Effect of a Critical Coronary Stenosis on Myocardial Neutrophil Accumulation During Ischemia and Early Reperfusion in Dogs

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In many experimental models of ischemia and reperfusion, reperfusion is performed abruptly, allowing full reactive hyperemia to occur. In the clinical setting, however, reperfusion after thrombolysis is often limited by residual stenosis. Some experimental models attempt to mimic this situation with a “critical stenosis” (defined as a coronary constriction sufficient to abolish reactive hyperemia without altering baseline flow). The purpose of this study was to determine whether preventing reactive hyperemia during the initial phase of reperfusion would modify the transmural distribution of myocardial blood flow or the myocardial accumulation of polymorphonuclear leukocytes (PMNs). The left circumflex artery was occluded for 90 minutes and then reperfused for 60 minutes in anesthetized, open-chest dogs. Autologous PMNs were isolated, labeled with $^{111}$In, and reinjected 1 hour before coronary occlusion. $^{125}$I-labeled albumin was injected simultaneously to correct for $^{111}$In associated with plasma proteins and to permit calculation of the number of PMNs in the inner, middle, and outer thirds of nonischemic and ischemic-reperfused tissue. The presence of a critical stenosis abolished reactive hyperemia during the first 5 minutes of reperfusion, but did not substantially affect blood flow measured after 55 minutes of reperfusion. In both groups, there was a significant accumulation of PMNs in all layers of the ischemic-reperfused bed compared with the nonischemic bed, and the magnitude of this PMN accumulation was not altered by the presence of a critical stenosis. Moreover, infarct size, estimated by triphenyl tetrazolium chloride (TTC) loss after 60 minutes of reperfusion, was not affected by the presence of a critical stenosis. Thus, the presence of a critical stenosis abolished the hyperemic blood flow after reperfusion but did not influence the early PMN response to ischemia and reperfusion or the early loss of TTC staining. (Circulation 1989;80:1805–1815)

The severity and duration of an ischemic interval are the major determinants of whether cardiac myocytes will recover or undergo necrosis after reperfusion. It has been postulated in recent years, however, that the fate of some myocytes may be determined, in part, by events occurring during the early period of reperfusion. Although conflicting results have been reported, some studies have provided evidence that modification of the conditions of reperfusion, per se, can limit myocardial infarct size, that is, that “reperfusion injury” occurs and can be attenuated or prevented by various therapies.

One potentially important variable aspect of reperfusion is the distal arterial pressure and consequent rate of flow through the microvasculature during the initial phase of reperfusion. In many experimental models, coronary occlusion is followed by an abrupt, complete reopening of the artery, permitting a marked hyperemic flow through the ischemically dilated microvasculature. In clinical circumstances, however, reperfusion is usually achieved more slowly (e.g., by thrombolytic therapy), and reactive hyperemia may be limited by the residual stenosis caused by underlying atherosclerotic plaque. Some investigators mimic this situation in their experimental models of ischemia and reperfusion by imposing a persistent partial constriction on the occluded and reopened artery. This “critical stenosis” permits restoration of baseline coronary blood flow but prevents most of the initial reactive hyperemia.

Recent studies have shown that polymorphonuclear leukocytes (PMNs) accumulate in substantial...
quantities in myocardium during the early reperfusion period after an episode of ischemia of sufficient duration to produce myocardial necrosis.\textsuperscript{5–8} We have observed, for example, a significant accumulation of these cells in the ischemic-reperfused region during the first 60 minutes of reperfusion after either 40 or 90 minutes of ischemia.\textsuperscript{9} Other studies have provided evidence that such an accumulation of neutrophils could cause additional injury of myocytes or the microvasculature, either by the passive plugging of capillaries,\textsuperscript{10} the production of toxic oxygen species such as the superoxide radical, or the release of a variety of degradative enzymes.\textsuperscript{11}

Whether the magnitude of PMN accumulation during the early phase of reperfusion is affected by the presence or absence of a critical coronary stenosis has not previously been determined. For example, it is possible that prevention of reactive hyperemia could prevent the washout of chemotactic factors present in the tissue, or the reduction of microvascular perfusion pressure could cause increased number of PMNs to be trapped in the microvasculature. Either effect could cause a more rapid accumulation of PMNs in the injured tissue.

If PMNs cause myocyte death, an increased PMN accumulation in the presence of a critical stenosis might be translated into a parallel increase in infarct size. This hypothetical effect of a critical stenosis on reperfusion injury could, if true, explain some of the conflicting results obtained among studies of the effect of different free radical scavengers, such as the enzyme superoxide dismutase (SOD), on myocardial infarct size. A critical stenosis was used, for example, in several studies in which SOD, with or without catalase, was administered during reperfusion after a 90-minute episode of ischemia.\textsuperscript{3,12–14} In each of those studies, infarct size was reportedly limited by therapy. Conversely, in a similar study, also of reperfusion after 90 minutes of ischemia, but in which no critical stenosis was used, we found no effect of SOD and catalase on infarct size.\textsuperscript{15} The difference in the mode of reperfusion is a possible reason for these discrepant results. A critical stenosis could exacerbate reperfusion injury, for example, by slowing blood velocity, increasing PMN adhesion to endothelium, and thereby increasing free radical production.\textsuperscript{15} If so, a critical stenosis could increase the proportion of an infarct attributable to free radical–mediated cell death, and thereby, potentially preventable by anti–free radical therapy. Alternatively, it is possible that reactive hyperemia increases PMN or free radical–induced reperfusion injury to such an extent that it overcomes the potential beneficial effects of an anti–free radical therapy.

Thus, this study was done to determine whether reperfusion, in the presence of a critical stenosis to prevent reactive hyperemia, alters the magnitude of early myocardial PMN accumulation or the initial extent of myocyte necrosis, assessed by triphenyl tetrazolium chloride (TTC) macrochemistry. These indexes were measured at the end of the first 60 minutes of reperfusion after a 90-minute episode of ischemia in dogs.

\section*{Methods}

\subsection*{Animal Preparation}

Experiments were conducted according to the criteria defined in the multicenter Animal Models for Protecting Ischemic Myocardium study.\textsuperscript{16} Forty adult mongrel dogs of either sex, weighing 14–20 kg, were anesthetized with sodium pentobarbital and ventilated using a Harvard ventilator (Harvard Apparatus, South Natick, Massachusetts) with room air supplemented with low-flow oxygen. The right femoral artery and vein were catheterized, a left thoracotomy was performed in the fourth intercostal space, and the left circumflex coronary artery was isolated distal to its atrial branch but proximal to its first marginal branch. A transit-time ultrasonic flow probe (Transonic Systems, Ithaca, New York) was placed around the artery. Two pneumatic occluders also were placed around the artery—one to be used to occlude the artery and the other to produce a critical stenosis. The left atrial appendage was catheterized for measurement of left atrial pressure and injection of radioactive microspheres. Arterial blood gases were maintained within physiologic range by adjusting ventilatory parameters as needed.\textsuperscript{16} Lead II of the electrocardiogram, arterial and left atrial blood pressure, pericardial temperature, and circumflex coronary blood flow were measured continuously with a Gould eight-channel recorder (Gould, Cleveland, Ohio). The animals were allowed at least 30 minutes of equilibration between completion of the surgery and injection of the labeled cells. To assess whether labeled PMNs responded to chemotactic stimuli in vivo, PMN influx was measured in cutaneous lesions produced by intradermal injection of about 20 million staphylococci (\textit{Staphylococcus aureus}, ATCC 29213, provided by Mary J. Rieman, Department of Microbiology, Duke University Medical Center) subcutaneously 1 hour after injection of labeled PMNs. Normal saline was injected in another area of skin as a control.

\subsection*{Isolation and In-Labeling of Neutrophils}

Before anesthesia, a total of 50 ml venous blood was drawn into two syringes, each containing 20 ml 6% Hetastarch in normal saline and 5 ml 3.8% Na citrate. The samples were allowed to sediment for 1 hour at room temperature (24–26° C). The leukocyte-rich plasma was then layered over four 5-ml cushions of Percoll in 1.058 g/ml NaCl (Pharmacia, Piscataway, New Jersey) (specific gravity, 1.077 g/ml; osmolality, 330 mosm). After 30 minutes of centrifugation at 800g, the upper layers containing plasma, platelets, monocytes, and lymphocytes were discarded. The bottom layers containing neutro-
phils and a few contaminating red blood cells were resuspended, and the contaminating erythrocytes were lysed with 20 ml of ice-cold 0.2% NaCl for 20 seconds; isotonicity was restored by adding 20 ml of ice-cold 1.6% NaCl. The remaining cells were pelleted at 200g for 10 minutes at room temperature and resuspended in 5–6 ml of a physiologic solution (pH 7.4) containing the following ionic concentrations (mM): 142 Na⁺, 2 K⁺, 1.5 Ca²⁺, 132 Cl⁻, and 14 lactate. The cells were then incubated with 150–300 µCi ¹¹¹In-oxine (Amersham, Arlington Heights, Illinois) for 30 minutes at room temperature. The labeled cell suspension was centrifuged at 200g for 5 minutes; the supernatant containing unbound ¹¹¹In-oxine was removed, and the labeled cells were resuspended in 3 ml of the previously described solution. ¹¹¹In activity then was measured in the cells and the supernatant to assess labeling efficiency (fraction of total ¹¹¹In taken up by the cells). Automatic blood counts and manual differentials were performed on samples of the original blood and isolated PMN preparation to assess the purity and yield of the isolate. In four of the 40 experiments, the PMNs failed to label with ¹¹¹In. These four dogs were excluded from the study. Immediately after the final resuspension, the labeled cells were reinjected slowly into the dog through a catheter placed into the femoral vein.

**Determination of Cellular Versus Plasma ¹¹¹Indium**

Pilot studies showed that, immediately after injection, ¹¹¹In was distributed in both the cellular and the plasma compartments of the blood. Plasma ¹¹¹In is known to be chelated by transferrin, a protein that is roughly equivalent in molecular weight to albumin. Hence, around 5 µCi radioiodinated (¹¹¹I-labeled) serum albumin (RISA) (Mallinckrodt, St. Louis, Missouri) was injected simultaneously along with the labeled PMNs to measure myocardial albumin space and correct for tissue ¹¹¹In activity associated with plasma proteins in either the intravascular or extravascular locations. Serial blood samples were obtained at 5, 10, 15, and 30 minutes after the injection of labeled cells, and then every 30 minutes until completion of the experiment.

**Experimental Design**

The dogs were assigned to one of two groups: full reperfusion (control group, n = 22) or reperfusion through the critical stenosis (group with critical stenosis, n = 14). In the animals assigned to the group with critical stenosis, the distal occluder was adjusted before injection of the labeled cells to produce a coronary stenosis sufficient to eliminate the hyperemic response to a 10-second occlusion without altering baseline flow. Control animals received similar 10-second test occlusions.

One hour after injection of the labeled cells, the circumflex coronary artery was occluded by inflating the balloon occluder. The occluder was released after 90 minutes of occlusion. In all dogs, the regional distribution of myocardial blood flow was measured with the microsphere technique 10 minutes into the 90-minute occlusion. In 18 dogs (10 controls and eight with critical stenosis), blood flow was also measured 5 and 55 minutes into the reperfusion. Hearts were excised after 1 hour of reperfusion. Fifteen seconds before excision of the heart, the circumflex artery was reoccluded, and the fluorescent dye Thioflavins S (TS) (Sigma Chemical, St. Louis, Missouri) (1.0 ml/kg of a freshly centrifuged 4% solution) was injected intravenously to demarcate the nonischemic myocardium (brightly fluorescent) from the previously ischemic nonfluorescent region (area at risk [AAR]). We used this method in the present study to avoid flushing PMNs from the microvasculature by postmortem coronary dye perfusion. Although weak TS fluorescence may be observed in the subepicardial zone of the ischemic region when substantial collateral blood flow is present, such fluorescence rarely obscures the ischemic-nonischemic interfaces.

**Regional Blood Flow Measurements**

At each time indicated above, 2–3 million microspheres (10±2 µm) radiolabeled with ⁴⁶Sc, ⁴¹Ce, or ⁹⁵Nb and suspended in 0.05% Tween 80 and 10% Dextran (New England Nuclear, Boston, Massachusetts) were injected through the left atrial catheter and followed by a 20-ml saline flush. A reference blood flow was withdrawn from the femoral artery at a rate of 7.75 ml/min for 2.5 minutes beginning just before injection of the spheres.

**Postmortem Studies**

**Tissue sampling, isotope counting, and macroscopic determination of infarct size.** Immediately after excision, the atrial, valvular, and right ventricular tissue were discarded, and the left ventricle was sectioned from base to apex in six transverse slices in a plane parallel to the atrioventricular groove. The most apical slice was discarded. Slices 1, 3, and 5 (from base to apex) were used to measure isotope radioactivity, whereas slices 2 and 4 were used for the measurement of AAR and macroscopic infarct size.

Tissue slices for isotope counting were divided under ultraviolet light into nonischemic (fluorescent) and central ischemic zones, the latter consisting of 50–75% of the ischemic (nonfluorescent) bed. Lateral and septal borderzones were eliminated to avoid contamination of ischemic samples with nonischemic tissue. The nonischemic and ischemic samples were divided into inner, middle, and outer thirds (i.e., subendocardium, midmyocardium, and subepicardium). Excision biopsies from staphylococcal and control skin lesions were obtained. The weight of each sample was recorded, and the radioactivity of tissue and blood samples from the five isotopes (¹¹¹In, ¹²⁵I, ⁴⁶Sc, ⁹⁵Nb, and ⁴¹Ce) was counted...
immediately in a Packard 5912 gamma counter with corrections made for overlap of isotope spectra.

The sections used for measurement of AAR and TTC-based estimation of infarct size were examined under ultraviolet light through a yellow filter. The borders of the sections and of the AAR (defined as the nonfluorescent area) were traced on an acetate sheet superimposed on the surface of the section. These sections were then immersed for 20 minutes at 37°C in a buffer containing 1.0% TTC (Sigma) in 0.09 M sodium phosphate buffer (pH 7.4) and 8% dextran (mol wt, 77,800) for determination of the extent of necrosis. After being fixed in phosphate-buffered formalin for at least 72 hours, the apical sides of the sections were photographed. Additionally, the most apical of the two slices was processed for histology and duplicate sections were cut; one was stained with hematoxylin and eosin and the other with Heidenhain’s variant of Mallory’s connective tissue stain. These histologic slices were evaluated under a microscope for the presence of contraction band necrosis, and the approximate transmural extent of infarction was estimated. To quantify infarct size on the TTC-stained slices, an enlarged section of each photograph was traced, and the areas of necrotic and viable tissue were determined using a SUMMAKETCH PLUS digitizing tablet (Summagraphics, Fairfield, Connecticut) interfaced to an IBM PC-compatible computer with appropriate measurement software (SIGMACAN, Jandel Scientific, Corte Madera, California). The AAR also was digitized using photocopies of the acetate sheets, and infarct size was calculated as a percentage of the AAR.

Analysis of blood samples and determination of PMN-specific activity. For each of the heparinized blood samples collected, automated blood counts and manual differentials were performed to determine an average number of circulating PMNs divided by milliliters of blood at each time point. A 3-ml portion of each sample was centrifuged, 1 ml plasma was withdrawn, and both fractions were counted separately for radioactivity to determine whole blood and plasma 111In activity, as well as plasma 125I activity. Using blood samples obtained during occlusion and reperfusion only, a linear regression analysis of plasma values versus time was done to estimate 111In and 125I/ml plasma at the time of heart excision. Cell-associated 111In activity was calculated at each time point using the formula: cell-associated 111In counts/ml whole blood=(total 111In counts/ml whole blood)−(1−hematocrit/100)×(111In counts/ml plasma). The PMN-specific activity was calculated at each time point during occlusion and reperfusion as cell-associated 111In counts per milliliter of blood divided by number of PMNs per milliliter of blood. An average PMN-specific activity was calculated and used as a reference for tissue 111In activity.

Calculation of regional blood flow, albumin space, and PMN accumulation. Regional myocardial blood flow was calculated from the three microsphere measurements using the formula: Tissue flow is equal to tissue counts multiplied by reference flow divided by reference counts (ml/min/g wet wt). Myocardial albumin space was calculated as tissue 125I counts divided by 125I counts per milliliter of plasma at the time of excision (ml/100 g wet wt).

Because part of the 111In activity measured in each tissue sample was associated with plasma proteins, it was necessary to subtract the activity associated with those plasma proteins from the measured 111In activity to obtain cellular 111In activity. This was done using the following formula: Cellular 111In counts/mg tissue = (total 111In counts/mg tissue) − (ml albumin space/mg tissue) (111In counts/ml albumin space at time of heart excision). The number of tissue PMNs was then calculated using the formula: Tissue PMN/mg = (cellular 111In counts/mg)/average 111In counts/PMN.

Statistical Analysis

Data are expressed as group mean±SEM. Statistics were performed using a STATGRAPHICS STATISTIC SOFTWARE (STSC, Rockville, Maryland). Intragroup comparisons were made by a paired Student’s t test, whereas intergroup comparisons were made using an unpaired t test. To control for the variability in infarct size due to collateral blood flow, the TTC-based estimates of infarct size were compared between the two groups using an analysis of covariance with infarct size as a dependent variable and collateral blood flow as a covariate. A p value less than 0.05 was considered statistically significant.

Results

Mortality and Animal Exclusion

Of the 36 dogs initially entered into the study, four (three controls and one with critical stenosis) died of intractable ventricular fibrillation during the first 15 minutes of occlusion. Another three dogs (two controls and one with critical stenosis) developed reflow ventricular fibrillation. One of them (control) was successfully cardioverted, and results from it were included in the study. The analysis was then performed on the remaining 30 dogs (20 controls and 10 with critical stenosis). Four of these dogs (three controls and one with critical stenosis) were found to have subendocardial collateral blood flow of more than 0.15 ml/min/g. These dogs were excluded for group comparison of PMN accumulation, albumin space, infarct size, and hemodynamics, to establish groups with equivalent and relatively uniformly severe subendocardial ischemia. However, data from all animals were included when studying the relation between infarct size and collateral blood flow.

Characteristics of PMN Preparations

PMN preparations (n=30) obtained in the animals that completed the study exhibited the following
characteristics: The yield, expressed as a percentage of the total number of PMNs present in the original blood sample, was 52.5±3.5%; the average purity, expressed as a percentage of PMNs among the total number of isolated cells, was 90.2±0.9%. Contamination was due to monocytes, lymphocytes, and eosinophils; no red blood cell contamination was ever observed. The average labeling efficiency after incubation, expressed as a percentage of $^{111}$In incorporated into cells, was 77.4±3.1%. No significant differences were observed between the two groups when these parameters were analyzed separately for each group.

**$^{111}$In-Labeled Kinetics in the Circulation**

Figure 1 shows the $^{111}$In activity in the circulation as a function of time after injection of the labeled cells. The top curve represents the $^{111}$In present in the whole blood, whereas the bottom curve represents the cell-associated $^{111}$In activity. It is clear from these curves that a portion of the $^{111}$In was present outside of the cellular compartment, as represented by the difference between the two activity curves. Whole blood and cell-associated activity showed a rapid peak, followed by a rapid decline within the first 30–60 minutes after injection. After 60 minutes (i.e., time of occlusion) and until the end of the experiment, both activities were stable. Thus, the $^{111}$In-specific activity of PMNs remained nearly constant throughout the occlusion-reperfusion period. At the time of excision, an average of 67.4±0.4% of the whole blood counts were cell associated.

**Albumin Space in the Myocardium**

Figure 2 shows the albumin space in the nonischemic and ischemic-reperfused myocardium for the two groups. There was a marked increase in the albumin space in all layers of the ischemic-reperfused myocardium for both groups, compared with the corresponding nonischemic layers ($p<0.01$ for each layer). The location of the increased tissue albumin has not been determined; thus, the reasons for the increased albumin space after ischemia and reperfusion are not known with certainty. However, it seems likely that albumin entered the extravascular compartment due to altered endothelial permeability or to overt microvascular damage, especially in the subendocardial layer. Whatever the reason for the increased albumin space, there were no significant differences in this parameter between the two groups in any layer.

**Hemodynamics and Arrhythmias**

Hemodynamic results are summarized in Table 1. There were no significant differences between the two groups for any parameter measured. It must be noted that in most cases, hemodynamic measurements during reperfusion were obtained during episodes of sustained ventricular tachycardia because both groups exhibited a high incidence of transient or sustained reperfusion-induced ventricular tachycardia (75% of dogs in the control group and 70% in the group with critical stenosis). When only dogs with subendocardial flow less than 0.15 ml/min/g were taken into account, this incidence was 82.4% in the control group and 77.7% in the group with critical stenosis. Thus, unlike what has been suggested in some studies,6,13,17 reperfusion in the presence of a critical coronary stenosis did not influence the incidence of reperfusion-induced ventricular tachycardia.
Myocardial Blood Flow

**Left circumflex coronary blood flow.** Figure 3 shows left circumflex coronary artery blood flow (measured by an ultrasonic flow probe) before ischemia and during reperfusion. Preischemic flow was not significantly different between the two groups (control group, 32.3±3.8 ml/min; group with critical stenosis, 26.2±2.7 ml/min; p=NS). Immediately after reflow, there was a marked reactive hyperemic response in the control group (peak reactive hyperemia, 146.9±11.4 ml/min). No hyperemia was observed in the group with critical stenosis (peak reactive hyperemia, 27.4±2.4 ml/min). There was a rapid reduction of hyperemic blood flow in the control group, such that after 5 minutes of reperfusion (i.e., at the time of the second microsphere injection), blood flow was down to 57.7±8.9 ml/min. Further reduction occurred during the reperfusion period, and after 55 minutes of reperfusion, blood flow was similar to preischemic levels (26.9±4.0 ml/min). There also was a gradual decline in blood flow during reperfusion in the group with critical stenosis, and after 55 minutes of reperfusion, blood flow was significantly reduced compared with the preischemic levels (16.4±2.2 ml/min).

**Regional myocardial blood flow.** Ischemia: Figure 4, left, shows that both groups were subjected to comparable, severe ischemia, with the expected gradient of collateral flow, increasing from the subendocardium to the subepicardium. There were no significant differences between the two groups.

Five-minute reperfusion: Figure 4, middle, shows myocardial blood flow in the nonischemic bed and the ischemic-reperfused bed after 5 minutes of reperfusion. Blood flow to the nonischemic region was unexpectedly lower in the group with critical stenosis compared with the control group. This is unlikely to be an effect of the critical stenosis but is probably a consequence of random selection of animals with higher nonischemic flows in the control group compared with the group with critical stenosis. In the control group, blood flow was significantly higher in the epicardial portion of the ischemic-reperfused bed, compared with the nonischemic epicardial layer, whereas no such difference was observed in the midmyocardium and subendocardium. In the group with critical stenosis, blood flow was significantly lower in the subendocardium and midmyocardium of the ischemic-reperfused bed compared with the nonischemic tissue. Additionally, blood flow in the ischemic-reperfused region was markedly lower in all layers in the group with critical stenosis compared with the control group.

Fifty-five-minute reperfusion: Comparison between Figures 4, middle, and 4, right, shows that reperfusion blood flow in the ischemic-reperfused region decreased between 5 and 55 minutes of reperfusion in the control group, probably as a result of disappearance of reactive hyperemia, although this difference reached statistical significance only in the subepicardial region. In contrast, blood flow to the ischemic-reperfused regions in the group with critical stenosis remained relatively constant. Consequently, differences in blood flow between the control and the animals with critical stenosis were less marked after 55 minutes of reper-

**TABLE 1. Hemodynamic Data**

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Values are mean±SEM. HR, heart rate (beats/min); SBP, systolic blood pressure (mm Hg); RPP, rate-pressure product (HR*SBP/1,000). There were no significant differences between the two groups for any parameter at any time point.

**FIGURE 3.** Plots of left circumflex coronary artery blood flow before ischemia and during reperfusion. Preischemic flows were similar in the two groups. Immediately after reperfusion, there was marked hyperemic response in control group. Hyperemic response progressively disappeared during 1-hour period of reperfusion. Hyperemic response was abolished in group with critical stenosis. Data are group mean±SEM; control dogs, n=15; dogs with critical stenosis, n=9.
Figure 4. Plots of regional myocardial blood flow. Bars represent mean±SEM. *p<0.05 and **p<0.01, ischemic-reperfused myocardium versus nonischemic myocardium; $p<0.05$ and $$p<0.01$, control dogs versus dogs with critical stenosis. Left panel: Collateral blood flow (measured 10 minutes into ischemia) in subendocardial, midmyocardial, and subepicardial thirds of control dogs and dogs with critical stenosis. No significant differences were observed between the two groups in any layer. Control dogs, n=17; dogs with critical stenosis, n=9. Middle panel: Regional blood flow measured 5 minutes into reperfusion. Blood flow to nonischemic region was lower in dogs with critical stenosis compared with control dogs. In control dogs, reactive hyperemia was seen only in subepicardial third of ischemic-reperfused region. In dogs with critical stenosis, blood flow was significantly lower in subendocardial and midmyocardial thirds of ischemic-reperfused regions, compared with nonischemic tissue. Control dogs, n=6; dogs with critical stenosis, n=7. Right panel: Regional blood flow measured 55 minutes into reperfusion. Blood flow in subendocardial third of ischemic-reperfused bed was significantly lower in dogs with critical stenosis compared with the control dogs. In both groups, blood flow to subendocardial and midmyocardial thirds of ischemic region were lower than corresponding nonischemic layers.

fusion than after 5 minutes, reaching statistical significance only in the subendocardial layers. At 55 minutes, blood flow to the subendocardial and midmyocardial of the ischemic-reperfused region was significantly reduced compared with nonischemic flow, both in the control group and the groups with critical stenosis.

Area at Risk and Macroscopic Infarct Size

Figure 5 shows the average AAR, as a percentage of the left ventricle, and TTC-based estimates of infarct size, expressed relative to the left ventricle and to the AAR. One dog in the group with critical stenosis was not analyzed for infarct size because of difficulties in determining the borders of the TTC negative tissue. The size of the AAR, an important baseline predictor of infarct size, was not significantly different between the two groups, averaging 41.2±0.8% in control dogs (n=16) and 41.8±1.1% in dogs with critical stenosis (n=8). Similarly, analysis of infarct size, expressed as a percentage of the anatomic AAR, indicated no statistical differences between the two groups: 42.0±2.8 in control dogs and 44.8±5.0 in the animals with critical stenosis. Thus, by direct comparison, critical stenosis had no effect on the early loss of TTC staining. However, it is well known that infarct size, even when expressed as a percentage of the AAR, is highly variable among dogs and that most of this variation is due to variation in the amount of collateral blood flow. To control for this variability, we analyzed the relation between infarct size and collateral blood flow (Figure 6). There was an inverse correlation between infarct size and collateral blood flow in both groups. The presence of critical stenosis did not shift this relation. This visual observation was confirmed by an analysis of covariance, using infarct size as the dependent variable and collateral blood flow as a covariate (F=0.053; p=NS). Thus, the presence of a critical stenosis during reperfusion did not modify myocardial infarct size, as assessed by the early loss of TTC staining.

PMN Accumulation

PMN content in control skin averaged 1,636±323 PMNs/mg (n=30); accumulation was greater in the skin inoculated by staphylococci, averaging 13,613±5,517 PMNs/mg. The ratios of PMNs present in inflamed to control skin averaged 8.8±1.9. This ninefold accumulation indicates that PMNs isolated and labeled with our technique responded normally to chemotactic stimuli in vivo. No statistically significant differences in PMN accumulation...
in the skin were observed between the control group and the group with critical stenosis.

Figure 7 shows PMN accumulation in the inner, middle, and outer thirds of the nonischemic and the ischemic-reperfused tissue in both control dogs and dogs with critical stenosis. In the nonischemic myocardium, the number of PMNs per milligram of myocardium remained quite uniform among layers in both groups, averaging approximately 3,300–4,100 PMN/mg. In both groups, there was a marked accumulation of PMNs ($p<0.01$ in all layers) in the ischemic-reperfused myocardium, when compared with the nonischemic myocardium. The accumulation was the most marked in the subendocardial regions, averaging $11,982\pm1,487$ PMN/mg in control dogs ($n=17$) and $10,371\pm1,611$ PMN/mg in the dogs with critical stenosis ($n=9$). As observed previously, there was a transmural gradient of PMN accumulation, the accumulation being the greatest in the subendocardium, and decreasing toward the subepicardium. Average PMN accumulation was $9,259\pm1,384$ (control dogs) and $8,377\pm1,316$ (dogs with critical stenosis) in the midmyocardium, and $5,377\pm600$ (control dogs) and $6,612\pm1,038$ (dogs with critical stenosis) in the subepicardium. There were no significant differences in PMN accumulation between the two groups in any layer. Thus, the presence of a critical coronary stenosis during reperfusion did not influence PMN accumulation.

**Discussion**

**Hypothetical Effects of Critical Stenosis**

When a normal coronary artery is suddenly occluded and then abruptly reperfused, the initial phase of reperfusion is associated with marked reactive hyperemia due to vasodilation of the ischemic vasculature. However, this reactive hyperemic response may be infrequent in a clinical setting of myocardial infarction because reperfusion of an obstructed artery by thrombolysis occurs slowly, and maximum flow is often limited by the presence of a residual stenosis. The effect of the presence or absence of a flow-limiting critical stenosis on the myocardial response to reperfusion is not known. A number of effects, however, can be postulated, including: 1) The presence of a critical stenosis might be beneficial if it limited reperfusion-induced damage to the microvasculature (reperfusion injury), for example, by preventing the transmission of arterial perfusion pressure to the capillaries, or by reducing a reperfusion-induced burst of oxygen-derived free radicals. 2) The prevention of hyperemia could be detrimental if this reduced the washout of chemotactic factors for PMNs or caused increased capillary plugging by PMNs because of the lower microvascular perfusion pressure. These potential effects of a critical stenosis could in turn lead to an increased reperfusion injury. 3) A critical stenosis could cause persistent subendocardial ischemia due to a redistribution of flow from the subendocardium to the subepicardium despite normal mean flow.

**Observed Effects of Critical Stenosis**

The present study was done to determine the likelihood of the various hypotheses outlined above. Thus, we evaluated the effects of a critical stenosis on myocardial blood flow, macroscopic myocardial infarct size, and PMN accumulation in the ischemic-reperfused region at the end of the first hour of reperfusion. The results with $^{111}$In-labeled PMNs confirm our previous observation that there is a marked accumulation of PMNs in the ischemic-reperfused region compared with the nonischemic bed. The imposition of a partial constriction on the
occluded and reperfused artery (critical stenosis), however, did not alter the magnitude of this PMN accumulation in response to the episode of ischemia and reperfusion. In addition, the presence of the critical stenosis did not alter infarct size estimated by TTC staining at the end of the first hour of reperfusion. These results, that is, no effect of a critical stenosis on PMN accumulation or infarct size, do not substantiate any of the previously mentioned hypotheses. Our results suggest that the presence of a critical stenosis to prevent reactive hyperemia has neither beneficial nor detrimental effects. One implication of these results is that discrepancies regarding the effect of free radical scavengers such as SOD on infarct size, are unlikely to be due to the presence or absence of a critical stenosis during reperfusion. Other experimental differences need to be considered.

Comparison With Previous Studies

Regional blood flow during reperfusion. As expected, the presence of a critical coronary stenosis markedly limited myocardial blood flow measured after 5 minutes of reperfusion, when compared with the control group. In addition, in the presence of a critical stenosis, myocardial blood flow measured after 5 minutes of reperfusion was significantly lower in the ischemic-reperfused bed than in the nonischemic region, especially in the subendocardium (Figure 4, middle panel). This is consistent with results from several other laboratories. It would be tempting to conclude from these results that critical stenosis caused an important maldistribution of blood flow, resulting in persistent subendocardial ischemia during the early reperfusion period. It should be noted, however, that the subendocardial zone undergoes necrosis even in the setting of abrupt reperfusion. Moreover, the presence of reduced perfusion does not necessarily imply the presence of ischemia (i.e., blood flow insufficient to meet metabolic requirements) because dead myocytes consume no oxygen and even viable myocytes may have contractile dysfunction (i.e., be stunned) and consequently have a reduced demand for myocardial blood flow. Thus, the pathophysiologic importance of an early reduction in subendocardial perfusion seems questionable.

Infarct size. Our data presented in Figures 5 and 6 show that reperfusion in the presence of a critical stenosis does not modify the size of myocardial infarcts estimated from loss of TTC staining after 90 minutes of ischemia and 1 hour of reperfusion. This is consistent with the results obtained in other studies; however, three recent studies reported larger infarcts in the presence of critical stenosis. The reason for these conflicting results is not clear. In one of these studies, in which infarct size (expressed as a percentage of the AAR) was reported to be larger in the group with critical stenosis than in the control group, infarct size was highly variable in both groups and the difference was not statistically significant. Much of the variability of infarct sizes within experimental groups of dogs usually can be attributed to variation in the amount of collateral blood flow among the dogs. However, in this study, the authors did not compare infarct size between groups by an analysis of covariance using collateral blood flow as a covariate. In this regard, it is interesting to note that, in one of the studies in which the authors concluded that critical stenosis did not alter infarct size, average infarct size was also slightly larger in the group with critical stenosis than in the control group. However, this difference virtually disappeared when two control animals with high subendocardial collateral blood flow were excluded; also, a regression analysis of infarct size versus collateral blood flow showed no difference between the two groups. In the two other studies reporting increased infarct size in the presence of a residual stenosis, the stenosis that was applied during reperfusion was much more severe (i.e., reduction of flow to 35–50% of baseline flow) than in the present study or in other studies where infarct size was unaffected. It is possible that a more severe constriction of the coronary artery can lead to more severe impairment of flow during reperfusion and to increased infarct size. However, in neither study was infarct size analyzed with respect to collateral flow. Moreover, in one of these two studies, the difference in infarct size between the two groups was very small, and infarct size was highly variable in both groups. It must be noted, however, that conclusions regarding infarct size in the present study are limited by the suboptimal methods of estimating infarct size that were necessitated by the nature of the study. In our previous experiments to test the efficacy of an intervention on infarct size, infarct size always had been assessed by histologic techniques after 3 or 4 days of reperfusion. We believe that histologic analysis provides the most accurate and definitive means of quantifying infarct size. In the present experiment, we could not measure both 1) early PMN accumulation and 2) subsequent histologic infarct size. Because myocardial necrosis cannot be quantified easily by histology after a short duration, for example, 1 hour of reperfusion, we estimated infarct size based on loss of TTC staining. Moreover, because a substantial proportion of the left ventricle was required for isotope measurements, infarct size was measured only in two representative slices of the left ventricle. Loss of TTC staining may not be a sensitive index of cell death in all conditions. In addition, the methods of quantifying the extent of TTC loss in this study were less accurate than our usual histologic methods. We did compare the extent of infarction apparent by TTC, however, with the transmural extent of contraction-band necrosis evident by microscopic examination. From this semiquantitative comparison, we conclude that loss of TTC...
staining provided reasonable estimates of the extent of necrosis in both groups.

It also must be recognized that infarct size measured after 1 hour of reperfusion may not be a valid index of final infarct size if, as hypothesized by some investigators, 14,27 delayed reperfusion injury causes additional necrosis hours or days after reperfusion. In this regard, we cannot rule out the possibility that a critical stenosis might cause differences in the degree or delayed reperfusion injury that could translate into differences in infarct size after longer periods of reperfusion. This scenario seems unlikely, however, because the major effect of a critical stenosis on myocardial blood flow was the transient attenuation of reactive hyperemia during the first 5 minutes of reperfusion. Thus, any effect of critical stenosis on PMN accumulation or myocyte injury should already have occurred after 1 hour of reperfusion.

Despite the aforementioned concerns, average infarct size obtained in this study for the control group (42.01 ± 2.79% of the AAR) was similar to that obtained in a previous study where we measured infarct size by histology after 90 minutes of ischemia and 4 days of reperfusion (40.7 ± 5.3). 15 The regression lines between infarct size and collateral blood flow obtained in these two studies were superimposable with similar scatter. These observations suggest that 1) the methods of estimating infarct size in the present study were reliable and 2) most, if not all, of the myocyte death that is observed after 90 minutes of ischemia and 4 days of reperfusion has already occurred by the end of the first hour of reperfusion.

PMN accumulation. The present study is the first to compare directly the effects of the presence or absence of a critical stenosis on PMN accumulation in the ischemic-reperfused myocardium. Several studies, however, have measured myocardial PMN accumulation during ischemia, reperfusion, or both, with or without a critical stenosis. Significant early accumulation of PMNs has been consistently reported using either 111In-labeled PMNs5,6,8 or assays of the PMN-specific enzyme, myeloperoxidase.7 In a previous study using an experimental protocol very similar to the one used in the present experiments, we reported significant PMN accumulation in the first hour of reperfusion after a 90-minute episode of ischemia.9 It must be noted, however, that the number of PMNs calculated to be present in both nonischemic or ischemic-reperfused myocardium is higher in the present study than in our initial study. We believe that, because of technical limitations, we underestimated the number of PMNs present in both regions in the previous study. In that study, the average circulating half-life of the injected cells was very short (25.8 ± 2.8 minutes), and the PMN–specific activity decreased progressively over the 60-minute period of reperfusion. Because calculation of the number of PMNs in the myocardium depends on knowing the circulating PMN–specific activity when accumulation is occurring, the inconstant specific activity necessitated that assumptions be made about the kinetics of accumulation.9 In the present study, we obtained preparations of labeled PMNs with a longer half-life, primarily by replacing one isolation medium (Ficoll-Hypaque, or Histopaque, Sigma Chemical, St. Louis, Missouri) by another (Percoll, Pharmacia Biotechnology Products, Piscataway, New Jersey). In pilot studies, we estimated the half-life of the labeled cells (measured after the initial peak) to be more than 6 hours. A plateau of activity was reached within the first hour after the injection of the labeled cells (i.e., at the time of occlusion) (see Figure 1). Consequently, 111In-specific activity was nearly constant throughout the occlusion-reperfusion period, and some of the uncertainties of the previous study were avoided. We believe that the consistently higher number of PMNs in the present study, both in the ischemic and nonischemic regions, is likely to be more accurate.

Conclusions and Implications

In this study, we found that prevention of reactive hyperemia by a critical stenosis during reperfusion does not influence the early PMN response or extent of myocardial injury assessed after 90 minutes of myocardial ischemia and 60 minutes of reperfusion. Our results suggest that conflicting results among studies done to test the effect of anti–free radical interventions on infarct size are unlikely to be explained by the presence or absence of a critical stenosis during reperfusion.

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