Reduced IP receptors in STZ-induced diabetic rat kidneys and high-glucose-treated mesangial cells

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Nasrallah, Rania, and Richard L. Hébert. Reduced IP receptors in STZ-induced diabetic rat kidneys and high-glucose-treated mesangial cells. Am J Physiol Renal Physiol 287: F673–F681, 2004. First published May 25, 2004; 10.1152/ajprenal.00025.2004.—Mesangial cells (MG) are an important source of renal PGE2 and PGI2. The purpose of this study was to examine the effects of cicaprost (CCP; PGI2 analog) on MG function and the expression of IP receptors in streptozotocin (STZ)-diabetic rats and glucose-treated MG cells. CCP increased cellular cAMP in immortalized rat MG cells. Both glucose and anisomycin attenuated CCP-cAMP, but not PMA, angiotsenin II, or transforming growth factor-β. Also, IP receptor protein was reduced in response to glucose. While CCP decreased the levels of the cell cycle inhibitor p27, it did not alter thymidine or leucine incorporation. However, CCP reduced fibronectin levels by 40% and increased matrix metalloproteinase-2 levels threefold, a key enzyme in matrix degradation. Finally, IP receptors were significantly reduced in the outer medulla of 4- and 12-wk STZ-diabetic rats and in the cortex, outer, and inner medullary regions in 6-mo uninephrectomized STZ-diabetic rats. The changes in the CCP/IP system observed in this study suggest that IP may serve as an alternate therapeutic target in diabetes.

cAMP; cikaprost/IP; immortalized mesangial cells; streptozotocin-diabetic rats

PROSTACYCLIN ALTERS CELL FUNCTION by two distinct mechanisms (13, 24, 41): increasing intracellular cAMP via cell surface IP receptors and binding peroxisome proliferator-activated receptors (PPARβ/δ). While the significance of the PGI2/PPAR pathway to renal physiological or pathological processes is still undefined, the PGI2/IP/cAMP system is certainly an important regulator of renal hemodynamics and tubular transport properties. Our group and others (20, 27, 31) have localized IP receptors to various cells in the kidney (endothelial, epithelial, mesangial, interstitial), but species differences have been noted in terms of expression patterns. However, using the selective IP agonist cikaprost (CCP), we have clearly demonstrated that distinct IP receptors mediate the signaling responses to PGI2 in rat and rabbit collecting ducts (30, 31), a major area of prostanooid synthesis in the kidney. While the glomerular mesangial cells are also an important source of renal prostaglandins, the effects of the PGI2/IP/cAMP system on mesangial cell function is poorly defined. Furthermore, most studies thus far have utilized nonselective PGI2 analogs (iloprost, carboxoprosta- cyclin, beraprost sodium), therefore failing to distinguish between IP receptor and PPAR involvement.

Changes in mesangial cells are recognized as a major hallmark of diabetic nephropathy: proliferation, hypertrophy, and mesangium expansion occur in the early phases of the path to glomerulosclerosis (2, 43). The disturbances are multifaceted, but the underlying link between different factors has yet to be defined. Glucose is surely a key determinant of the mesangial alterations, but cross talk does occur between numerous pathways during the course of the disease. Important systems include the polyol pathway, the renin-angiotensin system, PKC, MAPK, and transforming growth factor (TGF)-β, to name a few (4, 17, 42). While the role of prostaglandins in diabetes is well recognized, their individual contribution to specific renal changes has not been thoroughly addressed. Levels of prostaglandins will fluctuate throughout the course of the disease (8–10), but how that affects the overall cell response in specific nephron segments is unclear. Surely, a better understanding of how the individual prostanooid pathways, receptor expression as well as prostaglandin synthesis, are changing throughout different stages of diabetes is needed to clarify their involvement and provide more specific targets for therapeutic intervention.

Renal PGI2 production is dependent on the activity of two enzymes: cyclooxygenases (COX-1 and -2) and PGI2 synthase (24, 35). We have recently shown that primary cultures of rat mesangial cells have diminished levels of PGI2 synthase in response to prolonged high glucose but increased levels of COX-2 (28). Similar findings were reported in human aortic endothelial cells exposed to high glucose, with increased COX-2 and thromboxane A2 synthesis but reduced PGI2 release (7). We also showed that prostacyclin analogs (CCP, iloprost) increase cAMP in mesangial cells without altering intracellular calcium levels and that prolonged exposure to glucose reduced CCP-cAMP without altering IP mRNA levels (28). The effect of CCP on mesangial cell function was not investigated.

Countless studies have implicated alterations in prostacyclin levels in the pathogenesis of diabetic nephropathy. Villa et al. (37) administered CCP to 8-mo uninephrectomized STZ rats and showed comparable benefits (normalized glomerular filtration rate, reduced proteinuria) to the angiotensin-converting enzyme inhibitor fosinopril. Also, long-term administration of beraprost sodium (prostacyclin analog) to diabetic patients has proven to be beneficial in the prevention of diabetic complications by decreasing albuminuria (32, 39). In another study, beraprost sodium attenuated glomerular hyperfiltration and macrophage infiltration in STZ-diabetic rats by modulating nitric oxide synthase expression (44). While these studies suggest a defect in prostacyclin signaling, to date there is no evidence of changes in IP receptor levels in diabetic kidneys.
Therefore, the purpose of this study was to examine the effects of CCP in immortalized rat mesangial cells, looking at the expression of IP receptor mRNA and protein, growth responses (thymidine, leucine incorporation), the expression of p27, matrix proteins (fibronectin), and matrix metalloproteinase (MMP-2) levels. Additionally, the last part of this work examines the changes in IP mRNA levels in the three regions of the kidney at different stages of STZ-diabetes in rats (2–16 wk) and in advanced disease in uninephrectomized 6-mo STZ-diabetic rat kidneys. Deciphering the regional differences in receptor expression will facilitate the investigation of the contribution of this system to the nephropathy and the link to specific alterations throughout the nephron. A better understanding of these changes could lead to better avenues for the treatment or prevention of individual complications as they arise.

MATERIALS AND METHODS

Diabetic rat model. Tissue from three kidney regions (cortex, outer medulla, inner medulla) was isolated from 200-g Sprague-Dawley rats under bright light using a dissecting microscope. The diabetic model utilized in the studies is STZ-diabetes, a widely used model resembling type 1 diabetes mellitus. STZ is an N-nitroso derivative of β-glucosamine that is utilized to induce diabetes in a variety of experimental animals and to determine the long-term complication of diabetes. It basically functions as a toxin that selectively destroys the insulin-producing cells of the pancreas, rendering the rat diabetic experimental animals, and to determine the long-term complication of diabetes. It basically functions as a toxin that selectively destroys the insulin-producing cells of the pancreas, rendering the rat diabetic experimental animals, and to determine the long-term complication of diabetes. It basically functions as a toxin that selectively destroys the insulin-producing cells of the pancreas, rendering the rat diabetic experimental animals, and to determine the long-term complication of diabetes. It basically functions as a toxin that selectively destroys the insulin-producing cells of the pancreas, rendering the rat diabetic experimental animals, and to determine the long-term complication of diabetes.

Six different groups of rats were employed:
1) Vehicle-treated control (administration of 0.1 mol/l sodium citrate buffer, pH 4.0)
2) STZ-diabetic rats [administration of 65 mg/kg STZ (Sigma, St. Louis, MO) in 0.1 mol/l sodium citrate buffer, pH 4.0, with hyperglycemia being maintained between 17 and 25 mmol/l with daily subcutaneous injections of 1–2 U of insulin]
3) STZ-insulin rats (same as in group 2 except for maintenance of euglycemia by subcutaneous implantation of a sustained release insulin implant; Linplant, Linshin, Scarborough, ON)
4) Right unilateral nephrectomy (NX)
5) NX/STZ: STZ administration (same as in group 2) + unilateral nephrectomy
6) NX/STZ-insulin: same as group 3 + unilateral nephrectomy

The day after STZ administration, a urine analysis was performed for glucose and ketones using a Keto-Diastix reagent strip (Bayer, Etobicoke, ON), and animals with sustained glucosuria are assigned to the STZ-diabetic, NX/STZ, or NX/STZ-insulin groups. Experiments were performed on STZ rats in early stages of diabetes (2, 4, 8, 12, and 16 wk) and in later stages of the disease (24 wk) in uninephrectomized STZ rats. See Table 3 for a comparison of the kidney weights and body weights of the 6-mo uninephrectomized rat groups.

Mesangial cell culture. Rat mesangial (rMG) cells immortalized with pSV3-Neo at passage 8 were obtained from the American Type Culture Collection (ATCC; Manassas, VA). These cells are characterized as desmin-negative, vimentin-negative, and negative for cytokeratin 8. In our laboratory further confirmed their origin by immunocytochemical analysis of α-smooth muscle actin. For all experiments, rMG cells were cultured on petri dishes in DMEM (Sigma) containing 15% fetal bovine serum and 0.4 mg/ml genitin (G-418; Gibco) and maintained at 37°C and 10% CO2. After the media were replaced every 2 days to maintain a constant growth environment, and for cAMP and thymidine/leucine incorporation experiments the media were replaced by DMEM without serum 24 h and 3 days before stimulation of the cells, respectively.

cAMP measurements. Confluent rMG cells were pretreated for 15 min with 10 μM indomethacin and 0.5 mM 3-isobutyl-1-methylxanthine and then stimulated for 10 min at timed intervals with 0.1 μM CCP or PGE2. cAMP levels were measured using a radioassay kit. For experiments with high glucose, the cells were cultured in the presence of 5.6 mM glucose (control) or 25 mM glucose for 24 h before stimulation with prostanoids. Mannitol was used as an osmotic control. Data are presented as means (fold of control) ± SE. To examine the effect of different factors on the CCP-IP-cAMP system, we measured the cAMP response to CCP in cells treated with 1 μM angiotensin II, 2 ng/ml TGF-B, 100 nM anisomycin (MAPK activator, Sigma), and 100 nM PMA (PKC activator, Sigma) for various times.

RT-PCR. Total RNA was isolated from cultured rMG cells using the TRizol method (Gibco) and DNase treated to prevent amplification of genomic DNA. RNA was reverse transcribed into cDNA and amplified by PCR using specific primers (listed in Table 1) for the IP receptor (407 bp) and each of the PGE2 receptor subtypes: EP1 (336 bp), EP2 (401 bp), EP3 (437 bp), and EP4 (423 bp). Samples were loaded onto a 1.5% agarose gel containing ethidium bromide for visualization of the amplified products. To confirm product identity, each fragment was cloned and sequenced.

Western blotting. Cell lysates were obtained by sonicating cells in RIPA buffer containing various phosphatase and protease inhibitors. Twenty-five micrograms of each sample were resolved by SDS-PAGE on a polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking O/N in 5% milk/TBS-T, the membrane was incubated for 3 h with a human anti-IP polyclonal antibody (gift from Dr. R. M. Nüssing) at a dilution of 1:4,000. After incubation with a horsedarish peroxidase-conjugated goat anti-rabbit IgG secondary antibody, enhanced chemiluminescence was performed for signal detection. The IP level was compared in cells exposed to either control

Table 1. Primer pairs and probes used for RT-PCR and real-time RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Probe</th>
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</thead>
<tbody>
<tr>
<td>IP: FF1 + FF2</td>
<td>cgggacacgagggatgagttacc</td>
<td>gcacagggcagacagtcaagtgcag</td>
<td>NA</td>
</tr>
<tr>
<td>EP1 receptor</td>
<td>cgccagcggcttacacgca</td>
<td>ccctgcgtgggactaatcg</td>
<td>NA</td>
</tr>
<tr>
<td>EP2 receptor</td>
<td>agggctctgtgctctcatctc</td>
<td>caagccctccatctcttcaat</td>
<td>NA</td>
</tr>
<tr>
<td>EP3 receptor</td>
<td>cggcggcagctgtgctctcat</td>
<td>tggagctgctctctgtctgctc</td>
<td>NA</td>
</tr>
<tr>
<td>IP receptor</td>
<td>gcctctgctgctctctgtctgctc</td>
<td>cgtgctgctctctgtctgctc</td>
<td>NA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>gaagatggagtcac</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

For each IP and PGE2 (EP) receptor subtype, the sequence for the upstream and downstream primers used for amplification by RT-PCR are provided. Also, the primers and probes used to amplify the IP receptor and GAPDH mRNA by real-time RT-PCR are shown. NA, not applicable.
(5.6 mM glucose), 25 mM glucose, or mannitol. A single band of 52 kDa was obtained. For detection of fibronectin, p27, and MMP-2, cells were treated for 24 h with 1 μM CCP. Specific antibodies for each protein were obtained from Calbiochem.

Northern blotting. Total RNA was isolated from rMG cells, using the TRizol method, and was DNase treated. Cultured rMG cells were exposed to control, 25 mM glucose, or mannitol, and 10 μg of total RNA from each sample were loaded onto a formaldehyde gel. RNA was then transferred to a nitrocellulose membrane. After baking for 2 h at 80°C in a vacuum, the membrane was incubated O/N with a [32P]dCTP-labeled human IP cDNA (Cayman Chemical) or mouse EP4 cDNA probe (a gift from M. D. Breyer, Vanderbilt University) and then exposed to film for 1 wk. To normalize the samples, the expression of β-actin was determined for the same membrane. Denitometric analysis was used to compare the relative expression of IP and EP4 mRNA in each sample. Data are presented as means (arbitrarily units or fold of control) ± SE (n = 3). For quantification of IP mRNA in STZ-diabetic rats, kidneys were removed from control, diabetic, and insulin-treated rats at 2–6 wk after STZ injection. Total RNA was isolated from samples of cortex, outer, and inner medulla using the TRizol method, as described by the manufacturer (GIBCO BRL), and was DNase treated (Boehringer Mannheim) to reduce genomic DNA. Ten micrograms of total RNA from each sample were used, IP mRNA was performed as described above, and the data are summarized in Table 2.

1[3H]thyymidine and 1[3H]leucine incorporation. rMG cells were cultured in serum-containing growth media on 24-well plates to 50% confluence and serum-starved for 3 days, after which 1 M CCP or 15% FBS was added to the DMEM for an additional 4 or 24 h. 1[3H]thyymidine (2 μl/ml) was added to each well during the last 4 or 24 h at 37°C, or 1[3H]leucine for 24 h. Then, the cells were washed 4× in ice-cold PBS and solubilized in 1 N NaOH for 15 min at 37°C. The samples were then transferred to scintillation fluid and counted. To determine whether CCP altered the effect of FBS on DNA or protein synthesis, 10 nM-1 μM CCP was added to the samples in the presence of FBS.

Real-time RT-PCR. Total RNA was isolated using TRizol (GIBCO) from different preparations of cortex, outer, and inner medulla from four groups of rats (control, NX-STZ, NX-STZ-insulin, NX) at 24 wk of diabetes. The relative quantity of each target nucleic acid in different samples was determined by analyzing the cycle-to-cycle change in fluorescence signal as a result of amplification during PCR. To quantify the amount of RNA in each sample, a relative standard curve was prepared by diluting a stock of control RNA. GAPDH mRNA is detected as an internal control to standardize the amount of sample RNA added to a reaction. The availability of distinguishable reporter dyes makes it possible to amplify and detect the target message and GAPDH in the same tube. The RT-PCR was performed using a TaqMan PCR Core Reagents Kit and GAPDH Control Reagents Kit providing a VIC-labeled GAPDH probe. The following parameters were employed: 48°C for 30 min then 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and 60°C for 1 min. Primers and probes were selected and obtained from the Custom Oligonucleotide Synthesis Service of Applied Biosystems. The upstream and downstream primers as well as fluorescent probes for IP and GAPDH are listed in Table 1. The data were analyzed by a computer using the ABI prism 7000 sequence detection system and are expressed as the IP mRNA/GAPDH ratio.

Statistics. SigmaPlot software for Windows version 4.0 (1986–1997) was used to analyze data. Results are expressed as means ± SE (P < 0.05). Statistical significance was determined using an unpaired t-test or one-way ANOVA followed by Tukey’s test.

RESULTS

EP4 receptor, but not IP receptor, mRNA is increased in glucose-treated rMG cells. To determine whether IP receptors are expressed in immortalized rMG cells, we first examined the expression of IP and EP receptor subtypes by RT-PCR. Table 1 lists the primer pairs used for amplification of each receptor fragment. As shown in Fig. 1, IP as well as EP1 and EP4 receptor fragments were amplified and their identity was confirmed by cloning and sequence analysis. Although a band for EP2 mRNA was detectable, the sequence did not match that expected by 10.220.33.2 on April 8, 2017 http://ajprenal.physiology.org/ Downloaded from

![Fig. 1. IP receptor mRNA and PGE2 receptor subtypes EP1 and EP4 are detected in immortalized rat mesangial (rMG) cells. RT-PCR was performed using specific primers to amplify fragments from each of the IP and EP receptors, and the amplified products were confirmed by cloning and sequencing.](http://ajprenal.physiology.org/)

### Table 2. Summary of Northern blot densitometric analysis of IP mRNA levels at different stages of STZ-diabetes

<table>
<thead>
<tr>
<th>Cortex, wk</th>
<th>Control</th>
<th>Diabetic</th>
<th>Insulin</th>
</tr>
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<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>2 ± 0.6</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1.3 ± 0.3</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>2.3 ± 0.8</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>2.6 ± 0.8</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0.87 ± 0.34</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>2 ± 0.6</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Outer, wk</th>
<th>Control</th>
<th>Diabetic</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>1 ± 0.3</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>0.7 ± 0.1*</td>
<td>1 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>1 ± 0.3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0.98 ± 0.24</td>
<td>0.99 ± 0.35</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0.7 ± 0.08*</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>1 ± 0.3</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Inner, wk</th>
<th>Control</th>
<th>Diabetic</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1</td>
<td>0.85 ± 0.09</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1 ± 0.2</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>0.7 ± 0.2</td>
<td>1.1 ± 0.8</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1.9 ± 0.9</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>0.85 ± 0.09</td>
<td>0.9 ± 0.2</td>
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Values are means ± SE expressed as fold of control; n = 4–5. Total RNA was isolated from cortex, outer, and inner medullary regions of the kidney from 2-, 4-, 6-, 8-, 12-, and 16-wk streptozotocin (STZ)-rats. Northern blot analysis was performed to quantify levels of IP mRNA in each region using a human IP cDNA probe. *P < 0.05.

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consistent with our work in primary cultures of mesangial cells showing no change in IP mRNA after 5 days' culture in high glucose (28). On the other hand, a 2.2-fold increase in EP4 mRNA was obtained (Fig. 2C), although a similar increase is seen with mannitol. Whether this change in mRNA is due to an osmotic effect is noteworthy, and ongoing work in our laboratory will further examine this issue. Interestingly, in primary cultures of mesangial cells, Ishibashi et al. (15) observed no change in EP4 mRNA in response to high-glucose exposure. In contrast, we have previously reported an approximately two-fold increase in both EP1 and EP4 in cultured rat inner medullary collecting duct exposed to high glucose for 4 days (29).

IP receptor protein levels are decreased in rMG cells exposed to high glucose. Because no change in IP mRNA in response to high glucose was obtained, we examined whether glucose altered IP protein levels by Western blotting. As shown in Fig. 3, a 50% reduction in IP protein was noted in response to high-glucose exposure. In contrast, we have previously reported an approximately two-fold increase in both EP1 and EP4 in cultured rat inner medullary collecting duct exposed to high glucose for 4 days (29).

IP receptor protein levels are decreased in rMG cells exposed to high glucose. Because no change in IP mRNA in response to high glucose was obtained, we examined whether glucose altered IP protein levels by Western blotting. As shown in Fig. 3, a 50% reduction in IP protein was noted in response to high-glucose exposure. This decrease was not obtained when glucose was substituted by mannitol, eliminating a possible osmotic effect on IP protein levels. Whether this decrease is due to enhanced degradation or reduced synthesis is not clear at this time.

CCP-stimulated cAMP is attenuated by 24-h glucose and anisomycin. As can be seen in Fig. 4, both CCP and PGE2 stimulated cAMP to a similar extent in cultured rMG cells, up to 56 ± 4 and 52 ± 5%, respectively. When cells were cultured in the presence of 25 mM glucose or mannitol for 24 h, the stimulatory response to CCP was reduced by over 50% and to PGE2 by ~40%. Whether this effect of glucose is entirely osmotic requires further investigation, but the finding that glucose and not mannitol altered IP protein levels (see above) argues for possible glucose effects independently of osmotic changes. While the effect on both receptors was similar in response to glucose, we noted differences in the response to other glucose-dependent factors. While CCP-cAMP stimulation was greatly attenuated by 24-h anisomycin treatment (Fig. 5A) from 56 ± 4%, PMA treatment only slightly
reduced the cAMP response to CCP to 37.5 ± 8% (Fig. 5B). On the other hand, a significant inhibition of PGE2-stimulated cAMP to 14 ± 1.5 from 52 ± 5% was obtained in response to 15-min PMA pretreatment. Of interest, both angiotensin II and TGF-β had no effect on the cellular response to prostanoids (data not shown).

**CCP decreases the levels of p27 and fibronectin and increases MMP-2 in rMG cells.** To determine whether CCP could regulate the growth of rMG cells or matrix protein production by these cells, we measured the levels of p27 and fibronectin in cells exposed to CCP for 24 h. As indicated in Fig. 6, both p27 and fibronectin levels were significantly reduced by CCP treatment by 45 and 55%, respectively. Consistent with the decrease in fibronectin levels, a threefold increase in MMP-2 levels was obtained. This matrix metalloproteinase is a gelatinase important in the degradation of matrix proteins such as fibronectin and collagen IV (26, 38), which are major components of the mesangial matrix.

**CCP does not alter growth responses in rMG cells.** Inhibitors of cyclin-dependent kinases such as p21 and p27 are important regulators of growth responses in many cell types, including mesangial cells. Both of these factors are important in the progression of diabetic nephropathy (2, 23) and are thought to play a role in the mesangial cell changes (proliferation, hypertrophy) that are seen in diabetes. Despite a reduction in the levels of p27 in response to CCP, we did not obtain any changes in both thymidine or leucine incorporation by rMG cells. As shown in Fig. 7, FBS increased the level of thymidine incorporation 1.5-fold after 24 h. Not only was there no effect of CCP alone on these cells, CCP did not alter the response to FBS either. In other cell models, an inhibition of FBS-stimulated proliferation has previously been reported in response to prostanoids, including PGE2 (14). Furthermore, CCP did not affect protein synthesis in rMG cells, but FBS treatment increased leucine incorporation by ~2.5-fold.

**IP receptor mRNA is decreased in diabetic rats.** The role of prostaglandins in diabetes is surely multifaceted. However, the nature of their involvement in diabetic nephropathy remains evasive to this day. Certainly, they contribute to the pathology, but they may also serve to antagonize other harmful agents. Although numerous studies have suggested a change in the levels of prostanoids or their metabolites in diabetes, to date there is very little information regarding the expression of prostanoid receptors in diabetics. Northern blotting and real-time RT-PCR analysis were utilized to measure the relative expression of IP mRNA in different regions of the kidneys of STZ-treated rats at different stages of diabetes as well as in 6-mo uninephrectomized STZ-diabetic rats. As shown in Fig. 8, a 30% decrease in IP mRNA was only seen in the outer medulla at 4 and 12 wks of STZ-diabetes. A summary of other stages of diabetes is provided in Table 2. As can be noted, there is no significant change in IP expression at any other stage, and none in the cortex or inner medulla. A major drawback with the STZ model is the lack of resemblance to type I diabetes in terms of renal pathology. Also, the progression of the disease is variable among animals. To hasten the development of diabetic complications, the STZ-diabetic rats were subjected to

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Fig. 5. Anisomycin (ANI) but not PMA altered CCP-stimulated cAMP in rMG. Confluent rMG cells were serum starved and stimulated for 10 min with 0.1 μM CCP, and intracellular cAMP levels were measured. A: effect of 100 nM ANI for 24 h on the cAMP response to CCP. ANI alone does not alter cAMP levels. B: effect of 15 min PMA (100 nM) pretreatment on the response to 100 nM CCP and PGE2. Values are means ± SE expressed as cAMP (%stimulation); n = 3. *P < 0.05. **P < 0.001.

Fig. 6. Twenty-four-hour CCP decreases fibronectin and p27 levels but increases matrix metalloproteinase (MMP)-2 protein in rMG cells. Western blot analysis of p27, fibronectin, and MMP-2 was performed in rMG cells treated with 1 μM CCP for 24 h. Densitometric analysis of protein levels is shown. Values are means ± SE expressed as fold-control; n = 3–5. *P < 0.05.
additional stress by undergoing a unilateral nephrectomy after confirmation of their diabetic state, and IP mRNA levels were determined after a 6-mo period. To assess the impact of this procedure on the kidney, we compared the average kidney sizes to the body weights of the different rat groups. As shown in Table 3, the ratio of kidney to body weight was 3.5-fold higher in the diabetic group compared with controls. By real-time RT-PCR, we observed comparable basal levels of IP mRNA in the cortex and inner medulla (Fig. 9) but about one-third less in the outer medullary region of the kidney. However, a 50% reduction of IP expression in uninephrectomized STZ-diabetic rats was consistently seen in all kidney regions. Maintaining euglycemia in insulin groups reversed the decrease in IP mRNA to levels comparable to those in controls. Only in the cortex was there an equivalent reduction of IP mRNA in uninephrectomized controls, with tendencies toward an increase in mRNA levels in the medullary regions for this group. However, because only two of the four animals initially assigned to this group survived to 6 mo, a statistical analysis could not be performed for this group.

### DISCUSSION

This study examined the role of the CCP/IP/cAMP system in immortalized rMG cells. We show changes in rMG function in response to CCP, including decreased p27 and fibronectin

![Graph](http://example.com/graph1.png)  
Fig. 7. CCP does not alter [3H]thymidine (A) or [3H]leucine (B) incorporation by rMG cells. rMG cells were serum starved for 3 days and then cultured in the presence of DMEM containing either 15% FBS or 1 μM CCP (6 CCP) for 24 h. To determine whether CCP altered the response to FBS, cells were treated with 10 nM (8C), 100 nM (7C), and 1 μM (6C) CCP in the presence of 15% FBS. Values are means ± SE expressed as fold-control; n = 4.*P < 0.001.

![Graph](http://example.com/graph2.png)  
Fig. 8. IP mRNA is decreased in the outer medullary region of streptozotocin (STZ)-diabetic rats. Northern blot analysis of IP mRNA expression was performed on tissue from 3 regions of the kidney at different stages of STZ-diabetes. Densitometric analysis comparing IP mRNA levels in control, diabetic, and insulin-treated rats with 4 (A) and 12 wk (B) of diabetes is shown. Values are means ± SE expressed as fold-control; n = 5. *P < 0.05.

### Table 3. Comparison of kidney and body weights of 6-mo controls and uninephrectomized animals

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<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Wt, g</th>
<th>Kidney Wt, g</th>
<th>Kidney Wt/Body Wt, &lt;sup&gt;×10&lt;sup&gt;-3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>732±9</td>
<td>49±3</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>NX/STZ-diabetic</td>
<td>6</td>
<td>433±21†</td>
<td>3.8±0.2*</td>
<td>2.5</td>
</tr>
<tr>
<td>Insulin</td>
<td>6</td>
<td>619±23‡</td>
<td>3.7±0.1*</td>
<td>8.8</td>
</tr>
<tr>
<td>NX</td>
<td>2</td>
<td>649</td>
<td>2.4</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats. NX, uninephrectomized. Daily measurements of body weights were recorded for each animal in all 4 groups (control, NX/STZ-diabetic, insulin, NX). The mean for each group is presented, as well as the corresponding kidney weights. The ratio of kidney weight to body weight is increased in diabetic compared with control and insulin groups throughout the course of the study. *P < 0.05, †P < 0.001, ‡P < 0.05 (diabetic vs. insulin).
levels as well as elevated MMP-2. However, CCP did not alter growth responses in rMG cells. Examining the literature available on the CCP/IP system systemically and in the kidney can provide insight into the possible significance of these findings. To date, most reports on extrarenal PGI₂ focus on its importance in balancing the constrictor effects of thromboxane A₂ (TxA₂). Actually, the cardiovascular complications associated with the use of selective COX-2 inhibitors may be due to a decrease in PGI₂ synthesis, leaving TxA₂ unopposed (5).

Similar to this role, the IP system in glomeruli may serve a protective function in diabetes as a homeostatic modulator of constrictors such as TxA₂ and angiotensin II. Several studies indicate that both of these factors are elevated in mesangial cells in response to high glucose and in diabetic glomeruli, contributing to hyperfiltration (1, 4, 8), hypertrophic events, and accumulation of matrix proteins (9, 16). Therefore, targeting IP may slow the progression of these complications, because both fibronectin and p27 are known to be elevated in diabetes and are important players in the development of glomerulosclerosis. Interestingly, Awazu et al. (2) reported a defect in fibronectin translation in p27-null mice. Whether the decrease in fibronectin levels that we observed in rMG cells in response to CCP is partly due to its effects on p27 requires further investigation. In this regard, it is interesting that new avenues of p27 research are emerging, diversifying its role within the cell beyond the regulation of cell-cycle progression (6), and the mechanisms linking the CCP/IP/cAMP system to these events may be elucidated in the future.

An investigation of the progression of diabetic complications in STZ-diabetic mice that are deficient in p27 (2) indicates that it is key in several aspects of nephropathy. Despite the importance of p27 in regulating proliferation and hypertrophy of cells, other inhibitors of cyclin kinases such as p21 are elevated in diabetic glomeruli and are also key to the development of diabetic hypertrophy (23). Perhaps in the rMG cells used for this study, there is compensation by these other regulators of cell-cycle progression, and this would explain the lack of effect of CCP on thymidine or leucine incorporation by these cells. More work is needed to verify this possibility.

Although the expansion of the mesangium in diabetes is mainly due to enhanced matrix protein synthesis and accumulation, there is also a decrease in protein degradation. Various matrix metalloproteinases are responsible for the cleavage of collagen, fibronectin, laminins, and other components of the matrix. Because CCP in this study increased the levels of MMP-2, it could prove to be useful in reducing the accumulation of matrix proteins and slowing the development of glomerulosclerosis. This possibility is further supported by the reduction in fibronectin observed in response to CCP in rMG cells. However, it is not yet clear whether the decrease in fibronectin levels is due to defects in synthesis (dependent on p27, as mentioned above), to enhanced degradation, or both.

Numerous studies allude to the putative role of prostanoids (PGs) in diabetic nephropathy (10), but a major controversy exists as to the nature of their involvement: whether they propagate the complications or serve to antagonize the deleterious effects of other agents. Moreover, current work has examined the beneficial effects of specifically targeting certain prostanoid pathways to alleviate the manifestations of the disease, including EP1-receptor antagonists (25) and IP-receptor agonists (18, 37, 44). However, the underlying mechanisms of PG involvement remain uncertain. In diabetic kidneys, it has been clearly demonstrated that COX enzymes are elevated, and glomerular PG production in most species is increased (11, 15, 36), as well as in STZ rats (22, 33). We recently reported elevated PGE₂ and PGI₂ synthesis in glucose-treated rat inner medullary collecting duct, as well as elevated COX-1 and -2 in medullary regions of 4- to 6-wk STZ-diabetic rat kidneys (29).

Furthermore, selective inhibitors of COX-2 (NS-398) have been used to reverse some of the renal complications of STZ-diabetes, such as altered glomerular filtration rate, without affecting mean arterial pressure or renal plasma flow (19). Altogether, the work thus far tends to indicate that the alterations in prostaglandins are mainly at the level of synthesis, without any detailed studies of actual cell responses and changes in expression of different prostaglandin receptors. In the current study, we characterized the expression of IP receptors in diabetic kidneys at different stages of the disease. We show that in the STZ-diabetic model, a reduced expression of IP is seen after 4 and 12 wk of diabetes, but no changes in expression were detected at other time points. Surprisingly, significant changes were only detected in the outer medullary regions. The renal manifestations or cellular events coinciding with this decrease in IP mRNA, and the significance to the nephropathy at these stages of diabetes, remain unclear at this time. On the other hand, when the diabetic kidney was subjected to additional stress to hasten the development of renal pathology, a reduction in IP expression was noted throughout the kidney. Future studies will be aimed at unravelling the significance of the decrease in IP to nephron dysfunction in diabetes, especially changes in the distal nephron: altered electrolyte and water transport, acid-base regulation, interstitial fibrosis, and tubular atrophy.

Consistent with the decrease in IP expression in vivo, we report a reduced level of IP in cultured rMG cells exposed to high glucose. Accordingly, the cAMP response to CCP was attenuated in high-glucose-treated cells. While no change in IP mRNA levels was noted in vitro, an increase in EP₄ mRNA levels was detected in response to glucose. The mechanisms underlying the inconsistent response to glucose in terms of
increasing mRNA levels vs. decreased PGE2-cAMP responses require further investigation, especially since Ishibashi et al. (15) reported no change in EP2 mRNA in primary mesangial cell cultures but showed the same attenuation of PGE2-cAMP. An extensive study of how individual EP receptors are altered throughout the course of diabetes in vivo is lacking and may clarify the discrepancies obtained in the two in vitro models: primary vs. immortalized mesangial cells. This highlights the importance of carefully extrapolating information from one specific model and applying it to clinical practice.

In addition to glucose, many other glucose-dependent diabetic factors have been shown to modulate cyclooxygenases and increase prostaglandin synthesis, including the glucose-induced activation of PKC as well as stimulation of MAPK pathways. An increase in both arachidonic acid release and eicosanoid production has been reported in mesangial cells in response to glucose-induced PKC activation (3, 21, 40). Similarly, IL-1β, an important cytokine, increased in mesangial cells in response to high glucose. Both JNK/SAPK and p38 MAPK have been implicated in COX-2 and PGE2 increases in response to IL-1β in rMG cells (12). However, as shown in the present study, although both PKC and MAPK are involved in altered prostanooid synthesis, they tend to attenuate the cAMP responses to both PGE2 and CCP, respectively. The mechanisms involved need further clarification. Although there is no perfect animal model mimicking human diabetic nephropathy, and isolating mesangial cells from their glomerular setting and matrix environment surely alters their response, there is clearly sufficient evidence that the PGF2/EP/cAMP system is attenuated in vitro. Future work examining the expression of IP protein in diabetic kidneys will confirm whether this defect also reflects the in vivo diabetic environment, thus providing a target for therapeutic intervention.

In summary, the present study demonstrates that IP receptor expression is diminished in diabetic kidneys. Although no change in IP mRNA is detectable in vitro, a significant decrease in IP protein was seen in response to high glucose. Also, on exposure to high glucose, signaling for CCP and PGE2 signaling is attenuated in rMG cells. We show a decrease in p27 and fibronectin in response to CCP, as well as an increase in MMP-2 levels. Further studies will clarify the mechanisms involved in these responses and the significance of the attenuation of this pathway to the glomerular and tubular pathology seen in diabetes.

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GRANTS

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REFERENCES


