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Prostaglandin E₂ Mediates Connecting Tubule Glomerular Feedback

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Fig S1. Effect of exogenous PGE<sub>2</sub> added to the bath on the diameter of isolated preconstricted Af-Arts. PGE<sub>2</sub> dose dependently relaxed the Af-Art (\( * P < 0.05, ** P < 0.01 \) vs. preconstricted Af-Art diameter). At 10<sup>-7</sup> mol/L, exogenous PGE<sub>2</sub> dilated the Af-Art to about 50% of its diameter, which is similar to the dilation achieved by endogenous PGE<sub>2</sub> released during CTGF. The vasodilatory effect of PGE<sub>2</sub> added to the Af-Art at concentrations up to 10<sup>-7</sup> mol/L was completely blocked by the EP4 blocker L161982 (10<sup>-5</sup> mol/L; ### \( P < 0.001 \) with vs. without L161982).