SUPPLEMENTAL L-ARGININE DURING CARDIOPLEGIC ARREST AND REPERFUSION AVOIDS REGIONAL POSTISCHEMIC INJURY

Unenhanced hypothermic cardioplegia does not prevent postischemic endothelial and contractile dysfunction in hearts subjected to antecedent regional or global ischemia. This study tested the hypothesis that supplementing blood cardioplegic solution and reperfusion with the nitric oxide precursor L-arginine would preserve endothelial function, reduce infarct size, and reverse postcardioplegia regional contractile dysfunction by the L-arginine–nitric oxide pathway. In 23 anesthetized dogs, the left anterior descending coronary artery was ligated for 90 minutes, after which total bypass was established for surgical “revascularization.” In 10 dogs, unsupplemented multidose hypothermic blood cardioplegic solution was administered for a total of 60 minutes of cardioplegic arrest. In eight dogs, L-arginine was given intravenously (4 mg/kg per minute) and in blood cardioplegic solution (10 mmol) during arrest. In five dogs, the nitric oxide synthesis blocker N ω -nitro-L-arginine (1 mmol) was used to block the L-arginine–nitric oxide pathway during cardioplegia and reperfusion. Infarct size (triphenyltetrazolium chloride) as percent of the area at risk was significantly reduced by L-arginine compared with blood cardioplegic solution ($28.2\% \pm 4.1\%$ versus $40.5\% \pm 3.5\%$) and was reversed by N ω -nitro-L-arginine to $68.9\% \pm 3.0\%$ ($p < 0.05$). Postischemic regional segmental work in millimeters of mercury per millimeter (sonomicrometry) was significantly better with L-arginine (92 ± 15) versus blood cardioplegic solution (28 ± 3) and N ω -nitro-L-arginine (26 ± 6). Segmental diastolic stiffness was significantly lower with L-arginine (0.46 ± 0.06) compared with blood cardioplegic solution (1.10 ± 0.11) and was significantly greater with N ω -nitro-L-arginine (2.70 ± 0.43). In ischemic-reperfused left anterior descending coronary arterial vascular rings, maximum relaxation responses to acetylcholine, the stimulator of endothelial nitric oxide, was depressed in the blood cardioplegic solution group ($77\% \pm 4\%$) and was significantly reversed by L-arginine ($92\% \pm 3\%$). Smooth muscle function was unaffected in all groups. We conclude that cardioplegic solution supplemented with L-arginine reduces infarct size, preserves postischemic systolic and diastolic regional function, and prevents arterial endothelial dysfunction via the L-arginine–nitric oxide pathway. (J THORAC CARDIOVASC SURG 1995;110:302-14)

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Prolonged regional ischemia produces myocardial necrosis within 1 hour of occlusion despite reperfusion.¹ In addition, previous studies have demonstrated that both regional and global myocardial ischemia and reperfusion injure the coronary artery endothelium, manifested as impaired coronary vascular responses to various endothelium-dependent vasodilators.¹⁻⁶ This endothelial dysfunction may be physiologically expressed as a reduced production or release of endothelium-derived relaxing factor. Endothelium-derived relaxing factor is synonymous

with nitric oxide (NO) itself or a nitroso intermediate compound⁷ and acts not only as an endogenous nitrovasodilator⁸ but also as an inhibitor of platelet aggregation,⁹ an antineutrophil autacoid reducing adherence to the vascular endothelium,¹ and a direct diradical coupling agent of superoxide anions.¹⁰ Because the antineutrophil and antioxidant activities of NO released by the coronary endothelium are important cardioprotective mechanisms that prevent injury to the endothelium and myocytes, the loss of this endogenous cardioprotection subsequent to endothelial damage may be an important component in the pathophysiologic progression of myocardial ischemia-reperfusion injury.

Protecting the myocardium during cardiac operations with hypothermic cardioplegic solutions has been widely used to both prevent ischemic injury during elective cardiac arrest and reduce injury after subsequent reperfusion. In clinical cardiac operations, the heart is exposed not only to protected ischemia, but also to antecedent unprotected ischemia (coronary artery occlusion, oxygen supply/demand mismatch) and reperfusion during infusion of cardioplegic solution or removal of the aortic crossclamp. The potential for injury exists at each of these points. The cardioplegic solution would be an ideal vehicle for the delivery of agents that target the perpetrators of endothelial and myocyte injury during elective cardiac arrest and subsequent reperfusion. Replacement of NO by an exogenous NO donor or, alternatively, supplementation with a precursor of NO (that is, L-arginine)¹¹ may prevent endothelial damage, reduce necrosis, and preserve postischemic function after regional ischemia.

This study tested the hypothesis that adjuvant L-arginine, the precursor of NO, administered during hypothermic cardioplegic arrest and reperfusion would attenuate endothelial dysfunction, reduce infarct size, and reverse postcardioplegia regional dysfunction by the L-arginine–NO pathway in dogs subjected to 1.5 hours of collateral-deficient regional ischemia,¹ multidose blood cardioplegia, and reperfusion.

Material and methods

The dogs were handled in compliance with the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985). The institutional Animal Care and Use Committee approved the study protocol.

Surgical procedure. Heartworm-free adult mongrel dogs weighing 19.0 to 35.7 kg (average 23.2 kg) of either sex were initially anesthetized with intravenous sodium

thiamylal (20 mg/kg). A bolus injection of fentanyl citrate (350 μ g) and diazepam (5 mg) was given followed by continuous infusion of fentanyl citrate (0.3 μ g/kg per minute) and diazepam (0.03 mg/kg per minute) during the experiment. Each dog was endotracheally intubated and the lungs were ventilated with oxygen-enriched room air to maintain arterial oxygen tension greater than 100 mm Hg with use of a volume-cycled respirator (Harvard Apparatus, South Natick, Mass.). The right femoral artery and vein were cannulated for arterial blood sampling and for fluid administration, respectively. Arterial carbon dioxide tension was maintained between 35 and 45 mm Hg by adjustment of ventilatory rate, and pH was adjusted between 7.37 and 7.43 with intravenous sodium bicarbonate as necessary.

The surgical preparation used in the present study for assessing regional segmental function by sonomicrometry³ and creating collateral-deficient regional ischemia¹ has been presented in detail elsewhere. After dissection of the proximal portion of the left anterior descending (LAD) coronary artery, a small right ventricular branch of the LAD distal to the point of ligation was dissected free for arteriotomy and retrograde venting of collateral blood flow to minimize collateral perfusion during LAD occlusion.¹ The dogs were systemically heparinized with heparin sodium 300 U/kg, supplemented at 300 U/kg every 90 minutes. The left subclavian artery was cannulated for aortic perfusion. Superior and inferior vena caval cannulas were inserted transatrially into the right atrium and poised in the atrium so not to impair venous return.

Hemodynamic and segmental function data were obtained at baseline with the circulation intact. Each dog then received a bolus injection of lidocaine (1.2 mg/kg), after which the LAD and obvious collateral vessels encroaching on the territory perfused by the LAD were ligated for 90 minutes of regional ischemia. Immediately after LAD ligation, a small right ventricular branch of the LAD was transected to divert collateral blood flow from the ischemic zone. This technique has been shown to result in the uniform depletion of collateral flow to all layers of the canine myocardium and has been shown to produce a consistent degree of necrosis within the area at risk.¹ Ventricular fibrillation, when encountered, was converted by direct-current countershocks of 15 watt-seconds. After 90 minutes of regional ischemia, hemodynamic and segmental function data were collected again. Cardiopulmonary bypass was instituted as previously described.¹²

Experimental protocol. Dogs were randomly divided into three groups on the basis of the exclusion or inclusion of the NO-precursor L-arginine (L-Arg group) or the NO synthesis blocker N ω -nitro-L-arginine (L-NA; L-NA group) in blood cardioplegia and as intravenous supplement. In the BCP group (BCP group, $n = 10$), unmodified standard blood cardioplegia⁵ was used. In the L-Arg group ($n = 8$), L-arginine was added to blood cardioplegia to achieve a final concentration in the blood cardioplegic solution of 10 mmol/L³ and administered intravenously (4 mg/kg per minute infusion rate) from the application of the aortic crossclamp to the end of the experiment. In the third group (L-NA, $n = 5$) L-NA was added to blood cardioplegia (1 mmol/L) and infused systemically by bolus intravenous injection (16.8 mg/kg body weight) just before

Table I. Hemodynamic data at baseline, ischemia, and reperfusion

	Baseline	90 Minutes of ischemia	Reperfusion
HR (beats/min)			
BCP	104 ± 7	124 ± 6*	149 ± 4*
L-Arg	91 ± 9	131 ± 7*	139 ± 6*
L-NA	105 ± 8	127 ± 5*	131 ± 4*†
MAP (mm Hg)			
BCP	105 ± 2	85 ± 3*	82 ± 2
L-Arg	97 ± 4	92 ± 2*	87 ± 2
L-NA	94 ± 4†	87 ± 1*	85 ± 2*
PRP (mm Hg/ min × 10 ³)			
BCP	12.0 ± 0.8	11.0 ± 0.7*	14.2 ± 0.5*
L-Arg	9.8 ± 1.2	12.7 ± 0.8*	14.7 ± 0.9*
L-NA	10.2 ± 0.5	11.6 ± 0.6*	12.9 ± 0.7*

Values are given as means plus or minus the standard error of the estimate. HR, Heart rate; MAP, mean arterial pressure; PRP, pressure-rate product.

* $p < 0.05$ versus previous period.

† $p < 0.05$ versus BCP group.

infusion of L-arginine (10 mmol/L) and each hour thereafter. Preliminary experiments showed that a 1 mmol/L concentration of L-NA blocked the endogenous NO release stimulated by acetylcholine for the duration of the experiment.

Cardioplegic arrest. After the aorta was crossclamped, blood cardioplegic solution was delivered by the warm-cold induction modality¹³ and terminal warm infusion. Potassium level was 20 to 25 mmol/L during warm infusions and 10 mmol/L during cold infusions. Calcium level was 0.2 to 0.3 mmol/L, pH 7 to 9 at 37° C, and osmolality 360 to 380 μ Osm. Hematocrit value ranged from 0.14 to 0.18. The LAD vessel loop was removed and the site of coronary artery collateral venting closed before the 20-minute administration of supplemental cardioplegia, which thereby allowed delivery of blood cardioplegic solution down the LAD. Systemic temperature was maintained at 28° C. After the final infusion of blood cardioplegic solution, systemic rewarming to 37° C was completed, and the crossclamp was removed immediately after completion of the terminal blood cardioplegic infusion. Mean aortic pressure was gradually increased from 50 mm Hg to 80 mm Hg after electromechanical reanimation was observed. If fibrillation occurred, direct-current counter-shocks of 10 watt-seconds were applied. The heart was maintained in the vented total bypass state for the initial 30 minutes of reperfusion. After systemic blood Ca²⁺ content was corrected to baseline values,¹⁴ the dog was weaned off bypass and allowed to support the circulation for an additional 30 minutes in the working state. After postischemic hemodynamic and segmental function data were acquired at the end of 60 minutes of reperfusion, the heart was excised for the subsequent study of coronary artery endothelial function (description of in vitro study follows) by the organ bath technique.³

Determination of area at risk and infarct size. The interrupted left circumflex artery (LCx) segment was bridged with a polyethylene tube to reestablish perfusion

continuity, and Unisperse blue dye (Ciba-Geigy, Inc., Newport, Del.) was perfused through the aortic root to stain the normally perfused region blue. The area at risk was demarcated by the lack of blue staining. The area of necrosis and infarct size were determined by triphenyltetrazolium chloride vital stain and gravimetric analysis as previously described.^{3, 15}

Data acquisition and analysis. Hemodynamic data were acquired and processed by computer as detailed previously.^{3, 16} Measurements were taken before coronary artery occlusion (control), after 90 minutes of occlusion, and after 60 minutes of reperfusion in the working state. Hemodynamic and cardiodynamic data were averaged and output was obtained from no fewer than 15 beats. The pressure-rate product, used as an index of myocardial oxygen demands, was calculated as the product of heart rate and peak left ventricular systolic pressure. Percent segmental shortening, segmental work, and the characteristics of segmental stiffness were determined as previously described.⁵

Plasma creatine kinase activity. Blood samples for measuring creatine kinase activity were withdrawn from the femoral artery at baseline, after 90 minutes of regional ischemia, at the end of 1 hour of cardioplegic arrest, at the end of beating-empty (30 minutes) reperfusion, and at the end of 60 minutes of reperfusion. The plasma was analyzed spectrophotometrically for creatine kinase activity (CK-10 kit; Sigma Diagnostic, St. Louis, Mo.) and protein concentration (Sigma Diagnostic). Creatine kinase activity was expressed as international units per microgram of protein.

Cardiac myeloperoxidase activity. Tissue samples weighing approximately 0.4 gm were taken from the nonischemic zone and from the nonnecrotic and necrotic areas of the ischemic zone for spectrophotometric analysis of myeloperoxidase activity as an assessment of neutrophil accumulation in myocardium as described previously.¹

In vitro coronary artery ring studies. Both the ischemic-reperfused LAD and the nonischemic LCx were carefully dissected from the heart after the experiment, cut into four rings of approximately 2 mm in length, connected to isometric force transducers (model TR; Radnoti 001, Monrovia, Calif.), and placed in organ chambers filled with 20 ml of 37° C Krebs-Henseleit solution with the following composition (in millimoles per liter): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, 12.5 NaHCO₃, and 10 glucose. Changes in isometric force were digitized at 3 Hz with use of an analog-to-digital converter and an IBM-PC AT computer (IBM Corp., Armonk, N.Y.). Isometric force data were analyzed with use of a videographics program developed in our laboratory.¹⁷ After 60 minutes of equilibration, the rings were placed at the optimal point of their length-tension relationship with the use of potassium chloride 30 mmol/L. After indomethacin 10 μ mol/L was used to prevent vascular responses to endogenous prostacyclin, the optimal dose of U46619 was determined. Cumulative concentration-response curves to acetylcholine, a muscarinic receptor-mediated endothelium-dependent stimulator of NO; the calcium ionophore A23187, a receptor-nonmediated endothelium-dependent stimulator of NO; and acidified (pH 2.0) sodium nitrite, an endothelium-

independent smooth muscle relaxing agent, were sequentially performed. Drug concentrations are expressed as the final concentration in the organ chamber.

In addition, the heart was excised from six normal dogs and ring studies were done to obtain control data.

Statistical analysis. Dogs were not included in the final analysis if (1) the cardioplegic solution was not within specified delivery pressure, pH, potassium concentration, or osmolality, (2) ventricular fibrillation was unconverted after two direct-current shocks, or (3) the protocol was not satisfactorily completed.

All data were analyzed with the Statistical Analysis System program (PC-SAS; SAS Institute, Cary, N.C.). Time-related differences and group-time interactions were analyzed by two-way analysis of variance for repeated measures adjusted for baseline values. Infarct size and myeloperoxidase data were compared among the three groups by one-way analysis of variance. Responses to vasodilators in rings contracted with U46619 are expressed as percent changes of tension from the precontracted levels, and those data are compared among the four groups at each concentration by one-way analysis of variance. EC_{50} , the dose of the drug required to effect relaxation to 50% of maximum precontracted levels, was calculated and expressed as the negative log of the drug concentration. Holms' sequentially rejective method¹⁸ was used to reduce the chance of type 1 errors in multiple comparisons at a probability level of 0.05. All data are presented as means plus or minus the standard error of the mean.

Results

One dog in the BCP group had heartworms and was excluded. In one dog in the L-Arg group, the cardioplegic solution was not delivered within specified delivery pressures and the dog was excluded. Twenty-three dogs (BCP = 10; L-Arg = 8; L-NA = 5) were entered into the final data analysis of infarct size and regional function.

Blood gas data obtained before bypass and after stabilization on bypass were not significantly different among the groups.

Hemodynamics. In the standard BCP group, direct-current countershock was required to convert ventricular fibrillation in four dogs during ischemia. In the L-Arg group, only two dogs required cardioversion during ischemia, and in the L-NA group, two dogs required cardioversion during ischemia. There were no group differences in the number of cardioversions required during the early period of reperfusion (BCP = 1, L-Arg = 1, L-NA = 1).

Hemodynamic data for the three groups at baseline and during ischemia and reperfusion are summarized in Table I. There was a slight but significant difference in baseline mean arterial pressure between the BCP and L-NA groups. During coronary occlusion, heart rate was significantly increased and

Table II. Blood cardioplegia delivery volume in milliliters

Group	Warm infusion	Cold infusion	20-Minute infusion	40-Minute infusion	Terminal
BCP	471 ± 64	422 ± 37*	224 ± 27*	205 ± 22	348 ± 23*
L-Arg	600 ± 78	449 ± 44*	264 ± 15*	267 ± 21	416 ± 17*
L-NA	400 ± 36	276 ± 12*†	176 ± 12*†	169 ± 17†	307 ± 44*†

Values are given as mean plus or minus the standard error of the estimate.

* $p < 0.05$ versus previous period.

† $p < 0.05$ versus L-Arg group.

mean arterial pressure decreased significantly for all groups compared with the baseline value. Pressure rate product increased significantly in both the L-Arg and L-NA groups but decreased in the BCP group compared with the previous period. There were no group differences in any of the parameters during ischemia. During reperfusion, heart rate remained elevated in all groups and there was a significant difference between the BCP and L-NA groups. Mean arterial pressure did not change compared with the ischemic period. Pressure rate product during reperfusion increased significantly from ischemia for all groups, but with no group differences.

Myocardial temperature and delivery of blood cardioplegia. Myocardial temperatures were measured in the anterior (ischemic) and posterior (nonischemic) walls of the left ventricle after each infusion of blood cardioplegic solution. Myocardial temperature was significantly higher in the ischemic zone than in the nonischemic zone during the cold induction phase when distribution of cardioplegic solution was impaired by the ligature. After release of the ligature before the 20-minute infusion, temperature in the ischemic zone was still significantly higher than that in the nonischemic zone by an average of 8° C, but there were no group differences. In addition there was a gradient between the nonischemic and previously ischemic zone after the terminal cardioplegia delivery. These persistent temperature gradients suggest an impairment of microvascular perfusion because no obvious epicardial coronary constriction was evident at the site of occlusion.

The volume of blood cardioplegic solution delivered during the cold phase of induction and during the intermittent infusions was significantly less than the initial warm induction volume (Table II). The volume of cold cardioplegic solution was particularly diminished in the L-NA group. Delivery volume increased significantly during the longer, normo-

Table III. Segmental length data for ischemic segment

	Baseline	90 Minutes of ischemia	Reperfusion
EDL (mm)			
BCP	15.3 ± 0.7	18.1 ± 0.8*	15.7 ± 0.8*
L-Arg	16.1 ± 0.7	19.0 ± 0.4*	16.7 ± 0.9*
L-NA	19.9 ± 0.7†	21.9 ± 0.5*†	18.0 ± 1.1*
ESL (mm)			
BCP	12.8 ± 0.8	19.3 ± 0.8*	15.5 ± 0.8*
L-Arg	13.3 ± 0.6	20.4 ± 0.5*	15.9 ± 0.9*
L-NA	17.1 ± 0.9†	22.6 ± 0.5*†	18.1 ± 1.1*

Values are given as means plus or minus the standard error of the estimate.
EDL, End-diastolic length; ESL, end-systolic length.

* $p < 0.05$ versus previous period.

† $p < 0.05$ versus other two groups.

thermic terminal infusion relative to the 40-minute intermittent dose. There were no significant group differences in blood cardioplegia composition.

Segmental systolic and diastolic function. At baseline and ischemia, end-diastolic and end-systolic segment lengths in the L-NA group were greater than those in the other two groups possibly because of placement of the ultrasonic transducers farther apart (Table III). Ischemia caused paradoxical systolic expansion so that end-systolic length exceeded end-diastolic length in all groups. Both the magnitude of systolic shortening and segmental work showed severe dyskinesia. Although dysfunction assessed by systolic shortening was not as severe in the L-NA group as in the other groups, segment work was equally depressed in all groups. During reperfusion, a significant decrease in segmental shortening and segmental work persisted in all groups, but the L-Arg group showed a significantly greater restoration of segment function compared with the other groups (Fig. 1).

The β -coefficient (modulus of diastolic segmental stiffness) of the exponential end-diastolic pressure-segment length relation is summarized in Fig. 2. LAD occlusion slightly increased the segmental stiffness above the respective baseline values in all groups; segmental stiffness in the L-NA group was significantly greater than that in the other two groups. During reperfusion, segmental stiffness was significantly less in the L-Arg group than in the BCP and L-NA groups, whereas stiffness in the L-NA group was significantly greater than that in the other two groups. Therefore both systolic and diastolic

postischemic functions were improved by L-arginine treatment.

Area at risk and infarct size. The mass of the left ventricle and area at risk were similar among the BCP, L-Arg, and L-NA groups. The area placed at risk by coronary occlusion expressed as a percentage of the left ventricular mass was comparable among all groups (Fig. 3). When necrosis was expressed as a percentage of left ventricular mass, there was a strong tendency ($p = 0.08$) for the L-Arg group to have a smaller infarct relative to the BCP group. In contrast, infarct size was significantly ($p < 0.05$) increased in the L-NA group compared with that in the BCP and L-Arg groups.

The area of necrosis, expressed as a percentage of the area at risk, shown in Fig. 3, was 30.4% less ($p < 0.05$) in the hearts protected with L-arginine compared with that in the BCP group. In contrast, L-NA treatment significantly ($p < 0.05$) increased infarct size relative to that in the BCP group and that in the L-Arg group. Therefore L-arginine supplementation reduced infarct size by the L-arginine-NO pathway and blockade of this pathway not only reversed this effect, but also exacerbated infarct size.

Plasma creatine kinase activity. There was no significant difference in plasma creatine kinase activity at baseline among the three groups (Table IV). All three groups showed a similar and significant increase in plasma creatine kinase activity during LAD occlusion but no further increase during blood cardioplegia delivery. After 60 minutes of reperfusion, all three groups showed increased plasma creatine kinase activity, which was significantly greater in the L-NA group than in the other two groups, consistent with the larger infarct size in that group. There was a tendency ($p = 0.12$) for the L-Arg group to have a lower creatine kinase activity compared with that of the BCP group.

Cardiac myeloperoxidase activity. To validate the correlation between myeloperoxidase activity and neutrophil population, myeloperoxidase activity was assessed in a range (4.5×10^7 to 8.0×10^3 cells/ml) of neutrophils. The correlation coefficient was 0.962 for the following relationship: $\log PMN = 4.278 (MPO) + 33.13$, where PMN is the number of neutrophils per milliliter and MPO is the myeloperoxidase activity of the test PMN solution in units per milliliter. Therefore myeloperoxidase activity was highly correlated with the numbers of neutrophils in the test sample. In addition, the presence of the red triphenyltetrazolium chloride precipitate on viable

Fig. 1. Segmental shortening and segmental work in ischemic-reperfused zone before (*Control*) and at end of coronary occlusion (*Ischemia*) and after total of 60 minutes of reperfusion in working heart. * $p < 0.05$ versus other two groups.

Fig. 2. Segmental stiffness, indexed as β -coefficient or modulus of stiffness from exponential end-diastolic pressure-segment length relationship. * $p < 0.05$ versus previous value; ‡ $p < 0.05$ L-NA group versus BCP and L-Arg groups; † $p < 0.05$ versus other two groups.

tissue did not interfere with the myeloperoxidase assay.

Cardiac myeloperoxidase activities (expressed in units per gram tissue) in tissue samples from nonischemic, ischemic but nonnecrotic (triphenyltetrazolium chloride positive), and necrotic (triphenyltetra-

zolium chloride negative) areas of myocardium are shown in Fig. 4. Myeloperoxidase activity in the nonischemic zone was low and comparable among the BCP, L-Arg, and L-NA groups. Myeloperoxidase activity in the ischemic nonnecrotic zone increased significantly higher than the values in the nonisch-

Fig. 3. Mass of area at risk versus mass of left ventricle as percentage (*Ar/LV*, top panel), mass of area of necrosis versus mass of left ventricle as percentage (*An/LV*, middle panel), and area of necrosis as percent of area at risk (*An/Ar*, bottom panel). Although area placed at risk was comparable among groups, area of necrosis/area at risk ratio was significantly reduced in L-Arg group and significantly augmented in L-NA group. * $p < 0.05$ versus BCP and L-NA groups; ** $p < 0.05$ versus BCP and L-Arg groups. Bars represent mean $\pm$ standard error; circles represent individual values for each group.

emic zone in the three groups. However, in hearts treated with L-arginine, myeloperoxidase activity was significantly ($p < 0.05$) reduced by 65.2% of that in the BCP group, whereas blockade of NO synthase activity with L-NA increased myeloperoxidase activity. Therefore L-arginine prevented neutrophil accumulation in the nonnecrotic myocardium. However, in the necrotic zone, myeloperoxidase activity in the three groups was less than that in the ischemic nonnecrotic zone with no difference among the

three groups and no difference from that in the nonischemic zone.

In vitro coronary ring data. Our preliminary experiments showed that a 1 mmol/L concentration of L-NA was sufficient to block acetylcholine-stimulated, NO-induced relaxation. However, preliminary studies also showed that the antagonist effects of L-NA in normal coronary rings persist despite up to 3 hours of continuous washout (data not shown), which is manifested as a 70% decrease in the maximal extent of relaxation, a dramatic right shift in the dose-response curve, and an increase in the EC_{50} . Therefore L-NA infused in the experimental L-NA group would have influenced the ring analysis and created a drug-induced rather than injury-induced depression of relaxation responses. Therefore we excluded the L-NA group from in vitro coronary ring data analysis. The optimal passive length determined from length-tension responses to potassium chloride in the LAD was comparable among all groups.

Endothelium-dependent relaxations. Fig. 5 shows vasodilator responses to acetylcholine (upper panel) in the LAD and LCx rings, expressed as a percentage of U46619-induced precontraction. Although acetylcholine caused concentration-dependent relaxation responses in all three groups, differences in the magnitude of relaxation were observed among groups. There were no differences in the responses between LAD and LCx arteries in the control group. The concentration response curves in LAD rings exposed to both occlusion and cardioplegic ischemia from the BCP and L-Arg groups were markedly shifted to the right compared with the curve from the control group, with a reduction in the maximum relaxation compared with that in the control group. However, the responses in the L-Arg group were significantly ($p < 0.05$) higher than those in the BCP group at the third to the sixth concentrations of acetylcholine (0.085 to 1.685 $\mu\text{mol/L}$). EC_{50} ($-\log[\text{mol}]$), the dose of the drug required to effect relaxation to 50% of precontracted levels, also increased in the BCP (6.75 ± 0.04) and L-Arg (6.93 ± 0.03) groups compared with those in the control group (7.52 ± 0.07), but the increase in EC_{50} in the L-Arg treatment group was significantly ($p < 0.05$) less compared with that in the BCP group (Fig. 6). The concentration response curves to acetylcholine in the LCx rings exposed to cardioplegic arrest only from the BCP and L-Arg groups were also markedly shifted to the right relative to those from the control group; the maximum relaxation was significantly

Fig. 4. Myeloperoxidase (MPO) activity in nonischemic zone (NIZ), ischemic but nonnecrotic zone (IZ), and ischemic-necrotic zone (NEC). * $p < 0.05$ versus BCP and L-NA groups.

Table IV. Plasma creatine kinase activity (international units per milligram protein) during the time course of the experiment

Group	Preischemia	Ischemia	Blood cardioplegia	Reperfusion (BE)	Reperfusion (BW)
BCP	0.79 ± 0.18	6.64 ± 0.83*	9.17 ± 0.70	15.64 ± 1.26*	20.71 ± 1.73*
L-Arg	0.75 ± 0.13	7.21 ± 1.17*	7.98 ± 1.06	13.48 ± 1.73*	17.62 ± 3.36*
L-NA	0.51 ± 0.04	7.50 ± 0.36*	7.47 ± 0.64	18.25 ± 4.53*	24.04 ± 7.84*†

BE, Beating empty; BW, beating working.

* $p < 0.05$ versus previous period.

† $p < 0.05$ versus the other two groups.

reduced compared with that in the control group. In contrast to the ischemic-reperfused LAD, there were no differences in the acetylcholine response curves between the L-Arg and BCP groups (Fig. 5) in the LCx rings exposed to cardioplegia-induced ischemia. Although the EC_{50} in the BCP and L-Arg groups was significantly increased over that of the control group, the response was significantly less in the L-Arg group (Fig. 6).

The endothelium-dependent nonreceptor-mediated vasodilator A23187 also caused concentration-dependent relaxation in all three groups (Fig. 5). There were some minor differences in relaxation responses between groups; however, overall the three groups were comparable and maximal relaxations were complete ($\geq 100\%$ of precontracted tension) although maximal relaxation was less in the BCP group than in the control or L-Arg groups. A similar pattern in relaxation response to A23187 was observed in the nonischemic LCx rings. EC_{50} in the LAD ring was slightly smaller in the control group (7.72 ± 0.06) compared with that in the BCP (7.87 ± 0.04) and L-Arg (7.86 ± 0.03) groups; similarly, EC_{50} in the LCx ring was slightly smaller in

the control (7.80 ± 0.04) and BCP (7.94 ± 0.06) groups compared with that in the L-Arg group (7.90 ± 0.03). These data suggest that endothelium-dependent injury in this model is confined primarily to receptor-mediated processes, whereas nonreceptor-mediated relaxation responses remained largely intact.

Endothelium-independent smooth-muscle relaxations. Acidified sodium nitrite was used to induce endothelium-independent relaxation of vascular smooth muscle (Fig. 6). In the LAD rings, there were some depressed relaxation responses in the BCP and L-Arg groups relative to control group rings and the EC_{50} values were significantly smaller, indicating a smaller degree of smooth muscle dysfunction. However, maximal relaxations were comparable among all groups. In the LCx, there were no significant differences among groups. These data indicate that L-arginine treatment protects the coronary receptor-mediated endothelial function from ischemia and reperfusion damage, whereas neither endothelium-dependent nonreceptor-mediated vasodilation nor smooth muscle function was adversely affected. These data also suggest that superimposed

Fig. 5. Vasodilator responses in LAD and LCx vascular rings to acetylcholine, the receptor-dependent, endothelium-dependent stimulator of NO (*upper panel*); receptor-independent, endothelium-dependent NO-stimulator calcium ionophore A23187 (*middle panel*); and smooth muscle relaxing NO donor agent acidified NaNO₂ (*bottom panel*). Relaxation responses are expressed as percentage of U46619-induced precontraction. **p* < 0.05 versus control group (CTRL); †*p* < 0.05 versus CTRL, BCP; ‡*p* < 0.05 versus L-Arg, CTRL.

coronary artery occlusion-induced ischemia and global cardioplegia-induced ischemia in the LAD produce more severe injury than cardioplegic arrest alone in the LCx.

Discussion

The present study tested the hypothesis that the physiologic precursor to NO, L-arginine, in blood cardioplegic solution and as intravenous infusate during reperfusion would reduce postischemic injury by a NO-related mechanism. We found that L-arginine used as an adjunctive agent attenuated infarct size by 30.4% compared with an unsupplemented blood cardioplegic solution, preserved post-

ischemic systolic and diastolic function in the area at risk, and reduced neutrophil accumulation in the ischemic-reperfused myocardium. This cardioprotective effect of L-arginine was also accompanied by greater preservation of coronary vascular endothelial function related to stimulated production of NO. Our observations suggest that reduction of acute myocardial infarct size and avoidance of segmental systolic and diastolic dysfunction may be attributed in part to inhibition of neutrophil activities including accumulation possibly by direct inhibition of neutrophil adherence to coronary endothelium, superoxide production by activated neutrophils,¹⁹ and attenuation of neutrophil-dependent endothelial cell dam-

Fig. 6. EC_{50} values calculated from concentration relaxation curves for each experiment. *Top panel*, EC_{50} for relaxation responses to acetylcholine; *middle panel*, EC_{50} for relaxation responses to calcium ionophore A23187; *bottom panel*, EC_{50} for relaxation responses to NO donor agent acidified NaNO₂. * $p < 0.05$ versus other two groups; † $p < 0.05$ versus control group (CTRL).

age.^{1, 20} The observed cardioprotection most likely involved NO-related mechanisms because the NO-synthase blocker L-NA reversed the beneficial effect of L-arginine completely. Therefore supplementation of blood cardioplegia and reperfusion reduced postischemic injury by mechanisms involving the L-arginine–NO pathway. This study adds to the growing number of studies that ascribe a beneficial role to NO in reducing the pathologic manifestations of ischemia-reperfusion injury.^{3, 21, 22}

Previous studies have demonstrated that both regional and global myocardial ischemia and reperfusion impair coronary vascular endothelial function, characterized as reduced vasodilator responses to endothelium-dependent stimulation of nitric oxide.^{1–6} In the present study, the ischemic-reperfused

LAD sustained significantly greater endothelial damage than did the LCx, which underwent only cardioplegic arrest and reperfusion. This is consistent with the concept that normothermic ischemia sets the stage for injury, whereas endothelial injury is expressed primarily during reperfusion.^{2, 3, 23, 24} The data also suggest that otherwise normal coronary arteries tolerate 1 hour of cardioplegic arrest well. Endothelial dysfunction in the LAD of the untreated BCP group was reversed by L-arginine, consistent with the dose-dependent reversibility shown by others^{25, 26} and dose-dependent intracellular transport of L-arginine.²⁶ Results of the present study are consistent with the reversibility of ischemically impaired endothelial dysfunction by supplemental L-arginine.^{3, 21} These findings suggest that

endothelial dysfunction can be attenuated with myoprotective strategies that preserve the coronary endothelium at the time of cardioplegic infusion or blood reperfusion. Although previous studies from our laboratory have shown that NO-donor agents are cardioprotective against global postischemic dysfunction,⁶ more studies need to be done with models of acute coronary artery occlusion that simulate surgical revascularization of evolving infarction to determine optimal doses and applications of L-arginine, as well as the mechanisms involved in endothelial preservation.

In the present study we found that neutrophil accumulation in the nonischemic myocardium (assessed by myeloperoxidase activity) was reduced by 65.2% in dogs treated with L-arginine. However, there were no differences in myeloperoxidase activity in the necrotic zone among the three groups. Previous studies by Chatelain and associates,²⁷ Engler and Covell,²⁸ and Dreyer and associates²⁹ have shown that reperfusion markedly enhances the infiltration of neutrophils into the ischemic-reperfused myocardium. One mechanism of the cardioprotective and endothelial protective effects of L-arginine may be related to the antineutrophil properties of NO.^{1, 10, 30, 31} The present study suggests that L-arginine treatment may be an effective therapy directed against neutrophil activity in surgical revascularization of acute myocardial infarction. The observation that myeloperoxidase activity was not increased in the necrotic region of the BCP and L-NA groups is in contrast to results of our previous studies in which L-arginine and NO-donor agent SPM-5185 reduced myeloperoxidase activity in the necrotic zone.^{1, 3} This may suggest that neutrophil accumulation is not a mechanism associated with necrosis or that necrosis occurred before reperfusion-induced neutrophil accumulation in the surgical model. Further studies are needed to resolve this issue.

Reperfusion of total coronary occlusion is accompanied by severe systolic and diastolic contractile dysfunction^{3, 15} even in the absence of infarction.³²⁻³⁴ Surgical reperfusion with use of a period of blood cardioplegia-induced arrest allows partial restoration of regional postischemic dysfunction, but significant hypokinesia persists.^{35, 36} In the present study, we found that ischemic-reperfused segments treated with blood cardioplegic solution enhanced with L-arginine demonstrated significantly better postischemic systolic and diastolic function than hearts treated with unenhanced blood cardioplegic

solution. This functional preservation was not only reversed but exacerbated by the NO synthase antagonist L-NA. These data confirm involvement of the L-arginine-NO synthase pathway and are consistent with previous reports from this laboratory in which L-arginine produced temporary recovery of regional postischemic function after nonsurgical reperfusion³ and NO donor agents increased regional or global functional recovery after nonsurgical and surgical reperfusion.^{1, 6, 37} In the nonsurgical models,^{1, 3} preservation of postischemic contractile performance may be associated with a concomitant reduction in infarct size, as in the present study. However, because even noninfarcted myocardium exhibits postischemic dysfunction (stunning),³³ protective mechanisms with L-arginine treatment may also involve preservation of the contractile apparatus or myocyte calcium kinetics.^{38, 39} Further studies would be required to elucidate the exact mechanism or mechanisms responsible for preservation of postischemic systolic and diastolic function with L-arginine treatment. However, inhibition of neutrophils and neutrophil-mediated damage to the contractile apparatus may be implicated from the present study and those of others.^{40, 41}

In summary, L-arginine used as an adjunct to blood cardioplegia and as intravenous therapy reduced infarct size and restored partial postischemic systolic and diastolic function in the ischemic-reperfused segment. Blockade of these beneficial effects by the NO synthase inhibitor suggests that this cardioprotection was expressed through the L-arginine-NO pathway and most likely involved inhibition of neutrophil accumulation within the area at risk and preservation of the coronary vascular endothelium. The results of this study suggest that (1) the endogenous L-arginine-NO pathway acts as an inherent cardioprotective mechanism unmasked by L-NA and (2) L-arginine may act by recruiting endogenous NO, presumably from the vascular endothelium. The results are in contrast with data from Matheis and associates⁴² that suggest that NO is a mediator of deleterious processes in models of hypoxia-reoxygenation injury, possibly via conversion to peroxynitrite and hydroxyl radicals.^{43, 44} The present study supports the use of adjunctive L-arginine during cardiac operations in addition to exogenous donors of NO. As suggested by the study of Cohen and colleagues,⁴⁵ the L-arginine-NO pathway may be recruited by pretreatment with precursor L-arginine before cardiac operation or by oral doses before operation.

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REFERENCES

1. Lefer DJ, Nakanishi K, Johnston WE, Vinten-Johansen J. Antineutrophil and myocardial protection actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion in dogs. *Circulation* 1993;88:2337-50.
2. Tsao PS, Aoki N, Lefer DJ, Johnson G III, Lefer AM. Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. *Circulation* 1990;82:1402-12.
3. Nakanishi K, Vinten-Johansen J, Lefer DJ, Fowler WC III, McGee DS, Johnston WE. Intracoronary L-arginine during reperfusion improves endothelial function and reduces infarct size. *Am J Physiol* 1992; 263:H1650-8.
4. Lefer AM, Tsao PS, Lefer DJ, Ma X. Role of endothelial dysfunction in the pathogenesis of reperfusion injury after myocardial ischemia. *FASEB J* 1991;5:2029-34.
5. Nakanishi K, Zhao ZQ, Vinten-Johansen J, Lewis JC, McGee DS, Hammon JW Jr. Coronary artery endothelial dysfunction after ischemia, blood cardioplegia, and reperfusion. *Ann Thorac Surg* 1994;58:191-9.
6. Lefer DJ, Nakanishi K, Vinten-Johansen J. Endothelial and myocardial cell protection by a cysteine-containing nitric oxide donor after myocardial ischemia and reperfusion. *J Cardiovasc Pharmacol* 1993; 22:S34-43.
7. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for biological activity of endothelium-derived relaxing factor. *Nature* 1987;327:525-6.
8. Furchgott RF, Carvalho MH, Khan MT, Matsunaga K. Evidence for endothelium-dependent vasodilation of resistance vessels by acetylcholine. *Blood Vessels* 1987;24:145-9.
9. Furlong B, Henderson AM, Lewis MJ, Smith JA. Endothelium-derived relaxing factor inhibits in vitro platelet aggregation. *Br J Pharmacol* 1987;90:687-92.
10. Gryglewski RJ, Palmer RMJ, Moncada S. Superoxide anion is involved in the breakdown of endothelium-derived relaxing factor. *Nature* 1986;320:454-60.
11. Palmer RMJ, Rees DD, Ashton DS, Moncada S. L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem Biophys Res Commun* 1988;153: 1251-6.
12. Hudspeth DA, Williams MW, Zhao ZQ, et al. Pre-treatment pentostatin augments interstitial fluid adenosine and prevents postischemic dysfunction in canine hearts protected with blood cardioplegia. *Ann Thorac Surg* 1994;58:719-27.
13. Rosenkranz ER, Vinten-Johansen J, Buckberg GD, Okamoto F, Edwards H, Bugyi H. Benefits of normothermic induction of blood cardioplegia in energy-depleted hearts, with maintenance of arrest by multiple cold blood cardioplegic infusions. *J THORAC CARDIOVASC SURG* 1982;84:667-77.
14. Yokoyama H, Julian JS, Vinten-Johansen J, et al. Postischemic $[Ca^{2+}]$ repletion improves cardiac performance without altering oxygen demands. *Ann Thorac Surg* 1990;49:894-902.
15. Vinten-Johansen J, Lefer DJ, Nakanishi K, Johnston WE, Brian CA, Cordell AR. Controlled coronary hydrodynamics at the time of reperfusion reduces postischemic injury. *Coron Artery Dis* 1992;3:1081-93.
16. Vinten-Johansen J, Nakanishi K, Zhao ZQ, McGee DS, Tan P. Acadesine improves surgical myocardial protection with blood cardioplegia in ischemically injured canine hearts. *Circulation* 1993;88(Suppl): II350-9.
17. Tan P, Klopfenstein HS, Vinten-Johansen J. Interactive software for analysis of global or segmental cardiac systolic and diastolic performance by pressure-volume (dimension) relations [Abstract]. *J Am Coll Cardiol* 1993;21:15A.
18. Holm S. A simple sequentially rejective multiple test procedure. *Scand J Stat* 1979;6:65-70.
19. Clancy RM, Leszczynska-Piziak J, Abramson SB. Nitric oxide, an endothelial cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. *J Clin Invest* 1992;90:1116-21.
20. Sato H, Zhao ZQ, McGee DS, Hammon JW Jr., Vinten-Johansen J. L-Arginine supplementation during surgical revascularization of myocardial ischemia reduces infarction and coronary endothelial injury [Abstract]. *FASEB J* 1994;8:A854.
21. Weyrich AS, Ma X, Lefer AM. The role of L-arginine in ameliorating reperfusion injury after myocardial ischemia in the cat. *Circulation* 1992;86:279-88.
22. Johnson G III, Tsao PS, Lefer AM. Cardioprotective effects of authentic nitric oxide in myocardial ischemia with reperfusion. *Crit Care Med* 1991;19:244-52.
23. Siegfried MR, Erhardt J, Rider T, Ma X, Lefer AM. Cardioprotection and attenuation of endothelial dysfunction by organic nitric oxide donors in myocardial ischemia-reperfusion. *J Pharmacol Exp Ther* 1992; 260:668-75.
24. Nakanishi K, Zhao ZQ, Vinten-Johansen J, Hudspeth DA, Lewis JC, Hammon JW. The effects of blood cardioplegia on the coronary vascular endothelium [Abstract]. *FASEB J* 1993;7:703.
25. Gold ME, Bush PA, Ignarro LJ. Depletion of arterial L-arginine causes reversible tolerance to endothelium-

- dependent relaxation. *Biochem Biophys Res Commun* 1989;164:714-21.
26. Bogle RG, Coade SB, Moncada S, Pearson JD, Mann GE. Bradykinin and ATP stimulate L-arginine uptake and nitric oxide release in vascular endothelial cells. *Biochem Biophys Res Commun* 1991;180:926-32.
 27. Chatelain P, Latour J, Tran D, De Lorgeril M, Duras G, Bourassa M. Neutrophil accumulation in experimental myocardial infarcts: relation with extent of injury and effect of reperfusion. *Circulation* 1987;75:1083-90.
 28. Engler R, Covell JW. Granulocytes cause reperfusion ventricular dysfunction after 15-minute ischemia in the dog. *Circ Res* 1987;61:20-8.
 29. Dreyer WJ, Michael LH, West MW, et al. Neutrophil accumulation in ischemic canine myocardium: insights into time course, distribution, and mechanism of localization during early reperfusion. *Circulation* 1991;84:400-11.
 30. McCall T, Whittle BJR, Broughton-Smith NK, Moncada S. Inhibition of FMLP-induced aggregation of rabbit neutrophils by nitric oxide [Abstract]. *Br J Pharmacol* 1988;95:517P.
 31. Kubes P, Suzuki M, Granger N. Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc Natl Acad Sci* 1991;88:4651-5.
 32. Vinten-Johansen J, Carroll PJ, Johnston WE, et al. Reversal of dyskinesia by increased end diastolic segment length in ischaemic-reperfused myocardium. *Cardiovasc Res* 1989;23:810-20.
 33. Vinten-Johansen J, Gayheart PA, Johnston WE, Julian JS, Cordell AR. Regional function, blood flow, and oxygen utilization relations in repetitively occluded reperfused canine myocardium. *Am J Physiol* 1991;261:H538-47.
 34. Braunwald E, Kloner RA. The stunned myocardium: prolonged, post-ischemic ventricular dysfunction. *Circulation* 1982;66:1146-9.
 35. Vinten-Johansen J, Edgerton TA, Howe HR, et al. Immediate functional recovery and avoidance of reperfusion injury with surgical revascularization of short-term coronary occlusion. *Circulation* 1985;72:431-9.
 36. Vinten-Johansen J, Edgerton TA, Hansen KJ, Carroll P, Mills SA, Cordell AR. Surgical revascularization of acute (1 hour) coronary occlusion: blood versus crystalloid cardioplegia. *Ann Thorac Surg* 1986;42:247-54.
 37. Nakanishi K, Zhao ZQ, Vinten-Johansen J, Hudspeth DA, Hammon JW Jr. Blood cardioplegia enhanced with an organic nitric oxide donor reverses postischemic ventricular dysfunction and coronary endothelial damage. *Surg Forum* 1993;44:245-7.
 38. der Toit EF, Opie LH. Modulation of severity of reperfusion stunning in the isolated rat heart by agents altering calcium flux at onset of reperfusion. *Circ Res* 1992;70:960-7.
 39. Kusuoka H, Marban E. Role of altered calcium homeostasis in stunned myocardium. In: Kloner RA, Przyklenk K, eds. *Stunned myocardium: properties, mechanisms, and clinical manifestations*. New York: Marcel Dekker, 1993:197-213.
 40. Bolli R, Jeroudi MO, Patel BS, et al. Marked reduction of free radical generation and contractile dysfunction by antioxidant therapy begun at the time of reperfusion: evidence that myocardial "stunning" is a manifestation of reperfusion injury. *Circ Res* 1989;65:607-22.
 41. Mullane K, Engler R. Proclivity of activated neutrophils to cause postischemic cardiac dysfunction: participation in stunning? *Cardiovasc Drugs Ther* 1991;5:915-24.
 42. Matheis G, Sherman MP, Buckberg GD, Haybron DM, Young HH, Ignarro LJ. Role of L-arginine-nitric oxide pathway in myocardial reoxygenation injury. *Am J Physiol* 1992;262:H616-20.
 43. Radi R, Beckman JS, Bush KM, Freeman BA. Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Arch Biochem Biophys* 1991;288:481-7.
 44. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci* 1990;87:1620-4.
 45. Cohen NM, Tarry WC, Wise RM, Makhoul RG, Damiano RJ Jr. Chronic administration of L-arginine, a nitric oxide precursor, protects the heart from ischemic-reperfusion injury [Abstract]. *Circulation* 1993;88(Suppl):I330.