Ischemic Preconditioning Reduces Infarct Size in Swine Myocardium

Robert J. Schott, Sven Rohmann, Ellen R. Braun, and Wolfgang Schaper

We evaluated the hypothesis that stunning swine myocardium with brief ischemia reduces oxygen demand in the stunned region and increases tolerance of myocardium to longer periods of ischemia. Wall function was quantified with ultrasonic crystals aligned to measure wall thickening, and stunning was achieved with two cycles of left anterior descending coronary artery (LAD) occlusion (10 minutes) and reperfusion (30 minutes), after which the LAD was occluded for 60 minutes and reperfused for 90 minutes. Infarct size (as a percent of risk region) was then determined by incubating myocardium with para-nitro blue tetrazolium. Regional oxygen demand was measured as myocardial oxygen consumption before the 60-minute LAD occlusion in the stunned region; tracer microspheres were used to determine blood flow, and blood from the anterior interventricular vein and left atrium was used to calculate oxygen saturations. After the second reperfusion period, wall thickening in the stunned region was reduced to 1.4±2.4% compared with 36.7±2.5% (mean±SEM) before ischemia (p<0.001). Regional myocardial oxygen consumption after stunning (3.1±0.7 ml O₂/min/100 g) was no different from regional myocardial oxygen consumption before stunning (3.7±0.6 ml O₂/min/100 g). In the nine pigs “preconditioned” by stunning, infarct size was 10.4±6.3% of the risk region compared with 48.0±12.7% in the six control pigs subjected to 60 minutes of ischemia without prior stunning (p<0.005). The risk regions were similar (14.4±1.5% vs. 14.6±1.9% of the left ventricle, preconditioned vs. control pigs, respectively). We conclude that stunning swine myocardium with two cycles of a 10-minute LAD occlusion followed by reperfusion increases ischemic tolerance but that changes in regional demand in stunned myocardium do not predict the marked reduction in infarct size that follows a subsequent 60-minute period of ischemia. (Circulation Research 1990;66:1133–1142)

Myocardium subjected to continuous, severe ischemia does not suffer irreversible injury if reperfused within 15–20 minutes.1,2 Repeated periods of ischemia of less than 15 minutes do not result in a cumulative injury if myocardium is reperfused between episodes of ischemia.1,3 Paradoxically, repeated ischemia-reperfusion cycles render the myocardium more resistant to infarction during subsequent longer episodes of ischemia, a phenomenon that has been termed “ischemic preconditioning.” The mechanisms for ischemic preconditioning are not completely understood, although a number of important observations have been made. An initial period of brief ischemia followed by reperfusion has been shown to retard the consumption of high-energy phosphates during subsequent ischemic episodes.4–8 Murry et al3–5 have also observed a reduction in lactate accumulation in ischemic myocardium preconditioned with prior episodes of ischemia; this observation has led them to hypothesize that the increased ischemic tolerance accrues from preservation of energy resources during ischemia and/or from a reduction in the accumulation of toxic catabolites. This same group has implicated oxygen free radicals in the mechanism of preconditioning.9 In a preliminary report, the protective effects of ischemic preconditioning were blocked by the administration of superoxide dismutase and catalase. This suggests that free radical generation during the initial ischemia-reperfusion cycles conditions the canine myocardium to the subsequent damage inflicted by 60 minutes of ischemia.9

Brief episodes of ischemia and reperfusion also produce transient contractile dysfunction,10 termed “stunned myocardium,”11 which in some models correlates with decreased myocardial oxygen consumption (MVO₂) in the ischemic region during reperfusion.12,13 In swine, the effects of stunning on MVO₂ are controversial. Some investigators14 have observed reduced MVO₂; others15 have reported no changes in

From the Max Planck Institute, Department of Experimental Cardiology, Bad Nauheim, FRG.
Address for correspondence: Robert J. Schott, MD, Max Planck Institute, Benekestrasse 2, D-6350 Bad Nauheim, FRG.
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M\(\text{VO}_2\) of stunned swine myocardium. We assessed M\(\text{VO}_2\) in stunned myocardium as a measure of regional demand; we hypothesized that the depressed contractile dysfunction that characterizes stunned myocardium reduces oxygen demand and that this contributes to the reduction in infarct size observed with ischemic preconditioning. Implicit in this hypothesis is the assumption that changes in regional demand before occlusion affect the evolution of necrosis during a subsequent ischemic episode, when metabolism becomes anaerobic and contractile effort is rapidly supplanted by systolic bulging.\(^{10}\) However, it is known that variations in global left ventricular demand at the onset of occlusion affect the extent of necrosis during a subsequent ischemic episode.\(^{16}\)

We selected swine myocardium because of its similarity to human myocardium in two important respects: there are few native collaterals,\(^{17}\) and swine myocardium has no detectable xanthine oxidase, which is one likely source of oxygen free radicals.\(^{18,19}\) Therefore, we subjected stunned swine myocardium to 60 minutes of ischemia to determine whether tissue without xanthine oxidase activity would show an increase in ischemic tolerance and to determine if an increase in ischemic tolerance could be predicted by a decrease in demand associated with stunned myocardium.

**Materials and Methods**

**Experimental Preparation**

Male mixed-breed Landrace-type domestic pigs weighing 19–23 kg were sedated with 2 mg/kg i.m. azaperone (Stresnil, Janssen Pharmaceutica, Neuss, FRG) 30 minutes before anesthesia with 30 mg/kg i.v. pentobarbital. After tracheostomy, the pigs were mechanically ventilated with a respirator (Mark 7, Bird Products, Palm Springs, California) on room air supplemented with 2 l oxygen per minute. Frequent arterial blood gases were measured to guide adjustment of ventilator settings. Both internal jugular veins were cannulated with polyethylene catheters. Anesthesia was maintained with continuous infusion of pentobarbital at 3 mg/min through one jugular catheter; the other was used for fluid and drug administration. The right femoral artery was cannulated with a polyethylene catheter, which was advanced to approximately the midaorta for continuous recording of arterial pressure and withdrawal of reference blood samples. The heart was exposed through a midline thoracotomy and suspended in a pericardial cradle. The left anterior descending coronary artery (LAD) was gently dissected free of surrounding tissue after the second branch. A loose ligature was placed around the vessel, which was subsequently occluded with a small vascular clip. A 27-gauge butterfly needle was introduced into the epicardial veins for collection of the blood samples used for calculation of regional venous oxygen saturations. The anterior interventricular vein, adjacent to the occlusion site, was cannulated in a similar fashion to obtain oxygen saturations from the stunned region. A catheter was placed in the left atrium for injection of tracer microspheres and intermittent monitoring of left atrial pressure, which was used to guide fluid replacement. A stiff polyethylene catheter with multiple side holes was advanced over a wire into the right atrium and inserted retrograde between 1 and 2 cm into the terminal segment of the coronary sinus to allow collection of blood for the calculation of coronary sinus oxygen saturation. A micromanometer (Millar Instruments, Houston, Texas) was advanced via the carotid artery into the left ventricle for the continuous measurement of left ventricular pressure. Rectal temperature was monitored throughout the experiment, and the chest cavity was covered with a plastic sheet and kept warm with a heat lamp. Myocardial function in the ischemic area was measured with 5-MHz ultrasonic crystals aligned transmurally to measure wall thickness.\(^{20}\) A brief (<10-second occlusion) was performed to identify the ischemic region, and the crystal pair was located centrally in the ischemic region. The inner crystal was advanced to the endocardium tangentially through a stab wound in the epicardium. The epicardial crystal, attached to a Dacron patch, was then positioned on the epicardium with the aid of an oscilloscope and secured with two sutures.

**Experimental Design**

The experimental design is depicted in Figure 1. The pigs were divided into two groups: preconditioned and control. Both groups underwent a 30-minute control period. Preconditioned pigs were then subjected to two 10-minute occlusions followed by 30-minute reperfusions before the final 60-minute occlusion and 90-minute reperfusion. Control pigs were subjected to only the 60-minute occlusion followed by 90 minutes of reperfusion.
Myocardial Blood Flow Measurements

Blood flow measurements were made with 10 μm tracer microspheres (New England Nuclear, Boston, Massachusetts) by using the reference withdrawal method. Approximately 2 million spheres labeled with 113Sn, 109Ru, 46Sc, 141Ce, 57Co, and 95Nb were injected into the left atrium in random order. Reference blood was withdrawn at a constant rate (20 ml/min) from the aortic catheter. After risk region and infarct size assessments were made at the conclusion of the experiment, each ring of the left ventricle (approximately 1 cm in width) was placed in formalin. After allowing at least 72 hours for fixation, the rings were removed from formalin and cut into 4–16 sections depending on the size of the ring. Each section was further divided into approximately equal endocardial, midmyocardial, and epicardial samples, which were weighed (generally 50–300 mg) and placed into labeled tubes for gamma counting in a germanium solid-state well-type detector. The compound spectrum was processed by a PDP-11/24 computer for generation of blood flow maps for each ring. From the first microsphere injection during ischemia, three perfusion patterns were discerned: normally perfused tissue, ischemic tissue (which averaged <10 ml/min/100 g of flow during occlusion), and mixed tissue. Blood flow during reperfusion in the previously identified ischemic tissue was used for calculation of regional ischemic MVO2, whereas blood flow from all tissue was used for calculation of global MVO2.

Risk Region and Infarct Size

After 90 minutes of reperfusion, the LAD was reoccluded, and 10 ml of 10% fluorescein dye was injected into the left atrial catheter. After 1–2 minutes, the pig was killed with a bolus injection of KCl, and the heart was excised. Warm 2% agarose was injected into the left ventricle via the aortic root, and the heart was placed on ice. After the agarose gelled, the right ventricle was cut away, and the left ventricle was “breadloafed” into 4–6 rings approximately 1 cm wide perpendicular to the LAD. The rings were weighed, and the risk region was traced onto an acetate sheet under a black light, which sharply defined the borders of the risk area, which was not perfused with fluorescein. Subsequently, the rings were incubated for 30–45 minutes in 0.125 g para-nitro blue tetrazolium (p-NBT) per liter of phosphate buffer (pH 7.1) at 37°C; p-NBT stains noninfarcted tissue deep blue and leaves infarcted tissue pale. Rings were then photographed with a Polaroid MP-4 camera, and acetate tracings of the ring and infarcted region were planimetered with a graphics tablet. With these data, the percent risk region (area at risk divided by area of ring) was calculated for the top and bottom of each ring, and the average value for each ring was multiplied by the weight. The weights of the regions were summed and divided by the weight of the left ventricle to yield the percent of the left ventricle at risk and percent of the left ventricular risk infarcted.

Myocardial Oxygen Consumption

MVO2 was determined in three regions of preconditioned hearts at three time points: during the control period, at 25 minutes after the first reperfusion, and at 25 minutes after the second reperfusion. The blood flow for calculation of control MVO2 was obtained from normal region flow during the first 10-minute occlusion (Figure 1). Oxygen saturation for global MVO2 determination was obtained from the coronary sinus and left atrium. Global and regional MVO2 at the first and second reperfusion were determined from blood flow measurements made concomitantly with the oxygen saturation measurements. The regional MVO2 represents oxygen consumption in the stunned region. Oxygen saturations were obtained from the anterior interventricular vein at the level of the occlusion. In six preconditioned pigs, local nonischemic MVO2 was obtained by calculating the oxygen saturations in a small vein draining the anterolateral wall of the left ventricle and by using the blood flow data from the first ring, which in all cases was outside the risk region. This served as a second control MVO2. All MVO2 values were calculated in the following fashion:

\[
\text{O}_2 \text{ saturation} = \frac{\text{oxyhemoglobin} \times \text{oxyhemoglobin}}{\text{hemoglobin}}
\]

\[
\text{O}_2 \text{ content} = 1.34 \times \text{hemoglobin} \times \text{O}_2 \text{ saturation}
\]

\[
\text{O}_2 \text{ extraction} = \text{left atrial O}_2 \text{ content} - \text{venous O}_2 \text{ content}
\]

\[
\text{O}_2 \text{ consumption} = \text{O}_2 \text{ extraction} \times \text{blood flow}
\]

The hemoglobin was measured on a Sysmex microcell counter (model CC180, Digitana AG, Hamburg, FRG) and was measured at the time of the second reperfusion. Blood samples for oxyhemoglobin concentrations were kept on ice until measured on blood gas analyzer (model AVL 995, Bad Homburg, FRG) within 10 minutes from the time the sample was drawn.

Biochemical Analysis

At the conclusion of the final reperfusion period, biopsies from the risk and the control area were taken with a high-speed biopsy drill and frozen within 10 seconds in liquid nitrogen until analysis. One half of the specimens was randomly selected from both the preconditioned and control groups for measurement of ATP, creatine phosphate (CP), ADP, and AMP by ion-paired high-pressure liquid chromatography. Briefly, the column used was a μBondapak C-18 (250 mm × 4 mm, 10 μm thick)(Waters, Millipore, Milford, Massachusetts). Buffers were 100 mM ammonium phosphate with 6 mM tetrabutylammonium hydrogen sulfate as buffer A and 30% acetonitrile plus 70% buffer A as buffer B (as modified by K. Hashizume in this laboratory). Step 1 was 98% buffer A and 2% buffer B for 5.5 minutes isocratically. Step
2 was from 98% to 0% buffer A and from 2% to 100% buffer B for 15 minutes on a linear gradient. Step 3 was 100% buffer B for 4.5 minutes isocratically, and then the column was reequilibrated with the same buffer as in step 1 for about 35 minutes.

Data Analysis

Continuous recording of hemodynamic and wall thickness data was made on a Graphtec recorder (Watanabe Instruments, Japan). Recordings at time points of interest were made at a paper speed of 50 mm/sec, and the data were collected from the record by hand. Wall thickness variables were averaged over five cycles at each time point and included end-diastolic wall thickness (EDWT, defined at 20 msec before peak negative dP/dt) and end-systolic wall thickness (ESWT, defined at the onset of peak positive dP/dt). These data were used to calculate the extent of wall thickening with the following formula: \[
\frac{(\text{ESWT} - \text{EDWT})}{\text{EDWT}} \times 100.
\]
Other variables include peak systolic pressure, mean arterial pressure, and heart rate. The double product was calculated by multiplying peak systolic pressure by heart rate.

Data are reported as mean±SEM. For comparison of variables across time between preconditioned and control groups, a two-factor analysis of variance (ANOVA) for repeated measures was used. When the ANOVA was significant, pairwise comparisons were made with Student's t test with the significance level for the p values corrected with the Bonferroni method. For comparison across time in the preconditioned group, a one-factor ANOVA of repeated measures was employed, with an identical post hoc testing strategy as for the two-factor ANOVA. A two-sample rank-sum test (Mann-Whitney U test) was used to test the differences between preconditioned and control animals with regard to risk region and the percent of the risk region infarcted.

Results

Twenty-five pigs were randomly divided between a control group (n=12) and a preconditioned group (n=13). Three preconditioned pigs (23%) were excluded from the study because of intractable fibrillation. A fourth preconditioned pig fibrillated at the very end of the study as the fluorescein was injected, after biopsy. Because the risk region could not be determined, this pig was not included in the infarct analysis. (The heart was cut and incubated with p-NBT and noted to have minimal infarction.) Two preconditioned pigs were excluded from MVo2 analysis because of technical problems with measurements (one with blood flow and the other with oxyhemoglobin concentrations). Of the 10 preconditioned pigs included in the study, nine were analyzed for infarct size, and eight were analyzed for oxygen consumption. Two control pigs were excluded because of fibrillation (16.7%); one was excluded for poor wall function in the control state, and the other was excluded because the risk area was only 6% of the left ventricle (13% of the risk region was noted to be infarcted in this pig). Two control pigs were excluded because of failure to demonstrate reperfusion with the microspheres; the pattern of reperfusion was consistent with spasm or arterial thrombus in one animal, and in the second animal, the gamma counter failed to detect activity above background in any of the tissue specimens with reperfusion. (The experiment was otherwise completed, and 34% of the risk region was infarcted.) This left six pigs, which formed the control group.

Hemodynamic and Wall Function

Hemodynamic and wall thickness data are summarized in Table 1. The preconditioned pigs remained remarkably stable throughout each of the occlusions and reperfusions and did not vary significantly from the control pigs immediately before and during the 60-minute occlusion with regard to heart rate, mean arterial pressure, or double product. For preconditioned pigs, heart rate, mean arterial pressure, or double product did not vary from control values at any time point throughout the entire experiment. The changes in wall function through each of the brief occlusions and reperfusions for the preconditioned pigs are presented in Figure 2.

Myocardial Blood Flow

Myocardial blood flow values are summarized in Table 2. Myocardial blood flow for preconditioned and control pigs was not different during the 60-minute occlusion and subsequent reperfusion in either ischemic or nonischemic regions. There was no difference across time in endocardial, epicardial, or transmural blood flow by ANOVA in the nonischemic regions in both preconditioned and control pigs. For ischemic region myocardial blood flow comparisons within the preconditioned group, the reperfusion time points were grouped separately from the occlusion time points. Within the ischemic region, no differences were detected in endocardial, epicardial, or transmural blood flow during the three occlusions. The repeated-measures ANOVA was significant for the three reperfusion time points in the epicardial, endocardial, and transmural sections of ischemic tissue. Pairwise testing with the Bonferroni correction for differences during reperfusion in the ischemic region of preconditioned pigs revealed a significant reduction in endocardial (p<0.005), epicardial (p<0.05), and transmural blood flow (p<0.01) between the first and second reperfusion periods. Thus, as the myocardium became nearly akinetic in the second reperfusion period, blood flow decreased transmurally to this region.

Myocardial Oxygen Consumption

Global MVo2, local nonischemic MVo2, and regional (stunned) MVo2 is represented for preconditioned pigs before occlusion and at the end of the first and second reperfusion periods in Figure 3. Although a tendency for global and local nonischemic MVo2 to increase and for regional MVo2 to
Table 1. Hemodynamic and Wall Function Data

<table>
<thead>
<tr>
<th>Time period</th>
<th>HR (beats/min)</th>
<th>MAP (mm Hg)</th>
<th>DP (100)</th>
<th>EDWT (mm)</th>
<th>ESWT (mm)</th>
<th>ΔWT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preconditioned pigs (n=10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>96.0±3.1</td>
<td>61.7±2.7</td>
<td>72.6±3.8</td>
<td>5.2±0.2</td>
<td>7.0±0.3</td>
<td>36.7±2.5</td>
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<tr>
<td>TCO1</td>
<td>94.0±2.7</td>
<td>58.6±2.4</td>
<td>72.3±5.5</td>
<td>4.4±0.4</td>
<td>4.2±0.3*</td>
<td>-4.0±4.8*</td>
</tr>
<tr>
<td>REP1</td>
<td>96.5±2.4</td>
<td>57.1±3.6</td>
<td>68.7±4.8</td>
<td>5.1±0.2</td>
<td>5.9±0.3*</td>
<td>14.8±3.3*</td>
</tr>
<tr>
<td>TCO2</td>
<td>94.4±2.9</td>
<td>54.9±3.5</td>
<td>65.1±4.5</td>
<td>4.6±0.2</td>
<td>4.1±0.2*</td>
<td>-12.6±2.3*</td>
</tr>
<tr>
<td>REP2</td>
<td>100.5±4.9</td>
<td>54.9±4.6</td>
<td>67.5±6.6</td>
<td>5.2±0.2</td>
<td>5.3±0.2*</td>
<td>1.4±2.4*</td>
</tr>
<tr>
<td>TCO3</td>
<td>101.7±7.1</td>
<td>53.7±5.0</td>
<td>68.9±6.7</td>
<td>4.8±0.4</td>
<td>4.4±0.3*</td>
<td>-7.6±2.4*</td>
</tr>
<tr>
<td>REP3</td>
<td>118.9±6.3</td>
<td>49.9±6.8</td>
<td>78.0±10.9</td>
<td>6.8±0.5</td>
<td>5.4±0.8</td>
<td>-10.2±2.8*</td>
</tr>
<tr>
<td>Control pigs (n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>95.0±6.2</td>
<td>62.3±4.4</td>
<td>70.1±8.0</td>
<td>5.7±0.7</td>
<td>7.3±0.8</td>
<td>28.0±6.2</td>
</tr>
<tr>
<td>TCO</td>
<td>99.2±6.2</td>
<td>57.5±4.7</td>
<td>61.1±10.3</td>
<td>4.7±0.8</td>
<td>4.2±0.8</td>
<td>-13.0±3.5</td>
</tr>
<tr>
<td>REP</td>
<td>107.5±6.6</td>
<td>57.0±5.7</td>
<td>66.2±11.8</td>
<td>7.8±0.8</td>
<td>7.4±0.9</td>
<td>-6.0±4.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. HR, heart rate; MAP, mean arterial pressure; DP, double product (peak systolic pressure times HR); EDWT and ESWT, end-diastolic and end-systolic wall thickness, respectively, measured with ultrasonic crystals; ΔWT, change in wall thickness calculated by [(ESWT−EDWT)/EDWT]×100. Preconditioned and control pigs underwent a 30-minute control period (Control). The preconditioned group was then subjected to a 10-minute coronary occlusion (TCO1) followed by a 30-minute reperfusion (REP1) and a second 10-minute occlusion (TCO2) followed by a second 30-minute reperfusion (REP2); the group was then subjected to a final 60-minute occlusion (TCO3) followed by a 90-minute reperfusion (REP3). The control group was subjected only to a 60-minute occlusion (TCO) followed by a 90-minute reperfusion (REP). Values were obtained at 5 minutes into TCG1 and TCO2, at 25 minutes into REP1 and REP2, at 30 minutes into TCO3 and TCO, and at 15 minutes into REP3 and REP.

*Significant decrease at p<0.001 compared with all time points after the control period.
$Significant decrease at p<0.005 compared with all time points after the control period.
\$Significant decrease at p<0.0001 compared with control.

decrease is evident, the values at the first and second reperfusion periods were not significantly different from the control period for the stunned region. This finding is consistent with previously reported swine data.\textsuperscript{15} To evaluate the dissociation evident in M\textsubscript{Vo2}, we calculated the regional/global M\textsubscript{Vo2} ratio. This ratio was 1.0±0.1 before occlusion and decreased to 0.7±0.1 at the end of both the first and second reperfusion periods (p<0.005 by ANOVA of repeated measures). The relation between regional wall function and regional M\textsubscript{Vo2} was evaluated by plotting the M\textsubscript{Vo2} measured at each time point (the control period and first and second reperusions) against the simultaneously obtained regional wall thickening, which is presented in Figure 4. The relation is described by linear regression with a slope of 5.0 (95% CI 0.7–9.4) and a y intercept of 2.1 (r=0.47, p<0.05).

A linear regression of M\textsubscript{Vo2} against regional wall thickening during only the first and second reperusions when the myocardium is stunned gives a similar slope (slope=5.2, r=0.62).

Figure 2. Graph showing ischemic region wall thickening in preconditioned hearts measured with ultrasonic crystals. Wall thickening is calculated as follows: % ΔWT = [(ESWT−EDWT)/EDWT]×100, where ESWT and EDWT are end-systolic and end-diastolic wall thickness, respectively (see “Materials and Methods”). CON (control) is the 30-minute period before the 10-minute coronary occlusions (TCO1 and TCO2). REP1 and REP2 are the 30-minute reperfusion periods. TCO3 is the 60-minute occlusion followed by the final 90-minute reperfusion (REP3). Ischemic region wall function recovers immediately with reperfusion after the 10-minute occlusions, but at the onset of the 60-minute occlusion, this region is akinetic (stunned).

Risk Region and Infarct Size

The risk region and infarct size expressed as a percentage of risk region are presented in Figure 5. The risk regions were virtually identical (14.4±1.5% for controls vs. 14.6±1.9% for preconditioned pigs, p=NS). The risk area was kept intentionally small by occluding the LAD after the second branch, because large risk areas were associated with an unacceptably high incidence of fibrillation in pilot studies with 45 minutes of LAD occlusion. The percent of the risk region infarcted was 48.0±12.7% for controls and 10.4±6.3% for preconditioned pigs (p<0.005 by Mann-Whitney U test). In preconditioned myocardium, the infarcted tissue was generally spotty and scattered throughout the risk region.

High-Performance Liquid Chromatography Data

Biopsy specimens from all experiments were divided randomly into two groups for biochemical
TABLE 2. Myocardial Blood Flow Determined With Tracer Microspheres

<table>
<thead>
<tr>
<th></th>
<th>Nonischemic blood flow (ml/min/100 g)</th>
<th>Ischemic blood flow (ml/min/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ENDO</td>
<td>EPI</td>
</tr>
<tr>
<td>Preconditioned pigs (n=9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCO1</td>
<td>103.8±16.1</td>
<td>111.2±8.7</td>
</tr>
<tr>
<td>REP1</td>
<td>123.7±32.1</td>
<td>115.0±16.0</td>
</tr>
<tr>
<td>TCO2</td>
<td>110.7±25.4</td>
<td>106.8±14.6</td>
</tr>
<tr>
<td>REP2</td>
<td>107.9±25.5</td>
<td>97.8±14.1</td>
</tr>
<tr>
<td>TCO3</td>
<td>86.0±20.3</td>
<td>87.8±12.8</td>
</tr>
<tr>
<td>REP3</td>
<td>120.6±26.4</td>
<td>112.6±21.5</td>
</tr>
<tr>
<td>Control pigs (n=6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCO</td>
<td>92.1±10.6</td>
<td>103.8±7.1</td>
</tr>
<tr>
<td>REP</td>
<td>106.2±10.2</td>
<td>97.1±5.4</td>
</tr>
</tbody>
</table>

Values are mean±SEM. ENDO, endocardial blood flow; EPI, epicardial blood flow; TRANS, transmural blood flow.

For preconditioned pigs, values were obtained at 8 minutes into the first and second 10-minute coronary occlusions (TCO1 and TCO2, respectively), at 25 minutes into the first and second 30-minute reperfusions (REP1 and REP2, respectively), at 55 minutes into the 60-minute occlusion (TCO3), and at 25 minutes into the 90-minute reperfusion (REP3). For control pigs, values were obtained at 55 minutes into the 60-minute occlusion (TCO) and at 25 minutes into the 90-minute reperfusion (REP). Occlusion and reperfusion data were grouped separately for analysis.

*p<0.05 vs. REP1.

Discussion

Ischemic Preconditioning and Ischemic Tolerance

The primary objective of this study was to assess the effects of stunning on ischemic tolerance in swine myocardium. Myocardium was subjected to two 10-minute ischemic episodes, each followed by 30 minutes of reperfusion, which produced regional akinesia by the end of the second reperfusion period. Myocardium "preconditioned" in this fashion has less than one quarter of the necrosis of myocardium that has not been stunned, despite the additional 20 minutes of ischemia in the preconditioned pigs. The differences in infarct size between preconditioned and control pigs are not attributable to differences in the traditional determinants of infarct size: risk regions and hemodynamics between the two groups.

**Figure 3.** Bar graph showing myocardial oxygen consumption (MVO$_2$) calculated from the entire left ventricle (global), a nonischemic control region in the circumflex distribution, and from the "stunned" region. Control is the 30-minute period before the first occlusion. REP 1 and REP 2 are the 30-minute reperfusion periods after the 10-minute occlusions. MVO$_2$ is measured at 25 minutes into REP 1 and REP 2.

**Figure 4.** Scatterplot of simultaneously obtained regional myocardial oxygen consumption (MVO$_2$) versus wall thickening (% ΔWT) for preconditioned pigs before ischemia (control) and at 25 minutes into each 30-minute reperfusion period (Rep 1 and Rep 2) after each 10-minute occlusion. Each experiment is represented by three time points.
were similar, and collateral transmural blood flow to the ischemic region in swine was minimal (<10 ml/min/100 g in these experiments). These results are in close agreement with preconditioning effects reported by Murry et al. They observed a 75% reduction in infarct size after 40 minutes of LAD occlusion in dogs preconditioned with four 5-minute occlusions followed by 5-minute reperfusions. Li et al. have also observed a substantial (10-fold) decrease in infarct size in dogs preconditioned with as little as 5 minutes of occlusion followed by 5 minutes of reperfusion before a 60-minute circumflex occlusion.

The mechanism of preconditioning is not completely understood although a number of researchers have observed that ATP depletion is slowed after an initial episode of ischemia. Murry et al. have speculated that this, coupled with reduced catabolite accumulation after the initial periods of ischemia, could account for the protective effect of preconditioning. Furthermore, CP stores exhibit an “overshoot” with reperfusion after brief periods of ischemia, making more energy stores available when entering a long period of ischemia. However, CP is depleted within minutes after the onset of ischemia, and the additional CP from the overshoot is probably not sufficient to sustain viability through 60 minutes of ischemia. Recently, it has been reported that preadministration of superoxide dismutase and catalase can blunt the preconditioning effects of brief ischemia; this finding suggests that oxygen free radical species produced during the initial episodes of brief ischemia might be important in the development of tolerance to subsequent longer episodes of ischemia. Our demonstration of a preconditioning effect in myocardium devoid of xanthine oxidase activity suggests either another source of free radicals (i.e., neutrophils) or another mechanism for the preconditioning effects of brief ischemia in pigs.

We determined infarct size in myocardium reperfused for 90 minutes after the final occlusion period by incubating the myocardium with p-NBT. This established technique relies on the conversion of the tetrazolium salt to formazan in the presence of cofactors (nicotinamide adenine dinucleotides), which stain viable myocardium deep blue. Infarcted tissue loses these cofactors during reperfusion and remains pale and easily distinguishable from viable myocardium. The duration of reperfusion periods used with this technique has been quite variable; 30 minutes of reperfusion is the minimum time reported for the delineation of infarcted tissue. Recently, Fujiwara et al. compared 1-, 3-, and 7-hour reperfusion times after 1 hour of distal LAD occlusion in domestic swine and reported that 1 hour of reperfusion was adequate for the delineation of infarct size when cut specimens were incubated with p-NBT. In that study, infarct size was also determined with an immunohistochemical technique employing myoglobin antibodies, as well as with hematoxylin-eosin and Masson’s trichrome staining of the myocardium. We have previously reported on the adequacy of p-NBT technique after 90 minutes of reperfusion, with ultrastructural verification of infarction.

### Myocardial Oxygen Consumption in Stunned Myocardium

A second objective of this study was to measure M\(\text{Vo}_2\) in stunned myocardium. We hypothesized that preconditioning might result from stunning; with ischemic disengagement of the contractile apparatus, oxygen demand would be lowered entering a subsequent ischemic period. This requires an important assumption: variations in regional demand under aerobic conditions at the onset of occlusion affect the evolution of myocardial necrosis under anaerobic conditions during occlusion. Previous experiments have demonstrated that variations in global demand before the onset of ischemia can alter the rate of necrosis after arterial occlusion, whereas alterations in M\(\text{Vo}_2\) midway through a 90-minute occlusion do not affect the extent of necrosis. This finding suggests that the period immediately after occlusion in virgin myocardium is critical, which is supported by the observation of Neely and Feuvray that there is a burst of anaerobic glycolysis immediately after occlusion in virgin myocardium, with slowing of aerobic

### Table 3. High-Energy Phosphates

<table>
<thead>
<tr>
<th>Group</th>
<th>Creatine phosphate</th>
<th>ATP</th>
<th>ADP</th>
<th>AMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preconditioned pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>33.8±2.1</td>
<td>5.5±0.1</td>
<td>2.3±0.4</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Nonischemic</td>
<td>35.9±3.0</td>
<td>20.4±0.3</td>
<td>2.9±0.4</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>Control pigs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemic</td>
<td>13.2±2.1*</td>
<td>4.0±0.9</td>
<td>1.9±0.2</td>
<td>0.2±0.1</td>
</tr>
<tr>
<td>Nonischemic</td>
<td>27.9±4.2</td>
<td>20.3±1.4</td>
<td>3.0±0.4</td>
<td>0.3±0.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM and are in nanomoles per milligram of dry weight. Ischemic biopsies were taken from the center of the ischemic region 90 minutes after the final reperfusion; nonischemic specimens were taken from the lateral wall at the same time. *p<0.005 vs. preconditioned ischemic region myocardium.
glycolytic flux as ischemic catabolites (lactate, nicotinamide adenine dinucleotide, and \( H^+ \)) accumulate.\(^{32,33}\) The data of Murry et al\(^2\) indicate that the rate of ATP depletion and lactate production are greatest in the first 10 minutes after arterial occlusion. We hypothesized that continued contractile effort after occlusion was responsible for the accelerated anaerobic flux and that the rate of anaerobic flux could be slowed by stunning the myocardium. Our failure to observe a significant change in \( \text{MVO}_2 \) in stunned myocardium suggests that despite near akinesis, oxidative metabolism remains relatively constant. Thus, the marked protective effect cannot be explained by an appreciable decrease in regional demand entering the ischemic period.

\( \text{MVO}_2 \) of stunned myocardium has been a continuing source of controversy, with groups reporting that \( \text{MVO}_2 \) is decreased,\(^{12-14}\) unchanged,\(^{15,34}\) or increased.\(^{35}\) This may reflect species differences as well as the heterogeneity found in the contractile effort in myocardium that is labeled “stunned.” A recent brief report suggests that regional \( \text{MVO}_2 \) is minimally reduced until function is depressed to the onset of systolic bulge, at which point \( \text{MVO}_2 \) falls off rapidly.\(^{36}\) The akinetic stunned myocardium in the experiments reported here retains some residual contractile effort: the myocardium bulges \( 12\% \) during the second occlusion but “recovers” to akinesis with the second reperfusion. Therefore, in addition to the basal metabolic requirements of myocardial tissue, which may account for one fourth to one third of resting \( \text{MVO}_2 ,^{37} \) there is the contractile effort to maintain akinesis.

With the necessity for small risk regions in these experiments, some function in the stunned region may artifactually result from “tethering” to normal myocardium, although Gallagher et al\(^38\) have shown that this “functional border zone” extends for less than 0.5 cm in either direction from the perfusion boundary when ultrasonic crystals are aligned to measure wall thickness.

Although we report that regional \( \text{MVO}_2 \) did not change significantly from the \( \text{MVO}_2 \) before stunning, the trend was for gradually decreased \( \text{MVO}_2 \) in the stunned myocardium. In addition to (stunned) regional \( \text{MVO}_2 \), we calculated left ventricular global \( \text{MVO}_2 \) from the coronary sinus oxygen saturation and mean blood flow to the entire left ventricle. In a subset of six pigs, a second control \( \text{MVO}_2 \) was measured in a normally contracting region of myocardium in the left circumflex distribution. \( \text{MVO}_2 \) in these control regions tended to increase after coronary occlusion. A change in hemodynamics did not account for this, although it is known that the nonischemic regions of the myocardium compensate with increased contractility during occlusion\(^{39}\) and that this may in turn account for the increased \( \text{MVO}_2 \) in this region. When (stunned) regional \( \text{MVO}_2 \) was compared with the simultaneously obtained global \( \text{MVO}_2 \), regional \( \text{MVO}_2 \) fell from 100% of global \( \text{MVO}_2 \) before occlusion to 70% of the global \( \text{MVO}_2 \) with stunning (\( p<0.005 \)). This was accompanied by a fall in transmural blood flow from the first to the second reperfusion period; this fall is consistent with down-regulation of blood flow in response to decreased oxygen demand.\(^{13}\) Regional \( \text{MVO}_2 \) was weakly correlated with regional wall function (\( r=0.47, p<0.05 \)).

\( \text{MVO}_2 \) before occlusion is relatively low (3.5±0.4 ml O\(_2\)/min/100 g) in this model. This is less than we have previously reported for swine using the Bretschneider equation,\(^24\) although it is consistent with other reports of measured \( \text{MVO}_2 \) in swine.\(^{14}\) This may be a function of an open-chest preparation\(^34\) but also may reflect inclusion of small quantities of right ventricular venous effluent in our measurements of oxyhemoglobin from the interventricular vein and coronary sinus. The right ventricle has a lower oxygen consumption,\(^{40}\) which may have elevated the oxyhemoglobin concentrations that we measured. In addition, oxygen saturations from the stunned region are included in the calculations of global \( \text{MVO}_2 \) during the first and second reperfusion periods.

**High-Energy Phosphates**

\( \text{CP} \) levels from the preconditioned myocardium at 90 minutes of reperfusion were twice that of myocardium subjected to a single 60-minute episode of ischemia, despite an additional 20 minutes of ischemia. Our conclusion is that preconditioned myocardium is viable after 60 minutes of ischemia and is able to resume oxidative phosphorylation and replete \( \text{CP} \) stores. It is less likely that preconditioned myocardium can preserve significant quantities of \( \text{CP} \), which under normal circumstances is rapidly depleted after the onset of ischemia.\(^{33}\) \( \text{ATP} \) and \( \text{ADP} \) were depleted to the same degree at 90 minutes after the 60-minute occlusion in both preconditioned and control myocardium. Regeneration of creatine kinase occurs quickly in stunned myocardium;\(^{28}\) however, \( \text{ATP} \) and \( \text{ADP} \) resynthesis requires days after brief ischemia.\(^{41}\)

**Summary**

In summary, we demonstrated that two brief cycles of ischemia and reperfusion increase the ischemic tolerance of swine myocardium during a subsequent 60-minute coronary occlusion. In addition to reducing the volume of infarcted myocardium, preconditioning with ischemia was associated with restoration of \( \text{CP} \) stores 90 minutes after a 60-minute episode of ischemia. \( \text{MVO}_2 \) in stunned myocardium was decreased when compared with simultaneously measured global left ventricular \( \text{MVO}_2 \); however, it was not decreased when compared with preischemic regional \( \text{MVO}_2 \). This suggests that increases in ischemic tolerance are independent of changes in regional oxygen demand associated with stunning. The precise mechanisms by which ischemically primed myocardium can protect itself remain unclear. The extent of the infarct size reduction exceeds any that has been produced with pharmacological interventions and warrants further careful investigation.
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