Na⁺/H⁺ Exchange Inhibitor Cariporide Attenuates Cell Injury Predominantly During Ischemia and Not at Onset of Reperfusion in Porcine Hearts With Low Residual Blood Flow

Hermann H. Klein, MD; Sibylle Pich, PhD; Rainer M. Bohle, MD; Stephanie Lindert-Heimberg; Klaus Nebendahl, VMD

Background—This study investigated whether myocardial protection by inhibition of Na⁺/H⁺ exchange (NHE) occurs during ischemia and/or during reperfusion.

Methods and Results—The left anterior descending coronary artery was occluded in 32 pigs for 60 minutes and then reperfused for 24 hours. Infarct sizes (nitroblue tetrazolium [NBT] stain, histology) were determined at the end of the experiments. An extracorporeal bypass was used to achieve a constant residual blood flow of 3 mL/min in the myocardium at risk during ischemia. The NHE-1 inhibitor cariporide or distilled water was infused into the extracorporeal bypass system. In group 1, active treatment was administered from the onset of ischemia until 10 minutes of reperfusion (n = 8). In group 2, active treatment was infused during the first 30 minutes of ischemia only (n = 8). The group 3 animals (n = 8) received intracoronary cariporide after 45 minutes of ischemia until 10 minutes of reperfusion. The control animals (group 4, n = 7) were treated similarly to group 1 animals, with the cariporide solution being replaced by distilled water. Infarct sizes of group 1 (NBT stain, 41.5 ± 20%; histology, 44.6 ± 12%) and group 2 (NBT stain, 33.5 ± 14%; histology 34.9 ± 15%) differed significantly (at least P = 0.012) from infarct sizes of group 3 (NBT stain, 71.6 ± 15%; histology, 69.2 ± 12%) and the control group (NBT stain, 76 ± 9%; histology 72.4 ± 12%). Cariporide treatment in group 1 and group 2 significantly improved functional recovery after 24 hours of reperfusion.

Conclusions—Myocardial protection by cariporide is predominantly achieved by NHE inhibition during ischemia and not during early reperfusion. (Circulation. 2000;102:1977-1982.)

Key Words: infarction ■ ischemia ■ reperfusion ■ ions

Na⁺/H⁺ exchange (NHE) inhibition has been shown to substantially protect the ischemic, reperfused myocardium in different species and in a great variety of preparations (summarized in Reference 1). It is still a matter of controversy whether myocardial protection by Na⁺/H⁺ exchange inhibition takes place during ischemia and/or during reperfusion. Preischemic intravenous treatment with the NHE-1 inhibitors HOE694,2,3 cariporide4–6 and EMD 851317 in regionally ischemic, reperfused pig, rabbit, and dog hearts reduced infarct size to a highly significant extent.2–7 Three of these 6 studies did not find a significant decrease in infarct size by an intravenous treatment at the time of reperfusion,2,4,5 whereas the other 3 studies5–7 reported a significant reduction in infarct size under these circumstances. Although these results favor myocardial protection by NHE-1 inhibition during ischemia, it cannot not be ruled out that the positive effects of preischemic treatment were achieved at the time of reperfusion and that NHE-1 inhibition induced by a treatment at the time of reperfusion does not occur fast enough to counteract the rapid changes in Na⁺/H⁺ exchange on reperfusion. This study in regionally ischemic, reperfused porcine hearts with low residual blood flow was designed to clarify whether myocardial protection by cariporide (HOE642, 4-isopropyl-3 methylsulphonylbenzoyl-guanedine methanesulphonate) takes place during ischemia or at the onset of reperfusion.

Methods

The experiments were approved by the government of Lower Saxony, Germany, and in accordance with institutional guidelines.

General Experimental Setup

Anesthesia, medication, and the general experimental setup have been described in previous studies.2,8 Thirty-seven farm pigs (37 to 51 kg) were used in this study. Each was premedicated with azaperon, metamidate hydrochloride, and atropine. General anesthesia was maintained with metamidate hydrochloride and intravenous piritramide. Artificial ventilation was performed with nitrous oxide and oxygen. All animals were anticoagulated with intravenous
heparin (10 000 IU) before the first arterial catheter was introduced. Another 10 000 IU heparin was given intravenously before the extracorporeal bypass system was introduced.

After median thoracotomy, the left anterior descending coronary artery (LAD) was dissected free distal to the first diagonal branch. Left ventricular (LV) pressure and its first derivative (dP/dt) were measured with a 5F Millar catheter-tipped manometer. A 5F catheter was placed into the ostium of the great cardiac vein. The LAD was occluded at the prepared site for 60 minutes and then reperfused for 24 hours. Forty-five minutes after the onset of reperfusion, the chest was closed in layers and the animal was allowed to recover. On the following day, the pig was reanesthetized and the thoracotomy was repeated.

Measurement of Global Hemodynamics, Regional Systolic Shortening, and LAD Blood Flow

Global hemodynamic variables, including LV peak pressure (LVPP), LV end-diastolic pressure (LVEDP), dP/dt max, and heart rate were monitored continuously and recorded before coronary artery occlusion, at 5 minutes intervals during ischemia, during the first 45 minutes of reperfusion, and at the end of the experiment.

One pair of ultrasonic crystals was implanted in the midmyocardial layer of the risk region. The end-diastolic distance of the crystals (EDD) was determined at the onset of ventricular systole. The end-systolic distance (ESD) was defined by peak negative dP/dt. Relative systolic shortening (SS%) was assessed as the difference of EDD minus ESD over EDD×100. SS% was recorded before treatment, immediately before coronary artery occlusion, immediately before reperfusion, after 45 minutes of reperfusion, and at the end of the experiment after thoracotomy had been repeated. EDD was additionally recorded after 10 minutes of reperfusion. EDD values were normalized to 100% before ischemia.

The LAD blood flow (mL/min) was recorded with a 1- or 2-mm ultrasonic flow probe (T-106 Flowmeter, Transonic Systems Inc) at the same time intervals as global hemodynamic parameters. The flow probe was placed around the coronary artery distal to the site of occlusion.

Plasma Concentrations of Cariporide

Coronary venous plasma concentrations of cariporide were determined after 30 minutes of ischemia (groups 1 and 2), immediately before reperfusion, and after 1 minute of reperfusion. In addition, venous plasma concentrations of cariporide were assessed immediately after reperfusion. Measurements were performed by Aventis, with a high-performance liquid chromatography method.

Histochemical Measurement of Infarct Size

After 24 hours of reperfusion, the heart was excised after occlusion of the coronary artery at exactly the same site and after administration of a central venous injection of 10 mL 10% fluorescein sodium solution. The heart was cut into slices of ~5-mm width parallel to the anteriocentric groove. All slices containing reperfused myocardium were weighed and photographed under ultraviolet light. The slices proximal to the inserted crystals (in general, 5) were stained with nitroblue tetrazolium (NBT) solution to assess the infarcted area at risk of necrosis was cut out from the second proximal slice toward the apex under fluorescent illumination. The tissue was routinely paraffin-embedded, serially sectioned, stained with hematoxylin and eosin (HE), andquantitatively analyzed by light microscopy. Infarct size was calculated as the sum of necrotic fields in relation to the entire risk region and expressed as a percentage of the risk region.

Extracorporeal Bypass System

An extracorporeal bypass was used to achieve a constant residual blood flow of 3 mL/min during ischemia. A 5F catheter was introduced into the femoral artery and connected through the silicone tubing of a laboratory pump with a 4F end-hole catheter that was advanced into the LAD ~0.3 cm distal to the planned site of occlusion. A 3-way stop cock was placed between the femoral catheter and the pump tubing to allow a continuous infusion of heparin (500 IU/h). An in-line ultrasonic flow probe (T-106 Flowmeter, Transonic Systems Inc) was positioned between the pump tubing and the coronary catheter to constantly adjust the pump delivery blood into the LAD to exactly 2.8 mL/min during ischemia. Two 3-way stop cocks were positioned distal to the flowmeter for infusions of cariporide or distilled water. Residual blood flow together with the treatment solution or distilled water was administered into the proximal part of the LAD distal to the site of occlusion.

Experimental Protocol

Cariporide (0.22 mg/kg body wt) was dissolved in 100 mL distilled water. Four groups with 8 animals each were formed. The animals of group 1 were treated with an intracoronary infusion of 0.2 mL/min of the cariporide solution and 2.8 mL/min of arterial blood during the whole ischemic period (cariporide dose: 44 ng/kg per minute). At the onset of reperfusion, the coronary catheter was withdrawn into the ostium of the LAD, the extracorporeal bypass system was stopped, and the cariporide solution was infused into the proximal LAD at an infusion speed of 2 mL/min for another 10 minutes. The pigs in group 2 were treated in the same way as in group 1 for the first 30 minutes of ischemia. At that time, active treatment was replaced by distilled water. In group 3, active treatment with cariporide was started after 45 minutes of ischemia and was then identical to group 1. In the control group (group 4), which was treated according to the protocol of group 1, cariporide solution was replaced by distilled water.

Relation Between Cariporide Blood Levels and Infarct Size Reduction

The effective plasma concentration of cariporide to reduce infarct size was studied in another 5 pigs subjected to 45 minutes of distal LAD occlusion and 24 hours of reperfusion. Five minutes before ischemia, 1 pig each was treated intravenously with cariporide doses of 0.5 mg/kg, 0.25 mg/kg, 0.1 mg/kg, 0.05 mg/kg, and placebo. Venous plasma concentrations of cariporide were determined at the onset of ischemia and infarct sizes at the end of the experiments.

Statistics

All data are presented as mean±SD. Weights of the left ventricles and ischemic regions, residual blood flows, and infarct sizes among the 4 groups were compared with the Kruskal-Wallis H test followed by a Mann-Whitney U test. Hemodynamic data and parameters of regional myocardial function were analyzed among the groups with 2-way repeated-measures ANOVA. When ANOVA indicated a significant difference, the Student-Newman-Keuls post hoc test was applied to further compare the data. Statistical significance was accepted at the 5% level and in case of multiple comparisons (Mann-Whitney U test) at the 0.017 (3 comparisons) and 0.0125 level (4 comparisons).

Results

General Results

One animal of group 4 died in the postoperative night. Thus, the analyses of the control group are based on 7 experiments. Ventricular fibrillation occurred in 4 pigs (group 1, 2 pigs; group 3, 2 pigs) during ischemia and in 1 animal of group 2 during reperfusion. It was immediately terminated by electrical countershocks.
Plasma Concentrations of Cariporide
In the treated groups, venous plasma concentrations of cariporide were \( \approx 30 \) to 40 nmol/L at the time of reperfusion. Coronary venous (cv) plasma levels of cariporide were similar in group 1 (0.92 ± 0.18 μmol/L) and group 2 (0.97 ± 0.29 μmol/L) after 30 minutes of ischemia. Immediately before reperfusion, cv cariporide concentrations of group 1 (0.91 ± 0.21 μmol/L) and group 3 (0.82 ± 0.07 μmol/L) did not differ and were significantly higher than in group 2 (0.24 ± 0.1 μmol/L, \( P<0.004 \)). After 1 minute of reperfusion, cv cariporide levels were significantly higher (\( P<0.004 \)) in group 1 (1.82 ± 0.56 μmol/L) and group 3 (1.97 ± 0.81 μmol/L) compared with group 2 (0.31 ± 0.06 μmol/L).

Global Hemodynamic Variables

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Before Ischemia</th>
<th>During Ischemia</th>
<th>During 45 min of Reperfusion</th>
<th>24 h of Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LVPP, mm Hg</td>
<td>101 ± 11</td>
<td>92 ± 11</td>
<td>89 ± 11</td>
<td>85 ± 11</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>98 ± 7</td>
<td>92 ± 5</td>
<td>82 ± 12</td>
<td>79 ± 14</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>96 ± 14</td>
<td>90 ± 11</td>
<td>81 ± 9</td>
<td>80 ± 15</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>104 ± 7</td>
<td>95 ± 5</td>
<td>84 ± 2</td>
<td>87 ± 17</td>
</tr>
<tr>
<td>1</td>
<td>dP/dt max, mm Hg/s</td>
<td>1812 ± 318</td>
<td>1440 ± 307</td>
<td>1332 ± 227</td>
<td>875 ± 149</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>2038 ± 239</td>
<td>1526 ± 277</td>
<td>1299 ± 233</td>
<td>950 ± 274</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>1875 ± 320</td>
<td>1521 ± 417</td>
<td>1436 ± 245</td>
<td>1138 ± 392</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>1943 ± 585</td>
<td>1525 ± 265</td>
<td>1426 ± 247</td>
<td>1086 ± 212</td>
</tr>
</tbody>
</table>

Group 1 indicates treatment during ischemia as well as during 10 minutes of reperfusion; group 2, treatment during the first 30 minutes of ischemia; group 3, treatment after 45 minutes of ischemia until 10 minutes of reperfusion; group 4, control group. Values are mean ± SD.

Regional Myocardial Function
The EDD of the implanted crystals did not differ among the groups before ischemia and before reperfusion. EDD amounted to 10.5 ± 2.6 mm (group 1), 10.4 ± 2.8 mm (group 2), 10.4 ± 1.6 mm (group 3), and 10.7 ± 1.6 mm (group 4) before ischemia. After 10 minutes of reperfusion, the alterations in end-diastolic distance of the reperfused myocardium of groups 1 and 2 indicated no contracture (group 1, 102 ± 14%; group 2, 106 ± 10%) compared with baseline values. In contrast, EDD was reduced in group 3 to 85 ± 11% and in the control group to 66 ± 10%. Groups 1 and 2 differed significantly from groups 3 and 4. Contracture of EDD in group 3 was significantly less compared with the control group. A similar result was observed after 45 minutes of reperfusion, except for the insignificant difference between groups 3 and 4. This result indicates that treatment of groups 1 and 2 exhibited the same protection from myocardial contracture during early reperfusion, which was less pronounced when treatment was started shortly before reperfusion (group 3).

SS% did not differ among the groups before ischemia, before reperfusion, and after 45 minutes of reperfusion. Recovery of regional systolic shortening was significantly better in groups 1 and 2 compared with both other groups at the end of the experiments (Figures 1 and 2).

Histochemical and Micromorphometric Infarct Sizes
Left ventricular weights (group 1, 1336 ± 14.5 g; group 2, 1223 ± 16.8 g; group 3, 1244 ± 6.2 g; group 4, 1193 ± 11.4 g) and risk regions (group 1, 32.0 ± 7.9 g; group 2, 28.3 ± 3.2 g; group 3, 29.2 ± 4.7 g; group 4, 29.7 ± 3.6 g) did not differ significantly among the groups. Histochemical infarct sizes amounted to 41.5 ± 20% (group 1), 33.5 ± 14% (group 2), 71.6 ± 15% (group 3), and 76 ± 9% (group 4). Micromorpho-
metric infarct sizes were 44.6±12% (group 1), 34.9±15% (group 2), 69.2±12% (group 3), and 72.4±12% (group 4). Salvage of jeopardized myocardium was mainly observed in the midmyocardial and subepicardial layers. The continuous intracoronary treatment during ischemia and early reperfusion (group 1) as well as the active treatment during the first 30 minutes of ischemia (group 2) reduced histochemical and micromorphometric infarct sizes highly significantly compared with groups 3 and 4 (group 1 versus group 3, \( P = 0.003 \) [histology]; group 1 versus group 4, \( P = 0.0026 \) [NBT stain], \( P = 0.008 \) [histology]; group 2 versus group 3, \( P = 0.0008 \) [NBT stain], \( P = 0.002 \) [histology]; group 2 versus group 4, \( P = 0.0012 \) [NBT stain], \( P = 0.0012 \) [histology]). The differences between groups 1 and 2 as well as between groups 3 and 4 were not significant. These results demonstrate that continuous treatment during ischemia and during early reperfusion was not more effective than therapy with cariporide during the first half of ischemia. Of particular note is that cariporide administration during late ischemia and early reperfusion did not result in a significant protection from cell death compared with the control group. The mean infarct size of group 3 was 6% (NBT stain) or 4% (histology) smaller than in the control group. These findings show that cariporide predominantly attenuates cell death caused by ischemia and reperfusion by NHE inhibition during ischemia and not at the onset of reperfusion (Figure 3).

**Cariporide Plasma Concentration and Infarct Size Reduction**

In the pigs subjected to 45 minutes of ischemia and 24 hours of reperfusion, infarct size was reduced from 64% (control) to 4% (cariporide concentration 1.21 \( \mu \text{mol/L} \)) and to 18% (cariporide concentration 0.7 \( \mu \text{mol/L} \)). Lower plasma concentrations (0.4 and 0.16 \( \mu \text{mol/L} \)) were associated with infarct sizes of 60% and 65%.

**Discussion**

**Limitations of the Study**

This investigation was performed to clarify to what extent cariporide protects the myocardium during ischemia and/or during reperfusion. Because an intravenous treatment before ischemia can be expected also to be active at the onset of reperfusion and because an intravenous treatment before reperfusion may not be fast enough to immediately block the NHE system at the onset of reperfusion, cariporide was infused into the myocardium at risk of necrosis at different time intervals. It is known from earlier experiments that a residual blood flow of 3 mL/min results in a distinct transmural perfusion gradient with the level of subendocardial flow only being 30 to 40% of subepicardial flow.8

The dose of cariporide was chosen to achieve a coronary venous level of \( \approx 1 \mu \text{mol/L} \) during the specified ischemic intervals and a higher level at the onset of reperfusion. A concentration of 1 \( \mu \text{mol/L} \) has been shown to almost completely block the amiloride-sensitive sodium influx in rabbit erythrocytes and the NHE system in human platelets.10 This concentration was also effective in reducing infarct size in the dose-response evaluation in this study. Thus, it can be expected that the myocardial concentration of cariporide in our experiments was high enough to effectively block the myocardial NHE system at the time of active treatment. Although the intracoronary infusion of cariporide in group 2 was restricted to the first 30 minutes of ischemia, the low residual blood flow prevented a complete myocardial wash-
out of the compound during the second half of ischemia. It cannot be ruled out that this lower myocardial concentration of cariporide still offered some protection from ischemia-reperfusion injury. In contrast to almost all other groups who have investigated the effect of NHE inhibition on experimental infarct size, this study used a reperfusion long enough to delineate the necrotic myocardium by histochemical as well as histological means.

**Myocardial Protection by Na⁺/H⁺ Exchange Inhibition**

Five or even 6 isoforms of the Na⁺/H⁺ exchanger have been described. NHE-1 has been found in every tissue examined and represents the isoform involved in myocardial cytoplasmic pH and volume regulation.²,³

Characteristic features of myocardial ischemia include increased intracellular proton generation resulting in intracellular acidosis and accumulation of intracellular sodium accompanied by an increase of intracellular free calcium.⁴,⁵ Reperfusion is associated with an initially further increase followed by a decrease in intracellular sodium and an additional accumulation of intracellular calcium. It is generally accepted that intracellular calcium overload can induce cell death in myocardial ischemia and reperfusion.⁶ As early as 1985 it was hypothesized that intracellular calcium overload during reperfusion is the consequence of intracellular sodium accumulation. During myocardial ischemia, the intracellular and extracellular pH drop to low levels, rendering the NHE system inactive. At the onset of reperfusion, the pH gradient across the cell membrane results in the maximum activation of the NHE system. Intracellular protons are exchanged with extracellular sodium, with the consequence of intracellular sodium overload. Intracellular sodium is cycled with extracellular calcium by the Na⁺-Ca²⁺ exchanger, leading to calcium overload and cell death during reperfusion.⁶

**Revised Concept of Myocardial Protection From Ischemia-Reperfusion Injury by NHE Inhibition in Intact Animals**

The results of this and of previous studies of our laboratory²,³,⁶ that used the specific NHE inhibitors HOE 694 and cariporide form the basis for a revised concept of myocardial protection by NHE inhibition in regional ischemia and reperfusion in intact animals. The best protection by NHE inhibition is clearly achieved by a treatment before ischemia and in the case of a low residual blood flow, also during early ischemia. This protection cannot be ascribed to NHE inhibition at the onset of reperfusion because NHE inhibition during late ischemia⁶ and at the onset of reperfusion, group 3 in this study, did not reduce infarct size significantly in this preparation. The high coronary venous levels of cariporide in group 3 immediately before and after reperfusion can almost rule out that cariporide was not available in the myocardium at risk of necrosis at an effective concentration. Thus, attenuation of cell death is predominantly achieved by NHE inhibition during ischemia and not at the onset of reperfusion. Although extracellular-intracellular ionic shifts were not determined in our studies, the consistency of our results allows the formulation of a revised concept of myocardial protection by NHE inhibition. From the onset of ischemia, intracellular acidosis activates the NHE system, which results in acid extrusion and an increase in intracellular sodium. The rise in intracellular sodium is accompanied by an increase of intracellular free calcium.¹⁷,¹⁸ When ischemia continues, the decrease in extracellular pH attenuates the activity of the NHE system, reducing the sodium influx. Studies of cardiac Purkinje fibers suggest that acid efflux by the NHE system can still occur even when the chemical gradient for protons is reversed from an outward to an inward gradient.¹⁹ Thus, the activity of the NHE system will decrease during ischemia; however, a complete inactivation of NHE is not likely to occur. This view is supported by the results of our study,² which demonstrated that the intravenous use of cariporide 15 minutes after the onset of ischemia reduced infarct size in a preparation with low residual blood flow. The kinetics of intracellular sodium gain in the presence of NHE inhibition in ischemia of longer duration have not been reported. The attenuation of intracellular sodium accumulation by NHE inhibition is substantial during 30 minutes of ischemia.²⁰ It is very likely that NHE inhibition is able to delay the increase in intracellular sodium and probably intracellular free calcium only a limited time because infarct sizes after 45 minutes of ischemia without NHE inhibition were similar to those after 70 minutes of ischemia in the presence of NHE inhibition in regionally ischemic, reperfused porcine hearts.¹⁶ Although reperfusion immediately establishes a large transmembrane pH gradient that activates the NHE system, recovery of intracellular pH is mainly mediated by lactate and CO₂ washout and the gain in intracellular sodium is more likely caused by Na⁺-HCO₃⁻ co influx than sodium coupled acid extrusion.²¹ Because NHE inhibition at the time of reperfusion (group 3) attenuated myocardial contracture after 10 minutes of reperfusion but did not reduce infarct size significantly, it is speculated that intracellular free calcium accumulation during early reperfusion might have been reduced to some extent. However, this favorable action prevented cell death in only an insignificant amount of myocardium (4% to 6% of infarct size) in this study. Although the contribution of many details of NHE inhibition on attenuation of cell death in ischemia and reperfusion cannot be quantified, for example, delayed recovery of intracellular pH during reperfusion,²⁰,²² the delay of the increase in intracellular sodium during ischemia associated with a reduced intracellular free calcium accumulation during ischemia and to some extent during early reperfusion appears to be the key mechanism for myocardial protection by NHE inhibition.

**Conclusions**

This study in regionally ischemic, reperfused porcine hearts with low residual blood flow suggests that NHE inhibition by cariporide protects the myocardium predominantly during ischemia and not at the time of reperfusion. It is assumed that myocardial injury is mainly attenuated by a delayed increase in intracellular sodium and free calcium during ischemia and to a much lesser extent by a reduced calcium accumulation during reperfusion.
Acknowledgment

Cariporide (HOE642) was kindly provided by Aventis Pharma, Frankfurt/Main, Germany.

References


Na⁺/H⁺ Exchange Inhibitor Cariporide Attenuates Cell Injury Predominantly During Ischemia and Not at Onset of Reperfusion in Porcine Hearts With Low Residual Blood Flow
Hermann H. Klein, Sibylle Pich, Rainer M. Bohle, Stephanie Lindert-Heimberg and Klaus Nebendahl

doi: 10.1161/01.CIR.102.16.1977

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2000 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/102/16/1977

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

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

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