Heme oxygenase (HO) is the rate-limiting enzyme for the degradation of protoheme IX to biliverdin, which is a precursor of bilirubin, the terminal heme-degrading product. Three isoforms of HO have been identified: HO-1 is inducible, whereas HO-2 and HO-3 are constitutively expressed. Three isoforms of HO have been identified: HO-1 is inducible, whereas HO-2 and HO-3 are constitutively expressed. Carbon monoxide (CO) is another HO product of the HO reaction, which has well been recognized as a physiologically important vasoactive substance rather than a toxic waste product. This gaseous monoxide is necessary to maintain microvascular patency in the liver under both unstimulated and stress conditions. Inhibition of the HO/CO system prevents balloon injury–induced neointimal development and systemic induction of the system attenuates vascular tone and proliferative responses, we herein established transgenic (Tg) mouse strains that chronically overexpress HO-1 site specifically in VSMCs. The current study has provided evidence that phenotypes of these mice are characterized by increased systemic blood pressure and reduced vasodilatory responses through mechanisms involving impairment of nitric oxide (NO)–mediated activation of soluble guanylate cyclase (sGC), a major receptor sensing gaseous monoxides in the cells.
Materials and Methods
All experiments were performed in accordance with the Declaration of Helsinki and were approved by the University of Tokyo and Saitama Medical School Ethical Committee for Animal Experiments.

Transgenic Construct
An SM22-α promoter region that contains a 1.4-kb fragment of the murine SM22-α promoter including a 64-bp sequence of exon 1 was isolated from mouse genomic DNA, amplified with Pfu polymerase, and cloned into the TA cloning vector pCR TOPO (Invitrogen). The 5′ and 3′ primer we used for isolation are the identical sequences described previously with an additional XhoI and SalI as follows: 5′-AATTCTGGGATCCGACGATATCCGTTTG-3′ and 5′-AAGTCGACGCGCTCTGTCTTTGTGGACTG-3′, respectively. An entire protein-coding region of human HO-1 cDNA cloned into pH HO vector12 was cut out by digest with HindIII and inserted into the HindIII site of the pBluescript sk (−) vector of which the NotI site was converted to the XhoI site. The XhoI cassette of HO-1 cDNA and a 0.85-kb SV40-derived sequence with an intron and poly-A were cloned into the NotI and XhoI sites of the pCR TOPO vector, respectively.

Generation and Identification of Transgenic Mice
The 2.4-kb SM22-α 5′-flanking promoter region-HO-1 cDNA construct was liberated from the vector by XhoI digestion and purified by agarose gel electrophoresis and a GeneClean kit (BIO101). Purified DNA was dissolved in 5 mmol/L Tris (pH 7.4) plus 0.1 mmol/L EDTA before pronuclear injection. Final injection concentration was calculated as 500 copies per pL. Donor eggs were prepared from B6C3 F1 mice. Microinjected eggs were then transferred into the oviducts of pseudopregnant ICR foster mothers and allowed to develop to term. Founder mice were identified by Southern blot analysis of tail DNA. For Southern blot analysis, a 300-bp fragment of human HO-1 cDNA digested with HindIII sites of the pCR TOPO vector, respectively.

RNA Analysis
Total tissue RNA was prepared from aorta and heart by guanidinium isothiocyanate extraction as previously reported.13 Fifteen micrograms of total RNA loaded in each lane was fractionated by formaldehyde gel electrophoresis and transferred onto nitrocellulose membranes. The filters were hybridized with cDNA probes specific for rat HO-1 and HO-2.14 The mouse and human SM22-α genes were amplified by the GeneAmp RNA PCR kit, and the primers used to amplify a murine SM22-α promoter region were identical to the primers we used for isolation.

Determination of Heme Oxygenase Activity
Heme oxygenase activity in microsomes was determined in aorta, femoral artery, and heart (left ventricle) from Tg mice as compared with that in age-matched nontransgenic (Ntg) littermates.18 The activity was expressed as nmol bilirubin formed per hour per mg of protein. The protein concentration was determined by a dye binding assay (Bio-Rad).

Immunohistochemistry
HO-1 immunoactivity was detected in aorta using rabbit polyclonal antibody against human HO-1 (1:1000 dilution) purchased from StressGen. Briefly, sections were trypsinized with 1% vol/vol trypsin in PBS at 37°C for 60 minutes and washed with PBS. Non-specific protein binding was blocked with 0.1% vol/vol horse normal serum at room temperature for 30 minutes. The sections were then incubated overnight at 4°C with the primary antibody to visualize immunoreactivities.7

Western Blot Analysis
Frozen tissue was homogenized in lysis buffer (containing, in mmol/L, NaCl 150, Tris [pH 7.5] 30, and PMSF 1; 0.25 mol/L sucrose; 5 μg/mL leupeptin; and 1.9 μg/mL aprotinin). Protein concentrations were determined with a dye binding assay (Bio-Rad) using BSA as a standard. Total tissue protein (50 μg) was prepared in sample buffer for Western blot analysis.9 Polyclonal antibodies against endothelial NO synthase (eNOS) and sGC were purchased from StressGen and Calbiochem, respectively.

Measurements of Blood Pressure
Male Tg mice (6 to 14 weeks old) and their Ntg littermates used in the current study were obtained from heterozygote intercrosses. Systolic blood pressure (SBP) and heart rate were measured both directly and indirectly. For indirect measurements, SBP and heart rate were measured without anesthesia using a programmable sphygmomanometer connected with cuff probe for mouse (MCP-1, Softon) as described previously.17 For direct measurements, we anesthetized mice with an intraperitoneal injection of pentobarbital sodium at 0.08 mg/g body weight and cannulated their right femoral artery with polyethylene tubing. After mice were allowed to recover, blood pressure was recorded continuously with the mice conscious and unrestrained as described elsewhere.18 When necessary, sodium nitroprusside (SNP) was administered through the femoral vein catheter.

Determination of NOX− and NOx− (NOx) Concentrations in Urine
Mice were placed in individual metabolic cages for 24 hours for collecting urine, and concentrations of NOx were measured with a commercial kit (Cayman) before and after treatment with N-nitro-L-arginine (L-NNA) for 2 weeks.7

Ex Vivo Studies of Aortic Tone
The descending aorta was carefully removed and a cylindrical segment (3 mm long) was excised from aorta. The rings were equilibrated in an organ bath for 30 minutes under a resting tension of 0.3 g in carbogenated (95% O2/5% CO2) Krebs bicarbonate solution, pH 7.4 (containing, in mmol/L, NaCl 120, KCl 5.2, CaCl2 2.4, MgSO4·7H2O 1.2, NaHCO3 25, Na2EDTA 0.03, and dextrose [pH 7.4] 11). Subsequently, rings were contracted with phenylephrine (1 μmol/L), and the relaxant responses to a cumulative dose of acetylcholine (Ach); SNP; or 3-(5-furyl)-1-(2-thienyl)pentane-1,4-dinitrosoxide (YC-1), an allosteric reagent for sGC that activates the enzyme through NO-independent mechanisms,19 were assessed. The relaxation responses were expressed as mean+SEM of percent-age values showing reversal of the phenylephrine-induced contraction responses.

Determination of cGMP Content
Concentrations of cGMP were measured in extracts of aorta using a commercial ELISA kit (Amersham). Aortic rings were incubated for 15 minutes with vehicle, S-nitrosothioluate (GSNO; 50 μmol/L); SNP (300 nmol/L and 10 μmol/L); tin protoporphyrin IX (SnPP; 10 μmol/L and 1 mmol/L), an HO inhibitor; or YC-1 (30 μmol/L). At the end of experiments, samples were immediately frozen in liquid nitrogen and stored at −80°C until the cGMP assay was performed.15

Assessment of sGC Activity in Aortic Protein Extracts
After preparation of the aorta, samples were incubated in Krebs buffer (37°C in 5% CO2) in the presence or absence of the indicated reagents. After 15 minutes, the aorta was shock frozen, homogenized in liquid nitrogen, and dissolved in 200 mL of ice-cold lysis buffer (containing, in mmol/L, Tris-HCl 20, sucrose 0.25, EDTA 200, DTT
Reagents
All reagents were purchased from Sigma, unless otherwise specified. L-NNA and amlodipine were dissolved in drinking water to give a final dose of 60 and 5 mg/kg per day for 2 weeks, respectively. Clotrimazole (80 mg/kg body weight, IP), a reagent that blocks cytochrome P450 monooxygenases, was suspended in corn oil for administration once every 24 hours. SNP was dissolved with saline and administered intravenously at desired concentrations. When necessary, aortic rings were incubated with SnPP (Porphyrin Products) or L-NNA as described previously.

Data Analysis
Results are expressed as mean±SEM. Unless otherwise stated, each of the studies was performed in a minimum of 10 to 14 mice. Statistical significance was assessed by one-way ANOVA combined with the Fisher multiple comparison test. *P<0.05 was considered statistically significant.

Results
Establishment of the HO-1 Tg Mice
The Tg mice in the current study overexpressed HO-1 under the control of SM22-α promoter. The construct was shown in Figure 1A, and the restriction fragment comprising promoter and HO-1 cDNA was purified and used for microinjection. Microinjection of the transgenic construct into fertilized mouse eggs gave rise to 28 live-born offspring. Among them, 8 were founders carrying the transgene; the founders were identified by Southern blot analysis of genomic DNA. The copy number of the transgene incorporated into the germline varied from one to nine as estimated by slot hybridization. Three independent lines with the highest copy numbers (≈7

Figure 1. A, Generation of HO-1 transgene. cDNA insert (shaded) from an HO-1 cDNA was placed downstream of the SM22-α promoter (black). XhoI/SalI site was used for microinjection to generate HO-1 Tg mice. Poly A fragment is shown in white. B, Representative Northern blot analyses of HO-1 and HO-2 in aorta and heart of Tg mice (upper half). Blots were probed consecutively with HO-1 (top panels), HO-2 (middle panels), and β-actin, used as control for loading (bottom panels). Lower half, Densitometry of relative mRNA levels from 10 animals for each group. Column 1, Ntg aorta; column 2, Tg aorta; column 3, Ntg heart; column 4, Tg heart. ***P<0.001 vs corresponding Ntg. C, Upper panels, Representative Western blot analyses of HO-1 protein. Mr indicates molecular marker (kDa). Lower panel, Mean protein levels relative to Ntg control measured from 10 animals for each group. Column 1, Ntg aorta; column 2, Tg aorta; column 3, Ntg heart; column 4, Tg heart. **P<0.01 vs corresponding Ntg. D, Differences in heme oxygenase activities in aorta, femoral artery, and heart between Tg and Ntg mice. Values are mean±SEM for 10 to 14 animals in each group. E, Localization of HO-1 protein in aorta. Detection of HO-1 protein of Tg (panels a and c) and Ntg (panels b and d) was performed by immunostaining (scale, ×80 in panels a and b, ×400 in panels c and d).
to 9) were selected for further analysis and characterization as described below. Figure 1B showed Northern blot analysis of HO mRNA in aorta and heart from Ntg and Tg mice. The HO-1 gene in aorta from Tg mice expressed 5- to 6-fold greater than that from Ntg without any changes in HO-2 expression, whereas there were no notable changes in message levels in the heart between Ntg and Tg mice. This correlated with a 4- to 5-fold induction of the HO-1 protein as judged by Western blot analysis in aorta from Tg mice (Figure 1C) and coincided with an increase in the HO activity in vessels from Tg as compared with Ntg (Figure 1D). Immunohistochemistry revealed that the HO-1 immunoreactivities were evident in the medial smooth muscle layer of aorta from Tg mice, whereas there were few if any in the Ntg aorta from Ntg (Figure 1E).

**HO-1 Tg Mice Exhibit High Blood Pressure**
The Tg mice showed no changes in appearance and no changes in body weight at 6 weeks of age (20.0±1.2 g [Tg] versus 20.1±1.1 g [Ntg]). SBP of 6-, 10- and 14-week-old mouse was measured both indirectly and directly. SBP of Tg mice as measured by tail cuff was significantly higher than that of Ntg littermates, and this result was reproducible when SBP was measured directly at all indicated time periods (indirect and direct measurements, respectively, were 118±6 and 116±6 mm Hg [Tg] versus 94±4 and 92±3 mm Hg [Ntg] at 6 weeks; 120±4 and 118±3 mm Hg [Tg] versus 94±3 and 92±4 mm Hg [Ntg] at 10 weeks; and 118±4 and 116±3 mm Hg [Tg] versus 94±5 and 92±4 mm Hg [Ntg] at 14 weeks; *P*<0.01). However, no significant difference in heart rate was observed at all indicated periods (data not shown). We therefore analyzed the parameter using 6-week-old mice for further analyses of contractile function of aorta.

**Responses of Blood Pressure to L-NNA and SNP Are Reduced in Tg Mouse**
Two-week treatment with amloidipine normalized blood pressure in Tg mice to the levels of Ntg mice (before, 116±4 mm Hg; after, 94±3 mm Hg). Treatment with clotrimazole caused a slight decrease in blood pressure of Ntg and Tg mice, but with no statistical significance as compared with the Ntg group (Figure 2A). L-NNA administration increased blood pressure specifically in Ntg but not in Tg mice. As a result of the 1- and 2-week treatment, difference in blood pressure between the two groups was canceled out (Figure 2A). Intravenous administration of SNP (24 to 192 nmol/kg) reduced SBP in Ntg mice, but the response of the decreased pressure was significantly blunted in Tg mice. As seen in Figure 2B, the reduction of SBP in Ntg mice peaked as early as 30 seconds after injection and lasted within 5 minutes at all doses, whereas that in Tg mice was markedly suppressed and did not last more than 3 minutes. These results suggest that nitrovasodilatory responses were significantly reduced by the VSMC-directed overexpression of HO-1.

**Increased NO Generation and Overexpression of eNOS in Aorta and Kidney of Tg Mouse**
To examine whether reduced production of NO could result in the increased blood pressure of Tg mice, we measured urinary excretions of NOx before and after the 2-week treatment with L-NNA. As seen in Figure 3A, the basal urinary excretion of NOx from Tg mice was significantly greater than that of Ntg mice. This event coincided with the increased expression of eNOS protein in aorta and kidney as judged by Western blot analysis (Figure 3B). At 14 days after L-NNA treatment, urinary NOx levels were not significantly different between both strains (Figure 3A).

**Desensitization of NO-Mediated Vasorelaxation in Tg Mice**
To examine mechanisms for impairment of nitrovasodilatory response of Tg mice, endothelium-dependent and endothelium-independent relaxation of aortic rings precontracted with phenylephrine were tested. EC<sub>50</sub>, which indicates the negative logarithm of 50% relaxation of the aortic ring segment for Ach and SNP, was significantly greater in Tg as compared with Ntg mice (6.21±0.03 [Ach] and 6.84±0.03 [SNP] in Tg mice versus 5.9 ±0.02 [Ach] and 5.99±0.2 [SNP] in Ntg mice; n=10; *P*<0.01). In contrast, the EC<sub>50</sub> for YC-1 was not significantly different between Tg and Ntg mice (6.18±0.05 in Tg and 6.02±0.04 in Ntg mice; n=10; *P*>0.05) (Figure 4). These results suggest that the impaired vasodilator response of Tg results from reduced sensitivity of sGC to NO rather than from decreased bioavailability of sGC by itself.

**Reduction of cGMP Contents in Aorta of Tg Mice**
We found that basal contents of cGMP in aorta from Tg mice were significantly reduced as compared with those measured...
in Ntg. Treatment with L-NNA decreased cGMP contents specifically in Ntg mice, but not in Tg mice. As a result, cGMP contents in Tg mice were significantly greater than those in Ntg mice. Treatment with SnPP dose-dependently increased aortic cGMP contents in Tg to levels similar to those observed in control Ntg mice. Treatment with GSNO or SNP did not cause a notable increase in aortic cGMP contents in either control Tg or amlodipine-treated Tg mice as compared with that observed in Ntg mice (Figure 5B). However, blockade of the HO reaction by SnPP significantly and dose-dependently restored the SNP-induced cGMP elevation in the Tg aorta, although L-NNA did not improve it. On the other hand, treatment with YC-1 resulted in a marked increase in cGMP levels in Tg, which was comparable with that in Ntg (Figure 5B). These results suggest that contents of functionally intact sGC were unchanged in Tg, whereas the sensitivity of the enzyme to NO was impaired through mechanisms involving the catalytic activities of HO.

SnPP Restored the Reduced sGC Activity in Aorta From Tg Mice

The basal and SNP-stimulated sGC activities in aorta from Tg mice were significantly reduced as compared with Ntg mice (Figure 6A), although amounts of sGC expressed in situ were almost identical between Tg and Ntg mice, as judged by RT-PCR and Western blot analysis (Figure 6B). SnPP dose-dependently restored the SNP-induced increase in the sGC activity in aorta from Tg mice. Incubation with catalase (500 U/mL) or superoxide dismutase (100 U/mL) did not improve SNP-induced increase in the sGC activity (Figure 6A).

Discussion

In this study, we have established Tg mice that overexpress HO-1 specifically in VSMCs. To overexpress the exogenous human HO-1 gene in a VSMC-selective manner, we have used the mouse 1.4-kb SM22-α promoter region. SM22-α is known to be a VSMC-specific protein, and its expression in adult is restricted to smooth muscle–containing tissues, whereas in the early stages of development, it is also present in skeletal and cardiac muscle tissues. Experiments using transgenic mice harboring SM22-α-lacZ transgenes revealed that transgene expression was changed in a time course
similar to those of endogenous SM22-α. The Tg mice herein established turned out to express markedly HO-1 gene and protein in vessels with 3-fold increase in its catalytic activity. Immunohistochemistry revealed that intensive HO-1 immunoreactivities occur in VSMCs, but not in endothelial cells, indicating that SM22-α promoter used in the current study worked successfully. These findings indicate that Tg mice overexpress HO-1 in a VSMC-specific manner concurrently with an actual increase in the catalytic activity.

Observation that the site-specific HO-1 gene transfer into VSMCs reduces nitrosodiatory responses led us to shed light on a novel biological effect of CO on the NO-mediated vasorelaxing mechanisms. Vasoactive properties of CO derived from HO have been shown to be ascribable to its ability to relax smooth muscle cells by activating sGC; this enzyme contains a prosthetic heme that acts as the receptor not only for NO but also for CO. However, previous investigation in vitro showed that NO and CO activate the cyclase by distinct mechanisms.24 NO binds to the heme and proceeds to break the proximal histidine-iron bond, forming a 5-coordinate nitrosyl heme complex that is thought to result in conformational changes and a 100-fold increase in cGMP generation with its excess amounts. By contrast, CO, while binding to the heme of the cyclase with high affinity, forms a 6-coordinated heme complex with the histidine-iron bond remaining intact.24 The potency of CO is thus far less than that of NO. Considering such a discrepancy of the cyclase-activating properties between the gases, the current results led us to hypothesize that CO endogenously generated from VSMCs could modulate the cyclase activities through competing with NO released from endothelial cells. Such a hypothesis is supported by the observation that vasorelaxation mediated by NO was significantly suppressed, whereas the expression of the cyclase seems unchanged in the HO-1 Tg mice. Another possible mechanism by which HO-1 overexpressed in VSMCs counteracts NO-mediated vasorelaxation is involvement of accelerated degradation of heme required for the cyclase in VSMCs. However, this scenario appears implausible, because the VSMCs were able to up-regulate sGC activities normally in response to YC-1, the reagent sensitizing the enzyme to endogenous CO. In other words, sGC expressed in the arterial system of Tg mice appears to be functionally intact, and the CO-mediated impairment of vasorelaxation is an NO-dependent event. CO overproduced through the HO reaction has recently been shown to alter cytochrome P450–mediated synthesis of vasoactive prostanooids and could also explain the alterations in systemic blood pressure. This possibility is also unlikely, inasmuch as the treatment with the enzyme inhibitor clotrimazole exhibited no effects, at least in the current study. These facts led us to hypothesize that CO competes with NO on sGC and may function as a partial agonist for the enzyme; this gaseous monoxide stimulates the enzyme when NO levels are low (eg, in liver microcirculation), whereas it inhibits the enzyme when NO levels are sufficient. Such a possibility was previously discussed elsewhere and is supported by the present results showing differences in aortic cGMP contents between Tg and Ntg mice; mechanisms for a decrease in aortic cGMP contents in Tg mice could be ascribable to a partial blockade of NO through CO overproduction. Tg mice did not greatly alter the cGMP contents on administration of the NO synthase (NOS) inhibitor, because endogenously overproduced CO restores the cyclase activity. On the other hand, the treatment of the same inhibitor reduced the cGMP contents markedly in Ntg mice because of a relative shortage of CO. Furthermore, in vitro blockade of HO by the enzyme inhibitor restores the cGMP contents in Tg mice, because CO-mediated blockade of NOS is canceled.

Although previous studies using disease models have not fully addressed cellular components responsible for the HO-1 induction, microvascular endothelial cells have been shown to induce HO-1 on inflammatory stimuli and could alter adhesivity of inflammatory cells. On the other hand, atherosclerosis has been shown to account for such a disease condition that causes the HO-1 induction in cells of the arterial wall. It has also been shown that VSMCs constitute a major site for the HO-1 induction in the model of arterial balloon injury. These findings indicate that the
cellular site responsible for the enzyme induction and subsequent biological effects on vascular functions could vary among different disease models. Because of such a multiplicity and heterogeneity of the HO-1 induction, it has been difficult to address precisely the consequences of HO-1 overexpression on functions of vascular systems in vivo. Thus, previous methods to induce or overexpress HO-1 through pharmacological interventions or nonspecific gene transfer into tissues have not allowed us to examine consequent outcomes of the enzyme induction.

In this respect, the current genetic engineering approach provided evidence for HO-1–mediated functional remodeling of NO-mediated regulation of the arteriolar tone that is triggered primarily through VSMCs. It should be noted that eNOS is upregulated concurrently with elevated urinary excretions in Tg mice, although mechanisms for this event are unknown. The induction of HO-1 has been known to alter local NO generation through multiple mechanisms, as follows: competitive consumption of NADPH between the two enzyme systems, degradation of the prosthetic heme for NOS, and CO binding to the heme. However, all of these mechanisms did not explain upregulation of endothelium-derived NO generation in the Tg mice. Considering that local wall shear stress plays a crucial role in the expression of eNOS, it is not unreasonable to speculate that the elevated NO generation in Tg mice could result from reduced sensitivity of sGC to endothelial NO and subsequent impairment of vasodilation that causes in turn the elevated shear force in the arterial endothelium. Such interactions between endothelial NO generation and HO-1 in VSMCs deserve further study given the evidence for differences in local hemodynamic forces between Tg and Ntg mice.

Finally, the current study shed light on clinical implications for HO-1–mediated desensitization of sGC to NO in the vascular systems that may serve as a putative mechanism for resistance to nitrosodilators. Previous studies have shown that exposure to greater doses of exogenous NO decreases the sensitivity of VSMCs to NO donors through downregulating amounts of the cyclase expression. However, considering that excess amounts of NO could induce HO-1 in various experimental models, the current study raised a possibility that such an NO-mediated desensitization of the cyclase could occur through CO-mediated mechanisms even when the expression amounts of sGC are not downregulated. Further investigation as to whether repeated administration of nitrosodilators could induce HO-1 in and around VSMCs should obviously be necessary to address roles of the HO/CO system in the modulation of NO-mediated vascular responses in human cases. Our mouse model provided new insights into the in vivo mechanisms of cross talk between NO and CO for regulation of the activity of sGC, serving as a useful model to explore the pathophysiological roles of CO in cardiovascular diseases.

Acknowledgments
This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan, a grant from Maruki Memorial Foundation, and by the Japan Cardiovascular Disease Research Foundation (to T.M.). We thank Junko Tatebe for her technical assistance.

References
Vascular Smooth Muscle Cell–Directed Overexpression of Heme Oxygenase-1 Elevates Blood Pressure Through Attenuation of Nitric Oxide–Induced Vasodilation in Mice
Tomihiko Imai, Toshisuke Morita, Takayuki Shindo, Ryozo Nagai, Yoshio Yazaki, Hiroki Kurihara, Makoto Suematsu and Shigehiro Katayama

Circ Res. 2001;89:55-62; originally published online June 21, 2001; doi: 10.1161/01.HGR.0000691198.06867.26

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/89/1/55

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research 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 Research is online at:
http://circres.ahajournals.org//subscriptions/