Cerebral Microvascular Responses to Hypercholesterolemia
Roles of NADPH Oxidase and P-Selectin


Abstract—Although hypercholesterolemia is widely accepted as a major risk factor for coronary artery and peripheral vascular diseases, its role in the pathogenesis of stroke is controversial. The objectives of this study were to determine how hypercholesterolemia affects the cerebral microcirculation under resting conditions and after ischemia-reperfusion (I/R). Platelet- and leukocyte-endothelial cell interactions and oxidant production (using the oxidant-sensitive fluorochrome dihydrorhodamine-123) were monitored by intravital videomicroscopy in the cerebral microvasculature of mice placed on either a normal (ND) or cholesterol-enriched diet (HCD). Platelets labeled with carboxyfluorescein diacetate succinimidyl ester (CFDASE) and leukocytes labeled with rhodamine 6G were seen to roll and firmly adhere, with a corresponding increase in oxidant production, in venules on HCD, but not ND. Immune neutralization of P-selectin attenuated the platelet- and leukocyte-endothelial cell interactions and the enhanced oxidant production associated with HCD. A GPIIb/IIIa blocking antibody did not alter the blood cell-vessel wall interactions to HCD. Mice deficient in the NADPH oxidase subunit gp91phox exhibited significantly blunted platelet and leukocyte recruitment responses to HCD. Focal I/R also elicited inflammatory and prothrombogenic responses in cerebral venules and these were exaggerated in mice on HCD. These results implicate an oxidant-dependent, P-selectin–mediated mechanism in the brain cell-vessel wall interactions induced by hypercholesterolemia in the brain and demonstrate that the deleterious effects of I/R on the brain are exacerbated by this cardiovascular risk factor. (Circ Res. 2004;94:239-244.)

Key Words: platelet adhesion ■ leukocyte adhesion ■ P-selectin ■ cerebral ischemia ■ GPIIb/IIIa

Hypercholesterolemia is a recognized risk factor for coronary artery and peripheral vascular diseases because its effects on large arterial vessels (atherosclerosis) increase the likelihood that tissues will experience an ischemic episode.1 However, there is a growing body of evidence that hypercholesterolemia also leads to microvascular dysfunction long before the appearance of atherosclerotic lesions in large vessels and that the altered microvascular function during hypercholesterolemia produces exaggerated tissue injury responses to ischemia and reperfusion and other stimuli (e.g., endotoxemia).2,3 The microvascular dysfunction induced by hypercholesterolemia is manifested in arterioles as impaired endothelium-dependent vasodilation and in postcapillary venules as an accumulation of rolling and adherent leukocytes as well as platelets, whereas both vascular segments (arterioles and venules) exhibit an oxidative stress.2,3 The activation of multiple cell types (endothelial cells, leukocytes, platelets) and involvement of different segments of the microcirculation suggest that circulating soluble mediators (e.g., cytokines) and/or cell adhesion–dependent signaling contribute to the microvascular alterations of hypercholesterolemia. Furthermore, hypercholesterolemia appears to be one of a growing list of pathological states in which there is an interface between inflammation and thrombosis.4

Although epidemiological and experimental studies have provided strong support for the view that elevated cholesterol is associated (and correlated) with an increased incidence of coronary and peripheral vascular diseases, no such correlation has been firmly established for the brain.5,6 The pathophysiological basis for the poor predictive value of serum cholesterol for stroke risk remains unclear, particularly in light of numerous reports that describe reductions in carotid atherosmas and risk for ischemic stroke in patients treated with the cholesterol-lowering statins.7,8 Some investigators have suggested that the beneficial effects of statins in stroke may be due in part to lipid-lowering properties, with the primary benefit derived from improved endothelial function as well as the antiinflammatory and antithrombotic actions of the drugs.8 However, others have suggested that the cerebral microvasculature may have inherent biological processes that differ from peripheral vascular beds with respect to endothelial cell function.6 Although hypercholesterolemia is known to induce a proinflammatory and prothrombogenic state in the microvasculature of different tissues, including skeletal mus-
Circulation Research  February 6, 2004

Intravital Fluorescence Microscopy and Video Analysis

Postcapillary venules (30 to 40 μm, diameter) in the cerebral microcirculation were observed with an upright fluorescence microscope as previously described. CFSE-labeled platelets and rhodamine 6G-labeled leukocytes were monitored at a final magnification of ×560 or ×1120 using appropriate filter sets. The microscopic images were recorded on video tape for later analysis. Both platelets and leukocytes were classified according to the quality or duration of their interaction with the venular wall as either free-flowing, rolling, or adherent. Rolling platelets and leukocytes were defined as cells crossing the 100 μm venular segment at a velocity that was significantly lower than the centerline velocity; their numbers are expressed as cells per 30 seconds per mm² of venular surface (calculated from diameter and length, assuming cylindrical vessel shape). Adherent platelets and leukocytes were stationary for >2 and >30 seconds, respectively, and were expressed as the number of cells per mm² of venular surface.

In a separate group of mice, the oxidant-sensitive fluorescent probe dihydrorhodamine-123 (DHR, Molecular Probes Inc, 10 μmol/L) was dissolved in artificial CSF and superfused on the brain surface (the dura mater was cut to allow for direct exposure) via the closed cranial window. Brain tissue was exposed to DHR for 15 minutes, followed by perfusion with plain artificial CSF. DHR oxidation was visualized and quantified using previously described procedures. DHR fluorescence intensity was monitored in a region of brain surface that was equivalent to twice the area of the 100 μm venular segment under study and in a rectangular area (100×50 μm) immediately outside the vessels.

Materials and Methods

Animal Preparation

Experiments were performed on male C57Bl/6J mice and gp91<sup>phox</sup>-deficient mice backcrossed 12 times on a C57Bl/6 background (Jackson Laboratories, Bar Harbor, Maine), weighing 21 to 25 g, using procedures previously described. The animals were anesthetized with an IP injection of α-chloralose (60 mg/kg) and urethane (600 mg/kg), whereas lidocaine (1%) was used for local anesthesia. All mice were tracheotomized with a polyethylene catheter (PE90, Becton Dickinson) and artificially ventilated (Harvard Rodent Ventilator, Model 683, Harvard Apparatus) with room air during observation with intravital microscopy. The femoral artery and vein were cannulated with a polyethylene catheter (PE10) to monitor mean arterial blood pressure, sample arterial blood for blood gas analysis, and for intravenous administration of labeled platelets and rhodamine 6G. Rectal temperature was monitored and maintained at 36.5°C to 37.5°C.

Brain Preparation

The head of each mouse was fixed in a plastic frame in the sphinx position. The left parietal bone was exposed by a midline skin incision, followed by a craniotomy (diameter: 2.5 mm) 1 mm posterior from the bregma and 4 mm lateral from the midline, using a drill. The dura mater remained intact while analyzing leukocyte and platelet adhesion. A 12-mm glass cover slip was placed over the exposed brain tissue, which was suffused with artificial cerebrospinal fluid (CSF). The experimental procedures described above were reviewed and approved by the LSU Health Sciences Center Institutional Animal Care and Use Committee.

Platelet Preparation

Platelets were isolated from donor mice and labeled with carboxy-fluorescein diacetate succinimydyl ester (CFDA-SE; Molecular Probes) as previously described. Manual blood cell counts yielded less than 0.05% leukocytes in the platelet suspension. Platelet recipient mice each received 100×10<sup>6</sup> platelets infused over 5 minutes, yielding approximately 10% of the total platelet count. The platelets were allowed to circulate for a period of 5 minutes before recording. Flow cytometric analysis of the isolated platelets has previously revealed no significant changes in the expression of platelet P-selectin and GPIbIIa, indicating that minimal cell activation occurs during the platelet isolation procedure. Although every effort was made to minimize activation of donor platelets during the labeling process, we cannot rule out the possibility that the exogenous platelets are “primed” for activation when administered to the recipient mice.

Intravital Fluorescence Microscopy and Video Analysis

Postcapillary venules (30 to 40 μm, diameter) in the cerebral microcirculation were observed with an upright fluorescence microscope as previously described. CFSE-labeled platelets and rhodamine 6G-labeled leukocytes were monitored at a final magnification of ×560 or ×1120 using appropriate filter sets. The microscopic images were recorded on video tape for later analysis. Both platelets and leukocytes were classified according to the quality or duration of their interaction with the venular wall as either free-flowing, rolling, or adherent. Rolling platelets and leukocytes were defined as cells crossing the 100 μm venular segment at a velocity that was significantly lower than the centerline velocity; their numbers are expressed as cells per 30 seconds per mm² of venular surface (calculated from diameter and length, assuming cylindrical vessel shape). Adherent platelets and leukocytes were stationary for >2 and >30 seconds, respectively, and were expressed as the number of cells per mm² of venular surface.

In a separate group of mice, the oxidant-sensitive fluorescent probe dihydrorhodamine-123 (DHR, Molecular Probes Inc, 10 μmol/L) was dissolved in artificial CSF and superfused on the brain surface (the dura mater was cut to allow for direct exposure) via the closed cranial window. Brain tissue was exposed to DHR for 15 minutes, followed by perfusion with plain artificial CSF. DHR oxidation was visualized and quantified using previously described procedures. DHR fluorescence intensity was monitored in a region of brain surface that was equivalent to twice the area of the 100 μm venular segment under study and in a rectangular area (100×50 μm) immediately outside the vessels.

Focal Ischemia Model

In some experiments, focal cerebral ischemia was induced by occlusion of the middle cerebral artery (MCA) using the intraluminal filament method, previously described. The blunt tip of a 6-0 nylon monofilament was advanced to the level of the carotid bifurcation via the internal carotid artery. The nylon thread was advanced until light resistance was felt, the distance from the nylon thread tip to the internal carotid artery-ptyerogypalatine artery bifurcation was slightly more than 6 mm and the distance to the bifurcation of the internal and external carotid arteries was slightly less than 9 mm. The nylon thread was removed after a 30-minute or 1-hour occlusion period. In the sham group, these arteries were visualized but not disturbed.

Experimental Protocols

Diet

Wild-type or gp91<sup>phox</sup>-deficient mice at 6 to 8 weeks of age were placed on either a normal diet (ND) or a high-cholesterol diet (HCD) (Teklad 90221 containing 1.25% cholesterol, 15.8% fat, and 0.125% choline chloride; Harlan Teklad) for a period of 1 (I/R experiments) or 2 (hypercholesterolemia without I/R) weeks. In some experiments, mice were placed on a diet enriched with 0.125% choline chloride, but without the added cholesterol of Teklad 90221.

Platelets and Leukocytes

Labeled platelets were infused and their interactions recorded in five randomly selected venular segments. Rolling and adherent leukocytes were then monitored and recorded in the same venular segments. The latter was achieved by IV administration of 50 μL of 0.02% rhodamine 6G (Sigma Chemicals), followed by a continuous infusion (2 mL/h) of the fluorochrome at the same concentration for 5 to 10 minutes.

Groups

In the first series of experiments, platelets isolated from ND or HCD (2 weeks) mice were infused into either ND or HCD (2 weeks) recipients, and blood cell/vessel wall interactions were monitored. Based on the results from these experiments, further groups were studied using platelets from donors that matched the recipient mice with respect to diet as follows: (1) gp91<sup>phox</sup>-deficient mice on HCD...
for 2 weeks; (2) 2-week HCD mice that received 2 mg/kg (IV) anti-murine monoclonal antibody (mAb) directed against P-selectin (RB40.34) 30 minutes before quantification of blood cell adhesion; (3) 2-week HCD mice that received 2 mg/kg (IV) anti-murine mAb directed against GPIIb/IIIa (1B5, provided by Dr. Barry Coller from Rockefeller University, New York, NY) 30 minutes before quantification of blood cell adhesion.

DHR oxidation was monitored in three randomly selected venular segments of each animal in separate groups of mice placed on either a ND or HCD for 2 weeks, with or without P-selectin mAb RB40.34. The effects of HCD on the blood cell/vessel wall interactions after middle cerebral artery occlusion (MCAO) and reperfusion were studied in another series of experiments. For these experiments, mice were placed on either ND or HCD for 1 week before the experiment and then subjected to 30 minutes of MCAO and 4 hours of reperfusion. DHR oxidation was monitored after 1 hour of MCAO and 4 hours of reperfusion.

**Statistics**

Data were analyzed using an analysis of variance and Fisher’s post hoc test. The data are reported as mean±SE. Statistical significance was set at P<0.05.

**Results**

Blood pressure (87.1±0.7 mm Hg), blood pH (7.33±0.01), Po₂ (103±2 mm Hg), and PCO₂ (34.1±0.6 mm Hg) (obtained after platelet and leukocyte adhesion measurements) showed no significant differences between all experimental groups. Serum cholesterol values were 79±4 mg/dL in ND mice and were significantly elevated to 154±11 mg/dL in 1-week HCD mice and 180±7 mg/dL in 2-week HCD mice.

Figure 1 compares the leukocyte (panel A) and platelet (panel B) adhesion responses in cerebral venules between wild-type mice placed on either a normal or cholesterol-enriched diet. Whereas minimal leukocyte rolling and adhesion were noted in venules of ND mice, HCD mice exhibited profound increases in both variables. Mice placed on a 0.125% choline chloride–supplemented diet (same diet as used in this study to induce hypercholesterolemia, but without the cholesterol enrichment) for 2 weeks yielded resting numbers of adherent leukocytes (43.2±9.1 cells per mm²) and platelets (30.8±8.1 cells per mm²) that did not differ from values obtained in ND mice (34.2±6.7 and 25.2±16.3 cells per mm², respectively). When the adhesion (rolling and firm adhesion) of ND platelets was monitored in ND recipients, low level interactions were noted (Figure 1B). However, when ND platelets were monitored in HCD recipients, greatly elevated levels of platelet rolling and adhesion were observed. Similar adhesion responses were noted when HCD platelets were monitored in HCD recipients. Much smaller adhesion responses were noted when HCD platelets were monitored in ND recipients, suggesting that cholesterol also exerts a direct effect on platelets.

The increases in leukocyte rolling and adhesion induced by HCD were significantly attenuated after treatment with a mAb against P-selectin, but not GPIIb/IIIa (Figure 2A). Similar responses were noted for platelet adhesion, with the P-selectin mAb (but not the GPIIb/IIIa mAb) completely preventing the firm adhesion of platelets induced by HCD. Whereas the P-selectin mAb was equally effective in preventing the rolling of platelets in HCD, the GPIIb/IIIa mAb also exerted a significant attenuating effect on platelet rolling.

Figure 3 illustrates that the oxidation of DHR, an index of oxidative stress, is significantly elevated in cerebral venules of HCD mice, compared with their normocholesterolemic counterparts (ND). The elevated DHR oxidation was particularly evident in the perivascular compartment. The HCD-induced increase in DHR oxidation was attenuated by the P-selectin blocking mAb RB40.34.

Figure 4 compares the platelet and leukocyte recruitment responses to HCD in cerebral venules of wild-type mice placed on (ND) and hypercholesterolemic (HCD) diets. ND→ND indicates both platelet donor and recipient on ND; ND→HCD, platelets from ND mice and recipient on HCD; HCD→HCD, both the platelet donor and recipient mice were HCD; and HCD→ND, platelets from HCD and recipient on ND. In each group, 8 to 10 animals were studied. *P<0.05 relative to the ND→ND group; #P<0.05 relative to the ND→HCD group.

Figure 5 compares the responses of leukocyte and platelet adhesion to MCAO/reperfusion in cerebral venules of wild-type mice placed on either a normal or cholesterol-enriched diet for 1 week. The leukocyte recruitment responses (rolling and adherence) to 30 minutes of MCAO and 4 hours of reperfusion in ND mice were comparable to those induced by
1 week of HCD (Figure 5A) and the combination of MCAO/reperfusion and HCD elicited leukocyte responses that appeared to be additive of the individual responses. The platelet rolling and adhesion responses to either 1 week of HCD or MCAO/reperfusion were relatively small. However, MCAO/reperfusion produced large and highly significant increases in platelet rolling and adherence in HCD mice.

Figure 2. Effect of P-selectin and GPIb/IIa monoclonal antibodies (mAb) on rolling and adhesion of leukocytes (A) and platelets (B) in hypercholesterolemic wild-type mice (HCD). ND refers to mice on normal diet. In each group, 6 to 8 animals were studied. *P<0.05 relative to the ND group; #P<0.05 relative to the nontreated HCD group.

Discussion

The results of this study indicate that diet-induced hypercholesterolemia causes the cerebral microvasculature to undergo oxidative stress and to assume a proinflammatory and prothrombogenic phenotype. Enhanced leukocyte-endothelial cell adhesion in response to hypercholesterolemia has been demonstrated in venules of mouse cremaster muscle,9,10 rat mesentery,13,18 rabbit mesentery,19 and in the rabbit coronary artery.20 Increased platelet adhesion in HCD mice has been previously shown in intestinal venules,12 whereas HCD-induced oxidative stress has been demonstrated in arterial endothelium21 and postcapillary venules of mouse cremaster.10 Our observation that the cerebral microcirculation responds to HCD like other vascular beds is inconsistent with the view that brain endothelial cells are uniquely insensitive to the deleterious effects of elevated blood cholesterol.6

Figure 3. Dihydrorhodamine (DHR) oxidation (oxidative stress) in wild-type mice on a normal (ND) and cholesterol-enriched (HCD) diet, and the effects of pretreatment with a P-selectin mAb. DHR oxidation was monitored within and surrounding cerebral venules. Six animals were studied in each group. *P<0.05 relative to the ND group; #P<0.05 relative to the HCD group.

Figure 4. Effects of hypercholesterolemia on the rolling and adhesion of leukocytes (A) and platelets (B) in wild-type (WT) and gp91phox-deficient (gp91phox−/−) mice. In each group, 6 to 8 animals were studied. *P<0.05 relative to the WT ND; #P<0.05 relative to the WT HCD group.

Figure 2. Effect of P-selectin and GPIb/IIa monoclonal antibodies (mAb) on rolling and adhesion of leukocytes (A) and platelets (B) in hypercholesterolemic wild-type mice (HCD). ND refers to mice on normal diet. In each group, 6 to 8 animals were studied. *P<0.05 relative to the ND group; #P<0.05 relative to the nontreated HCD group.

Figure 3. Dihydrorhodamine (DHR) oxidation (oxidative stress) in wild-type mice on a normal (ND) and cholesterol-enriched (HCD) diet, and the effects of pretreatment with a P-selectin mAb. DHR oxidation was monitored within and surrounding cerebral venules. Six animals were studied in each group. *P<0.05 relative to the ND group; #P<0.05 relative to the HCD group.

Figure 4. Effects of hypercholesterolemia on the rolling and adhesion of leukocytes (A) and platelets (B) in wild-type (WT) and gp91phox-deficient (gp91phox−/−) mice. In each group, 6 to 8 animals were studied. *P<0.05 relative to the WT ND; #P<0.05 relative to the WT HCD group.
An interesting and novel observation of our studies on the platelet adhesion responses of the brain microcirculation to HCD is that the blood vessel wall, rather than platelets per se, must be exposed to the hypercholesterolemic milieu in order to realize the complete prothrombogenic response. This is supported by the observation (Figure 1) that hypercholesterolemic recipients of platelets derived from either ND or HCD mice exhibit a robust platelet adhesion response, whereas normocholesterolemic recipients of HCD platelets exhibit only a modest platelet adhesion response. This indicates that the cell activation and accompanying increase in P-selectin expression that has been described for platelets isolated from hypercholesterolemic human subjects \(^{22}\) and mice \(^{12}\) cannot alone explain the prothrombogenic state induced by HCD. It appears likely that the extracellular milieu in hypercholesterolemia may contain substances that either promote platelet-endothelial cell adhesion directly or inhibit the production of agents that normally act to prevent platelet adhesion (e.g., nitric oxide).

Our study also provides novel insights into the mechanisms that underlie the leukocyte and platelet recruitment induced by HCD in the cerebral microcirculation. Our findings suggest that the oxidative stress induced by HCD involves the superoxide-producing enzyme NADPH oxidase, because the HCD-induced recruitment of platelets and leukocytes in cerebral venules was dramatically reduced in mice that were deficient in gp91phox, a critical subunit of the NADPH oxidase protein complex. Increased superoxide formation by vascular endothelial cells in response to hypercholesterolemia has been previously reported.\(^ {23,24}\) Furthermore, we have shown that mice deficient in p47 phox, another subunit of NADPH oxidase required for enzyme function, exhibit an attenuated HCD-induced leukocyte recruitment in cremasteric venules.\(^ {10}\) Bone marrow chimeras produced by transplanting p47 phox-deficient marrow into wild-type (WT) recipients or WT marrow into p47 phox-deficient recipients revealed a role for both vessel wall and blood cell NADPH oxidase in mediating the enhanced leukocyte adhesion response to hypercholesterolemia.\(^ {10}\) It is possible that the two sources of NADPH oxidase also contribute to the leukocyte and platelet adhesion induced by HCD in cerebral venules. The oxidative stress induced by HCD may directly reflect the accumulation of oxidant generating neutrophils, the activation of endothelial cells by adherent, activated leukocytes, or both. In either instance, one might expect P-selectin blockade to blunt the oxidative stress.

Although a GPIIb/IIIa mAb had no effect on the HCD-induced cerebral microvascular responses in our study, immunoneutralization of P-selectin exerted a profound attenuating effect on the elevated rolling and adhesion of platelets and leukocytes seen in this model. The observation that both blood cell adhesive interactions were blunted by P-selectin blockade is consistent with reports describing increased P-selectin expression on both circulating platelets\(^ {12,22}\) as well as endothelial cells\(^ {18}\) and suggests that both the platelets and leukocytes utilize P-selectin for adhesion in the brain microcirculation during HCD. Although the platelets and leukocytes may directly bind to venular endothelium using P-selectin, it is also possible that endothelial P-selectin mediates the attachment of leukocytes to endothelial cells, whereas platelets utilize P-selectin to bind to PSGL-1 (a ligand for P-selectin) that is constitutively expressed on the surface of leukocytes. Our observation that a P-selectin mAb and gp91phox deficiency yield similar attenuation of the HCD-induced leukocyte and platelet adhesion raises the possibility that NADPH oxidase-derived superoxide may contribute to the increased P-selectin expression on endothelial cells and/or platelets. This possibility is supported by reports demonstrating a
linkage between oxidative stress and P-selectin mediated leukocyte adhesion both in vitro and in vivo.

Leukocytes and platelets have both been implicated in the pathogenesis of I/R injury in a variety of organs, including heart, brain, liver, and intestine. Studies on peripheral vascular beds like the mesentery, liver, and skeletal muscle have demonstrated that the inflammatory responses to I/R are greatly exacerbated in the presence of hypercholesterolemia. This has led to the proposal that this risk factor not only renders tissues more likely to experience an ischemic episode, but it also increases the vulnerability of organs to microvascular dysfunction and tissue injury when exposed to an ischemic insult. The results of the present study provide the first evidence for a similar influence of hypercholesterolemia on the brain microcirculation. We found that the recruitment of leukocytes and particularly platelets were exaggerated after focal ischemia and reperfusion in the presence of hypercholesterolemia. Because the enhanced blood cell recruitment occurred in the absence of a further increase in DHR oxidation within and surrounding the cerebral venules, it appears unlikely that a greater oxidative stress can account for the exaggerated response. Alternatively, the more intense inflammatory response may reflect an enhanced generation of inflammatory mediators (lipid mediators, cytokines) whose production is not linked to oxidative stress.

Our results are interesting and potentially important in light of the generally held view that serum cholesterol is a poor predictor for stroke risk. It would appear from our work that the cerebral microcirculation responds to either I/R and/or hypercholesterolemia in a manner that is quite similar to other vascular beds. The unexpected beneficial effects of statins in stroke patients may reflect an action of these agents to prevent the exaggerated inflammatory and prothrombogenic responses, and consequent microvascular dysfunction that results from the combination of I/R and hypercholesterolemia.

Acknowledgments

This work was supported by a grant from the National Heart, Lung, and Blood Institute (HL26441).
Cerebral Microvascular Responses to Hypercholesterolemia: Roles of NADPH Oxidase and P-Selectin

_Circ Res._ 2004;94:239-244; originally published online December 11, 2003;
doi: 10.1161/01.RES.000011524.05779.60

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 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/94/2/239

_Permisos_: 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/