Involvement of endogenous prostaglandin E₂ in tubular epithelial regeneration through inhibition of apoptosis and epithelial-mesenchymal transition in cisplatin-induced rat renal lesions

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Summary. In the kidney, prostaglandin (PG) E₂ is the main PG, playing important roles in maintaining homeostasis or development of pathological settings. Roles of PGE₂ in renal lesions remain to be clarified. The expression patterns of PGE₂ synthesis enzymes such as cyclooxygenase (COX)-1, COX-2 and microsomal PGE synthase (mPGES)-1, and PGE₂ receptors (EP2 and EP4) were examined in cisplatin-induced rat renal failure. The immunoexpressions for COX-1, mPGES-1 and EP4 receptor were increased exclusively in the affected renal tubules, but those of COX-2 and EP2 receptor were not detected; increased expression of COX-1 was confirmed at mRNA level. Using rat renal epithelial cell line (NRK-52E), the effects of PGE₂ on cell proliferation were investigated. The addition of PGE₂ or 11-deoxy-PGE₁ (EP4 receptor agonist) to NRK-52E increased the cell number, indicating the effects of PGE₂ via EP4 receptor. Furthermore, 11-deoxy-PGE₁-treated NRK-52E cells underwent the G₀/G₁ arrest and decreased apoptosis. NRK-52E treated with transforming growth factor (TGF)-β1, an inducer of epithelial-mesenchymal transition (EMT), in the presence of 11-deoxy-PGE₁ decreased the mRNA expression of α-smooth muscle actin (a marker of myofibroblasts). Collectively, the present study shows that COX-1 plays more important roles than dose COX-2 in cisplatin-induced rat renal failure; the product, PGE₂, may regulate renal epithelial regeneration via EP4 receptor through inhibition of apoptosis and EMT.

Key words: Cisplatin, Cyclooxygenase-1, PGE₂, Rat, Renal tubular regeneration

Introduction

Prostaglandin (PG), mainly PGE₂, plays important roles in renal hemodynamics, renin release and tubular sodium/water re-absorption (Breyer and Breyer, 2000; Kotnik et al., 2005). In pathological settings such as diabetic nephropathy, PGE₂ synthesis is elevated; the increased PGE₂ may influence cell proliferation, differentiation or apoptosis (Sanchez and Moreno, 2002; Moore et al., 2005; Zhang et al., 2006). Such functions may be related to regeneration after tubular epithelial cell injury in the kidney. However, the functional roles of PGE₂ in renal failure are not fully understood (Vukicevic et al., 2006).

Cisplatin (CDDP; cis-diaminedichloroplatinum) has been widely used as an anticancer drug in human clinic, but it is well known that the chemical has nephrotoxicity as a side effect (Hanigan and Devarajan, 2003). CDDP injection to rats causes renal tubular epithelial cell injury, especially in the lower straight part (P₃ segment) of the proximal tubules in the corticomedullary junction, and, after injury, renal epithelial cells regenerate; however, when the basal lamina is completely damaged by injury, incomplete regeneration occurs (Yamate et al., 1995, 2000). Incompletely regenerating renal epithelial cells may undergo epithelial-mesenchymal transition (EMT) leading to interstitial fibrosis (Iwano et al., 2002). Cisplatin-induced renal lesions are useful for studies on the processes of renal tubular regeneration or the
pathogenesis of subsequent renal interstitial fibrosis (Yamate et al., 1995, 2000, 2005). In CDDP-induced renal lesions the expression patterns of PGE$_2$ synthesis-related enzymes remain to be investigated.

PGE$_2$ is synthesized from arachidonic acid through two-step reactions. The arachidonic acid is first converted to an unstable intermediate PGH$_2$ via the prostaglandin E synthase (PGES) (Kotnik et al., 2005; Kudo and Murakami, 2005). The constitutive COX-1 is thought to be responsible for basal PG production for homeostasis in normal conditions, whereas COX-2 expression is primarily associated with inflammatory reactions (Bondesen et al., 2004). In the kidney, however, COX-2 is expressed constitutively, and seems to regulate various physiological conditions (Harris et al., 1994; Câmpean et al., 2003). Because EP2 and EP4 receptors are related to the pathogenesis of subsequent renal interstitial fibrosis (Vukicevic et al., 2006), particularly, we focused on expressions of EP2 and EP4 receptors in association with renal fibrosis.

**Materials and methods**

**Animals and CDDP-induced renal lesions**

The following experiments were in compliance with our institutional guideline for animal care. Fifty-two 6-week-old male F344/DuCrj rats (Charles River Japan, Hino, Shiga, Japan), weighing 125-150 g, were used after a one-week acclimatization period. They were housed in an animal room controlled at 22±3°C and with 12 hours-light-dark cycle, being allowed free access to a standard commercial diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan) and tap water. CDDP (Nippon Kayaku Co. Ltd., Tokyo, Japan) was injected intraperitoneally into forty-four rats at a single dose of 6 mg/kg body weight. Four to eight animals were sacrificed each on days 1, 3, 5, 7, 9, 12 and 15 after CDDP dosing. The remaining eight rats were sacrificed on day 0, and served as controls.

**Histopathology and immunohistochemistry**

Renal tissues were fixed in 10% neutral buffered formalin and periodate-lysine-paraformaldehyde (PLP) fixatives. Formalin-fixed specimens were processed routinely and embedded in paraffin; PLP solution-fixed specimens were embedded in paraffin with the Amelx method (PLP-Amelx method) (Suzuki et al., 2000). Formalin-fixed, paraffin-embedded samples were cut at 3-4 µm thickness, and stained with hematoxylin-eosin (HE) for morphological observations.

For immunohistochemistry, specimens processed in the PLP-Amelx method were cut at 4-µm thickness, deparaffined with xylene, re-hydrated with graded ethanol, and washed in water. These sections were boiled in a micro-wave for 5 minutes for antigen retrieval, and then endogenous peroxidase was blocked using 3% H$_2$O$_2$ for 10 minutes at room temperature. Primary antibodies used were monoclonal anti-COX-1 (1:200) (Cayman Chemical Co., Ann Arbor, MI, USA), polyclonal anti-COX-2 (1:300) (Cayman Chemical), polyclonal anti-mPGES-1 (1:200) (Cayman Chemical), polyclonal anti-EP2 receptor (1:500) (Cayman Chemical.), and polyclonal anti-EP4 receptor (1:500) (Upstate Biotechnology Inc. Lake Placid, NY, USA). Tissue sections were incubated with the primary antibody overnight (12-14 hours) at 4°C. Thereafter, sections were washed three times with phosphate-buffered saline (PBS) and incubated for 45 minutes with the secondary antibody (Kit system; Histofine Simple Stain MAX PO (R) or (M); Nichirei Co., Tokyo, Japan). Positive reactions were visualized with 3,3'-diaminobenzidine (DAB). Sections were lightly counterstained with hematoxylin. Sections incubated with normal rat or mouse serum instead of the primary antibody served as negative controls.

**Real-time reverse transcriptase-polymerase chain reactions (RT-PCR)**

Total RNA was extracted from renal tissues using Trizol Reagent™ (Invitrogen Co., Carlsbad, CA, USA) and SV Total RNA Isolation System (Promega, Osaka, Japan). The RNA was reverse-transcribed to cDNA using SUPERSCRIPT First-Strand Synthesis SystemTM (Invitrogen). All PCR experiments were performed with SYBR Green Real-time PCR Master Mix (Toyobo). The amplification program consisted of 1 cycle at 95°C with a 1-minute hold followed by 45 cycles at 95°C with a 15-second hold, 65°C (62°C for COX-2) of annealing temperature with a 15-second hold, and 72°C with a 30-
second hold. Melting curve analysis to verify the accuracy of the PCR products followed amplification. The PCR-primer sequences were shown in Table 1. β-actin was used as an internal control (Tsujino et al., 1997). Value was indicated as ∆∆Ct value with comparative Ct method.

In vitro studies

Cell line and cell culture

NRK-52E cells (Dainippon-Sumitomo Pharma Co., Esaka, Japan), which were established from normal rat proximal renal tubules (Lash et al., 2002) were used. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Nichirei Co., Tokyo, Japan) as the growth medium. The cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere, and cells were serially subcultured by treatment with a mixture of 0.1% trypsin and 0.02% ethylendiaminetetraacetic acid in PBS.

Influences of PGE₂ on cellular growth of NRK-52E Cells

NRK-52E cells (1x10⁴/ml) were seeded in 24 well plates (Corning Incorporated, Costar, NY, USA) and grown for 24 hours in the growth medium. After washing twice in PBS, the growth medium was changed to 1% FBS-supplemented DMEM and then the cells were incubated for 24 hours. After the incubation, PGE₂ (1, 10 or 100 nM, diluted in ethanol; Funakoshi Co. Ltd., Tokyo, Japan), forskolin (10 µM, diluted in ethanol; Sigma-Aldrich Co. St. Louis, MO, USA), H89 (a protein kinase A (PKA) inhibitor; 1 µM, diluted in ethanol; Biomol International, Plymouth meeting, PA, USA), 11-deoxy-PGE₁ (EP4 receptor agonist; 1, 10 or 100 nM, diluted in ethanol; Cayman Chemical) (Takeuchi et al., 2003) or vehicle (ethanol control) were added in the medium. After incubation for 48 hours, cells were trypsinized and collected by centrifugation at 1,500 rpm for 5 minutes and re-suspended in 1% FBS-supplemented DMEM. Cell viability was assessed by trypan blue (0.5% in PBS) dye exclusion, and the cell number was counted using a hemocytometer.

Flow cytometry analysis of cell cycle and quantitation of apoptosis

NRK-52E cells were treated with CDDP (25 µM) with or without 11-deoxy-PGE₁ (100nM) for 24 hours (Price et al., 2006). As a control, NRK-52E cells were incubated in the absence of CDDP and 11-deoxy-PGE₁. After the treatment, the cells were washed twice with PBS, trypsinized, and suspended in PBS containing 2% FBS. Then, the cells were rinsed and centrifuged at 1,500 rpm for 5 minutes. Finally, the cells were re-suspended in PBS containing 2% FBS. Cell concentrations were adjusted to 1x10⁶ cells/ml, and 1 ml of the cell suspension was centrifuged at 1,500 rpm for 5 minutes. Supernatant (0.9 ml) was removed, and cells were re-suspended in the remaining 0.1 ml fluid. Next, 1 ml of ethanol was added and the cells were fixed at 4°C for 1 hour. After the fixation, cells were centrifuged at 1,500 rpm for 5 minutes, suspended in the propidium iodide (PI) solution (50 µg/ml) (Invitrogen) containing RNase A (0.5 mg/ml; Roche Diagnostics Co. Ltd., Tokyo, Japan), and incubated for 30 minutes at room temperature (Lash et al., 2002). Samples were analyzed using a flow cytometer (FACSCalibur: BD Biosciences, Tokyo, Japan). PI staining enables us to estimate the sub-G₀/G₁ population as apoptotic cells and the distribution of cell cycle population. The percentage of apoptotic cells was measured by estimation of the sub-G₀/G₁ peak in the PI staining (Oyaizu et al., 2001).

RT-PCR analyses for α-SMA and E-cadherin in NRK-52E cells

NRK-52E cells were incubated with transforming growth factor (TGF)-β1, a cytokine with fibrogenic properties (Fan et al., 1999; Zeisberg and Kalluri, 2004; Rhyu et al., 2005). After being seeded in 25 mm² flasks

<table>
<thead>
<tr>
<th>Name (Ref.)</th>
<th>Oligo.</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>COX-1(Cheuk et al., 2000)</td>
<td>Forward</td>
<td>5'-TGGAGAAGTGCCAGCCCAACTCCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGGGCAGGTCTTGGTGTTGAGGCA-3'</td>
</tr>
<tr>
<td>COX-2 (Guo et al., 2006)</td>
<td>Forward</td>
<td>5'-TTTGTGATGTCATCCACCAGAAGAT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACGATGTTAAGGTTACGGGAGAAG-3'</td>
</tr>
<tr>
<td>α-SMA (Morioka et al., 2004)</td>
<td>Forward</td>
<td>5'-CACGGCATCATTCCAACTCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGCGGAGCTCTTATAGAAAGG-3'</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Forward</td>
<td>5'-GAAGGTCCTAAACGCAACAGGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CGTGTACACAGGTACACACAGC-3'</td>
</tr>
<tr>
<td>β-actin (Tsujino et al., 1997)</td>
<td>Forward</td>
<td>5'-TAAAGACCTCTATGGCAACACAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TGCTTGGCTGATCCACAT-3'</td>
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coated with collagen type I (BD Biosciences, Franklin Lakes, NJ, USA), cells grown to sub-confluence in the growth medium were incubated with 1% FBS-containing DMEM for 24 hours. After the incubation, 11-deoxy-

\( \text{PGE}_2 \) (0 or 100 nM) was added to the culture in the studies was 

Immunoexpression for cyclooxygenase (COX)-1 in the cortico-

in vitro 

semiquantitatively; -, no change; ±, faintly positive for COX-1, mPGES-1 and EP4 receptor were assessed 

analysis of variance (ANOV A). The immunoreactivities 

real-time RT-PCR methods were presented as mean ± 

these immunohistochemical examinations and by the 

COX-2 was counted per 20 glomeruli. Data obtained in 

clearly evident staining; 3+, markedly positive staining. CDDP, cisplatin.

Table 2. Immunohistoexpression for cycloooxygenase (COX)-1 and mPGES-1, as well as EP2 and EP4 receptors

<table>
<thead>
<tr>
<th>Days after CDDP injection</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>2+</td>
</tr>
<tr>
<td>5</td>
<td>2+</td>
</tr>
<tr>
<td>7</td>
<td>2+</td>
</tr>
<tr>
<td>9</td>
<td>3+</td>
</tr>
<tr>
<td>12</td>
<td>3+</td>
</tr>
<tr>
<td>15</td>
<td>3+</td>
</tr>
</tbody>
</table>

The degrees for COX-1 were assessed semiquantitatively as follows: +: moderately positive staining; 2+: more clearly evident staining; 3+: marked staining. CDDP, cisplatin.

Results

CDDP-induced Renal Lesions In Vivo

Histopathological Findings

In control kidneys, no histopathological finding was seen (Fig. 1A). On day 1, the renal tubules, particularly the proximal tubules in the cortico-medullary junction, showed nuclear alterations, such as dispersed, segmented or condensed heterochromatin; some epithelial cells underwent apoptosis, demonstrable by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method (data not shown). On day 3, epithelial cells in the affected renal tubules were desquamated into tubular lumina; some cells had swollen cytoplasm and enlarged nucleus with dispersed chromatin, indicating necrosis. On day 5, renal epithelial cells with such nuclear and cytoplasmic changes were more apparent (Fig. 1B), with extension of these lesions towards the cortex. In addition to these changes, on day 7, slight dilation of the affected tubules lined by flattened or cuboidal epithelial cells with basophilic cytoplasm (indicating regenerating epithelial cells) was observed, being accompanied with infiltration of mononuclear cells around the tubules and within their lumina (Fig. 1C). On days 9, 12 (Fig. 1D) and 15, renal tubules with variously-sized lumina were seen in the cortico-medullary junction; these tubules were rimmed by flattened, polygonal or cuboidal regenerating epithelial cells; some epithelial cells had a large nucleus with irregularly dispersed chromatin. In the interstitium around the affected renal tubules, fibrotic areas consisting of spindle-shaped myofibroblastic cells and mononuclear cells began to be seen (Fig. 1D), indicating development of fibrosis.
days 3 to 7 (Fig. 3B), and it recovered to control levels on days 12 and 15 (Fig. 3C). The number of COX-2-positive cells in the macula densa also began to be significantly decreased on day 1; on day 3 onwards, the decreased number became more prominent and the number did not recover until day 15 (Fig. 3D). COX-2 immunoreexpression was not detected in the affected proximal renal tubules.

In control kidneys, an immunoreaction for mPGES-1 was seen in renal epithelial cells to various degrees (Table 3); the reactivity was faint in the proximal tubules in the cortex (±) (Fig. 4A) and was not seen in the cortico-medullary junction (-) (Fig. 4C), whereas it was stronger in the thin limbs of loop of Henle and collecting ducts in the medulla (2+) (Fig. 4E). In CDDP-injected

<table>
<thead>
<tr>
<th>Site</th>
<th>Days after CDDP injection</th>
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<tbody>
<tr>
<td>Cortex</td>
<td>0 1 3 5 7 9 12 15</td>
</tr>
<tr>
<td>Cortico-medullary junction</td>
<td>- - - - ± + + +</td>
</tr>
<tr>
<td>Medulla</td>
<td>2+ 2+ 2+ 2+ + + + +</td>
</tr>
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</table>

The degrees for mPGES-1 were assessed semiquantitatively as follows: -: negative staining; ±: faintly positive staining; +: moderately positive staining; 2+: clearly evident staining.

Fig. 1. Histopathological findings of kidneys of control (A) and cisplatin (CDDP)-injected rats (B-D). A. Cortico-medullary junction of control rat. Proximal tubules in the cortico-medullary junction show normal histology. B. Day 5 after CDDP dosing; epithelial cells in the cortico-medullary junction desquamate into tubular lumina, and some epithelial cells have a condensed hyperchromatic nucleus. C. Day 7; slight dilation of the affected renal tubules lined by flattened or cuboidal epithelial cells is observed, and desquamated epithelial cells are seen in the lumina. D. Day 12; renal tubules with variously sized lumina are seen; some epithelial cells have a large nucleus with irregular dispersed chromatin; spindle-shaped myofibroblastic cells and mononuclear cells are seen around the affected tubules. HE stain.
**Fig. 2.** Immunohistochemistry for cyclooxygenase (COX)-1 in kidneys of control (A) and CDDP-injected rat (B). A. Control; epithelial cells in the distal renal tubules in the cortex react to COX-1 (arrowheads). B. Day 12 after CDDP dosing; epithelial cells in the affected proximal renal tubules, especially in the regenerating epithelial cells in the cortico-medullary junction give a strong reaction to COX-1 (arrowheads). Immunohistochemical staining, counterstained with hematoxylin.

**Fig. 3.** Immunohistochemistry for COX-2 in kidneys of control (A) and CDDP-injected rats (B), and the kinetic of COX-2-positive cell number in the distal renal tubules (x 400 field) (C) or in macula densa (per 20 glomeruli) (D). A. Control; COX-2-positive reactions are seen in the epithelial cells of the distal renal tubules (x 400 field) (C) or in macula densa (per 20 glomeruli) (D). A. Control; COX-2-positive reactions are seen in the epithelial cells of the distal renal tubules (arrowhead) and macula densa (arrow). B. Day 5 after doing; in contrast to control, the number of cells reacting to COX-2 is smaller (arrowhead), indicating decreased number of the positive cells in CDDP-injected rats. C. The COX-2-positive cell number is significantly decreased on days 3, 5 and 7. D. The number of COX-2-positive cells in the macula densa remains significantly decreased until day 15. A and B, immunohistochemical staining, counterstained with hematoxylin. C and D, *, significantly different from day 0 (controls) at P<0.05.
Fig. 4. Immunohistochemistry for microsomal prostaglandin E synthase (mPGES)-1 in kidneys of control (A, C, E) and CDDP-injected (B, D, F) rats. A. Control; a faint positive reaction to mPGES-1 is seen in epithelial cells (arrow) of the proximal tubules in the cortex. B. Day 9 after doing; a strong reaction to mPGES-1 is seen in epithelial cells (arrow) in the proximal tubules in the cortex. C. Control; no positive reaction to mPGES-1 is seen in the cortico-medullary junction. D. Day 9; a marked reaction to mPGES-1 is seen in epithelial cells (arrow) in the affected renal tubules in the cortico-medullary junction. E. Control; there are positive reactions to mPGES-1 in the thin limbs of loop of Henle (arrow) and collecting ducts (arrowhead) in the medulla. F. Day 9; the reactivity to mPGES-1 is decreased in the thin limbs of loop of Henle (arrow) and collecting ducts (arrowhead) in the medulla, as compared with Figure 4F. Immunohistochemical staining, counterstained with hematoxylin.
rungs, the mPGES-1 reactivity in the proximal tubules in the cortex became stronger (Fig. 4B) on days 3 to 15 (+ - 2+) (Table 3); in addition, epithelial cells in the dilated proximal renal tubules in the affected cortico-medullary junction showed a clearly positive reaction to mPGES-1 on days 7 to 15 (± - +) (Fig. 4D, Table 3). The intensity of positive reaction seen in the thin limbs of loop of Henle and collecting ducts in the medulla was decreased on days 7 to 15 (+) (Fig. 4F, Table 3).

No positive reaction for EP2 receptor was seen in control kidneys (Fig. 5A). In CDDP-injected rats, epithelial cells of a few collecting ducts in the medulla or papilla showed a positive reaction for EP2 receptor (Fig. 5B), whereas no reaction was seen in the affected renal tubules of the cortico-medullary junction. In control kidneys, although epithelial cells of the distal renal tubules in the cortico-medullary junction (+) and collecting ducts in the medulla (2+) reacted to EP4 receptor (Fig. 5C, Table 4), epithelial cells of the proximal renal tubules in the cortico-medullary junction did not show a positive reaction. In CDDP-injected rats, the positive reaction to EP4 receptor began to be seen in the affected, dilated renal tubular epithelial cells on days 7 to 15 (2+) (Fig. 5D, Table 4).

mRNA Expressions of COX-1 and COX-2

COX-1 mRNA expression in CDDP-injected rats was significantly increased on days 3 and 5 (Fig. 6A). In contrast, in CDDP-injected rats, COX-2 mRNA

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**Fig. 5.** Immunohistochemistry for PGE$_2$ receptors (EP2 and EP4 receptors) in kidneys of control (A, C) and CDDP-injected (B, D) rats. A. Control; no positive reaction to EP2 receptor is seen in the medulla. B. Day 9; there are a few epithelial cells (arrowhead) reacting to EP2 receptor in the collecting ducts in the medulla. C. Control; EP4 receptor-positive cells are seen in the distal tubules in the cortico-medullary junction, whereas epithelial cells of the proximal tubules are negative to EP4 receptor (arrowheads). D. Day 7; epithelial cells in the affected, dilated renal tubules react strongly to EP4 receptor (arrowheads) in the cortico-medullary junction. Immunohistochemical staining, counterstained with hematoxylin.
expression was markedly decreased with a significant change on days 3, 5, and 15 (Fig. 6B). Expression patterns for both COX-1 and COX-2 mRNAs generally agreed with those of immunohistochemical stainings.

In vitro studies

Influences of PGE\(_2\) through Cyclic Adenosine Monophosphate (cAMP) Dependent Pathway on Cell Growth of NRK-52E Cells

The addition of PGE\(_2\) to NRK-52E cells significantly increased the cell number at 10 and 100 nM (Fig. 7A). The addition of H89, an inhibitor of PKA, to PGE\(_2\)-incubated NRK-52E cells significantly abolished the effects of PGE\(_2\) (Fig. 7B). The treatment with forskolin, which activates AC, resulted in a significantly increased number of NRK-52E cells (Fig. 7C). NRK-52E cells incubated with 11-deoxy-PGE\(_1\) (1, 10 or 100 nM), an agonist of EP4 receptor, showed a dose-dependent, significant increase in the cell number (Fig. 7D).

Effects of 11-deoxy-PGE\(_1\) on apoptosis and cell cycle of NRK-52E cells

The CDDP (25 \(\mu\)M) treatment increased apoptosis in NRK-52E cells, and significantly decreased G_0/G_1 phase population (Fig. 8A,B). These alterations induced by CDDP treatment were inhibited by the addition of 11-deoxy-PGE\(_1\) (Fig. 8A,B). There was no difference in the

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Table 4. Immunoexpression for PGE\(_2\) receptor (EP)4.

<table>
<thead>
<tr>
<th>Site</th>
<th>Days after CDDP injection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Cortico-medullary junction</td>
<td>+</td>
</tr>
<tr>
<td>Medulla</td>
<td>2+</td>
</tr>
</tbody>
</table>

The degrees for EP4 receptor were assessed semiquantitatively as follows: –, negative staining; ±, faintly positive staining; +, moderately positive staining, 2+, clearly evident staining.

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Fig. 6. mRNA expressions of COX-1 and COX-2 in control (on day 0) and CDDP-injected rats (on days 1-15) by the real-time RT-PCR method. A. Expression level of COX-1 mRNA is significantly decreased on days 3, 5, and 15. *; significantly different from day 0 (controls) at P<0.05.  

Fig. 7. Effects of PGE\(_2\) (A), H89 (B), forskolin (C) and 11-deoxy-PGE\(_1\) (D) on NRK-52E cells. A. The addition of PGE\(_2\) (0, 1, 10 or 100 nM) to NRK-52E cells significantly increases the cell number at 10 and 100 nM. B. The addition of H89 (1 \(\mu\)M), an inhibitor of protein kinase A, to NRK-52E cells in the presence and absence of PGE\(_2\) or endogenous PGE\(_2\), respectively. C. The treatment with forskolin (0 or 10 \(\mu\)M), which activates adenylate cyclase, results in significantly increased number of NRK-52E cells. D. NRK-52E cells incubated with 11-deoxy-PGE\(_1\) (0, 1, 10 or 100 nM), an agonist of EP4 receptor, show a dose-dependent, significant increase in the cell number. *, significantly different from controls (A, C, D) or PGE\(_2\) (B) at P<0.05.
cell population at S or G$_2$/M phase. There are two potential mechanisms for the increased cell number: stimulation of cell proliferation or inhibition of cell death (Aoudjit et al., 2006). The present findings suggested that the mechanism for the increased cell number in NRK-52 cells treated with 11-deoxy-PGE$_1$ might be due to the inhibition of cell death.

Effects of 11-deoxy-PGE$_1$ on the mRNA Expression of $\alpha$-SMA and E-cadherin of NRK-52E Cells Treated with TGF-ß1

TGF-ß1-treated NRK-52E cells increased $\alpha$-SMA mRNA expression and decreased E-cadherin mRNA expression (data not shown), indicating loss of epithelial phenotype and acquisition of myofibroblastic phenotype. The $\alpha$-SMA mRNA expression was significantly inhibited by the addition of 11-deoxy-PGE$_1$ (Fig. 9A), whereas E-cadherin mRNA expression was not changed in TGF-ß1-treated NRK-52E cells in the presence of 11-deoxy-PGE$_1$ (Fig. 9B).

Discussion

In control kidneys, immunoexpressions for COX-1 and COX-2 were seen in the distal renal tubules (for COX-1 and COX-2) or macula densa (for COX-2), indicating constitutive expressions of these factors in the kidney (Câmpean et al., 2003). CDDP-induced rat renal lesions were characterized by epithelial necrosis/apoptosis and subsequent regeneration, as well as interstitial fibrosis in the cortico-medullary junction; the histopathological findings in the present study were in agreement with those described previously (Yamate et al., 1995, 2000). In CDDP-induced renal lesions, COX-1-immunoreaction appeared stronger in the affected renal tubules and the degree, particularly in regenerating epithelial cells, was increased with the observation period; furthermore, along with the increased immunoreaction, increased mRNA level of COX-1 was confirmed in the early stages after injection. On the other hand, COX-2 immunoexpression seen in the distal tubules and macula densa were decreased after injection, and COX-2 mRNA expression also remained decreased until day 15. It has been reported that COX-1 deficient mice showed morphological abnormality of the renal tubule, whereas COX-2-deficient mice underwent abnormalities characterized by overabundance of immature glomeruli and dysplastic tubules (Langenbach et al., 1995; Hirose et al., 1998). COX-1 and COX-2 play important roles in the genesis of renal tubules and in the development of both glomeruli and renal tubules, respectively. Renal tubular regeneration has been considered to repeat the course of renal tubulo-genesis (Lin et al., 2005). In the CDDP-induced renal failure, increased expression of COX-1 might be related to the regeneration of the proximal renal tubules after injury.

Generally, COX-2 expression is increased in correlation with inflammatory reactions after injury and in fact, increased COX-2 expression has been reported in rat renal failure models such as 5/6 renal ablation, passive Heymann nephritis and unilateral ureteral obstruction (Blume et al., 1999; Sanchez et al., 1999; Harding, 2004). The reason why the COX-2 expression
was decreased in the present CDDP-induced rat renal failure was unknown. TGF-β1 is known to decrease COX-2 formation (Jones and Budsberg, 2000). Because TGF-β1 has been reported to be increased in fibrotic kidneys (Yamate et al., 2004), the increased expression of TGF-β1 might be related in part to the decreased expression of COX-2. As another possibility, the CDDP-specific pathophysiological conditions, in which PG production is decreased (Safirstein and Deray, 1998), may be involved in the down-regulated COX-2 expression in CDDP-treated rats. The present study at least showed that expression patterns of COX-1 and COX-2 in CDDP-induced rat renal failure were different from those in other rat renal failure models.

Arachidonic acid is converted to PGH₃ by either COX-1 or COX-2, followed by formation of PGE₂ via PGES (Kotnik et al., 2005; Kudo and Murakami, 2005). In CDDP-induced rat renal failure, immunoeexpressions for COX-1 and mPGES-2 became much greater in the affected renal tubules, whereas COX-2 immunoreaction was not detected in the affected cortico-medullary junction. These findings show that PGE₂ produced via COX-1 rather than COX-2 plays important roles in the development of CDDP-induced renal pathological changes.

Epithelial regeneration in the proximal renal tubules of the affected cortico-medullary junction and subsequent development of myofibroblasts are responsible for the progression of CDDP-induced renal failure; the myofibroblasts may be formed by the EMT (Iwano et al., 2002; Yamate et al., 2005). Epithelial cells in the affected renal tubules showed a strong reaction to EP4, but not to EP2. The dual immunohistochemistry revealed that some affected epithelial cells positive to EP4 receptor also reacted to cyclin D1 or 5'-bromo-2'-deoxyuridine (BrdU) (data not shown), indicating proliferating activity of the affected renal cells. Taken together, EP4 receptor expression might be related to regeneration of tubular epithelial cells after injury in CDDP-induced rat renal failure.

Dedifferentiation and proliferation are important aspects in the regeneration of renal tubular epithelial cells (Maeshima et al., 2003; Zeisberg and Kalluri, 2004; Lin et al., 2005). The activity of PGE₂ is carried out partly via EP4 receptor and the receptor is associated with stimulation of AC (Breyer and Breyer, 2000). The present in vitro studies indicated that the addition of PGE₂ to NRK-52E cells increased the cell number and that the increased number occurred through the cAMP-PKA pathway. The treatment of NRK-52E cells with 11-deoxy-PGE₂ showed the same effects as PGE₂, indicating that PGE₂ is capable of increasing the cell number of NRK-52E cells via EP4 receptor. These in vitro results are likely to reflect the epithelial aspects (epithelial regeneration after injury as mentioned above) seen in the affected renal tubules in CDDP-induced rat renal failure.

Flow cytometry analysis using the PI staining revealed that the addition of 11-deoxy-PGE₁ to CDDP-treated NRK-52E cells inhibited apoptosis and increased the G₀/G₁ cell population, indicating G₀/G₁ arrest. It has been considered that G₀/G₁ arrest is important to protect cells from effects of chemotherapeutic agents (Chen et al., 2000). The affected epithelial cells in CDDP-induced rat renal failure might have been arrested at the G₀/G₁ phase, which aspect might be regulated by activated PGE₂ in association with increased expressions of COX-1, mPGES-1 and EP4. Furthermore, because it has been reported that apoptosis plays an important role in the development of CDDP-induced renal failure (Zhou et al., 1999; Yamate et al., 2000; Sheikh-Hamad et al., 2004), PGE₂ might ameliorate CDDP-induced renal failure through inhibition of apoptosis.

Cells arrested at the G₀/G₁ phase are not only associated with cell protection, but also with differentiation (Massagué, 2004). The addition of 11-deoxy-PGE₁ to TGF-β₁-incubated NRK-52E cells caused the decrease in mRNA expression of α-SMA, a representative marker of myofibroblasts which might be developed by EMT (Rhyu et al., 2005). On the other hand, the mRNA expression of E-cadherin, an epithelial marker of EMT (Yamashita et al., 2005), did not alter in PGE₂-treated NRK-52E cells pre-incubated with TGF-β₁. These findings suggest that PGE₂ inhibits conversion of renal epithelial cells into myofibroblastic cells. PGE₂ produced in renal tubular epithelial lesions may be an inhibitor of EMT in which renal epithelial cells acquire a myofibroblastic nature leading to interstitial fibrosis (Zeisberg and Kalluri, 2004).

In conclusion, the present study has shown that COX-1 plays more important roles than does COX-2 in the tubular epithelial alterations in CDDP-induced rat renal failure; furthermore, PGE₂, activated by increased COX-1 and mPGES-1 expressions, affects epithelial regeneration via the up-regulated EP4 receptor in the affected renal tubules; additionally, the regulation of PGE₂ on epithelial regeneration involves the suppression of apoptosis and EMT. Collectively, it was demonstrated that endogenous PGE₂ participated in the complicated pathological processes of regeneration of tubular epithelial cells in CDDP-induced rat renal failure. Microenvironmental conditions (such as inflammatory cytokines and adhesion molecules) evoked via cell-cell or cell-matrix interaction is crucial for repair after tissue injury (Bonventre, 2003). It is interesting to pursue the relationship of these factors with PGE₂ production for the sake of explorations of therapeutic strategies for chronic renal insufficiency, because incomplete epithelial regeneration of renal tubules leads to EMT resulting in progressive interstitial fibrosis.

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PGE$_2$ in rat renal tubular regeneration

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