Prevention of Diabetic Nephropathy in Rats by Prostaglandin E Receptor EP1-Selective Antagonist

HISASHI MAKINO,* ISSEI TANAKA,* MASASHI MUKOYAMA,* AKIRA SUGAWARA,* KIYOSHI MORI,* SEIJI MURO,* TAKAYOSHI SUGANAMI,* KENSEI YAHATA,* RIEKO ISHIBASHI,* SHUICHI OHUCHIDA,‡ TAKAYUKI MARUYAMA,* SHUH NARUMIYA,† and KAZUWA NAKAO*

*Department of Medicine and Clinical Science, †Department of Pharmacology, Kyoto University Graduate School of Medicine, Kyoto, Japan; ‡Discovery Research Laboratories, Minase Research Institute, Ono Pharmaceutical Co., Ltd., Osaka, Japan.

Abstract. Local production of prostaglandins (PGs) in the kidney is increased in clinical and experimental diabetic nephropathy, but the role of PGs in the pathogenesis and progression of diabetic nephropathy has remained unclear. It is here shown that an orally active antagonist selective for the PGE receptor EP1 subtype potently prevents the progression of nephropathy in streptozotocin-induced diabetic rats. The effects are shown by ameliorated renal and glomerular hypertrophy, decreased mesangial expansion, inhibited transcriptional activation of transforming growth factor-β (TGF-β) and fibronectin, and complete suppression of proteinuria. In vitro, this agent completely inhibits TGF-β and fibronectin upregulation in mesangial cells cultured under high-glucose conditions. These data indicate that the PGE2-EP1 system plays a crucial role in the development of diabetic renal injury in rats. It is further shown that both the EP1 antagonist and aspirin, a nonselective PG synthase inhibitor, markedly attenuate mesangial expansion, whereas only the EP1 antagonist inhibits glomerular hypertrophy and proteinuria, which suggests that these changes are caused by different mechanisms. This study reveals a potential usefulness of selective EP1 blockade as a novel therapeutic strategy for diabetic nephropathy and also brings a new insight into our understanding of this disease.

Diabetic nephropathy is a major cause of end-stage renal disease, and there has been a continuous increase in its incidence worldwide in the past two decades (1). Diabetic nephropathy is characterized by microalbuminuria, renal and glomerular hypertrophy, mesangial expansion with glomerular basement membrane thickening, arteriolar hyalinosis, and global glomerular sclerosis, which ultimately cause the progression of proteinuria and renal failure (2–4). Hyperglycemia is a necessary precondition for the development of the lesions (1,2,5,6), whereas systemic hypertension is an equally important aggravating factor of the disease (1,7). Other mechanisms, including glomerular hypertension with hyperfiltration, increased advanced glycation end products, sorbitol and protein kinase C (PKC) pathway activation, growth factors and cytokines such as transforming growth factor-β (TGF-β), and genetic susceptibility, have been identified as important deteriorating factors (2), but the precise mechanisms through which diabetic renal injury progresses remain to be resolved.

Local production of prostaglandins (PGs) in the kidney is increased in clinical and experimental diabetic nephropathy. Indeed, PG synthesis is augmented in glomeruli isolated from diabetic rats induced by streptozotocin (STZ), an animal model for type 1 diabetes (8) and in mesangial cells cultured under high-glucose concentrations (9–11). Overproduction of PGE2 has especially been shown to be associated with increased GFR in the early stages of diabetic nephropathy (11–14), suggesting a pathogenic role in hemodynamic alterations. Aspirin as well as indomethacin, a nonselective inhibitor of PG synthase or cyclooxygenase (COX) that inhibits the synthesis of PGE2 together with other PGs, was reported to cause partial amelioration in STZ-induced diabetic rats (11–13) or in diabetic patients with nephropathy (14).

PGE2, a major PG in the kidney, plays an important role in renal physiology, including the regulation of vascular smooth muscle tonus, glomerular filtration, renin release and tubular salt and water transport (15). Multiple actions of PGE2 are mediated via specific G protein-coupled cell surface receptors (15,16). On the basis of pharmacologic investigations and recent molecular studies (16), E-prostanoid (EP) receptors are now divided into four subtypes, EP1, EP2, EP3, and EP4. Previously we reported that EP1 and EP4 receptors are expressed in rat glomeruli and cultured mesangial cells (17). We further demonstrated that enhanced mesangial cell proliferation under high-glucose conditions in vitro was almost completely abolished by administration of EP1 antagonists (17). We therefore hypothesized that selective blockade of the EP1 receptor...
in vivo may be beneficial for preventing diabetic renal injury. Here we compare the effects of aspirin and a selective EP1 antagonist, ONO-8713, on the progression of renal injury in STZ-induced diabetic rats. We reveal that an EP1 antagonist markedly suppresses the progression of proteinuria, glomerular hypertrophy, and mesangial expansion in diabetic rats, suggesting that selective EP1 blockade may be a novel therapeutic strategy to treat diabetic nephropathy.

Materials and Methods

Induction of Diabetes and Drug Treatment

On the basis of the structures of known prostanoid receptor ligands, we performed an extensive screening to identify and optimize a selective EP1 antagonist, and found ONO-8713 (4-[2-[(N-isobutyl-N-(2-furylsulfonyl)amino)-5-trifluoromethylphenoxy)methyl] cinnamic acid) (18). Selectivity of ONO-8713 as to binding affinity and agonistic and antagonistic activities against PG receptors and thromboxane A2 receptor has been described elsewhere (18). In brief, ONO-8713 exhibited a Kᵢ value of 0.3 nM for both mouse and human forms of the EP1 receptor and more than 1000 nM for all other types of prostanoid receptors. This compound inhibited PGE2-induced calcium rise with IC50 values of 0.46 and 0.14 μM in mouse and human EP1-expressing cells, respectively, but showed no agonistic or antagonistic actions on other types of prostanoid receptors (18).

All animal experiments were conducted in accordance with our institutional guidelines for animal research. Male Wistar rats weighing 200 to 250 g were given free access to water and regular laboratory chow. Diabetes was induced in rats by single intraperitoneal injection of STZ (60 mg/kg body wt) (Sigma, St. Louis, MO) in citrate buffer. Diabetes was induced in rats by single intraperitoneal injection of STZ (60 mg/kg body wt) (Sigma, St. Louis, MO) in citrate buffer.

Renal Histology and Morphometric Analysis

Kidney sections were fixed by immersion in Carnoy solution followed by 4% buffered formaldehyde and embedded in paraffin. Two-micrometer sections were stained with periodic acid-Schiff and examined by light microscopy. Measurement of the glomerular cross-sectional area and the mesangial area of 30 glomeruli randomly selected in each rat by scanning of the outer cortex was performed with a computer-aided manipulator (KS-400; Carl Zeiss Vision, Munich, Germany) (19).

Cell Culture

Mesangial cells were established from glomeruli isolated from 10-wk-old male Sprague-Dawley rats with a differential sieving method (17) and used at passages 7 to 10. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies BRL, Grand Island, NY) containing 20% fetal calf serum (FCS; Sanko Junyaku, Tokyo, Japan) with 100 U/ml of penicillin and 100 μg/ml of streptomycin. As the cells reached 80% confluence, cells were grown in DMEM containing 20% FCS supplemented with 5.6 mM glucose (normal glucose) or 25 mM glucose (high glucose) for 5 d. Medium was then changed to DMEM containing 0.2% FCS supplemented with 5.6 mM glucose or 25 mM glucose in the presence or absence of ONO-8713 (1 μM) or aspirin (0.1 mg/ml), and the cells were further incubated for 24 h.

Northern Blot Analyses

Total RNA from isolated glomeruli and cultured mesangial cells were extracted by the acid guanidinium-phenol-chloroform method using Trizol reagent (Life Technologies BRL) (17,20). For analysis of EP receptors and COX-2, poly(A)⁺ RNA was prepared with PolyATtract (Promega, Madison, WI). Northern blot analyses were performed as described (17,20). In brief, 25 μg of total RNA was electrophoresed on 1.1% agarose gels containing 2.2 M formaldehyde, and RNA was transferred to nylon membrane filters (Pall BioSupport, Port Washington, NY). The cDNA fragments for rat TGF-β1 (nucleotides 1142–1546), fibronectin (nucleotides 619–1082), COX-1 (nucleotides 1297–1637), and COX-2 (nucleotides 843–1637), which were prepared by reverse transcription-PCR (RT-PCR) using rat kidney mRNA, were used as probes (21–23). The filter was hybridized with 32P-labeled probes, and autoradiography was performed by BAS-2500 (Fuji Photo film, Tokyo, Japan). For EP receptors, rat EP2, mouse EP3 and rat EP4 cDNA probes, and rat EP1 antisense RNA probe were used (17,20). The filters were rehybridized with human GAPDH cDNA probe (Clontech, Palo Alto, CA) for normalization.

In Situ Hybridization

The localization of EP1 receptor expression in the rat kidney was analyzed by in situ hybridization. Kidneys were frozen in isopentane at −50°C. and 8-μm cryosections were mounted on poly-L-lysine-coated slides. Samples were briefly air-dried and kept at −80°C until use. In situ hybridization was performed as described previously (24). Briefly, a radiolabeled cRNA probe for rat EP1 receptor was synthesized in vitro by T7 RNA polymerase after linearization of template DNA in the presence of [35S]CTP to a specific activity of 1.0 × 10⁹ cpm/μg. Hybridization was carried out at 58°C for 6 h in a solution containing 50% formamide, 2× SSC, 10 mM Tris-HCl (pH 7.5), 1× Denhardt’s solution, 10% dextran sulfate, 0.2% sodium dodecyl sulfate, 100 mM dithiothreitol, 500 μg/ml salmon sperm DNA, and 250 μg/ml yeast tRNA. Control hybridization experiments were carried out in adjacent sections using the same riboprobe with excess of unlabeled cRNA. Slides were exposed to β-max films (Amersham, Buckinghamshire, UK) at 4°C for 2 wk or dipped into autoradiographic emulsion NTB-2 (Eastman Kodak, Rochester, NY), exposed for 4 wk, and counterstained with methyl green pyronine stain solution (Nacalai Tesque, Kyoto, Japan).

Statistical Analyses

Data were expressed as the mean ± SEM. Statistical analyses were performed using ANOVA followed by Scheffe’s test. A P value < 0.05 was considered statistically significant.
**Results**

**Characteristics of Experimental Animals**

At 4, 8, and 12 wk after induction of diabetes by STZ, blood glucose was elevated and body weight gain was reduced similarly in untreated diabetic, aspirin-treated diabetic, and ONO-8713-treated diabetic rats (Table 1). PGE2 has both vasodilating and vasoconstricting properties, and EP1 and EP3 may mediate the vasoconstricting action (25,26). Treatment of rats with ONO-8713 resulted in transient and slight reduction in BP at 4 wk as compared with untreated or aspirin-treated rats (27). Previous studies have shown that treatment with COX inhibitors in diabetic models resulted in only a limited effect on proteinuria (11). We here evaluated the effect of selective EP1 blockers on proteinuria in STZ-induced diabetic rats. After induction of diabetes, urinary protein excretion normalized with creatinine increased gradually in untreated diabetic rats. After induction of diabetes, urinary protein excretion increased gradually in untreated diabetic rats. After induction of diabetes, urinary protein excretion increased gradually in untreated diabetic rats. After induction of diabetes, urinary protein excretion increased gradually in untreated diabetic rats.

Urinary excretion of PGE2 was significantly increased by 3.5- and 4.5-fold in diabetic rats as compared with nondiabetic controls at 4 and 12 wk, respectively (Table 2), which is consistent with previous investigations (9–13). Aspirin treatment abolished this enhancement (0.83-fold of controls), suggesting that the dose used in this study was sufficient to correct the overproduction of PGE2 in diabetic rats throughout the course. Treatment with ONO-8713 showed a slight reduction of urinary PGE2 excretion (approximately threefold of controls), but the change was not statistically significant (Table 2).

**Effects of EP1 Antagonist on Proteinuria and Renal Function**

Table 2. Urinary PGE2 excretion in control and diabetic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>4 wk (ng/d)</th>
<th>12 wk (ng/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.9 ± 0.5</td>
<td>5.5 ± 2.2</td>
</tr>
<tr>
<td>STZ</td>
<td>10.2 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.9 ± 2.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + aspirin</td>
<td>2.4 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.6 ± 1.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + ONO-8713</td>
<td>8.6 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.3 ± 4.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are expressed as the mean ± SEM. Each group has n = 4 at 4 wk and n = 6 at 12 wk.
<sup>b</sup> P < 0.01 versus nondiabetic controls.
<sup>c</sup> P < 0.01 versus STZ alone.

Table 1. Effects of an EP1 antagonist and aspirin on blood glucose, body weight, BP, and urine volume in streptozotocin (STZ)-induced diabetic rats<sup>a</sup>

<table>
<thead>
<tr>
<th>Variables</th>
<th>0 wk</th>
<th>4 wk</th>
<th>8 wk</th>
<th>12 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose (mg/dl)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>142 ± 35</td>
<td>200 ± 15</td>
<td>182 ± 3</td>
<td>176 ± 17</td>
</tr>
<tr>
<td>STZ</td>
<td>145 ± 14</td>
<td>510 ± 35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>436 ± 23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>556 ± 45&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + aspirin</td>
<td>127 ± 14</td>
<td>414 ± 30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>436 ± 37&lt;sup&gt;c&lt;/sup&gt;</td>
<td>577 ± 20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + ONO-8713</td>
<td>166 ± 36</td>
<td>520 ± 31&lt;sup&gt;c&lt;/sup&gt;</td>
<td>510 ± 58&lt;sup&gt;c&lt;/sup&gt;</td>
<td>478 ± 71&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>213 ± 4</td>
<td>463 ± 18</td>
<td>512 ± 25</td>
<td>527 ± 18</td>
</tr>
<tr>
<td>STZ</td>
<td>223 ± 3</td>
<td>324 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>333 ± 14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>374 ± 19&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + aspirin</td>
<td>214 ± 6</td>
<td>274 ± 21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>325 ± 19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>388 ± 54&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + ONO-8713</td>
<td>212 ± 3</td>
<td>288 ± 14&lt;sup&gt;c&lt;/sup&gt;</td>
<td>327 ± 18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>401 ± 40&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>121 ± 4</td>
<td>126 ± 6</td>
<td>123 ± 1</td>
<td>133 ± 5</td>
</tr>
<tr>
<td>STZ</td>
<td>123 ± 2</td>
<td>125 ± 7</td>
<td>129 ± 3</td>
<td>122 ± 2</td>
</tr>
<tr>
<td>STZ + aspirin</td>
<td>124 ± 5</td>
<td>128 ± 5</td>
<td>130 ± 9</td>
<td>130 ± 3</td>
</tr>
<tr>
<td>STZ + ONO-8713</td>
<td>117 ± 4</td>
<td>109 ± 4&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>118 ± 2</td>
<td>124 ± 4</td>
</tr>
<tr>
<td>Urine volume (ml/d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>22 ± 3</td>
<td>34 ± 6</td>
<td>18 ± 8</td>
<td>32 ± 8</td>
</tr>
<tr>
<td>STZ</td>
<td>18 ± 4</td>
<td>143 ± 36&lt;sup&gt;c&lt;/sup&gt;</td>
<td>118 ± 44&lt;sup&gt;c&lt;/sup&gt;</td>
<td>102 ± 27&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + aspirin</td>
<td>22 ± 1</td>
<td>77 ± 8&lt;sup&gt;bd&lt;/sup&gt;</td>
<td>80 ± 20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100 ± 25&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>STZ + ONO-8713</td>
<td>22 ± 4</td>
<td>113 ± 16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>148 ± 22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100 ± 20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Male Wistar rats were made diabetic using streptozotocin (STZ) (8) and treated orally with aspirin or ONO-8713, a selective EP1 antagonist (18). Values are expressed as the mean ± SE for control (n = 6), STZ (n = 10), STZ plus aspirin (n = 7), and STZ plus ONO-8713 (n = 10).
<sup>b</sup> P < 0.05.
<sup>c</sup> P < 0.02 versus nondiabetic controls.
<sup>d</sup> P < 0.05 versus STZ alone.
diabetic rats (2.2- and 4.6-fold at 4 and 8 wk, respectively, compared with nondiabetic controls) and became 7.0-fold at 12 wk ($P < 0.01$) (Figure 1A). Aspirin treatment partially attenuated urinary protein excretion (1.6-, 3.3- and 5.8-fold at 4, 8, and 12 wk, respectively), but the effects were not statistically significant. On the other hand, ONO-8713 treatment completely abrogated proteinuria, showing no significant difference from nondiabetic controls throughout the study period (1.0-, 0.8-, and 1.5-fold at 4, 8, and 12 wk, respectively) (Figure 1A). Therefore, the selective EP1 blockade with ONO-8713 is much more effective to prevent proteinuria in diabetic rats as compared with COX inhibitor treatment. Similar results were obtained when daily urinary protein excretion was compared (Figure 1B).

At 12 wk, there was no significant difference in creatinine clearance among four groups (control, 4.71 ± 0.53 ml/min; diabetic, 5.00 ± 0.41 ml/min; aspirin treatment, 5.23 ± 0.75 ml/min; ONO-8713 treatment, 5.12 ± 0.53 ml/min; $n = 6$). There was, however, a significant increase in blood urea nitrogen in diabetic rats as compared with nondiabetic controls (30.1 ± 2.3 versus 19.9 ± 1.0 mg/dl; $n = 6$; $P < 0.05$).

Interestingly, treatment with ONO-8713 significantly inhibited this increase (23.8 ± 2.2 mg/dl; $n = 6$; $P < 0.05$ versus untreated diabetic rats). Aspirin treatment also inhibited this increase (22.6 ± 1.4 mg/dl; $n = 6$; $P < 0.05$ versus untreated diabetic rats).

**EP1 Antagonist Ameliorates Renal and Glomerular Hypertrophy and Suppresses Mesangial Expansion**

The early phase of clinical and experimental diabetic nephropathy is characterized by hypertrophic changes of all renal compartments, including an increase of renal as well as glomerular size (2,27). In STZ-induced diabetic rats, renal hypertrophy was evident at 4 wk and remained significant at 12 wk, as shown by an increase in the kidney weight per body weight (3.30 ± 0.15, 5.41 ± 0.10, and 6.01 ± 0.26 mg/g at 0, 4, and 12 wk, respectively; $n = 10$) (Figure 2A). The treatment with ONO-8713 significantly attenuated the increase of kidney weight (5.07 ± 0.27 and 5.04 ± 0.52 mg/g at 4 and 12 wk, respectively; $n = 10$; $P < 0.05$ versus STZ alone) (Figure 2A). Aspirin treatment tended to have a milder effect compared with ONO-8713 treatment. Similar results were obtained when the gross kidney weights were compared (data not shown).

Microscopic examination at 12 wk after induction of diabe
tes revealed a remarkable difference in glomerular histology among the four groups (Figure 3). Untreated diabetic rats exhibited marked glomerular hypertrophy and mesangial expansion (Figure 3B), which are typical changes in diabetic nephropathy (2–4). Aspirin treatment attenuated mesangial expansion, but it failed to decrease the glomerular size (Figure 3C). In contrast, diabetic rats treated with ONO-8713 (Figure 3D) showed glomerular histology almost indistinguishable from that of nondiabetic controls (Figure 3A). To evaluate these histologic changes quantitatively, we measured the glomerular mesangial area and glomerular cross-sectional area and performed morphometric analyses (Figure 2, B and C). The data clearly demonstrate that the selective EP1 blockade markedly suppresses the glomerular injury in both parameters, whereas aspirin treatment lessens the mesangial expansion but not glomerular hypertrophy.

**EP1 Antagonist Inhibits Glomerular Upregulation of TGF-β and Fibronectin Expression**

The upregulation of TGF-β is postulated to play a pivotal role in facilitating matrix gene activation and subsequent glomerulosclerosis in diabetic glomerular injury (28,29). We therefore examined the glomerular expression of TGF-β and fibronectin genes (Figure 4). TGF-β1 and fibronectin mRNA expression in the glomeruli isolated from diabetic rats exhibited approximately threefold increases compared with nondiabetic controls at 4 wk after induction of diabetes ($P < 0.01$). Treatment with ONO-8713 and also with aspirin significantly attenuated the upregulation of TGF-β1 (1.1-fold and 1.4-fold differences in their expression).

---

*Figure 1. Urinary protein excretion of control and diabetic rats at 0, 4, 8, and 12 wk after induction of diabetes. Urinary protein levels normalized with creatinine (A) and daily protein excretion levels (B) are shown for control ($n = 6$), diabetic ($n = 10$), aspirin-treated diabetic ($n = 7$), and ONO-8713–treated diabetic rats ($n = 10$). Treatment with EP1 antagonist completely abrogated proteinuria. Values are expressed as the mean ± SE. *$P < 0.05$, **$P < 0.01$ versus control; #$P < 0.05$, ###$P < 0.01$.*
of controls, respectively; \( P < 0.01 \) versus untreated diabetic rats) and fibronectin mRNA levels (1.3-fold each of controls; \( P < 0.01 \) versus untreated diabetic rats) (Figure 4B), which were comparable to the levels in nondiabetic controls. Such inhibition by ONO-8713 was also noted at 12 wk, with similar effects by aspirin (Figure 4B). These results suggest that the ameliorated mesangial expansion in diabetic rats treated with ONO-8713 or aspirin (Figure 2B) was caused by inhibited TGF-\( \beta \) upregulation in the glomeruli.

Localization of EP1 in Rat Kidney

We next examined the localization of EP1 receptor expression in the rat kidney by in situ hybridization. Strong hybridization signals for EP1 mRNA were observed in the glomeruli as well as the tubular structures from the inner part of the cortex through the papilla (Figure 5A). Control experiments with excess of unlabeled probes gave only faint signals (Figure 5B). Microscopic examination of the glomeruli revealed significant labeling in the mesangial area (Figure 5C), and that of the outer medulla revealed intense labeling in tubular epithelial cells, presumably the collecting ducts (Figure 5D).

We further examined the gene expression of EP receptor subtypes in the isolated glomeruli of control and diabetic rats by Northern blot analyses. We found the presence of EP1 and EP4 messages but little EP2 or EP3 expression (Figure 6). High expression of the EP3 receptor in the whole kidney reflects its abundance in the tubules (15). In the glomeruli of diabetic rats, the expression of both EP1 and EP4 receptors increased significantly (EP1, 1.7-fold of controls; EP4, 1.8-fold of controls; \( n = 4 \); \( P < 0.05 \)). These findings suggest that the PGE2-EP system is activated in diabetic glomeruli.

Role of Autocrine PGE2-EP1 in Activating TGF-\( \beta \) Cascade in Mesangial Cells

We have already shown that EP1 and EP4 receptors are the predominant EP receptor subtypes expressed in cultured mesangial cells (17,20). To determine the mechanisms underlying the beneficial effects of ONO-8713 on glomerular histology and gene expression, we examined its effects on high glucose-induced gene activation in cultured mesangial cells. As shown in Figure 7A, high glucose conditions significantly augmented TGF-\( \beta 1 \) and fibronectin mRNA expression (1.8-fold of each control; \( P < 0.02 \)). Such upregulation was effectively abolished by the addition of ONO-8713 (\( P < 0.02 \)) or aspirin (\( P < 0.05 \)) (Figure 7, A and B). It is therefore suggested that PGE2 upregulates the TGF-\( \beta \)-fibronectin cascade in mesangial cells via the EP1 receptor under high-glucose conditions. Given that mesangial production of PGE2 is increased by high glucose (10,17), the present study reveals the importance of the autocrine PGE2-EP1 system in high glucose–induced matrix gene activation. Among PG-generating COX enzymes, an inducible isofrom COX-2 has been implicated in the glomeruli of diabetic nephropathy (30). We therefore next examined the gene expression of COX-2 in mesangial cells. Mesangial COX-2 expression showed a significant threefold increase in high-glucose culture (Figure 8A). Of note, this induction of COX-2 tended to be attenuated by ONO-8713 (Figure 8A). COX-1 gene expression, on the other hand, showed no significant change under high-glucose conditions. We further examined COX-2 gene expression in isolated glomeruli. We found significant COX-2 induction in diabetic rats, and such induction of COX-2 in diabetic states in vivo also appeared to be attenuated by ONO-8713 treatment (Figure 8B). Together with partial reduction in urinary PGE2 excretion by ONO-8713 treatment in diabetic rats (Table 2), these findings imply that the COX-2-PGE2-EP1 system organizes a vicious cycle in diabetic milieu and that the selective EP1 blockade disrupts this cycle.

Discussion

Previous studies have shown enhanced PG production in diabetic nephropathy (9–14), and attempts to suppress PG production by COX inhibitors resulted in partial amelioration of disease progression (11–14). We previously demonstrated that enhanced proliferation of mesangial cells under high-glucose conditions is completely abolished by selective EP1
To further explore the role of the EP1 receptor in diabetic states, we here investigated the effects of blocking the specific PGE2-EP1 pathway in vivo.

We demonstrate that oral administration of a selective EP1 antagonist ONO-8713 markedly ameliorates progression of nephropathy in a rat model of type 1 diabetes. The beneficial effects were clearly shown by attenuated renal and glomerular hypertrophy, and inhibited mesangial expansion and matrix gene activation, with complete suppression of proteinuria (Figures 1 to 4). There was no significant difference in creatinine clearance among diabetic groups, with a decreased level of blood urea nitrogen by ONO-8713 treatment. Treatment of diabetic rats with ONO-8713 did not seem to produce adverse effects during the period studied here (Table 1). These findings provide the first in vivo evidence that chronic inhibition of the PGE2-EP1 system protects the kidney from developing complications in diabetic states. Aspirin treatment, on the other hand, attenuated only mesangial expansion and matrix gene activation, without correcting glomerular hypertrophy or proteinuria. These results indicate that selective blockade of the PGE2-EP1 system is superior to inhibition of all actions of PGE2 and other prostanoids in preventing glomerular hypertrophy and proteinuria in diabetic rats. The data also importantly imply that glomerular hypertrophy and proteinuria in diabetes may occur without mesangial expansion or matrix gene activation, suggesting that these changes may be caused by different mechanisms. It has been consistently reported that anti-TGF-β antibody treatment alleviated mesangial expansion and renal insufficiency but failed to reduce proteinuria in a mouse model of diabetes (31). The importance of podocyte injury has recently been proposed in diabetic glomerular injury and proteinuria (32). Of note, EP1 and EP4 receptors are expressed in mouse podocytes in culture (33). The functional role of EP receptors in podocytes in the regulation of glomerular barrier function should await further clarification.

Factors contributing to glomerular enlargement in diabetic nephropathy include increased intraglomerular pressure, growth of glomerular cells, and accumulation of extracellular matrix (28). Considering differential effects of the EP1 antagonist and aspirin in the current study, these drugs may exert

![Figure 3](image_url) Glomerular histology of control and diabetic rats at 12 wk after induction of diabetes. Periodic acid-Schiff stain. A, Control; B, diabetes; C, diabetes plus aspirin; D, diabetes plus ONO-8713.

![Figure 4](image_url) Representative Northern blots for TGF-β1 and fibronectin (FN) mRNA expression (A) and their quantitative analysis (B) in the glomeruli isolated from control and diabetic rats at 4 and 12 wk after induction of diabetes. 1, control (n = 6); 2, diabetes (n = 6); 3, diabetes plus aspirin (n = 6); 4, diabetes plus ONO-8713 (n = 5). Values are expressed as the mean ± SE. *P < 0.01 versus control; #P < 0.05, ##P < 0.01.
different actions on glomerular hemodynamics. In this study, we revealed the expression of the EP1 message within the glomeruli, mostly in the mesangial area, by in situ hybridization (Figure 5), but its expression in renal microvessels was not apparent. Recently, it has been shown that the EP1 receptor is expressed in isolated preglomerular microvessels in rats by RT-PCR (34). Moreover, a recent report on immunohistochemical localization of EP receptors in human kidney revealed that the EP1 receptor is present in renal microvessels, including afferent and efferent arterioles (35). Therefore it is conceivable that the selective EP1 blockade affects glomerular hemodynamics, modulating intraglomerular pressure. In addition, in the microperfused rabbit glomerulus, indomethacin treatment significantly augmented angiotensin II-induced vasoconstriction of the efferent arteriole with orthograde perfusion, suggesting that PGs released from the glomerulus act to dilate the efferent arteriole (36). It is thus possible that the PGE2-EP1 system may act to inhibit glomerular hypertrophy by maintaining efferent arteriolar dilatation.

Upregulation of TGF-β plays a crucial role in the pathogenesis of mesangial matrix accumulation under diabetic milieu (28,29). Several mechanisms for this upregulation have been proposed. Among them, activation of PKC by hyperglycemia has been shown to stimulate TGF-β expression together with enhanced PGE2 production in mesangial cells (10,11,37). In the present study, both ONO-8713 and aspirin inhibited the increase of TGF-β gene expression in diabetic rat glomeruli (Figure 4). Moreover, upregulation of TGF-β expression in cultured mesangial cells under high-glucose conditions was also effectively abolished with either drug (Figure 7, A and B). These data suggest that the autocrine PGE2-EP1 system plays an important role in regulating the TGF-β–fibronectin cascade in mesangial cells. Consistent with the increased PG production (10,17), we observed augmented COX-2 expression in mesangial cells under high-glucose culture as well as in diabetic rat glomeruli, and such induction appeared to be attenuated by selective EP1 blockade (Figure 8). Our findings imply a role of the COX-2–PGE2–EP1 system in organizing a vicious cycle in diabetic states. Phorbol esters, PKC activators, have been shown to activate COX-2 expression (23); our results therefore raise a possibility that diabetic states cause mesangial COX-2 activation in a PKC-dependent pathway. EP1 receptor signals via calcium mobilization in mesangial cells (17); therefore, stimulation of this receptor potentially causes PKC activation. The pathogenic role of PKC in matrix gene activation, hemodynamic abnormalities, and proteinuria has recently been shown in STZ-induced diabetic rats using the PKC inhibitor (37,38). The link between PKC activation and the EP1 receptor is not clear at present, but it is possible that the PKC inhibitor may at least partially exert its effect by inhibiting PKC stimulated by the EP1 receptor, leading to the inhibition of TGF-β and fibronectin upregulation in diabetic glomeruli.

In summary, the present study demonstrates the treatment with a selective EP1 antagonist prevents glomerular hypertrophy, mesangial expansion, and matrix gene activation, with complete suppression of proteinuria in diabetic rats. Aspirin treatment exerted partial amelioration but did not prevent glomerular hypertrophy or proteinuria. These data indicate that activation of the PGE2-EP1 system plays a key role in the progression of diabetic renal injury. Whether this treatment represents a new therapeutic option for patients with diabetic nephropathy remains to be established in further studies.
Acknowledgments

We thank Dr. M. Haneda (Shiga University of Medical Science, Shiga, Japan) for valuable technical advice, Ms. J. Nakamura, Ms. A. Wada, and Ms. Y. Oki for technical assistance, and Ms. S. Doi and Ms. A. Sonoda for secretarial assistance. This work was supported in part by research grants from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, the Japanese Ministry of Health, Labor, and Welfare, “Research for the Future (RFTF)” of Japan Society for the Promotion of Science, Japan Foundation for Aging and Health, ONO Medical Research Foundation, Smoking Research Foundation, and the Salt Science Research Foundation.

References


Figure 6. Northern blot analyses for EP receptor subtypes in rat kidney. (A) Representative Northern blots for EP receptor subtypes in the glomeruli of control and diabetic rats at 4 wk, and in the whole kidney of control rats. (B) Quantitative analyses of EP1 and EP4 expression in the glomeruli of control (n = 4) and diabetic rats (n = 4) at 4 wk. Values are expressed as the mean ± SE. *P < 0.05 versus control.

Figure 7. Representative Northern blots for TGF-β1 and FN mRNA expression (A) and their quantitative analyses (B). 1, low glucose (5.6 mM); 2, low glucose plus 1 μM ONO-8713; 3, high glucose (25 mM); 4, high glucose plus 0.1 mg/ml aspirin; and 5, high glucose plus 1 μM ONO-8713. Values are the mean ± SE. *P < 0.02 versus low-glucose control; #P < 0.05, ##P < 0.02.

Figure 8. Representative Northern blots and quantitative analyses for COX gene expression in cultured rat mesangial cells (A) and those for COX-2 in isolated glomeruli of control and diabetic rats at 4 wk (B). (A) 1, low glucose (5.6 mM); 2, low glucose plus 1 μM ONO-8713; 3, high glucose (25 mM); 4, high glucose plus 0.1 mg/ml aspirin; 5, high glucose plus 1 μM ONO-8713. Values are the mean ± SE. n = 4; *P < 0.05, **P < 0.02 versus low-glucose control. B: 1, control (n = 4); 2, diabetes (n = 5); 3, diabetes plus ONO-8713 (n = 4). Values are the mean ± SE. *P < 0.05 versus control.