Putative Mechanisms of the Impairment of Endothelium-Dependent Relaxation of the Aorta With Atheromatous Plaque in Heritable Hyperlipidemic Rabbits

Hirofumi Tagawa, Hitonobu Tomoike, and Motoomi Nakamura

Attenuation of acetylcholine-induced endothelium-dependent relaxation of thoracic aortas excised from Watanabe heritable hyperlipidemiac (WHHL) rabbits linearly correlated with the percent area coated with atheromatous plaque. To elucidate mechanisms related to this reduced endothelium-dependent relaxation in the presence of atherosclerosis, the acetylcholine-induced release of endothelium-derived relaxing factor (EDRF) was assessed functionally as a percent relaxation of the precontracted detector strips obtained from the tunica media beneath the intact intima or the atheromatous plaque in the same aortic ring preparation. Relaxations of the normal detectors to effluents containing EDRF of thoracic aortas during stimulation by acetylcholine (3 × 10⁻⁶ M) in heterozygous and homozygous WHHL rabbits were 73±5% and 59±9% (p<0.01) of the phenylephrine-induced preconstriction, respectively. Relaxations of the atherosclerotic detectors to effluents (EDRF) through the aortas during stimulation by acetylcholine (3 × 10⁻⁶ M) in heterozygous and homozygous WHHL rabbits were 16±4% and 14±5%, respectively—values significantly smaller than those seen in the normal detectors. When superoxide dismutase was added to the perfusate of the donors from homozygous and heterozygous WHHL rabbits, atherosclerotic detectors relaxed by effluents stimulated by acetylcholine to 73% and 65% (p<0.01 versus before the addition of superoxide dismutase) of the normal detector, respectively. Relaxations induced by sodium nitroprusside as well as the contractions by acetylcholine, phenylephrine, and KCl (118 mM) were comparable in detector strips from the normal and atherosclerotic portions. Thus, not only is the amount of EDRF released by acetylcholine reduced in the presence of atherosclerosis, the tunica media beneath the atheromatous plaque is also to some extent responsible for the superoxide-induced inactivation of EDRF. (Circulation Research 1991;68:330–337)

A ugmented constrictive responses to serotonin, histamine, and ergonovine have been observed in vivo in coronary arteries after balloon denudation of the endothelium and hypercholesterolemia in pig and dog models.1,2 Enhanced vasoconstriction to serotonin and norepinephrine was demonstrated in vivo in the hind limb of athero-

sclerotic monkeys.3 Since Furchgott and Zawadzki4 reported the obligatory role of endothelial cells in the vascular relaxation induced by acetylcholine, the relation between increased sensitivity to vasoactive agents of the atherosclerotic vessel and the endothelial function has received much attention.5 The impairment in endothelium-dependent relaxation has been noted on vessels exposed to hypercholesterolemia or atherosclerosis in the rabbit aorta, monkey iliac artery, pig coronary artery, and human coronary artery in organ chamber experiments.6–9 Mechanisms that may account for the augmented vascular tone in pathological states are dysfunction of receptors in the endothelium, an intimal thickening barrier to diffusion of endothelium-derived relaxing factors (EDRF) from the endothelium to the vascular media, and a reduced sensitivity of medial smooth muscles to EDRF.10–12

We examined the mechanisms of impairment in cases of endothelium-dependent relaxation, the ex-
tent of EDRF release, and the response of smooth muscles to EDRF. The amount of EDRF released has been assessed functionally as the extent of relaxation induced by efluxes via a tube preparation of atherosclerotic and control aortas; however, findings in rabbit aortas were controversial.\textsuperscript{13,14} Accordingly, we used homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits to obtain donor vessels for the acetylcholine-induced release of EDRF, because the atheromatous plaque in this model covers more than 50\% of the luminal surface of the thoracic aorta. In addition, these characteristics of atheromatous invasion in 10–13-month-old WHHL rabbits are equivalent to the state of progressive atherosclerosis in adult humans with familial hyperlipidemia.\textsuperscript{15}

In the study of Bossaller et al.,\textsuperscript{9} the calcium ionophore A23187 and substance P were potent in relaxing the rabbit aorta when acetylcholine was ineffective. Accordingly, acetylcholine is one of the most sensitive probes to assess functionally the level of alterations in diseased states such as hypercholesterolemia and atherosclerosis.\textsuperscript{9} Thus, we examined the characteristics of acetylcholine-induced endothelium-dependent relaxation in WHHL rabbits by analyzing the release of EDRF and responses of medial smooth muscles to EDRF in bioassay experiments.

**Materials and Methods**

**Animal Model**

Homozygous and heterozygous WHHL rabbits were bred as described\textsuperscript{15} and housed in individual cages with temperature maintained at 20\degree C and relative humidity at 40–60\%. In this rabbit strain, an animal model of familial hypercholesterolemia, there is a spontaneously occurring hyperlipidemia along with generalized atherosclerotic lesions. When these rabbits reach the age of 10–13 months, an atheromatous plaque distributes heterogeneously over the luminal surface of the aorta (Figure 1), and the level of serum cholesterol is 385–634 mg/dl (n=8). As a control group to test the production of EDRF, heterozygous WHHL rabbits, age-matched to homozygous WHHL rabbits, were used, because the level of serum cholesterol was low (72–158 mg/dl; n=8), and there was no evidence of plaque lesions along the thoracic aorta.

**Endothelium-Dependent Relaxation**

Using a small razor blade, we divided the aortic ring into paired strips, covered with or without atheroma, as seen on gross inspection (Figure 1). These strips were mounted to isometric transducers (UL-50, Shinko Co., Ltd., Tokyo), placed in 20-ml chambers, and bathed in solution at 37\degree C. The bathing fluid was Krebs’ solution of the following composition (mM): NaCl 120, KCl 4.7, CaCl\textsubscript{2} 2.5, MgSO\textsubscript{4} 1.2, KH\textsubscript{2}PO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 24, and glucose 5.5. The solution was gassed continuously with 95\% O\textsubscript{2}-5\% CO\textsubscript{2} (pH 7.4). All preparations were first equilibrated for 60 minutes, and the basal tension was set to 2 g before drugs were added. These strips precontracted with phenylephrine (0.3 \mu M) relaxed in the presence of acetylcholine (0.001–3 \mu M) when the endothelial cells were intact. After the endothelium was removed with a cotton swab, acetylcholine did not relax these vascular strips. To assess the relation between the extent of atherosclerotic lesion and the impairment of endothelium-dependent relaxation, the acetylcholine-induced endothelium-dependent relaxation was examined using 33 strips of the lower thoracic aorta from eight homozygous WHHL rabbits. The percent surface covered with atheromatous plaque varied 0–100\% in these 33 strips. The response to acetylcholine is expressed as a percent of the initial contractions induced by phenylephrine (0.3 \mu M).
Bioassay of Endothelium-Derived Relaxing Factor

Two pieces of aortic tube 2 cm long were prepared from the thoracic aorta: one from homozygous WHHL rabbits with in-vitro atheroma exceeding 80% of the aortic luminal surface (atherosclerotic donor) and one from age-matched and body weight–matched heterozygous WHHL rabbits with normal serum cholesterol levels, as described, and no evidence of atherosclerotic lesions (control donor). These aortic tubes were perfused continuously with oxygenated (95% O_2-5% CO_2) Krebs’ solution at pH 7.4 and 37°C, and an aortic medial strip (detector strip) was superfused with effluents of aortic tube preparation (Figure 2). The detector strip was prepared from the tunica media of the same aortic ring preparation obtained from the thoracic aorta—one beneath the intact intima (normal detector) and the other beneath the atheroma (atherosclerotic detector) —using a binocular microscope. These detector strips were 3–4 mm long, 0.4–0.5 mm wide, and 0.3–0.4 mm thick. They were mounted vertically, and the isometric tension was measured at a preload of 1 g. The aortic tube preparation was mounted as closely as possible to the detector strip to minimize transit time from the tube (less than 1 second). After an equilibration period of 60 minutes, phenylephrine (0.3 μM) was added to the perfusate (Figure 2, solution A). When the contraction produced by phenylephrine had reached a steady state, acetylcholine was added to the perfusate, cumulatively in doses of 0.03, 0.1, 0.3, 1, and 3 μM (Figure 2, solution B).

In five of eight rabbits in each group (homozygous or heterozygous WHHL rabbits), effects of superoxide dismutase (SOD) were examined with regard to responses of the detector strips to EDRF. When the detector strips were precontracted by phenylephrine (0.3 μM) and then relaxed by acetylcholine (3×10⁻⁶ M), SOD (15 units/ml), a specific scavenger of superoxide anions, was added to the perfusates (Figure 2, solution C), and this effluent was superfused onto the detector strips of both normal and atherosclerotic detectors taken from the same ring preparations in homozygous WHHL rabbits (n=5).

Histological Examination

After the end of the study, the tissue specimen was immersed in a solution of Sudan IV, and the percent sudanophilic area measured with a planimeter was considered the percent area of the atheromatous plaque present on the strip surface.

Statistical Analysis

Data are given as mean±SD. Statistical significance of difference in relaxation of detector strips by perfusate from normal and atherosclerotic donors was evaluated by two-way analysis of variance. Effects of SOD on the relaxation of detector strips were compared within groups by the paired t test. The level of statistical significance was p<0.05. Correlation between the maximum relaxation induced by acetylcholine and the percent area of sudanophilia was examined by a least-squares method.

Results

As shown in Figure 3, the acetylcholine-induced relaxation was much less in strips from the atheromatous plaque (AS) than in those free of plaque (NL). ED₅₀ to acetylcholine in AS and NL was 637±607 and 52±64 nM (n=7 rabbits, p<0.05), and the maximal relaxations were 21.4±12.8% and 83.8±15.2% of the precontracted state (n=7, p<0.01), respectively. There was an inverse linear relation between the
maximal relaxation elicited by acetylcholine and the extent of the atheroma ($y=-0.85x+90.4$, $r=-0.94$, $n=33$ strips, $p<0.01$) (Figure 4).

The contractile responses of the medial strips to phenylephrine, per se, did not differ significantly between the atherosclerotic and normal sites (Table 1). Acetylcholine (30 nM to 3 μM) increased tension of the precontracted strips by 0.3 μM phenylephrine when the donors were absent (direct perfusion). These increases in tension were $4.4\pm4\%$ and $5.2\pm4.5\%$ in normal and atherosclerotic strips, respectively (not significant). Production of EDRF and responses of detector strips to EDRF were examined, as shown in Figures 2 and 5. When the detector strips were taken from the intact (plaque-free) site (normal detector), the acetylcholine-induced relaxation was larger in cases of heterozygous than in homozygous WHHL aortic tube preparations ($p<0.01$) when the dose of acetylcholine exceeded $10^{-6}$ M (Figure 6, left panel). However, this large relaxation noted in the homozygous or heterozygous WHHL aortas was never evidenced in medial smooth muscle preparations taken from the plaque site (atherosclerotic detector) (Figure 6, right panel).

Responses to sodium nitroprusside were similar in atherosclerotic and normal detectors (Table 1). When the detector strips were continuously superfused with SOD (15 units/ml), the relaxing responses to acetylcholine-induced EDRF in the atherosclerotic detector were significantly augmented ($p<0.01$, $n=5$) in perfusates from either atherosclerotic or control donors (Figure 7). The relaxing responses to the peak dose of acetylcholine ($3\times10^{-6}$ M) in the normal detector were not potentiated by SOD ($n=5$) (Figure 7) or by the solvent of SOD. The SOD-assisted recovery of the EDRF-induced relaxation of the atherosclerotic detectors was 27% and 35% less than that of the normal detector by EDRF from atherosclerotic and control donors, respectively.

### Discussion

In the present study, we obtained evidence that 1) there is an inverse linear relation between the percent area covered with atheromatous plaque and the

### Table 1. Basal Characteristics of Detector Strips

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Atherosclerotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction by 118 mM KCl (mg)</td>
<td>266±39</td>
<td>300±26</td>
</tr>
<tr>
<td>Contraction by phenylephrine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum response (% of 118 mM KCl)</td>
<td>151±14</td>
<td>146±15</td>
</tr>
<tr>
<td>$ED_{so}$ (nM)</td>
<td>253±206</td>
<td>240±109</td>
</tr>
<tr>
<td>Relaxation by sodium nitroprusside</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximal response (% of 0.3 μM phenylephrine)</td>
<td>89±3</td>
<td>90±6</td>
</tr>
<tr>
<td>$ED_{so}$ (nM)</td>
<td>90±65</td>
<td>83±60</td>
</tr>
</tbody>
</table>

Values are mean±SD.

---

**FIGURE 3.** Heterogeneous relaxing responses of rabbit aortic strips to acetylcholine at sites with (AS) or without (NL) atheromatous plaque. AS and NL strips were obtained from the same aortic ring preparation in a homozygous Watanabe heritable hyperlipidemic rabbit. Acetylcholine-induced relaxation is much smaller in AS than in NL. PE, phenylephrine; ACh, acetylcholine; NG, nitroglycerin, W, washout. The concentration of each drug in the chamber is expressed as the $-\log$ of the molar concentration.

**FIGURE 4.** Responses to acetylcholine of aortic strips with different degrees of atheromatous involvement. Extent of relaxation was expressed as a percent of level in the case of a precontraction with 0.3 μM phenylephrine. An inverse linear relation was noted ($r=-0.94$, $p<0.01$, 33 strips, eight homozygous Watanabe heritable hyperlipidemic rabbits).

**FIGURE 5.** Aortic ring.
extent of acetylcholine-induced endothelium-dependent relaxation, 2) the acetylcholine-induced release of EDRF is decreased in the atherosclerotic aortas of homozygous WHHL rabbits, 3) there is a marked inactivation of EDRF at the tunica media beneath the plaque, and 4) impairment in the acetylcholine-induced relaxation of the tunica media taken from the plaque site is partly recovered when SOD is included in the perfusate. These findings have implications regarding mechanisms underlying the localized augmentation of the response of the atherosclerotic vessels, including coronary arteries, to vasoactive agents such as serotonin, histamine, or ergonovine.¹⁷,¹⁸

The attenuation of endothelium-dependent relaxation was noted in angiographic studies of patients

![Figure 5](image-url)  
**Figure 5.** Bioassay of endothelium-derived substance in normal and atherosclerotic aortas taken from heterozygous and homozygous Watanabe heritable hyperlipidemic (WHHL) rabbits, respectively. A pair of circumferential fibers of the tunica media beneath the intact and atherosclerotic portions was prepared from the same lower thoracic aorta of homozygous WHHL rabbits and used to detect endothelium-derived substance. These detectors were superfused with Krebs' solutions perfused through the aortic tubes (donors) of heterozygous and homozygous WHHL rabbits; the former for control and the latter for the atherosclerotic donor. PE, phenylephrine; Ach, acetylcholine; W, system washout. The concentration of each drug in the perfusate is expressed as the −log of the molar concentration.

![Figure 6](image-url)  
**Figure 6.** Acetylcholine cumulative dose–response relation of normal (left panel) and atherosclerotic (right panel) detectors superfused via perfusates through control (○) and atherosclerotic (●) donor aortas obtained from heterozygous (hetero) and homozygous (homo) Watanabe heritable hyperlipidemic (WHHL) rabbits, respectively. Ordinate: relaxation expressed as a percentage of the contraction produced by phenylephrine (0.3 μM); abscissa: concentration of acetylcholine (M) on a log scale. Dose–response curves are significantly different between normal and atherosclerotic donors (p<0.01, n=8). Data are mean±SD. *p<0.01 between control and atherosclerotic donor aortas.

![Figure 7](image-url)  
**Figure 7.** Effects of superoxide dismutase (SOD) (15 units/ml) on acetylcholine (Ach)-induced relaxations of the aortic medial smooth muscles beneath normal and plaque sites. Role of endothelium-derived relaxing factor (EDRF) released from the homozygous (homo) (left panel) and heterozygous (hetero) (right panel) Watanabe heritable hyperlipidemic (WHHL) rabbit aortic tube preparations was assayed biologically as the extent of relaxations of the detector from the tunica media of thoracic aortas. Acetylcholine concentration was 3×10⁻⁶ M. Data are mean±SD.
with atherosclerotic coronary arteries\textsuperscript{19} or in those with variant angina.\textsuperscript{20} The intracoronary injection of acetylcholine caused a further narrowing of atherosclerotic coronary arteries, yet it dilated normal coronary arteries.\textsuperscript{19} Accordingly, alterations in endothelial functions of the vessel with atherosclerosis or exposed to hypercholesterolemia require attention. The WHHL rabbit strain provides a useful model to elucidate the relation between atherosclerosis and endothelial dysfunction, because effects of varying degrees of atheromatous plaque on vascular reactivity can be examined in rabbits of various ages.\textsuperscript{21} To ensure that the responses of medial smooth muscles to EDRF were not related to topological differences along the aorta,\textsuperscript{22} we dissected two pieces of muscle preparations from the tunica media in the same aortic ring of the thoracic aorta. Plausible artifacts induced by the procedures used to isolate the tunica media beneath the intact intima and atheromatous plaque of the same thoracic aortic ring were negligible in the present study, because contractile responses of the medial smooth muscles to KCl or to phenylephrine as well as relaxation in the presence of nitroprusside (endothelium independent) were comparable between normal and atherosclerotic detectors and also were similar qualitatively to those of the preparation with the endothelium.

EDRF is chemically labile and some EDRFs proved to be nitric oxide.\textsuperscript{23} In the present study, the amounts of EDRF were assessed functionally but not chemically with regard to the capability of the isolated vessels to relax.\textsuperscript{4,24} Studies by Jayakody et al\textsuperscript{13} and Verbeuren et al\textsuperscript{14} noted impairment of the acetylcholine-induced endothelium-dependent relaxation in aortas from rabbits fed a 2\% and 0.3\% cholesterol diet for 4 weeks (cholesterol levels greater than 2,000 mg/dl) and 8–16 weeks. Verbeuren et al\textsuperscript{14} found a significant correlation between the maximum relaxation evoked by acetylcholine and the degree of fatty streak formation in vessels, including aortic arch, abdominal aorta, and brachiocephalic and pulmonary arteries. In the present study, the reduced relaxation of the precontracted atherosclerotic aortas to acetylcholine seemed to be directly related to the extent of surface involvement by the atheromatous plaque. This attenuation of the endothelium-dependent relaxation by acetylcholine may be closely related to pathological changes specific to atherosclerosis. Putative processes linked to the impairment of the EDRF-related relaxation include 1) disruptions of endothelial acetylcholine receptor function caused by hypercholesterolemia, atherosclerosis, or both,\textsuperscript{9,25} 2) a reduced production of EDRF,\textsuperscript{24} 3) inactivation of EDRF caused by transit time\textsuperscript{26} or biologically\textsuperscript{27} at the thickened intima and tunica media, and 4) impaired responsiveness of medial smooth muscle to EDRF.\textsuperscript{28}

Although Srecharan et al\textsuperscript{24} found no differences in the sensitivity to EDRF in detectors taken from control and cholesterol-fed rabbits, they did note a significant reduction in the production of EDRF in hypercholesterolemic rabbits. In the present study, to minimize possible differences in the amount of EDRF released among aortas from different rabbit strains, we compared effluents from donor aortas of homozygous and heterozygous WHHL rabbits. The bioassay of EDRF by normal detectors in the present study also revealed that the $10^{-6}$ M acetylcholine-induced release of EDRF from the atherosclerotic aorta of homozygous WHHL rabbits was reduced by 25\% compared with that of aorta in heterozygous WHHL rabbits. However, such a reduction was not evident when the donor tissue was a segment of the abdominal aorta.\textsuperscript{14} This discrepancy no doubt relates to the fact that the cholesterol-fed rabbit model of atherosclerosis has fewer lesions in the abdominal than in the thoracic aorta.\textsuperscript{24}

Marked reduction of endothelium-dependent relaxation was noted in microvessels less than 25 $\mu$m in diameter of the cremaster in atherosclerotic rabbits,\textsuperscript{29} in small subepicardial arteries (286±18 $\mu$m) of the left anterior descending coronary artery in cholesterol-fed rabbits,\textsuperscript{30} and in coronary microvessels (122–220 $\mu$m) exposed to hypercholesterolemia in Malaysian cynomolgus monkeys.\textsuperscript{31} Osborne et al\textsuperscript{30} noted the same magnitude of contractions developed by acetylcholine in resistance vessels devoid of plaque but with ultrastructurally intact endothelium as in vessels with plaque; therefore, the plaque, per se, is not primarily responsible for changes in response to acetylcholine in vessels taken from hypercholesterolemic rabbits. Accordingly, there may be some defect in endothelial function regarding release or production of EDRF rather than the presence of intimal barrier in these small arteries belonging conceptually to resistance vessels. Hypercholesterolemia, per se, may play an important role in abnormalities of endothelium-dependent relaxation. Whether the conduit and resistance vessels differ with regard to EDRF release in response to acetylcholine remains to be elucidated.

Reductions of endothelium-dependent relaxation also can be explained by a reduced sensitivity of the medial smooth muscles to EDRF or by the inactivation of EDRF. A similar extent of relaxation to nitroprusside as well as contractions to acetylcholine in normal and atherosclerotic detectors suggests that the guanylate cyclase system or the acetylcholine-related contractile apparatus is intact in the smooth muscle cell interior. Because the mechanism of EDRF-related relaxation of vascular smooth muscle is similar to that in the case of nitroglycerin\textsuperscript{23} or nitroprusside, the sensitivity of medial smooth muscles to EDRF may remain unaltered. Nevertheless, responses of the atherosclerotic detectors to EDRF were markedly reduced in the present experiment. Most of the activity of EDRF was restored after SOD was added to the perfusate. In isometric tension studies, the release of superoxide anion depressed the endothelium-mediated relaxation to acetylcholine.\textsuperscript{32} Accordingly, the oxidative modification of EDRF is a plausible mechanism.
Previous studies revealed that the half-life of EDRF can be prolonged by SOD; however, the activity of EDRF was not augmented by SOD at transit times of 1 or 4 seconds.\textsuperscript{32,33} Thus, for a sensitive assay of EDRF, the transit time between the donor segment and the detector strip in our perfusion system was set at less than 1 second. In addition, our detectors were so thin that EDRF may have diffused quickly to the entire strip. In this preparation, SOD was effective only under conditions in which the degradation of EDRF was abnormally increased at the atherosclerotic detector, probably because of the activity of superoxide anion.

Because the presence of atheromatous plaques correlated with the accelerated inactivation of EDRF regionally across the tunica media, the augmented metabolic state, including production of superoxide anion, may be one mechanism worthy of consideration. This speculation is in accord with the increased regional distribution of a diffusible tracer across the media of atherosclerotic aorta in rabbits fed a 1% cholesterol diet after mechanical injury of the intima.\textsuperscript{34} Components related to atherogenesis, such as endothelial cells\textsuperscript{32} and inflammatory cells,\textsuperscript{35} produce superoxide anion, and this anion once generated modifies low density lipoprotein at the extracellular space.\textsuperscript{36} Modified low density lipoprotein along the elastic fibers of the aortic media of WHHL rabbits has been identified immunohistochemically.\textsuperscript{37} Thus, inactivation of EDRF at the tunica media caused by increased superoxide anion production not only augments vascular tone but also accompanies the generation of cytotoxic substances such as modified low density lipoprotein, events that may lead to an acceleration of atherosclerosis.\textsuperscript{38}

Acknowledgments

We thank Dr. Y. Watanabe (Kobe University, Japan) for providing the WHHL rabbits, Mrs. T. Nakashima for breeding the animals, Drs. J. Sadoshima and H. Shimokawa for discussion, and M. Ohara for comments.

References

24. Sreeharan N, Jayakody RL, Senaratne MJP, Thomson ABR, Kappagoda CT: Endothelium-dependent relaxation and

KEY WORDS • acetylcholine • superoxide dismutase • atherosclerosis • endothelium-derived relaxing factor
Putative mechanisms of the impairment of endothelium-dependent relaxation of the aorta with atheromatous plaque in heritable hyperlipidemic rabbits.

H Tagawa, H Tomoike and M Nakamura

doi: 10.1161/01.RES.68.2.330

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 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/68/2/330

**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/