Prostaglandin E₂ promotes cell survival of glomerular epithelial cells via the EP4 receptor

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Visceral glomerular epithelial cells (GEC; also known as podocytes) are intrinsic components of the kidney glomerulus and are crucial for glomerular perme selectivity and structural integrity (18). Recent development in GEC biology strongly supports the concept that many proteinuric human diseases including minimal change disease and focal segmental glomerulosclerosis are caused by GEC injury (10, 13, 18). In some experimental proteinuric models, as well as in human glomerular diseases, apoptosis of GEC has been observed in glomerulosclerosis (10, 13, 14, 18). For example, in the rat model of acute and chronic puromycin aminonucleoside nephrosis (PAN), which mimics human minimal change disease and focal segmental glomerulosclerosis, respectively, increasing GEC apoptosis and loss of GEC were observed with the progression of the glomerular lesion (26). It has been proposed that GEC apoptosis may lead to “podocytepenia” and consequently to sclerosis in focal segmental glomerulosclerosis (10, 13, 14, 18). Therefore, apoptosis/survival of GEC is likely to have an important role in glomerular perme selectivity in proteinuric glomerular diseases.

Cyclooxygenase (COX) is a key enzyme in arachidonic acid metabolism, converting arachidonic acid to bioactive lipid mediators (prostanoids). There are two iso forms, i.e., COX-1 and COX-2. Traditionally, studies on the role of COX in kidney function have been performed with pharmacological inhibitors of COX (nonsteroidal anti-inflammatory drugs) and mainly focused on hemodynamics and tubular functions. Consequently, little was known about the role of COXs in GEC function and glomerular perme selectivity, independent from glomerular hemodynamics. We previously reported that COX-1 is constitutively expressed in cultured rat GEC, while expression of COX-2 is minimal but is significantly upregulated by complement C5b-9 (29). In normal rat glomeruli, both COX-1 and COX-2 are detectable by immunoblotting (29). In the passive Heymann nephritis (PHN) model of membranous nephropathy, GEC injury is induced by complement C5b-9 and both isoforms of COX are upregulated, most likely in GEC (29). A COX-2-selective inhibitor reduces proteinuria in PHN; however, the effect is much weaker than that of the nonselective COX inhibitor indomethacin (30). In cultured rat GEC, indomethacin and a COX-1-selective inhibitor protected GEC from complement-mediated cell injury, while a COX-2-selective inhibitor had only minor impact (30). Thus COX-1 and COX-2 appear to have distinct roles in GEC function. We have shown that COX-2 preferentially couples to prostaglandin (PG) E₂ generation, compared with thromboxane (TX) A₂, when GEC are stimulated with complement (30). This differential coupling to different prostanoids may in part explain different roles of the two COX isoforms.

Previously, it was shown that cultured rat GEC can generate PGE₂, PGF₂α, PIG₂ and TXA₂ but not PGD₂ (15, 22). Among these prostanoids, PGE₂ and TXA₂ are quantitatively most prominent (22). PGE₂ and TXA₂ generation were also shown in rat glomeruli (30, 36). These prostanoids generally exert their biological functions by binding to their specific cell surface receptors, which belong to the G protein-coupled receptor superfamily. While most prostanoids have only one isoform of their receptors, the PGE₂ receptor is known to have four isoforms, i.e., EP1-EP4 (5). EP1 is coupled to phospholipase C and activates inositol trisphosphate/diacylglycerol signaling pathways. EP2 and EP4 are coupled to adenylate cyclase and increase cAMP generation. EP3 has several splice variants and decreases cAMP generation or activates other signaling pathways (5). In immortalized mouse podocytes, mRNA for the TXA₂ receptor (TP), PGF₂α receptor (FP), EP1, and EP4 were identified (3). Therefore, it is highly likely that prostanoids generated by GEC act in an autocrine or paracrine fashion.
It is well established that inhibitors of COX such as aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), or recently developed COX-2-selective inhibitors have proapoptotic/anti-proliferative effects in certain cell types, in particular gastrointestinal epithelial cells (7). In fact, NSAIDs are now used for chemoprevention of colon cancer (7). In colon cancer cells, COX-2 expression is markedly upregulated and it is generally believed that these drugs exert their proapoptotic/anti-proliferative effects via inhibiting PGE2 generation by COX-2, although possibility remains for other mechanisms (34). Thus it is reasonable to hypothesize that COX, in particular COX-2, would have anti-apoptotic/proliferative action in GEC via its products, in particular PGE2.

In the current study, we addressed the role of COX-2 and its product PGE2 in GEC survival. We demonstrate that induction of COX-2 expression in GEC leads to PGE2 production, which exerts anti-apoptotic effects on GEC via its EP4 receptor. In addition, the EP4 receptor antagonist augmented GEC apoptosis and proteinuria in the acute PAN model of GEC injury.

**MATERIALS AND METHODS**

**Materials.** Tissue culture media and Ecdysone-inducible mammalian expression system [including pVgRXR, pIND/Hygro (+), and ponasterone A] were purchased from Invitrogen Life Technologies (Burlington, ON). PGE2, puromycin aminonucleoside (PA), caspase-3 substrate, forskolin, and other standard biochemicals were from Sigma (St. Louis, MO). Protease inhibitor cocktail, reagents for reverse transcription-polymerase chain reaction, and 5-bromo-2'-deoxy-uridine (BrDU) labeling and detection kit were from Roche Diagnostics (Laval, QC). Rabbit anti-COX-2 antibody, NS-398 (COX-2 inhibitor) and AH-6809 (EP1/EP2 receptor antagonist) were from Cayman Chemical. Antibodies for phospho-extracellular signal-regulated kinase (ERK: Thr202/Tyr204), ERK, phospho-Akt (Ser473), Akt, cleaved/activated caspase-3 (5A1 rabbit monoclonal), and other standard biochemicals were from Sigma (St. Louis, MO). Protease inhibitor cocktail, reagents for reverse transcription-polymerase chain reaction, and 5-bromo-2'-deoxy-uridine (BrDU) labeling and detection kit were from Roche Diagnostics (Laval, QC). Rabbit anti-COX-2 antibody, NS-398 (COX-2 inhibitor) and AH-6809 (EP1/EP2 receptor antagonist) were from Cayman Chemical. Antibodies for phospho-extracellular signal-regulated kinase (ERK: Thr202/Tyr204), ERK, phospho-Akt (Ser473), Akt, cleaved/activated caspase-3 (5A1 rabbit monoclonal), and other standard biochemicals were from Sigma (St. Louis, MO). Protease inhibitor cocktail, reagents for reverse transcription-polymerase chain reaction, and 5-bromo-2'-deoxy-uridine (BrDU) labeling and detection kit were from Roche Diagnostics (Laval, QC).

**Establishment of COX-2 induction in cultured GEC.** Culture, characterization, and transfection of rat GEC were described previously (4, 9, 29). For establishment of COX-2-inducible GEC, COX that grow on plastic substrata (GEC-pl) were used. In some experiments, a subclone of COX-2 transfected GEC were stimulated with an insect hormone ponasterone A, a COX-2 activator. The activated receptor protein receptor encoded by pVgRXR is activated. The activated receptor then induces the transcription of COX-2. Ponasterone A is not known to have any impact on mammalian cells. In some experiments, a subclone of GEC that stably express pVgRXR alone (GEC-RXR) was used as control to rule out nonspecific effect of ponasterone A in GEC.

**Quantification of apoptosis.** Quantification of apoptosis was performed as described previously (4). In brief, cells were stained with Hoechst33422 and propidium iodine. The percentage of apoptotic cells (Hoechst positive, propidium iodine negative) was quantified from Hoechst33422 and propidium iodine. The percentage of apoptotic cells (Hoechst positive, propidium iodine negative) was quantified from Hoechst33422 and propidium iodine. The percentage of apoptotic cells (Hoechst positive, propidium iodine negative) was quantified from Hoechst33422 and propidium iodine. The percentage of apoptotic cells (Hoechst positive, propidium iodine negative) was quantified from Hoechst33422 and propidium iodine.

**Identification of prostanoid receptors in GEC.** Identification of prostanoid receptors in GEC. RNA was purified from cultured rat GEC (GEC-pl), rat mesangial cell, and normal rat kidney (1-mm horizontal slice at the midportion, containing cortex and medulla) using TRIzol reagent (Invitrogen) and cDNA was synthesized by reverse transcriptase following the standard protocol. Using cDNA as template, PCR was performed using the following primers (all in 5′→3′): TXA2 receptor (TP)-sense: ggggccttggg-actgct, TP-antisense: ggggtcagaaacacgagga, EP1-sense: tccatcactccaacagc, EP1-antisense: gggggagggggaagattg, EP2-sense: gcactatgggcttct, EP2-antisense: tcctcaggaggaagtt, EP3-sense: cggggggagggcaagggg, EP3-antisense: gaggggaggggaagattg, EP4-sense: gcgggaggggaggtgact, EP4-antisense: gaggggaggggaagattg, β-actin-sense: atggctcgtggctgtgacttc, β-actin-antisense: agcctggagggaggggaagattg, α-gaacttggactggatgtgacttc, α-gaacttggagggaggggaagattg, α-gaacttggagggaggggaagattg, α-gaacttggagggaggggaagattg. Cycle number of PCR was 35 cycles, except for β-actin (25 cycles).

**Caspase-3 activity assay.** Samples were prepared by lysing GEC or rat glomeruli in buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, and 100 μM EDTA. Assay was carried out with 0.2 mM of caspase-3 substrate (N-acetyl-Asp-Glu-Val-Asp p-nitroanilide, Sigma) and 70 μg of samples in buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 100 μM EDTA, and 1% glyceral in the volume of 400 μl. After incubating samples at 37°C for 10–15 min in the dark, samples were placed in a spectrophotometer and the absorbance at 405 nm was measured.
recorded every 5 min for 2 h. Change of absorbance (405 nm) per minute was used as relative caspase-3 activity. Buffer and substrate alone (without samples) were used to determine background. In some experiments, caspase-3 activity was quantified by immunoblotting by detecting and quantifying cleaved/activated caspase-3 using 5A1 rabbit monoclonal antibody, which recognizes the cleaved/activated fragment of caspase-3 (∼19 kDa) but not the full-length caspase-3 (Cell Signaling Technology).

**Induction of PAN and treatment with L-161982.** PAN was induced in male Sprague-Dawley rats (150 g body wt, Charles River, St. Constant, QC) by a single intravenous injection of puromycin amiononucleoside (PA, 80 mg/kg) as described previously (4). Rats develop significant proteinuria 14 days after the injection (4). Rats were divided into three groups and were treated as follows between day 7 and day 14 by intraperitoneal injections (once per day); group 1: control (non-PAN); group 2: PAN treated with vehicle (saline); group 3: PAN treated with L-161982 (10 mg·kg⁻¹·day⁻¹). The schedule of L-161982 was decided based on the report from Merck-Frosst (16). Urine was collected on day 14 and rats were killed on day 7 (1 wk). All studies were approved by the McGill University Animal Care Committee.

**Immunohistochemistry of COX-2.** Immunohistochemistry of COX-2 was performed following the protocol published previously, using rabbit anti-mouse COX-2 affinity-purified IgG (160126; Cayman Chemical) (24).

**RESULTS**

**Induction of COX-2 results in increased cell number of GEC.** We previously reported that COX-2 is upregulated by complement C5b-9 in GEC in culture and in vivo (29). In the current study, we aimed to study the impact of COX-2 induction on GEC function, independent from complement-mediated GEC injury. To this end, we established subclones of GEC, which overexpress COX-2 in an inducible manner utilizing Ecdysone-inducible mammalian expression system (see MATERIALS AND METHODS). COX-2 expression was induced by the insect hormone ponasterone A, which has otherwise no known impact on mammalian cells. COX-2 expression was strongly induced by ponasterone A at the dose of 1–8 μM (Fig. 1). In contrast, when cells were treated with vehicle (ethanol), COX-2 expression was either not detectable or minimal (Fig. 1). COX-2 induction was detectable as early as 4 h after ponasterone stimulation and persisted at least for 72 h (Fig. 1). Two other subclones were tested with similar results (not shown).

While establishing the basic characteristics of COX-2-inducible GEC, we noted that when COX-2 was induced with ponasterone A, GEC appeared to be more confluent, compared with control cells that were treated with vehicle. Thus we next quantified the cell number of COX-2-inducible GEC in the presence or absence of ponasterone A (Table 1). When cells were stimulated with ponasterone A in serum-free medium for 48 h, cell number was 71% greater, compared with control cells treated with vehicle (control: 6.6 ± 1.6 × 10⁴/well, ponasterone A: 11.3 ± 2.8 × 10⁴/well, P < 0.01, n = 12). When cells were stimulated in serum-poor medium (0.5% FBS), the increase in cell number by ponasterone A was 61% (control: 10.5 ± 2.0 × 10⁴/well, ponasterone A: 17.5 ± 2.7 × 10⁴/well, P < 0.01, n = 12). When cells were stimulated in K1 (serum replete) medium, the increase in cell number by ponasterone A was 33% (control: 22.1 ± 5.1 × 10⁴/well, ponasterone A: 29.6 ± 6.4 × 10⁴/well, P < 0.01, n = 12). When similar experiments were performed with a subclone of GEC that stably express pVG-RXR but not pIND-COX-2 (GEC-RXR), ponasterone A did not affect the cell number (not shown). These results indicate that the induction of COX-2 results in increased cell number in GEC and that this impact is most evident in serum-deprived conditions.

**Dose response of COX-2 induction**

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<th>Ponasterone A</th>
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<td>24 h stimulation</td>
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<td><strong>Time course of COX-2 induction</strong></td>
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<td>4h</td>
<td>Etoh</td>
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**Dose response of COX-2 induction**

Fig. 1. Establishment of cyclooxygenase (COX)-2 induction in cultured rat glomerular epithelial cells (GEC). Cells were established as in MATERIALS AND METHODS and COX-2 expression was induced by the insect hormone ponasterone A (Pona). Dose response (top) and time course (bottom) of COX-2 induction were tested by immunoblotting in one of the subclones. Two other subclones were tested with similar results (not shown). Etoh, ethanol.

Next, we studied the impact of COX-2 induction on GEC apoptosis (Fig. 2). When COX-2-inducible GEC were cultured in serum-free medium for 48 h, 14.6 ± 0.9% of the cells underwent apoptosis. When cells were treated with ponasterone A, the number of apoptotic cells decreased significantly to 7.9 ± 0.8% (P < 0.01, n = 3). Anti-apoptotic effect of ponasterone A was abolished by the COX-2-selective inhibitor NS-398 (18.9 ± 3.2% apoptosis) but not by the COX-1-selective inhibitor SC-560 (9.9 ± 8.0% apoptosis). These results indicate that induction of COX-2 causes increased cell proliferation and decreased apoptosis in serum-deprived conditions, thereby leading to increased cell number.

**Induction of COX-2 stimulates the ERK pathway.** The ERK pathway is known to mediate cell proliferation and survival (8). To begin to address the mechanisms of proliferative and anti-apoptotic effect of COX-2 induction, we next studied...
In addition, it was recently shown that PGE2 transphosphorylates and activates the epidermal growth factor (EGF) receptor via Src, thereby activating the ERK pathway and promoting cell survival/proliferation (6, 19, 31). Therefore, we hypothesized that the anti-apoptotic effect of COX-2 induction is mediated by PGE2 generation via COX-2. To test this hypothesis, we first tested whether GEC in culture generate PGE2 under serum-deprived conditions, and if so, whether this PGE2 generation is increased by COX-2 induction (Fig. 4A).

After COX-2-inducible GEC were cultured in serum-free medium for 48 h, concentration of PGE2 in culture media was 592 ± 97 pmol/0.1 ml. When cells were stimulated with ponasterone A under the same conditions, PGE2 concentration increased to 1,021 ± 244 pmol/0.1 ml (P < 0.05, n = 4).

We have previously established that the amounts of PGE2 in culture media represent the total amounts of PGE2 generated by the cells. Thus GEC generate detectable amounts of PGE2 under serum-deprived conditions, which increases by twofold when COX-2 expression is induced.

Cultured rat GEC express PGE2 EP4 receptor. There are four isoforms of the PGE2 receptor, i.e., EP1–4. It was previously reported that immortalized cultured mouse podocytes express mRNA for EP1 and EP4, but not for EP2 and EP3 (3). We next studied which isoforms of the PGE2 receptor are expressed in cultured rat GEC by reverse transcription-polymerase chain reaction (Fig. 4B). Signals for the EP4 receptor (TP) were strongly positive in rat GEC, in agreement with the results from mouse podocytes (3). EP1 mRNA was also detectable but the signal was much weaker than EP4 and TP. EP2 and EP3 mRNA were not detectable in rat GEC. These results led us to hypothesize that PGE2 mediates anti-apoptotic/proliferative effects of COX-2 induction in GEC most likely via the EP4 receptor.

### Table 1. Induction of COX-2 results in increased cell number of GEC

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<th>Vehicle</th>
<th>Ponasterone A</th>
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<tr>
<td>Serum free</td>
<td>6.6±1.6</td>
<td>11.3±2.8*</td>
</tr>
<tr>
<td>Serum poor (0.5% FBS)</td>
<td>10.5±2.0</td>
<td>17.5±2.9*</td>
</tr>
<tr>
<td>K1 (serum replete)</td>
<td>22.1±5.1</td>
<td>29.6±6.4*</td>
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Values are means ± SE. Cyclooxygenase (COX)-2-inducible glomerular epithelial cells (GEC) were plated in 24-well plates at 4 × 10⁴ cells/well in serum-poor (0.5% FBS) medium. After 24 h, culture media were changed to either serum-free, serum-poor, or K1 (serum replete) medium. Either ponasterone A (2 μM) or vehicle (ethanol) was added to culture media. After additional 48 h, cells were trypsinized and cell numbers were quantified using a hemocytometer. Units are ×10⁴ cells/well. *P < 0.01 vs. vehicle; n = 12.

![Fig. 2. COX-2 induction reduces serum deprivation-induced apoptosis in GEC.](http://ajprenal.physiology.org/)

![Fig. 3. COX-2 induction activates ERK in GEC.](http://ajprenal.physiology.org/)
tosis. Caspase-3 is one of the execution caspases (27). In the next series of experiments, we used caspase-3 activity as a surrogate marker of apoptosis. When GEC (COX-2-inducible GEC) were cultured in serum-replete conditions, caspase-3 activity in cell lysates was not distinguishable from that of buffer alone (not shown). After GEC were cultured in serum-deprived conditions for 48 h, caspase-3 activity was clearly detectable in cell lysates (Control, normalized to 100 in Fig. 5A). PGE2 significantly inhibited serum deprivation-induced caspase-3 activity in a dose-dependent manner [PGE2, 0.1 M: 91 ± 11, PGE2, 1 M: 51 ± 10 (P < 0.05 vs. control), PGE2, 5 M: 50 ± 6 (P < 0.05 vs. control), arbitrary units, n = 3 each; Fig. 5A].

To test whether the EP4 receptor mediates the anti-apoptotic effect of COX-2 induction, we next studied the impact of the EP4-specific antagonist L-161982 on serum deprivation-induced caspase-3 activity (Fig. 5B). When COX-2 expression was induced with ponasterone A, caspase-3 activity decreased by ~50%, similar to the effect of PGE2. L-161982 abolished the effect of ponasterone A (Fig. 5B). Of interest, the EP1/EP2 receptor antagonist AH-6809 appeared to augment the anti-apoptotic effect of ponasterone A, suggesting that PGE2 may increase apoptosis of GEC via EP1 receptors, thus opposing the EP4 action (Fig. 5B). These results suggest that the anti-apoptotic effect of COX-2 induction is mediated by the EP4 PGE2 receptor.

PGE2 and COX-2 induction activate Akt-1. It was reported previously that PGE2 activates the Akt survival pathway, which could potentially explain the antiapoptotic effect of PGE2 in GEC. Thus we next studied effect of PGE2 on Akt activation in GEC. Phosphorylation of Akt at Ser473, which is known to correlate with its activity, was studied using phospho-

**Fig. 4.** Generation of PGE2 and expression of PGE2 receptors in GEC. A: COX-2-inducible GEC were plated as in Fig. 2 and stimulated with ponasterone A (2 μM) or vehicle (ethanol) in serum-free medium for 48 h. PGE2 content in culture medium was quantified by enzyme immunoassay. *P < 0.05 vs. EtOH, n = 4. B: RNA was extracted from rat mesangial cells, rat GEC-pl, or normal rat kidney. mRNA for the indicated prostanoid receptors (TP, EP1–4) and β-actin were amplified by reverse transcription-polymerase chain reaction (see MATERIALS AND METHODS).

**Fig. 5.** Serum deprivation-induced caspase-3 activity is inhibited by PGE2 or COX-2 induction. A: COX-2-inducible GEC were plated in K1 medium. After cells adhered, medium was changed to serum poor. One or 2 days later when cells are ~80% confluent, medium was changed to serum free and cells were stimulated with PGE2 (0.1, 1, or 5 μM) or vehicle [ethanol: control (contl)]. After 48 h, cells were lysed and were subjected to caspase-3 assay as in MATERIALS AND METHODS. *P < 0.05 vs. control, n = 3. B: COX-2-inducible GEC were plated as in A. When culture medium was changed to serum free, cells were stimulated with ponasterone A (2 μM) in the presence or absence of the EP1/EP2 receptor antagonist, AH-6809 (10 μM), or the EP4 receptor antagonist, L-161982 (10 μM). Control cells were treated with vehicle (ethanol) in the place of ponasterone A. After 48 h, cells were lysed and were subjected to caspase-3 assay. *P < 0.01 vs. control, n = 4.
phospho-specific antibody. When GEC were stimulated with PGE2, Akt phosphorylation at Ser473 was increased at various time points between 30 min and 24 h, compared with time-matched vehicle-treated cells (Fig. 6A). Thus, in addition to the ERK pathway, PGE2 activates another major survival pathway in GEC. Akt activation was also observed by COX-2 induction in GEC after 4 h, although activation was transient and much less marked than by exogenous PGE2 (Fig. 6B).

**PGE2 attenuates PA-induced apoptosis in GEC.** The above experiments studied serum deprivation-induced apoptosis in GEC. However, serum deprivation of GEC is not a condition that is directly relevant to any human diseases or animal models. Thus, to expand our findings to a model more relevant in vivo, we next studied the effect of PGE2 in PA-induced apoptosis in GEC (Introduction). For this series of experiments, to facilitate the experiments, we utilized a subclone of GEC (GEC-col), which is highly sensitive to PA. GEC-col are dependent on extracellular matrices for survival and proliferation (4). When GEC-col were plated on plastic substratum, caspase-3 activity was readily detectable and PA (50 μg/ml) caused a further approximately twofold increase in caspase-3 activity (control: 45 ± 12, PA: 100 ± 0 arbitrary units, P < 0.05, n = 4; Fig. 7A). These results are consistent with PA-induced apoptosis of GEC-col on plastic substratum we reported previously (4). Concomitant administration of PGE2 (1 μM) significantly attenuated PA-induced caspase-3 activity (PA + PGE2: 75 ± 9 arbitrary units, P < 0.05 vs. PA, n = 4; Fig. 7A). We also carried out similar experiments using COX-2-inducible GEC (derived from GEC-pl). However, these cells were much more resistant to PA and although similar results were observed, response to PA tended to be smaller and more variable (not shown). To validate the above results, we also studied caspase-3 activation using the antibody that specifically detects activated/cleared caspase-3 (Fig. 7B) and obtained results similar to Fig. 7A. In addition, we have quantified apoptosis by staining with Hoechst3342 and propidium iodine. When GEC were untreated, less than 1% of cells were apoptotic. GEC treated with PA showed 18 ± 5% of apoptosis, while concomitant treatment with PGE2 reduced apoptotic cells to 8 ± 1% (P < 0.05 vs. PA, n = 3). Taken together, these results indicate that PGE2 is anti-apoptotic not only in serum deprivation-induced apoptosis but also in PA-induced apoptosis in GEC.

The EP4 receptor is known to couple to adenylyl cyclase and increased intracellular cAMP (5). Thus it is possible that the anti-apoptotic effect of PGE2 is mediated by cAMP. To address this possibility, we studied the effect of forskolin, a known activator of adenylyl cyclase. As shown in Fig. 7C, forskolin attenuated PA-induced caspase-3 activity to a similar extent, compared with PGE2. These results are consistent with the notion that cAMP may be a mediator of the anti-apoptotic effect of PGE2/EP4 in GEC.

Upregulation of the proapoptotic Bcl-family protein Bax and its translocation to mitochondria is a well-established mechanism of the intrinsic apoptosis pathway. In contrast, down regulation of the anti-apoptotic Bcl-family protein Bcl-xL is known to contribute to apoptosis (23). We next studied how these Bcl-family proteins are regulated by PA and PGE2. When GEC were incubated with PA, Bax protein increased, while Bcl-x-L protein decreased significantly (Fig. 7, D and E). PGE2 partially reversed PA-induced Bax upregulation (Fig. 7D). PGE2 also partially reversed Bcl-xL downregulation consistently, however, the effect was small and not statistically significant (Fig. 7E, P = 0.08 vs. PA). These results suggest that the anti-apoptotic effect of PGE2 is mediated, at least in part, by regulation of the Bcl-family proteins.

**EP4 receptor antagonist augments proteinuria and increases caspase-3 activity in PAN.** The above results suggest that COX-2 induction and subsequent PGE2 generation would protect GEC from apoptosis in vitro potentially via the EP4 receptor. We proceeded to verify these results in vivo. PAN is a rat model of proteinuric glomerular disease, in which PA-mediated GEC injury leads to morphological changes of GEC and proteinuria. A number of studies indicate that podocytes are the cellular target of PA (17, 21, 26). Kim and colleagues (14) demonstrated TUNEL-positive apoptotic podocytes in glomeruli 5–7 days after a single injection of PA. In this report, the number of podocytes decreased significantly with repeated injection of PA, while the number of mesangial cells remained unchanged, indicating that PA-induced podocyte apoptosis is the important feature of this model. Thus we reasoned that this is an appropriate model to study anti-apoptotic effect of PGE2/EP4 in GEC in vivo.

We first studied if COX-2 expression is upregulated in this model. Fourteen days after a single injection of PA, glomerular expression of COX-2 by immunoblotting was markedly increased, compared with control rats, similar to what was seen in GEC with inducible COX-2 (Fig. 8A, top). Immunohistochemistry of the kidney section revealed distribution of COX-2 positive cells mainly at the periphery of the glomerular tufts, consistent with its upregulation in GEC (Fig. 8A, bottom). Glomerular expression of COX-1 was only weakly detectable by immunoblotting and was not increased in PAN (not shown). Glomerular caspase-3 activity was increased by approximately fourfold in glomeruli from rats with PAN, compared with normal control rats (control: 25 ± 13 vs. PAN: 100 ± 8 arbitrary units, P < 0.01, n = 6; Fig. 8B). Because GEC is the major cellular target of PA in the...
glomerulus, it is reasonable to assume that increased glomerular caspase-3 activity represents PA-induced podocyte apoptosis. Urinary protein excretion was also significantly increased in rats with PAN (on day 14, control: 4/11006 to 27 mg/day, \( P < 0.05 \), \( n = 6 \)). When rats with PAN were treated with the EP4 receptor antagonist L-161982 between day 7 and day 14, glomerular caspase-3 activity increased by 82% (182/11006 to 37 arbitrary units, \( P < 0.05 \) vs. PAN, \( n = 6 \)). L-161982 also caused a trend to increase proteinuria, although the difference was not statistically significant (110/11006 to 43 mg/day, not significant from PAN, \( n = 6 \)). When similar experiments were performed at day 7, where rats were treated with L-161982 between day 0 and day 7, PAN rats treated with L-161982 showed significantly more proteinuria, compared with PAN rats treated with saline (day 7 PAN: 165 ± 4 mg/day, PAN+L-161982: 196 ± 8 mg/day, \( P < 0.05 \), \( n = 4 \)). These results suggest that inhibition of the PGE2 EP4 receptor leads to exacerbation of apoptosis and increased proteinuria in rats with PAN.

**DISCUSSION**

The current study addresses the role of COX-2 and PGE2 in GEC survival. The results of the current study point to the important role of PGE2, in particular its EP4 receptor in mediating cell survival of GEC. In normal gastric and colon cancer epithelial cells, PGE2 transactivated the EGF receptor and the ERK pathway, a well-established antiapoptotic/survival pathway (19). In other colorectal cancer cells, PGE2 transactivated the EGF receptor and the Akt pathway, another well-known survival pathway (6). However, in these studies, the EP receptor isoform responsible for the effect of PGE2 was not addressed. Stenson and colleagues (12, 31) recently demonstrated that PGE2 reduces radiation-induced epithelial cell apoptosis via the EP2 receptor through a mechanism involving Akt activation and bax translocation. These previous reports are generally in agreement with the current results, supporting the antiapoptotic effect of PGE2 in epithelial cells. However, Takadera et al. (28) recently reported that PGE2 induces caspase-dependent apoptosis in hippocampal neural cells, probably via the EP2 receptor. Thus the effect of PGE2 and the role of the PGE2 receptor isoforms in cell survival/apoptosis may be cell/tissue specific and the same receptor isoform may have the opposite effects in different cells/tissues. In addition, it should be noted that rat GEC-pl we utilized in the current study do not lose their ability to proliferate in culture, unlike GEC in vivo, which do not proliferate except in pathological conditions (18). Thus we should interpret the current results, in particular those regarding cell proliferation, with some caution.
Increasing evidence suggests that different prostanoids and their receptors elicit a variety of cellular responses, some of which antagonize with each other. A very well-recognized example is that TXA2 and PGI2 are vasoconstrictor and vasodilator, respectively. It is also known that PGE2 constricts smooth muscles in certain tissues and relaxes them in the other tissues. It is believed that constriction is mediated via the EP1 receptor, while relaxation is mediated via the EP2 or EP4 receptors (5). The results of the current study underscore the evolving complexity of the COX-prostanoid network. Although nonselective COX inhibitors and COX-2-selective inhibitors were shown to reduce proteinuria in experimental models and humans (11, 30, 33, 36), it is likely that these inhibitors are also negating the beneficial effects of some of the products of COXs, thereby reducing their therapeutic value.

Agonists specific to the prostanoid receptors that are cell protective for GEC could be beneficial for prevention and treatment of proteinuric glomerular disease such as focal segmental glomerulosclerosis and diabetic nephropathy. For example, the current results would suggest a possibility that EP4 receptor agonists may have beneficial effects on GEC injury and proteinuria. Of interest, the EP4 agonist 4819-CD was shown to reduce ischemia-reperfusion injury in the heart (35). EP4/H11002/H11002/H11002 mice usually do not survive the postnatal period (25); however, using specific breeding methods, the same group also demonstrated that EP4/H11002/H11002/H11002 mice are more susceptible to coronary ischemia-reperfusion (35). Similar methods or GEC-specific gene deletion of EP4 will likely give more insights into the role of the EP4 receptor in GEC in vivo.

In the current study, we observed that the ERK and Akt pathways are activated by COX-2 induction and/or PGE2. Both ERK and Akt pathways are known to be antiapoptotic/survival pathways and could contribute to COX-2/PGE2-mediated antiapoptosis in GEC. Of particular interest, Tessner and colleagues (31) showed that the EP2-mediated antiapoptotic effect of PGE2 in radiation-induced epithelial apoptosis involves Akt activation and bax translocation. In these elegant studies, the authors demonstrated that PGE2 inactivates the proapoptotic protein bax via activation of Akt (31). In the current study, we also demonstrated that the protein expression of two Bcl-family proteins, Bax and Bcl-xL, were regulated by PGE2 (Fig. 7, D and E). Thus it is conceivable that mechanisms involving Akt and the Bcl-family proteins may contribute to the antiapoptotic effect of COX-2/PGF2; however, exact signaling pathways and cellular mechanisms regulating apoptosis/survival in GEC remain to be unraveled. The proposed pathways are summarized in Fig. 9.

We have extensively reported the role of phospholipase A2s (PLA2s) in GEC as the key enzymes to liberate free arachidonic acid from the membrane phospholipids. In the current study,
induction of COX-2 alone was sufficient to augment PGE$_2$ generation in GEC. These results would suggest that there is a basal turnover of membrane phospholipids, which provides substrate arachidonic acid to COX-2 and its downstream enzyme, PGE$_2$ synthase. Cultured rat GEC express a small amount of cytosolic PLA$_2$, which could contribute to this turnover (20). Alternatively, other isoforms, such as calcium-independent iPLA$_2$S may have a role in this basal turnover (1).

In summary, we demonstrated that COX-2 and its product PGE$_2$ have an antiapoptotic effect on GEC and that the EP4 receptor of PGE$_2$, at least in part, mediates this effect. Recent evidence suggests that COX-2 contributes to cell survival both in physiological and pathological conditions and COX-2-mediated cell survival is a renewed focus of recent research in lipid mediators. A clearer understanding of the role of COX-2 and PGE$_2$ in apoptosis prevention, whether beneficial or pathogenic, would not only contribute to the elucidation of the pathogenesis of GEC injury, but would also provide further insights into a wider range of diseases.

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