Modulation of Ischemia/Reperfusion-Induced Microvascular Dysfunction by Nitric Oxide

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Abstract Leukocyte–endothelial cell adhesion and an altered metabolism of endothelial cell–derived nitric oxide (NO) have been implicated in the microvascular dysfunction associated with ischemia/reperfusion (I/R). The objective of this study was to determine whether NO donors can attenuate the reperfusion-induced increase in venular albumin leakage via an effect on leukocyte–endothelial cell adhesion. Leukocyte adherence and emigration as well as albumin extravasation were monitored in single postcapillary venules in rat mesentery subjected to 20 minutes of ischemia followed by 30 minutes of reperfusion. This I/R protocol elicits significant leukocyte adherence and emigration as well as a profound albumin leakage response. Superfusion of the mesenteric microcirculation with the NO donors sodium nitroprusside, spermine-NO, and SIN1 significantly reduced the I/R-induced leukocyte adherence/emigration and albumin leakage in postcapillary venules, whereas neither spermine nor the NO synthase inhibitor N\textsuperscript{G}-nitro-L-arginine methyl ester affected the I/R-induced responses. Platelet-leukocyte aggregation and mast cell degranulation were also observed in the postischemic mesentery, and the responses were also attenuated by the NO donors. Plasma nitrate/nitrite levels in the superior mesenteric vein were significantly reduced by I/R. The results of this study indicate that I/R-induced microvascular dysfunction (albumin leakage) is attenuated by NO and that the protective effect of NO donors may be related to their ability to reduce leukocyte–endothelial cell and leukocyte-platelet interactions and/or mast cell degranulation. (Circ Res. 1994;74:376-382.)

Key Words • vascular permeability • leukocyte–endothelial cell adhesion • mast cell degranulation • platelet-leukocyte aggregation

There is a growing body of evidence that implicates neutrophils in the pathophysiology of ischemia/reperfusion (I/R) injury. Intravital microscopic studies of tissues exposed to I/R have revealed an acute inflammatory response that is characterized by enhanced protein efflux and an increased adherence and emigration of leukocytes in postcapillary venules.\textsuperscript{1-4} The latter observation, coupled to reports that monoclonal antibodies that prevent leukocyte–endothelial cell adhesion also attenuate I/R-induced increased microvascular permeability,\textsuperscript{5-7} has led to the recognition that leukocyte–endothelial cell adhesion may be a rate-limiting step in the pathogenesis of I/R-induced tissue injury. Although the inflammatory response elicited by I/R has been extensively characterized, the mechanisms underlying this phenomenon remain poorly understood. Several factors, including reactive oxygen metabolites,\textsuperscript{8-12} leukotrienes,\textsuperscript{2} platelet-activating factor,\textsuperscript{13} and nitric oxide (NO),\textsuperscript{14} have been implicated in the leukocyte–endothelial cell adhesion and vascular protein leakage associated with I/R. It has been proposed that enhanced superoxide production in postischemic tissues leads to inactivation of endothelial cell–derived NO,\textsuperscript{15-17} which in turn results in leukocyte–endothelial cell adhesion and vascular protein leakage.\textsuperscript{14,18-21} This contention is supported by the observation that the acute inflammatory response normally observed in postischemic tissue can be mimicked by NO synthase inhibitors.\textsuperscript{22}

The concept that NO inactivation contributes to the inflammatory response elicited by I/R has led to the proposal that NO donors may be useful in preventing or attenuating reperfusion-induced leukocyte–endothelial cell adhesion and vascular protein leakage. This possibility is supported by reports that NO donors significantly reduce the adhesion of neutrophils to isolated coronary arteries derived from postischemic feline myocardium.\textsuperscript{23} In the present study, we assessed the effectiveness of NO donors in ablating the I/R-induced inflammatory response within the mesenteric microcirculation. Specifically, we addressed the possibility that NO donors attenuate the leakage of albumin from postcapillary venules exposed to I/R by inhibiting leukocyte–endothelial cell adhesion and/or leukocyte-platelet aggregation within these microvessels. In addition, we examined the influence of NO donors on I/R-induced mast cell degranulation.

Materials and Methods

Surgical Procedure

Male Sprague-Dawley rats (200 to 250 g) were maintained on a purified laboratory diet and fasted for 24 hours before each experiment. The animals were initially anesthetized with pentobarbital (65 mg/kg body weight), then a tracheotomy was performed to facilitate breathing during the experiment. The right carotid artery was cannulated, and systemic arterial blood pressure was measured with a Statham P23A pressure transducer (Oxnard, Calif) connected to the carotid artery cannula. Systemic blood pressure and heart rate were continuously recorded with a Grass physiological recorder (Grass Instruments, Quincy, Mass). The left jugular vein was also cannulated for drug administration.

Intravital Microscopy

Rats were placed in a supine position on an adjustable Plexiglas microscope stage, and the mesentery was prepared
for microscopic observation as described previously. Briefly, the mesentery was draped over a nonfluorescent coverslip that allowed for observation of a 2-cm² segment of tissue. The exposed bowel wall was covered with Saran Wrap (Dow Chemical Co); then the mesentery was superfused with bicarbonate-buffered saline (37°C, pH 7.4) that was bubbled with a mixture of 5% CO₂ and 95% N₂, which exposes mesenteric tissue to an oxygen tension of approximately 40 mm Hg.

An inverted microscope (TMD-2S, Diaphoto, Nikon, Tokyo, Japan) with a ×40 objective lens (Fluor, Nikon) was used to observe the mesenteric microcirculation. The mesentery was transilluminated with a 12-V, 100-W DC-stabilized light source. A video camera (VK-C150, Hitachi, Ibaregi, Japan) mounted on the microscope projected the image onto a color monitor (PVM-2030, Sony, Tokyo, Japan), and the images were recorded with a videocassette recorder (NVHS950, Panasonic, Tokyo, Japan). A video time-date generator (WJ810, Panasonic) projected the time, date, and stopwatch function onto the monitor.

Single unbranched venules with diameters of 25 to 35 μm and length >150 μm were selected for study. Venular diameter was measured either on- or off-line with a video caliper (Microcirculation Research Institute, Texas A&M University, College Station, Tex). Red blood cell (RBC) centerline velocity was measured in venules with an optical Doppler velocimeter (Microcirculation Research Institute). The velocimeter was calibrated against a rotating glass disk coated with RBCs. Venular blood flow was calculated from the product of mean RBC velocity \( V_{mean} \) (centerline velocity/1.6) and microvascular cross-sectional area, assuming cylindrical geometry. Wall shear rate (γ) was calculated based on the Newtonian definition: \( \gamma = 8V_{mean}/D \), where D is diameter.

The number of adherent leukocytes was determined offline during playback of videotaped images. A leukocyte was considered to be adherent to venular endothelium if it remained stationary for a period ≥30 seconds. Adherent cells were expressed as the number per 100-μm length of venule. The number of emigrated leukocytes was also determined offline during playback of videotape images. Any interstitial leukocytes present in the mesentery at the onset of the experiment were subtracted from the total number of leukocytes that accumulated during the course of the experiment. Leukocyte emigration was expressed as the number per field of view surrounding the venule. Platelet-leukocyte aggregates visible within postcapillary venules were quantified and expressed as the number of aggregates crossing a fixed point within the venule over a 5-minute period. To visualize mast cells surrounding the mesenteric microvasculature, 0.1% toluidine blue was added onto the mesentery at termination of each experiment. The number of intact and degranulated mast cells was determined, and the percentage of degranulated mast cells was calculated.

To quantify albumin leakage across mesenteric venules, 50 mg/kg of FITC-labeled bovine albumin (Sigma Chemical Co, St Louis, Mo) was administered intravenously to the animals 15 minutes before each experiment. Fluorescence intensity (excitation wavelength, 420 to 490 nm; emission wavelength, 520 nm) was detected with a silicon-intensified target camera (C-2400-08, Hamamatsu Photonics, Shizuoka, Japan). The fluorescence intensity of FITC-albumin within three segments of the venule under study and in three contiguous areas of periluminal interstitium area was measured at various times after administration of FITC-albumin with a computer-assisted digital imaging processor (NIH Image 1.35 on a Macintosh computer). An index of vascular albumin leakage was determined from the ratio of interstitial intensity to venule intensity at specific intervals after reperfusion of the ischemic intestine.

### Experimental Protocols

After all parameters measured on-line were in a steady state, images from the mesenteric preparation were recorded on videotape for 10 minutes. Immediately thereafter, the superior mesenteric artery (SMA) was ligated with a snare created from polyethylene tubing. The mesentery was made ischemic for 0 (sham operation) or 20 minutes. After the ischemic period, the snare was gently released. In some experiments, either sodium nitroprusside (SNP) (100 μmol/L; Sigma), spermine-NO (100 μmol/L; supplied by Dr Larry Keefer, National Cancer Institute, Frederick, Md), SIN1 (100 μmol/L), or spermine (100 μmol/L; Sigma) was added to the superfusate beginning 15 minutes before SMA occlusion and lasting throughout the reperfusion period; otherwise, the same protocol was used. A similar protocol was used for experiments employing N⁴-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L; Sigma); however, since this agent is known to elicit an inflammatory response in normal rat mesentery, it was added to the superfusate at the time of reperfusion. In some experiments, plasma samples were obtained from the carotid artery and superior mesenteric vein under baseline conditions (before I/R) and at 30 minutes after reperfusion. These samples were used to determine the plasma nitrite and nitrate levels, an index of NO formation.

The use of a total arterial occlusion model of I/R injury limited our ability to assess the changes that are due to ischemia per se. Some measured variables such as leukocyte adherence and emigration, venular wall shear rate, and platelet-leukocyte aggregation could not be readily assessed immediately before reperfusion. However, in five additional experiments, we were able to stain mast cells by superfusion of the mesentery with toluidine blue to assess whether ischemia per se elicited mast cell degranulation.

### Plasma Nitrite and Nitrate Determinations

Plasma levels of nitrite and nitrate in fasted rats were determined with a minor modification of the method of Granger et al22 in which all nitrate is reduced to nitrite by use of Escherichia coli nitrate reductase. Nitrate reductase was induced in E coli (ATCC 25922; Difeo Bactrol Disks) grown anaerobically in the presence of high levels of nitrate. After an 18-hour incubation period, bacteria were washed by centrifugation with phosphate-buffered saline (PBS) to remove the excess nitrate. The bacterial cell pellet was then suspended in PBS at a concentration of 100 mg/mL and frozen at −70°C. Small aliquots (100 μL) of plasma were diluted fivefold by addition of 400 μL deionized water, and protein was precipitated by addition of 1.5% ZnSO₄. Precipitant was removed by centrifugation and the supernatant saved on ice. To the supernatants, HEPES buffer and ammonium formate were added to achieve final concentrations of 100 and 300 mmol/L, respectively. Twenty-five microliters of the thawed E coli was then added to each tube and incubated for 60 minutes at 37°C. Bacteria were removed by centrifugation, and total nitrite was determined by the Griess reaction.27

### Statistics

The data were analyzed by standard statistical analysis, ie, one-way analysis of variance and Scheffé’s (post hoc) test. All values are reported as mean±SEM from six rats, and statistical significance was set at \( P < .05 \).

### Results

In untreated (control) rats, the RBC velocity and wall shear rate in mesenteric venules were 3.1±0.18 mm/s and 535±6 seconds⁻¹, respectively, under baseline conditions (see Table). During occlusion of the SMA, blood flow ceased within mesenteric venules. Ischemic periods of up to 20 minutes were associated with significant and sustained reperfusion; ie, RBC velocity (1.94±0.13...
mm/s) and wall shear rate (328±16 seconds⁻¹) were restored toward normal values after release of the SMA occlusion. The values for RBC velocity and wall shear rate obtained at 30 minutes after reperfusion were not significantly different from the values measured at 10 and 20 minutes after reperfusion. Longer durations of ischemia (≥30 minutes) were not associated with a consistent reperfusion response, with flow rarely occurring to a significant extent after release of the SMA occlusion. Consequently, measurements of leukocyte–endothelial cell adhesion and albumin leakage were obtained only in mesenteric venules exposed to 20 minutes of ischemia. The NO donors SNP, spermine-NO, and SIN1, but not spermine or L-NAME, prevented the reductions in RBC velocity and wall shear rate normally observed in mesenteric venules after reperfusion.

Fig 1 presents the number of adherent (A) and emigrated (B) leukocytes and the albumin leakage (C) responses elicited by 20 minutes of ischemia followed by reperfusion. The numbers of adherent and emigrated leukocytes were significantly elevated at 10 minutes after reperfusion and increased progressively thereafter. In animals subjected to 20 minutes of sham ischemia (manipulation of the SMA) and 30 minutes of “reperfusion,” leukocyte adherence was 2.6±0.8 per 100 μm, with 1.4±0.7 emigrated leukocytes per field and an albumin leakage index of 8.3±1.6%. Corresponding values obtained in mesenteric preparations exposed to 20 minutes of ischemia and 30 minutes of reperfusion were 18.4±1.0 per 100 μm, 8.8±0.8 per field, and 48.1±4.0%, respectively. Spermine-NO and SIN1 reduced leukocyte adherence by about 60%, and SNP reduced adherence by 40%. No significant changes in leukocyte adherence were noted in I/R preparations exposed to either spermine or L-NAME (Fig 1A). A similar pattern of effectiveness in reducing leukocyte emigration (Fig 1B) was observed with the different NO donors; ie, SNP, spermine-NO, and SIN1 reduced the number of emigrated leukocytes by 29% to 57%, 64% to 71%, and 68% to 75%, respec-
tively, whereas spermine and L-NAME had no effect. The number of adherent or emigrated leukocytes observed at 30 minutes after I/R was found to be inversely related to wall shear rates (adherence versus wall shear rate, \( y = 28.306 - 0.04Q_x, r = .601, P < .05 \); emigration versus wall shear rate, \( y = 13.281 - 0.02Q_x, r = .549, P < .05 \)). Fig 1C illustrates that the large increase in albumin leakage induced by I/R was significantly attenuated by SNP (46% to 63%), spermine-NO (70%), and SIN1 (69% to 71%) at both 10 and 30 minutes after reperfusion. Spermine and L-NAME had no effect on I/R-induced albumin leakage.

Fig 2 illustrates the dependence of I/R-induced albumin leakage in single venules on the number of adherent (A) and emigrated (B) leukocytes. All values were derived from the 30-minute values presented in Fig 1. Albumin leakage was highly correlated with both leukocyte adherence \( (r = .800, P < .05) \) and leukocyte emigration \( (r = .746, P < .05) \). Albumin leakage was greater in regions of the venule that exhibited a high level of leukocyte adherence/emigration than in regions exhibiting little or no adherence/emigration. The tight coupling between the magnitude of I/R-induced albumin leakage and the number of adherent or emigrated leukocytes suggests that the effectiveness of NO donors in blunting the albumin leakage response is related to their ability to attenuate leukocyte-endothelial cell adhesion.

Exposure of the rat mesentery to I/R was frequently associated with the appearance of large platelet-leukocyte aggregates that filled the venule lumen and coursed rapidly through the vessel with flowing blood. These aggregates have been previously described by other investigators as “flying thrombi”22 and have been observed in mesenteric venules exposed to the NO synthase inhibitor L-NAME.22 Although such aggregates were never observed during control conditions, 12.2 ± 1.4 aggregates per 5 minutes were observed in venules exposed to 20 minutes of ischemia and 30 minutes of reperfusion. Fig 3 summarizes the effects of NO donors on I/R-induced formation of platelet-leukocyte aggregates. Reductions in aggregate formation were noted in animals treated with either SNP, spermine-NO, or SIN1, but not with spermine or L-NAME.

Fig 4 presents the percentage of degranulated mast cells along the postcapillary venules at 30 minutes after reperfusion. In the control group (sham-operated rats), degranulated cells represented <5% (4.2 ± 1.9%) of total mast cell population situated along postcapillary venules. Twenty minutes of ischemia without reperfusion did not significantly increase the number of degranulated mast cells (5.2 ± 2.2%) above the value obtained in the control group. At 30 minutes after reperfusion, degranulated mast cells increased to approximately 35%. NO donors including SNP, spermine-NO, and SIN1 significantly inhibited the I/R-induced degranulation of mast cells, whereas neither spermine nor L-NAME exerted a significant influence on this response.

Fig 5 compares the plasma concentrations of nitrite and nitrate in carotid artery and superior mesenteric vein before and after I/R. The sum of nitrite and nitrate in superior mesenteric vein was 25.8 ± 1.8 μmol/L under baseline conditions, which decreased to 15.7 ± 2.7 μmol/L at 30 minutes after reperfusion; however, the nitrite/nitrate concentration in the carotid artery was unaltered.
by intestinal I/R (20.3±4.2 versus 15.5±1.8 μmol/L). An approximation of the decline in NO production resulting from I/R can be derived from the changes in the product of venular blood flow and the arteriovenous difference in nitrite/nitrate concentrations estimated before and after I/R. This approach predicts that I/R elicits a 98% reduction of NO production in rat mesentery.

**Discussion**

Depletion and/or inactivation of NO has been implicated as a key event in eliciting the acute inflammatory response observed in tissues exposed to I/R. A major objective of this study was to determine whether restoration of NO levels in posts ischemic rat mesentery with NO donors will result in an attenuation of the microvascular alterations normally observed in this model of acute inflammation. I/R of the rat mesentery is associated with an increased number of adherent and emigrated leukocytes, increased albumin extravasation, and the formation of platelet-leukocyte aggregates within postcapillary venules. The mast cells surrounding mesenteric microvessels also respond to I/R by degranulating. The results of the present study indicate that all of these inflammatory responses to I/R are significantly attenuated by exposure of the mesentery to NO donors.

In the present study, nitrite/nitrate levels in the superior mesenteric vein were used to determine whether I/R affects NO production in posts ischemic intestine. Our results indicate that 20 minutes of occlusion of the SMA and subsequent release led to a reduction in nitrite/nitrate levels, suggesting that I/R probably results in an inhibition or inactivation of NO synthase. The enhanced superoxide production that occurs in posts ischemic tissues would also reduce the bioavailability of endothelial cell–derived NO, although this effect would not be reflected as a change in plasma nitrite/nitrate. Consequently, one would predict that I/R should significantly reduce tissue NO levels as a result of both NO synthase inactivation and superoxide-mediated NO inactivation. Such a profound reduction in NO levels would explain why L-NAME, the NO synthase inhibitor, failed to exacerbate the leukocyte–endothelial cell adhesion and vascular protein leakage elicited by I/R.

An important observation of the present study is that NO donors effectively attenuate the leukocyte adherence and emigration in postcapillary venules elicited by I/R. These observations are consistent with in vitro studies that indicate that NO is an antiadhesive molecule. For example, when endothelial monolayers were exposed to constant shear rates in ex vivo superfusion flow chambers, high concentrations of l-arginine (2 mmol/L), the substrate for NO, reduced baseline leukocyte adhesion by approximately 25%. Based on the premise that L-arginine allowed for increased NO production , the authors hypothesized that NO attenuates baseline neutrophil–endothelial cell interactions under shear conditions. Another group of investigators has reported that basal (unstimulated) monocyte adhesion to porcine aortic endothelial cell monolayers is inhibited by exposure of the leukocytes to 90% pure NO (100 μmol/L). The mechanisms by which NO donors decrease leukocyte–endothelial cell adhesion remain unclear, but several possibilities have been proposed, including inactivation of superoxide, which is known to promote leukocyte adhesion, preventing neutrophil activation and adhesion molecule expression and/or by increasing vascular shear rate.

Previous studies from our laboratory indicate that I/R-induced leukocyte adhesion in mesenteric venules involves an interaction between CD11/CD18 on neutrophils with intercellular adhesion molecule-1 on endothelial cells. Our observation that NO donors attenuate I/R-induced adhesion raises the possibility that these agents interfere with the expression and/or function of these adhesion molecules. The NO donors either could directly affect the leukocytes and/or endothelial cells or may act by inhibiting the production of inflammatory mediators by other cell types, such as mast cells and platelets.

It is well known that alterations in venular shear rate can exert a significant influence on leukocyte adhesion in postcapillary venules. Reductions in venular shear rate tend to promote leukocyte adhesion, whereas in-
creases in shear rate would be expected to decrease leukocyte adhesion.\textsuperscript{35} In the present study, we noted a significant (approximately 40\%) reduction in venular shear rate after I/R. Furthermore, all of the NO donors prevented this reduction in shear rate. These observations raise the possibility that NO donors attenuate I/R-induced adhesion by increasing wall shear rate. Data obtained from feline mesenteric venules suggest that the 40\% reduction in wall shear rate observed in this study is not likely to elicit a significant adhesion response.\textsuperscript{30} However, it is possible that rat mesenteric venules may sustain a higher level of leukocyte adherence at low shear rates compared with those of cats. Indeed, our observation that the magnitude of the leukocyte adhesion (adherence and emigration) response elicited by I/R is inversely related to wall shear rate suggests that this possibility cannot be readily dismissed.

One of the major effects of I/R on postcapillary venules is the formation of platelet-leukocyte aggregates. Our previous work indicates that the formation of these aggregates involves an interaction between P-selectin on platelets and its ligand on leukocytes.\textsuperscript{7,22} We have shown that exposure of rat mesenteric venules to the NO synthase inhibitor L-NAME elicits the formation of platelet-leukocyte aggregates, which is also attenuated by a P-selectin–specific monoclonal antibody.\textsuperscript{22} The latter observation indicates that NO is an important factor that serves to prevent platelet-leukocyte aggregation under normal physiological conditions and that conditions associated with reduced NO production or enhanced NO inactivation are associated with an enhanced rate of formation of these aggregates. Thus, it is not entirely surprising that platelet-leukocyte aggregates are formed in postischemic venules and that NO donors are so effective in preventing the formation of these aggregates. Thus, the results of the present study, coupled with our previous work, would suggest that the NO donors interfere with either the expression or function of P-selectin on the surface of I/R-activated platelets. The contribution of the platelet-leukocyte aggregates to the pathobiology of I/R injury remains unclear; however, it is conceivable that these structures exacerbate the low microvascular perfusion observed in postischemic tissues and that they may contribute to the distant organ injury observed after reperfusion of the ischemic intestine.\textsuperscript{36}

An additional observation in this study was the significant mast cell degranulation elicited by I/R and the ability of NO donors to prevent this degranulation response. Previous reports have demonstrated that NO synthase inhibitors cause mast cell degranulation.\textsuperscript{37} Furthermore, in vitro studies indicate that NO donors may stabilize mast cells.\textsuperscript{38} Consequently, our results suggest that the reduction in NO production and the NO inactivation associated with I/R ultimately results in destabilization of mast cell membranes and the subsequent release of mast cell products in the vicinity of postcapillary venules. Mast cells are known to release a variety of substances that could elicit the leukocyte–endothelial cell adhesion, leukocyte-platelet aggregation, and vascular protein leakage associated with I/R. These include platelet-activating factor, histamine, leukotrienes, and the superoxide anion.\textsuperscript{39–41}

A major finding in the present study was that the NO donors effectively attenuated the increased albumin leakage elicited by I/R. This protective effect of NO donors is consistent with our observation that NO synthase inhibitors cause an increased leakage of albumin in mesenteric venules.\textsuperscript{22} We also demonstrated that L-NAME–induced vascular protein leakage is significantly reduced by prior administration of monoclonal antibodies that prevent leukocyte–endothelial cell adhesion.\textsuperscript{19,22} The latter observation raises the possibility that NO donors reduce vascular protein leakage by virtue of their ability to attenuate leukocyte adhesion and emigration. The coupling between I/R-induced vascular protein leakage and either the number of adherent or emigrated leukocytes (Fig 2) lends credence to this interpretation. However, the data presented in Fig 2 also suggest that the protective effect of the NO donors cannot be attributed entirely to inhibition of leukocyte–endothelial cell adhesion. It is conceivable that substances derived from activated platelets and/or mast cells may also contribute to the vascular leakage response and that the NO donors may afford protection by stabilizing or inactivating these cells. We previously reported that L-NAME–induced albumin leakage in rat mesenteric venules is slightly but significantly attenuated by an antibody against P-selectin,\textsuperscript{22} which virtually abolished the formation of platelet-leukocyte aggregates. This observation suggests that platelets may make only a small contribution to the vascular leakage observed in conditions associated with reduced NO production and/or NO inactivation.

**Acknowledgments**

This study was supported by a grant from the National Heart, Lung, and Blood Institute (HL-26441). The authors thank Michael Gibson for his technical assistance with the nitrite/nitrate assays.

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Modulation of ischemia/reperfusion-induced microvascular dysfunction by nitric oxide.
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Circ Res. 1994;74:376-382
doi: 10.1161/01.RES.74.3.376
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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