Myocardial ischemia and reperfusion have been shown to impair coronary vasorelaxation to endothelium-dependent vasodilators. To examine the time course of this dysfunction, occlusion of the left anterior descending (LAD) coronary artery (90 minutes) was followed by reperfusion for 0, 2.5, 5, 20, 180, or 270 minutes. Coronary arterial rings from the ischemic LAD and control left circumflex (LCx) arteries were tested for responsiveness to the endothelium-dependent receptor-mediated vasodilator, acetylcholine (ACh), and the endothelium-dependent nonreceptor-mediated vasodilator, A23187, as well as the endothelium-independent vasodilator, NaNO₂. ACh relaxation was not impaired after 90 minutes of ischemia without reperfusion. However, 2.5 minutes of reperfusion resulted in depressed ACh responses (36±10% of control) that was further reduced to 16±6% at 20 minutes, and remained comparably depressed at every time thereafter. A23187 vasodilator responses were also attenuated after reperfusion, although the reduced response occurred later (that is, at 20 minutes). There was no significant decrease in response to NaNO₂ in the LAD at any time or to any vasodilator in LCx control rings. Treatment with recombinant human superoxide dismutase (hSOD, 5 mg/kg/hr, that is, 15,545 SOD units/kg/hr), starting 10 minutes before reperfusion, preserved the vasodilator response to ACh (82±6%) and A23187, but treatment with the hydroxyl ion scavenger N-(2-mercapto propionyl)-glycine (MPG) (8 mg/kg/hr) only protected the A23187 response. No damage to the surface of the endothelium was observed by scanning electron microscopy at any time point. Myocardial cell damage increased with time of reperfusion as assessed by increasing plasma CK activities and amounts of necrotic tissue indexed to area at risk. Significant myocardial injury occurred at 3 hours after reperfusion. These findings suggest that endothelial dysfunction resulting in reduced endothelium-derived relaxing factor release occurs before the development of myocardial cell necrosis and may be due to oxygen-derived free radicals produced rapidly on reperfusion. (Circulation 1990;82:1402–1412)

Myocardial ischemia initiated by occlusion or blockade of a major coronary artery leads to a complex series of cellular events that can result in myocardial cell death. Although reperfusion can produce salvage of ischemic tissue, it may also contribute to myocardial cellular injury. Reperfusion can accelerate necrosis in irreversibly injured myocytes because of an increase in cell swelling, disruption of cell ultrastructure, formation of contraction bands, and deposition of intramitochondrial calcium phosphate granules. Sarcolemmal damage may also occur, leading to impairment of fluid regulation and ionic flux balance.

Since Furchgott and Zawadzki first described the obligatory role of the endothelium in the regulation of vascular tone to a variety of vasodilators, considerable effort has been devoted to investigation of the physiological and pathophysiological roles of the endothelium. Damage to the endothelium may impair release of vasoactive substances such as endothelium-derived relaxing factor (EDRF) and may predispose vessels to vasoconstriction.

Studies in myocardial ischemia and reperfusion have shown marked alterations in endothelium-dependent relaxation of the coronary vasculature. Earlier studies by Ku and later by van Benthuyzen et
al and Mehta et al have shown a decrease in endothelium-dependent relaxation of isolated coronary artery rings to a variety of endothelium-dependent vasodilators. Preliminary data suggest that this impairment of endothelium-dependent relaxation is attributable to oxygen-derived free radical release upon reperfusion.10,11 However, the precise time course of this injury, the identity of the free radical species, and the temporal relation between endothelial injury and myocardial damage is not clear. Therefore, the purposes of the present investigation were to 1) elucidate the time course of endothelial dysfunction and assessment of endothelial surface features after myocardial ischemia and reperfusion, 2) clarify which free radical species is responsible for the endothelial injury, and 3) determine the temporal relation between endothelial dysfunction and myocardial tissue damage.

Methods

Experimental Procedure

Sixty-six adult male cats (weight, 2.5–3.5 kg) were anesthetized with sodium pentobarbital (30 mg/kg body wt i.v.). An intratracheal tube was inserted through a midline incision, and all cats were given intermittent positive-pressure ventilation by a Harvard small animal respirator (Harvard Apparatus, South Natick, Mass.). A polyethylene catheter was inserted into the external jugular vein, and the right femoral vein was cannulated and connected to a Statham P23AC pressure transducer (Spectramed, Inc., Critical Care Division, Oxnard, Calif.) for the measurement of arterial blood pressure. A midline thoracotomy was performed, the pericardium was opened, and the heart was exposed. A 2-0 silk suture was carefully placed around the left anterior descending coronary artery (LAD) 10–12 mm from its origin. Heart rates and ST segment changes were obtained from standard lead II of the scalar electrocardiogram every 20 minutes. Arterial blood pressure and the electrocardiogram were continuously recorded on a Grass model 7 oscillographic recorder (Grass Instrument Co., Quincy, Mass.). The pressure-rate index, calculated as the product of the mean arterial blood pressure and heart rate divided by 1,000, was used as an approximation of myocardial oxygen demand.

Experimental Protocol

After a 30-minute period of stabilization after thoracotomy, myocardial ischemia was initiated by complete ligation of the LAD. This was designated as time 0. After 1.5 hours of ischemia, the ligature was untied, and the ischemic myocardium was reperfused for 0, 2.5, 5, 10, 180, or 270 minutes in 8, 6, 6, 8, 6, and 6 cats, respectively. In additional experiments, hSOD (3109 SOD units/mg protein) (Grunenthal GmbH, Aachen, F.R.G.) (5 mg/kg/hr) or N-(2-mercapto propionyl)-glycine (MPG) (Sigma Chemical Co., St. Louis, Mo.) (8 mg/kg/hr) was given as a constant infusion starting 10 minutes before reperfusion for 30 minutes in five cats in each group. These experiments were terminated 20 minutes after reperfusion (that is, at the end of the infusion of drug or vehicle). An additional series of five cats were given 5 mg/kg/hr hSOD for 30 minutes but were observed for the full 270-minute postreperfusion period and compared with five cats given an equal volume of 0.9% NaCl and observed for the full 270-minute postreperfusion period. Four sham-operated control cats were subjected to all of the procedures except that the LAD ligature was not tightened.

Arterial blood samples were drawn immediately before ligation and hourly thereafter. In experiments where reperfusion times did not end on an hourly interval, a final blood sample was taken at the end of the prescribed reperfusion time. These blood samples were collected in tubes containing 200 IU heparin sodium. Samples were centrifuged at 2,000g and 4°C for 20 minutes. The plasma was decanted and analyzed spectrophotometrically for CK activity according to the method of Rosalki,12 and protein concentration was determined by the biuret method.13 Plasma CK specific activity was expressed as international units per milligram protein×1,000.

At the end of the experimental period, the ligature around the LAD was retightened. Thirty milliliters of 5% Evans Blue dye was injected into the left atrium to stain the area of the myocardium perfused by the patent coronary arteries. The area at risk was, therefore, determined by negative staining. The atria, right ventricle, and major blood vessels were subsequently removed from the heart. The left ventricle was then sliced into parallel sections 3-mm thick along the atrioventricular groove. The unstained portion of myocardium (the area at risk) was separated from the stained portion (the area not at risk). The unstained portion was again sliced into smaller sections 1 mm thick and incubated in 0.1% solution of nitroblue tetrazolium stain in phosphate buffer at pH 7.4 and 37°C for 15 minutes to detect the presence of coenzyme and dehydrogenase. The necrotic portion of the myocardium that did not stain was separated from the stained portion (the nonnecrotic area at risk). Samples from all three portions of left ventricular myocardium (nonischemic, ischemic nonnecrotic, and ischemic necrotic) were weighed and stored at −70°C for subsequent assay of myeloperoxidase (MPO) activity.

Studies on Coronary Ring Responses

After injection of Evans Blue dye, hearts were excised and placed in warmed, oxygenated Krebs-Henseleit buffer. Both the left circumflex (LCx) and the LAD were carefully isolated, and 10–12-mm long segments were removed and placed into warmed Krebs-Henseleit consisting of (in 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. Wire hooks were fed through the rings and...
suspended in tissue baths filled with 20 ml Krebs-Henseleit buffer warmed to 37°C. Ring suspensions were connected to Grass FT-03 force-displacement transducers (Grass Instrument Co., Quincy, Mass.), and responses were recorded on a Grass model 7 oscillographic recorder. Coronary rings were initially stretched to give a preload of 0.5 grams of force and allowed to equilibrate for 1 hour. Preloads of 1 gram or higher caused injury to the endothelium and thus could not be used. Coronary rings were then exposed to 300 nM U46619 (The Upjohn Co., Kalamazoo, Mich.), a thromboxane A₂ mimic to generate about 0.5 g of developed force. The average developed force was 477±25 mg in all rings, no group being significantly different from this value. Once a stable contraction was observed, 0.1, 1, 10, and 100 nM acetylcholine (ACh) was added to the bath. After the response stabilized, the rings were washed and allowed to equilibrate to baseline once again. The procedure was repeated with A23187 (0.01, 0.1, 1, and 10 μM) and then to NaNO₂ (1, 10, and 100 μM). NaNO₂ was prepared by dissolving the compound in 0.1 N HCl and titrating it to pH 2.0. Titrating distilled water to pH 2.0 and adding aliquots to the buffer in the bath did not produce any vasorelaxation.

To determine whether the Evans Blue dye injection could alter vasorelaxant properties to ACh A23187 or NaNO₂, we tested six cat LCx coronary rings after injecting the cats with methylene blue and compared these to six LCx rings taken from cats not exposed to methylene blue. Percent relaxations were as follows: ACh (100 nM) 96±5 versus 95±4%, A23187 (10 μM) 97±5 versus 98±2%, and NaNO₂ (100 μM) 99±4 versus 97±2%. None of these responses was significantly different from another. Thus, Evans Blue dye did not influence vasorelaxation responses. Moreover, these concentrations of acetylcholine, A23187 and NaNO₂ were taken as the highest concentrations used because these concentrations yielded responses close to 100% relaxation.

Additional experiments were performed in two cats subjected to 90 minutes of ischemia followed by 2.5 minutes of reperfusion. LAD coronary rings were removed and studied as above except that the rings were dissected and equilibrated in the muscle chambers in the absence of oxygenation. The Po₂ of these muscle chambers was 50–60 mm Hg. After 1 hour of incubation, 300 nM U46619 was added to the chambers. The rings contracted 425–475 mg and then relaxed 29±3% to 100 nM ACh. This compares to a percent relaxation of 36±8% in LAD rings obtained from cats subjected to ischemia and 2.5 minutes of reperfusion but dissected and incubated in 95% O₂–5% CO₂ according to our usual methods. These values were not significantly different from each other and indicate that the impairment of vasorelaxation to ACh is not an artifact of “reoxygenation” of the coronary artery rings in the muscle baths.

MPO Activity

The activity of MPO, an enzyme specific for neutrophils, was determined in myocardial tissue by the method of Bradley et al, as modified by Mullane et al. MPO activity was used as an index of neutrophil accumulation in the heart. Tissue was stored at −70°C until homogenization. Myocardial tissue samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide (HTAB) (Sigma Chemical Co., St. Louis, Mo.), dissolved in 50 mmol potassium phosphate buffer at pH 6 using a Polytron (PCU-2) homogenizer (Kinemutica GmbH, Luzern, Switzerland) for 15 seconds×2 at 7,000 rpm. Homogenates were centrifuged for 20 minutes at 12,000g and 2°C. The supernatants were decanted and added to 0.167 mg/ml O-dianisodine dihydrochloride (Sigma Chemical Co.), and 0.005% H₂O₂ in 50 mmol phosphate buffer at pH 6. The change in absorbance was measured spectrophotometrically at 460 nm. One

![Figure 1. Plot of plasma creatine phosphokinase (CK) activity expressed as IU/mg protein×10⁻³ measured at various times during the ischemia (−90 to 0 minutes) and reperfusion (2.5 to 270 minutes) protocol. All values are mean±SEM for 5–7 cats/group. Plasma CK activity rises slightly after ischemia and as after short periods of reperfusion (2.5 to 20 minutes). Progressively larger increases in plasma CK activity are seen with longer periods of reperfusion.](image-url)
unit of MPO activity is defined as the quantity of enzyme degrading 1 μmol peroxide/min at 25°C.

Scanning Electron Microscopy

In additional experiments (that is, two or three cats/group), hearts were rapidly removed at 0, 20, 180, and 270 minutes after reperfusion after intravenous injection of 1,000 units/kg heparin sodium. Isolated hearts were perfused in a Langendorff apparatus at 50 cm H₂O at room temperature with 2.5% glutaraldehyde for 30 minutes. Coronary arteries (LAD and LCx) were then dissected free and placed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 24 hours. Arteries were then kept in 0.1 M phosphate buffer for 2–7 days until they were air dried overnight, spatter-coated with gold-palladium alloy, and viewed in a Phillips model 501 scanning electron microscope. Photomicrographs were taken at magnifications of ×1,250 and ×2,500.

Statistical Analysis

All values in the text and figures are presented as mean±SEM of n independent experiments. Data were analyzed by Kruskal-Wallis nonparametric analysis of variance because of inequalities of variances among the groups. Differences between specific means were tested by post-hoc analysis using the Mann-Whitney U test. A value of p less than 0.05 was accepted as being significant.

Results

Effects of Different Times of Reperfusion on Myocardial Tissue Injury

After occlusion, the PRI was reduced comparably in all of the groups. From 2 hours onward, the PRI increased slightly and remained relatively stable. There were no significant differences among the groups for heart rates, mean arterial blood pressure, or PRI, indicating myocardial oxygen demand was similar. PRI values were between 21 and 24 mm Hg (beats/min)/1,000 initially in all groups and were 18–22 mm Hg (beats/min)/1,000 at the end of the experiments. None of these changes was statistically significant. ST segment elevations (data not shown) were not different among the groups, indicating that the severity of ischemia was comparable among all groups of cats subjected to myocardial ischemia. All groups of cats developed an ST segment elevation of 0.09 to 0.15 mV at 20–40 minutes after occlusion.

Figure 1 illustrates changes in plasma CK activity in all of the untreated groups studied. Gradual increases in CK activity were observed throughout the ischemic and reperfusion periods. With increas-
Cat LAD Coronary Artery Rings

<table>
<thead>
<tr>
<th>Minutes Post-Reperfusion</th>
<th>ACh</th>
<th>NaNO₂</th>
</tr>
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<tbody>
<tr>
<td>0 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 min</td>
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<tr>
<td>5 min</td>
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<td>20 min</td>
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<td>180 min</td>
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<tr>
<td>270 min</td>
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**Figure 4.** Representative recordings of endothelium-dependent vasodilator, acetylcholine (ACh), and endothelium-independent vasodilator, NaNO₂, relaxation of precontracted (U46619) ischemic and reperfused coronary artery rings. Arrows indicate addition of U46619; dots on top indicate addition of ACh or NaNO₂. ACh gives almost full relaxation after ischemia alone. However, with reperfusion, ACh-induced relaxation is blunted with maximal decrement seen after 20 minutes of reperfusion. All rings show full relaxation to NaNO₂.

During the ischemic period as well as throughout the shorter periods of reperfusion (2.5, 5, and 20 minutes), there was no significant accumulation of neutrophils in any of the three areas of the myocardium studied. However, with longer periods of reperfusion there was a significant increase in MPO activity in both ischemic zones (the nonnecrotic
Effects of Different Times of Reperfusion on Coronary Ring Relaxation

Isolated coronary artery rings were studied to determine the integrity of endothelial function as assessed by the ability of the endothelium to release EDRF. The thromboxane A₂ (TXA₂) mimetic U46619 was used to contract coronary artery rings, and the rings were tested with endothelium-dependent (ACh, A23187) and endothelium-independent (NaNO₂) vasodilators. Figure 4 illustrates typical recordings of the responses to ACh and NaNO₂ in rings obtained from the ischemic LAD after different periods of reperfusion. ACh added to rings undergoing 90 minutes of ischemia without reperfusion gave a normal relaxation response. However, 2.5 minutes of reperfusion resulted in responses to ACh that were significantly depressed (50.4±18.5%) (p<0.01). Longer periods of reperfusion resulted in further decrements in responses to ACh compared with paired LCx controls (Figures 5 and 6). A similar pattern was obtained with the nonreceptor mediated endothelium-dependent dilator A23187 except that the decrease in vasodilator response was not significant until 20 minutes after reperfusion (Figures 7 and 8). With longer periods of reperfusion (20, 180, and 270 minutes), a severe decrement in vasorelaxation to A23187 was shown compared with paired LCx controls. In contrast to these effects, both ischemic and nonischemic coronary rings gave similar responses to the endothelium-independent vasodilator NaNO₂. These data are summarized in Figure 9. Thus, there appears to be a significant defect in endothelium-dependent relaxation to ACh in the coronary vasculature occurring soon after reperfusion (at 2.5 minutes) and progressing to near maximum values at about 20 minutes after reperfusion, thus preceding myocardial necrosis.
Effects of hSOD and MPG pretreatment

In an attempt to examine the role of oxygen-derived free radicals in the endothelial dysfunction, we infused the superoxide ion scavenger (hSOD) or the hydroxyl ion scavenger (MPG) intravascularly 10 minutes before reperfusion. In contrast to ischemic LAD coronary rings prepared from cats receiving only the vehicle, coronary rings obtained from cats pretreated with hSOD showed normal vasorelaxation to the highest concentrations of the endothelium-dependent vasodilators (ACh at 100 nM and A23187 at 10 μM), as well as to the highest concentration of the endothelium-independent vasodilator NaNO2 at 100 μM (Figure 10). However, pretreatment with the hydroxyl radical scavenger, MPG, did not protect the response to ACh (Figure 10), although MPG did preserve the A23187-induced vasorelaxation. Thus, superoxide ions appear to be more important in mediating the early postreperfusion endothelial injury than hydroxyl ions.

In an effort to determine whether the endothelial preservation effect of hSOD could exert an influence on the degree of myocardial injury after reperfusion, we infused 5 mg/kg/hr hSOD beginning 10 minutes before reperfusion until 20 minutes after reperfusion, and analyzed the extent of myocardial dysfunction 270 minutes after reperfusion. Table 1 summarizes these results compared with cats receiving the vehicle for hSOD (0.9% NaCl). Cats receiving hSOD exhibited markedly lower plasma CK activity than those receiving vehicle and also showed a markedly greater degree of preservation of coronary endothelial integrity as assessed by the ability to dilate to acetylcholine. Importantly, hearts of cats receiving hSOD developed much lower accumulation of myeloperoxidase activity indicating a lesser degree of neutrophil adherence. All these effects translated into a marked degree of cardioprotection because the hSOD cats developed a dramatically lower degree of cardiac necrosis 270 minutes after reperfusion (p<0.001 from vehicle controls). These results suggest that endothelial dysfunction occurring early after reperfusion may be an important marker that is indicative of early events leading to myocardial damage after ischemia and reperfusion.

Results of Scanning Electron Microscopy

At various times after reperfusion, coronary arteries were prepared to assess the surface changes of endothelial cell morphology by scanning electron microscopy. Figure 11 (A–D) illustrates representative examples of these micrographs. Coronary vessels obtained from ischemic cats subjected to reperfusion of 0, 20, 180, and 270 minutes did not show evidence of any surface ultrastructural damage.
(denudation, blebbing, or adherence of significant numbers of blood cells). In fact, the endothelial lining from these vessels undergoing ischemia and reperfusion showed no ultrastructural difference to nonischemic LCx vessels at any time point (0–4.5 hours postreperfusion). In all cases, intact endothelial cells with raised nuclei can be observed.

**Discussion**

Recent studies by Ku and Van Benthuyzen et al have shown that endothelial dysfunction can occur after ischemia and reperfusion in large canine coronary vessels. The data presented in this study not only show that coronary occlusion and reperfusion can cause such impaired responses, but that this defect in endothelium-dependent vasorelaxation is demonstrable within 2.5 minutes of reperfusion and progressively worsens with increasing durations of reperfusion. In our preparation, ischemia alone (without reperfusion) produced no impairment of endothelium-dependent relaxation to ACh and A23187. However, with increasing times of reperfusion, there was a greater loss of the relaxation response, with the impairment at 20 minutes being close to maximum. Because relaxation to the endothelium-independent dilator, NaNO₂, was preserved, this vasorelaxant defect seems specific to endothelium-dependent processes rather than to a defect in coronary vascular smooth muscle. It should be emphasized that acetylcholine and A23187 are research tools and do not necessarily reflect physiological stimuli. Interestingly, this endothelial dysfunction is not mirrored by alterations in injury to the surface of the endothelium. Scanning electron micrographs showed that even at the longest reperfusion time that produced severe loss of endothelial dependent relaxation, there was no evidence of injury to the endothelial cell surface. These findings differ from previous studies in dogs in which ultrastructural damage to the endothelium was observed.

**FIGURE 9.** Concentration-response curves to acidified NaNO₂ (1–100 µM) in left anterior descending coronary artery (LAD) rings (left panel) and left circumflex coronary artery (LCx) rings (right panel). None of the groups are different from the 0 minutes at any concentration in either LAD or LCx rings.

**FIGURE 10.** Bar graphs of mean responses of control and treated coronary artery rings after 90 minutes of ischemia and 20 minutes of reperfusion to vasodilators 100 nM acetylcholine, 10 µM A23187, and 100 µM NaNO₂ for 8–12 rings obtained from four or five cats. Rings treated with hSOD show full relaxation to endothelium-dependent dilators, whereas vehicle- and MPG-treated rings show severe decrement in relaxation. All rings dilate fully to the endothelium-independent dilator NaNO₂. *p<0.05 from hSOD; **p<0.01 from hSOD.
TABLE 1. Protective Effects of hSOD in Myocardial Ischemia Reperfusion in Anesthetized Cats

<table>
<thead>
<tr>
<th>Variable</th>
<th>MI Reperfusion</th>
<th>Vehicle</th>
<th>MI Reperfusion + hSOD*</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma creatine kinase activity (IU/mg protein×10⁻³)</td>
<td>45.5±5.5</td>
<td>15.0±1.4</td>
<td></td>
<td>&lt;0.01</td>
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<tr>
<td>Vasorelaxation to ACh (percent relaxation)</td>
<td>24±8</td>
<td>76±4</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cardiac myeloperoxidase activity (Units/100 mg tissue)</td>
<td>1.09±0.28</td>
<td>0.26±0.16</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cardiac necrotic area (% area at risk)</td>
<td>20.4±3.0</td>
<td>1.8±1.0</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*hSOD was given intravenously at 5 mg/kg/hr during a 30-minute period beginning 10 minutes before reperfusion.

All values are mean±SEM taken at 4.5 hours after reperfusion for five cats in each group.

MI, myocardial infarction; ACh, acetylcholine.

difference may be due to species or methodological differences because we did not study transmission electron microscopy, a method that can more readily detect edema of endothelial cells.

An interesting aspect of the endothelial dysfunction is its relation to cellular damage of myocytes. Myocardial tissue damage, as assessed by either elevated plasma CK activities or increasing mass of necrotic tissue, is evident only after longer reperfusion times (3–4.5 hours). Thus, the onset of this myocardial necrosis (3 hours) does not occur until well after the defect in endothelial cells is manifested (20 minutes).

There are a variety of mechanisms that have been proposed to explain the myocardial injury observed after periods of ischemia and reperfusion. Recent studies have demonstrated that production of oxygen-derived free radicals by neutrophils, monocytes, myocytes, and endothelial cells contribute to myocardial cell injury. Free radicals, particularly superoxide radicals, have been shown to inactivate EDRF, initiate lipid peroxidation, and alter membrane permeability to ions, all of which can lead to damage of the endothelium as well as myocytes. This mechanism is supported by several studies demonstrating beneficial effects of superoxide radical scavengers on myocardial tissue undergoing ischemia and reperfusion, as well as the actual measurement of free radicals in the posts ischemic heart. Data

FIGURE 11. Representative scanning electron micrographs of the luminal surface of cat left anterior descending coronary artery removed from cats subjected to 90 minutes of ischemia at (A) 0, (B) 20, (C) 180, and (D) 270 minutes of reperfusion. Marker indicates 100 μm. There was no sloughing of or significant adhesion or injury to the endothelial surface at any time studied.
from this study, as well as from other laboratories, show that superoxide dismutase may also provide protection against reperfusion-induced injury to coronary endothelial cells.10,11 Surprisingly, however, the hydroxyl radical scavenger, MPG, did not have as broad a protective effect on endothelium-dependent dilation as hSOD. Thus, even though reperfusion may produce bursts of different forms of oxygen free radicals, superoxide radicals appear to be more important in the functional injury to endothelial cells than other oxygen-derived free radicals. Moreover, this preservation of endothelial integrity early after reperfusion appears to protect against neutrophil adherence and myocardial necrosis later in the post-reperfusion period.

Despite these encouraging effects of hSOD, other well-conducted studies have failed to find a reduction in infarct size with SOD.26,27 This subject has recently been reviewed by Engler and Gilpin,28 who concluded that no definitive statement on the effectiveness of SOD in myocardial ischemia can be made at present due to differences in species, methods, dose and type of SOD, analysis of covariability, and existence of undefined factors in each series of experiments. Our studies are the first experiments of which we are aware of an effect of SOD in the ischemic feline myocardium.

This mechanism of superoxide production may also provide an explanation for the differences in vasorelaxant responses of ACh and A23187 after the shorter periods of reperfusion (1, 2.5, and 5 minutes). Because oxygen free radicals have been shown to initiate lipid peroxidation of cell membranes, it would follow that membrane elements could be targets for damage. Such a target may be endothelial cell membrane binding sites for such agonists as ACh. Free radicals may alter endothelial cell membrane binding sites for vasodilators, thereby restricting the signal for the endothelial cell to produce EDRF, as suggested by Pieper and Gross.29 Because A23187 enters directly into the endothelial cell to initiate EDRF production, its vasorelaxant effects may be undisturbed for longer periods of reperfusion. Our data are consistent with this hypothesis, but because we did not fully investigate the pharmacology of the muscarinic receptor (maximal effects in the presence and absence of specific muscarinic receptor antagonists), we cannot make any definitive statement about specific receptors.

Considerable evidence supports the role of neutrophils in reperfusion injury to myocardial tissue. In fact, studies that limit the activation and adherence of neutrophils in the ischemic tissue, either by depletion of neutrophils30 or inhibition of the recruitment or adherence of neutrophils,31-33 have shown significant reduction in myocardial damage after experimental coronary occlusion. Moreover, neutrophils are known for their ability to produce large amounts of superoxide radicals.16,34 Therefore, neutrophils may not only produce a significant amount of myocardial tissue damage, they may also contribute to endothelial cell dysfunction. To study the possible role of neutrophils in these processes, we measured MPO activity in different regions of the myocardium as an estimation of neutrophil accumulation. Significantly higher myocardial MPO activity is seen after longer periods of reperfusion (180 and 270 minutes) when myocardial tissue damage is evident. However, after the shorter periods of reperfusion (0, 2.5, 5, and 20 minutes) when endothelial function has already been compromised, no significant accumulation of neutrophils was observed. Yet, even at these shorter periods of reperfusion, hSOD protects against reperfusion-induced endothelial dysfunction. Therefore, it seems that superoxide radicals from other cell sources, possibly endothelial cells themselves,24,25 are contributing to the endothelial cell injury.

In conclusion, our study shows that reperfusion-induced endothelial dysfunction resulting in reduced EDRF release occurs in the absence of morphological evidence of overt injury to the endothelial cell membranes and before myocardial cell necrosis. This endothelial dysfunction can be attenuated by pretreatment with hSOD and to a lesser extent by MPG, suggesting that oxygen-derived free radicals play an important role in the endothelial dysfunction observed during ischemia and reperfusion.

Acknowledgments
We sincerely acknowledge the expert technical assistance of Philip Taylor III in the chemical procedures used during the course of these investigations, and Dr. Richard G. Taylor, Department of Pathology, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina, for performing the scanning electron microscopy. We also thank Dr. Johannes Schneider of Grunenthal AG, Aachen, F.R.G., for the generous supply of hSOD.

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**KEY WORDS** • acetylcholine • myocardial damage • superoxide anions • endothelium-derived relaxing factor • myeloperoxidase activity • scanning electron microscopy
Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat.
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Circulation. 1990;82:1402-1412
doi: 10.1161/01.CIR.82.4.1402

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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