The Role of L-Arginine in Ameliorating Reperfusion Injury After Myocardial Ischemia in the Cat

Andrew S. Weyrich, PhD; Xin-liang Ma, MD, PhD; and Allan M. Lefer, PhD

Background. Myocardial ischemia followed by reperfusion results in endothelial dysfunction characterized by a reduced release of endothelium-derived relaxing factor (EDRF). Because EDRF has been characterized as nitric oxide, we examined the ability of L-arginine, the substrate for nitric oxide synthesis, to protect in a feline model of myocardial ischemia plus reperfusion.

Methods and Results. The effects of L-arginine were investigated in a 6-hour model of myocardial ischemia and reperfusion in pentobarbital-anesthetized cats. A bolus administration (30 mg/kg) of L-arginine, or its enantiomer D-arginine, was given followed by a continuous infusion of 10 mg/kg/min for 1 hour starting 10 minutes before reperfusion. Myocardial ischemia plus reperfusion in cats receiving D-arginine resulted in severe myocardial injury and endothelial dysfunction characterized by marked myocardial necrosis, high cardiac myeloperoxidase activity in ischemic cardiac tissue, and loss of acetylcholine- and A-23187-induced endothelium-dependent relaxation in coronary artery rings. In contrast, myocardial ischemia plus reperfusion cats treated with L-arginine exhibited a reduced area of cardiac necrosis (16±2% versus 41±5% of area at risk, p<0.01), lower myeloperoxidase activity in the ischemic region (0.3±0.08 versus 0.8±0.10 units/100 mg tissue, p<0.05), and significant preservation of acetylcholine- (p<0.01) and A-23187- (p<0.01) induced endothelial-dependent relaxation.

Conclusions. These results demonstrate the ability of L-arginine to reduce necrotic injury in a cat model of myocardial ischemia plus reperfusion, and this reduction in infarct size is associated with the preservation of endothelial function and attenuation of neutrophil accumulation in ischemic cardiac tissue. (Circulation 1992;86:279–288)

Key Words • endothelium • necrosis, myocardial • nitric oxide • coronary artery

Recent investigations have characterized endothelium-derived relaxing factor (EDRF) as nitric oxide (NO) or a labile nitroso compound that spontaneously liberates NO.1-4 In the vascular system, NO release from endothelial cells accounts for vasorelaxation in vessels5 as well as inhibition of platelet6 and neutrophil7 aggregation. NO is produced from the semi-essential amino acid L-arginine as it is converted to L-citrulline by NO synthase in endothelial cells.8-11 Furthermore, cultured endothelial cells synthesize NO from the terminal guanidino nitrogen of L-arginine.8

Whether exogenous administration of L-arginine can modulate vascular tone in vivo is equivocal.12-14 Several investigators have demonstrated that sufficient amounts of endogenous L-arginine are available to saturate NO synthase and that the addition of exogenous L-arginine does not enhance endothelium-dependent relaxation in normal vessels.14-16 However, in reperfusion injury, endothelium-dependent relaxation is impaired, characterized by decreased release of NO after 2.5 minutes of reperfusion.17 Furthermore, NO donors given during the course of reperfusion preserve endothelial function and significantly decrease myocardial injury.18 Thus, maintaining NO levels during reperfusion can effectively minimize endothelial and myocardial damage associated with ischemia/reperfusion.

Because L-arginine is the substrate of NO synthesis and NO release is significantly attenuated after reperfusion, we hypothesized that the administration of exogenous L-arginine would maintain NO levels and thereby attenuate endothelial dysfunction and myocardial necrosis after ischemia/reperfusion. Accordingly, the objectives of this study were to evaluate the cardioprotective effects of exogenous L-arginine administration in myocardial ischemia/reperfusion, and determine the mechanisms of any observed cardioprotective effects of L-arginine, particularly as they relate to endothelial function.

Methods

Adult male cats were anesthetized with sodium pentobarbital (30 mg/kg i.v.). An intratracheal cannula subsequently was inserted through a midline incision, and all cats were placed on intermittent positive-pressure ventilation (small animal respirator; Harvard Apparatus, Dover, Mass.). A polyethylene catheter was...
inserted through the right femoral artery for measurement of mean arterial blood pressure (MAP) via a strain-gauge pressure transducer (Statham P23AC; Gould, Inc., Cleveland, Ohio). Additional polyethylene catheters were inserted through the right external jugular vein for infusion of anesthesia and through the right femoral vein for infusion of test agent or its vehicle. A midsternal thoracotomy was performed, and the pericardium was opened to allow placement of a 3-0 silk ligature around the left anterior descending coronary artery (LAD) 8–10 mm from its origin. Standard lead II of the scalar ECG was used to record ST segment elevation and heart rate. The ECG and MAP were recorded continuously on a Grass Instrument model 7 oscillographic recorder (Quincy, Mass.). ST segment elevations were determined from an ECG recording at 25 mm/sec every 20 minutes.

**Experimental Protocol**

All cats were allowed to stabilize for 30 minutes, and a baseline reading of ECG and MAP was recorded. Myocardial ischemia was produced by tightening the ligature that was previously placed around the LAD to completely occlude the vessel. This was designated as time 0. After 1.5 hours of ischemia, the LAD ligature was untied, and the ischemic myocardium was reperfused for 4.5 hours, resulting in a total postocclusion period of 6 hours. Ten minutes before reperfusion, a bolus (30 mg/kg) of the semisential amino acid L-arginine (Sigma Chemical, St. Louis, Mo.) or its enantiomer D-arginine (Sigma Chemical) was injected intravenously, followed immediately by an infusion of 10 mg/kg·min⁻¹ that continued for 60 minutes (i.e., terminated 50 minutes into the reperfusion period). This dose of L-arginine is very similar to those of Girerd et al.¹³ and Cooke et al.¹⁴ which were used in hypercholesterolemic rabbits. The animals were randomly divided into three groups: sham myocardial ischemia plus L-arginine (subjected to all experimental manipulations except that the ligature around the LAD was not tightened, n=5), myocardial ischemia plus L-arginine (n=6), or myocardial ischemia plus D-arginine (n=5).

**Myocardial Tissue Analysis**

At the end of the 6-hour experimental period, the ligature around the LAD was retightened, and 25 ml of 0.5% Evans blue was injected into the left atrium and circulated to the area of the myocardium that was perfused by the patent coronary arteries. Thus, the area at risk was determined by negative staining. The heart was excised rapidly and placed in warm, oxygenated Krebs-Henseleit solution consisting of (mmol/l): NaCl, 118; KCl, 4.75; CaCl₂·2H₂O, 2.54; KH₂PO₄, 1.19; MgSO₄·7H₂O, 1.19; NaHCO₃, 12.5; and glucose, 10.0. The left circumflex coronary artery (LCx) and LAD were carefully excised for subsequent study of coronary artery ring vasoactivity. The right ventricle and the great vessels were removed, and the left ventricle was sliced parallel to the atrioventricular groove in 3-mm-thick sections. The unstained portion of the left ventricle (i.e., total area at risk) was separated from the Evans blue-stained portion of the left ventricle (i.e., area not at risk). The area at risk was again sectioned into 1-mm-thick slices and incubated in 0.1% nitroblue tetrazolium in phosphate buffer (pH 7.4) at 37°C for 15 minutes. The tetrazolium dye forms a blue formazan complex in the presence of coenzymes and dehydrogenases. The irreversibly injured necrotic portion of the myocardium at risk that did not stain was separated from the stained portion of the myocardium (i.e., the ischemic but nonnecrotic area). All three portions of the left ventricular myocardium (i.e., nonischemic, ischemic nonnecrotic, and ischemic necrotic) were weighed; the results were expressed as the area at risk indexed as a percentage of the total left ventricular mass, the area of necrotic tissue was computed as a percentage of the area at risk, and the area of necrotic tissue was computed as a percentage of the total left ventricular mass. The three portions of cat myocardium were stored at −70°C for later assay of myeloperoxidase activity. In three additional cats receiving a saline vehicle instead of L- or D-arginine, the above procedures were repeated except that half of the area at risk was incubated with 3 mM L-arginine to rule out the possibility that L-arginine altered the staining properties of the nitroblue tetrazolium. The area of necrotic tissue computed as a percentage of the area at risk was 46.4±7.2% in the control area at risk samples and 44.5±4.7% in the area at risk samples incubated with L-arginine, indicating that L-arginine had no effect on nitroblue tetrazolium-staining properties.

**Determination of Tissue Myeloperoxidase**

Myocardial activity of myeloperoxidase, an enzyme occurring virtually exclusively in neutrophils, was determined using the method of Bradley et al.¹⁹ as modified by Mullane et al.²⁰ The myocardium was homogenized in 0.5% hexadecyltrimethyl ammonium bromide (Sigma Chemical) and dissolved in 50 mM potassium phosphate buffer (pH 6) using a Polytron homogenizer (PCU-2). Homogenates were centrifuged at 12,500g at 2°C for 30 minutes. The supernatants then were collected and reacted with 0.167 mg/ml O-dianisidine dihydrochloride (Sigma Chemical) and 0.0005% H₂O₂ in 50 mmol phosphate buffer (pH 6.0). The change in absorbance was measured spectrophotometrically at 460 nmol. One unit of myeloperoxidase was defined as that quantity of enzyme hydrolyzing 1 mmol peroxide/min at 25°C. The assays were performed without knowledge of the group to which each cat heart belonged.

**Myeloperoxidase Activity of Polymorphonuclear Leukocytes**

To quantify the amount of myeloperoxidase activity present in cat polymorphonuclear leukocytes (PMNs), neutrophils were isolated from whole blood. In six cats, peripheral blood (80 ml) was collected from the femoral artery and anticoagulated with citrate-phosphate-dextrose solution (Sigma Chemical) (1.4:10 [vol:vol] anticoagulant to whole blood). The blood was placed into round-bottom polycarbonate centrifuge tubes (Nalge, Rochester, N.Y.), and PMNs were isolated by a procedure modified from Lafrado and Olsen.²¹ Platelet-rich plasma (PRP) was obtained by centrifuging blood at 400g for 20 minutes in an Econospin Tabletop Swinging Bucket Centrifuge (Sorvall Instruments, Wilmington, Del.). PRP was decanted and centrifuged at 2,500g for 10 minutes to obtain platelet-poor plasma (PPP). PPP then was mixed with isotonic Percoll (Sigma Chemical) (9:1 [vol:vol] Percoll to 1.5 M NaCl) to produce Percoll-
PPP density gradients of 80%, 62%, and 50%. Eight milliliters of 6% dextran (average molecular weight, 60,000–90,000; Sigma Chemical) and 20 ml of phosphate-buffered saline (PBS) were added to the erythrocyte-leukocyte pellet from the initial 400g centrifugation. After mixture by inversion, the erythrocytes were allowed to settle for 50 minutes. The pellets were resuspended in 1 ml of 0.9% NaCl and layered onto the Percoll-PPP gradient. Centrifugation then was performed at 400xg for 40 minutes at 4°C in a Sorvall RC2-B refrigerated centrifuge. PMNs were collected from the 62–82% interface and washed twice with PBS before being assayed for viability with trypan blue exclusion. PMN preparations obtained by this method were typically >95% pure and >95% viable, as previously reported. The PMNs were counted, digested, and subsequently assayed spectrophotometrically for myeloperoxidase activity as described previously.

**PMN Adherence to Coronary Artery Endothelium**

In three additional cats, PMNs were isolated as described previously and labeled with Zynaxis PHK2 fluorescent dye according to the method of Yuan and Fleming. One milliliter of PKH2-GL dye (4 μM) was added to the cell suspension and then mixed for 5 minutes by inversion. Two milliliters of PBS (containing 10% PPP in PBS) was added to stop the reaction, and an additional 5 ml of PBS was underlayered in the suspension. Cells then were centrifuged at 400g for 10 minutes at room temperature. The supernatant was removed, and the cells were resuspended in PBS and then recounted. Labeling procedure yields cells possessing normal morphology and function. During this PMN isolation-and-labeling period, hearts from each cat were removed and placed in warm, oxygenated Krebs-Henseleit buffer. Both the LAD and LCx were removed and cut into 2- to 3-mm segments. The arteries were opened and placed into a cell culture dish filled with 3 ml of Krebs-Henseleit buffer. Isolated labeled PMNs (400,000 PMNs/ml) were incubated for 30 minutes with 3 mM D- or L-arginine. After this 30-minute period, the PMNs were washed with PBS and subsequently added to the cell culture dish containing normal coronary arteries. The PMNs were immediately activated with leukotriene (LT)B₄ (100 nM), and coronary segments were removed 20 minutes later to examine PMN adherence to coronary artery endothelium using fluorescence microscopy. The number of PMNs per square millimeter of coronary endothelium were counted in duplicate for each experiment.

**Isolated Coronary Ring Studies**

Both LAD and LCx segments were removed and placed into warmed Krebs-Henseleit solution. Isolated coronary vessels were cleaned and cut into rings 2–3 mm long. The rings then were mounted onto stainless-steel hooks, suspended in 10-ml tissue baths, and subsequently connected to FT-03 force displacement transducers (Grass) to record changes in force on a Grass model 7 oscillographic recorder. The baths were filled with 10 ml of Krebs-Henseleit solution and aerated at 37°C with a gas mixture of 95% O₂–5% CO₂. Coronary rings initially were stretched to give a preload of 0.5 g of force and equilibrated for 60 minutes. During this period, the Krebs-Henseleit solution in the tissue baths was replaced every 20 minutes. After equilibration, the rings were exposed to 100 nM U46619 (BIOMOL Research Laboratories Inc., Plymouth Meeting, Pa.), a thromboxane A₂ mimic, to generate about 0.5 g of developed force. Once a stable contraction was obtained, acetylcholine was given to yield a concentration of 0.1, 1, 10, and 100 nM in the bath. After the response stabilized, the rings were washed and allowed to equilibrate to baseline again. The procedure was repeated with the calcium ionophore A-23187 (Calbiochem, La Jolla, Calif.) in amounts that yielded 1, 10, 100, and 1,000 nM and then with NaNO₂ (Sigma Chemical) in amounts that yielded 0.1, 1, 10, and 100 μM. NaNO₂ was prepared by dissolving the compound in 0.1N HCl and titrating it to pH 2.0.

In six additional cats, the LAD and LCx were removed and placed into warmed Krebs-Henseleit solution after 90 minutes of ischemia and 20 minutes of reperfusion to examine whether in vitro administration of L-arginine could reverse endothelial dysfunction associated with reperfusion. The coronary rings were stabilized and equilibrated as described previously. Endothelium-dependent relaxation was tested with acetylcholine and A23187, and endothelium-independent relaxation was tested with NaNO₂. After these responses were determined, the coronary rings were incubated with 3 mmol L-arginine or D-arginine for 30 minutes. The coronary rings then were washed three times with Krebs-Henseleit solution, and vasorelaxation to acetylcholine, A23187, and NaNO₂ was repeated. In addition, eight LAD and LCx rings were denuded by gently rubbing a thin cotton tip back and forth to mechanically remove the endothelium. The denuded rings were studied in the same manner as coronary rings, with an intact endothelium subjected to 90 minutes of ischemia and 20 minutes of reperfusion.

**Vascular Effects of L-Arginine**

The vascular effects of L-arginine were examined in six additional cats. In the first set, the ischemic LAD and nonischemic LCx were isolated, removed, and cut into 2–3-mm rings. The coronary rings were suspended in 10-ml tissue baths and stretched to give a preload of 0.5 g of force as described previously. The arteries were equilibrated for 60 minutes; 3 mmol of L- or D-arginine was then added to the baths, and the effect of L- or D-arginine on isometric force was examined. The rings were then reequilibrated for 60 minutes. After this period, the L-arginine was removed, and the rings were reequilibrated for 60 minutes before another 3 mmol of L- or D-arginine was added to the baths. The rings were then allowed to equilibrate for 60 minutes before recording was continued.

**FIGURE 1.** Bar graph of peak ST segment elevation after left anterior descending coronary artery occlusion. Height of bars represents mean values; brackets represent ±SEM values for five or six cats. MI, myocardial ischemia.
TABLE 1. Mean Arterial Blood Pressure for Cats Treated With Sham Myocardial Ischemia Plus L-Arginine, Myocardial Ischemia Plus D-Arginine, and Myocardial Ischemia Plus L-Arginine for the Course of the Experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Sham myocardial ischemia plus L-arginine</td>
<td>111±12</td>
</tr>
<tr>
<td>Myocardial ischemia plus D-arginine</td>
<td>94±2</td>
</tr>
<tr>
<td>Myocardial ischemia plus L-arginine</td>
<td>104±5</td>
</tr>
</tbody>
</table>

All values are mean±SEM (mm Hg) for five or six cats in each group.

D-arginine on basal tone was recorded for 60 minutes. In a second set of studies, hearts from anesthetized cats were excised rapidly, transferred to a perfusion apparatus, and perfused retrogradely via the aorta with oxygenated (95% O₂-5% CO₂) Krebs-Henseleit buffer at pH 7.4 and 37°C according to the Langendorff procedure.²⁴ The hearts were adjusted to perfusion conditions at a constant pressure of 50 mm Hg for 5 minutes and then switched to a constant flow (i.e., 30–40 ml/min) at a perfusion pressure of 50 mm Hg. The coronary perfusion pressure was measured continuously from a side-arm in the aortic inflow tract connected to a Statham P23 pressure transducer and a Grass model 7 oscillographic recorder. After a 5-minute equilibration at constant flow, the effect of L-arginine on coronary vascular resistance was examined. A constant infusion of 3 mM L-arginine was administered for 30 minutes, and coronary perfusion pressure was recorded continuously during this time. At the end of this 30-minute period, the heart was perfused with Krebs-Henseleit buffer without L-arginine for 10 minutes. The ability of the coronary vasculature to vasodilate was tested using the endothelium-independent vasodilator nitroglycerin (50 μg/ml of flow).

**Statistical Analysis**

All values are presented as mean±SEM of n independent experiments. All data were subjected to ANOVA testing followed by the Bonferroni correction for post hoc t test analysis. Values of p<0.05 were considered statistically significant.

**Results**

**Cardiac Electrophysiological and Hemodynamic Changes**

In five cats subjected to sham myocardial ischemia, we observed that intravenous administration of L-arginine administered as a bolus of 30 mg/kg immediately followed by a continuous infusion of 10 mg/kg·min⁻¹ for 1 hour had no detectable effect on any of the measured hemodynamic or ECG variables. Furthermore, there were no significant differences in any of the variables observed initially between the two myocardial ischemia groups of cats. However, in all cats subjected to coronary artery occlusion, within 10 minutes of LAD occlusion, the ST segment became elevated and peaked at 40 minutes after coronary occlusion. After reperfusion, the ST segment declined to nearly control values. There was no significant difference in peak ST segment elevation between the two myocardial ischemia groups, indicating that the ischemic insult was similar in these cats (Figure 1). At reperfusion, there was a noticeable increase in the incidence of premature ventricular contractions (PVCs). Two cats in each myocardial ischemia group developed ventricular fibrillation that was successfully cardioverted to a normal sinus rhythm. There was no overall difference between the myocardial ischemia groups in the number of PVCs occurring after reperfusion, indicating that L-arginine or D-arginine did not exert any overt antiarrhythmic effects. Immediately after coronary occlusion, MAP decreased in all cats. There were no significant differences between the two myocardial ischemia groups at any of the hourly MAP or heart rate readings, suggesting that neither L-arginine nor D-arginine exerted any significant effect on systemic hemodynamics (Tables 1 and 2).

**Effect of L-Arginine on Reperfusion Myocardial Injury**

In every cat subjected to myocardial ischemia, we measured the area at risk of the ischemic heart and the amount of necrotic cardiac tissue expressed as a percentage of both the area at risk and the total left ventricular mass. There was no significant difference between ischemic groups in the area at risk expressed as a percentage of total left ventricular mass, indicating

<table>
<thead>
<tr>
<th>Group</th>
<th>Time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Sham myocardial ischemia plus L-arginine</td>
<td>171±18</td>
</tr>
<tr>
<td>Myocardial ischemia plus D-arginine</td>
<td>160±5</td>
</tr>
<tr>
<td>Myocardial ischemia plus L-arginine</td>
<td>178±6</td>
</tr>
</tbody>
</table>

All values are mean±SEM (beats per minute) for five or six cats in each group.

---

Downloaded from http://circ.ahajournals.org/ by guest on April 20, 2017
that the severity of ischemia was similar in both myocardial ischemia groups. However, the necrotic area expressed as a percentage of area at risk or of total left ventricular mass was significantly lower ($p<0.01$ and $p<0.05$, respectively) in cats treated with L-arginine, indicating that L-arginine significantly attenuated myocardial damage induced by ischemia and reperfusion (Figure 2).

**Prevention of PMN Accumulation in Ischemic Myocardial Tissue by L-Arginine**

Infiltration of the ischemic region by neutrophils during reperfusion is considered one of the major mechanisms responsible for reperfusion injury. We measured myeloperoxidase activity in the three different portions of the myocardium as a marker for neutrophil accumulation in ischemic tissue. It is evident that in the nonischemic myocardium (i.e., area not at risk), myeloperoxidase activity was very low in both myocardial ischemia groups and there was no significant difference between them, indicating that few neutrophils accumulated in the nonischemic myocardium. However, myocardial ischemia cats receiving D-arginine exhibited a marked increase in myeloperoxidase activity in the necrotic myocardium. In contrast, L-arginine–treated ischemic cats exhibited a significantly lower myeloperoxidase activity in necrotic myocardial tissue (Figure 3).

**Myeloperoxidase Activity of PMNs**

To quantify the amount of neutrophil activity present in cat PMNs, neutrophils isolated from peripheral whole cat blood were counted, digested, and assayed for myeloperoxidase activity. A concentration curve for myeloperoxidase activity is shown in Figure 4. With an increasing number of neutrophils, there was a linear increase in myeloperoxidase activity. The regression line had a significant $r$ coefficient of 0.965 ($p<0.001$). The amount of myeloperoxidase activity found in the necrotic myocardium of cats subjected to D-arginine was equal to approximately $1.6 \times 10^6$ PMNs/100 mg tissue in the necrotic zone. In contrast, the myeloperoxidase activity found in the necrotic myocardium of cats treated with L-arginine was equal to approximately $4.0 \times 10^6$ PMNs/100 mg tissue.

**PMN Adherence to Coronary Artery Endothelium**

Figure 5 summarizes the effects of L-arginine on PMN adherence. As shown in Figure 5, PMNs incubated with D-arginine for 30 minutes and subsequently activated with LTB$_4$ exhibited a significant ($p<0.01$) increase in PMN adherence to coronary artery endothelium compared with PMNs that were not activated with LTB$_4$. Similarly, PMNs incubated with L-arginine for 30 minutes and subsequently activated with LTB$_4$ had a significant increase in PMN ($p<0.01$) adherence to coronary artery endothelium compared with PMNs that were not activated with LTB$_4$. There was no significant difference in PMN adherence to coronary artery endothelium between PMNs incubated with L- or D-arginine.

**Effect of L-Arginine on Endothelial Dysfunction**

Because endothelial dysfunction is an early event in reperfusion injury, we also tested endothelial function by comparing vasoactivity of coronary artery rings treated with the endothelium-dependent vasodilators acetylcholine and A-23187 or the endothelium-indepen-
Data are mean±SEM for seven to 10 artery segments. LTB₄, leukotriene B₄.

dent vasodilator NaNO₂. Figure 6 presents typical recordings of LAD rings obtained from a sham–myocardial ischemia cat or from myocardial ischemia cats receiving D- or L-arginine. The coronary rings isolated from the sham–myocardial ischemia cat exhibited complete relaxation to the endothelium-dependent vasodilator acetylcholine and to the endothelium-independent vasodilator NaNO₂. However, the vasorelaxation response to acetylcholine in the coronary ring obtained from the myocardial ischemic cat receiving only D-arginine was almost totally abolished at 4.5 hours after reperfusion, although this ring relaxed completely in response to the endothelium-independent vasodilator NaNO₂. In contrast, the endothelium-dependent vasodilation of the ring obtained from a cat treated with L-arginine was significantly preserved.

Figure 7 summarizes the vasorelaxant responses to acetylcholine, A-23187, and NaNO₂ in isolated cat LAD rings. Clearly, the response of ischemic LAD rings to the endothelium-dependent vasodilators was preserved significantly in the L-arginine–treated ischemic cats. Figure 8 illustrates the vasorelaxant response of paired nonischemic LCx rings studied as a control. The nonischemic LCx rings, whether obtained from sham–myocardial ischemia cats or myocardial ischemia cats receiving D- or L-arginine, showed complete relaxation in response to all three dilators. Thus, there were no significant differences in response to any of the vasodilators studied between the groups of LCx rings.

In additional coronary ring studies, we examined the ability of in vitro administration of L-arginine to reestablish endothelial function after 90 minutes of ischemia followed by 20 minutes of reperfusion. Figure 9 illustrates typical recordings taken from these experiments. The complete vasorelaxant responses of LAD rings after 90 minutes of ischemia and 20 minutes of reperfusion are summarized in Table 3. Before incubation with L- or D-arginine, the vasorelaxation responses to acetylcholine and A-23187 were significantly attenuated in the ischemic-reperfused coronary rings and totally abolished in the endothelial-denuded ischemic-reperfused coronary rings. Coronary rings exposed to the...
endothelium-independent vasodilator NaNO₂ however, exhibited full relaxation in all rings. After 30 minutes of incubation with L-arginine, ischemic-reperfused coronary rings exerted significant increases in vasorelaxation in response to acetylcholine and A-23187. This increased vasorelaxant response to acetylcholine did not occur in ischemic-reperfused coronary rings incubated with D-arginine or endothelial-denuded coronary rings incubated with L-arginine. All rings completely relaxed in response to NaNO₂ after incubation with either L- or D-arginine. In LCx, maximal relaxation was obtained in all groups before and after administration of L-arginine.

**Vascular Effects of L-Arginine**

The vascular effects of L-arginine also were examined in ischemic LAD and nonischemic LCx rings as well as in isolated, perfused hearts. In isolated coronary artery rings, L-arginine did not significantly alter the basal tone of ischemic LAD rings (Δforce [g]=0.08±0.04 after 60 minutes) or nonischemic LCx rings (Δforce [g]=0.04±0.02 after 60 minutes) over a 60-minute incubation period. Similarly, L-arginine administered for 30 minutes in isolated, perfused hearts had no significant effect on coronary vascular resistance. These results are summarized in Table 4.

**Discussion**

The data obtained in this study clearly demonstrate significant cardioprotective effects of exogenous L-arginine infusion in a feline model of myocardial ischemia/reperfusion. The myocardial ischemia–plus–D-arginine and myocardial ischemia–plus–L-arginine groups exhibited similar degrees of ischemic insult as indicated by comparable increases both in ST segment elevation and in areas at risk of the left ventricle. Despite these similar degrees of ischemia, L-arginine decreased the necrotic area by approximately 50% of that developing in the D-arginine groups. Although the degree of necrosis appears to be large in the D-arginine control groups, D-arginine did not significantly increase the percentage of necrosis compared with recent studies using saline vehicle controls (40.9±5.1 for D-arginine versus 33.6±4.1 and 37.7±4.5 for saline vehicles, respectively). Earlier findings regarding myocardial ischemia in cats from our laboratory and others indicate that there is very little collateral blood flow in the ischemic cat myocardium. In this regard, cat myocardial blood flow declines 92–93% after LAD occlusion. Because cat coronary collateral blood flow is minor and L-arginine does not significantly alter coronary basal tone, it is unlikely that L-arginine administered for only the last 10 minutes of the 90-minute ischemic period would have increased coronary collateral blood flow to an extent that would significantly decrease myocardial necrosis. Therefore, the myocardial salvage afforded by L-arginine probably was not the result of any direct vasoactive effects of L-arginine. Although it is unlikely, we cannot definitively rule out the possibility that higher native collateral perfusion existed in ischemic cats given L-arginine.

**Figure 9.** Representative recordings of rings isolated from cat left anterior descending coronary arteries subjected in vivo to 90 minutes of ischemia and 20 minutes of reperfusion. The rings were precontracted with the thromboxane A₂ mimetic U46619, and responses to the endothelium-dependent vasodilator acetylcholine (ACH) at 100 nM and the endothelium-independent vasodilator NaNO₂ (100 μM) were obtained before (pre-) and after (post-) incubation with 3 mmol L- or D-arginine. The arrows indicate addition of U46619. Dots indicate addition of ACh and NaNO₂.

**Table 3.** Percent Relaxation of Left Anterior Descending Coronary Artery Rings After 90 Minutes of Ischemia and 20 Minutes of Reperfusion Before and After Incubation With L- or D-Arginine

<table>
<thead>
<tr>
<th></th>
<th>L-Arginine</th>
<th>D-Arginine</th>
<th>Deendothelialized plus L-arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td></td>
<td>40.2±4.1</td>
<td>71.3±4.4*</td>
<td>38.5±3.6</td>
</tr>
<tr>
<td></td>
<td>43.8±3.9</td>
<td>88.6±3.7*</td>
<td>42.3±4.1</td>
</tr>
<tr>
<td></td>
<td>92.3±3.0</td>
<td>93.1±2.9</td>
<td>90.6±3.1</td>
</tr>
</tbody>
</table>

All values are mean±SEM for eight to 11 rings in each group.

*p<0.01 compared with before values of the same group.
Perhaps the most intriguing finding of this investigation is that L-arginine preserved endothelial function in ischemic-reperfused arteries. Recent evidence suggests that endothelial dysfunction, which becomes significant 2.5 minutes after reperfusion, is the initial triggering mechanism that allows neutrophil adherence and diapedesis into the ischemic myocardium, which augments postreperfusion injury. Endothelial dysfunction associated with reperfusion injury is characterized by a markedly decreased release of EDRF in response to endothelium-dependent vasodilators. EDRF has been identified as NO or a labile nitroso compound that liberates NO and is produced from the conversion of the semiessential amino acid L-arginine into L-citrulline by NO synthase in endothelial cells. In the vascular system, the biosynthesis of NO by endothelial cells produces relaxation of vessels, inhibition of platelet aggregation, and attenuation of neutrophil adherence, all of which may be salutary in reperfusion injury.

Because ischemia/reperfusion results in diminished NO release from endothelial cells, attempts have been made to supplement the reduced NO to attenuate myocardial injury associated with ischemia/reperfusion injury. In this regard, Johnson et al. reported that direct infusion of authentic NO significantly decreased myocardial necrosis and neutrophil accumulation in ischemic tissue. Second, administration of organic NO donors, such as the sydnonimines, also provide significant cardioprotective effects. Specifically, Siegfried et al. demonstrated that SIN-1 or C87-3754, two sydnonimine NO donors, attenuated both endothelial dysfunction and myocardial necrosis in a feline model of myocardial ischemia/reperfusion. Furthermore, the cardioprotective effects of either authentic NO or the organic NO donors occurred at doses below the vasodilator threshold and thus were not associated with their vasodilator activity. A third approach to maintaining NO levels during reperfusion is to administer L-arginine, the substrate of NO synthesis. To our knowledge, this is the first study to demonstrate that exogenous infusion of L-arginine but not its enantiomer D-arginine significantly reduces endothelial dysfunction, neutrophil migration to ischemic tissue, and myocardial necrosis in a model of myocardial ischemia/reperfusion.

The exact cardioprotective mechanisms of L-arginine are not known with certainty, but if NO release is maintained close to the site of injury, it could have cytoprotective effects by inhibiting neutrophil aggregation and adherence. McCauley et al. reported that NO inhibits neutrophil aggregation in vitro. Furthermore, Kubas et al. demonstrated that endogenous NO release attenuates leukocyte adherence to endothelial cells. When basal release of NO was inhibited with either N\textsuperscript{0}-monomethyl-L-arginine (L-NMMA) or N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME), neutrophil adherence to postcapillary venular endothelium was increased by 15-fold. This increased neutrophil adherence was completely restored with exogenous L-arginine, suggesting that endothelium-derived NO is an important intrinsic modulator of leukocyte adherence.

Our finding that myeloperoxidase activity in the necrotic area is reduced with L-arginine administration reinforces these observations and provides further evidence that NO may exert antineutrophil properties. However, because PMNs incubated with L-arginine did not significantly decrease PMN adherence to coronary artery endothelium, the neutrophil-inhibiting properties of L-arginine are most likely due to the attenuation of endothelial function during reperfusion rather than to any direct effect of L-arginine on PMNs.

Increased NO release with L-arginine administration also may be cardioprotective through the action of directly quenching superoxide free radicals. Superoxide radicals are known to be one of the most important inactivators of NO and conditions associated with enhanced production of superoxide, by neutrophils or endothelial cells, have been shown to increase neutrophil adherence. Viewed another way, increased NO levels may inactivate superoxide radicals, thereby decreasing neutrophil adherence and offering protection to endothelial cells. Thus, a key aspect of the cardioprotective effect of L-arginine (i.e., increased NO level) could be to prevent endothelial cell or cardiac myocyte injury resulting from toxic substances such as superoxide radicals.

Another possible mechanism whereby L-arginine could produce cardioprotective effects in myocardial ischemia/reperfusion would be through coronary vasoconstriction. This does not appear to be the case, however, because L-arginine administration did not decrease MAP in the sham myocardial ischemia–plus-L-arginine group. Furthermore, 3 mM L-arginine did not alter the basal tone of ischemic or nonischemic coronary arteries or coronary vascular resistance in isolated, perfused hearts. These results confirm the findings of Cooke et al., who found that 3 mM L-arginine did not relax normal rabbit aortic rings and provided further evidence that the cardioprotective effects of L-arginine were not due to coronary vasodilation.

Although L-arginine clearly has cardioprotective effects, systemic administration of L-arginine also exerts several biological effects other than the generation of NO. These effects include the increased release of insulin and glucagon from pancreatic cells as well as the secretion of growth hormone and prolactin from the pituitary. Because L-arginine enhances insulin as well as glucagon secretion, this should offset the effect of insulin on blood glucose levels. Girerd et al. demonstrated that at a concentration equal to that used in our study (i.e., 10 mg/kg·min\textsuperscript{-1}), D-arginine as well as L-arginine induced comparable increases in insulin levels.
levels in rabbits, thus suggesting that the insulin-releasing effect of L-arginine is irrelevant to its cardioprotective action. In this connection, we found that D-arginine failed to attenuate endothelial dysfunction, neutrophil accumulation in the ischemic tissue, or myocardial necrosis.

In addition, L-arginine increases the secretions of growth hormone and prolactin from the pituitary. However, the effects of these hormones would most likely be long term (i.e., longer than the 6-hour observation period used in this study), and even if these effects occurred, they would have little or no cardioprotective effect. Although we cannot definitively eliminate the systemic effects of L-arginine on the endocrine system, our in vitro findings of a direct endothelial protective effect of L-arginine but not of D-arginine suggest that at least one component of the cardioprotective effect of L-arginine in vivo is preservation of NO release by endothelial cells. This endothelial-protective effect of L-arginine appears to occur early during reperfusion (i.e., when 65–75% of endothelial dysfunction occurs) because L-arginine can reverse endothelial dysfunction after 20 minutes of reperfusion. The ability of L-arginine to reverse endothelial dysfunction after reperfusion coincides with the results of others who have found that L-arginine restores endothelial function in hypercholesterolemic humans and rabbits, respectively.

In conclusion, we have demonstrated significant cardioprotective effects after the administration of exogenous L-arginine in a 6-hour model of myocardial ischemia/reperfusion in cats. The necrotic area was reduced by 50% compared with D-arginine–treated cats. Neutrophil accumulation was significantly decreased in the ischemic tissue, and coronary rings isolated from these cats treated with L-arginine exhibited preserved endothelial function as evidenced by a significant increase in their ability to respond to the endothelium-dependent vasodilators ACh and A-23187. Although our data do not allow us to unequivocally state the exact protective mechanism of L-arginine, it appears that L-arginine preserves coronary vascular endothelial release of NO, a finding that previously has been shown to be cardioprotective in this model of ischemia/reperfusion. Further studies are necessary to determine the exact mechanism by which L-arginine preserves endothelial function and attenuates myocardial necrosis.

Acknowledgment
We would like to acknowledge the expert technical assistance of Jin-ping Guo in conducting the biochemical assays used in these studies.

References
24. Tsao PS, Ma X-I, Lefer AM: Activated neutrophils aggravate endothelial dysfunction after reperfusion of the ischemic feline myocardium. Am Heart J (in press)
The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat.
A S Weyrich, X L Ma and A M Lefer

Circulation. 1992;86:279-288
doi: 10.1161/01.CIR.86.1.279

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/86/1/279