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Prostaglandin E_2 increases proximal tubule fluid reabsorption, and modulates cultured proximal tubule cell responses via EP_1 and EP_4 receptors

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Renal prostaglandin (PG) E_2 regulates salt and water transport, and affects disease processes via EP_{1-4} receptors, but its role in the proximal tubule (PT) is unknown. Our study investigates the effects of PGE_2 on mouse PT fluid reabsorption, and its role in growth, sodium transporter expression, fibrosis, and oxidative stress in a mouse PT cell line (MCT). To determine which PGE_2 EP receptors are expressed in MCT, qPCR for EP_{1-4} was performed on cells stimulated for 24 h with PGE_2 or transforming growth factor beta (TGFβ), a known mediator of PT injury in kidney disease. EP_1 and EP_4 were detected in MCT, but EP_2 and EP_3 are not expressed. EP_1 was increased by PGE_2 and TGFβ, but EP_4 was unchanged. To confirm the involvement of EP_1 and EP_4, sulprostone (SLP, $EP_{1/3}$ agonist), ONO8711 (EP_1 antagonist), and EP_1 and EP_4 siRNA were used. We first show that PGE_2, SLP, and TGFβ reduced H^3-thymidine and H^3-leucine incorporation. The effects on cell-cycle regulators were examined by western blot. PGE_2 increased p27 via EP_1 and EP_4, but TGFβ increased p21; PGE_2-induced p27 was attenuated by TGFβ. PGE_2 and SLP reduced cyclinE, while TGFβ increased cyclinD1, an effect attenuated by PGE_2 administration. Na-K-ATPase α1 (NaK) was increased by PGE_2 via EP_1 and EP_4. TGFβ had no effect on NaK. Additionally, PGE_2 and TGFβ increased fibronectin levels, reaching 12-fold upon co-stimulation. EP_1 siRNA abrogated PGE_2-fibronectin. PGE_2 also increased ROS generation, and ONO-8711 blocked PGE_2-ROS. Finally, PGE_2 significantly increased fluid reabsorption by 31 and 46% in isolated perfused mouse PT from C57BL/6 and FVB mice, respectively, and this was attenuated in FVB-EP_1 null mice. Altogether PGE_2 acting on EP_1 and EP_4 receptors may prove to be important mediators of PT injury, and salt and water transport.

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Prostaglandin (PG) E_2 is a major product of cyclooxygenase activity in the kidney. It has a substantial role in maintaining hemodynamics, salt and water homeostasis, and affects growth, inflammation, oxidative stress, and fibrotic responses (reviewed in refs. [1–3]). Four EP receptors (EP_{1-4}) mediate the signalling responses to PGE_2, by altering intracellular cAMP and/or Ca^{2+} levels. Renal cells often simultaneously express multiple EP receptors, and their relative levels determine the cell's response. Although the contribution of the proximal tubule (PT) to overall renal prostaglandin synthesis is minimal, the role of PGE_2 in PT transport function has been considered. For instance, PGE_2 stimulates cAMP and activates protein kinase A, and in turn regulates basolateral organic anion uptake.[4,5] In contrast, long-term exposure to PGE_2 inhibits its excretion by decreasing the levels of basolateral organic anion transporters that are responsible for PGE_2 uptake in rat renal PT cells.[6] The underlying regulatory pathways are not completely understood, but short-term *vs* long-term exposure to PGE_2 has opposite effects on the overall cell response. PGE_2 also reduces phosphate transport in mouse proximal convoluted tubules.[7] Though very little is known about PT salt and water transport in response to PGE_2, a recent study by Herman *et al*[8] describes a mechanism involving both protein kinase C and cAMP, by which PGE_2 regulates Na^+-K^+-ATPase in primary cultures of rabbit renal PT cells. PGE_2 can also mediate the responses of other hormonal systems on PT sodium transport function. For instance, Lopes *et al*[9] showed that Angiotensin-(1–7)-induced Na^+-ATPase activity is inhibited by bradykinin via PGE_2.

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Though it is clear that PGE_2 could influence the transport properties of the PT, to date the specific contribution of each PGE_2/EP receptor pathway to these responses has not been thoroughly investigated.

In addition to its role in regulating tubular transport, PGE_2 contributes to injurious processes in a variety of renal cells, and thereby influences the initiation and progression of kidney injury. The PT is the primary tubular segment affected in a number of kidney diseases. A recent emphasis is placed on the role of the tubulointerstitium in kidney disease, and its contribution to both initial events perturbing kidney function, but also in sustaining renal injury.[10–12] A tubular hypothesis whereby PT growth promotes PT hyper-reabsorption, which in turn contributes to hyperfiltration and hypertension, leading to further renal injury, is now given significant recognition in the literature (reviewed in Vallon and Thomson[12]). Transforming growth factor beta (TGFβ) is a key regulator of PT changes, promoting and maintaining alterations in growth and fibrotic responses.[13,14] Since PGE_2 regulates renal growth and tubular transport, it is essential to examine the contribution of specific PGE_2/EP receptor pathways to PT injury and clarify the effects on salt and water transport in this segment of the nephron. A study by Mohamed *et al*[15] showed that COX-2 derived PGE_2 is elevated in the PT and mediates inflammatory and apoptotic responses that contribute to injury in streptozotocin diabetic mice, and this is alleviated with netrin-1 overexpression. The exact mechanisms were not clarified. But overall, in the kidney PGE_2 acting on EP_4 receptors prevents injury,[16–18] whereas EP_1 contributes to injurious events.[19,20] Our study investigates the effects of PGE_2 on PT water transport, growth, sodium transporters, fibrosis, and ROS generation, and the interaction with TGFβ-mediated responses in a mouse PT cell line (MCT). Having confirmed that only EP_1 and EP_4 receptors are expressed in MCT cells, various approaches to distinguish EP_1 *vs* EP_4 mediated responses were employed, including: comparisons between PGE_2 and the $EP_{1/3}$ agonist sulprostone (SLP), EP_1 antagonist ONO8711, and EP_1 and EP_4 siRNA. A better understanding of the specific contribution of EP_1 and EP_4 receptors could prove to be important for kidney disease intervention strategies.

MATERIALS AND METHODS

MCT Cell Culture

The mouse renal tubular epithelial cell line (MCT) was a generous gift from Dr Eric Neilson (Northwestern University, Chicago, IL). These cells were characterized and shown to possess many of the characteristics of the *in vivo* PT.[21] MCT cells were grown in DMEM: F-12 (1:1) media supplemented with 10% FBS, 1% Penicillin-Streptomycin, and 1% L-glutamine and were maintained at 5% CO_2 and 37 °C during culture and treatment. Sub-confluent cells were maintained in serum-free media for 24 h and stimulated with 1 μM PGE_2 and 2 ng/ml of TGFβ for 24 h. If a response to 1 μM PGE_2 was observed, MCT cells were then stimulated for 24 h with 1 nM PGE_2 as well as 1 μM and 1 nM SLP (Cayman, Ann Arbor, MI, USA). Sulprostone is an $EP_{1/3}$ receptor agonist, but since EP_3 receptors are not expressed in MCT cells (as indicated below), SLP was used to determine the involvement of EP_1 receptors in the various responses. EP_1 receptors were antagonized with 100 nM ONO-8711 (Cayman) or blocked by EP_1 siRNA transfections, EP_4 receptors were blocked with EP_4 siRNA transfections (see below).

EP siRNA Transfections

MCT cells were cultured for 24 h and then transient transfections with EP_1 or EP_4 siRNA (Qiagen, Germany) were carried out as previously described.[22] Briefly, EP siRNA was prepared according to the manufacturer's instructions to a final concentration of 5 nM in 100 μl DMEM-F12 and HiPerfect Transfection reagent (Qiagen), and cells were maintained for a total of 48 h. A universal scrambled siRNA (Qiagen) was used as a negative control for these experiments. PGE_2 stimulations (1 μM) were carried out during the final 24 h transfection. Protein lysates were isolated, as well as total RNA to confirm the inhibition of EP_1 and EP_4 following siRNA transfections by quantitative PCR, as described below.

RNA Isolation and Quantitative PCR

Total RNA was isolated from MCT with TRIzol reagent (Life Technologies, Grand Island, NY, USA) and treated with DNAse I (Life Technologies). Quantitative PCR was performed to measure $EP_{1–4}$ receptors in samples using specific Taqman probes and primers from Applied Biosystems using the TaqMan One-step RT-PCR master mix reagents and the ABI Prism 7000 sequence detection system as previously described.[23] A 6-point standard curve was performed for each experiment as a control using mouse cortex RNA from 4.7 to 100 ng. Expression was normalized to GAPDH, detected with the TaqMan Rodent GAPDH control reagent kit (Life Technologies).

^{3}H-Thymidine and ^{3}H-Leucine Incorporation

To study the effects of PGE_2 and TGFβ on cell DNA and protein synthesis, we measured the incorporation of ^{3}H-thymidine and ^{3}H-leucine, respectively. MCT cells were cultured in 24-well plates, grown to 70% confluence and then cultured in serum-free media for 24 h stimulated with 1 μM or 1 nM PGE_2 or SLP, or 2 ng/ml of TGFβ (R&D Systems, Minneapolis, MN, USA). ^{3}H-thymidine or ^{3}H-leucine (0.5 μCi/ml) was added to each well for the duration of the stimulation. The plates were then washed four times in ice-cold PBS. Next, the cells were permeabilized in 500 μl of 1 N NaOH at 37 °C for 30 min, and the amount of ^{3}H-thymidine or ^{3}H-leucine, in counts per minute, was measured using a scintillation counter. Samples were done in triplicate and incorporation was expressed as fold of control.

Western Blotting

Confluent MCT cells were lysed in RIPA buffer containing: 0.5 mM PMSF, 1% protease inhibitor cocktail, 1 mM sodium

pyrophosphate, 10 mM sodium fluoride and 100 μM sodium orthovanadate, and sonicated for 5 s. Protein was quantified with Bradford reagent (Bio-Rad, Hercules, CA, USA). Samples were denatured at 70 °C for 15 min, electrophoresed and transferred onto a nitrocellulose membrane. Membranes were blocked in 10% milk/TBS-T for 90 min and incubated overnight with primary antibody followed by secondary antibody for 90 min. The following antibodies were used: anti-p27 (Calbiochem, San Diego, CA, USA), anti-p21 (Santa Cruz, Dallas, TX, USA), anti-cyclinD1 and cyclinE (Santa Cruz), anti-Na-K-ATPase-α1 (Millipore Canada, Etobicoke, ON, Canada), and anti-fibronectin (Sigma-Aldrich, St Louis, MO, USA). Super Signal West Pico Chemiluminescent reagents (Thermo Scientific, Rockford, IL, USA) were applied and β-actin was detected as a loading control for densitometry.

Lucigenin Assay

The lucigenin assay was used to measure ROS production in MCT cells in response to PGE_2. Cultured cells were stimulated for 24 h and scraped in lysis buffer containing KH_2PO_4, EGTA, aprotinin, pepstatin, leupeptin, and PMSF. In all, 50 µl of each sample was assayed using the lucigenin-derived chemiluminescence assay, in 175 μl assay buffer (KH_2PO_4, EGTA, and Sucrose) containing 1 mM lucigenin. Following basal measurements for each sample in a 96-well plate, NADPH (0.1 mM) was added to each well and luminescence was measured every 1.8 s for 30 cycles in a microplate luminometer (Orion II, Berthold Detection System), using the Simplicity 4.2 Program as previously described.[24] Activity was expressed as relative chemiluminescence units per µg protein. Apocynin (50 μM) was used to inhibit NADPH oxidase as previously done in our laboratory.[24]

Microdissections of PTs

Male C57BL/6 and FVB mice 4–6 months of age were killed by decapitation. The kidneys were quickly removed, and 1–2 mm coronal slices were placed in chilled dissection dishes for freehand dissection of PTs, distinguished from other segments based on various properties: diameter difference, cell heterogeneity, and transluscency.[21] The microdissected PTs were then transferred to a chamber for *in vitro* perfusions and measurement of net fluid reabsorption (Jv). To confirm the involvement of EP_1 receptors in fluid reabsorption, PTs were also isolated from mice lacking the EP_1 receptor (FVB-EP_1 null mice), which our group generated and thoroughly characterized.[20]

Measurement of Net Fluid Reabsorption (Jv)

In vitro microperfusion of isolated mouse PTs was performed as previously described.[25,26] Microdissected tubules (described above) were transferred to a thermostatically controlled chamber of 1 cm^3 volume and cannulated using concentric micropipettes. Bath solution was continuously exchanged at 0.5 ml/min by infusion pump (Harvard Apparatus, Holliston, MA, USA) and was maintained at 37 °C. The dissecting solution consisted of (in mM): NaCl, 137; $MgCl_2$, 1; $MgSO_4$, 0.8; KCl, 5; $CaCl_2$, 0.25; Tris-HCl, 10; Na_2HPO_4, 0.33; glutamine, 2; KH_2PO_4, 0.44; and L-lactate, 2. The perfusate composition was (in mM): NaCl, 125; $NaHCO_3$, 22; KCl, 5; $CaCl_2$, 1; $MgSO_4$, 1.2; glucose, 10.5; glutamine, 2; L-lactate, 2; and phosphoric acid, 1.2. The composition of bath medium was (in mM) NaCl, 101; $NaHCO_3$, 22; KCl, 5; $CaCl_2$, 1; $MgSO_4$, 1.2; glucose, 10.5; glutamine, 2; L-lactate, 2; phosphoric acid, 1.2, and Hepes, 32.5 (pH 7.4, osmolality 300 mOsmol). Bovine serum albumin (5%, Sigma, St Louis, MO, USA) was also added before dissections. The perfusate, which contained H^3-inulin (75 µCi/ml) as a volume marker, was collected into a constriction pipette of known volume (between 90 and 130 nl) and counted for H^3-inulin (New England Nuclear, Boston, MA, USA). The perfusion rate was maintained between 12 and 20 nl/min by adjusting the hydrostatic pressure. At this perfusion rate, osmotic equilibration between bath and lumen does not occur. In control studies, 30 min of equilibration was allowed and then, three collections were made for calculation of basal Jv in nl/mm/min. Tubules with a negative basal Jv were discarded. Then, 0.1 μM or 1 nM PGE_2 (Cayman) was added to the bath and 5 timed collections were made to determine net volume reabsorption (Jv). In the experimental period, the three highest collections of five were used to calculate mean Jv as the difference between the perfusion rate V_o and the collection rate V_L, both in nl/min, normalized to tubule length (L, in mm): $Jv = (V_o - V_o)/L$, where $V_o = V_L\ (C_L/C_o)$, where C_L and C_o are perfusate and collected fluid concentrations in cpm/nl, respectively.

Statistics

GraphPad Prism v4.03 (La Jolla, CA, USA) was used to plot and analyze the data. Values are expressed as means ± standard error of the mean (s.e.m.). A one-way ANOVA was performed with Tukey post-test. We also used an unpaired t-test to assess statistical significance between selected experimental groups. Additionally, a one-sample unpaired t-test was performed against a hypothetical value of 1.0 for all groups as the values represented fold controls, where the control group all had values of 1.0, $P < 0.05$.

RESULTS

EP_1 Receptors are Increased in MCT by PGE_2 and TGFβ

As shown in Figure 1a–d, both EP_1 and EP_4 receptors are expressed in MCT cells. EP_2 and EP_3 subtypes were not detected in MCT cells, but were detectable previously with the same probes and primers in other renal cell lines and mouse cortex and medulla samples.[23] In our experiment a standard curve was produced using mouse cortex as a control, further confirming the lack of expression of these two receptors in the MCT samples. The expression of EP_1 and EP_4 receptors in response to 24 h PGE_2 and TGFβ treatments were further characterized. Both PGE_2 and TGFβ increased EP_1/GAPDH by 2.5- and 3.8-fold, respectively (Figure 1a), but EP_4

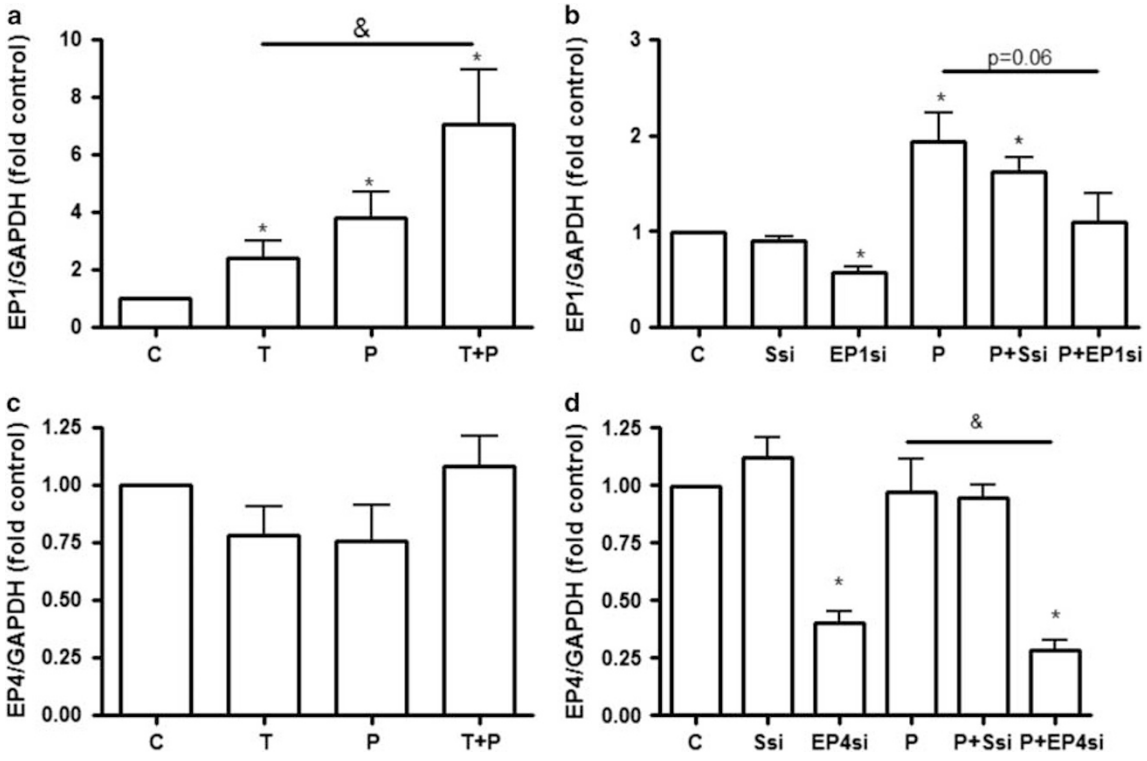

Figure 1 PGE_2 and TGFβ increase EP_1 but not EP_4 expression in MCT cells. MCT cells were stimulated for 24 h with TGFβ and PGE_2 in the presence or absence of TGFβ, and EP_1 (**a**) and EP_4 (**c**) receptors were detected by quantitative PCR. MCT cells were transfected for 48 h with EP_1 (**b**) and EP_4 (**d**) siRNA. EP/GAPDH presented as mean ± s.e.m., fold of control ($C = 1$), $n = 6$ and 8, respectively. $^*P < 0.05$ *vs* C; $^{\&}P < 0.05$ *vs* T or P. EP_1si or EP_4si, EP_1 or EP_4 siRNA (5 nM); P, PGE_2 (1 μM); P+EP_1si, PGE_2 and EP_1 siRNA; P+EP_4si, PGE_2 and EP_4 siRNA; Ssi, scrambled siRNA; P+Ssi, PGE_2 and scrambled siRNA; T, transforming growth factor β (TGFβ, 2 ng/ml); TP, TGFβ+PGE_2.

receptors remained unchanged (Figure 1c). Co-stimulation with both PGE_2 and TGFβ resulted in a 7-fold increase in EP_1 receptor expression, which was significantly greater than TGFβ alone, but not greater than the response to PGE_2 alone. Transfection of MCT cells with EP_1 (Figure 1b) and EP_4 (Figure 1d) siRNA resulted in a 50 and 75% reduction of EP_1 and EP_4 mRNA levels, respectively. However, scrambled siRNA had no effect on EP_1 or EP_4. EP_1 siRNA also reduced the PGE_2-mediated increase in EP_1 receptors ($P = 0.06$) back to control levels (Figure 1b).

PGE_2 Inhibits Cell-Cycle Progression in MCT Cells

To study the effect of PGE_2 on MCT growth responses, we first examined ^{3}H-thymidine and ^{3}H-leucine incorporation. As shown in Figure 2, 3H-thymidine incorporation (Figure 2a) was reduced by 47 and 68% in response to PGE_2 and TGFβ, respectively, and reduced by 76% upon co-stimulation. Though the co-treatment was significantly less than the inhibition by PGE_2 alone, the inhibition upon co-treatment was not significant compared with TGFβ alone. Since EP_3 receptors were not detected in MCT cells (discussed above), SLP was used as an EP_1 selective agonist to determine the extent of EP_1 involvement. As shown in Figure 2c, SLP, like PGE_2, also reduced thymidine incorporation by about 50%, and this effect was not influenced by the concentration of PGE_2 or SLP used (1 μM *vs* 1 nM treatments). Similarly, H^3-leucine incorporation (Figure 2b) was reduced by 40 and 65% in response to PGE_2 and TGFβ, and by 73% upon co-stimulation. The effect of co-treatment with PGE_2 and TGFβ was statistically significant compared with PGE_2 alone, but not different from TGFβ alone. Sulprostone and 1 nM PGE_2 reduced leucine incorporation by 25% (Figure 2d). To further explore the mechanism of growth inhibition, we examined the effects of PGE_2 on cell-cycle regulators, compared with TGFβ. Both p27 and p21 inhibit the cell cycle as cyclin-dependent kinase inhibitors,[27] and are key regulators of growth responses in renal cells. A representative blot for each and the corresponding β-actin are shown (Figure 3). As seen in Figure 3a, 1 μM PGE_2 increased p27 levels by 1.75-fold and SLP by 1.5-fold (Figure 3c). Transfection of MCT cells with EP_4 siRNA (Figure 3d) and EP_1 siRNA (Figure 3e) attenuated the p27 response to PGE_2, though only significant with the EP_4 siRNA (Figure 3d). The scrambled siRNA however had no effect on p27 levels, though the co-stimulation with PGE_2 and scrambled siRNA was only numerically increased by 1.5-fold (Figure 3d). Interestingly, EP_4 siRNA alone reduced p27 levels by 35% (Figure 3d), but EP_1 siRNA alone had no effect (Figure 3e). In contrast, TGFβ reduced p27 by 56% (Figure 3a), and significantly attenuated the response to PGE_2 upon co-stimulation. PGE_2 did not affect p21 levels

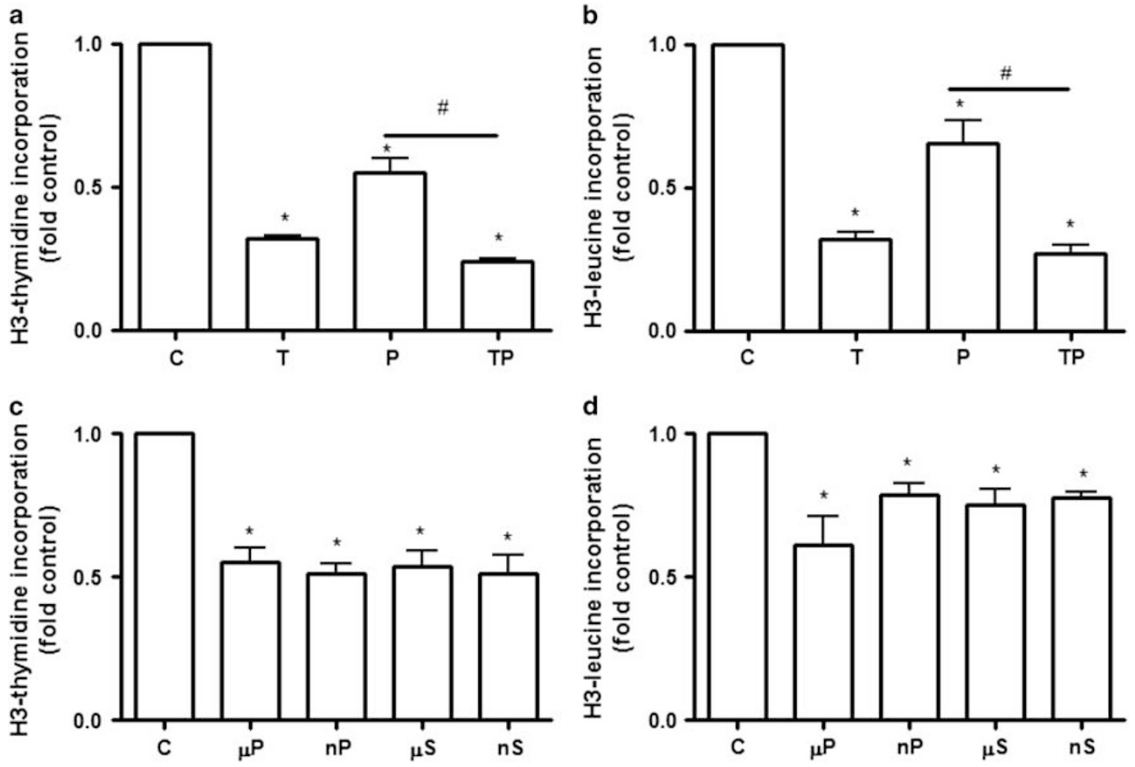

Figure 2 PGE_2 inhibits DNA and protein synthesis in MCT cells. MCT cells were stimulated for 24 h with TGFβ and PGE_2 in the presence or absence of TGFβ, and DNA and protein synthesis were assessed by ^{3}H-thymidine (**a**) and ^{3}H-leucine (**b**) incorporation. Comparisons were made between PGE_2 and SLP, at 1 μM or 1 nM for thymidine (**c**) and leucine (**d**). Data presented as mean ± s.e.m., fold control ($C=1$). $^*P<0.05$ *vs* C, $^{\#}P<0.05$ *vs* P, $n=5$.
C, control; P, PGE_2 (1 μM); PGE_2 1 μM (μP) and 1 nM (nP); SLP 1 μM (μS) and 1 nM (nS); T, transforming growth factor β (TGFβ, 2 ng/ml); TP, TGFβ+PGE_2.

(Figure 3b), but TGFβ increased p21 levels by 4.4-fold (Figure 3b). The PT growth responses are also regulated by cyclinD1/CDK4 and cyclinE/CDK2 complexes.[10] While PGE_2 did not alter cyclin D1 on its own, TGFβ increased cyclinD1 by 4.6-fold and PGE_2 attenuated this response to 1.6-fold (Figure 4a). PGE_2 did however decrease cyclinE by 25% (Figure 4b), whereas TGFβ had no effect on cyclinE levels. Similarly, SLP and 1 nM PGE_2 reduced cyclinE levels by 50–75% (Figure 4c). A representative blot for each of cyclinD1 and cyclinE, and their corresponding β-actin, are also shown in Figure 4.

PGE_2 Increases the Expression of Sodium Transporters in MCT Cells and Stimulates PT Fluid Reabsorption

PGE_2 has an important role in regulating salt and water transport along the nephron, but its effect on PT sodium and water transport is unknown. In MCT cells, PGE_2 increased Na^+-K^+-ATPase α1 (NaK) subunit by 2.4-fold (Figure 5a), whereas TGFβ alone had no effect. However, TGFβ significantly attenuated the stimulatory effect of PGE_2 to 0.5-fold. A representative blot showing NaK and the corresponding β-actin is shown for each experiment (Figure 5). Sulprostone (1 μM) only numerically increased NaK levels by 1.35-fold, $P>0.05$ (Figure 5b). Transfection of MCT cells with EP_4 siRNA abrogated the PGE_2-mediated NaK response from 1.95-fold to 1.3-fold, though not statistically significant (Figure 5c). Scrambled siRNA had no effect on NaK levels, and the PGE_2 effect in the presence of the scrambled siRNA was unchanged at 1.87-fold. As shown in Figure 5d, EP_1 siRNA also attenuated the PGE_2-induced NaK response to 1.2-fold. The majority of PT sodium transport is mediated by the sodium-hydrogen exchanger, NHE1 on the basolateral side and NHE3 on the apical membrane.[28] Unlike the increase in basolateral NaK shown here, NHE1 and NHE3 levels were unchanged following 24 h PGE_2 (unpublished data).

As shown in Figure 6, in isolated perfused mouse proximal convoluted tubules from C57BL/6 mice, PGE_2 increased net fluid reabsorption (Jv) in response to 1 nM and 0.1 μM PGE_2 (post-treatment), by 119% ($n=5$, $P=0.07$) and 131% ($n=5$, $P<0.05$) of control ($C=100$, pre-treatment), respectively (Figure 6a). The time course control was unchanged. In FVB mice, 0.1 μM PGE_2 stimulated Jv by 146% of control ($C=100$, $P<0.05$), and this effect was completely abolished in mice lacking EP_1 receptors to 96% of control (Figure 6b).

PGE_2 Increases Fibronectin in MCT Cells Via EP1 Receptors

To further characterize the PGE_2 response in MCT cells, the effect of PGE_2 upon fibronectin levels was assessed as previously described.[29] As shown in Figure 7a, fibronectin

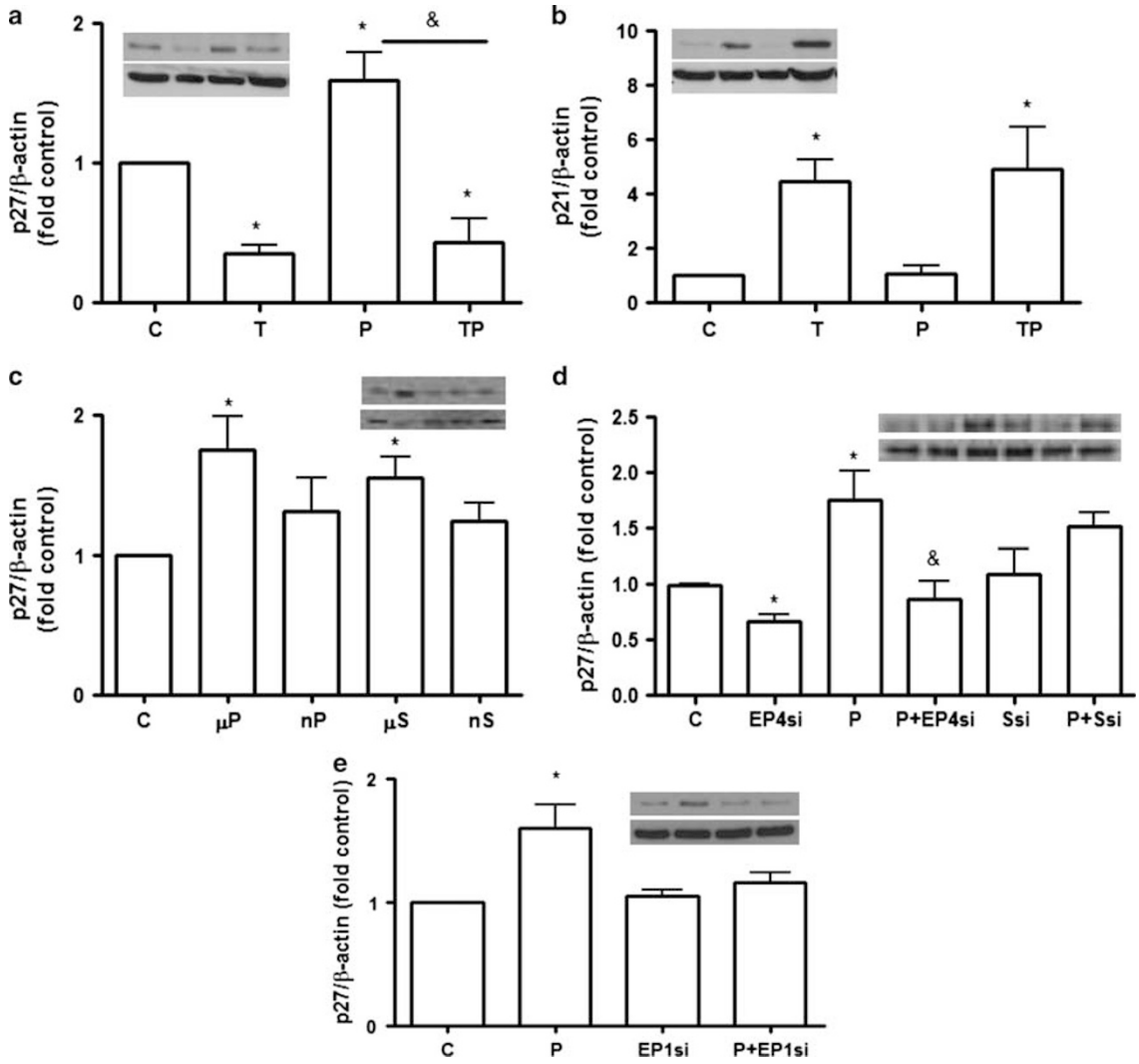

Figure 3 PGE_2 increases p27 levels in MCT cells via EP_1 and EP_4 receptors. MCT cells were stimulated for 24 h with TGFβ and PGE_2 in the presence or absence of TGFβ, and p27 (**a**) and p21 (**b**) levels were assessed by western blotting. Representative blots of p27 or p21 (upper) and corresponding β-actin (lower) are shown for each experiment. In (**c**), MCT cells were treated with PGE_2 and the SLP (1 μM or 1 nM), and the densitometric analysis of p27/β-actin is shown. In (**d**), MCT cells were transfected for 48 h with EP_4 siRNA (EP_{4si}) or a universal scrambled siRNA (Ssi), and PGE_2 was added during the final 24 h. In (**e**), MCT cells were transfected with EP_1 siRNA (EP_{1si}). The densitometric analysis is shown with data presented as mean ± s.e.m., fold control ($C=1$). $^{*}P<0.05$ *vs* C, $^{\&}P<0.05$ *vs* P, $n=4$–8. C, control; EP_1si or EP_4si, EP_1 or EP_4 siRNA (5 nM); P, PGE_2 (1 μM); PGE_2 1 μM (μP) and 1 nM (nP); SLP 1 μM (μS) and 1 nM (nS); P+EP_1si, PGE_2 and EP_1 siRNA; P+EP_4si, PGE_2 and EP_4 siRNA; P+Ssi, PGE_2 and scrambled siRNA; Ssi, scrambled siRNA; T, transforming growth factor β (TGFβ, 2 ng/ml); TP, PGE_2 and TGFβ.

levels were significantly increased in response to PGE_2 and TGFβ stimulation for 24 h, 1.9- and 6.5-fold, respectively, and then upon co-stimulation a greater response was observed, reaching 12-fold (Figure 8a). This was significantly greater than the response to PGE_2 alone, but not that of TGFβ alone. EP_1 siRNA reduced fibronectin, and significantly attenuated the response to PGE_2 (Figure 7b). As shown scrambled siRNA had no effect on fibronectin levels or the stimulatory response to PGE_2. On the other hand, EP_4 siRNA had the opposite effect of EP_1 siRNA, slightly increasing fibronectin by 1.55-fold (not statistically significant), and numerically enhanced the PGE_2 response from 2.1-fold to 2.96-fold (Figure 7c). Though TGFβ increased NFκB/GAPDH by almost 2-fold, PGE_2 had no effect on nuclear factor κB mRNA (Figure 7d).

PGE_2 Increases ROS Generation in MCT Cells Via EP1 Receptors

Since PGE_2 acting on EP_1 receptors increased fibronectin, and oxidative stress responses have a key role in the fibrotic process in PTs,[10,30], we studied whether EP_1 receptors are involved in ROS generation using the lucigenin-enhanced chemiluminescence assay. As shown in Figure 8, stimulation for 24 h with PGE_2 increased ROS production in MCT cells by 1.8-fold; and this response was attenuated by the EP_1

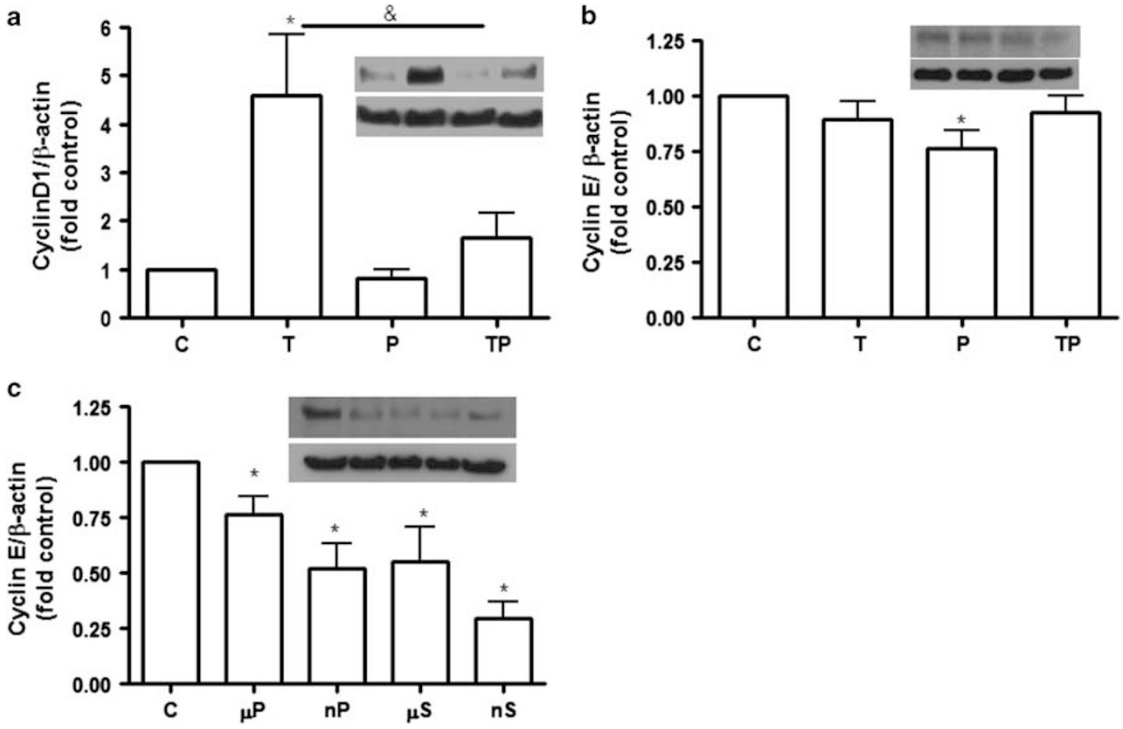

Figure 4 PGE_2 and SLP reduce cyclin E expression in MCT cells. MCT cells were stimulated for 24 h with TGFβ and PGE_2 in the presence or absence of TGFβ, and cyclin D1 (**a**) and cyclin E (**b**) levels were assessed by western blotting. In (**c**), MCT cells were treated with PGE_2 and SLP (1 μM or 1 nM), and cyclin E levels were assessed. A representative blot of cyclin (upper) and β-actin (lower) is shown for each. The densitometric analysis is presented with data expressed as mean ± s.e.m., fold control ($C=1$). $^{*}P<0.05$ *vs* C, $^{\&}P<0.05$ *vs* T, $n=6$. C, control; P, PGE_2 (1 μM); PGE_2 1 μM (μP) and 1 nM (nP); SLP 1 μM (μS) and 1 nM (nS); T, transforming growth factor β (TGFβ, 2 ng/ml); TP, TGFβ+PGE_2.

antagonist ONO-8711 to 1.2-fold. Apocynin a known inhibitor of NADPH oxidase completely abrogated the lucigenin response, suggesting that NADPH oxidase may be the source of ROS production by PGE_2.

DISCUSSION

In the kidney, PGE_2 is recognized for its vascular effects and glomerular hemodynamic responses, its role in distal tubule and collecting duct transport, and renin secretion at the level of the macula densa (reviewed in Nasrallah *et al*[31]). We examined whether EP receptors are present in the MCT and characterized the responses to PGE_2. The major findings are that MCT cells only express EP_1 and EP_4 receptors, and EP_1 is increased by both PGE_2 and TGFβ. An inhibition of the cell cycle (DNA and protein synthesis) was observed in response to PGE_2 and TGFβ in MCT cells, but this was achieved through distinct pathways: p27 *vs* p21, and cyclinE *vs* cyclinD1, respectively. Figure 9 summarizes the MCT growth response to PGE_2, upon activation of EP_1 or EP_4 receptors, compared with TGFβ. PGE_2 and TGFβ also have opposite effects on sodium transporters: PGE_2 increases the levels of NaK via EP_1 and EP_4, and the PGE_2 response is attenuated by TGFβ. In the isolated perfused PT, PGE_2 increased fluid reabsorption via EP_1 receptors. PGE_2 also increases MCT cell fibronectin and ROS generation via EP_1.

Though EP receptor protein levels along the nephron have proven difficult to detect, relying mainly on pharmacological responses to selective agonists and antagonists, a study reported the expression of all four EP receptor subtypes in rabbit PTs, which were isolated with iron oxide to separate glomeruli.[8] In our study, only EP_1 and EP_4 were detected in MCT cells at the mRNA level. Similarly, we previously isolated rat PTs by Percoll gradient and detected $EP_{(1,\ 3,\ and\ 4)}$ in the tubule suspensions, but when the suspensions were cultured under selective conditions for PT growth, the EP_3 receptor was no longer detectable.[32] Distal tubule contamination of the suspensions, that abundantly express EP_3, probably accounts for the detection of EP_3 receptors in these rat preparations, and could explain its detection in rabbit PTs,[8] alternatively species differences that might account for the discrepancy.

In kidney disease, the PT undergoes changes in growth, and is subjected to oxidative stress that alters cell fate and promotes fibrosis. It is increasingly recognized that the tubular-interstitial alterations are associated with progression of diabetic kidney disease. In a recent review by Tang and Lai[30] the pathogenic role of the PT epithelial cell and its activation by a number of substances is elaborately described, explaining the resulting cell-cycle arrest, inflammatory, and fibrotic responses, oxidative stress as well as altered transport function.[10] In the PT, CDK2/cyclinE and CDK4/cyclinD1

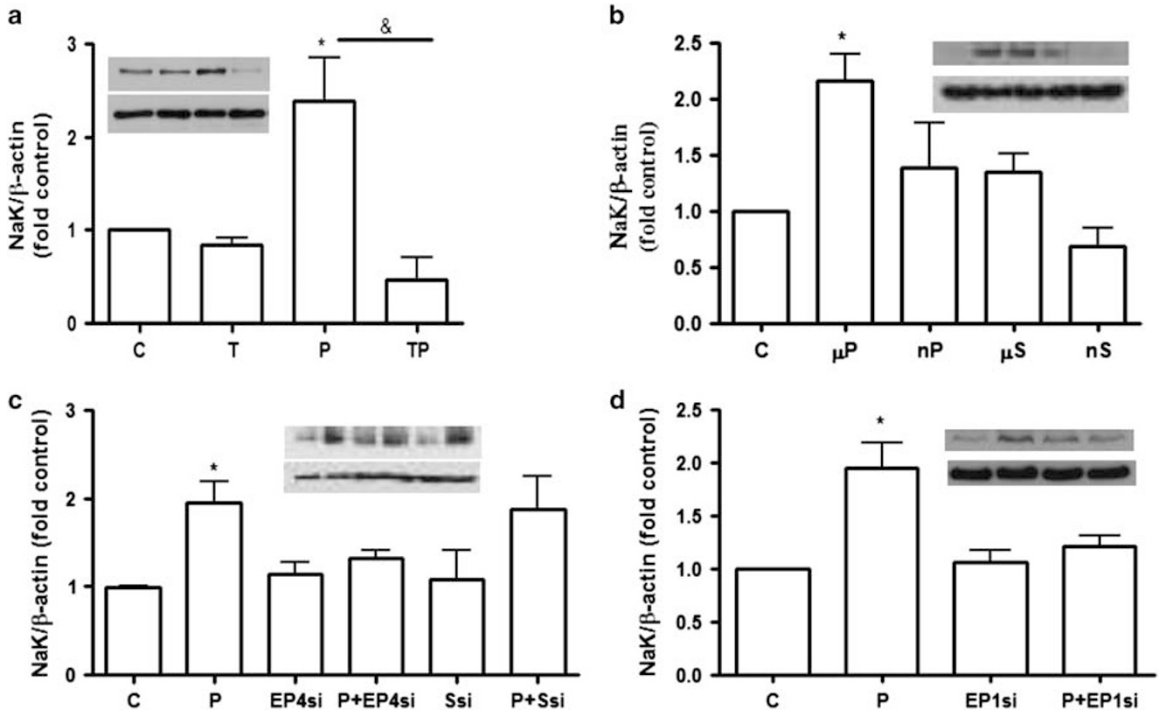

Figure 5 PGE_2 increases sodium potassium ATPase expression in MCT cells via EP_1 and EP_4 receptors. In (**a**), MCT cells were stimulated for 24 h with TGFβ and PGE_2 in the presence or absence of TGFβ, and sodium potassium ATPase α1 (NaK) levels were assessed by western blotting. A representative blot is shown for NaK (upper) and corresponding β-actin (lower) for each experiment. The densitometric analysis of NaK/β-actin is shown with data presented as mean ± s.e.m., fold control ($C = 1$). In (**b**), MCT cells were treated with PGE_2 and SLP (1 μM or 1 nM). In (**c**), MCT cells were transfected for 48 h with EP_4 siRNA (EP_{4si}) or scrambled siRNA (Ssi), and PGE_2 was added during the final 24 h. In (**d**), MCT cells were transfected with EP_1 siRNA (EP_{1si}). C, control; EP_1si or EP_4si, EP_1 or EP_4 siRNA (5 nM); P+ EP_1si, PGE_2 and EP_1 siRNA; P+EP_4si, PGE_2 and EP_4 siRNA; P, PGE_2 (1 μM); PGE_2 1 μM (μP) and 1 nM (nP); SLP 1 μM (μS) and 1 nM (nS); P+Ssi PGE_2 and scrambled siRNA; Ssi, scrambled siRNA (5 nM); T, transforming growth factor β (TGFβ, 2 ng/ml); TP, PGE_2 and TGFβ. $^*P < 0.05$ *vs* C, $^{\&}P < 0.05$ *vs* P, $n = 4$–8.

regulate cell-cycle progression, and this is inhibited by cyclin-dependent kinase inhibitors p27 and p21.[10] The contribution of PGE_2 to regulation of cell cycle in healthy and injured renal cells has been described[16,33–36] but not in the PT. Our work showed that PGE_2 and SLP inhibit the cell cycle in MCT cells similar to TGFβ, reducing both protein and DNA synthesis. As depicted in Figure 9, the effects on regulators of cell-cycle progression in MCT cells were opposite for PGE_2 and TGFβ. While PGE_2 increased p27 via EP_1 and EP_4 receptors, TGFβ increased p21. Terada *et al*[37] previously reported this same growth inhibitory response to 12 h TGFβ treatment in renal tubular LLC-PK1 cells, but mediated by decreased cyclin D1 levels rather than increased p21.

TGFβ is most often a proliferation inhibitor, especially in epithelial cells.[38–42] Though our studies corroborate this effect, we also noted an increase in cyclinD1 levels in MCT cells stimulated with TGFβ. Since p21 binds CDK2 and inhibits the cell cycle, it is not unforeseen that cyclinD1 levels are transiently increased as part of a mechanism of cell adaptation to the quiescent state, but detailed time analysis is needed to confirm this possibility. Additionally, both PGE_2 and SLP reduced cyclin E, and PGE_2 attenuated TGFβ-mediated cyclinD1. Since transfection of MCT cells with EP_1 and EP_4 siRNA abrogated the increase in p27, the inhibition of the cell cycle in MCT cells is likely mediated by PGE_2 acting on both EP_1 and EP_4 receptors to increase p27. However, the underlying mechanisms appear to be species and cell type specific (tubular epithelial cells *vs* vascular smooth muscle cell-like mesangial cells), and highly dependent on the culturing conditions. They may also be related to the concentration of PGE_2 used, for instance, lower concentrations of PGE_2 also increased p27 in rat mesangial cells causing cell-cycle arrest via the EP_1 receptor, but increased protein synthesis.[43] We did not observe a p27 response to 1 nM PGE_2 in the MCT cells, though the inhibition of DNA synthesis was not concentration dependent and was noted with higher and lower concentrations of both PGE_2 and SLP. Another interesting observation is that we only observed a senescent-like (reduced DNA and protein synthesis) response to PGE_2 like TGFβ, without any cell hypertrophy. The typical hypertrophic response often described in kidney disease characterized by reduced DNA but increased protein synthesis is only observed under certain conditions in renal cells, and it appears to be delayed, requiring 48–96 h.[44,45] In a study looking at angiotensin II responses in PT cells, 48 h of angiotensin II treatment reduced thymidine but increased

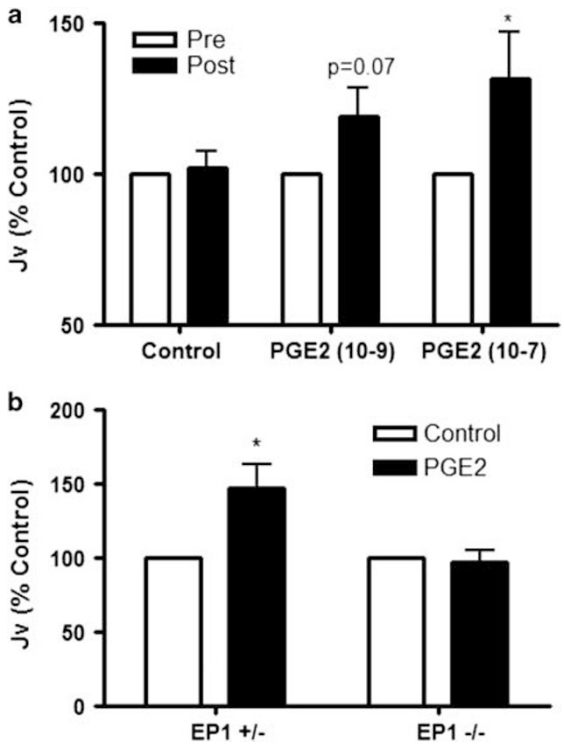

Figure 6 PGE_2 increases fluid reabsorption in mouse proximal tubules via EP_1 receptors. In (**a**), mouse isolated perfused tubules from C57BL/6 mice were treated with PGE_2 (10^{-9} and 10^{-7} M), and net fluid reabsorption (Jv: nl/mm/min) was determined. A time control is also shown (Control). Data are shown as mean ± s.e.m. of the three highest collections pre- (untreated, white bars) and post-PGE_2 treatment (black bars), and expressed as % of pre-treatment control (pre, white bars) which is presented at 100%. $N=5$. $^*P<0.05$ *vs* respective pre-treatment. In (**b**), tubules were obtained from FVB heterozygous (EP_1 +/−) or mice lacking EP_1 receptors (EP_1 −/−), and Jv is presented as mean ± s.e.m. for untreated (control, white bars) and PGE_2-treated tubules (black bars). The percentage of control ($C=100$) for $n=4$ and 5 tubules is shown, respectively. Results of paired *t*-test analysis are indicated. $^*P<0.05$ *vs* control.

leucine incorporation. TGFβ also induces this hypertrophic response following longer exposure in PT cells.[44,45] Therefore, it is possible that this senescent-like state described in our 24 h study is transient, and PGE_2 would have the same hypertrophic effect with longer stimulations. Further studies are needed to explore this possibility. Regardless of the exact mechanisms leading to cell-cycle arrest, the resultant senescence may have important implications in the context of renal disease progression, leading to inflammatory and fibrotic responses, and perturbed PT transport function (reviewed in Vallon and Thomson[12]). These findings lead to interesting avenues for future exploration.

It is proposed that PT growth promotes PT sodium hyperreabsorption in chronic kidney disease, which in turn contributes to hyperfiltration and renal injury.[46] Therefore, the contribution of PGE_2/EP receptors to changes in salt and water transporters in the PT is of great interest. Our work indicates that PGE_2 increases sodium transporter expression in PT cells via EP_1- and EP_4-dependent mechanisms. The increase in NaK is consistent with other reports showing that PGE_2 stimulates NaK via PKA (conceivably mediated by EP_4); however, PKC (probably EP_1)-dependent mechanisms were also reported in mouse and rabbit PTs.[8,15] Similarly, the stimulation of PT sodium hydrogen exchanger (NHE3) expression by PGE_2 was indirectly confirmed in a previous study showing that inhibition of COX-2 derived PGE_2 in a model of rat experimental colitis, using COX-2 antisense oligonucleotides, attenuated the rise in PT NHE3 associated with the inflammatory state.[47] We did not observe a change in NHE1 or NHE3 in response to PGE_2 in our cells (unpublished data).

In addition to increasing NaK in MCT cells, PGE_2 stimulated fluid reabsorption in isolated perfused mouse tubules. The mean response over baseline was 31% in C57BL/6 mice, and 46% in FVB mice; of importance, this is the first demonstration in mouse PT of a functional role for PGE_2 in this segment. We also confirmed the role of EP_1 receptors in the PGE_2 mediated fluid reabsorption, since the stimulatory effect was absent in EP_1 null tubules. However, we cannot rule out the involvement of EP_4 receptors at this time since global knockouts of EP_4 are not available due to lethality.[48] Interestingly, we had previously reported a minimal response to PGE_2 on baseline water transport in the isolated perfused rabbit cortical collecting duct. Only in the presence of vasopressin did PGE_2 greatly affect the transport properties of the collecting duct.[26] In a rat study of straight PT, Garvin[49] demonstrated a 20% response in fluid transport with angiotensin II, but to the best of our knowledge there are no other studies looking at hormonally stimulated water transport in the mouse PT. Interestingly, the minimal responses in the PT may be due to shifting of transport between paracellular and transcellular pathways, so that by altering aquaporin-1 mediated transcellular transport in response to angiotensin II or PGE_2, paracellular transport is reduced, and overall balance is maintained. A nice portrayal of these two fluid transport systems in the proximal has been elaborately presented by Schnermann *et al*,[50] using claudin-2/aquaporin-1 double knockout mice, showing the shift from paracellular (claudin-2 dependent) to transcellular (AQP-1 dependent) transport in single (AQP-1 or claudin-2) knockouts to compensate for the lacking transport pathway. Another possibility is that in the healthy PT fluid transport is not very dependent on hormonal stimulation, and in the challenged (kidney disease) PT there is greater hormone dependence. Studies are ongoing in our laboratory to validate this possibility.

A common feature of progressive kidney disease is fibrosis induction, characterized by cytoskeletal reorganization, matrix changes, and scarring. TGFβ is the main growth factor reported to mediate the initiation and maintenance of the fibrotic process.[14] In our study, we show that PGE_2 increases fibronectin levels in mouse proximal tubule MCT cells, and EP_1 but not EP_4 receptors are involved in this response. Numerous studies implicate oxidative stress in the PT changes

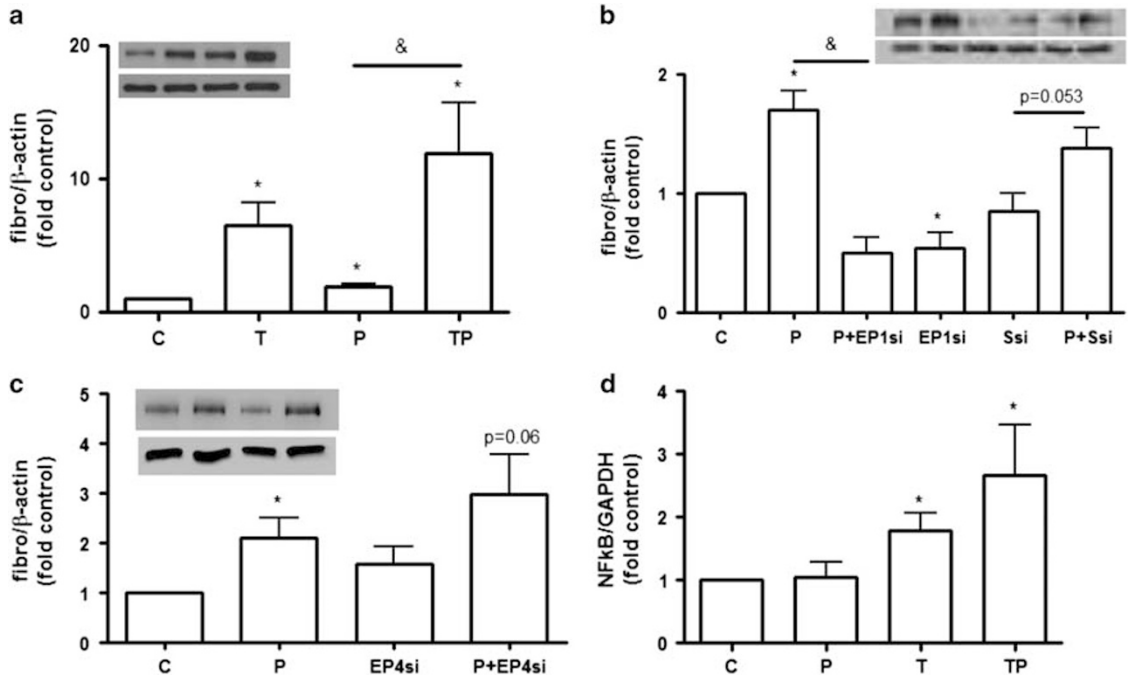

Figure 7 PGE_2 increases fibronectin levels in MCT cells via EP_1 receptors. MCT cells were stimulated for 24 h with TGFβ and PGE_2 in the presence or absence of TGFβ, and fibronectin levels (fibro) were assessed by western blotting (**a**). A representative blot is shown for fibronectin (upper) and corresponding β-actin (lower), and the densitometric analysis of fibro/β-actin is shown for each experiment. In (**b**), MCT cells were transfected for 48 h with EP_1 siRNA (EP1si) or scrambled siRNA (Ssi), and PGE_2 was added during the final 24 h. In (**c**), MCT cells were transfected with EP_4 siRNA (EP_{4si}). In (**d**), following 24 h stimulations, NFκB expression was assessed by quantitative PCR and normalized to GAPDH. Data are presented as mean ± s.e.m., fold control ($C=1$). $^*P<0.05$ *vs* C, $^{\&}P<0.05$ *vs* P. $N=4$–7. C, control; EP_1si or EP_4si, EP_1 or EP_4 siRNA (5 nM); NFκB, nuclear factor kappa B; P, PGE_2 (1 μM); P+EP_1si, PGE_2 and EP_1 siRNA; P+EP_4si, PGE_2 and EP_4 siRNA; P+Ssi PGE_2 and scrambled siRNA; Ssi, scrambled siRNA (5 nM); T, transforming growth factor β (TGFβ, 2 ng/ml); TP, TGFβ+PGE_2.

in response to high glucose, and reduction of ROS reduces fibrosis in this segment of the nephron.[51] Our group[29] recently demonstrated a role for NADPH oxidase NOX-4 in fibrotic events resulting from exposure of MCT cells to high glucose. PGE_2 also increased ROS generation in MCT cells, and like fibronectin EP_1 receptors mediate this effect. This finding is consistent with our recent work in diabetic EP_1 receptor null mice, where cortical fibronectin levels as well as ROS were increased in diabetic OVE26 mice, and reduced in diabetic-EP_1 null mice.[20] Further confirming that antagonism of EP_1 protects against PT injury.

Clinical Perspectives and Significance

In kidney disease, the PT is subjected to sodium and water transport challenges. Though basal PT water transport properties are not highly dependent on PGE_2, a more pronounced role may be observed in kidney disease for example in diabetes, where the transport behavior of this segment is heightened. We have clearly shown that PGE_2 acting on EP_1 and EP_4 receptors in MCT cells can modify growth responses and sodium transporter expression, and the

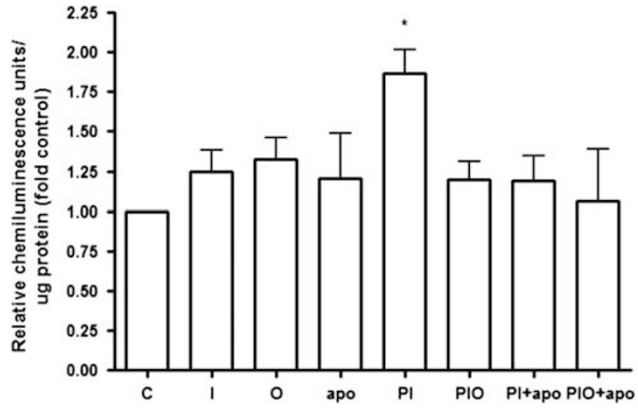

Figure 8 PGE_2 increases ROS generation in MCT cells via EP_1 receptors. Lucigenin assay following 24 h stimulations of MCT cells. Data are presented as relative chemiluminescence units per µg of protein. The mean ± s.e.m. are shown as fold control ($C=1$). $^*P<0.05$, $n=5$. apo, apocynin (NADPH oxidase inhibitor; C, control (DMEM); I, indomethacin (cyclooxygenase inhibitor; 10 μM); O, ONO-8711 (EP1 antagonist; 100 nM); PI, PGE_2 (1 μM) + indomethacin; PIO, PGE_2 +indomethacin+ONO-8711; 50 µM); PI+apo, PGE_2+indomethacin +apocynin; PIO+apo, PGE_2+indomethacin+ONO-8711+apocynin.

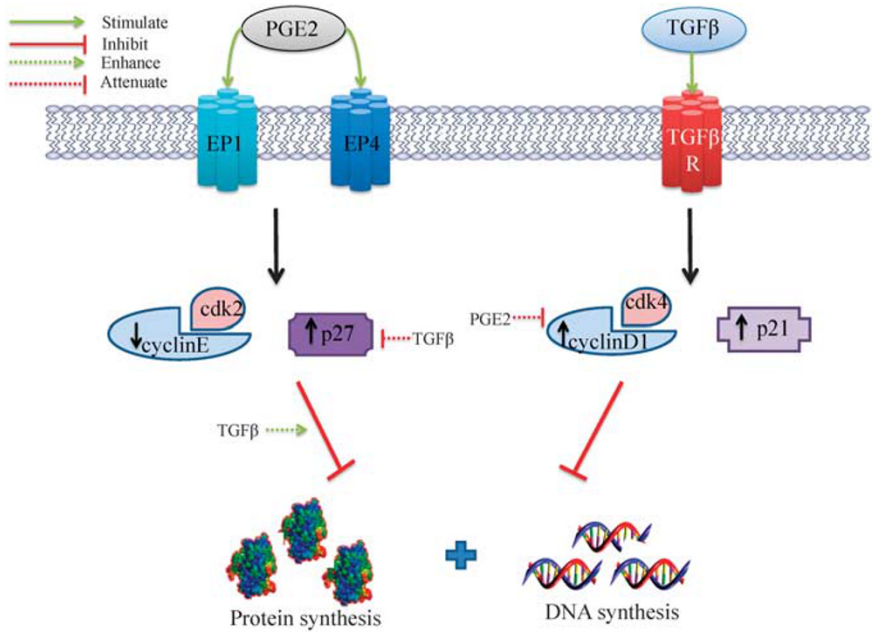

Figure 9 PGE_2 and TGFβ inhibit growth responses in MCT cells. As depicted in this summary, PGE_2 and TGFβ affect different cell-cycle regulators in MCT cells to inhibit protein and DNA synthesis. In proximal tubule cells, growth responses are mainly regulated by cyclin-dependent kinase inhibitors p27 and p21, or cyclinD1/CDK4 and cyclinE/CDK2 complexes. Our study shows that in MCT cells PGE_2 binds cell surface EP_1 or EP_4 receptors to increase p27 and reduce cyclinE, whereas TGFβ increases p21 and cyclinD1. PGE_2 attenuated the TGFβ-cyclinD1 response, and TGFβ attenuated the PGE_2-p27 response. TGFβ also enhanced the growth inhibitory response of PGE_2. But the overall effect is cell-cycle arrest.

EP_1 receptor contributes to fibrosis and oxidative stress. Since PT growth promotes PT sodium hyper-reabsorption in chronic kidney disease, which in turn contributes to hyperfiltration and renal injury, PGE_2 acting on PT EP receptors has the potential to influence PT sodium and water transport and injurious processes, and contribute to kidney disease.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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