JACC Vol. 21, No. 5
April 1993:1245–53

Myocardial Infarct Extension During Reperfusion After Coronary Artery Occlusion: Pathologic Evidence

ANDREW FARB, MD, FACC, FRANK D. KOLODGIE, MS, MARIE JENKINS, RENU VIRMANI, MD, FACC

Washington, D. C.

Objectives. The goal of this study was to demonstrate myocardial infarct extension during reperfusion within the same animal.

Background. Whether myocardial reperfusion can result in the extension of myocardial necrosis remains controversial. The transformation of reversibly injured myocytes into irreversibly damaged cells after reperfusion has been difficult to demonstrate pathologically.

Methods. New Zealand White rabbits (Group I, n = 10) were subjected to 30 min of coronary artery occlusion and 180 min of reperfusion. Horseradish peroxidase, a tracer protein that permeates the sarcolemma of irreversibly injured myocytes, was used to quantitate myocyte necrosis at the beginning of reperfusion. Within the same heart, infarct size was measured after 180 min of reperfusion by triphenyltetrazolium chloride (TTC) staining. In separate experiments to demonstrate the validity of the model, rabbits were subjected to 30 min of coronary occlusion, followed by intravenous infusion of horseradish peroxidase and rapid induction of death (Group II) or 30 min of occlusion, 180 min of reperfusion with horseradish peroxidase administered after 180 min of reperfusion and TTC staining after induced death (Group III).

Results. In Group I, infarct size at the onset of reperfusion, delineated by horseradish peroxidase, measured 45.3 ± 2.8% of the area of risk and was significantly less than TTC-delineated infarct size after 180 min of reperfusion (59.8 ± 3.3%, p = 0.0002). By electron microscopy, border areas within the ischemic bed demonstrated irreversibly injured horseradish peroxidase–positive myocytes adjacent to irreversibly injured horseradish peroxidase–negative myocytes, suggesting that further cell death occurred during reperfusion. In Group II, infarcts delineated by horseradish peroxidase after 30 min of coronary occlusion were similar in size to infarcts measured by this tracer in Group I. In Group III, infarcts delineated by horseradish peroxidase at 180 min of reperfusion were similar in size to infarcts measured by TTC and similar to TTC-delineated infarcts measured at 180 min of reperfusion in Group I.

Conclusions. These results provide evidence that there is a subset of myocytes in border areas within the ischemic region that are viable at the beginning of reperfusion but subsequently progress to irreversible injury during the reperfusion period.

(J Am Coll Cardiol 1993;21:1245–53)

Timely reperfusion after coronary artery occlusion is the most effective means of reducing mortality and preserving left ventricular function (1–3). However, it is controversial whether blood reperfusion has detrimental effects on the reperfused myocardium (reperfusion injury) and can transform reversibly injured myocytes into irreversibly injured cells (4). To date, there is only indirect evidence that reversibly injured myocytes within the reperfused ischemic region can be transformed into irreversibly injured cells (infarct extension). Various interventions (for example, perfluorochemicals [5–7], oxygen-derived free radical scavengers [8,9], adenosine [10,11], preconditioning [12,13], antibodies that inhibit neutrophil aggregation, adhesion and chemotaxis [14]) administered during reperfusion have been shown to reduce infarct size. In these studies, infarct size in one group of animals that received an experimental intervention was compared with infarct size in another group of animals treated with placebo. Documentation of infarct extension during reperfusion within a single animal has not been demonstrated pathologically.

Disruption of sarcolemmal membrane integrity and amorphous mitochondrial matrix densities are the hallmarks of irreversible myocyte injury (15–20). Horseradish peroxidase, a fine structural tracer protein molecule (molecular weight 40,000 d, estimated equivalent radius 25 to 30 Å [21]), has been used to detect ultrastructural changes in membrane permeability in experimental models of myocyte necrosis as a result of catecholamines (22,23) or focal ischemia (24). In a previous study, we utilized the ability of horseradish peroxidase to traverse the damaged sarcolemma to measure infarct size in rabbits immediately after 30 min of coronary occlusion or after 24 h of reperfusion (25); the sarcolemmal

The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

From the Department of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, D.C. This study was presented in part at the 63rd Annual Scientific Sessions of the American Heart Association, Dallas, Texas, November 1990. It was supported by the American Registry of Pathology, Washington, D.C.

Manuscript received July 20, 1992; revised manuscript received September 30, 1992, accepted October 5, 1992.

Address for correspondence: Renu Virmani, MD, Department of Cardiovascular Pathology, Armed Forces Institute of Pathology, Washington, D. C. 20306-6000.

0735-1097/93/$6.00

JACC Vol. 21, No. 5
April 1993:1245–53

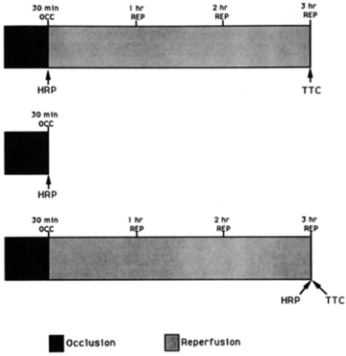


Figure 1. Experimental protocol in Groups I to III. HRP = horseradish peroxidase; OCC = occlusion; REP = reperfusion; TTC = triphenyltetrazolium chloride.

membrane of normal and nonirreversibly injured myocytes was impermeable to the tracer. In the present study, we used horseradish peroxidase to identify and quantitate myocytes irreversibly injured at the beginning of reperfusion after 30 min of coronary occlusion for comparison within the same animal, with infarct size at 180 min of reperfusion defined by triphenyltetrazolium chloride (TTC) staining. To further evaluate infarct extension during reperfusion, horseradish peroxidase was infused before induced death in rabbits subjected to 30 min of coronary occlusion and rabbits subjected to 30 min of occlusion followed by 180 min of reperfusion. These studies provided pathologic evidence of infarct extension during reperfusion.

Methods

Animal preparation. This study conformed to the guidelines specified in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Armed Forces Institute of Pathology Animal Care and Use Committee. A summary of the experimental protocols is shown in Figure 1.

Group I. Male New Zealand White rabbits were anesthetized with intravenous sodium pentobarbital (30 mg/kg body weight), intubated and ventilated with a small animal ventilator (Harvard Apparatus) with 100% oxygen. Arterial blood gas measurements were performed to assure an oxygen saturation of ≥98%. A lead II electrocardiogram (ECG) was monitored throughout the surgical procedure. A catheter was placed in the left or right femoral artery for blood pressure monitoring. A left thoracotomy and pericardiotomy were performed, and the left obtuse marginal artery was identified. A 4-0 cardiovascular silk ligature was placed around the left obtuse marginal artery near the base of the heart, and a snare was fashioned with polyethylene tubing. The snare was tightened and coronary artery occlusion was confirmed by the presence of cyanosis of the myocardium supplied by the occluded artery and ECG evidence of myocardial injury (ST segment elevation). After 30 min of occlusion, the snare was released, and reperfusion of the left obtuse marginal artery was visually confirmed.

After confirmation of reperfusion, 100 mg/kg of horseradish peroxidase (type II, Sigma Chemical Company) in 5 ml/kg of normal saline solution was administered intravenously over 1 min. At 180 min of reperfusion, heparin (1,000 U) was administered intravenously, followed by euthanasia with an overdose of sodium pentobarbital. Hearts were rapidly excised and perfused retrograde at 60 to 80 mm Hg through the aortic stump with lactated Ringer's solution for 1 min to clear the coronary circulation of blood. A 2% solution of TTC warmed to 37°C was perfused retrograde for 5 min. To define the area of risk, the left obtuse marginal artery was then reoccluded, and 10 mg of fluorescent zinc cadmium-sulfide particles (Duke Scientific) suspended in 10 ml of normal saline solution was infused into the aortic stump (26). The snare was released, and the hearts were perfusion-fixed for 10 min with 2% glutaraldehyde-2% formaldehyde in 0.1 mol/liter of sodium cacodylate buffer at 60 to 80 mm Hg. The ventricles were sliced transversely into four or five slices (each 2 mm thick), and the slices were immersed in the fixative described for 5 to 6 h at room temperature. On completion of fixation, the heart slices were washed overnight in 0.1 mol/liter of sodium cacodylate buffer containing 11% sucrose.

To demonstrate the validity of the experimental model, the following additional experiments were performed.

Group II. New Zealand White rabbits underwent the identical procedure of 30 min of coronary artery occlusion, release of the coronary snare and infusion of horseradish peroxidase. After the infusion, the animal was killed. The heart was excised and underwent TTC staining, identification of the area of risk and perfusion fixation as described.

Group III. New Zealand White rabbits underwent the identical initial operative procedure as Group I with 30 min of coronary artery occlusion and 180 min of reperfusion. However, horseradish peroxidase was administered after 180 min of reperfusion. Induced death, TTC staining, identification of the area of risk and perfusion fixation were performed as described for Group I.

Preparation of tissues. The right ventricle was removed and each slice of the left ventricle was weighed. Sections from ischemic and nonischemic areas were removed for electron microscopic processing. A whole-mount 8-μm frozen section was cut from each left ventricular slice on a freezing microtome (International Equipment). Ventricular slices were photographed to obtain TTC-measured infarct size, and photography was repeated using ultraviolet light to identify the risk region. For measurement of horseradish peroxidase-delineated infarcts, the brown peroxidase reaction product was developed as follows: frozen sections were incubated for 5 min at room temperature in 100 ml

of 0.05 mol/liter tris (hydroxymethyl) aminomethane (tris)-hydrochloride buffer, pH 7.6, containing 100 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical) and 33.3 μliter of 30% hydrogen peroxide.

For electron microscopy of tissues from Groups I and II, myocardial biopsy samples were taken from the central ischemic and nonischemic ventricular beds (divided into endocardial and epicardial halves). From these tissues, 50-μm myocardial sections (eight sections/rabbit) were cut with a vibrating microtome (Biorad, Microscience Division, UK). Further tissue processing followed the procedure outlined by Yunge et al. (23). Briefly, sections were incubated for 1 h in 20 ml of 0.05 mol/liter tris (hydroxymethyl) amino-methane (tris)-hydrochloride buffer, pH 7.6, containing 20 mg of 3,3'-DAB and 6.7 μliter of 30% hydrogen peroxide. Tissues were then postfixed in ferrocyanide-reduced 1% osmium tetroxide in Palade buffer, pH 7.4, containing 4.9% sucrose for 90 min at 4°C (27). Slices were then hand-processed in a series of graded ethanols and embedded in Epon. Unstained thick sections (2 μm) were examined by light microscopy. Border areas containing unstained myocytes and myocytes with intramyocyte brown peroxidase reaction product were selected for thin sectioning and staining with lead citrate and uranyl acetate. Sections were viewed on a Zeiss 109 electron microscope and were evaluated for ultrastructural evidence of irreversible injury (mitochondrial flocculent densities and sarcolemmal breaks) and the presence of dark gray electron-dense staining of contracted sarcomeres, representing the strongly positively charged horseradish peroxidase bound to hypercontracted myofilaments.

Infarct size quantification by horseradish peroxidase and triphenyltetrazolium chloride methods. Thirty-five–millimeter photographs of TTC-stained gross left ventricular slices were magnified (×6), and the outlines of the left ventricle, risk regions and infarcted myocardium were traced. Infarcted regions were identified by the absence of red staining. From horseradish peroxidase–stained frozen sections, left ventricular regions were magnified (×24), and infarcts were defined by the presence of intramyocyte brown peroxidase reaction product. Risk regions were superimposed over horseradish peroxidase–stained sections. For each method, infarcts, risk regions and left ventricular areas were measured by computer planimetry from tracings, and infarct size was expressed as a percent of the left ventricle and area of risk.

Myocardial blood flow. The rabbit heart has a poorly developed collateral network (28). In a separate group of rabbits, we sought to confirm this aspect of the rabbit myocardial circulation and to demonstrate tissue reperfusion on release of the coronary artery snare using the method just described. Male New Zealand White rabbits were anesthetized, ventilated and monitored as described. A left thoracotomy and pericardiotomy were performed, and a polyethylene catheter was placed in the left atrium for radioactive microsphere injection. The left obtuse marginal artery was identified and a snare was placed as described previously. The left obtuse marginal artery was occluded for 30 min, followed by reperfusion.

Transmural myocardial blood flow was determined using 15-μm radiolabeled microspheres: cerium-141, chromium-51 and strontium-85. Microspheres (2.0×10^5) were injected through the left atrial catheter at the following times: before coronary occlusion, at 30 min of occlusion and after 5 min of reperfusion. Just before and for 1 min after microsphere infusion, blood was withdrawn from the femoral artery catheter by constant withdrawal pump (Harvard Apparatus) at a rate of 2.5 ml/min as described previously (29). Rabbits were killed, and hearts were excised and perfused retrograde with TTC followed by a suspension of zinc-cadmium particles. Myocardial left ventricular samples from the nonfluorescent TTC-negative risk region and from the fluorescent TTC-positive nonrisk region were removed, separated into endocardial and epicardial halves and weighed. Reference blood samples and myocardial tissues were counted in a multichannel gamma counter (Minaxi-gamma, Autogamma 5000 Series, Packard Instrument), with background correction for overlapping radioactivity between radioisotopes (Compusphere Software, Packard Instrument). Myocardial blood flow was expressed in ml/min per g of myocardial tissue.

Statistical methods. Infarct size is reported as mean value ± SEM. Comparison of the methods of infarct size measurement were performed using repeated measures analysis of variance for Group I and nonpaired *t* tests for comparisons between groups.

Results

Group I: 30 min of occlusion, 180 min of reperfusion—horseradish peroxidase infused at onset of reperfusion (Table 1). Fourteen rabbits underwent this portion of the study.

Table 1. Group I: Infarct Size Delineated by Horseradish Peroxidase at the Onset of Reperfusion After 30 Minutes of Occlusion and by Triphenyltetrazolium Chloride After 180 Minutes of Reperfusion in the Same Animal

Rabbit No.	Horseradish Peroxidase			Triphenyltetrazolium Chloride		
	I/LV	I/AR	AR/LV	I/LV	I/AR	AR/LV
1	17.8	36.7	44.9	20.1	47.7	42.1
2	32.6	64.6	50.5	38.4	72.0	53.4
3	15.6	47.2	33.0	17.7	57.0	31.0
4	30.1	52.9	56.8	39.8	71.0	56.0
5	21.2	47.2	44.8	25.2	64.5	39.0
6	13.7	32.4	42.1	19.1	49.5	38.5
7	17.7	40.5	43.7	23.1	52.4	44.1
8	16.2	41.5	38.9	27.5	70.9	38.8
9	19.9	44.6	43.5	26.9	65.6	41.0
10	19.4	42.5	45.6	20.9	47.4	44.0
Mean	20.4	45.3*	44.4	25.9	59.8*	42.8
±SEM	2.0	2.8	2.0	2.4	3.3	2.3

*$p = 0.0002$, horseradish peroxidase versus triphenyltetrazolium chloride. All data are expressed as percent. AR = area at risk; I = infarct; LV = left ventricle.

JACC Vol. 21, No. 5
April 1993:1245–53

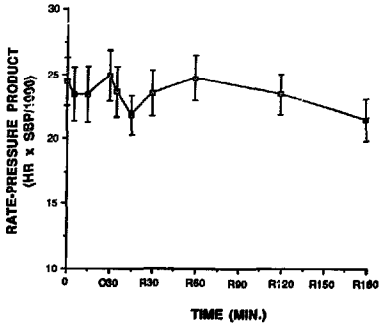
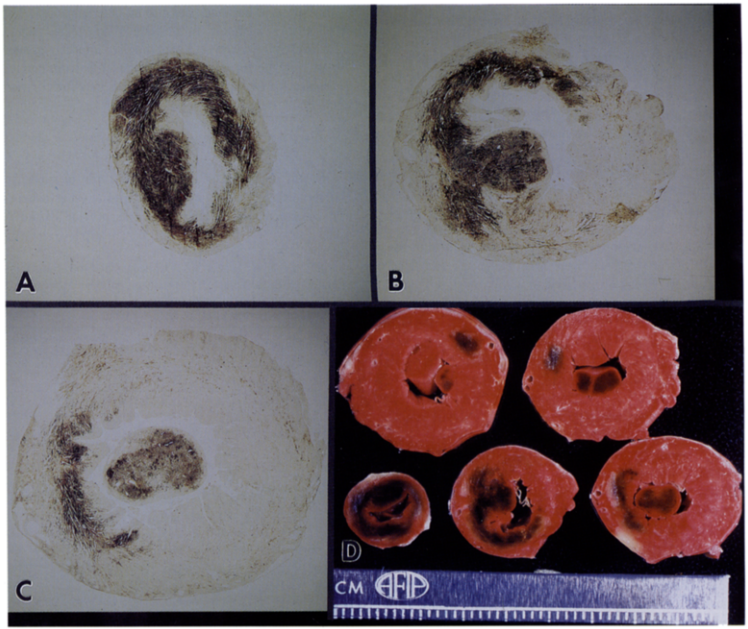


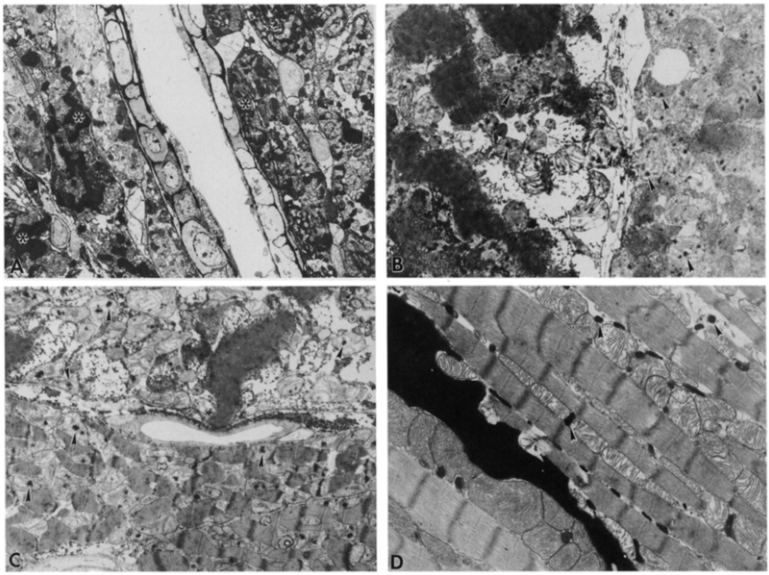
Figure 2 (above). Rate-pressure product during experimental protocol in Group I rabbits. HR = heart rate; O = occlusion; R = reperfusion; SBP = systolic blood pressure.

Four animals were excluded for the following reasons: wide complex tachycardia before coronary occlusion (n = 1), failure to identify the left obtuse marginal artery (n = 1) and very small infarcts without apical involvement (n = 2). In the 10 rabbits used for infarct analysis, hemodynamic variables (heart rate, blood pressure and rate-pressure product) remained stable throughout the periods of ischemia and reperfusion. Rate-pressure product data are presented in Figure 2.

By light microscopy, frozen sections processed for peroxidase staining demonstrated dark brown intramyocyte reaction product in cells within the ischemic bed (Fig. 3). The distribution of the horseradish peroxidase–positive cells was greatest in the central midmyocardium with relative

Figure 3 (below). A to C, Representative left ventricular frozen section slices from a Group I heart demonstrating brown horseradish peroxidase reaction product, identifying myocardium infarcted at the onset of reperfusion after 30 min of occlusion. D, Triphenyltetrazolium chloride–stained left ventricular slices from the same heart depicted in A to C showing tan hemorrhagic infarcted myocardium after 180 min of reperfusion. CM = centimeters.





sparing (horseradish peroxidase–negativity) in the subendocardium and subepicardium. Infarct size at the beginning of reperfusion, delineated by horseradish peroxidase, measured 45.3 ± 2.8% of the area of risk and was significantly smaller than infarct size after 180 min of reperfusion outlined by TTC (59.8 ± 3.3%, p = 0.0002).

By electron microscopy, myocytes containing intracellular horseradish peroxidase, consisting of electron-dense dark gray staining of contracted sarcomeres, exhibited mitochondrial flocculent densities and sarcolemmal breaks indicative of irreversible injury (Fig. 4A). Focally, in border areas within the ischemic bed, there was an admixture of irreversibly injured horseradish peroxidase–positive myocytes adjacent to horseradish peroxidase–negative myocytes that demonstrated irreversible injury (Fig. 4, B and C) on the basis of amorphous mitochondrial matrix densities. There were far greater numbers of irreversibly injured horseradish peroxidase–positive myocytes than irreversibly injured horseradish peroxidase–negative cells. Reversibly injured myocytes were horseradish peroxidase–negative (Fig. 4D) and showed mild edema, glycogen depletion, mitochondrial swelling, disruption of mitochondrial cristae and loss of normal granules. In these areas, peroxidase reaction product was present in the interstitium and myocyte T tubules.

Figure 4. Electron micrographs from central ischemic zone within the risk region in a Group I heart. **A** and **B** are from the endocardial half of the heart and **C** and **D** are from the epicardial half. **A,** Irreversibly injured myocytes containing dark gray intracellular horseradish peroxidase (HRP) staining of hypercontracted sarcomeres (*), indicative of cell death at the onset of reperfusion within the endocardial region. **B,** In border areas, an irreversibly injured horseradish peroxidase–positive myocyte **(left)** is adjacent to an irreversibly injured horseradish peroxidase–negative myocyte, indicative of infarct extension during reperfusion. Note mitochondrial flocculent densities in both cells **(arrowheads). C,** Photomicrograph of the border area in the epicardial half of the heart, demonstrating a horseradish peroxidase–positive irreversibly injured myocyte **(upper cell)** and a horseradish peroxidase–negative irreversibly injured adjoining myocyte **(lower cell).** Both cells contain intramitochondrial flocculent densities **(arrowheads). D,** Horseradish peroxidase–negative epicardial myocytes showing mild changes of reversible injury within the ischemic bed. Note dark horseradish peroxidase reaction product within the interstitium and in T tubules **(arrowheads).** (**A,** ×2700; **B,** ×11,000; **C,** ×8,900; **D,** ×14,300.)

Group II: 30 min of occlusion followed by horseradish peroxidase infusion and induced death (Table 2). Excluded cases consisted of two rabbits that died during anesthesia induction and one in which reperfusion was not achieved. Horseradish peroxidase–delineated infarcts after 30 min of

JACC Vol. 21, No. 5
April 1993:1245–53

Table 2. Group II: Horseradish Peroxidase–Delineated Infarct Size After 30 Minutes of Occlusion

Rabbit No.	I/LV	I/AR	AR/LV
1	29.5	57.9	50.9
2	28.0	52.4	53.4
3	11.0	23.9	46.6
4	8.7	17.9	48.6
5	27.6	57.1	48.3
Mean	21.0	41.8	49.6
SEM	4.6	8.7	1.2

Abbreviations as in Table 1. All data are expressed as percent.

occlusion measured 41.8 ± 8.7% of the area of risk, which was similar to the peroxidase-measured infarcts in Group I. Triphenyltetrazolium chloride staining failed to clearly differentiate infarcted from noninfarcted tissue in all hearts; therefore, infarct size determination by this method in this group could not be performed. Electron microscopy confirmed that intramyocyte peroxidase staining identified only irreversibly injured cells; irreversibly injured horseradish peroxidase–negative cells were not seen. Reversibly injured horseradish peroxidase–negative cells in the ischemic bed demonstrated glycogen depletion, focal myocyte and mitochondrial swelling and loss of normal granules in the absence of mitochondrial flocculent densities.

Group III: 30 min of occlusion, 180 min of reperfusion—horseradish peroxidase infused after 180 min of reperfusion (Table 3). Of six rabbits, one was excluded because reperfusion did not occur after release of the snare. Horseradish peroxidase–delineated infarct size after 180 min of reperfusion (n = 5) measured 58.5 ± 6.4% of the risk region, which was similar to values for TTC-delineated infarcts in the same animals (60.7 ± 3.6%) and in Group I (59.8 ± 3.3%). Infarct size defined by horseradish peroxidase in Group I was significantly smaller than both horseradish peroxidase– and TTC-delineated infarcts in Group III ($p < 0.05$ and < 0.01, respectively).

Infarct sizes in the three experimental groups measured by horseradish peroxidase and TTC are summarized in

Table 3. Group III: Horseradish Peroxidase and Triphenyltetrazolium Chloride–Delineated Infarct Size After 180 Minutes of Reperfusion and 30 Minutes of Occlusion

Rabbit No.	Horseradish Peroxidase			Triphenyltetrazolium Chloride		
	I/LV	I/AR	AR/LV	I/LV	I/AR	AR/LV
1	16.8	40.1	41.8	19.3	52.5	36.8
2	23.9	50.4	47.4	24.3	54.8	44.4
3	38.6	71.8	53.7	32.8	61.7	53.1
4	30.1	56.3	53.4	33.5	61.4	54.6
5	41.4	73.8	56.1	38.0	73.2	52.0
Mean	30.2	58.5	50.5	29.6	60.7	48.2
±SEM	4.6	6.4	2.6	3.4	3.6	3.3

Abbreviations as in Table 1. All data are expressed as percent.

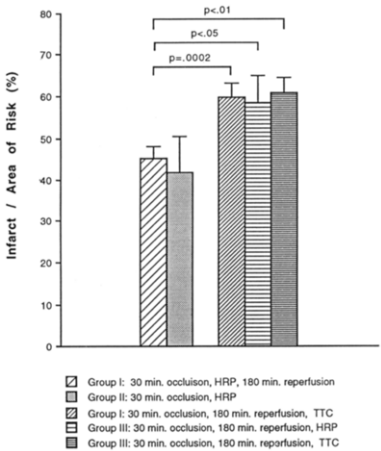


Figure 5. Progressive increase in infarct size after 30 min of occlusion versus 30 min of occlusion and 180 min of reperfusion. In Group I, horseradish peroxidase (HRP) defined infarcts at the beginning of reperfusion just after 30 min of occlusion, and triphenyltetrazolium chloride (TTC) staining delineated infarcts in the same animal after 180 min of reperfusion. Group II rabbits were subjected to 30 min of occlusion, infusion of horseradish peroxidase at the onset of reperfusion, followed by induced death. In Group III, horseradish peroxidase and TTC defined infarcts at 180 min of reperfusion after 30 min of occlusion. Infarct size measured by horseradish peroxidase at 30 min of occlusion was similar in Groups I and II. Infarct size at 180 min of reperfusion measured by TTC staining in Group I and by horseradish peroxidase and TTC staining in Group III was similar.

Figure 5. Risk regions as a percent of the left ventricle were similar among Groups I, II and III.

Myocardial blood flow. Six rabbits underwent this portion of the study. Myocardial blood flow measurements (Fig. 6) confirmed essentially no collateral blood flow in the area of risk and restoration of perfusion on release of the coronary artery snare.

Discussion

Present study. This study supports the hypothesis that a subset of myocytes reversibly injured after a defined period of ischemia may proceed to irreversible injury during the reperfusion period. In Group I, horseradish peroxidase infused at the beginning of reperfusion after 30 min of occlusion delineated irreversibly injured myocytes during the ischemic period. However, infarct size determined by

TTC staining 180 min after reperfusion was 15% larger. Electron microscopy confirmed that myocytes within the ischemic bed containing intracellular horseradish peroxidase and mitochondrial flocculent densities were irreversibly injured at the time of reperfusion. In border areas adjoining the irreversibly injured horseradish peroxidase–positive myocytes, horseradish peroxidase–negative myocytes also demonstrated lethal injury. These results suggest that horseradish peroxidase–negative myocytes with flocculent densities were injured during reperfusion, resulting in infarct extension after the restoration of blood flow.

To address whether the horseradish peroxidase accurately delineated only irreversibly injured myocytes, this agent was infused after 30 min of coronary occlusion (Group II). Electron microscopy demonstrated irreversible injury only in horseradish peroxidase–positive myocytes. In these animals, peroxidase-measured infarcts were similar in size to the horseradish peroxidase-delineated infarcts in Group I, in which horseradish peroxidase was infused after 30 min of occlusion with induced death at 180 min of reperfusion (Group I). When horseradish peroxidase was administered after 180 min of reperfusion (Group III), infarct size was similar to that of infarcts measured by TTC. Infarct size measured by TTC was similar in Group I and Group III hearts. These data further support the concept that infarct extension occurs within the ischemic risk region during the reperfusion period in the rabbit model (Fig. 5 and 7).

The difficulty in directly identifying infarct extension during reperfusion in an individual heart is secondary to limitations in identifying and especially quantitating irreversibly injured myocytes at the onset of reperfusion. Depending on the animal model, light microscopy and TTC staining require several hours before features of myocardial necrosis are readily observed. Electron microscopy, a sensitive technique for determining irreversible myocyte injury in early infarcts, is impractical and prohibitively costly for infarct size measurement. In an attempt to quantitate infarcted myocardium at the start of reperfusion, we utilized horseradish peroxidase in a new method of identifying irreversibly injured myocytes in early infarcts (25). After 30 min of coronary artery occlusion, horseradish peroxidase delineated a readily measurable region of infarcted myocardium that was confirmed by electron microscopy, with adjoining horseradish peroxidase–negative myocytes showing only mild reversible injury. In this study in 24-h old reperfused infarcts, horseradish peroxidase–measured infarct size correlated well with traditional staining methods (hematoxylin-eosin and TTC stains) (25).

Studies critical of reperfusion-induced infarct extension. Previous reports have attempted to address whether reperfusion itself can cause lethal injury to postischemic reperfused myocytes. Using two small- to medium-sized coronary arteries supplying separate vascular beds within the same dog, Hofmann et al. (30) found no extension of necrosis after reperfusion; however, myocardial blood flow was not determined to exclude the potential effects of collateral flow on

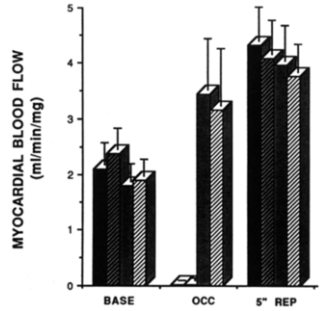
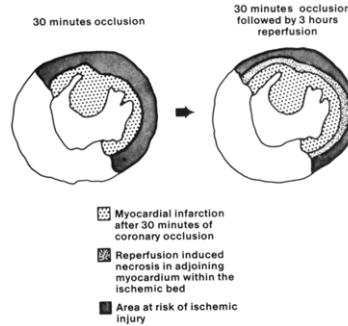


Figure 6. Myocardial blood flows in six rabbits subjected to 30 min of coronary artery occlusion and reperfusion (REP). Bars represent, from left to right, the ischemic region of the epicardium, ischemic region of the endocardium, nonischemic region of the epicardium and nonischemic region of the endocardium. BASE = baseline (before occlusion); OCC = end occlusion.

infarct size (31). Ganz et al. (31) demonstrated no increase in infarct transmurality after 5 min of reperfusion in the dog. As noted by Miura (32), the experimental model may have been insensitive to detect infarct extension. Further, 13 of 20 animals underwent 150 to 240 min of coronary occlusion, which may have resulted in infarcts nearing completion at the onset of reperfusion. It is also possible that 5 min of reperfusion was insufficient for the development of additional postreperfusion necrosis in this model. Reperfusion certainly accelerates the ultrastructural pathologic changes

Figure 7. Schematic drawing of infarct extension within the ischemic bed during reperfusion.



JACC Vol. 21, No. 5
April 1993:1245–53

of contraction band necrosis, cellular edema and granular mitochondrial densities in irreversibly injured cells (33–35). However, the transformation of reversibly injured myocytes into irreversibly injured cells may require prolonged periods of reperfusion, just as 20 to 40 min of regional ischemia in the dog (33) and 10 to 15 min in the rabbit (36) are necessary to detect irreversible injury. Ganz et al. (31) were limited to a brief period of reperfusion in their model because longer reperfusion would have subjected "control" areas to sustained ischemia.

Studies supportive of reperfusion-induced infarct extension. Frame et al. (37) administered a specific, radiolabeled anticardiac myosin antibody to dogs after 60 min of coronary artery occlusion and 45 min of reperfusion. In that study, antibody binding increased during reperfusion, indicative of progressive myocyte membrane disruption during reperfusion. In a study by Ambrosio et al. (38), there was a decrease in myocardial blood flow at 210 min of reperfusion after 90 min of coronary occlusion in the canine model despite a hyperemic response at 2 min of reperfusion. During ischemia, myocardial blood flow in thioflavine-negative areas was significantly less than in adjoining thioflavine-positive zones in animals killed after 2 min of reperfusion. The thioflavine-negative foci in dogs with reperfusion for 210 min demonstrated contraction band necrosis and severe endothelial injury in contrast to the coagulation necrosis found in thioflavine-negative areas in dogs reperfused for 2 min before induced death. These results suggest, in separate animals, that areas with higher collateral flow at the time of coronary occlusion undergo reperfusion-induced irreversible injury. The present study describes a method that allows for an extended period of reperfusion without further ischemia in the same heart and provides evidence for reperfusion injury.

In most other studies, the concept of myocardial infarct extension during reperfusion could only be inferred when an intervention administered at the time of reperfusion resulted in a reduction in infarct size. Numerous interventions have been utilized in experimental preparations of myocardial reperfusion in an attempt to maximize myocardial salvage. There have been conflicting data with regard to the benefit of oxygen-derived free radical scavengers; some studies (8,9) have shown infarct size reduction, whereas others (39) have not demonstrated myocardial salvage. There have been differences in the animal models used and times of death, and attention has focused on the limitations of gross histochemical techniques (TTC and nitroblue tetrazolium) to accurately identify infarcted myocardium with resultant underestimation of infarct size (40,41). It has been proposed that superoxide dismutase may reduce the washout of myocyte dehydrogenases, resulting in positive TTC staining of necrotic myocytes (41). Whether other interventions that have been reported to reduce infarct size after reperfusion (for example, adenosine, perfluorochemicals and preconditioning) affect the ability of TTC and nitroblue tetrazolium to define infarcted myocardium is unknown. Further studies utilizing standard light microscopic and horseradish peroxidase methods of infarct size measurement within the same heart in preparations of prolonged reperfusion after coronary occlusion may clarify the benefits of various interventions after myocardial reperfusion.

Study limitations. The present study was performed in a small animal model that has poor collateral circulation. It is possible that infarct extension may not occur in a well collateralized model in which blood flow exceeds a critical threshold during ischemia to preserve cellular viability and allow for hibernation with eventual recovery in contrast to irreversible injury during reperfusion. There was some variation in the risk regions among the experimental groups that did not reach statistical significance. Given the small size of the rabbit heart and variations in coronary artery branching patterns among animals, minor differences in the location of the coronary snare may result in relatively greater variability in the size of risk areas. This is potentially important because infarct size has been shown to vary directly with the amount of myocardium at risk (in the dog model) and may be due to the extent of collateral blood flow (42). Finally, the use of frozen sections and the expense of horseradish peroxidase would impose limitations in larger animal models.

Conclusions. It has been well established that critically timed reperfusion will salvage myocardium compared with permanent coronary artery occlusion. Although the overall benefits of myocardial reperfusion are certain, the present study supports the hypothesis that there are viable myocytes within the reperfused ischemic vascular bed that undergo lethal injury during the reperfusion period. These results provide pathologic evidence of infarct extension during reperfusion within a single heart and, therefore, provide a basis for continued exploration of interventions designed to salvage myocardium and limit reperfusion injury within the ischemic risk region after the restoration of coronary flow.

We appreciate the technical assistance of Rufus Seabron and Russell M. Jones.

References

1. Kloner RA, Ellis SG, Lange R, Braunwald E. Studies of experimental coronary artery reperfusion: effects on infarct size, myocardial function, biochemistry, ultrastructure, and microvascular damage. Circulation 1985;58(suppl I):I-1–8.
2. Ritchie JL, Cerqueira M, Maynard C, Davis K, Kennedy JW. Ventricular function and infarct size: the Western Washington intravenous streptokinase in myocardial infarction trial. J Am Coll Cardiol 1988;11:689–97.
3. ISIS-2 (Second International Study of Infarct Survival) Collaborative Group. Randomized trial of intravenous streptokinase, oral aspirin, both, or neither among 17,187 cases of suspected acute myocardial infarction. Lancet 1988;2:349–60.
4. Braunwald E, Kloner RA. Myocardial reperfusion: a double edged sword. J Clin Invest 1985;76:1713–9.
5. Bajaj AK, Cobb MA, Virmani R, Gay JC, Light RT, Forman MB. Limitation of myocardial reperfusion injury by intravenous perfluorochemicals: role of neutrophil accumulation. Circulation 1989;79:645–56.
6. Rice HE, Virmani R, Hart CL, Kolodgie FD, Farb A. Dose-dependent

reduction of myocardial infarct size with the perfluorochemical Fluosol-DA. Am Heart J 1990;120:1039–46.
7. Kolodgie FD, Virmani R, Farb A. Limitation of no reflow injury by blood-free reperfusion with oxygenated perfluorochemical (Fluosol-DA 20%). J Am Coll Cardiol 1991;18:215–23.
8. Ambrosio G, Becker LC, Hutchins GM, Weisman HF, Weisfeldt ML. Reduction in experimental infarct size by recombinant human superoxide dismutase: insights into the pathophysiology of reperfusion injury. Circulation 1986;74:1424–33.
9. Chi L, Tamura Y, Hoff PT, et al. Effect of superoxide dismutase on myocardial infarct size in the canine heart after 6 hours of regional ischemia and reperfusion: a demonstration of myocardial salvage. Circ Res 1989;64:665–75.
10. Olafsson B, Forman MB, Puett DW, et al. Reduction of reperfusion injury in the canine preparation by intracoronary adenosine: importance of the endothelium and the no-reflow phenomenon. Circulation 1987;76:1135–45.
11. Babbitt DG, Virmani R, Forman MB. Intracoronary adenosine administered after reperfusion limits vascular injury after prolonged ischemia in the canine model. Circulation 1989;80:1388–99.
12. Schott RJ, Rohmann S, Braun ER, Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. Circ Res 1990;66:1133–42.
13. Li GC, Vasquez JA, Gallagher KP, Lucchesi BR. Myocardial protection with preconditioning. Circulation 1990;82:609–19.
14. Simpson PJ, Todd RF, Mickelson JK, et al. Sustained limitation of myocardial reperfusion injury by a monoclonal antibody that alters leukocyte function. Circulation 1990;81:226–37.
15. Whalen DA, Hamilton DG, Ganote CE, Jennings RB. Effect of a transient period of ischemia on myocardial cells. I. Effects of cell volume regulation. Am J Pathol 1974;74:381–98.
16. Kloner RA, Ganote CE, Whalen DA, Jennings RB. Effect of a transient period of ischemia on myocardial cells. II. Fine structure during the first few minutes of reflow. Am J Pathol 1974;74:399–422.
17. Jennings RB, Ganote CE, Reimer KA. Ischemic tissue injury. Am J Pathol 1975;81:179–98.
18. Ashraf M, Halverson CA. Structural changes in the freeze-fractured sarcolemma of ischemic myocardium. Am J Pathol 1977;88:583–94.
19. Jennings RB, Reimer KA. Lethal myocardial ischemic injury. Am J Pathol 1981;102:241–55.
20. Farber JL, Chien KR, Mittnacht S. The pathogenesis of irreversible cell injury in ischemia. Am J Pathol 1981;102:271–81.
21. Karnovsky MJ. The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. J Cell Biol 1967;35:213–36.
22. Boutet M, Hüttner I, Rona G. Permeability alteration of sarcolemmal membrane in catecholamine-induced cardiac muscle injury: in vivo studies with fine structural diffusion tracer horseradish peroxidase. Lab Invest 1976;34:482–8.
23. Yunge L, Bruneval P, Sami Cokay M, et al. Perturbation of the sarcolemmal membrane in isoproterenol-induced myocardial injury of the rat: permeability and freeze-fracture studies in vivo and in vitro. Am J Pathol 1989;134:171–85.
24. Rona G, Dadonnel MC, Hüttner I, Bier C, Boutet M. Reperfusion effect upon ischemic myocardial injury. Exp Mol Pathol 1979;31:211–8.
25. Farb A, Kolodgie FD, Virmani R. Early detection and measurement of experimental myocardial infarcts with horseradish peroxidase. J Mol Cell Cardiol (in press).
26. Downey JM, Miura T, Eddy LJ, Chambers DE, Mellert T, Hearse DJ. Xanthine oxidase is not a source of free radicals in the ischemic rabbit heart. J Mol Cell Cardiol 1978;19:1053–60.
27. Karnovsky MJ. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy (abstr). Proceedings of the 11th Annual Meeting of the American Society for Cell Biology. J Cell Biol 1971;51:146a.
28. Schaper W. Experimental infarcts and the microcirculation. In: Hearse DJ, Yellon DM, eds. Therapeutic Approaches to Myocardial Infarct Size Limitation. New York: Raven, 1984:79–90.
29. Norton ED, Jackson EK, Virmani R, Forman MB. Effect of intravenous adenosine on myocardial reperfusion injury in a model with low myocardial collateral blood flow. Am Heart J 1991;122:1283–91.
30. Hofmann M, Hofmann M, Genth K, Schaper W. The influence of reperfusion on infarct size after experimental coronary artery occlusion. Basic Res Cardiol 1980;72:572–82.
31. Ganz W, Watanabe J, Kanamasa K, Yano J, Han DS, Fishbein MC. Does reperfusion extend necrosis? A study in a single territory of myocardial ischemia—half reperfused and half not reperfused. Circulation 1990;82:1020–33.
32. Miura T. Does reperfusion induce myocardial necrosis? Circulation 1990;82:1070–2.
33. Kloner RA, Ganote CE, Whalen DA, Jennings RB. Effect of a transient period of ischemia on myocardial cells. II. Fine structure during the first few minutes of reflow. Am J Pathol 1974;74:399–422.
34. Sommers HM, Jennings RB. Experimental acute myocardial infarction: histologic and histochemical studies of early myocardial infarcts induced by temporary or permanent occlusion of a coronary artery. Lab Invest 1964;13:1491–503.
35. Jennings RB, Ganote CE, Reimer KA. Ischemic tissue injury. Am J Pathol 1975;81:179–98.
36. Connelly C, Vogel WM, Hernandez YM, Apstein CS. Movement of necrotic wavefront after coronary artery occlusion in rabbit. Am J Physiol 1982;243:H682–90.
37. Frame LH, Lopez JA, Khaw BA, Fallon JT, Haber E, Powell WJ. Early membrane damage during coronary reperfusion in dogs: detection by radiolabelled anticardiac myosin (Fab')2. J Clin Invest 1983;72:535–44.
38. Ambrosio G, Weisman HF, Mannisi JA, Becker LC. Progressive impairment of regional myocardial perfusion after initial restoration of postischemic blood flow. Circulation 1989;80:1846–61.
39. Richard VJ, Murray CE, Jennings RB, Reimer KA. Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infarcts caused by 90 minutes of ischemia in dogs. Circulation 1988;78:473–80.
40. Horneffer PJ, Healy B, Gott VL, Gardner TJ. The rapid evolution of a myocardial infarction in an end-artery coronary preparation. Circulation 1987;76(suppl V):V-39–42.
41. Shirato C, Miura T, Ooiwa H, Toyofuku T, Wilborn WH, Downey JM. Tetrazolium artifactually indicates superoxide-induced salvage in reperfused rabbit heart. J Mol Cell Cardiol 1989;21:1187–93.
42. Lowe JE, Reimer KA, Jennings RB. Experimental infarct size as a function of the amount of myocardium at risk. Am J Pathol 1978;90:363–80.