Prostacyclin-Deficient Mice Develop Ischemic Renal Disorders, Including Nephrosclerosis and Renal Infarction

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Background—Prostacyclin (PGI2) is a short-lived endogenous inhibitor of platelet aggregation and a potent vasodilator and regulator of the growth of vascular smooth muscle cells. To study the role of PGI2 in the vascular system in vivo, PGI2-deficient (PGID) mice were established by genetic disruption of the PGI2 synthase gene.

Methods and Results—PGI2 synthase–null mice were generated by replacing the exons of PGI2 synthase gene that encodes for the catalytic site of the enzyme with a neomycin resistance gene. In these mice, PGI2 levels in the plasma, kidneys, and lungs were reduced, whereas thromboxane and prostaglandin E2 levels became elevated. Blood pressure and the amounts of urea nitrogen and creatinine in plasma of the PGID mice were significantly higher than those of wild-type mice (P<0.05). They developed progressive morphological abnormalities in the kidneys, accompanied by atrophy, surface irregularity, fibrosis, cyst, arterial sclerosis, and hypertrophy of vessel walls. Thickening of the thoracic aortic media and adventitia were observed in aged PGID mice. Importantly, these phenotypes have not been reported in PGI2 receptor–deficient mice.

Conclusions—PGI2 deficiency resulted in the development of vascular disorders with the thickening of vascular walls and interstitial fibrosis, especially in mouse kidneys. The findings demonstrated in vivo that PGI2 is important in the homeostasis of blood vessels. Our established PGID mice are useful for studies on the initiation and development of vascular diseases, such as ischemic renal disorders with arterial sclerosis and infarction, and also for studies on the novel signaling pathway of PGI2. (Circulation. 2002;106:2397-2403.)

Key Words: prostaglandins ■ thromboxane ■ kidney ■ arteriosclerosis ■ hypertension

Prostacyclin (PGI2), a major product of the arachidonate cascade in blood vessels, plays an important role in the maintenance of homeostasis in the vascular system.1,2 PGI2 synthase (PGIS), which catalyzes conversion from prostaglandin (PG) H2 to PGI2, is expressed in various tissues, especially vascular endothelial and smooth muscle cells.3–7 PGI2 inhibits both proliferation and growth of smooth muscle cells.8,9 PGI2 production and PGIS expression are decreased in the lungs of patients with pulmonary hypertension, and lung vascular PGI2 synthesis is impaired in severe pulmonary hypertension, including primary pulmonary hypertension (PPH), a progressive and fatal disease.10,11 Continual intravenous infusion of PGI2 can decrease pulmonary resistance and pulmonary pressure, improving survival in patients with PPH.12 Furthermore, we reported that the overexpression of PGIS by gene transfer significantly reduces neointimal formation in the rat endothelial cell–denuded carotid arteries,9 and also ameliorates monocrotamine-induced pulmonary hypertension in rats and attenuated hypertrophy of the vessel wall in rat lung.13 Pulmonary PGIS overexpression in transgenic mice also protects against the development of hypoxic pulmonary hypertension.14 However, no abnormality has been reported in the proliferation or growth of vascular smooth muscle cells in G protein coupled-PGI2 receptor (IP receptor)–null mice.15 Therefore, the roles and signal transduction mechanisms of a short-lived PGI2 in the vascular system remain unclear. To determine the physiological roles of PGI2 in vivo, we performed gene-targeting analysis of PGIS. Previously, we reported on cDNA; the primary structure of human, bovine, mouse, and rat PGIS; and the structure and characterization of the human PGIS gene.3–6 PGIS belongs to a cytochrome P-450 superfamily (CYP8A1),16 and the active sites of the enzyme have been characterized by site-directed mutagenesis.17 Recently, it has been reported that repeat polymorphism of the human PGIS gene seems to be a risk factor for higher pulse pressure and is consequently a risk factor for systolic hypertension in the Japanese population.18 In the present article, we show that PGI2-deficient
mice established by knocking out the PGIS gene, in a manner different from that of IP receptor-null mice, produced progressive morphological abnormalities in the kidneys, including vascular disorders. A thickening of the arterial walls was also observed in aged PGID mice.

**Methods**

**Generation of PGIS-Null Mice**

A clone of the murine PGIS gene including exon 7 to exon 10 (lmPGIS2) was isolated from the 129SVJ murine genomic library in the Lambda FIX II vector (Stratagene, La Jolla, Calif) with murine PGIS cDNA used as the probe. The targeting vector was designed to replace the XhoI-BamHI fragment including the end of exon 7 to exon 9 with a gene conferring neomycin resistance (Figure 1A). A 2.3-kb BamHI-XbaI fragment containing a part of exon 7 was inserted into the BamHI/XbaI site between hsv-thymidine kinase and the neomycin-resistant genes of pPTN plasmid. A 6-kb BamHI fragment containing exon 10 was inserted at the XhoI and NotI site, which is upstream of the neomycin resistance gene of the resulting pPTN plasmid.

**RNA Blot Analysis**

After etherization, mouse lungs were extirpated and pulverized in liquid nitrogen. Poly(A)^+ RNA (3 μg) was subjected to RNA blot and hybridization as described previously with 32P-labeled cDNA encoding mouse PGIS used as a probe.

**Immunoblot Analysis**

Aortas from 3 mice of each genotype were pulverized in liquid nitrogen and homogenized in 3 mL of 10 mmol/L Tris-HCl (pH 7.5) containing 5 mmol/L EDTA, 5 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L PMSF, and 2 μg/mL leupeptin (buffer A), and the

(Figure 1A) as described previously. The genotypes of the mice were also determined by polymerase chain reaction (PCR), with the genomic DNA isolated from mouse tails used as a template and p3 (5'-TGGTTTTTTTGTGATGCTAGG AT-3'), p4 (5'-TTAGATT CCAAAGCTGTAGAGATGTC-3') and neo-p2 (5'-GCTACCGGTG GATGTGGAATGTGTG-3') as primers (Figure 1A). The reaction was performed in a 50-μL reaction containing 10 mmol/L Tris-HCl, pH 8.3 at 25°C, 50 mmol/L KCl, 0.1% Triton X-100, 2.5 mmol/L MgCl₂, 0.32 mmol/L of each dNTP, 10% dimethylsulfoxide, 0.2 μmol/L of p3 and p4, 0.05 μmol/L of neo-p2, and 2.5 U of recombinant Taq DNA polymerase (Toyobo). PCR was carried out under the following conditions: denaturation at 94°C for 2 minutes, 30 cycles of 94°C for 30 seconds, 50°C for 20 seconds, 72°C for 1 minute, and 72°C for 7 minutes. Primers p3 and p4 amplify an 875-bp wild-type allele fragment, and primers p3 and neo-p2 amplify a 708-bp targeted allele fragment.
micromosomal fraction was isolated. The micromoles were solubilized with 100 μL of buffer A containing 1% Triton X-100 for 1 hour at 4°C and added to 100 μL of buffer A. After centrifugation at 105,000g for 1 hour, the supernatants were stored at −80°C until use. SDS-polyacrylamide gel electrophoresis was performed under reducing conditions on a 10% polyacrylamide gel, and then the resolved proteins were transferred onto the Immobilon polyvinylidene difluoride transfer membrane (Millipore). After blocking, the membrane was incubated with a peptide antibody that had been raised against human PGIS,9,15 followed by incubation with a goat anti-rabbit antibody conjugated to horseradish peroxidase (Daco, Denmark). Immunoreactive signals were detected with an enhanced chemiluminescence kit (Amersham). The membrane was exposed to x-ray film (Fuji Photo Film Co, Ltd) for 2 minutes.

Measurement of 6-Keto PGF1α, Thromboxane B2, and PGE2 Concentrations in Plasma and Tissues
Mouse blood was collected with anticoagulant and indomethacin from the postcaval vein after etherization and the plasma was stored at −80°C. Mouse lungs and kidneys were extirpated and pulverized in liquid nitrogen. Prostanoids were extracted with ethanol from pooled plasma from 10 to 15 mice, and lungs and kidneys from 3 to 4 mice. In each of three divided extracts, H-labeled 6-keto PGF1α, thromboxane (TX) B2, or PGE2 (5000 to 10,000 cpm) (Amersham) was added for calculation of the recovery factors. Each extract was partially purified with Sep-Pak Plus C-18 cartridge (Waters), and the eluate was subjected to high-pressure liquid chromatography (μ-Pondapak C-18 column, Waters).20 The radioactive fractions were pooled, the solvent was dried under nitrogen gas, and then each prostanoid was measured with the use of enzyme immunoassay kits (Cayman Chemical Company).

Measurement of Blood Urea Nitrogen and Creatinine
Mouse blood was collected with anticoagulant from the postcaval vein after etherization. Blood urea nitrogen (BUN) and creatinine (CRE) in plasma were measured with the detection kits UN-test Wako and CRE-test Wako, respectively (Wako).

Histological and Morphometric Analyses
Kidneys were extirpated from mice after etherization and fixed in 10% formalin in phosphate-buffered saline (pH 7.4). After they had been embedded in paraffin, the sections (1 or 3 μm thick) were stained by hematoxylin and eosin, periodic acid-Shiff, periodic acid-methenamine silver (PAM), or Masson’s trichrome (MTC) staining methods. Immunohistochemical analysis was carried out as described previously9,13 with the use of rabbit antibody raised against collagen type I, type IV, or fibronectin. The thoracic aorta was extirpated after perfusion fixation with 10% formalin neutral buffer solution (pH 7.4), divided into 4 equal pieces, and embedded in the same direction in a paraffin block. The sections (3 μm thick) were stained by MTC staining. The cross-sectional areas of medial and adventitial layers and the internal diameters were measured as described previously9 with the Analytical Imaging Station (Imaging Research Inc). The average of 4 sections was used as the value for each mouse.

Beraprost Treatment of the Mice
Beraprost sodium (0.1 mg/kg body weight per day; Toray) or saline was administered to 4-week-old PGID or wild-type mice by subcutaneous injection (n=8). After 7 weeks, morphological observation of the mouse kidney was carried out as described above.

Statistical Analysis
Values are expressed as mean±SD. The significance of differences between values was examined by Student’s t test, and differences resulting in P<0.05 were regarded as significant.

Results
Establishment of PGID Mice and Metabolic Changes in PG Production
To investigate vascular diseases under the PG1 deficiency in vivo, we developed PGIS-null mice. Because we previously had determined that the essential regions involved in the enzyme activity are encoded in exons 8 and 9 with the characterization of the human PGIS gene,3,17 a targeting vector was designed to replace the region including the end of exon 7 to exon 9 with a gene that confers neomycin resistance (Figure 1A). Two mouse lines, 172 and 183, were generated from two independent targeted embryonic cell clones. The genotypes of the mice were identified by Southern blot analysis and PCR (Figure 1B and 1C). To confirm whether the homozygous mice lacked PGIS, we analyzed the expression of mRNA and enzyme protein by RNA blot and immunoblot analyses, respectively (Figure 1D and 1E). The intact-sized PGIS mRNA expressed in the lung of wild-type mice was reduced in heterozygous mice and was absent in homozygous mice. Likewise, the normal enzyme protein was undetected in the micromosomal fraction from the lungs of homozygotes. Furthermore, production of 6-keto PGF1α, the stable hydrolytic product from PG12, was not detectable in the lung of homozygotes (Figure 1F). In contrast, there was no significant difference in the 6-keto PGF1α level between wild-type and heterozygous mice. These results demonstrate that the homozygotes of mutant PGIS gene lack PG12, and these mice were named PGID (PGI2-deficient) mice. It is assumed that the change in production of other PG in vivo is caused by a defect in PGIS. We then measured the amounts of TXB2, a stable metabolite of TXA2, and PGE2 in plasma, kidney, and lung (Figure 2). The content of TXB2 increased 3- to 5-fold in these tissues and in the plasma from PGID mice. The content of PGE2 also increased in PGID mice. These changes in arachidonate metabolism are probably due to conversion of PGH2, a common substrate of PG isomers, to other PGs such as TXA2 and PGE2 instead of PG12 as a result of PGIS deficiency.

BUN and Blood CRE Levels of PGID Mice
A serious abnormality was found in the kidneys of the PGID mice (Figure 3A). This phenotype was observed in all PGID mice, though the extent of the lesions and the period of onset differed among individuals. BUN and blood CRE levels of 10-week-old PGID mice were 41.7±16.0 mg BUN/dL plasma (n=21) and 0.610±0.13 mg CRE/dL plasma (n=16), and those of wild types were 25.5±7.3 mg BUN/dL plasma (n=16) and 0.502±0.096 mg CRE/dL plasma (n=14), respectively. BUN and CRE levels of PGID mice were significantly higher than those of wild types (P<0.001 and P<0.01). In contrast BUN and CRE values of the homozygotes were 23.2±5.5 mg BUN/dL plasma (n=22) and 0.528±0.059 mg CRE/dL plasma (n=16), respectively, and were not different from wild types.
Blood Pressure of PGID Mice

The blood pressure of 16-week-old PGID mice was significantly elevated in comparison with that of wild types, although the difference was about 15 mm Hg (Table). However, the blood pressure of PGID mice until 10 weeks old was the same as that of wild types (data not shown). The heart rates were not different between wild-type and PGID mice.

Renal Morphological Changes in PGID Mice

Various morphological changes were observed in the kidneys of PGID mice, such as atrophy, surface irregularity, and cysts (Figure 3A). In the section, fibrosis and necrosis lesions were distributed from the renal medulla to renal cortex in a deltaic shape, and the boundary between unaffected areas and lesions could be clearly observed (Figure 3B). Retraction of the renal cortex layer was also observed. Fibrosis was observed along renal tubules and vessels, and higher expression of collagen type IV was detected in the lesions by immunohistochemical staining (Figure 4). The same regions were weakly stained immunohistochemically with collagen type I and fibronectin antibodies (data not shown). Enlargement of Bowman’s space and deflux of the renal tubular cells were observed in the renal cortex (Figure 3D through 3F), which may have been induced by restricted occlusion of renal tubules and small or capillary vessels caused by fibrosis. The fusion of these cavities may result in the large cysts observed (Figure 3B). In addition, narrowing and infarction through thickening of the intimal or medial smooth muscle layers were also observed in the arterioles and the interlobular, arcuate, and renal arteries (Figure 3F and 3H through 3J). These findings suggest that PG12 deficiency induces fibrosis and vascular injury in kidneys. A stable PGI2 analogue, beraprost sodium, was

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<th>Heart Rate and Blood Pressure of Wild-Type and PGID Mice</th>
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<td>Wild Type (n=11)</td>
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Heart rates and systolic, diastolic, and mean blood pressures (BPs) of 16-week-old wild-type and PGID mice were measured as described in Methods.

*P<0.05, †P<0.005, ‡P<0.001 vs wild type.
administered to 4-week-old PGID mice by subcutaneous injection for 7 weeks. No significant improvement in the renal morphological abnormalities was observed compared with saline-treated PGID mice (data not shown), although we need more detailed studies.

Thickening of Media and Adventitia of Thoracic Aorta in Aged PGID Mice

No morphological changes in aged mice were observed externally in other tissues such as lung and heart under normal conditions. To examine the possibility of arteriosclerosis, sections of the thoracic aorta containing the aortic arch from 23- to 25-week-old mice were examined. The areas of media and adventitia of the aorta from PGID mice were about 1.3-fold wider than corresponding areas in wild types (0.151 ± 0.029 mm² versus 0.118 ± 0.013 mm², *P* = 0.00095) and 2.2-fold wider than those of wild type mice (0.068 ± 0.020 mm² versus 0.031 ± 0.003 mm², *P* = 0.000011), respectively (Figure 5). Neointimal formation was not observed in the sections of the aorta, and no significant difference was observed between the internal

diameters of the aorta in either PGID mice or wild types (Figure 5C). These findings may suggest that PGI₂ deficiency induces the thickening of arterial and small blood vessel walls and that PGI₂ is important in maintaining the normal state of vascular walls. Systolic blood pressures of the PGID mice and wild types were 100.5 ± 11.7 mm Hg and 88.1 ± 6.3 mm Hg, respectively. The difference in blood pressure between both groups was 12 mm Hg and was not greater than the difference found between groups at 16 weeks (15 mm Hg, Table). The severity of renal disorders in the PGID group varied between individuals, and a definite correlation between renal and aortic morphological change and blood pressure has not yet been found. However, the possibility that high blood pressure influenced the thickening of vessel walls in PGID mice cannot be excluded.

**Discussion**

Renal disorders, including cystic fibrosis and arteriosclerosis and thickening of the small arterial and aortic walls, were developed in PGID mice through targeted disruption of the PGIS gene. The renal abnormalities of PGID mice were found to be somewhat similar to those reported in cyclooxygenase (COX)-2-deficient mice.²¹⁻²³ There were changes in the arachidonate metabolism in the PGID mice attributable to PGIS deficiency, and TXA₂ and PGE₂ production was in-
creased in comparison to wild-type mice. This increase in TXA₂ production may enhance platelet aggregation and vasoconstriction in PGID mice, which might lead to small injuries to the vascular walls being aggravated more easily. The levels of PGE₂ production were decreased in COX-2–deficient primary embryonic fibroblasts and macrophages from both COX-2– and COX-1–null mice. In contrast, basal PGE₂ production was higher in lung fibroblasts from COX-2– and COX-1–null mice compared with that of wild-type mice, and COX-1–deficient cells produced PGE₂ at a higher level than did COX-2–deficient cells after treatment with interleukin-1. The renal abnormalities found in PGID mice, however, were not observed in COX-1–null mice. Considering these results, it seems that the increase in PGE₂ in PGID mice did not cause renal abnormalities. Nevertheless, a detailed study on the effect of PGE₂ is needed, because PGE₂ receptor subtypes, EP1 to 4, are expressed in the kidney and contribute to glomerular vascular tone, vasodilation and constriction, vasodilation of the vasa recta, and the regulation of NaCl absorption via the thick ascending limb and collecting duct.

As described above, COX-2 deficiency induced renal abnormalities, some of which are similar to those of PGID mice. The kidneys of PGID mice usually develop normally in terms of size, but we occasionally found mice with only one kidney or with one that was very small and undeveloped. In these mice, however, normal areas existed in the remaining kidney, and the mice survived. These findings suggest that a low level of PGI₂ may partly participate in renal abnormalities caused either by COX-2 deficiency or by the specific COX-2 inhibitors.

IP receptor is expressed in the interlobular arteries and glomerular arterioles, but not in the juxtaglomerular cells in mouse kidneys. Interestingly, no renal abnormalities were reported in IP receptor–deficient mice. So far, the administration of beraprost did not improve the renal abnormalities of PGID mice, suggesting that the signaling pathway through the IP receptor is not significantly involved in the development of renal disorders. Recently, it has been reported that endogenously produced PGI₂ and exogenously administered carbaprostacyclin, a stable PGI₂ analogue, are ligands of PPARδ and we also found that intracellular PGI₂ produced by expressing PGIS, controls cell death by activating the endogenous PPARδ. Therefore, it is possible that another signaling pathway through a novel PGI₂ receptor such as PPARδ is involved in the development of renal abnormalities and the thickening of arterial walls.

In the present article, we demonstrated, using our established PGID mice, that PGI₂ deficiency induces vascular disorders in the kidney and aorta. Although the initiation mechanism is unclear, PGI₂-deficient vascular endothelial and smooth muscle cells might be sensitive to stresses in comparison with wild-type cells. The blood pressure of PGID mice increases with age, and this result lends support to the contention that polymorphism of the human PGIS gene, which decreases the promoter activity, seems to be a risk factor for higher pulse pressure and is consequently a risk factor for systolic hypertension in the Japanese population. The PGID mice are a useful animal model for vascular disorders, especially ischemic renal diseases such as arterial sclerosis and infarction. Furthermore, it seems that the PGID mice have the potential to be a PPH model because PGI₂ production and PGIS expression are decreased in the lungs of PPH patients. Although we did not observe any abnormality in the lungs of PGID mice under normal conditions to date, the PGID mice might be sensitive to hypoxia compared with wild-type mice and prove to be valuable when studying the mechanisms of hyperplasia of vascular walls. In addition to the vascular system, PGIS is expressed in various tissues in the reproductive, immune, and nervous systems. However, the precise functions of PGIS are still unclear, and these PGID mice will also be useful in the investigation of the novel action mechanisms of PGI₂ in these systems.

Conclusions

We have established PGI₂-deficient mice by PGIS gene targeting and have demonstrated that PGI₂ deficiency leads to the development of renal vascular disorders in mice.

Acknowledgments

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