Diminished Basal Nitric Oxide Release After Myocardial Ischemia and Reperfusion Promotes Neutrophil Adherence to Coronary Endothelium

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We measured changes in basal release of nitric oxide and its effect on polymorphonuclear leukocyte (PMN) adherence to endothelial cells (ECs) in a feline model of myocardial ischemia (90 minutes) and reperfusion. Basal release of nitric oxide from the left anterior descending coronary artery (LAD) after myocardial ischemia/reperfusion and from the control left circumflex coronary artery (LCX) was assessed by $N^\text{G}$-nitro L-arginine methyl ester (L-NAME)–induced vasoconstriction. L-NAME induced a significant EC-dependent vasoconstriction in control LCX rings (0.28±0.04 g), which was fully reversed by L-arginine but not d-arginine. L-NAME–induced vasoconstriction of LAD rings was not significantly changed after 90 minutes of myocardial ischemia without reperfusion. However, 10 minutes of reperfusion reduced the L-NAME–induced vasoconstriction to 0.13±0.04 g (p<0.05), and this was restored by addition of 3 mM l-arginine but not d-arginine. Longer periods of reperfusion progressively decreased L-NAME–induced vasoconstriction. After 270 minutes of reperfusion, L-NAME–induced vasoconstriction was virtually abolished. Myocardial ischemia without reperfusion did not increase PMN adherence to ECs. However, PMN adherence to LAD ECs was significantly increased after 20 minutes of reperfusion (39±6 to 105±9 PMNs/mm², p<0.01), and incubation of LAD segments with l-arginine significantly attenuated this increase in PMN adherence. After 270 minutes of reperfusion, PMN adherence to LAD ECs was further increased to 224±10 PMNs/mm² (p<0.001). This increase in PMN adherence was almost completely blocked by MAb R15.7, a monoclonal antibody against CD18 of PMNs, and was significantly attenuated by MAb RR1/1, a monoclonal antibody against intercellular adhesion molecule-1 of ECs (p<0.01). These results indicate that decreased basal release of endothelium-derived relaxing factor after myocardial ischemia/reperfusion precedes enhanced PMN adherence to the coronary endothelium, which may lead to PMN-induced myocardial injury.

(Circulation Research 1993;72:403–412)

KEY WORDS • $N^\text{G}$-nitro L-arginine methyl ester • isolated coronary artery rings • L-arginine • polymorphonuclear leukocytes • endothelium-derived relaxing factor

The endothelium produces a variety of vasodilator and vasoconstrictor substances that regulate vascular tone and exert a number of other important cardiovascular effects.1,2 One of the most important of these vasodilators is endothelium-derived relaxing factor, which has been identified as nitric oxide (NO).3,4 NO also inhibits platelet aggregation5 and may have antineutrophil properties.6 Recent studies have shown that there is basal NO release from normal endothelial cells that can be enhanced when the endothelium is stimulated with endothelium-dependent vasodilators.5,7 Evidence is accumulating that vasodilator-stimulated NO release from coronary vascular endothelium is significantly impaired in myocardial ischemia followed by reperfusion.8–10 However, basal release of NO in myocardial ischemia and reperfusion has not been studied in this setting.

It is now well recognized that polymorphonuclear leukocytes (PMNs) play an important role in myocardial injury associated with ischemia and reperfusion.11 Accumulating evidence suggests that PMNs also contribute to endothelial dysfunction after reperfusion of the coronary vasculature.12 Recently, NO has been shown to be an endogenous inhibitor of leukocyte chemotaxis,13 adherence,6 and activation.14 Since normal endothelial cells release NO basally and this NO may prevent PMNs from adhering to endothelial cells, it is conceivable that reduced basal NO release after myocardial reperfusion may promote or allow PMN adherence to endothelial cells, leading to neutrophil-induced reperfusion injury. Therefore, the purposes of this study were 1) to determine the time course of changes in basal NO release from coronary vascular endothelium after myocardial ischemia with reperfusion and 2) to relate basal NO release to alterations in PMN adherence to coronary vascular endothelial cells.
Materials and Methods

Adult male cats (2.5–4.0 kg) were anesthetized with sodium pentobarbital (30 mg/kg i.v.). An intratracheal cannula was inserted through a midline incision, and all cats were placed on intermittent positive-pressure ventilation (small animal respirator, Harvard Apparatus, South Natick, Mass.). A polyethylene catheter was inserted into the right external jugular vein for supplemental addition of sodium pentobarbital as needed. Another polyethylene catheter was positioned in the abdominal aorta through the left femoral artery for measurement of mean arterial blood pressure via a Statham P23AC pressure transducer. After a midsternal thoracotomy, the pericardium was opened, and a 2-0 silk ligature was placed around the left anterior descending coronary artery (LAD) 8–10 mm from its origin. Standard lead II of the scalar electrocardiogram (ECG) was used to determine heart rate and ST-segment elevation. The ECG and mean arterial blood pressure were continuously recorded on an oscillographic recorder (model 7, Grass Instrument Co., Quincy, Mass.). ST-segment elevations were determined manually from an ECG recording at 50 mm/sec every 20 minutes. The pressure–rate index, an approximation of myocardial oxygen demand, was calculated as the product of mean arterial blood pressure and heart rate divided by 1,000.

In five additional cats, we measured the LAD flow before coronary occlusion, during coronary occlusion, and after reperfusion by using an electromagnetic flowmeter (model ME 501, Carolina Medical Electronics Inc., King, N.C.) connected to Grass model 7 oscillographic recorder. The cat LAD was isolated carefully, and an EP-102 flow probe was placed around the LAD.

Experimental Protocol

After completing all surgical procedures, cats were allowed to stabilize for 30 minutes, at which time a baseline reading of ECG and mean arterial blood pressure was taken. Myocardial ischemia was produced by tightening the previously placed reversible ligature around the LAD to completely occlude the vessel. This was designated as time 0. After 90 minutes of ischemia, the LAD ligature was untied, and the ischemic myocardium was reperfused for 0, 10, 20, 120, and 270 minutes. Five to six cats were studied at each time point.

Isolated Coronary Ring Studies

At the end of each experiment, both LAD and left circumflex coronary artery (LCX) segments (0.3–0.5 mm i.d.) were dissected out and placed in warmed Krebs-Henseleit (K-H) buffer consisting of (mM) NaCl 118, KCl 4.75, CaCl2·2H2O 2.54, KH2PO4 1.19, MgSO4·7H2O 1.19, NaHCO3 12.5, and glucose 10.0. Isolated coronary vessels were cleaned and cut into rings 2–3 mm in length. The rings were then mounted on stainless-steel hooks, suspended in 10-ml tissue baths, and subsequently connected to Grass FT-03 force displacement transducers to record changes in force using a Grass model 7 oscillographic recorder. The baths were filled with 10 ml K-H buffer and aerated at 37°C with a gas mixture of 95% O2–5% CO2. Coronary rings were initially stretched to give a preload of 0.5 g of force and equilibrated for 60–90 minutes. A preload of 0.5 g has been proved to be optimal in cat coronary artery rings, and 1 g of preload often induces endothelial injury. During this period, the K-H buffer in the tissue baths was replaced every 20 minutes. After equilibration, the rings were then exposed to 100 nM 11-epoxymethanoprostaglandin H2 (U-46619, Biomol Laboratories, Plymouth Meeting, Pa.), a thromboxane A2 mimic, to generate approximately 0.5 g of developed force. Once a stable contraction was obtained, 0.1, 1, 10, and 100 nM acetylcholine (ACH), a typical endothelium-dependent vasodilator, was added to the bath to assess the changes in agonist-stimulated NO synthesis and release after myocardial ischemia and reperfusion. After the response stabilized, the rings were washed, and developed force was allowed to return to baseline. The procedure was then repeated with NaNO2, an endothelium-independent vasodilator (0.1, 1, 10, and 100 μM). These rings were washed and allowed to equilibrate to baseline again.

Cat PMN Isolation and Labeling

Autologous PMNs were isolated by a procedure modified from Lafrado and Olsen. Peripheral blood (20 ml) was collected from the femoral artery before coronary artery occlusion and anticoagulated with citrate-phosphate-dextrose solution (anticoagulant: whole blood, 1:4:10 [vol:vol]; Sigma Chemical Co., St. Louis, Mo.) into round-bottom polycarbonate centrifuge tubes (Nalge Co., Rochester, N.Y.). Platelet-rich plasma was obtained by centrifuging blood at 400g for 20 minutes in an Ecospin tabletop centrifuge (Sorvall Instruments, Wilmington, Del.). Platelet-rich plasma was decanted and centrifuged at 2,500g for 10 minutes to obtain platelet-poor plasma. Platelet-poor plasma was then mixed with isotonic Percoll (9 vol Percoll:1 vol 1.5 M NaCl, Sigma) to produce Percoll–platelet-poor plasma density gradients of 80%, 62%, and 50%. Eight milliliters of 4% dextran (average molecular weight, 60,000–90,000; Sigma) was added to the erythrocyte-leukocyte suspension from the initial 400g centrifugation. After mixture by inversion, the erythrocytes were allowed to settle for 50 minutes. The upper suspension was recentrifuged, and the pellet was resuspended in 1 ml of 0.9% NaCl and layered onto the Percoll–platelet-poor plasma gradient. Centrifugation was then performed at 400g 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 phosphate-buffered saline (PBS) before being assayed for viability by trypan blue exclusion. PMN preparations obtained by this method were typically >95% pure and >95% viable.

Isolated PMNs were then labeled with Zynaxis PKH2 fluorescent dye according to the method of Yuan and Fleming. One milliliter of diluent was added to a loose cell pellet containing approximately 10 million cells. 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% platelet-poor plasma in PBS) was added to stop the reaction, and another 5 ml PBS was then added subsequently to the suspension. Cells were then centrifuged at 2,500g for
10 minutes at room temperature. The supernatant was removed, and the cells were resuspended in PBS and then recounted. This labeling procedure yields cells possessing normal morphology and function. The labeling efficiency was evaluated by comparing the number of PMNs counted under light microscopy with the number counted under epifluorescent microscopy. After completing the labeling procedure, PMNs were first counted under light microscopy, and a total value of PMNs per milliliter (i.e., both labeled PMNs and unlabeled PMNs) was obtained. The same samples of PMNs were then counted under epifluorescent microscopy. In eight separate experiments, the number of PMNs per milliliter was 24.5 ± 1.2 million/ml under light microscopy and 24.1 ± 1.3 million/ml under epifluorescent microscopy. These results indicate that the efficiency of the labeling procedure used in this study was 98%.

**PMN Adherence to Coronary Artery Endothelium**

Ischemic/reperfused LAD and control nonischemic/reperfused LCX segments were carefully isolated and cut into rings 2–3 mm in length. These rings then were opened carefully and placed endothelial side up in 5-ml round cell-culture dishes containing 3 ml K-H solution. After 10 minutes of preincubation of the coronary segments, autologous unstimulated PMNs (4 × 10^6 cells/ml) were added and incubated for 20 minutes. Coronary artery segments were then removed from culture dishes and dipped three to four times in fresh K-H solution. These coronary segments were then placed on a glass slide with the endothelial side up. The number of PMNs adhering to the endothelial surface in five separate microscopic fields was counted under epifluorescent microscopy at a magnification of ×100, and the number of adherent PMNs per square millimeter of endothelial surface area was calculated.

**Validation of Cross-reactivity of Monoclonal Antibodies to Cat Cells**

In some experiments, monoclonal antibodies (MAbs) against adhesion molecules (MAb R15.7 against CD18 and R11/1 against intercellular adhesion molecule-1 [ICAM-1]) were used (Dr. R. Rothlein, Boehringer-Ingelheim Pharmaceuticals). The binding of MAb R15.7 to freshly isolated cat PMNs was determined by flow cytometric analysis. Neutrophils were isolated from peripheral arterial blood (20–40 ml) in five cats, as described above. Suspensions of 1.0 × 10^6 cat PMNs in Dulbecco's PBS were treated with human block immunoglobulin G (4.0 mg/ml, Sigma), and then MAb R15.7 (20 μg/ml) or human immunoglobulin G1 (20 μg/ml, Coulter Corp., Hialeah, Fla.) was added to the cat PMNs. The PMNs were then placed on ice for 30 minutes. The cells were spun in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N.Y.) for 30 seconds at 7,000 rpm, and the supernatant was removed. The PMNs were then washed in Dulbecco's PBS to remove any excess of primary antibodies. F(ab')2 fragments of a goat anti-mouse immunoglobulin G–phycoerythrin conjugate (Tago, Inc., Burlingame, Calif.) was used as the secondary antibody reagent at a 1:100 dilution. The stained neutrophils were fixed in 1% paraformaldehyde and then analyzed on a Coulter EPICS flow cytometer.

The cross-reactivity of RR1/1 to cat vascular endothelium was previously assessed by immunohistochemical localization of ICAM-1. At a dilution of 1:500, RR1/1 strongly cross-reacted with ICAM-1 selectively on ischemic/reperfused cat coronary endothelial cells. A slight degree of basal reaction to RR1/1 was observed in control nonischemic cat coronary endothelium. However, control antibodies failed to react in either control or ischemic/reperfused coronary vessels.

**Statistical Analysis**

All values in the text, table, and figures are presented as mean ± SEM of n independent experiments. All data were subjected to analysis of variance followed by the Bonferroni correction for post hoc t test comparison. Values of p ≤ 0.05 were considered to be statistically significant.

**Results**

**Cardiac Electrophysiological and Hemodynamic Changes**

In all groups of cats studied, there were no significant differences in any of the variables observed before coronary occlusion. A few minutes after LAD occlusion, the ST segment of the ECG became significantly elevated and peaked at 20–40 minutes after coronary occlusion. After reperfusion, the ST segment decreased to nearly control values, indicating that significant reperfusion occurred. There were no significant differences in peak ST-segment elevation among the ischemic groups, indicating that the ischemic insult was similar in the ischemic groups. At reperfusion, there was a noticeable increase in the incidence of premature ventricular contractions in all cats. There was no obvious overall difference between the ischemic groups in the number of premature ventricular contractions occurring after reperfusion. In all groups of ischemic/reperfused cats, the pressure–rate index decreased significantly after coronary occlusion and gradually returned to nearly control values after reperfusion. There were no significant differences between the ischemic groups at any of the hourly pressure–rate index readings.

Control LAD blood flow before occlusion was 5.1 ± 0.8 ml/min. Ligating the LAD induced a complete occlusion (i.e., blood flow decreased to zero). Untying of the ligature around the LAD after 90 minutes of occlusion resulted in successful reperfusion in all five cats. Immediately after reperfusion, LAD blood flow increased to 8.6 ± 1.3 ml/min, indicating that reperfusion was complete, including the occurrence of reactive hyperemia. After 3–5 minutes of reperfusion, LAD blood flow gradually returned to control levels and was maintained at this value for the whole observation period. These results clearly indicate that the reperfusion was successful in our cat model of myocardial ischemia and reperfusion.

**L-NAME-Induced Endothelium-Dependent Vasoconstriction in Cat Coronary Arteries**

L-NAME is a potent inhibitor of endothelial NO synthase. When infused in vivo, L-NAME induces a dose-dependent increase in mean arterial blood pressure. Addition of L-NAME in vitro produces an endothelium-dependent contraction and an inhibition of
endothelium-dependent relaxation induced by ACh. In the present study, we observed that L-NAME (1 mM) resulted in a significant endothelium-dependent vasoconstriction in cat coronary arteries that was reversed by L-arginine (3 mM) but not by d-arginine (3 mM). In endothelium-denuded rings, L-NAME did not induce any detectable coronary vasoconstriction, and L-arginine did not induce coronary vasorelaxation (Figure 1). Moreover, in coronary arterial rings isolated from cats subjected to 90 minutes of ischemia and 270 minutes of reperfusion, significant endothelial dysfunction occurred that was characterized by the loss of ACh-induced endothelium-dependent vasodilation and a decreased L-NAME–induced vasoconstriction (Figure 2). These results indicate that there is a basal release of NO in normal cat coronary endothelium that opposes basal tone. When basal synthesis and release of NO was inhibited by L-NAME, a significant vasoconstriction resulted because of the unopposed basal vascular tone. However, in coronary rings in which the endothelium had been mechanically denuded or subjected to ischemia/reperfusion, addition of L-NAME produced little or no vasoconstriction (Figure 2).

Effect of Reperfusion Duration on L-NAME–Induced Vasoconstriction in Cat Coronary Artery Rings

Isolated coronary rings were studied to assess basal NO release by measuring the L-NAME–induced vasoconstriction. In control coronary rings, addition of 1 mM L-NAME induced 0.25–0.30 g of force (Figure 3). L-NAME added to rings isolated from cats subjected to 90 minutes of ischemia without reperfusion also resulted in a comparable contractile response to L-NAME. However, ischemia followed by 20 minutes of reperfusion resulted in significantly depressed L-NAME–induced vasoconstriction. Longer periods of reperfusion caused further decrements in the contractile response to L-NAME. At 270 minutes of reperfusion, the L-NAME response was almost totally abolished and was not significantly different from that observed with endothelium-denuded coronary rings. However, the vasoconstrictor responses to U-46619 and KCl were not decreased at 20 and 270 minutes after reperfusion (Table 1), indicating that vasoconstrictor response of vascular smooth muscle was not decreased after reperfusion, and the loss of L-NAME–induced vasoconstriction could not be attributed to the general vascular smooth muscle injury. Moreover, the L-NAME–induced vasoconstriction in coronary rings isolated from control nonischemic LCX rings remained unchanged after reperfusion, indicating that endothelial dysfunction after reperfusion is specific for the LAD. These data are summarized in Figure 3. Thus, there appears to be a significant loss of basal release of NO from the coronary endothelium soon after reperfusion (i.e., as early as 10 minutes) that progresses to near maximum values at 120 minutes after reperfusion.

Restoration of L-NAME–Induced Vasoconstriction by L-Arginine

Since L-arginine is the substrate for NO synthesis, we tested the effects of adding L-arginine in vitro on L-NAME–induced vasoconstriction. In the rings undergoing 20 minutes of reperfusion, the L-NAME–induced vasoconstriction was significantly decreased (Figure 4). Repeated addition of L-NAME after 20 minutes caused

![Figure 1](image1.png)

**Figure 1.** Typical recordings of vasoconstriction induced by N⁶-nitro L-arginine methyl ester (L-NAME, 1 mM) in control cat coronary artery rings with endothelial cells (+EC) or without endothelial cells (−EC). For +EC, L-NAME induced significant vasoconstriction, and this vasoconstriction was totally reversed by L-arginine (3 mM) but not by D-arginine (3 mM). For −EC, L-NAME (1 mM) did not produce any significant vasoconstriction.

![Figure 2](image2.png)

**Figure 2.** Representative recordings of acetylcholine (ACh)–induced and NaNO₂-induced vasorelaxation (left two recordings) and N⁶ nitro L-arginine methyl ester (L-NAME, 1 mM)–induced contraction (right recordings). Arrows in the left two recordings indicate the addition of U-46619; dots on top indicate the addition of ACh (0.1, 1, 10, and 100 nM) or NaNO₂ (0.1, 1, 10, and 100 μM). Control coronary arterial rings exhibited significant ACh- and NaNO₂-induced relaxation and L-NAME–induced vasoconstriction. In coronary arterial rings exposed to 90 minutes of myocardial ischemia (MI) and 270 minutes of reperfusion (R), ACh-induced vasorelaxation was almost completely lost, and L-NAME–induced contraction was not observed, indicating significant endothelial dysfunction.
Inhibition of PMN adherence was significantly restored (Figure 4). In contrast, addition of D-arginine did not restore the L-NAME response. Similarly, addition of L-arginine to LAD rings from the cats subjected to 270 minutes of reperfusion had no effect on L-NAME–induced vasocontraction. These results imply that loss of basal NO release occurs early after reperfusion and may become refractory to substrate addition later in reperfusion.

**Inhibition of Basal NO Release From Normal Coronary Artery Endothelial Cells Resulting in Increased PMN Adherence**

In the present study, we observed the effect of inhibiting basal NO release from normal coronary arteries on PMN adherence to endothelium. L-NAME (1 mM) was incubated with control coronary artery segments for 20 minutes. These coronary segments were then transferred to fresh K-H solution that did not contain L-NAME. Unstimulated autologous PMNs were then incubated for another 20 minutes with these artery segments. In normal coronary artery segments, few PMNs adhered to the endothelium (Figure 5). However, when basal release of NO was inhibited by L-NAME, there was a twofold to threefold increase in PMN adherence to the coronary endothelium. Addition of 3 mM L-arginine simultaneously with L-NAME totally prevented this increase in PMN adherence to the coronary endothelium, whereas addition of 3 mM D-arginine had no effect (Figure 5). Moreover, pretreatment of coronary segments with other vasocontractors, such as vasopressin (1 μM) and KCl (20 mM), did not promote PMN adherence, indicating that L-NAME preincubation–induced increase in PMN adherence could not be attributed to the nonspecific vasocontractor effect. These results suggest that basal release of NO significantly inhibits PMN adherence to endothelial cells, but reduced basal NO release markedly enhances PMN adherence.

**Effects of Different Durations of Reperfusion on PMN Adherence to Ischemic/Reperfused Coronary Artery Endothelial Cells**

We found that basal NO release from ischemic/reperfused coronary artery endothelium was progressively decreased after reperfusion, and inhibition of basal NO release from normal arteries promotes PMN adherence to the endothelial cells. Thus, we studied the effects of different durations of reperfusion on PMN adherence to the ischemic/reperfused coronary artery endothelium. In nonischemic coronary arteries, few PMNs adhered to the endothelial surface (Figure 6). Ninety minutes of ischemia without reperfusion or 10 minutes of reperfusion did not change their adheriveness. However, after 20 minutes of reperfusion, PMN adherence to endothelium was increased threefold to fourfold (p<0.01) (Figure 6). A progressive increase in PMN adherence to ischemic/reperfused coronary artery endothelium occurred over time, such that at 270 minutes of reperfusion there was a sixfold to eightfold increase in PMN adherence (p<0.001) (Figure 6). PMN adherence to the nonischemic control LCX segments was not changed at any time points observed.

**Effects of L-Arginine Incubation on PMN Adherence to Ischemic/Reperfused Coronary Arteries**

L-Arginine (3 mM) or D-arginine (3 mM) was incubated with coronary artery segments isolated from cats after 20 or 270 minutes of reperfusion for 20 minutes. These coronary artery segments were then transferred to fresh K-H solution that did not contain either L- or D-arginine. Unstimulated PMNs were then incubated with the coronary ring segments for another 20 minutes, and PMN adherence was assessed. As shown in Figure 7, PMN adherence to the coronary endothelium subjected to 90 minutes of ischemia and 20 minutes of

**TABLE 1. Vasocontractor Response of Left Anterior Descending Coronary Artery Rings After Myocardial Ischemia and Reperfusion**

<table>
<thead>
<tr>
<th>Time after reperfusion (minutes)</th>
<th>Vasocontractor response (g)</th>
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<tbody>
<tr>
<td></td>
<td>U-46619 (100 nM)</td>
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<tr>
<td>-90 (before ischemia)</td>
<td>0.51±0.08</td>
</tr>
<tr>
<td>90</td>
<td>0.54±0.07</td>
</tr>
<tr>
<td>270</td>
<td>0.60±0.07</td>
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Values are mean±SEM for 10–12 rings.
FIGURE 4. Bar graphs summarizing vasocontraction (g) to N\textsuperscript{G}-nitro l-arginine methyl ester (L-NAME, 1 mM) in control left circumflex arterial rings (CON-LCX) and ischemic-reperfused left anterior descending arterial rings (IR-LAD) before and after the addition of either 3 mM L-arginine (IR-LAD+L-arginine) or 3 mM D-arginine (IR-LAD+D-arginine). The left panel indicates coronary arterial rings exposed to 90 minutes of ischemia and 20 minutes of reperfusion (90' MI+20' R), and the right panel indicates coronary arterial rings subjected to 90 minutes of ischemia and 270 minutes of reperfusion (90' MI+270' R). Bar heights are means; brackets indicate ±SEM; and numbers at the bottom of bars are numbers of coronary rings studied. As shown, L-NAME-induced contraction was significantly lost after reperfusion but was restored with L-arginine in rings subjected to only 20 minutes of reperfusion.

reperfusion was significantly increased. However, this enhanced PMN adherence to endothelial cells from ischemic/reperfused coronary artery segments was markedly attenuated by incubation with l-arginine for 20 minutes. In contrast, incubation of ischemic/reperfused coronary segments with D-arginine had no effect on PMN adherence. Moreover, l-arginine incubation had no effect on PMN adherence to the coronary endothelium subjected to 90 minutes of ischemia and 270 minutes of reperfusion (Figure 7). Furthermore,

FIGURE 5. Bar graph showing polymorphonuclear leukocyte (PMN) adherence to the endothelium of left circumflex coronary artery (LCX) ring segments. L-NAME, N\textsuperscript{G}-nitro l-arginine methyl ester. PMN adherence was observed in LCX segments alone (LCX+PMN) and in segments incubated with 1 mM L-NAME (LCX+L-NAME+PMN), 1 mM L-NAME and 3 mM L-arginine (LCX+L-NAME+L-arg.+PMN), 1 mM L-NAME and 3 mM D-arginine (LCX+L-NAME+D-arg.+PMN), 1 μM vasopressin (LCX+vasopressin+PMN), or 20 mM KCl (LCX+KCl+PMN). Data are expressed as numbers of adhered PMNs per endothelial surface area (mm\textsuperscript{2}). Bar heights are means; brackets indicate ±SEM; and the numbers at the bottom of bars are numbers of coronary ring segments studied. **p<0.01 compared with LCX+PMN group.
incubation of PMNs, rather than the coronary arteries, with L-arginine for 20 minutes did not significantly decrease PMN adherence to the coronary artery segments subjected to 20 minutes of reperfusion, indicating that the inhibitory effects of L-arginine on PMN adherence occurred on the endothelium.

Role of CD18/ICAM-1 Interaction in PMN Adherence to Ischemic/Reperfused Coronary Endothelium

The binding of MAb R15.7 to freshly isolated cat neutrophils was confirmed by flow cytometric analysis. Cat neutrophils (n=5) treated with MAb R15.7 (20 µg/ml) were 98.0±1.0% positive and exhibited a mean channel fluorescence value of 164.0±53.5. In contrast, aliquots of cat neutrophils (n=5) treated with an immunoglobulin G control (20 µg/ml) were 5.0±0.7% positive, with a mean channel fluorescence value of 3.8±1.0. These results clearly demonstrate that MAb R15.7 cross-reacts with CD18, the β chain of the cat neutrophil adherence glycoprotein complex. In some experiments, 20 µg/ml MAb R15.7 was incubated with 4×10⁷/ml PMN suspension for 5 minutes. Ischemic/reperfused coronary artery segments were then placed into this suspension. Similar studies were done with MAb RR1/1 (40 µg/ml), an MAb against ICAM-1, and incubated for 5 minutes with coronary artery segments rather than suspensions of PMNs. Unstimulated PMNs were then added and incubated for another 20 minutes. MAb R15.7 dramatically attenuated the increase in PMN adherence to the coronary endothelium subjected to 20 minutes of reperfusion (Figure 8). Although not as effective as MAb R15.7, MAb RR1/1 also significantly decreased PMN adherence (Figure 8). In contrast to the effects of L-arginine on PMN adherence, MAb R15.7 and MAb RR1/1 both significantly attenuated the increase in PMN adherence to the coronary endothelium subjected to 270 minutes of reperfusion (Figure 8). These results implicate the adherence glycoprotein complex of neutrophils and their primary counterreceptor ICAM-1 on the endothelium as being important factors in PMN-induced coronary vascular injury.
Discussion

There are two modes of NO release via constitutive NO synthase present in endothelial cells. Stimulated NO release occurs in response to a variety of endothelium-dependent dilators including ACh, adenine nucleotides (e.g., ADP), thrombin, bradykinin, substance P, and the calcium ionophore A23187. Another mode of endothelial NO production is via basal NO release. In unanesthetized animals, basal NO release is considered to be the major mode of NO production by vascular endothelial cells. This basal release of NO in vivo plays an important role in regulating vascular tone. When basal NO release is blocked by in vivo administration of NO synthase inhibitors (e.g., analogues of l-arginine such as Nω-monomethyl l-arginine [L-NMMA], Nω-iminoethyl l-ornithine [L-NIO], and L-NAME), a significant increase in mean arterial blood pressure occurs. Moreover, in vitro addition of L-NMMA, L-NIO, or L-NAME to isolated vascular rings produces endothelium-dependent vasoconstriction. Thus, it is now widely accepted that NO synthase inhibitor–induced vasoconstriction is a reliable index for estimating basal NO release.

Recent studies of myocardial ischemia and reperfusion have shown marked endothelial dysfunction of the coronary vasculature characterized by reduced endothelium-derived relaxing factor release in response to endothelium-dependent dilators. Earlier studies by Mehta et al. and Van Benthuysen et al. indicated a decrease in endothelium-dependent relaxation of isolated coronary artery rings to a variety of endothelium-dependent vasodilators, including ACh, ADP, bradykinin, thrombin, and A23187. These results indicate that stimulated NO synthesis or release is significantly decreased after reperfusion of ischemic coronary vasculature. However, the consequence of diminished basal NO release, which may play a more important role in vascular homeostasis, has not been studied. Our present results clearly show that the addition of L-NAME to control cat coronary artery rings induced a significant endothelium-dependent vasoconstriction that was reversed by L-arginine but not by D-arginine. This vasoconstrictor response was absent in endothelium-denuded coronary rings. The L-NAME–induced vasoconstriction followed a time course comparable to that previously shown for endothelial dysfunction characterized by decreased vasorelaxant responses to endothelium-dependent vasodilators. Thus, 90 minutes of ischemia without reperfusion did not change L-NAME–induced vasoconstriction, indicating that basal NO release was not impaired by ischemia alone. However, after 10 minutes of reperfusion, the L-NAME–induced vasoconstriction was already significantly decreased. Moreover, this endothelial dysfunction progressively worsened so that by 270 minutes of reperfusion L-NAME–induced vasoconstriction was almost totally abolished.

The mechanisms underlying the loss of basal NO release after myocardial ischemia and reperfusion are not clear. However, there are several potential mechanisms to explain reduced basal NO release after reperfusion. First, depletion of L-arginine, the substrate for NO synthesis, might occur after ischemia and reperfusion. Recent results show that normal endothelial cells can recycle L-citrulline, a coproduct of NO synthesis from L-arginine, to produce additional L-arginine, thus
maintaining a high concentration of L-arginine in endothelial cells. This recycling is significantly inhibited by L-glutamine. Although it is unlikely that so short a period of reperfusion as 10–20 minutes could critically deplete L-arginine levels, ischemia followed by reperfusion may block the recycling of L-citrulline to L-arginine, thereby decreasing the L-arginine concentration in endothelial cells. Second, NO synthase activity in coronary vascular endothelial cells may decrease after ischemia/reperfusion. It is well recognized that oxygen-derived free radicals (i.e., superoxide radicals) and oxygenezed lipoproteins significantly inhibit NO synthesis. In this connection, a large burst of free radicals is generated from endothelial cells shortly after reperfusion that could inhibit NO synthesis. Third, neutrophils once adhered and activated may release more free radicals that may inactivate NO and inhibit NO synthesis. In this connection, Ohlstein and Nichols reported that formyl-Met-Leu-Phe-activated rabbit PMNs induced significant endothelium-dependent vasoconstriction and reversed ACh- and A23187-induced vasorelaxation. These effects were significantly attenuated by the superoxide radical scavenger human superoxide dismutase. Fourth, Vallance et al. recently reported that two L-NMMA-like endogenous inhibitors of NO synthesis were found in patients suffering from renal failure. These endogenous inhibitors may also be present in ischemic/reperfused endothelial cells, therefore inhibiting NO synthesis.

The loss of basal NO release may have significant pathophysiological significance in reperfusion injury. First, decreased NO release may promote vasoconstriction, thus contributing to the "no-reflow phenomenon" after myocardial ischemia and reperfusion. Second, loss of basal NO release may facilitate platelet aggregation and release of platelet mediators (e.g., thromboxane A2 and platelet activating factor), which may exacerbate myocardial injury. Third, since NO is a potent endogenous inhibitor of PMN chemotaxis, adherence, and activation, decreased NO release may promote PMN adherence to the endothelium, which may contribute to endothelial and myocardial damage during the reperfusion period. In the present study, we observed that decreased basal NO release after reperfusion was associated with a significant increase in PMN adherence to ischemic/reperfused endothelium. This effect clearly is specific for reduced NO rather than being related to vasoconstriction, since L-NNAME induces increased adherence, but other vasoconstrictors (e.g., vasopressin and KCl) were without any proadhesion effect (Figure 5). These results have important potential pathological significance, since adherent PMNs may subsequently produce and release large amounts of free radicals, cytokines, and proteases, all of which may amplify endothelial dysfunction and promote cardiac damage. Therefore, the early decrease of basal NO release may promote PMN adherence to endothelial cells, which may aggravate endothelial dysfunction, leading to a vicious cycle.

In this study, we found that endothelial dysfunction manifested by decreased basal NO release was reversed by addition of L-arginine at 20 minutes but not at 270 minutes of reperfusion. These results suggest that, during the early period of reperfusion, NO synthesis is functionally abnormal, but this decreased NO release could be overcome by addition of additional substrate. When reperfusion is prolonged, dysfunction of the endothelial membrane L-arginine transfer system or of the endothelial NO synthetic system may occur progressively. Therefore, the severe endothelial dysfunction characterized by virtually complete loss of NO release could not be reversed by L-arginine.

Interactions of circulating leukocytes with the vascular endothelium are regulated by the balance of proadhesion factors and antiadhesion factors. Decreasing antiadhesion factors or increasing proadhesion factors would disturb the net balance and result in an increase in neutrophil adherence. In the nonischemic reperfused normal vascular endothelium, inhibiting NO production by L-NNAME could promote the interaction of basally expressed CD11/CD18 on the PMN surface with its major ligand, ICAM-1, which is constitutively expressed on the unstimulated endothelium at low levels of activity. During the early period of reperfusion, both adherence molecules (i.e., CD11/CD18 on PMNs and ICAM-1 on endothelial cells) can be significantly upregulated and NO production is significantly decreased. This increase in proadhesion factors together with decreases in antiadhesion factors can result in a significant increase in PMN adherence. Since reduced NO production is reversible by addition of L-arginine at this time, the increase in PMN adherence during the early reperfusion period could be attenuated by addition of L-arginine to reverse the NO production. However, in the later stages of reperfusion, endothelial dysfunction becomes largely irreversible, and NO production cannot be restored by L-arginine. Thus, endogenous NO synthetic mechanisms cannot be used to restore endothelial function in the late stages of reperfusion injury. Therefore, PMN adherence at this time could only be attenuated by directly adding NO, by giving an NO donor, or by addition of an antibody to CD18 or to endothelial ICAM-1. In this regard, our present results demonstrate that an MAb directed against either CD18 or ICAM-1 significantly attenuated the increase in PMN adherence to ischemic/reperfused coronary endothelium, providing direct evidence that CD18–ICAM-1 interaction plays a critical role in PMN–endothelium interaction after myocardial ischemia and reperfusion.

The effect of adhered PMNs on vascular activity exhibits significant species differences. Using rat PMNs, Mehta et al. and Lee et al. found that activated PMNs produce a vasorelaxation that may be mediated by neutrophil-derived NO. However, in other studies in which neutrophils were isolated from rabbit, dog, and cat, activated PMNs resulted in significant vasoconstriction in both aorta and coronary artery. It is very likely that in the cat, dog, and rabbit, any NO generated by PMNs is overriden by superoxide radicals, which contract vascular smooth muscle rather than relax it. Thus, NO generation from PMNs, if it occurs in cat PMNs, probably does not exert significant pathophysiological effects in reperfusion injury, as investigated in this study.

In summary, we have demonstrated that basal NO release was significantly decreased after myocardial ischemia and reperfusion. These responses occurred as early as 10 minutes after reperfusion, reaching near maximum effects approximately 120 minutes after...
reperfusion. It is well recognized that PMN chemotactic and activating agents such as platelet activating factor, complementa, and leukotriene B4, as well as endothelial stimulating factors such as tumor necrosis factor-α and interleukin-1β are significantly increased after myocardial ischemia and reperfusion, all of which may promote PMN adherence to endothelial cells. In the present study, we found that basal NO release was significantly decreased after myocardial ischemia and reperfusion, which may also promote PMN adherence to ischemic/reperfused endothelium via CD18/ICAM-1-dependent mechanisms and contribute to PMN-mediated endothelial and myocardial injury.

Acknowledgments

The authors thank Dr. Xiao-yong Li, Dr. Bruce Bochner, and Sherry Sterbinsky for their valuable assistance.

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Circ Res. 1993;72:403-412
doi: 10.1161/01.RES.72.2.403
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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