Antagonism of the prostaglandin E\(_2\) EP1 receptor in MDCK cells increases growth through activation of Akt and the epidermal growth factor receptor

Mary Taub, Robert Parker, Paremala Mathivanan, Muhamad Asnawi Mohd Ariff, and Trina Rudra

Biochemistry Department, University at Buffalo School of Medicine and Biomedical Sciences, Buffalo, New York

Submitted 17 September 2013; accepted in final form 18 June 2014

Taub M, Parker R, Mathivanan P, Ariff MA, Rudra T. Antagonism of the prostaglandin E\(_2\) EP1 receptor in MDCK cells increases growth through activation of Akt and the epidermal growth factor receptor. Am J Physiol Renal Physiol 307: F539–F550, 2014. First published July 9, 2014; doi:10.1152/ajprenal.00510.2013.—The actions of prostaglandin E\(_2\) (PGE\(_2\)) in the kidney are mediated by G protein-coupled E-prostanoid (EP) receptors, which affect renal growth and function. This report examines the role of EP receptors in mediating the effects of PGE\(_2\) on Madin-Darby canine kidney (MDCK) cell growth. The results indicate that activation of Gs-coupled EP2 and EP4 by PGE\(_2\) results in increased growth, while EP1 results in an increase in intracellular Ca\(^{2+}\), Akt phosphorylation, and the increased phosphorylation of PKC. All four subtypes of EP receptors are present in the kidney (9). Especially high levels of EP1, EP3, and EP4 are present in the collecting duct, where these receptors regulate salt and water reabsorption. While EP receptor levels are not necessarily as high elsewhere in the kidney, EP receptors nevertheless control a number of reabsorptive processes in other nephron segments, as exemplified by its regulation of Na\(^+\)-phosphate cotransporters in the renal proximal tubule (RPT) (49) and Na-K-ATPase in the distal tubule (58).

PGE\(_2\) and/or EP4 receptors reportedly are protective in a number of renal disease states, unlike EP1. Indeed, activation of EP4 (using the EP4 agonist CP-044, 519-02) reduced RPT necrosis and apoptosis in a mercuric chloride model of acute renal failure (57). Similarly, EP2 and EP4 receptor activation increased growth (and reduced apoptosis) in renal proximal and distal tubules in a rat model of chronic renal failure (57). In contrast, antagonism of EP1 (rather than activation of EP1 by agonists) has been reported to be protective. For example, EP1 antagonism (by the EP1-selective antagonist ONO-8713) prevented progressive renal damage in stroke-prone spontaneously hypertensive rats (SHRSP) (48). Similarly, the development of nephropathy in streptozotocin-induced diabetic rats was retarded by EP1 antagonists (6, 35, 36). It is unclear whether J) EP1 receptors have direct, or indirect roles in these processes, and 2) whether kidney epithelial cell growth is affected in these disease states, as a consequence of EP1 receptor activation.

Previously, we observed stimulatory effects of PGE\(_2\) on both the growth and transport of Madin-Darby canine kidney (MDCK) cells in hormonally defined serum-free medium (50, 55). However, the growth-stimulatory effects of PGE\(_1\) and PGE\(_2\) were no longer observed in PGE\(_1\)-independent MDCK cells with elevated intracellular cAMP levels (53). These latter observations can be explained by the involvement of EP2 and/or EP4 receptors in mediating the growth-stimulatory effects of PGE\(_1\) and PGE\(_2\). Similarly, the stimulatory effect of PGE\(_2\) on Na-K-ATPase was lost in dibutyryl cAMP-resistant MDCK cells defective in cAMP-dependent protein kinase (PKA), also consistent with the involvement of EP2 and EP4 receptors (due to their ability to activate AC) (14). Subsequently, we found that the stimulatory effects of PGE\(_2\) on Na-K-ATPase could be attributed to transcriptional control of the Na-K-ATPase \(\beta\)-subunit gene and that EP1 and EP2 were involved, although all 4 EP receptors are present in MDCK cells (38, 39, 50). Similarly, our studies indicated that EP1, EP2, as well as EP4 were involved in transcriptional regulation of the Na-K-ATPase \(\beta\)-subunit gene in primary rabbit RPT cells (25).

This report evaluates whether Gq-coupled as well as Gs-coupled EP receptors similarly mediate the growth-stimulatory effects of PGE\(_2\) on MDCK cells. Our results indicate that both
EP2 and EP4 are involved in mediating the growth-stimulatory effects of PGE2 in MDCK cells, while EP1 is growth inhibitory, placing a brake on EP2- and EP4-mediated control. Our results indicate that Akt as well as the EGFR receptor (EGFR) tyrosine kinase are both included among the signaling pathways responsible for growth regulation mediated via EP1.

MATERIALS AND METHODS

Materials. Hormones, human transferrin, and other chemicals were from Sigma-Aldrich (St. Louis, MO). DMEM, Ham’s F12 medium (F12), and soybean trypsin inhibitor were from Invitrogen (Carlsbad, CA). The rabbit polyclonal antibodies against p-Akt 1/2/3 (sc-33437), the goat polyclonal antibodies against Akt1 (sc-1618) and EGFR (sc-03-G), and the mouse monoclonal antibody against β-actin (sc-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody against human EP1 (LS-A962–50) was from Lifespan Biosciences. The goat anti-goose rhadish peroxidase (HRP) conjugate was from Abcam (Cambridge, MA). The goat anti-mouse HRP conjugate, the goat anti-rabbit HRP conjugate, the Immun-Star HRP Substrate, nitrocellulose, acrylamide, and other electrophoresis reagents were from Bio-Rad (Hercules, CA). LucentBlue X-ray film was from Advansta (Menlo Park, CA). The Prisma 6 program was obtained from GraphPad Software, (San Diego, CA). Lentiviral particles containing a pLKO.1 vector expressing short hairpin (sh) RNA against J) the EP1 receptor (TRCN0000415217) with the sequence CCGGATCATGGTGGTGGTGCGTCCTCGGAATTCAAAAGCAAGTTTTTTTTTT, which is present in both the human and dog EP1 receptor; 2) the EGFR (TRCN0000259791) with the sequence CCGTCCTCCAGAGGATGTTGACATTTTTTTTTTTT, present in both human and dog EP1; and 3) the empty vector were obtained from Sigma-Aldrich. The dominant negative EGFR mutant HER CD533 (26) in pcDNA3 or the empty vector (pcDNA3). Two days later, the cultures were resuspended in PBS and counted in a Coulter counter. After detachment, a soybean trypsin inhibitor was added. The cells were washed twice with PBS and incubated with EDTA/trypsin. After detachment, a soybean trypsin inhibitor was added. The cells were resuspended in PBS and counted in a Coulter counter. At the initiation of the growth study, MDCK cells were inoculated at 10^5 cells/dish into culture dishes containing DME/F12 supplemented with 5 μg/ml bovine insulin, 5 μg/ml human transferrin, and other effector molecules, as described in the experiment. The cultures were maintained in a humidified 5% CO2-95% air environment for 6 days, unless otherwise stated. After 6 days, the cells were removed from the culture dishes with EDTA-trypsin and counted in a Coulter counter. The average cell number in each experimental condition was calculated from triplicate determinations. The control cell number was the average cell number in present cultures grown in DME/F12 supplemented with 5 μg/ml insulin and 5 μg/ml transferrin, unless otherwise stated.

In the growth studies with MDCK cells with ibuprofen, ibuprofen concentrations were employed that inhibit PGE2 biosynthesis >85%, by RIA (13). In growth studies with first-passage (P1) rabbit RPT cells, the cells were plated at 1.8 × 10^6 cells/35-mm dish, while in the growth studies with M1 cells, M1 cells were plated at 10^5 cells/60-mm dish. To study colony formation by M1 cells, the cells were plated at 250 cells/dish. After 10 days in culture, cultures were washed with PBS, incubated with formalin for 5 min, and stained with crystal violet. Colonies were photographed with a Canon Powershot A630 camera. The number of colonies in each condition was averaged from triplicate determinations.

Preparation of cell lysates for electrophoresis. At the end of the incubation period, the monolayers were washed with ice-cold PBS at 4°C and solubilized at 4°C in a lysis buffer containing 20 mM Tris, pH 7.5, 1% Triton X-100, 120 mM NaCl, 1 mM EDTA (RIPA), as well as protease and phosphatase inhibitors, including 1 mM PMFS, 1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM Na+ orthovanadate and 1 mM NaF. Cell lysates were removed from the dishes with a rubber policeman and transferred into microfuge tubes at 4°C.

Western blot analysis. Samples were equalized with regard to protein, based upon Bradford protein determinations (7), and separated by electrophoresis through 7.5% SDS/polyacrylamide gels, in parallel with molecular weight markers. The proteins in the gels were transferred to nitrocellulose using a Trans-Blot Apparatus (Bio-Rad). Blots were blocked 1 h in Tris-buffered saline (TBS) containing 0.1% (vol/vol) Tween 20 (TTBS), followed by a 2-h incubation with primary antibody in TTBS. Subsequently, the blots were washed six
times with TTBS (10 min/wash), followed by a 45-min incubation with a horseradish peroxidase (HRP) conjugated-secondary antibody. After the incubation with a secondary antibody, the blots were washed seven times with TTBS (15 min/wash). Finally, the blots were incubated with Immuno-Star HRP Luminol/Enhancer (Bio-Rad), and bands were visualized using LucentBlue X-ray film. Blots probed initially with a primary antibody of interest were subsequently reprobed with an antibody against a loading control such as β-actin. In the case of anti-p-Akt antibody, the blots were reprobed with anti-Akt antibody. X-ray films of the blots were scanned with a Bio-Rad scanning densitometer, and the relative band intensity was quantitated using the Quantity One Program.

**Calculation of results of growth studies and statistics.**

The average cell number in each experimental condition, including the control condition, was divided by the average cell number in the control condition using the Prism 6 Program. Thus the control value was 1, unless otherwise stated. For each experimental condition, the “fold-control cell number” was the ratio obtained by dividing the cell number in each experimental condition by the control cell number, unless otherwise specified. To determine whether differences between conditions were statistically significant, t-tests were conducted using Prism 6 software. Differences were deemed significant when $P < 0.05$.

**RESULTS**

Both EP2 and EP4 receptors mediate the growth-stimulatory effect of PGE2. Previously, we reported that PGE1 and PGE2 stimulate MDCK cell growth in defined medium (51). To identify the EP receptors that are involved, the effects of a number of EP receptor-specific agonists and antagonists were examined. Initially, the effect of the EP4 receptor antagonist L161, 982 and the EP2 receptor antagonist AH6809 on the growth-stimulatory effect of PGE2 was examined. Figure 1A shows that L161, 982 inhibited the PGE2 stimulation by 2.4-fold at $5 \times 10^{-7}$ M. AH6809 also inhibited the PGE2 stimulation, as shown in Fig. 1B. The involvement of EP2 was examined further using an EP2-specific agonist. Figure 1C shows a significant growth-stimulatory effect of butaprost at concentrations ranging from $5 \times 10^{-8}$ to $5 \times 10^{-7}$ M.

**Role of EP1 receptors:** effect of SC51089 and ONO-8711.

To determine whether Gq-coupled EP1 is also involved, the effect of the EP1 antagonist SC51089 was examined in two different culture conditions, including 1) medium supplemented with insulin and transferrin, while lacking PGE2; and 2) medium further supplemented with 70 nM PGE2. Figure 2A shows results when cultures were grown in the control condition (lacking PGE2). SC51089 was added at the beginning of the growth study, along with the other supplements. Under these conditions, 2 μM SC51089 increased growth 1.8 ± 0.1-fold in the absence of PGE2 (relative to the control value in medium lacking PGE2). Similarly, 70 nM PGE2 increased MDCK cell growth 2.2 ± 0.2-fold relative to the control condition (i.e., the culture condition lacking PGE2 and SC51089). MDCK cell growth increased even further when 2 μM SC51089 was present as well as PGE2 [growth increased 3.2 ± 0.2-fold relative to control (lacking PGE2) and 1.8 ±
0.1-fold relative to cultures grown with PGE2 but in the absence of SC51089. These results can be explained if 1) EP1 receptor activation results in growth inhibition (rather than a stimulation of growth), and 2) SC51089 prevents the growth inhibition caused by EP1 receptor activation, by preventing the interaction of PGE2 with EP1. Consistent with this hypothesis, Fig. 2B shows that another EP1 antagonist, ONO-8711, increased MDCK cell growth both in the presence of PGE2 (a 2.2 ± 0.3-fold increase relative to cultures with PGE2 and lacking ONO-8711) as well as in the absence of PGE2 [a 1.9 ± 0.1-fold increase relative to control MDCK cells (grown in the absence of both ONO-8711 and PGE2)].

**Effect of EP1 knockdown on the ONO-8711 stimulation.** To determine whether the stimulatory effect of ONO-8711 is indeed due to its interaction with the EP1 receptor (thereby preventing EP1 activation by PGE2), MDCK cells were transduced with lentiviral particles containing the pLKO.1 expression vector with EP1 shRNA, in parallel with transductions with the empty vector pLKO.1. Figure 3A shows the expression of the 41.8-kDa EP1 receptor in MDCK cells transduced with the empty vector. In MDCK cells with EP1 shRNA, the level of the EP1 receptor was reduced by 82 ± 1%, compared with MDCK with the empty vector.

The effect of ONO-8711 on growth was examined in MDCK cells with this EP1 knockdown (KD), relative to MDCK cells with the empty vector. Figure 3B shows that in the absence of PGE2 30 nM ONO-8711 caused a 1.9 ± 0.2-fold increase in the growth of MDCK cells transduced with the empty vector relative to untreated, control EV-MDCK cells. In the presence of PGE2, a 1.8 ± 0.1-fold increase in growth was also observed in MDCK cells with the empty vector (relative to the growth obtained with PGE2 alone). In contrast, a significant growth stimulatory effect of 30 nM ONO-8711 was not observed in MDCK cells with lentiviral EP1 shRNA, when they were maintained either in the presence of PGE2 or in the absence of PGE2. These results support the hypothesis that the growth-stimulatory effect of ONO-8711 is a consequence of its interaction with the EP1 receptor.

**Studies with M1 collecting duct cells and rabbit RPT cells.** To determine whether the growth-stimulatory effect of ONO-8711 is a common property shared by other kidney tubule epithelial cell culture systems, two additional kidney cell culture systems were examined, including the mouse M1 cortical collecting duct cell line and primary rabbit RPT cells. Figure 4A shows that the EP1 receptor is expressed in M1 cells at a level equivalent to that observed in MDCK cells, while in primary RPT cells EP1 expression is 1.5-fold higher.

The effect of 30 nM ONO-8711 and 70 nM PGE2 on colony formation as well as the growth of M1 cells was examined. As shown in Fig. 4B and C, 30 nM ONO-8711 and 70 nM PGE2 both increased the number of M1 colonies significantly relative to cultures in the control condition [that is, cultures that were not treated with ONO-8711 (i.e., that were untreated and without PGE2); 11.1 ± 0.7- and 11.1 ± 0.5-fold, respectively]. ONO-8711 also significantly increased the number of M1 colonies in the presence of PGE2 (1.4 ± 0.1-fold relative to cultures with PGE2 alone). As shown in Fig. 4D, 30 nM ONO-8711 and 70 nM PGE2 similarly increased the overall growth of M1 cells [8.1 ± 0.0- and 6.9 ± 0.4-fold, respectively, relative to the control value (i.e., the average number obtained with untreated M1 cells without PGE2)]. When ONO-8711 was added with PGE2, ONO-8711 also significantly increased growth above the level obtained in the presence of PGE2 alone.

The effect of ONO-8711 and PGE2 on the growth of rabbit RPT cells was also studied. As shown in Fig. 4E, 30 nM ONO-8711 and 70 nM PGE2 both significantly increased the growth of rabbit RPT cells when added individually (a 1.5 ± 0.05- and 1.2 ± 0.05-fold increase, respectively, relative to the control value, i.e., the value in untreated cultures without PGE2). A further increase was observed when ONO-8711 was added in the presence of PGE2 (1.4 ± 0.1-fold relative to the PGE2 control). Thus these results indicate that the growth-stimulatory effect of ONO-8711 is not a response specific to the MDCK cell line but instead is a more generalized characteristic of cultured kidney tubule epithelial cells.

**Effect of ibuprofen on ONO-8711 stimulation.** The studies described above suggest that EP1 antagonists may alleviate growth inhibition caused by EP1 receptors both in the presence and in the absence of exogenous PGE2. Presumably, EP1 receptor-mediated growth inhibition in the absence of exogenous PGE2 is a consequence of endogenously produced PGE2.

To evaluate this hypothesis, the effect ONO-8711 was studied in the presence of increasing concentrations of ibuprofen, a cyclooxygenase (COX) 1,2 inhibitor (in the absence of exogenous PGE2). Figure 5A shows that the ONO-8711 stimulation was gradually lost, as the ibuprofen concentration was increased to 100 μM. The effect of exogenous PGE2 on growth was also examined in the presence of ibuprofen, because the growth stimulation caused by effectors (such as ONO-8711) may possibly be lost as a consequence of nonspecific inhibitory
effects of ibuprofen on EP receptor signaling. Figure 5B shows that PGE2 (0.070 μM) was still growth stimulatory in the presence of ibuprofen (relative to untreated MDCK cells with ibuprofen). Thus these results can be interpreted as indicating that the growth-stimulatory effects of ONO-8711 are lost when EP1 receptors were no longer activated by PGE2. Therefore, these results support the hypothesis that the increased growth caused by ONO-8711 was due to the ability of ONO-8711 to block the interaction of PGE2 with EP1 receptors, thereby preventing growth inhibition caused by EP1 receptor activation.

Involvement of Akt in mediating the effect of ONO-8711 and insulin. EP1 receptor antagonists reportedly cause activation of Akt in hippocampal slices in vitro (62). Similarly, insulin has been reported to cause the phosphorylation and activation of Akt in mammalian systems (24). Thus the involvement of Akt in mediating the effects of insulin and ONO-8711 in MDCK cells was examined by Western blot analysis. Figure 6 shows that the level of phosphorylated Akt (pAkt) increased following a 30-min incubation with either 5 μg/ml insulin or 30 nM ONO-8711 (by 4.4 ± 0.3, and 1.9 ± 0.1-fold, respectively). The level of pAkt did not increase further when insulin and ONO-8711 were added in combination. The latter results may be explained if 1) the growth-stimulatory effects of ONO-8711 and insulin depend upon a threshold level of phosphorylation of Akt (without requiring an additive effect), and thus 2) other signaling pathways are involved.

Fig. 4. Effect of ONO-8711 on the growth of mouse M1 collecting duct cells and rabbit kidney proximal tubule cells. A: expression of EP1 receptor in MDCK, M1, and primary rabbit renal proximal tubule (RPT) cells. The expression of the EP1 receptor is shown in a Western blot of MDCK cells, M1 mouse collecting duct cells, and primary rabbit RPT cells. Bands have been rearranged to show the 3 cell types as adjacent. β-Actin is shown as a loading control. B: colony formation by mouse M1 collecting duct cells. M1 collecting duct cells were plated at a low density (250 cells/dish) either in control medium (supplemented with insulin and transferrin) or in control medium further supplemented with 30 nM ONO-8711, 70 nM PGE2, or 30 nM ONO-8711 and 70 nM PGE2 in combination. Subsequently, colony formation by mouse M1 collecting duct cells was visualized by crystal violet staining, as described in MATERIALS AND METHODS. C: effect of ONO-8711 on the frequency of colony formation by mouse M1 collecting duct cells. The number of colonies was determined in M1 cell cultures maintained as described above. D: effect of ONO-8711 on M1 cell growth. M1 cell growth was examined (vs. untreated cultures) both in the presence of 70 nM PGE2, and in the control condition (i.e., in the absence of PGE2). E: effect of ONO-8711 on proximal tubule cell growth. Proximal tubule cell growth was examined under the conditions described in above. Values are averages ± SE of triplicate determinations. *P < 0.05 relative to the untreated control. †P < 0.05 relative to untreated cultures maintained with PGE2.

Fig. 5. Effect of ibuprofen on the growth response to ONO-8711. A: effect of increasing concentrations of ibuprofen (0 –100 μM) on growth in the presence and absence of 30 nM ONO-8711. The cells were counted after 8 days in culture. #P < 0.05 relative to the control value (i.e., the value obtained in the absence of both ONO 8711 and ibuprofen). B: effect of ONO-8711 and 70 nM PGE2 on growth, either in the presence or the absence of 100 μM ibuprofen. The cells were counted after 5 days in culture. Values are the averages ± SE of triplicate determinations. *P < 0.05 relative to the control value (i.e., the value obtained with untreated cells grown in the absence of ibuprofen). ‡P < 0.05 relative to the value obtained with untreated cells grown in the presence of ibuprofen.
The effect of ibuprofen on Akt phosphorylation was also studied. Figure 6B shows that the level of pAkt increased 2.4 ± 0.1-fold in the presence of ibuprofen. This latter observation can be explained if ibuprofen (like ONO-8711) prevents the inhibition of Akt phosphorylation, resulting from EPI activation by endogenous PGE₂.

To examine the involvement of Akt in the growth response to insulin and ONO-8711, further studies were conducted with the Akt inhibitor MK-2206. Figure 7A shows that the growth-stimulatory effect of insulin was lost as the MK-2206 concentration increased to 0.5 μM. Figure 7B shows that in addition 1 μM MK-2206 completely inhibited the growth-stimulatory effect of ONO-8711 (in the presence, as well as in the absence of insulin), in addition to reducing growth overall. These results are consistent with the hypothesis that the growth-stimulatory effects of both insulin and ONO-8711 are mediated, at least in part, via Akt.

Dependence of the growth-stimulatory effect of ONO-8711 upon insulin and phosphatidylinositol 3-kinase. The studies described above indicate that the growth-stimulatory effects of both ONO-8711 and insulin are dependent upon Akt. Thus, the hypothesis was investigated that the cellular response to ONO-8711 was dependent upon insulin, and for this was Akt dependent.

To evaluate this hypothesis initially, the dependence of the ONO-8711 stimulation upon insulin concentration was studied. Transferrin was present alone in these experiments in the control condition, because the effect of insulin itself was being studied (unlike the other growth studies described above, where serum-free medium was supplemented with both 5 μg/ml insulin as well as 5 μg/ml transferrin in control). Figure 8A shows a significant growth-stimulatory effect of ONO-8711, even in the absence of insulin (1.5 ± 0.1-fold vs. the control value, without ONO-8711 and without insulin). However, ONO-8711 had a maximal growth-stimulatory effect in the presence of 5 μg/ml insulin (the insulin concentration that causes a maximal growth-stimulatory effect on MDCK cells) (51).

Insulin, like a number of growth factors, activates phosphatidylinositol 3-kinase (PI3K), which produces PtdIns (3,4,5) P3, so as to initiate a growth response (1). Activation of PI3K is limited by PTEN (phosphatase and tensin homolog), which dephosphorylates PtdIns (3,4,5) P3, and in this manner limits the effect of PI3K. To determine whether the growth-stimulatory effect of insulin and ONO-8711 in MDCK cells involves PTEN (and thus PI3K), the effect of the PTEN inhibitor bpV(phen) was examined.

Figure 8B shows the stimulatory effect of bpV(phen) on MDCK cell growth as a function of bpV(phen) concentration. The amplitude of the growth-stimulatory effect of bpV(phen) continued to increase as the bpV(phen) concentration was increased to 7.5 nM. The growth-stimulatory effect of bpV(phen) was observed in medium supplemented with insulin, as well as in medium supplemented with ONO-8711 and insulin. However, the overall magnitude of the bpV(phen) stimulation did not increase significantly when ONO-8711 was present in addition to insulin. The slope of the best-fit line to the experimental results obtained in the absence of ONO-8711 was a “2.4 ± 0.2-fold increase (relative to the control cell number)/log[nmole bpv(phen)].” The slope of the best-fit line to the experimental results did not change significantly in the presence of ONO-8711.

As shown in Fig. 8C, the growth-stimulatory effect of bpV(phen) was not observed in the absence of insulin (even when ONO-8711 was present). Thus the increased growth caused by the PTEN inhibitor bpV(phen) can be attributed to the ability of bpV(phen) to increase the growth response to insulin, irrespective of the presence of ONO-8711. These results are consistent with the hypothesis that the growth-stimulatory effect of ONO-8711 does not depend upon PI3K but instead is a consequence of signaling events occurring downstream from PI3K.
Role of Akt and the EGFR in mediating the response to SC51089. The effect of the EP1 antagonist SC51089 on Akt phosphorylation was also examined. Figure 9A shows that the level of phospho-Akt increased 7 ± 1- and 13 ± 1-fold following a 30-min incubation with either SC51089 or insulin, respectively. The level of Akt phosphorylation did not increase further when SC51089 and insulin were added in combination. Previously, the EP1 receptor was reported to transactivate the EGFR, increasing the phosphorylation of the EGFR (22). Thus the effect of SC51089 on EGFR phosphorylation was examined. Figure 9B shows that the level of phospho-EGFR increased 4.8 ± 0.2-fold in the presence of SC51089 (P < 0.05), unlike the case with insulin.

To further examine the role of Akt and the EGFR in mediating the growth response to SC51089, the Akt inhibitor MK-2206 and the EGFR kinase inhibitor AG1478 were studied. Figure 10A shows that MK-2206 prevented the SC51089-mediated increase in MDCK cell growth, while the growth-stimulatory effect of PGE2 remained (indicating involvement of another mechanism). Figure 10B shows that AG1478 (at either 0.1 or 0.25 μM) inhibited the growth-stimulatory effects of both SC51089 and ONO-8711. However, 0.1 μM AG1478 only partially inhibited the growth-stimulatory effect of EGF, 0.25 μM AG1478 being required for a complete inhibition of the growth-stimulatory effect of EGF.

To further evaluate whether EGFR activation is required to obtain a growth-stimulatory effect of ONO-8711, growth studies were conducted with MDCK cells that had been transduced with a lentivirus containing pLKO.1 with shRNA against the EGFR. As shown in Fig. 11A, the level of the EGFR was reduced by 94 ± 2% in MDCK cells transduced with EGFR shRNA (i.e., MDCK cells with an EGFR KD). Figure 11B shows that ONO-8711 and EGF were not growth stimulatory to MDCK cells with an EGFR KD, unlike their growth-stimulatory effects observed in MDCK cells transduced with pLKO.1 (i.e., the empty vector in this experiment).

Growth studies were also conducted with HERCD533-MDCK cells, permanently expressing a vector (pcDNA3) encoding for a dominant negative EGFR (HERCD533), which prevents EGFR-mediated signaling (26). Figure 11C shows that HERCD533-MDCK cells lack the growth-stimulatory effect of EGF, unlike MDCK cells which permanently express the empty vector (EV-MDCK), pcDNA3. Figure 11C also shows that HERCD533-MDCK cells lack the growth-stimulatory response to ONO-8711, unlike the EV-MDCK. These results are consistent with the hypothesis that EGFR activation is required to elicit the growth-stimulatory effect of ONO-8711.

To determine whether the growth response to EGF is similarly dependent upon the EP1 receptor, the effect of increasing concentrations of EGF (0 –20 ng/ml) on the growth on MDCK cells with an EP1 knockdown was examined in parallel with MDCK cells with pLKO.1 (EV-MDCK cells). As shown in Fig. 11D, EGF increased the growth of EV-MDCK cells as high as 2.0 ± 0.2-fold (at 10 ng/ml EGF), as the EGF concentration was increased to 20 ng/ml. In contrast, Fig. 11D shows that in MDCK cells with an EP1 KD, the maximum stimulation obtained with EGF was 1.3 ± 0.04-fold (also at 10 ng/ml EGF). These observations suggest that signaling via the
EGFR is indeed dependent upon the EP1 expression level in MDCK cells.

**DISCUSSION**

Activation of the EP1 receptor has been associated with the progression of a number of renal disease states (3, 31, 43, 48), unlike the EP2 and EP4 receptors, whose activation has been found to be protective (2, 56, 57). Indeed, the EP1 antagonist ONO-8713 decreased renal damage in stroke-prone spontaneously hypertensive rats (SHRSPs) (48). In addition, ONO-8713 reduced mesangial expansion, as well as glomerular and tubular hypertrophy, in streptozotocin-induced diabetic rats (36).

![Fig. 10. Effect of Akt inhibitor MK-2206 and EGFR inhibitor AG1478 on the growth response to EGFR antagonists.](image)

*P < 0.05 relative to the control value. †P < 0.05 relative to untreated cultures lacking MK-2206, but lacking MK-2206. ‡P < 0.05 relative to untreated MK-2206.

Fig. 11. Genetic studies indicating the involvement of the EGFR and the EP1 receptor in the growth response to ONO-8711, and EGF, respectively. A: effect of an EGFR KD on expression of the EGFR. A Western blot shows expression of the EGFR in MDCK cells transduced with lentiviral pLKO.1 (EV), or with EGFR shRNA. α-Tubulin is shown as a loading control. B: effect of an EGFR KD on growth. The effect of 30 nM ONO-8711, 70 nM PGE2, and 5 ng/ml EGF on growth was examined both in MDCK cells transduced with lentivirus containing either the EV (pLKO.1) or pLKO.1 with shRNA against the EGFR. The control value was the average cell number in untreated cultures lacking the EGFR KD for each cell type. C: effect of dominant negative (DN) EGFR on growth. The effect of 30 nM ONO-8711, 70 nM PGE2, and 5 ng/ml EGF on growth was examined both in MDCK cells transduced with lentivirus containing either the EV (pLKO.1) or pLKO.1 with shRNA against the EGFR. The control value was the average cell number in untreated cultures lacking the EGFR KD for each cell type. D: effect of EP1 KD on the growth response to EGF. The effect of EGF (0-20 ng/ml) on growth was examined both in MDCK cells transduced with lentivirus containing either the EV (pLKO.1) or pLKO.1 with shRNA against the EP1 receptor. The control value was the average cell number obtained in untreated cultures (in the absence of EGF) for each cell type. In B, C, and D, MDCK cell cultures were counted after 6 days in culture. Values are averages ± SE of triplicate determinations. *P < 0.05 relative to the control value of MDCK cells transduced with EV. #P < 0.05 relative to the control value of MDCK cells transduced with either lentiviral EGFR shRNA, EGFR DN, or EP1 shRNA.

AJP-Renal Physiol • doi:10.1152/ajprenal.00510.2013 • www.ajprenal.org
Renal functions which may be affected under these conditions include Na$^+$ transport and vasoconstriction (28, 42). In contrast, EP4 receptor activation reduced glomerular sclerosis and increased the proliferation of proximal convoluted epithelial cells in a rat model of chronic renal failure (2, 57). Similarly, the activation of both EP2 and EP4 receptors increased the survival of distal as well as proximal tubules, in a nephrotoxic mercuric chloride rat model of acute renal failure (57).

Our previous studies indicate that PGE$_2$ is growth stimulatory to MDCK cells, as well as primary cultures of baby mouse kidney epithelial cells in serum-free medium (51, 54). The results presented here support the hypothesis that the growth-stimulatory effects of PGE$_2$ are mediated by Gs-coupled EP2 and EP4 receptors, unlike Gq-coupled EP1 receptors, whose activation results in growth inhibition. Consistent with this hypothesis, AH6809 and L161,982 (agonists of EP2 and EP4, respectively) inhibited the growth-stimulatory effect of PGE$_2$, unlike the EP1 antagonists ONO-8711 and SC51089, which were growth stimulatory. Consistent with the involvement of EP1 receptors in the growth response to these EP1 antagonists, the growth-stimulatory effect of ONO-8711 was not observed in MDCK cells with an EP1 receptor KD.

In our initial studies concerning the growth of MDCK cell cells in hormonally defined medium, we found that five growth supplements, insulin, transferrin, T$_3$, hydrocortisone, and PGE$_2$, permitted MDCK cells to grow at the same rate as in serum-supplemented medium (51). To determine whether the requirement for the five growth supplements was unique to MDCK cells, or was common to other types of kidney tubule epithelial cells, we investigated the ability of the defined medium for MDCK cells to promote the growth of primary cultures of baby mouse kidney epithelial cells (51, 54). Our initial results indicated that primary cultures of baby mouse kidney epithelial cells grew in the defined medium for MDCK cells without fibroblast overgrowth (51). Subsequent studies indicated that the primary cultures of baby mouse kidney epithelial cells grew in response to each of the five supplements in the defined medium for MDCK (54). However, PGE$_2$ and transferrin had larger effects on growth of the primary baby mouse kidney epithelial cells than the other three supplements, similar to the observations made with MDCK.

In this report, PGE$_2$ was similarly observed to be growth stimulatory to mouse M1 collecting duct cells and rabbit RPT cells in defined medium. In addition, ONO-8711 was observed to increase the growth of M1 cells and rabbit RPT cells, as well as MDCK cells. These latter observations suggest that these findings reflect a true in vivo process rather than an aberrant observation made with a single established kidney cell line.

The growth-stimulatory effects of ONO-8711 and SC51089 were observed both in the presence and in the absence of exogenously added PGE$_2$. Presumably, the increased growth caused by these EP1 antagonists in the absence of exogenous PGE$_2$ is due to their ability to block EP1 receptor activation by endogenously produced PGE$_2$. Consistent with this hypothesis, ONO-8711 did not increase MDCK cell growth in the presence of ibuprofen, a COX1 and COX2 inhibitor.

Previously, Kawano et al. (27) reported that EP1 receptor activation was responsible for the neurotoxicity caused by the COX2-derived PGE$_2$, which is produced during cerebral ischemia. The inhibition of EP1 receptor activation was found to provide neuroprotection by a mechanism involving inhibition of PTEN and activation of Akt (62). Our results indicate that the EP1 antagonists ONO-8711 and SC51089 similarly increase the level pAkt in MDCK cells (indicative of Akt activation). Insulin also increased Akt phosphorylation in MDCK cells, presumably as a consequence of the interaction of insulin with the IGF$_1$ and/or IGF$_2$ receptors expressed in MDCK cells (insulin receptors are not present in MDCK) (29). However, when ONO-8711 was added in combination with insulin, no further increase Akt phosphorylation was observed under our experimental conditions. These latter results may nevertheless still be explained by the involvement of pAkt, if the growth-stimulatory effects of ONO-8711 and insulin depend upon the cells achieving a threshold level of Akt phosphorylation. Presumably, under the incubation conditions of the Western blot study, this threshold level was achieved by the addition of insulin alone.

The involvement of Akt as a mediator of the growth-stimulatory effect of ONO-8711 during longer-term incubations is supported by the observation that the Akt inhibitor MK2206 prevented the growth-stimulatory effects of ONO-8711, SC51089, and insulin. The Akt phosphorylation that occurs under these conditions may be initiated by signaling events originating from IGF receptors (which ultimately result in the growth response to insulin). In this case, the EP1 receptor, when activated, would presumably function so as to reduce the Akt phosphorylation (and activation) that occurs in response to insulin and insulin-like growth factors. In this case, EP1 receptor antagonists, including ONO-8711 and SC51089, would act so as to prevent this consequence of EP1 receptor activation.

The increased growth that occurs in response to insulin very likely involves the activation of PI3K, and as a consequence Akt (24). However, other growth-stimulatory factors (such as ONO-8711 and SC51089) may also increase growth by their ability to inhibit PTEN, a regulatory protein which inhibits PI3K (24). Indeed, the PTEN inhibitor bpV(phen) stimulated MDCK cell growth, indicating that PI3K does indeed limit the growth of MDCK cells in serum-free medium. Consistent with this hypothesis, the increased growth observed in the presence of bpV(phen) was dependent upon the presence of insulin in the culture medium, presumably due to a requirement for the activation of PI3K (by insulin). However, the growth-stimulatory effect of bpV(phen) did not increase significantly when ONO-8711 was present in addition to insulin, indicating that ONO-8711 did not cause the further activation of PI3K/PTEN.

The involvement of the EGFR signaling pathway in EP1-mediated growth control in MDCK cells was also indicated in our studies. Interrelationships between EGF and PGE$_2$ have been reported previously in the kidney, where both growth factors are produced in large quantities (5, 45). Following the production of EGF by the kidney, EGF binds to EGFRs, localized on the surface of many types of renal cells (10, 18). One consequence of the activation of the EGFR is the activation of PLA$_2$, and the subsequent production of PGE$_2$, which ultimately affects renal function (32, 37). For example, PGE$_2$ produced in response to EGF in RPT cells, stimulates basolateral organic anion transport (47), while PGE$_2$ produced in response to EGF in mesangial cells, causes decreased expression of cyclin D3, and hypertrophy (43). In addition to stimulating PGE$_2$ production, activation of the EGFR has other functional consequences, including inhibition of the epithelial
sodium channel (ENaC) in the collecting duct, unlike PGE₂ which targets Na-K-ATPase in this nephron segment (40).

A number of renal hormones and effectors, including arginine vasopressin, aldosterone, and ANG II, “transactivate” the renal EGFR (15, 20, 30). One mechanism for the transactivation is the release of heparin-binding EGF (HB-EGF), or transforming growth factor-α (TGF-α), from the surface of renal cells. The released HB-EGF (and/or TGF-α) subsequently binds to the EGFR, resulting in EGFR phosphorylation and activation. Both HB-EGF and TGF-α can be released from the surface of renal cells following the activation of a disintegrin and metalloproteinase (ADAM) (21, 30). Indeed, ADAM-dependent EGFR transactivation is reportedly induced by G protein-coupled receptors (GPCRs), including Gq-coupled receptors. The binding of ligands to such GPCRs has been observed to cause the phosphorylation and activation of ADAM family members, resulting in the release of EGFR ligands from the plasma membrane. This mechanism is of particular concern in polycystic kidney disease (PKD), because the EGFR (normally localized basolaterally) relocates to the apical membrane. EGFR and related EGFR ligands (released by ADAMs) are present in the lumen of the nephron, where they interact with the apical EGFRs in PKD, activating EGFR signaling pathways, leading to increased growth and Na⁺ transport (61). Indeed suppression of EGFR transactivation has become a target of therapy in PKD (4).

In MDCK cells, the activation of EP1 receptors apparently results in the inhibition, rather than the activation, of the EGFR. Instead, EGFR activation apparently occurs in response to EP1 antagonists. One possible mechanism of activation (described above) is the release of HB-EGF by ADAMs in response to EP1 antagonism. In this case, activation of EP1 receptors by PGE₂ instead results in the inhibition of ADAMs. Indeed, tissue inhibitors of metalloproteinases (TIMPs) are inhibitors of ADAMs, and TIMPs may be activated as a consequence of EP1 receptor activation.

EGFR transactivation can also occur independently of the release of HB-EGF by ADAMs. Indeed, the EGFR reportedly interacts with EP1 receptors in other cell types, including human CCLP1 cholangiocarcinoma cells (23). In this case, the affinity of the EGFR for the EP1 receptor is altered by the binding of ligands to the EP1 (23). If this mechanism occurs in MDCK cells, the binding of EP1 antagonists to the EP1 receptor would be expected to cause an increase in EGFR phosphorylation and activation, by altering EGFR/EP1 receptor interaction.

Previous studies have indicated that PGE₂ plays a role in promoting cystogenesis in renal tubules. PGE₂ is present in cyst fluid of human ADPKD (19) and induces cystogenesis via the activation of EP2 and EP4 receptors (16, 17). As a consequence, increased growth and transport are observed in ADPKD tubular cells (33). PGE₂ similarly increases growth and Na⁺ transport in MDCK cells, as well as chloride secretion in MDCK cells, the murine M-1 cortical collecting duct cell line, and polycystin 1-deficient murine inner medullary collecting duct cells (33, 46, 50, 51, 59). We do not yet know whether these PGE₂-mediated signaling events are altered in ADPKD. However, the results presented in this report indicate that EP1 activation dampens EP2- and EP4-mediated proliferative events, which are required for cystogenesis. Activation of EP1 may do so by affecting the Akt and EGFR signaling pathways. In addition, EP1 may also prevent the increased growth that occurs in ADPKD cells by preventing the activation of the B-Raf/Erk pathway that normally occurs in response to cAMP, presumably as a consequence of EP2 and EP4 activation (60). To achieve this cAMP-dependent growth-stimulated phenotype, renal cells must be in a Ca²⁺-restricted state. However, activation of EP1 raises intracellular Ca²⁺ (as a consequence of PLC activation), thereby preventing renal cells from achieving the necessary Ca²⁺-restricted state required for activation of B-Raf/ERK. Further studies are necessary to evaluate these hypotheses.

ACKNOWLEDGMENTS

We thank Dr. Sylvain Meloche for the expression vector for the dominant negative EGFR.

GRANTS

Funding for this work was obtained from National Heart, Lung, and Blood Institute Grant 1RO1-HK-69676-01 to M. Taub.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


