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

Chieko Yokoyama, PhD; Tomoko Yabuki, PhD; Manabu Shimonishi, PhD; Masayuki Wada, MSc; Toshihisa Hatae, PhD; Susumu Ohkawara, PhD; Junji Takeda, MD, PhD; Taroh Kinoshita, PhD; Masaru Okabe, PhD; Tadashi Tanabe, PhD

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 monocrotaline-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

Received May 14, 2002; revision received August 6, 2002; accepted August 6, 2002.

From the Department of Pharmacology, National Cardiovascular Center Research Institute (C.Y., T.Y., M.S., M.W., T.H., S.O., T.T.), Division of Microcirculatory Kinetics (M.W., T.T.), and Department of Social and Environmental Medicine (J.T.), Graduate School of Medicine, Research Institute for Microbial Diseases (T.K.), and Genome Information Research Center (M.O.), Osaka University, Suita, Osaka, Japan. 
Correspondence to Tadashi Tanabe, PhD, Department of Pharmacology, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. E-mail tanabe@kiko.go.jp
© 2002 American Heart Association, Inc.
Circulation is available at http://www.circulationaha.org DOI: 10.1161/01.CIR.0000034733.93020.BC

2397
(PGID) 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). The targeting vector was transfected into the R1 embryonic stem cells by electroporation, and two independent clones of the targeted embryonic stem cell were selected. Chimeric mice were generated as described previously and were backcrossed 7 times with C57BL/6J (Clea Japan Inc, Japan). The mice were maintained in a specific pathogen-free facility at The National Cardiovascular Center Research Institute. For identification of the genotypes, the mouse genomic DNA was digested with EcoRI and separated by 0.7% agarose gel electrophoresis. DNA was transferred to Biodyne A nylon membrane (Pall BioSupport) and hybridized with 32P-labeled 5/H11032 or 3/H11032 specific probes (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’-TGGTTTGTGTTGATGCTAGGG AT-3’), p4 (5’-TTCAGATT CCAAGCTTGTAAGAGTC-3’) and neo-p2 (5’-GCTACCGGG TATGTTGGAATGTGTG-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.

**RNA Blot Analysis**

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

**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
micromosal fraction was isolated. The microsomes 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 105,000 g 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,9,13 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 PGF _α_1 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 PGF _α_1, thromboxane (TX) B _2_, or PGE _2_ (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 Pressure

Mice were restrained in a mouse pocket and maintained at 37°C. Heart rate, both systolic and diastolic, and mean blood pressures were measured 5 times continuously for each animal at 37°C by the tail-cuff method with a Softron BP-98A noninvasive automatic blood pressure analyzer, and the results were used as the mean.

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). The content of TXB _2_, a stable metabolite of TXA _2_, and PGE _2_ in plasma, caused by a defect in PGIS. We then measured the amounts of TXB _2_ in homozygotes. Furthermore, production of 6-keto PGF _α_1, the stable hydrolytic product from PGI _2_, was not detectable in the lung of homozygotes (Figure 1F). In contrast, there was no significant difference in the 6-keto PGF _α_1 level between wild-type and heterozygous mice. These results demonstrate that the homozygotes of mutant PGIS gene lack PGI _2_, and these mice were named PGID (PGI _2_-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 TXB _2_, a stable metabolite of TXA _2_, and PGE _2_ in plasma, kidney, and lung (Figure 2). The content of TXB _2_ increased 3- to 5-fold in these tissues and in the plasma from PGID mice. The content of PGE _2_ also increased in PGID mice. These changes in arachidonate metabolism are probably due to conversion of PGH _2_, a common substrate of PG isomerases, to other PGs such as TXA _2_ and PGE _2_ instead of PGI _2_, 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 heterozygotes 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.

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 PGI _2_ 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 microsomal fraction from the lungs of homozygotes. Furthermore, production of 6-keto PGF _α_1, the stable hydrolytic product from PGI _2_, was not detectable in the lung of homozygotes (Figure 1F). In contrast, there was no significant difference in the 6-keto PGF _α_1 level between wild-type and heterozygous mice. These results demonstrate that the homozygotes of mutant PGIS gene lack PGI _2_, and these mice were named PGID (PGI _2_-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 TXB _2_, a stable metabolite of TXA _2_, and PGE _2_ in plasma, kidney, and lung (Figure 2). The content of TXB _2_ increased 3- to 5-fold in these tissues and in the plasma from PGID mice. The content of PGE _2_ also increased in PGID mice. These changes in arachidonate metabolism are probably due to conversion of PGH _2_, a common substrate of PG isomerases, to other PGs such as TXA _2_ and PGE _2_ instead of PGI _2_, as a result of PGIS deficiency.

The renal vascular lesions of PGID mice were characterized by a significant reduction in the medullary and interstitial areas, 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.
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 defluxion 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).

Heart Rate and Blood Pressure of Wild-Type and PGID Mice

<table>
<thead>
<tr>
<th></th>
<th>Wild Type (n=11)</th>
<th>PGID (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>596.5±72.8</td>
<td>580.4±85.4</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>111.4±4.0</td>
<td>126.5±10.6‡</td>
</tr>
<tr>
<td>Mean BP, mm Hg</td>
<td>86.1±4.5</td>
<td>96.9±11.9†</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>73.5±5.7</td>
<td>80.5±10.6*</td>
</tr>
</tbody>
</table>

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. There were changes in the arachidonate metabolism in the PGID mice attributable to PGIS deficiency, and TXA₂ and PGE₂ production was in-

---

**Figure 4.** Fibrosis lesion of kidneys of PGID mice and expression of collagen type IV. The sections (3 μm thick) of kidneys from 14-week-old PGID (A, B) and wild-type (C) mice were stained with MTC (A) and immunostaining was performed with collagen type IV antibody (B, C). Scale bars represent 50 μm.

**Figure 5.** Thickening of aorta of aged PGID mice. Thoracic aortas from 23- to 25-week-old male mice were observed under the microscope (A). Scale bars represent 500 μm (upper panels) and 50 μm (lower panels). The areas of media and adventitia (B) and internal diameters (C) were compared with wild-type and PGID male mice as described in Methods. Open bar, wild type (n=15); closed bar, PGID (n=13). *P<0.001, **P<0.00005.
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.21,22,24 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-1.25 The renal abnormalities found in PGID mice, however, were not observed in COX-1–null mice.24 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 4 PGE2 receptor subtypes, EP1 to 4, are expressed in the kidney26,27 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.28

As described above, COX-2 deficiency induced renal abnormalities,21–23 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.29 Interestingly, no renal abnormalities were reported in IP receptor–deficient mice.15 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 carbasprostacyclin, a stable PGI2 analogue, are ligands of PPARδ.30,31 and we also found that intracellular PGI2 produced by expressing PGIS, controls cell death by activating the endogenous PPARδ.15 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.18 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**

This work was supported by grants from the Ministry of Health, Labour and Welfare (Japan); grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology (Japan); and a grant from the Takeda Medical Research Foundation. We thank Tamiko Sugimoto and Setsuko Bandoh for their technical assistance.

**References**

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

Chieko Yokoyama, Tomoko Yabuki, Manabu Shimonishi, Masayuki Wada, Toshihisa Hatae, Susumu Ohkawara, Junji Takeda, Taroh Kinoshita, Masaru Okabe and Tadashi Tanabe

_Circulation_. 2002;106:2397-2403
doi: 10.1161/01.CIR.0000034733.93020.BC
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/106/18/2397

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/