Influx of Neutrophils Into the Walls of Large Epicardial Coronary Arteries in Response to Ischemia/Reperfusion

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**Background.** There are several clinical situations in which large epicardial coronary arteries are deprived of blood flow, such as occurs when an obstructing thrombus or embolus lodges within a vessel or during coronary dissection. There is little information concerning the effect of flow deprivation on large epicardial coronary arteries.

**Methods and Results.** We studied a model in which a segment of a large epicardial coronary artery was deprived of blood flow using both proximal and distal clamps for 3 hours followed by reperfusion. On examination by light microscopy of cross sections of the arteries, 19±6 neutrophils were present in the intima of ischemic/reperfused vessels, whereas only a mean of 4±3 (SEM) were present in the intima of nonischemic vessels (p<0.02). On average, there were 17±9 neutrophils just under the elastic lamina in ischemic/reperfused vessels versus none in the nonischemic vessels (p<0.05); there were 16±10 neutrophils present within the media of ischemic/reperfused vessels, and none (p<0.05) in the nonischemic vessels. Electron microscopic analysis revealed that neutrophils in the ischemic/reperfused vessels were often "sandwiched" between the endothelial cells and the elastic lamina. Ultrastructural abnormalities within the myocardium also revealed damage to the microvasculature, including the presence of neutrophils within the vessels and erythrocyte stasis. To rule out the possibility that findings in the large epicardial arteries were due to toxic substances from static blood within the isolated arterial segment, a protocol was performed in which blood was removed from the isolated segment. Again, neutrophil infiltration into the vessel was observed. Resting mean epicardial coronary artery blood flow before coronary occlusion was 19±3 ml/min; mean coronary blood flow 2.5 hours after reperfusion was identical at 19±3 ml/min. Response to both endothelial-dependent vasodilation (acetylcholine) and endothelial-independent vasodilation (nitroglycerin) challenges was normal early after reperfusion but was depressed late after reperfusion, suggesting progressive vascular dysfunction and hence a form of vascular reperfusion injury in this model.

**Conclusions.** When large epicardial coronary arteries are deprived of blood flow, followed by reperfusion in this model, neutrophils migrate into the vessel wall as well as into the microvasculature. These abnormalities are associated with reduced endothelial-dependent and endothelial-independent coronary vasodilator reserve. (Circulation 1991;84:1758–1772)

Theoretically, endothelial damage to blood vessels, induced by ischemia, could lead to worsening ischemia. Although microvascular morphology and the no-reflow phenomenon within the myocardium have been studied in detail in experimental models of ischemia and reperfusion,1–3 there is less information available concerning the effect of ischemia on large epicardial coronary arteries. There are several clinical situations in which large epicardial coronary arteries are deprived of blood flow; one example is when an obstructing thrombus or embolus lodges within a vessel, and another is during coronary dissection.

The purposes of the present study were to determine whether ischemia, followed by reperfusion, causes morphological abnormalities or stimulates neutrophil infiltration into the ischemic/reperfused segment of an epicardial coronary artery, to char-
characterize the nature of any morphological abnormalities, and to assess whether there are any physiological abnormalities in coronary blood flow or coronary vascular reserve associated with the abnormal morphology.

Therefore, we used an experimental model in which a segment of epicardial coronary artery was isolated from blood flow using proximal and distal atraumatic clamps. Studies with electron and light microscopy were carried out to assess the extent of neutrophil infiltration into the large epicardial coronary arteries; in separate experiments, Doppler flow probes were then used to assess coronary flow and coronary flow reserve.

**Methods**

**Protocol 1: Effect of Ischemia/Reperfusion on Neutrophil–Epicardial Coronary Artery Interaction: Electron Microscopic Analysis**

Mongrel dogs of either sex were anesthetized with sodium pentobarbital (30–35 mg/kg i.v.) to maintain a surgical depth of anesthesia, intubated, and ventilated. Additional anesthesia was administered as needed. A left thoracotomy was performed through the fifth left intercostal space, and the pericardium was incised to expose the surface of the heart. A segment of the left anterior descending coronary artery or a diagonal branch approximately 1–2 cm long and a segment of the circumflex artery or marginal branch of the circumflex artery were isolated. There were no branches along the isolated segments. The left anterior descending coronary or diagonal artery segment was then occluded with both proximal and distal atraumatic Schwartz vascular clamps for 3 hours. The clamps were then removed, and the vessel was reperfused for 2½ hours. With the use of both proximal and distal clamps, a segment of epicardial coronary artery was isolated from any blood supply from either intraluminal flow or flow through the vasa vasorum. The isolated circumflex or its marginal segment served as a nonischemic control. At the end of 2½ hours of reperfusion, the animals were euthanized under deep anesthesia with an intracardiac injection of potassium chloride. The midsection of the isolated coronary segments were carefully removed with a scalpel, with care taken to avoid the very proximal and distal portions of the segments where the clamps had been placed. Sections approximately 0.5–1.0 cm distal to the clamp were also obtained. Myocardial tissue from the left anterior and circumflex bed was also obtained for morphological analysis. The sections were immersed in Karnovsky’s fixative (2.5% glutaraldehyde and 2% formaldehyde) for a minimum of 3 hours, washed with buffer, osmicated for 1½ hours with 1% osmium, dehydrated in graded acetone, and embedded in Araldite. Thick sections (0.5–1.0 μm) were prepared and stained with 1% toluidine blue for light microscopy, and thin sections were stained with uranyl acetate and lead citrate for electron microscopic analysis.

The number of neutrophils per 70 μm of artery circumference was determined in eight equidistant sites around the vessels by one of the investigators (F.G.) with the use of 0.5–1.0-μm sections and light microscopy. Electron microscopy also was performed to obtain qualitative evaluation of ultrastructural abnormalities of the blood vessels.

Sections 0.5–1.0 μm thick of three different blocks of the left ventricles from each of seven animals were examined (by F.G.) for quantitative purposes under light microscopy at a magnification of ×1,200. An ocular reticle (Carl Zeiss) was used to delineate a square field on which a uniform distribution of 42 sampling points was superimposed. Starting at the upper left corner of each section, two successive laterally adjacent fields, each completely filled by myocardial tissue, were brought into view. The number of sampling points overlying abnormal and unaltered myocardial profiles was counted to determine the volume fractions of altered myocytes recognizable by light microscopy. The total number of vessels was determined, and the percentage of vessels with stasis was calculated. The number of vessels containing two or more leukocytes as well as red blood cells and the pressure of leukocytes in the interstitium was also tabulated.

Throughout all of the protocols, this study conformed to the guidelines prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication NIH 85-23, revised, 1985).

**Protocol 2: Effect of Ischemia/Reperfusion on Number and Distribution of Neutrophils in Large Epicardial Coronary Arteries**

The purpose of this protocol was to determine whether the morphological abnormalities observed in protocol 1 could also be appreciated by routine light microscopic techniques.

In this protocol, the basic experimental design was similar to that of protocol 1, with the exception that acepromazine (4–6 mg i.m.) was administered before anesthesia. In addition, lidocaine (1.5 mg/kg i.v.) was administered just before coronary artery occlusion. A segment of the left anterior descending coronary artery or a diagonal branch was occluded by proximal and distal nontraumatic clamps for 3 hours and then reperfused for 2½ hours. A marginal branch of the circumflex artery served as a nonischemic control. At the end of the reperfusion period, the middle of the isolated segment of ischemic/reperfused and nonischemic epicardial artery was removed, fixed in 10% buffered formalin, and prepared for light microscopic analysis. Cross sections of the coronary arteries were stained with hematoxylin and eosin.

The total number of neutrophils present in a cross section of the coronary arteries was counted with the use of light microscopy. The locations of the neutroph-
Phil in the intima, media, and elastic lamina were also recorded. Neutrophil counts were performed in a blinded fashion.

Protocol 3: Is Neutrophil Infiltration Into the Walls of the Epicardial Coronary Arteries a Result of Blood Stasis?

In protocols 1 and 2, neutrophil infiltration into the walls of blood vessels occurred within segments isolated from the circulation and then reperfused. To determine whether neutrophil infiltration into large epicardial coronary arteries subjected to ischemia and reperfusion was due to stasis of blood (with toxic metabolites of the static blood leading to endothelial injury), we carried out an additional study. This study was similar to protocol 2 except that immediately after coronary artery occlusion, blood was removed from the isolated segment with a tuberculin syringe, and the segment was then washed with buffered saline. Thus, during the 3-hour occlusion of the isolated coronary segment, blood was not present within this segment of the coronary artery. At the end of 3 hours, reperfusion of blood was induced by removing the proximal and distal atraumatic clamps. At the end of reperfusion, the dogs were euthanized as described above, and the hearts were fixed in 10% buffered formalin. The coronary arteries (left anterior descending or diagonal, serving as ischemic/reperfused; circumflex or marginal branch of circumflex, serving as nonischemic control) were then prepared and sectioned in a transverse plane for light microscopy. Histological sections were stained with hematoxylin and eosin, and neutrophils were counted in a blinded fashion.

Protocol 4: Is This Model Associated With Reduced Blood Flow Through Large Epicardial Coronary Arteries?

While protocols 1, 2, and 3 dealt with morphological features of an isolated ischemic/reperfused segment of the epicardial coronary artery, protocols 4 and 5 assessed coronary blood flow in this model. In protocol 4, we sought to determine whether the model of isolating a segment of coronary artery with proximal and distal clamps was associated with abnormalities in epicardial coronary artery blood flow.

Dogs were prepared for surgery as described above. A cannula was placed into the carotid artery to monitor blood pressure and heart rate. A section of the left anterior descending or circumflex artery long enough to accommodate proximal and distal clamps with no side branches in between, as well as a flow probe, was isolated. A Doppler flow probe was used first to measure epicardial coronary blood flow preocclusion and then after 3 hours of coronary artery occlusion and 2½ hours of reperfusion.

Protocol 5: Is This Model Associated With Reduced Coronary Vasodilator Reserve as Assessed by Either Endothelial-Dependent or Endothelial-Independent Vasodilators?

Anesthetized open-chest dogs were intubated and ventilated. A proximal segment of either the left anterior descending or circumflex artery was isolated, and a Doppler flow probe was placed around the vessel just distal to the site to be occluded. A carotid arterial line was placed to continuously measure blood pressure. Heart rate was monitored with an electrocardiogram. Under fluoroscopic guidance, a catheter was positioned in the coronary artery to be occluded. During baseline readings, acetylcholine (an endothelial-dependent vasodilator; mean dose, 1.2 μg i.e.) was administered through the catheter, and the change in coronary blood flow was recorded. Once coronary blood flow had returned towards baseline, nitroglycerin (an endothelial-independent vasodilator; mean dose, 59 μg i.e.) was administered through the catheter. In one dog in which the ostium of the coronary was difficult to cannulate, the agents were administered into the left ventricular cavity (no difference in response was noted compared with the other dogs). Change in coronary blood flow was recorded, and blood flow was allowed to return to baseline. Lidocaine (1.5 mg/kg i.v.) was administered. The coronary arterial segment was then occluded, with proximal and distal atraumatic vascular clamps as described above, for 90 minutes followed by 2 hours of reflow.

Vasodilator reserve was again tested early after reperfusion (within 30 minutes in five dogs in which reactive hyperemia had largely subsided) by administration of the same doses of acetylcholine and then nitroglycerin as during baseline. At the end of 2 hours, vasodilator response in the coronary artery was again tested in all dogs.

Protocol 6

In an additional study in two anesthetized dogs, we examined the effects of dissection and manipulation on epicardial vessel coronary vasodilator reserve. The circumflex artery was dissected from epicardial fat, and a suture was placed around the artery without coronary occlusion. A Doppler flow probe was placed around the vessel just distal to the suture. The left anterior descending artery received a transient 15-minute occlusion. During two baseline readings, acetylcholine (10 μg in one dog and 80 μg in the other) and then nitroglycerin (100 μg) were injected intravenously. These doses were repeated four times over the next 3 hours. The increase in coronary blood flow was recorded.

Statistics

Wilcoxon rank sum test was used for comparing neutrophil counts in 70-μm intervals on thick sections of occluded/reperfused segments versus nonischemic control arteries and versus the distal portion of the occluded/reperfused artery. Wilcoxon rank sum test was used for comparing neutrophil counts in intima, subintimal elastic lamina, and media of control versus ischemic/reperfused arteries in protocols 2 and 3. Paired t statistic was used for comparing coronary blood flow values before occlusion and after reperfusion in protocol 4. Paired t statistic was used
to compare changes in response to acetylcholine and nitroglycerin at baseline with those at 2 hours after reperfusion (protocol 5). Analysis of variance was used to compare heart rate and mean systemic pressure throughout this protocol.

**Results**

**Protocol 1: Effect of Ischemia/Reperfusion on Neutrophil–Epicardial Coronary Artery Interaction: Electron Microscopic Analysis**

Ten animals were entered into this protocol. One animal died because of ventricular fibrillation during the coronary occlusion, and one animal died after induction of anesthesia. Data from eight animals were available for qualitative assessment; seven also had histological sections adequate for quantitative analysis.

Qualitative assessment revealed only occasional neutrophils adhering to the endothelium of the nonischemic coronary arteries (Figure 1). In contrast, in the arteries subjected to 3 hours of coronary artery occlusion and 2½ hours of reperfusion, numerous neutrophils appeared to be adhering to or associated with the intima (Figure 2). The neutrophils often appeared to be located underneath the endothelial cells, sandwiched between these and the elastic lamina (Figures 2 and 3). The endothelium overlying the neutrophils often appeared vacuolated; in some cases, platelets also were observed to be adhering to the endothelial cells (Figure 4). The neutrophils occasionally appeared to be penetrating the elastic lamina and entering the media; in some cases, the endothelium appeared denuded (Figure 5). There was an average of 2.0±0.8 (mean±SEM) neutrophils per 70-μm of coronary artery per dog in the occluded and reperfused coronary arteries versus 0.4±0.2 in the nonischemic control arteries ($p=0.03$). Examination of vessels distal to the distal clamp also revealed neutrophils adhering to the coronary artery, but these tended to be less numerous than within the previously occluded segment (1.2±0.6, $p=NS$ versus the occluded segment and nonischemic controls).

Ultrastructural data on the myocardium and microvasculature of the ischemic reperfused zone are shown in Table 1 and Figures 6 and 7. The myocardium from the region supplied by the ischemic/reperfused zone showed variable degrees of myocardial damage, including areas of necrosis with and without contraction bands, densities in mitochondria (Figure 6), and vacuolization. Microvessels demon-

![Figure 1](http://circ.ahajournals.org/)

**FIGURE 1.** Electron micrograph of nonischemic vessel. Endothelial cell (arrowhead) is intact. Nucleus of an endothelial cell is present (left). Elastic lamina (el) is present under endothelial cells. A portion of media appears on bottom, including a smooth muscle cell. Original magnification, ×14,500.
FIGURE 2. Electron micrograph of ischemic/reperfused coronary artery segment. A neutrophil (n) is present between endothelium (e) and elastic lamina (el). Original magnification, ×25,000.

strated various degrees of damage as well, including endothelial disruption, red cell plugging, platelet adhesion (Figure 7), and intraluminal blebs. Neutrophils were often observed within the microvessels, and occasional neutrophils were observed in the interstitium. Foci of microscopic hemorrhage were observed (Figure 7). Nonischemic myocardium and microvasculature from the circumflex territory appeared normal.

Protocol 2: Effect of Ischemia/Reperfusion on Number and Distribution of Neutrophils in Large Epicardial Coronary Arteries

Eight of 10 dogs entered into this protocol had adequate histological sections for neutrophil counting. The number of dogs with neutrophils attached to the endothelium was two of eight in the nonischemic circumflex marginal (Figure 8), but seven of eight in the ischemic/reperfused left anterior descending or diagonal arteries (p<0.05 by χ² analysis, Figure 9). Four of eight dogs had neutrophils present within the media of the ischemic/reperfused artery (Figure 10); neutrophils were not observed in the media of control, nonischemic vessels.

On average, there were 19.1±5.7 (mean±SEM) neutrophils attached to the intimal circumference of ischemic reperfused vessels on the light microscopic cross section compared with 3.5±2.7 neutrophils in the nonischemic control (p<0.02). On average, there were 16.8±9.0 neutrophils just under the elastic lamina (Figure 11) in the ischemic/reperfused vessel versus none (p<0.05) in the nonischemic vessel. There were 16.1±9.6 neutrophils within the media of the ischemic/reperfused vessels versus none (p<0.05) in the nonischemic vessels.

Protocol 3: Is Neutrophil Infiltration Into the Walls of the Epicardial Coronary Arteries a Result of Blood Stasis?

Four dogs entered and successfully completed the protocol. In the nonischemic epicardial coronary artery, there were an average of 2.2±1.0 (±SEM) neutrophils within the intima and none in the media; in contrast, in the ischemic/reperfused vessels, there were an average of 54±12 neutrophils in the intima and 47±40 neutrophils in the media. These results suggest that neutrophil infiltration into the walls of the epicardial arteries isolated
from the circulation and then reperfused is not due to stasis of blood within the ischemic segments.

**Protocol 4: Is This Model Associated With Reduced Blood Flow Through Large Epicardial Coronary Arteries?**

Seven dogs were entered into the protocol. Two died of ventricular fibrillation, and in a third dog, no blood flow values were available after 1 hour of occlusion because of flowmeter malfunction. Thus, successful experiments were performed in four dogs. Data are shown in Figure 12. Mean coronary blood flow before coronary artery occlusion was 19±3 ml/min; mean coronary blood flow 2½ hours after reperfusion was identical at 19±3 ml/min. Heart rate was 135±5 beats/min before coronary artery occlusion and 117±17 beats/min 2½ hours after reperfusion (p=NS). Mean arterial blood pressure was 120±10 mm Hg before occlusion and 114±8 mm Hg 2½ hours after reperfusion.

**Protocol 5: Is This Model Associated With Reduced Coronary Vasodilator Reserve as Assessed by Either Endothelial-Dependent or Endothelial-Independent Vasodilators?**

Of 15 dogs entered into the protocol, seven successfully completed the protocol. Seven dogs died of ventricular fibrillation during coronary artery occlusion. One dog was eliminated when a major branch of the left anterior descending coronary artery had to be permanently ligated because of bleeding. Successful vasodilator data were obtained in seven of seven dogs before occlusion and seven of seven dogs 2 hours after reperfusion; data were available in five of seven dogs early after reperfusion (within 30 minutes, at which time reactive hyperemia had fully subsided to baseline in four dogs and to about 40 ml/minute in a fifth dog.

The hemodynamic variables of heart rate and mean systemic arterial pressure remained similar throughout the duration of the protocol. At baseline, heart rate was 135±6 beats/min, and mean systemic pressure was 130±6 mm Hg. Soon after reperfusion (within 30 minutes), heart rate was 121±6 beats/min, and mean systemic pressure was 112±10 mm Hg. After 2 hours of reperfusion, heart rate was 122±6 beats/min, and mean arterial pressure was 111±9 mm Hg. There were no significant differences in either variable at any time point.

Mean resting coronary artery flow in this group was similar at baseline, within 30 minutes of reperfusion in five dogs in which reactive hyperemia resolved, and after 2 hours of reperfusion (16±3,
21±6, and 15±3 ml/min, respectively). However, changes in vasodilator response to challenge doses of acetylcholine and nitroglycerin altered with time. Increases in coronary blood flow after acetylcholine and nitroglycerin are shown in Table 2 and Figure 13.

Within 30 minutes after reperfusion, there was no change in vasodilator response for either acetylcholine (increase in coronary blood flow was 23±5 ml/min before occlusion and 24±10 ml/min at 30 minutes) or for nitroglycerin (increase in flow was 28±9 ml/min at baseline and 29±12 ml/min after 30 minutes). However, at 2 hours after reperfusion, vasodilator response showed an increase in flow with acetylcholine that was now only 12±5 ml/min ($p<0.03$ versus preocclusion baseline), and increase in flow with nitroglycerin was only 11±3 ml/min ($p=0.05$ versus preocclusion baseline).

Protocol 6

Baseline increases in blood flow in the nonoccluded circumflex artery ranged from 207% to 221% of control in response to acetylcholine and from 195% to 228% of control in response to nitroglycerin. During the ensuing 3 hours, increases in blood flow ranged from 173% to 255% of control in response to acetylcholine and from 200% to 257% of control in response to nitroglycerin, suggesting that manipulation of the artery per se did not impede vasoreactivity.

Discussion

The results of the present study show that deprivation of blood flow followed by reperfusion of large epicardial coronary arteries is a stimulus for neutrophil attachment to and migration into the walls of the arteries. The neutrophils appear to attach to the endothelium, then lodge themselves underneath the endothelium, and then penetrate the elastic lamina of the vessel. In some cases, they also were observed within the media. They were often associated with vacuolization of the endothelium, but in most cases the endothelium did not appear morphologically altered or necrotic. This raises the possibility that the neutrophils themselves may be contributing to damage of the vessel. It is well known that activated neutrophils release toxic substances, including oxygen free radicals and hypochlorous acid.\(^5\)

Could neutrophil infiltration have been secondary to endothelial damage induced by toxins associated with static blood trapped in the isolated arterial segment? This possibility is highly unlikely because when blood was removed from the isolated segment and the isolated segment was washed with buffered

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**FIGURE 4.** Ischemic/reperfused coronary segment. A neutrophil is present under endothelium. Endothelial cell appears vacuolated (v). Platelets (p) are attached to vacuolated endothelium. Original magnification, ×18,500.

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\(^5\) Reference to hypochlorous acid.
saline, we still observed neutrophil infiltration into the blood vessel. Most likely, ischemia of the isolated coronary segment itself followed by reperfusion was the stimulus for neutrophil infiltration.

Are the morphological observations of neutrophil infiltration into the epicardial vessels associated with any functional abnormality in epicardial coronary blood flow in vivo? Coronary blood flow in the large epicardial vessel was the same after 3 hours of occlusion and 2½ hours of reperfusion as it was at baseline. Therefore, the neutrophil infiltration into either the large blood vessels or microvasculature damage did not alter resting epicardial coronary artery blood flow levels. However, this model was associated with altered in vivo coronary vasodilator reserve. We tested vasodilator capacity in this model by administering acetylcholine (which is known to stimulate endothelium-derived relaxing factor and thus acts as an endothelial-dependent vasodilator) as well as nitroglycerin (which stimulates vasodilation by direct action on the smooth muscle cells and is therefore an endothelial-independent dilator). Before coronary occlusion, both agents induced substantial increases in coronary blood flow within the epicardial coronary artery. Soon after reperfusion both agents produced increases in coronary blood flow similar to those before occlusion. However, there was a substantial change in coronary vascular reserve after 2 hours of reperfusion, when coronary vasodilation with both acetylcholine and nitroglycerin was significantly blunted. These results suggest that flow reserve deteriorates over time within the first few hours of reperfusion.
Table I. Histological Abnormalities in Ischemic/Reperfused Subendocardium of Left Anterior Descending Coronary Artery Territory

<table>
<thead>
<tr>
<th>Dog</th>
<th>Vessels (n)</th>
<th>Vessels with RBC stasis</th>
<th>Extravascular RBCs</th>
<th>Microvessels with WBCs* (n)</th>
<th>WBCs in interstitium</th>
<th>Contraction band necrosis (vol%)</th>
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<tbody>
<tr>
<td>1</td>
<td>165</td>
<td>39 23</td>
<td>++</td>
<td>5</td>
<td>+</td>
<td>25</td>
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<td>2</td>
<td>168</td>
<td>22 13</td>
<td>+</td>
<td>9</td>
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<td>6</td>
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<tr>
<td>3</td>
<td>179</td>
<td>31 17</td>
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<td>8</td>
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<td>4</td>
<td>165</td>
<td>26 16</td>
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<td>5</td>
<td>143</td>
<td>13 9</td>
<td>+++</td>
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<td>6</td>
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<td>7</td>
<td>180</td>
<td>6 3</td>
<td>−</td>
<td>7</td>
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<td>11</td>
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<tr>
<td>Total</td>
<td>1,168</td>
<td>161 14</td>
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</tbody>
</table>

RBC, red blood cells; WBC, white blood cell.
+ , Present; ++, moderately frequent; +++ , frequent
*Number of vessels with two or more WBCs.
Observations are from 0.5–1 μm toluidine blue-stained slides.

The most likely explanation for deterioration of flow reserve is as a result of progressive microvascular damage, including neutrophil plugs and erythrocyte stasis. Blood flow measured through a large epicardial blood vessel reflects total blood flow to the territory supplied; this is mainly dependent on resistance in the microcirculation. Previously, researchers from our laboratory as well as others documented the finding of no-reflow areas within the myocardium when the duration of occlusion was maintained for 60–90 minutes or longer.1,7 This abnormality was associated with microvascular damage, including en-
dothelial gaps and blebs, erythrocyte stasis, and foci of hemorrhage. In the present study and our previously described studies, we observed neutrophil infiltration into the microvessels in such models. Engler et al postulated that the neutrophils may actually plug the microvasculature, leading to areas of no reflow or release toxic metabolites that could induce microvascular vasospasm. Neutrophil influx into the myocardium and capillary plugging have been implicated as contributing to impaired coronary vasodilator response in models of single coronary artery occlusion and reperfusion.

The fact that in the present study coronary flow response to vasodilators decreased between early after reperfusion and 2 hours after reperfusion is in agreement with a recent study suggesting that no reflow areas actually worsen over time after reperfusion of the epicardial coronary artery. This finding supports the concept that the vasculature may develop a form of "reperfusion injury." Furthermore, this injury may not be confined to the microvasculature but may also involve the large epicardial coronary arteries.

A recent study using a single coronary artery clamp ischemia/reperfusion model showed altered vascular reserve in the isolated microvessels but not the larger vessels. However, unlike this previous study, in our present study the arterial segment was isolated from blood flow with proximal and distal clamps.

It is possible that a smaller contribution to reduced in vivo vasodilator reserve was due to damage to the larger epicardial coronary arteries. Recent studies that have investigated isolated arterial rings have documented that previous exposure of these arteries to ischemia/reperfusion diminishing vasodilator response. Ku used a canine model of coronary occlusion and reperfusion and observed that coronary rings isolated from the occluded/reperfused epicardial vessels demonstrated impaired relaxation when exposed to thrombin. Van Benthuyzen et al studied coronary arterial rings removed from dogs that had undergone 60 minutes of ischemia and 60 minutes of reperfusion. They observed that the vasodilator response to the endothelium-dependent vasodilator acetylcholine was attenuated, whereas the response to the endothelium-independent vasodilator nitro-
prusside remained intact. Tsao and colleagues\textsuperscript{15} observed that arterial rings isolated from cats subjected to 90 minutes of ischemia followed by as long as 270 minutes of reperfusion exhibited depressed vasodilator response to the endothelial-dependent dilating agent acetylcholine. Thus, it is conceivable that alterations in the larger vessels may also contribute, although most likely to a lesser extent than defects in the intramural resistance vessels, to postischemic perfusion abnormalities.

Although the present study used a double-occlusion technique, many other studies,\textsuperscript{9,11,12} including our own,\textsuperscript{1,2,8} have used a single-occlusion technique. The purpose of the double-occlusion technique was to ensure that blood could neither enter nor leave the isolated arterial segment. This technique appeared to result in a more intense neutrophil infiltration into epicardial arteries than that described with a single-clamp method.\textsuperscript{14} In the clinical setting, it is likely that an obstructive thrombus would overlie a zone of atherosclerotic narrowing. Although an obstructive clot may not create as much ischemia in the wall of an epicardial vessel as double clamping, it could lead to more endothelial damage. Release of vasoactive components from the thrombus could promote artery spasm, leading to more erythrocyte stasis and propagation of the clot. A clot could also cause damage by producing microemboli into the distal vasculature. Although some investigators have used periods of ischemia less than 3 hours,\textsuperscript{14} we have observed more

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**TABLE 2. Increase in Flow After Acetylcholine and Nitroglycerin Injections**

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>After early reperfusion*</th>
<th>2 Hours after reperfusion</th>
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<tbody>
<tr>
<td><strong>Acetylcholine</strong></td>
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<td><strong>Mean</strong></td>
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<td><strong>SEM</strong></td>
<td>5</td>
<td>10</td>
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<td><strong>Nitroglycerin</strong></td>
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<td>26</td>
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<td>10</td>
<td>NA</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>18</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td>28</td>
<td>29</td>
<td>11‡</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>9</td>
<td>12</td>
<td>3</td>
</tr>
</tbody>
</table>

*Within 30 minutes of reperfusion.

$^\dagger p<0.03$ versus baseline.

$^\ddagger p=0.052$ versus baseline.

Flow given in ml/min.

FIGURE 10. Light micrograph of neutrophils within media of ischemic/reperfused coronary artery segment.
consistent microvascular damage and more reproducible and confluent infarcts with occlusions of this duration than with 40–60 minute occlusions.\textsuperscript{1,2,8} Vascular damage also tends to be more prominent with occlusions of 90 minutes or 3 hours than with shorter occlusions.\textsuperscript{1,2,8}

One limitation of the present study is that no matter how one occludes a coronary artery in an animal model, a certain degree of trauma will occur. This is true whether one occludes the artery with external clamps or occludes the artery from within, such as with an angioplasty balloon (which results in endothelial denudation). We tried to minimize trauma as much as possible by gently dissecting around the artery and using atraumatic clamps. Manipulation of the nonoccluded circumflex artery did not result in significant morphological damage to this vessel or the myocardium supplied by it. This fact and the fact that neutrophil accumulation was also observed (although to a lesser degree) up to at least 1 cm distal to the distal clamp suggest that trauma alone probably was not the sole cause for neutrophil accumulation; nevertheless, a certain degree of trauma was unavoidable. Also, in our protocols in which blood was removed from the isolated segment, trauma again was unavoidable. We cannot rule out the possibility that trauma may have contributed to greater neutrophil infiltration in this protocol than in protocol 2. However, an alternate explanation is that residual blood within the isolated vessel served as a buffer during ischemia and may have led to less damage to the endothelium than when the vessel was infused with saline.

Summary

We observed that isolating a segment of a large epicardial coronary artery from blood flow and then reperfusing stimulates neutrophil influx into the wall of the blood vessel and causes microvascular damage. Although this observation was associated with normal resting coronary flow, it was associated with
Acetylcholine  
Nitrogllycerin  
Pre Occlusion  

30 minutes Post Reperfusion  

2 hours Post Reperfusion  

20 seconds  

20 seconds  

FIGURE 13. Tracings of coronary flow (ml/min) in a dog before occlusion (top panels), 30 minutes after reperfusion (middle panels), and 2 hours after reperfusion (bottom panels) with acetylcholine (left panels) and nitroglycerin (right panels). Coronary flow increases considerably after acetylcholine or nitroglycerin before occlusion and 30 minutes after reperfusion; however, at 2 hours after reperfusion, increase in coronary flow in response to these agents is markedly blunted. Horizontal line through these tracings represents mean coronary blood flow.

depressed endothelial-dependent and endothelial-independent vasodilator response. Vasodilator response appeared to deteriorate between early reperfusion and 2 hours after reperfusion, supporting the concept of a vascular reperfusion injury. Although this most likely was related to progressive microvascular damage, our data and data from studies of isolated ischemic/reperfused coronary arteries suggest that a smaller component contributing to altered perfusion after ischemia may be related to macrovascular injury.

Acknowledgment

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References


**KEY WORDS** ischemia • reperfusion • neutrophils • coronary vasodilator reserve
Influx of neutrophils into the walls of large epicardial coronary arteries in response to ischemia/reperfusion.
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