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Granulocytes Cause Reperfusion Ventricular Dysfunction After 15-Minute Ischemia in the Dog

Robert Engler and James W. Covell

Regional ventricular dysfunction (the stunned myocardium) persists for several hours after 15 minutes of ischemia and reperfusion in the dog. Superoxide-radical--induced damage appears to be one of the mechanisms of this injury. We tested whether granulocytes were a direct source of injury in the stunned myocardium in the 15-minute ischemia dog model. Regional function during agranulocytic extracorporeal coronary perfusion (using Leukopak filters with ischemia and reperfusion was compared with function during a second period of ischemia and reperfusion after removal of the filters). Flow reduction and reperfusion flow, preload, afterload, and inotropic stimulation were the same during agranulocytic and granulocytopenic perfusion. During agranulocytic perfusion, stunning did not occur (>100% of preischemic function during reperfusion), but when the filters were removed and about 16% of the normal granulocyte count was present, stunning occurred with only 76% return of function at 60 minutes of reperfusion (p < 0.01). A second series of studied animals with extracorporeal perfusion and granulocyte replete perfusion all had less than 75% return of regional function, indicating that the agranulocytic perfusion and not the extracorporeal aspects of the experiment prevented stunning. We conclude that granulocytes are the direct source of the injury in stunned myocardium and apparently the main source of superoxide in the 15-minute ischemia dog model. Other possible granulocyte-related mechanisms of reperfusion injury include capillary no-reflow, causing microvascular ischemia and degranulation leading to enzyme-induced damage.

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Following 15 minutes of ischemia and reperfusion, myocardial function is depressed and returns to normal slowly over 6 hours despite the absence of cell death. Following longer periods of ischemia, full recovery takes days to weeks. The phenomenon of dysfunction without irreversible injury has been termed postischemic dysfunction or stunning. There are several potential mechanisms of stunning after 15 minutes of ischemia and reperfusion. First, adenosine 5'-triphosphate (ATP) is depleted during ischemia, and resynthesis might be impaired. This could be caused by oxygen radical damage to myocytes during ischemia. However, it appears that ATP synthesis and utilization is not the only limiting factor because prolonged inotropic stimulation with epinephrine results in sustained improvement; if ATP turnover were limiting, a sustained positive inotropic effect would not be possible. Supplying additional calcium also results in sustained improvement, which suggests that damage to some aspect of calcium metabolism is involved. Second, the no-reflow phenomenon may be a factor by causing continued ischemia. After the period of reactive hyperemia is over, reperfusion flow is decreased compared with normal myocardium that was not subject to ischemia. The capillary no-reflow phenomenon and edema in this model within the first hour of ischemia and reperfusion is caused by granulocytes. Finally, reperfusion injury is known to occur in the heart. It has been proposed that the mechanism of reperfusion injury is through resupply of molecular oxygen and metabolism to superoxide radical by xanthine oxidase in the endothelium. Superoxide radical is a potent oxidizing agent that can damage cell membranes and lipoproteins. In myocardial stunning, the hypothesis of superoxide-induced damage is supported by the improvement of postischemic dysfunction during administration of the scavenger enzymes, superoxide dismutase and catalase. However, the stunning is not completely prevented by pretreatment with superoxide dismutase and catalase, indicating incomplete scavenging or other mechanisms such as no-reflow or cellular injury by metabolite accumulation. Since granulocytes are the main cause of capillary no-flow in this situation and granulocytes are a potential source of superoxide radical, the hypothesis that granulocytes are a direct cause of stunning after 15 minutes of myocardial ischemia and reperfusion was tested in the dog model. Complete return of function (no myocardial stunning) was found in hearts perfused with agranulocytic blood. When the same heart was then perfused with blood that was granulocytopenic, prolonged postischemic ventricular dysfunction occurred. This occurrence indicates that granulocytes are the main direct cause of stunned myocardium after 15 minutes of ischemia and operate through several possible mechanisms, such as oxygen--radical production, capillary no-reflow, or other consequences of their presence and activation.

Materials and Methods

The goal of the preparation was to perfuse the heart with agranulocytic blood, to subject a region to 15

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minutes of ischemia and reperfusion, and to repeat the ischemia–reperfusion sequence with granulocytes present.

Seven mongrel dogs were anesthetized with sodium pentobarbital (25 mg/kg) and the heart exposed in the pericardial cradle through a left thoracotomy. A short, stiff polyethylene tube was inserted through the left ventricular apex for measuring left ventricular pressure and its derivative. Ultrasonic dimension gauges were placed at a depth of 5 mm in the anterior midwall halfway from base to apex and 5 mm deep in the lateral free wall one third of the way from the atroventricular groove to the apex. The most proximal segment of the circumflex and left anterior descending coronary artery were dissected free at their origin from the left main coronary artery and prepared for cannulation. A catheter was passed into the aorta through the femoral artery to measure aortic pressure.

The extracorporeal coronary perfusion apparatus was prepared as described previously10,11 (Figure 1). Carotid artery blood was pumped through Baxtor-Travenol Leukopak filters into a reservoir. A section of Tygon tubing that bypassed the filters was clamped shut. From the reservoir, blood could be pumped past an injection port, mixing chamber, and reference withdrawal port for radiolabelled-microsphere measurements of regional myocardial blood flow. The flow then was divided to 2 Sarns roller pumps for servo-controlled coronary perfusion. Pulse dampers consisted of a 10-cm section of rubber tubing. Coronary perfusion pressure was measured at a side port just proximal to the coronary cannula. The pressure was maintained at 155 mm Hg. At normal coronary flow rates, a pressure drop of 40 mm Hg occurs from the pressure side port to the cannula tip. The extracorporeal system was primed with blood from a donor dog.

Anticoagulation was begun with heparin 400 UI/kg body wt and maintained with 100 U/kg/hr. Anesthesia was maintained with pentobarbital, and the blood gases and pH were monitored frequently and maintained at physiologic values with sodium bicarbonate. Animals were ventilated with 100% oxygen.

First the circumflex and then the left anterior descending coronary artery were ligated, cannulated, and perfused through the extracorporeal circuit. The average occlusion time was approximately 90 seconds but was never more than 4 minutes. Servo-controlled perfusion pressure was 155 mm Hg. Lidocaine, 2–2.5 mg/kg, was given prior to cannulation. Additional doses of 1 mg/kg were given prior to each of the 2 coronary occlusions.

Data were recorded on an 8-channel Brush-Clivette recorder and on FM tape.

Experimental Procedure

Complete blood counts were performed on blood from the coronary perfusion circuit and the systemic circulation of the dog prior to each coronary occlusion. Preischemia measurements were recorded, and the pump perfusing the left anterior descending coronary artery was stopped for 15 minutes. Regional myocardial blood flow was measured by injection of approximately 450,000 15-microsphere (Sn-113, Nb-95, Ru-103, or Sc-46) at 10 minutes of ischemia, and reference withdrawal was continued for 2.5 minutes at 7 ml/min.17 After 15 minutes of ischemia, reperfusion was begun by starting the pump. Regional myocardial blood flow was measured at 50 minutes of reperfusion by a second injection of labelled microspheres. After 60 minutes of reperfusion, the Tygon tubing bypassing the filters was opened, and the filters were clamped. At this time, the animal was granulocytopenic with a granulocyte count of about 10% of the normal level. Ten minutes were allowed for equilibration.

![Figure 1. Extracorporeal perfusion circuit. Blood is pumped from carotid artery through either Leukopak filters or, after filter removal, plain Tygon tubing. Water baths maintain physiologic temperature. Mixing chamber is inverted tube in inverted vortex mixer. Cx, circumflex; LAD, left anterior descending coronary artery.](http://circres.ahajournals.org/doi/figure/S1)
tion of the granulocytes in the animal and the extracorpo-
real circuit, and the same protocol was repeated. In pil-
ot experiments, regional function in agranulocytic
animals percent shortening of the ultrasonic dimen-
sion gauge) returned completely to normal by 60
minutes. Previous investigators have found that 3 se-
quential 15-minute ischemia-reperfusion sequences
resulted in equivalent dysfunction and recovery. 

The first ischemia–reperfusion was always per-
formed with Leukopak filters in the circuit. The ex-
periment sequence of granulocyte free followed by
granulocytopenic perfusion could not be reversed be-
cause ventricular function does not return to normal in
1 hour with granulocytes present in this model.

Measurement of regional function data and pres-
sures were made at preischemia, 1, 2, 5, 10, and 15
minutes of ischemia; and 1, 2, 5, 30, 45, and 60 min-
utes of reperfusion. Following the granulocytopenic
blood perfusion sequence, a large overdose of pento-
barbital was given for euthanasia. The heart was ex-
cised with coronary cannula left in place. The 2 cannu-
lae were simultaneously perfused postmortem with
colored dye to identify the ischemic and normal areas.

End-diastolic segment length (EDL) was deter-
mixed at the rise of positive dP/dt. End-systolic seg-
ment length (ESL) was determined at peak negative
dP/dt. Percent segment shortening was calculated from
the formula: %SS = 100(EDL – ESL)/EDL.

Statistical analysis was performed by repeated
measures analysis of variance (BMDP) for the follow-
ing variables: ischemic segment EDL and %SS, normal
segment EDL and %SS, aortic pressure, and regional
myocardial blood flow. Regional function and loading
conditions were compared with repeated measures
ANOVA for the factors, granulocytopenic vs agranu-
locytic blood, and for the times, preischemia, and 1, 5,
30, and 60 minutes of recovery. Blood cell counts and
function and loading conditions at 15 minutes of ische-
emia were compared by Student’s paired t test. All data
are reported as mean ± SD.

Results

Blood Cell Counts

During Leukopak filtration, the white blood cell
count in the perfusion line was 620 ± 310/μl, of which
10 ± 10/μl were granulocytes. The platelet count was
reduced to 11,000 ± 9,000/μl, and hematocrit was not
reduced at 40.0 ± 5.5%. After the filters were re-
moved, the total leukocyte count in the extracorporeal
perfusion line was 1,710 ± 990/μl, and the granulocyte
count was 1,050 ± 840/μl, no different than the systemic
count in the dog but significantly greater than during
filtered perfusion (p < 0.01). After filter removal, the platelet count was 41,200 ±
15,400/μl, and the hematocrit was 39.4 ± 5.1. Thus,
during filtration, the coronary perfusate was essential-
ly granulocytic and thrombocytopenic, but after filter
removal, the animals were granulocytic (10% of
the average normal value in our laboratory). The ani-
mals remained thrombocytopenic.

Regional Myocardial Blood Flow

During agranulocytic and granulocytopenic perfu-
sion ischemia, the blood flow to the ischemic endocar-
dium was reduced to an equivalent degree, 0.18 ±
0.12 ml/min/g and 0.19 ± 0.12 ml/min/g, respec-
tively. Reperfusion flow and normal area flow also were
equal during agranulocytic and granulocytopenic per-
fusion (Figure 2). Epicardial flow changes were simi-
lar (Figure 2).

Regional Function

During coronary occlusion, ischemic dysfunction
was equal during agranulocytic and granulocytopenic
perfusion. There was a significant difference in func-
tion recovery during reperfusion (Figures 3 and 4).
During agranulocytic reperfusion, there was prompt
due and near-complete return of function within 1 minute
of reperfusion, which remained above 100% of preis-
chemia through 60 minutes of reperfusion. During
subsequent granulocytopenic perfusion, functional re-
covery was only 70 ± 28% of preischemia at 1 minute
and remained depressed at 76 ± 21% by 60 min-
utes (p < 0.01 vs agranulocytic perfusion) (Figures 3
and 4).

Because inotropic stimulation can enhance function
of the stunned myocardium,6 the normal area function
was compared to test for differences in catecholamine
stimulation of the whole heart between agranulocytic
and granulocytopenic reperfusion. Normal area shor-
tening increased during ischemia and reperfusion but
was not different during the two ischemia–reperfusion
sequences indicating equal degrees of positive inotro-
ic stimulation (Figure 5).

Loading Conditions

End-diastolic segment lengths were used in the is-
chemic area as an estimate of preload. End-diastolic
shows the change in end-diastolic segment lengths from preischemia. The slight trend toward longer end-diastolic segment length during agranulocytic reperfusion was not significant (*p > 0.5). Mean aortic pressure served as an index of afterload and was the same during agranulocytic and granulocytopenic perfusion (Figure 7). Thus, the loading conditions during recovery were equal during agranulocytic and granulocytopenic perfusion.

Extracorporeal Circulation

In the 4 animals subjected to ischemia and reperfusion with extracorporeal coronary perfusion using no filters (granulocyte-replete perfusion), regional myocardial blood flows were equal to the experiment group. Segment shortening in the anterior wall decreased from $15.7 \pm 4.2\%$ preischemia to $-31 \pm 2.9\%$ (-19% of preischemia) at 15 minutes of ischemia and recovered to $9.3 \pm 3.9\%$ (60% of preischemia; range, 32–75%) at 60 minutes of reperfusion. Thus, the extracorporeal circuit and heparinization does not prevent posts ischemic ventricular dysfunction; all 4 animals had 75% or less recovery of function at 60 minutes of reperfusion.

Note added in proof. Leukopak filters activate the complement system and cause the release of small amounts of adenosine from erythrocytes; the reservoir and long perfusion tubing allow for metabolism in the extracorporeal circuit prior to coronary perfusion.

Discussion

The main finding of this study is that agranulocytosis completely prevents ventricular dysfunction following 15 minutes of coronary occlusion and reperfusion in the dog. Previous experiments of myocardial ischemia using the dog model have consistently shown marked depression of contractile function following 15 minutes of ischemia and reperfusion.$^3,4,6,8,14,15,16$ Thus,
the consequences of a 15-minute coronary occlusion and reperfusion in the agranulocytic dog heart in the present study is dramatically different from all previous studies. To obtain matched control studies, the Leukopak filters were removed, and the coronary occlusion and reperfusion were repeated with granulocytopenic blood. At this time, following more than 90 minutes of extracorporeal filtration of total left coronary artery flow, the animals were partially depleted of granulocytes. Approximately 10% of the normal granulocyte count remained. The repeated 15-minute ischemia-reperfusion sequence resulted in 76% return of regional function at 60 minutes. This level of postischemic dysfunction in the control group is similar to or slightly better than the treatment group when other investigators used interventions such as oxygen-radical scavengers or inotropic stimulation. This remarkably good recovery observed is presumably due to granulocytopenia. However, the high capacity of granulocytes to cause injury also is suggested because these animals were depleted to 10% of normal and still showed postischemic dysfunction. Thus, partial depletion of granulocytes would be an incomplete test of their pathophysiologic role because of their high capacity to cause injury.

It is recognized that repeated episodes of brief ischemia may result in some conditioning of the myocardium so that it becomes resistant to the effects of subsequent ischemia. The effect of any preconditioning on the present study would be to improve function after the second (granulocytopenic) ischemia and reperfusion and, thus, could not have altered the chief results. Furthermore, coronary occlusion and reperfusion sequences result in the equal recovery of function following 3 sequential 15-minute occlusions. To be certain that the extracorporeal circulation alone did not pre-
vent postischemic dysfunction during a first but not a second episode of ischemia, the identical procedure was performed without any filtration in 4 granulocyte-replete animals. This was necessary because we could not randomize the Leukopak-filters/filters-removed sequence. Performing the first occlusion and reperfusion in granulocyte-replete animals resulted in 75% or less return of function in all 4 animals. Thus, granulocytes are necessary to produce myocardial stunning following 15 minutes of ischemia and reperfusion. Following longer periods of ischemia, other mechanisms also may be important.

Several mechanisms whereby the granulocyte might cause stunning are known. These mechanisms, which can be categorized into the 3 main consequences of granulocyte activation during ischemia, are 1) oxygen-radical production causing cell membrane, sarcoplasmic reticulum, or enzyme damage; 2) granulocyte-capillary plugging resulting in continued ischemia; and 3) degranulation leading to enzyme damage to cells.

**Oxygen Radicals**

Oxygen radicals as the cause of the injury in stunned myocardium has been supported in two studies. Both of these studies used superoxide dismutase and catalase pretreatment to improve functional recovery following 15 minutes of ischemia in dogs. In each case, ischemic dysfunction was improved by the treatment compared with placebo, but the return of function in treated animals was considerably less than 100% of preischemic function (20% in one case and 60% in the other). These experiments clearly indicate that these
oxygen-radical scavengers reduce postischemic dysfunction and imply that superoxide and/or its products are involved mechanisms. Until now, the source of the oxygen-radical superoxide was thought to be xanthine oxidase in the endothelial cell. In previous studies, a granulocyte source of superoxide radical cannot be excluded. Granulocytes are potential sources of large quantities of superoxide through the enzyme NADPH oxidase. Our studies demonstrate a 100% return of function, clearly indicating that the major source of superoxide radical causing stunning and scavenged in the previous studies was the granulocyte.

McCord has proposed that reperfusion injury is caused by production of oxygen radicals on reintroduction of molecular oxygen after ischemia. He also proposed that the alteration in the tissue that resulted in superoxide formation was conversion of xanthine dehydrogenase to xanthine oxidase (Figure 8). The findings in the current study suggest a modification of this hypothesis to include the granulocyte as a source of superoxide radical and possibly no-reflow, causing reperfusion injury (Figure 8).

The stimulus that activates granulocytes during reperfusion injury is unknown. One hypothesis is that the superoxide generated by xanthine oxidase in the endothelial cell could produce a tissue chemotactic factor that results in activation and trapping of granulocytes. It may be that the endothelial cell is one of the sources of the chemotactic factors that activate and trap granulocytes during ischemia and reperfusion. Some what more controversial evidence for the xanthine oxidase hypothesis comes from experiments performed with the xanthine oxidase inhibitor allopurinol. Some experiments have shown a reduction in irreversible myocardial injury following pretreatment with allopurinol, but others have failed to show an effect. Some of the discrepancies between the allopurinol studies may be explained by the necessity for pretreatment or by the absence of xanthine oxidase in some species. Whether allopurinol works indirectly, by preventing the production of the chemotactic substance and decreasing granulocyte accumulation, or directly, by actually reducing the xanthine oxidase production of superoxide, is unknown. There is one additional unexplored area whereby allopurinol could reduce ischemic injury. Allopurinol is metabolized to allopurinol ribotide, and as such, it can profoundly affect purine metabolism within cells. This altered purine metabolism might affect ATP degradation, ATP resynthesis, or the release of products such as adenosine, which is known to inhibit granulocytes. In other models of reperfusion injury such as buffer-perfused hearts where P02 is high or following longer periods of ischemia, additional sources of oxygen radicals such as mitochondrial cytochromes, catecholamine oxidation, or arachidonate metabolism might be important.

**Granulocyte-Capillary Plugging**

The second potential mechanism of granulocyte-induced reperfusion injury is granulocyte capillary plugging leading to continued microvascular ischemia. Granulocytes accumulate in myocardial capillaries during ischemia, and accumulation is dramatically enhanced on acute reperfusion. This phenomenon has been termed leukocyte capillary plugging and is the main mechanism of the capillary no-reflow phenomenon during the first 5 hours of myocardial ischemia. This phenomenon results from the rheologic properties of the granulocyte such that marked deformation is necessary for capillary transit, and because of natural adherence, there is a small safety margin for granulocyte transport through capillaries. We have shown that following 1 hour of ischemia and brief reperfusion, 24% of myocardial capillaries remain ob-

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**Figure 8** Diagram of hypothesis that xanthine oxidase, converted from xanthine dehydrogenase (XD) by a calcium-dependent protease during ischemia, produces superoxide and causes reperfusion injury as shown by solid lines in box. Addition of granulocyte as source of superoxide and toxic enzymes and cause of capillary no-reflow by capillary plugging is shown by dashed lines. One source of chemotactic factors that activate granulocytes could be xanthine oxidase-produced superoxide reacting with tissue or plasma proteins. NADPH Oxidase is membrane-bound enzyme in granulocytes that produces superoxide. Both hypochlorous acid and N-chloroamines also are produced by granulocytes.
stromed by granulocytes. Obstruction of this fraction of capillaries could result in considerable tissue underperfusion due to high oxygen consumption and, thus, the relatively short diffusion path for oxygen in myocardial tissue. Vasconstriction, edema, and hemorrhage also may increase vascular resistance, especially after longer periods of ischemia. Consistently, previous studies of the stunned myocardium have shown decreased reperfusion blood flow compared with the nonischemic tissue. In the studies by Becker et al., there was a slight reduction in reperfusion flow, even following 5 minutes of myocardial ischemia. It seems likely that this reduced reperfusion flow is due to granulocyte capillary plugging. The relative contribution of this mechanism to the stunned myocardium is not known at the present time. Furthermore, the persistence of granulocyte capillary plugging during reperfusion is not known. The phenomenon has not been observed by routine histologic methods, probably because single granulocytes obstruct individual capillaries in a stochastic fashion, which is quite different from leukocyte infiltration.

A third potential effect of granulocyte activation during myocardial ischemia is degranulation. The effect of enzymes contained within these granules in ischemic myocardium is unknown. Inotropic stimulation can improve function of the stunned myocardium. This finding could indicate either use of the inotropic reserve in essentially normal myocytes or improvement in function of stunned myocytes within the ischemic area as the stunned myocardium likely contains a mixed population of normal and injured cells. The finding that inotropic stimulation improves posts ischemic dysfunction is compatible with either of the mechanisms proposed above. Oxygen-radical damage could inhibit calcium handling or excitation-contraction coupling in the myocyte. Alternatively, the capillary no-reflow phenomenon might lead to severe dysfunction of some myocytes in the neighborhood of obstructed capillaries and to essentially normal function of other myocytes adjacent to perfused capillaries. Inotropic stimulation could augment function in those normally perfused myocytes and, thus, improve net function without actually affecting the subpopulation of stunned myocytes. This mechanism is possible because leukocyte capillary plugging is a stochastic process. Within individual histologic sections, a seemingly random distribution of perfused and nonperfused capillaries have been seen. These findings suggest the possibility of a nonuniform mechanism of the stunned myocardium within an ischemic area. Thus, some myocytes might be normal and some quite abnormal due to neighboring capillary no-reflow and/or oxygen-radical production by the granulocyte.

Platelets were reduced most during agranulocytic perfusion but also were reduced significantly during granulocytopenic perfusion. While platelet granulocyte interactions cannot be excluded, the platelet count difference could not explain our findings since platelets do not produce superoxide and do not obstruct capillaries in this model.

Our results provide conclusive evidence that granulocytes have a major effect on function in the first 15 minutes of acute myocardial ischemia and reperfusion. Following longer periods of ischemia, manipulation of granulocyte function has been shown to salvage ischemic myocardium. After 15 minutes of myocardial ischemia, it is unclear whether the damage (stunning) occurs during ischemia or on reperfusion. If the stunned myocardium is due to reperfusion damage, then reperfusion is in fact a double-edged sword.

During the first hour of ischemia, granulocytes are important in the injury process; we have shown that edema formation, arrhythmias, and blood flow are all improved dramatically by agranulocytosis. The present study provides increasing evidence that damage from acute myocardial ischemia is not solely due to the interruption of nutrient blood flow nor to the failure to remove metabolic products. Rather, ischemia initiates a cascade of reactions, including mechanical capillary obstruction by granulocytes, oxygen-radical formation, and perhaps other inflammatory events that markedly increase the injury (Figure 8). It is unfortunate that the inflammation that is triggered during ischemia, or perhaps immediately on reperfusion, continues during the reperfusion period. The duration of the persistent inflammation and granulocyte activation after reperfusion is unknown. A means to halt the inflammation (to block the inflammatory signals) might allow reperfusion with granulocyte-replete blood and prevent granulocyte-mediated injury. In the absence of such an intervention, means to remove or to inhibit granulocytes during acute reperfusion must be sought to prevent the stunned myocardium and reperfusion injury.

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