Decreased susceptibility to renovascular hypertension in mice lacking the prostaglandin I\textsubscript{2} receptor IP

Takayuki Fujino,¹ Naoki Nakagawa,¹ Koh-ichi Yuhki,¹ Akiyoshi Hara,¹ Takehiro Yamada,¹ Koji Takayama,¹ Shuhko Kuriyama,¹ Yayoi Hosoki,¹ Osamu Takahata,¹ Takanobu Taniguchi,² Jun Fukuzawa,³ Naoyuki Hasebe,³ Kenjiro Kikuchi,³ Shuh Narumiya,⁴ and Fumitaka Ushikubi¹

§Department of Pharmacology, ²Department of Biochemistry, and ³First Department of Internal Medicine, Asahikawa Medical College, Asahikawa, Japan.
⁴Department of Pharmacology, Faculty of Medicine, Kyoto University, Kyoto, Japan.

Persistent reduction of renal perfusion pressure induces renovascular hypertension by activating the renin-angiotensin-aldosterone system; however, the sensing mechanism remains elusive. Here we investigated the role of PGI\textsubscript{2} in renovascular hypertension in vivo, employing mice lacking the PGI\textsubscript{2} receptor (IP–/– mice). In WT mice with a two-kidney, one-clip model of renovascular hypertension, the BP was significantly elevated. The increase in BP in IP–/– mice, however, was significantly lower than that in WT mice. Similarly, the increases in plasma renin activity, renal renin mRNA, and plasma aldosterone in response to renal artery stenosis were all significantly lower in IP–/– mice than in WT mice. All these parameters were measured in mice lacking the four PGE\textsubscript{2} receptor subtypes individually, and we found that these mice had similar responses to WT mice. PGI\textsubscript{2} is produced by COX-2 and a selective inhibitor of this enzyme, SC-58125, also significantly reduced the increases in plasma renin activity and renin mRNA expression in WT mice with renal artery stenosis, but these effects were absent in IP–/– mice. When the renin-angiotensin-aldosterone system was activated by salt depletion, SC-58125 blunted the response in WT mice but not in IP–/– mice. These results indicate that PGI\textsubscript{2} derived from COX-2 plays a critical role in regulating the release of renin and consequently renovascular hypertension in vivo.

Introduction

In renovascular hypertension, the reduction of renal blood flow due to renal artery stenosis originating from obstructive vascular diseases, such as atherosclerosis or fibromuscular dysplasia, induces excessive activation of the renin-angiotensin-aldosterone (RAA) system and leads to hypertension (1). In patients and animal models of renovascular hypertension, expression of COX-2, a rate-limiting enzyme for prostanooid synthesis, has been reported to be increased in the kidneys (2, 3). In addition, production of PGE\textsubscript{2} and I\textsubscript{2} in the kidney has been reported to be increased during renovascular hypertension (4, 5), suggesting that the prostanooids play an important role in the pathogenesis of renovascular hypertension. The roles of the prostanooids in renovascular hypertension, however, have not yet been fully defined.

The RAA system plays an important role in the maintenance of vascular tone, circulating blood volume, and electrolyte balance in the body. Renin is a rate-limiting enzyme involved in the activation of the RAA system and is secreted from the granular cells of juxtaglomerular apparatus (JGA) in the kidney. It converts plasma angiotensinogen to Ang I, which is successively changed to Ang II, a powerful vasoconstrictor, by angiotensin-converting enzyme present on the epithelial cells of pulmonary vasculatures. Ang II acts on the adrenal cortex and stimulates the secretion of aldosterone, which facilitates sodium reabsorption in the kidney and expands the circulating blood volume. Thus, Ang II and aldosterone are thought to be important players in the control of BP; therefore, renin secretion is precisely regulated through two major sensing mechanisms, along with regulation by the sympathetic nervous system. One mechanism is the baroreceptor mechanism, which senses the reduction in renal perfusion pressure and increases renin secretion (6). This mechanism is thought to reside in the renal vasculature itself and to be independent of renal tubular elements, although its exact location remains unknown. The other is the macula densa mechanism, which senses the decrease in the concentration of chloride ions in glomerular filtrate at the macula densa cells and increases renin secretion (6). The macula densa cell, a differentiated tubular epithelial cell, is one of those composing the JGA. These two sensing mechanisms transmit their information to the granular cells via the respective mediators (6). The role of the prostanooids as such mediators, however, especially in vivo, remains to be determined.

It is well established that cAMP works as a second messenger of renin secretagogues, such as norepinephrine, in the granular cells of JGA and that the increase in intracellular cAMP concentration induces renin secretion (7). PGE\textsubscript{2} exerts its action through four subtype receptors, the EP\textsubscript{1}, EP\textsubscript{2}, EP\textsubscript{3}, and EP\textsubscript{4}, and PGI\textsubscript{2} acts on its receptor IP. Stimulation of the EP\textsubscript{2}, EP\textsubscript{3}, and IP increases intracellular cAMP concentration, indicating that these receptors could mediate the stimulatory signal for renin secretion. In contrast, stimulation of the EP\textsubscript{1} and EP\textsubscript{3} leads to the increase in intracellular Ca\textsuperscript{2+} concentration and the decrease in intracellular cAMP concentration, respectively (8, 9). In addition, PGE\textsubscript{2} and PGI\textsubscript{2} have been reported to stimulate renin secretion in cultured juxtaglomerular (JG) cells (10). These results suggest that PGE\textsubscript{2} and PGI\textsubscript{2} work as mediators of renin secretion acting directly on the granular cells, while their in vivo actions in the regulation of renin secretion are not known.
In the present study, we attempted to clarify the roles of PGE$_2$ and PGI$_2$ in the pathogenesis of renovascular hypertension employing a two-kidney, one-clip (2K1C) hypertension model using mice lacking the EP$_1$ (EP$_1$–/– mice), EP$_2$ (EP$_2$–/– mice), EP$_3$ (EP$_3$–/– mice), EP$_4$ (EP$_4$–/– mice), or IP (IP–/– mice).

**Results**

**Renovascular hypertension in 2K1C model.** At day 14 of 2K1C, the left kidney became atrophic and the right kidney showed compensatory hypertrophy (Figure 1A). The degree of atrophic change estimated by weights of the left kidneys was similar among WT, EP$_1$–/–, EP$_2$–/–, EP$_3$–/–, and IP–/– mice and between F2-WT and EP$_4$–/– mice (data not shown), suggesting that the clipping of the renal artery induced a similar degree of ischemic stress to the left kidney in each mouse group.

In WT and F2-WT mice, systolic BP (sBP) rose gradually and reached a plateau at day 5 of 2K1C, while there was no such increase in sBP in the sham-operated groups (Figure 1, B and C) measured by the tail-cuff method. In IP–/– mice, however, the increase in sBP was significantly lower than that observed in WT mice (Figure 1B), suggesting an important pathophysiological role of PGI$_2$ in renovascular hypertension. In contrast, there were no significant differences in the degree and time-course of hypertension in EP$_1$–/–, EP$_2$–/–, EP$_3$–/–, and EP$_4$–/– mice compared with those in their respective WT mice (Figure 1, B and C), suggesting that there is a minor role, if any, for PGE$_2$ in the present model of renovascular hypertension. When BP was measured directly through the cannula inserted into the carotid artery at day 7 of 2K1C, the increase in sBP was significantly lower in IP–/– mice compared with that in WT mice; these were 144 ± 12, 118 ± 6, and 104 ± 5 mmHg in WT, IP–/–, and sham-operated WT mice, respectively. This result confirmed the decreased susceptibility of IP–/– mice to renovascular hypertension as was observed by the tail-cuff method. The sBP in WT and F2-WT mice decreased gradually and returned to a preoperation level.

**Figure 2**

Activation of the RAA system in WT and IP–/– mice in 2K1C model. (A and B) Increases in PRA (A) and renin mRNA expression in the kidney (B) at day 7 of 2K1C. Each column represents mean ± SEM of 5–15 mice per group. In B values were expressed as a ratio of renin/β-actin mRNA of 2K1C mice to that of sham-operated mice. *P < 0.05 versus WT mice. (C) Immunohistochemical analysis of renin expression in the WT kidney in 2K1C model. The immunoreactivities of renin in the kidney from the sham-operated WT mouse were detected only in the cells of JGA (left). The immunoreactivities of renin in the kidney at day 7 of 2K1C were detected in the afferent arterioles adjacent to JGA in addition to in JG cells (right). Arrows indicate the renin immunoreactivities. Original magnification, ×400. (D) Increase in PAC at day 7 of 2K1C. Each column represents mean ± SEM of 5–15 mice per group. *P < 0.05 versus WT mice. Pre-ope, preoperation.
Figure 3
Expression of COX-2 mRNA in the WT kidney in 2K1C model. The expression of COX-2 mRNA increased significantly after the operation and reached a peak level at day 4 of 2K1C. Each column represents mean ± SEM of 5–12 mice per group. Values were expressed as a ratio of COX-2/β-actin mRNA of 2K1C mice to that of sham-operated mice.

level within 3 weeks after the operation, as the left kidney fell into severe atrophy. Although we performed the present study using female mice, the degree and time course of hypertension in male WT and IP−/− mice subjected to 2K1C were similar to those in female mice (data not shown), indicating that there was no gender difference in the present phenotype.

The basal levels of heart rate were not significantly different among WT, EP1−/−, EP2−/−, EP3−/−, and IP−/−, or between F2-WT and EP2−/− mice, and there were no significant changes in heart rate during the experiments in all groups of mice (data not shown). Plasma creatinine levels at day 14 of 2K1C were not significantly different among the mice groups (data not shown).

Activation of the RAA system in 2K1C model. To explore the mechanism whereby PGII induces hypertension in 2K1C model, we examined the role of PGII in renin secretion by examining plasma renin activity (PRA). In WT mice, PRA increased significantly at day 7 of 2K1C, while there was no such increase in sham-operated WT mice (Figure 2A). In IP−/− mice, however, the increase in PRA was significantly lower than that in WT mice, suggesting a stimulatory effect of endogenous PGII on renin secretion. In contrast, there were no significant differences in the degree of increases in PRA between WT and EP2−/− or F2-WT and EP2−/− mice (data not shown). In sham-operated WT and IP−/− mice, there were no significant increases in PRA compared with preoperation values; these were 4.3 ± 1.2 and 5.0 ± 1.6 ng Ang I/ml/h, respectively.

To determine if PGII has a stimulatory effect on renin production as well as secretion, we examined the expression of renin mRNA in the kidney. In WT mice, renal renin mRNA expression increased significantly at day 7 of 2K1C (Figure 2B). In IP−/− mice, however, the increase in the expression level was significantly lower than that in WT mice, suggesting that there is a stimulatory role of endogenous PGII in renin production. In contrast, there were no significant differences in the expression levels of renin mRNA among WT, EP1−/−, EP2−/−, EP3−/−, or between F2-WT and EP2−/− kidneys (data not shown). The immunoreactivities of renin were detected only in JG cells in WT kidney at day 7 of sham operation (Figure 2C). In the clipped WT kidney, however, the immunoreactivities were found in the vascular cells of afferent arterioles near the glomerulus in addition to JG cells, indicating that transitional cells began to produce renin after 2K1C.

In accordance with the increase in PRA, plasma aldosterone concentration (PAC) increased significantly in WT mice at day 7 of 2K1C (Figure 2D). In IP−/− mice, however, the increase in PAC was significantly lower than that in WT mice, suggesting the activation of the RAA system by endogenous PGII. In sham-operated WT and IP−/− mice, there were no significant increases in PAC compared with preoperation values; these were 19 ± 7 and 23 ± 4 pg/ml, respectively.

Expressions of COX mRNAs in the kidney and production of PGII in 2K1C model. We further examined the expression of COX, the key enzymes for prostanooid synthesis. In WT kidney, the expression level of mRNA for COX-2, an inducible isoform of COX, increased significantly after the operation of 2K1C (Figure 3), indicating that the expression level of COX-2 mRNA increased, along with the increased expression of renin mRNA and the elevation of BP. There was no significant difference in the expression levels of COX-2 mRNA between WT and IP−/− kidneys (data not shown), suggesting that a similar degree of prostanooid synthesis took place in WT and IP−/− mice after 2K1C. In contrast, the expression levels of COX-1 mRNA in the kidney did not change significantly after the operation in either WT or IP−/− mice (data not shown).

There were no significant differences in basal concentrations of 6-keto-PGF1α, a stable metabolite of PGII, in either urine or plasma between WT and IP−/− mice (Table 1). In both WT and IP−/− mice, concentrations of 6-keto-PGF1α in urine and plasma increased significantly at day 7 of 2K1C compared with the basal values. There were no significant differences in these increases between WT and IP−/− mice, indicating that the COX-PGII system was stimulated to a similar extent in WT and IP−/− mice subjected to 2K1C.

Effects of SC-58125 in the 2K1C model. To estimate the degree of participation of COX–2–derived PGII in the pathogenesis of renovascular hypertension, the effects of SC-58125 were examined. In WT mice, SC-58125 failed to affect BP in either WT or IP−/− mice, but SC-58125 significantly reduced the increases in PRA and renin mRNA expression in WT mice to levels similar to those in vehicle-treated IP−/− mice. In addition, SC-58125 had no effect on PRA or renin mRNA expression in IP−/− mice, suggesting that COX–2–derived PGII stimulated renin secretion and production. The concentrations of 6-keto-PGF1α in urine and plasma decreased significantly in both WT and IP−/− mice to a similar

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effects of SC-58125 in 2K1C model</th>
</tr>
</thead>
<tbody>
<tr>
<td>sBP (mmHg)</td>
<td>Vehicle</td>
</tr>
<tr>
<td>142 ± 6</td>
<td>129 ± 9</td>
</tr>
<tr>
<td>PRA (ng Ang I/ml/h)</td>
<td>15.5 ± 1.8</td>
</tr>
<tr>
<td>Renin mRNA</td>
<td>2.41 ± 0.25</td>
</tr>
<tr>
<td>6-keto-PGF1α</td>
<td></td>
</tr>
<tr>
<td>Urine (pg/mg creatinine)</td>
<td>5.32 ± 0.35</td>
</tr>
<tr>
<td>Plasma (pg/ml)</td>
<td>167 ± 12</td>
</tr>
</tbody>
</table>

Effects of SC-58125 on sBP, PRA, renin mRNA expression, and concentrations of 6-keto-PGF1α in urine and plasma were examined in WT and IP−/− mice at day 7 of 2K1C. Vehicle or SC-58125 (10 mg/kg/day) was injected at days 5 and 6 of 2K1C. Basal concentrations of 6-keto-PGF1α in urine were 1.72 ± 0.10 and 2.08 ± 0.10 pg/mg creatinine in WT and IP−/− mice, respectively. These values in plasma were 110 ± 4 and 112 ± 13 pg/ml, respectively. Values for renin mRNA expression were expressed as a ratio of renin/β-actin mRNA of 2K1C mice to that of sham-operated mice. Values represent mean ± SEM of 6–10 mice per group. *P < 0.05 versus vehicle-treated group.
extant under SC-58125 treatment. SC-58125 had no effect on BP or PRA in sham-operated WT and IP–/– mice (data not shown).

**Activation of the RAA system under low-salt diet with furosemide.** In WT mice subjected to a low-salt diet with furosemide (25 mg/kg, intraperitoneally) every day, PRA (A) and renin mRNA expression (B) increased significantly more than in control mice at day 7 of salt depletion. These increases were blunted significantly in IP–/– mice compared with those in WT mice. Each column represents mean ± SEM of 5–12 mice per group. Values in B were expressed as a ratio of renin/ß-actin mRNA of salt-depleted mice to that of control mice. *P < 0.05 versus WT mice.

**Table 2**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>SC-58125</th>
<th>IP–/–</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA (ng Ang 1/ml/h)</td>
<td>19.6 ± 2.2</td>
<td>10.8 ± 3.1 *</td>
<td>10.8 ± 2.4</td>
</tr>
<tr>
<td>Renin mRNA</td>
<td>1.44 ± 0.04</td>
<td>1.22 ± 0.06 *</td>
<td>1.31 ± 0.07</td>
</tr>
<tr>
<td>6-keto-PGF₁α</td>
<td>2.86 ± 0.14</td>
<td>2.46 ± 0.08 *</td>
<td>2.93 ± 0.11</td>
</tr>
<tr>
<td>Plasma (pg/ml)</td>
<td>115 ± 15</td>
<td>69 ± 10</td>
<td>120 ± 10</td>
</tr>
</tbody>
</table>

Effects of SC-58125 on PRA, renin mRNA expression, and concentrations of 6-keto-PGF₁α in urine and plasma were examined in WT and IP–/– mice at day 7 of salt depletion. Basal concentrations of 6-keto-PGF₁α in urine were 1.72 ± 0.10 and 2.08 ± 0.10 pg/mg creatinine in WT and IP–/– mice, respectively. These values in plasma were 110 ± 4 and 112 ± 13 pg/ml, respectively. Values for renin mRNA expression were expressed as a ratio of renin/ß-actin mRNA of salt-depleted mice to that of control mice. Values represent mean ± SEM of 4–10 mice per group. *P < 0.05 versus vehicle-treated group.
of the responsible prostanoids remain elusive, however. Here we showed that IP deficiency significantly suppressed the development of renovascular hypertension in vivo, demonstrating for the first time that endogenous PGJ2 plays an important role in the pathogenesis of renovascular hypertension by way of the IP. In contrast, the deficiency of EP1+/−, EP2+/−, EP4+/−, or EP2−/− did not attenuate at all the development of renovascular hypertension in the 2K1C model. This suggests that endogenous PGE2 does not participate in the pathogenesis of renovascular hypertension, at least in the present model of renovascular hypertension. There may be, however, some fixation of particular genes in EP−/− mice due to a selective bleeding as described. Therefore, it would be necessary to give care to the difference in genetic background when considering in vivo phenotype of EP−/− mice, because the factors responsible for BP response would be different according to a genetic background.

The pivotal role of the RAA system in the development of renovascular hypertension is well-established. In IP−/− mice, the increases in PRA and PAC were significantly lower compared with those in WT mice, indicating the stimulatory actions of endogenous PGJ2 on renin and succeeding aldosterone secretions. In addition, the expression level of renin mRNA was significantly elevated in the WT kidney after the operation, although the elevation was significantly blunted in IP−/− kidney, indicating that endogenous PGJ2 also stimulates renin production. These results suggest that the role of PGJ2 in the development of renovascular hypertension was mediated by activation of the RAA system.

Immunohistochemical analysis detected the renin immunoreactivities only in JG cells in the kidney from sham-operated mice. In the kidney of 2K1C, however, the renin immunoreactivities were additionally found in the afferent arterioles adjacent to the glomeruli. This expansion of renin immunoreactivities toward afferent arterioles corresponds to the fact that intermediated cells in the media of the afferent arteriole can produce renin granules upon a decrease in perfusion pressure of the kidney (6). We previously showed that the IP was expressed in smooth muscle cells of the afferent arterioles in the murine kidney (11). Taken together, these findings suggest that PGJ2 produced in the arterioles could act at the IP on intermediate cells transformed to contain renin granules as well as at the IP on JG cells.

We determined which isofrom of COXs is responsible for production of PGJ2 participating in the pathogenesis of renovascular hypertension. Expression of COX-2 mRNA, but not COX-1 mRNA, increased significantly after the operation in both WT and IP−/− kidneys to a similar degree. In addition, PRA and the expression level of renal renin mRNA in SC-58125-treated WT mice were significantly lower than those in vehicle-treated WT mice and were similar to those in vehicle-treated IP−/− mice. Moreover, SC-58125 failed to affect these parameters in IP−/− mice. These results suggest that COX-2-derived PGJ2 is responsible for the activation of the RAA system in renovascular hypertension. Nevertheless, SC-58125 failed to significantly suppress sBP. Several investigators have reported the effects of COX inhibitors in renovascular hypertension, although the results are controversial. Some reports suggested significant suppressive effects of COX-2 inhibitors on BP in renovascular hypertension (12, 13). In contrast, the absence of effects of these inhibitors on BP and renin production in the 2K1C model have been shown (14, 15). This discrepancy may be derived from the effects of COX-2 inhibitors on a variety of systemic actions of the prostanoids in the body irrespective of their potent inhibitory effect on renin secretion and production, as shown in this...
study. Otherwise, the failure of SC-58125 to suppress BP elevation in the present study may originate from its relatively moderate effect on PGI₂ production due to a mild dose protocol.

We examined whether PGI₂ also plays a role in the regulation of the RAA system through the macula densa mechanism under salt-deficient conditions. In WT mice, a low-salt diet with furosemide significantly increased PRA and renin mRNA expression, indicating an activation of the RAA system. In IP⁻/⁻ mice, however, these increases were significantly blunted, indicating that endogenous PGI₂ also participates in macula densa mechanism of renin release and production. In addition, SC-58125 significantly reduced salt deficiency–induced increases in PRA and renin mRNA expression in WT mice to a level similar to those in vehicle-treated IP⁻/⁻ mice. These inhibitory effects of SC-58125 on the RAA system, however, disappeared in IP⁻/⁻ mice, suggesting that COX-2–derived PGI₂ participates in the macula densa mechanism.

In both the 2K1C and salt restriction model, the activation of the RAA system in IP⁻/⁻ mice was significantly blunted compared with that in WT mice. The activity of the RAA system was still significantly higher in IP⁻/⁻ mice compared with that in control mice, however. This result suggests that the IP-independent mechanism through factors other than PGI₂, such as sympathetic nervous system and nitric oxide, would be involved in the activation of the RAA system in the pathogenesis of renovascular hypertension and under the condition of salt deficiency. In addition, possible contribution of other prostanoids in the regulation of the RAA system, such as PGD₂, PGE₃, and TXA₂, could not be excluded in the present study.

Finally, we examined whether PGI₂ acts directly at JG cells. In the cultured cells from WT mice, cicaprost exhibited a potent stimulatory effect on renin mRNA expression and renin activity, effects that disappeared completely in cultured cells from IP⁻/⁻ mice. In contrast, PGE₂ and selective EP agonists had no effect on renin mRNA expression and renin activity in the cultured cells from WT mice. In addition, cicaprost potently increased cAMP contents in the cultured cells in an IP-dependent manner. In accordance with this result, a stimulatory effect of PGI₂ on renin production and cAMP contents in cultured JG cells has been reported (10). These results indicate that PGI₂ is a potent stimulant of renin mRNA expression acting directly on JG cells and that the effect of PGI₂ is mediated by increased cAMP contents. In contrast to the present result, however, several reports have suggested a stimulatory effect of PGE₂ on renin mRNA expression in cultured JG cells (10, 16). Although the reason for this discrepancy is not clear, it may be derived from a species difference. In fact, a reported EC₅₀ value of PGE₂ in renin mRNA expression in cultured murine JG cells is more than two rank orders higher compared with that of PGI₂ (10), indicating a low potency of PGE₂ in mouse JG cells. Otherwise, this discrepancy may be attributed to a difference in the experimental condition.

In conclusion, COX-2–derived PGI₂ plays a critical role through the IP in regulating the release of renin and consequently renovascular hypertension in vivo. The mediatory role of PGI₂ also occurred in the macula densa mechanism of renin secretion, indicating that PGI₂ is a key molecule in the regulation of renin release.

Methods

Mice. Generation and maintenance of EP₁/⁻, EP₂/⁻, EP₁/⁻, EP₂/⁻, and IP⁻/⁻ mice have been reported (17–20). The EP₁/⁻, EP₂/⁻, EP₁/⁻, and WT control mice have a genetic background similar to C57BL/6 mice. Most EP₂/⁻ mice die postnatally as a result of patent ductus arteriosus and do not survive at all in the C57BL/6 background. Therefore, F2 progenies of surviving EP₂/⁻ mice and their WT littermates were maintained independently in the mixed genetic background of 129/Ola and C57BL/6. For the experiments using EP₁/⁻ mice, F2-WT mice having this genetic background were used as a control. All experiments, which were approved by the Ashikawa Medical College Committee on Animal Research, were performed using 10- to 20-week-old female mice.

The 2K1C procedure as a model of renovascular hypertension. The 2K1C procedure was performed according to the reported method (21) with some modification. Mice were anesthetized with ketamine (100 mg/kg, intraperitoneally) and xylazine (5 mg/kg, intraperitoneally), and were placed in a prone position under a control of body temperature. The left kidney was exposed through the posterolateral incision, and the renal artery was individualized from the renal vein and nerves over a short segment, along which a fluorocarbon thread (Siglon; Sunline Co. Ltd.) 5 mm long, with a diameter of 0.11 mm, was placed. A clip was then made by twisting 10 mm of copper wire around the renal artery and the thread, which was removed after the clipping. In a pilot experiment, the diameter of the stenosis was estimated by measuring the diameter of the clips, which were removed from mice 24 hours after the operation. Digitalized photographs of the clips captured by CCD camera were analyzed by the software program NIH image, and the diameter of the clips thus measured was 0.11 ± 0.01 mm (n = 9). Renal blood flow estimated by laser-Doppler method (ALF21; Advantec MFS Inc.) fell to a level of about 30% ± 1.6% of the preclipping value (n = 5). The procedure gave a fairly constant degree of elevation in BP, and 91% of WT mice subjected to 2K1C (n = 109) showed the increase in sBP greater than 50% of the mean increase of all WT mice. A sham procedure, which included the entire surgery with the exception of arterial clipping, was applied in control mice.

To examine the effect of SC-58125 (Cayman Chemical Co.), a selective COX-2 inhibitor (22, 23), in the 2K1C model we administered it at days 5 and 6 of 2K1C. SC-58125 dissolved in DMSO at a concentration of 20 mg/ml was injected intraperitoneally at a dose of 0.5 μg/g body weight (10 mg/kg body weight) using a microsyringe (no. 710; Hamilton Co.). The dose of DMSO had no effect on BP, PRA, and renin mRNA expression. We chose a dose protocol of SC-58125, 10 mg/kg for 2 days, because SC-58125 administered at 20 mg/kg for 2 days had an inhibitory effect on COX-1, which was estimated by the reduction of plasma level of TXB₂ (22), a COX-1–related metabolite. In addition, we found a sign of gastric damage by high doses of SC-58125, which was represented by reduction of gastric weight (23).

The incidence of minute renal infarction after the operation was estimated by examining the degree of sclerotic change in glomeruli at day 7 of 2K1C because no gross infarction was detected histologically. There was no significant difference in the percentage of sclerotic or collapsed glomeruli between WT and IP⁻/⁻ kidneys; these were 4.7% ± 1.8% (n = 10) and 5.0% ± 3.2% (n = 12), respectively. This result indicates that the tendency to thrombosis found in IP⁻/⁻ mice was not apparent in the present 2K1C model.

BP was measured by a method using tail plethysmography (Softron Co. Ltd.) as reported (24). We also measured BP directly from the carotid artery in some experiments in which the left carotid artery was cannulated with a polyurethane catheter (Instech Laboratories Inc.) at day 7 of 2K1C, and the BP was measured with a polygraph (San-Ei Instrument Co. Ltd.) after recovery from anesthesia.

Low-salt diet with furosemide. Mice were fed with a normal diet containing 0.33% NaCl or a low-salt diet containing 0.12% NaCl (Oriental Yeast Co. Ltd.) for 7 days. Mice on a low-salt diet also received an intraperitoneal injection of furosemide (25 mg/kg) everyday. At days 5 and 6 of salt depletion, 10 mg/kg of SC-58125 was injected intraperitoneally.

Measurements of PRA and PAC. PRA was measured according to the reported method (25) with some modifications. In short, blood (400 μl) was collected from the heart, and 8 μl of 0.5 M EDTA (pH 8.0) was added. After centrifugation at 1,500 g for 10 minutes, plasma was collected and stored at –80°C until use. To determine PRA, plasma (10 μl) was incubated for 1
hour at 37°C with 10 μl of plasma prepared from nephrectomized mice 36 hours after the operation plus 10 μl of phosphate buffer (50 mM, pH 6.6). The generated Ang I was measured by an enzyme immunoassay (EIA) kit (Peninsula Laboratory Inc.). Residual renin activity in plasma from nephrectomized mice was subtracted from the PRA of each sample. The PAC was measured by an EIA kit (Cayman Chemical Co.).

Examination of renin and COX mRNA expressions in the kidney. After the kidney was excised, the renal cortex was separated from the medulla along the inner stripe of outer medulla, frozen in liquid nitrogen, and stored at –80°C until use. Total RNA (2 μg), which was isolated from the renal cortex using Isogen (Nippon Gene Co. Ltd.), was reverse-transcribed as reported (26). The resulting cDNA was amplified by PCR using primer sets corresponding to the respective mRNA for renin, COX-1, COX-2, and β-actin (27, 28). The quantity of PCR product was determined by real-time PCR analysis using a Lightcycler apparatus (Idaho Technology Inc.) and DNA Master SYBR Green I (Roche Molecular Biochemicals).

Immunohistochemical analysis of renin expression in the WT kidney. After fixation by perfusion and immersion with 4% paraformaldehyde, the kidney was embedded in paraffin. Tissue sections (5 μm thick) prepared from paraffin-embedded kidneys were washed in a buffer containing 0.05% polyoxymethylene sorbitan monolaurate (Tween 20; ICN Biomedicals Inc.) and incubated with normal rabbit serum for 10 minutes at room temperature. These tissue sections were then incubated with the first Ab for renin (Swant) for 20 minutes at room temperature. After the sections were incubated with secondary Ab (Histofine; Nichirei Corp.) for 20 minutes at room temperature, the immunocomplexes were visualized using the strepto-avidin-biotin complex method (Nichirei Corp.). Each section was counterstained with hematoxylin (Wako Pure Chemical Industries Ltd.). As a negative control, we used preimmune mouse IgG (DAKO Corp.).

Measurements of 6-keto-PGFα and creatinine contents in plasma and urine. Plasma and urinary contents of 6-keto-PGFα, a stable metabolite of PGJ2, were measured by an EIA kit (Cayman Chemical Co.). Contents of creatinine in plasma and urine were measured by the method of Folin-Wu (described in ref. 29).

Isolation and culture of JG cells. JG cells were separated and cultured according to the reported method (30). The kidneys were removed, decapsulated, and minced with a surgical blade. After the minced tissue was incubated with 0.1% collagenase for 15 minutes at 37°C, it was filtered through a 100-μm nylon mesh. The filtered cells were put on 15 ml of 30% isosmotic Percoll solution (Amersham Biosciences) and centrifuged at 27,000 g for 20 minutes at 4°C. The cell layer enriched with JG cells near the surface was collected and washed (31). The cells (106) were then plated onto a collagen-coated dish (60 mm) in 5 ml of culture medium: RPMI-1640 supplemented with 2% FBS, transferrin (10 μg/ml), insulin (10 μg/ml), sodium selenite (0.67 ng/ml), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Invitrogen Corp.). The cells were cultured for 20 hours in a humidified atmosphere containing 5% CO2 at 37°C. After the culture medium was changed to a fresh one containing indomethacin (10 μM), various concentrations of cicaprost (Schering AG), an IP agonist, or PGE2 (Cayman Chemical Co.) were added, and the cells were incubated further for 20 hours. In some experiments, the cells were incubated with 1 μM of DI-004, AE1-259, AE-248, and AE1-329 (Ono Pharmaceutical Co.); these are the selective agonists for EPG, EP3, EP2, and EP1, respectively (32, 33). After the cell culture, total RNA was prepared, and RT-PCR analyses for renin mRNA expression were performed. For the examination of renin activity, the cells were disrupted by sonication and were centrifuged at 2,000 g for 5 minutes. Then the supernatant was used for the measurement of renin activity.

The JG cells were identified as the positive cells for renin immunoreactivity. We applied the prepared cells onto a glass slide optimized for tissue culture (Falcon; BD) and incubated them with an Ab for renin (Swant). After staining of the cells with hematoxylin, we counted a number of cells positive for renin immunoreactivity and determined the purity of the cells. The average content of the renin-positive cells was 26% of the total cells. The other 74% cells were mainly spindle-shaped and might represent fibroblastic or mesangial cells (31), although we could not identify the types of these cells.

Measurements of cAMP contents in the cultured cells. The isolated cells rich in JG cells (2 × 106) were plated onto a 24-well culture plate in 0.5 ml of the culture medium. After 24 hours of culture, the cells were washed twice with a serum-free medium, and 0.5 ml of RPMI-1640 containing 1 mM 3-isobutyl-1-methylxanthine and 0.1% BSA was added. After preincubation for 10 minutes at 37°C, the cells were stimulated with 1 μM of cicaprost for 30 minutes. The incubations were terminated by adding 0.5 ml of 6% perchloric acid, and the cAMP contents were measured as reported (26).

Data analysis. All data were expressed as mean plus or minus SEM. Statistical comparison of data were made with repeated two-way ANOVA followed by a Bonferroni/Dunn test for multiple comparison. Differences were considered significant if P-values were less than 0.05.

Acknowledgments

We thank K. Nakaya and T. Yokoyama for help in breeding and maintenance of mice, K. Nakamichi for experimental assistance, and Y. Takashima for secretarial assistance. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan and by the Research Grant for Cardiovascular Disease (14A-1) from the Ministry of Health and Welfare. This work was also supported by grants from Ono Pharmaceutical Co., the Smoking Research Foundation, and Hokkaido Heart Association.

Received for publication February 20, 2004, and accepted in revised form July 14, 2004.

Address correspondence to: Fumitaka Ushikubi, Department of Pharmacology, Ashikawa Medical College, Midorigaoka Higashi 2-1-1-1, Ashikawa 078-8510, Japan. Phone: 81-166-68-2362; Fax: 81-166-68-2369; E-mail: ushikubi@ashikawa-med.ac.jp.


