Ultrastructural assessment of myocardial necrosis occurring during ischemia and 3-h reperfusion in the dog

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Becker, Lewis C., Richmond W. Jeremy, Jutta Schaper, and Wolfgang Schaper. Ultrastructural assessment of myocardial necrosis occurring during ischemia and 3-h reperfusion in the dog. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H243–H252, 1999.—To determine whether myocardial necrosis may occur during posts ischemic reperfusion, electron microscopy was used to identify morphological features of irreversible injury in myocardial samples taken from anesthetized dogs with 90-min ischemia and 0-, 5-, 90-, or 180-min reperfusion. In samples without detectable collateral blood flow, necrosis was almost complete, whether or not the myocardium was reperfused. In samples with collateral flow, necrosis was more frequent after 180-min reperfusion than in the absence of reperfusion, despite similar collateral flows in the two groups. Excess of necrosis after 180-min reperfusion was evident in endocardium (ischemia only: 4 of 13, 180-min reflow: 14 of 20; P = 0.03) and midwall (ischemia only: 9 of 25, 180-min reflow: 29 of 45; P = 0.02). Multiple logistic regression with variables of collateral flow and transmural position was used to determine risk of irreversible injury in 111 samples from ischemic myocardium without reperfusion (model predictive accuracy = 75%, P < 0.00001) and to predict risk of necrosis in myocardium reperfused for 180 min. Of 65 samples from endocardium and midwall with detectable collateral flow, the model predicted necrosis in 23 samples but necrosis was observed in 43 samples (P < 0.01). Reperfusion duration was a determinant of frequency of irreversible injury. Multiple logistic regression for 186 samples from myocardium reperfused for 5, 90, or 180 min showed that reperfusion duration was an independent predictor of irreversible injury (P = 0.0003) when collateral flow and transmural location were accounted for. These findings are consistent with the occurrence of necrosis during reperfusion in myocardium exposed to substantial, prolonged ischemia but with sufficient residual perfusion to avoid necrosis during the period of flow impairment.

myocardium; cell death; electron microscopy; blood flow

Experimental studies show that reperfusion of ischemic myocardium causes major structural changes in injured myocytes (19, 23, 41, 46), whereas nonreperfused infarcts are characterized by relatively well-preserved myofibrils (35). Despite the apparent destructive effects of reperfusion on the structure of ischemic myocardium, it has been argued that these effects are confined to myocytes that are already irreversibly injured and that reperfusion does not result in necrosis of additional myocytes (9, 18, 22, 41).

This argument is challenged by numerous studies that showed that infarct size can be reduced by a therapeutic intervention administered shortly before, or at the time of, reperfusion. In particular, interventions directed against neutrophils were successful at limiting infarct size (24, 25, 29, 32, 43), but administration of oxygen free radical scavengers (2), adenosine (31), nitric oxide donors (42), and perfluorochemicals (5) were also successful. These intervention studies are not conclusive, however, because the evidence provided by these interventions for reperfusion-related necrosis is essentially indirect. Not all such studies were positive (13, 36, 45), and although methodological factors may account for negative results, these findings question the existence of additional myocardial necrosis occurring after reperfusion.

Conclusive proof of additional necrosis during reperfusion injury would require demonstration that a population of myocytes, which had suffered reversible injury but were viable at the end of the ischemic period, subsequently lost viability and developed irreversible structural damage during the reperfusion period. Unfortunately, such serial ultrastructural studies in the same population of myocytes are not presently possible. Nevertheless, some previous studies used histopathological methods serially in the same animals to address this issue and reported that necrosis may occur after blood flow is restored (14, 15). However, these conclusions have been questioned because of possible unreliability of the stains and tracers used to identify myocyte necrosis.

An alternate approach is to demonstrate in a group of animals that reperfused myocardium exhibits more necrosis than nonreperfused myocardium, after controlling for the severity of the preceding ischemia. In our study, we chose electron microscopy to identify necrotic myocytes because it represents the “gold standard” for such assessments. Electron microscopy is sensitive and equally applicable to nonreperfused and reperfused myocardium, and the morphological criteria for irreversible injury are well established (22, 23, 35, 39–41). The
The experiment was designed to account for the major determinants of ischemic necrosis (6, 27, 33, 37) and included a constant ischemia time of 90 min, several different durations of reperfusion (0–180 min), and a multivariate analysis that accounted for transmural progression of necrosis and collateral blood flow as well as duration of reperfusion. The results show that, in addition to collateral flow and transmural location, the duration of reperfusion is an important independent predictor of myocardial necrosis, confirming by a new and direct approach that myocyte necrosis may develop after restoration of blood flow.

**METHODS**

Experimental protocol. At Johns Hopkins University, adult mongrel dogs of either gender (25–30 kg) were anesthetized with intravenous sodium thiopental (12.5 mg/kg) and intramuscular urethane (136 mg/kg) containing α-chloralose (14 mg/kg). The dogs were ventilated with room air at positive pressure. A catheter was inserted into the aorta for microsphere sampling. The heart was exposed through a left lateral thoracotomy, and a catheter was placed in the left atrium for microsphere injections. A black silk suture was placed loosely around the circumflex artery, proximal to any major marginal branches. The coronary artery was occluded by sliding a small plastic tube over the suture, and regional myocardial blood flow was measured after 85-min ischemia with radionuclide-labeled microspheres. The total duration of ischemia was 90 min in each dog. Dogs were randomly assigned to ischemia without reperfusion or ischemia with different durations of reperfusion before the start of each experiment. Reperfusion was effected by release of the coronary snare.

The frequency of irreversible injury was first compared between myocardial samples taken from ischemic myocardium without reperfusion (n = 12 dogs) and from myocardium reperfused for 180 min (n = 12 dogs). The effect of duration of reperfusion on frequency of irreversible injury was studied in myocardial samples from a further five dogs (5-min reperfusion: n = 2, 90-min reperfusion: n = 3). At the conclusion of each experiment, the heart was arrested with potassium chloride, and immediately removed. The left ventricle was sectioned into five 1-cm-thick short-axis slices, and multiple transmural myocardial biopsies (2-mm wide and 2-mm deep) were rapidly excised from the apical surface of each slice. Most biopsies were taken from the ischemic myocardium in the circumflex territory, but control samples were also obtained from the nonischemic territory of the left anterior descending artery. Each transmural biopsy was divided into endocardial, midwall, and epicardial sections. Each of these samples was weighed (20–30 mg), immediately placed in plastic scintillation vials filled with cold 3% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.4), and subsequently counted in a gamma counter for calculation of regional myocardial blood flow. An average of 50 samples (range 30–75) were obtained from each heart. The number of samples taken depended on the size of the ischemic region.

Measurement of regional myocardial blood flow. Regional myocardial blood flow was measured during coronary artery occlusion with 15-μm-diameter radionuclide-labeled microspheres (DuPont, North Billerica, MA). After vascular agitation ~20 million microspheres were injected into the left atrium over 5–10 s, and a simultaneous reference blood sample was withdrawn from the femoral artery at a constant rate of 2.06 ml/min for 5 min. Blood and myocardial samples were counted in a gamma counter (Packard model 5986) at an appropriate energy window. Flow in each myocardial sample was calculated as Qs = Qr × Cs/Cr, where Qs is sample flow, Qr is reference flow rate, Cs is background-corrected sample counts per minute, and Cr is background-corrected reference blood counts per minute.

Fixation and selection of samples for electron microscopy. After myocardial samples were weighed and counted for radioactivity, they were kept in fixative at 4°C for 24 h, rinsed in cold 0.1 M cacodylate buffer (with saccharose added, pH 7.4), and stored at 4°C. The buffer was renewed daily until shipment by air express to the Max Planck Institute in Bad Nauheim, Germany, for electron microscopy. The glutaraldehyde-cacodylate buffer fixative was freshly prepared every 2 wk.

From each dog, 10–25 samples were selected for electron microscopy, according to the level of collateral blood flow to the sample during coronary occlusion. Samples with collateral flow >0.4 ml·min⁻¹·g⁻¹ were rejected because this level of flow does not usually result in myocyte necrosis during occlusion (37). About four samples from each heart were also selected from the anterior left ventricle to serve as a control for the tissue fixation and analysis procedure. Each sample was identified by an individual code number before shipment.

**Microscopic analysis.** All samples were studied by light and electron microscopy by one of the authors (J. Schaper) without knowledge of sample location, regional blood flow, or duration of reperfusion. The tissue samples were embedded in Epon using a Wakura automatic tissue processor, after fixation in 2% osmic acid anhydride, dehydrogenation in an ethanol serial, and substitution by propylene oxide.

Semi-thin (1–2 μm) sections were prepared and stained with toluidine blue. Artifact-free areas were selected for preparation of thin sections (50–60 nm) for electron microscopy. These sections were attached to uncoated copper grids, stained with uranyl acetate and lead citrate, and viewed in a Philips EM 300 electron microscope. For each myocardial sample, 30–40 micrographs were examined for features of irreversible myocyte injury, according to previously published and validated criteria (Table 1; Refs. 39, 40). Irreversible injury was identified by the presence of amorphous densities, matrix clearing and/or cristae breakage in the mitochondria, clearing and shrinkage of nuclei, and/or disruption of the sarclemma. The presence of contraction bands was not used as a criterion of irreversible injury. Each sample was deemed to have suffered irreversible injury if >90% of micrographs for that sample showed criteria for irreversible injury. In a few samples in which the micrographs revealed a heterogeneous appearance, additional sections were obtained and examined to resolve the issue for that sample.

**Table 1.** Electron microscopy criteria for irreversible myocardial injury

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Reversible</th>
<th>Irreversible</th>
</tr>
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<tbody>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amorphous densities</td>
<td>None</td>
<td>Present</td>
</tr>
<tr>
<td>Matrix clearing</td>
<td>+ to ++</td>
<td>+++</td>
</tr>
<tr>
<td>Cristae breakage</td>
<td>+ to ++</td>
<td>+++</td>
</tr>
<tr>
<td>Nuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clearing</td>
<td>None to +</td>
<td>+ to + to +++</td>
</tr>
<tr>
<td>Shrinkage</td>
<td>None to +</td>
<td>+ to + to +++</td>
</tr>
<tr>
<td>Myofilaments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraction bands</td>
<td>None to +</td>
<td>Severe with disorganization</td>
</tr>
<tr>
<td>Sarcolemma</td>
<td>Intact</td>
<td>Intact or disrupted</td>
</tr>
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</table>

Criteria are from Refs. 39 and 40. +, Mild; ++, moderate; ++++, severe.
Data analysis. Because reperfusion is associated with tissue edema and an increase in tissue wet weight of \( \sim 25\% \) (34), the measured blood flows in each sample from reperfused myocardium were corrected for a 25\% increase in wet weight during reperfusion and results are reported for corrected flows. Regional myocardial blood flows were compared between samples, grouped according to transmural location and duration of reperfusion, by analysis of variance (38). The frequency of samples meeting criteria for irreversible injury was compared by \( \chi^2 \)-square, with samples grouped according to collateral flow, transmural location, and duration of reperfusion. The relationships among the independent variables of collateral blood flow, transmural location, and duration of reperfusion and the dependent variable of risk of irreversible injury were determined for individual samples by multiple logistic regression (44a). All data are reported as means \( \pm \) SD, and a P value \( \leq 0.05 \) is considered significant.

RESULTS

Of 343 samples obtained from ischemic or reperfused myocardium, 176 (51\%) were judged to predominantly contain irreversibly injured myocytes. Another 30 samples from the nonischemic control region were also examined, of which only 1 was considered to contain irreversibly injured myocytes. Examples of normal and reversibly injured myocardium are shown in Fig. 1. Mild to moderate chromatin clumping and clearing of the nucleus or limited mitochondrial changes with broken cristae were not considered evidence of irreversible injury.
ible injury. Representative examples meeting criteria for irreversible injury are shown in Fig. 2 for samples without reperfusion (Fig. 2A), after 5-min reperfusion (Fig. 2B), and after 180-min reperfusion (Fig. 2C). In the absence of any reperfusion, irreversible injury was characterized by marked nuclear changes, amorphous densities in the mitochondria, and, sometimes, damage to the sarcolemma. Reperfusion for 5 min was associated with more severe morphological changes, including distortion of sarcomeres with formation of contraction bands and destruction of the sarcolemma. After 180-min reperfusion, irreversibly injured cells showed further structural deterioration, including contraction bands and destruction of mitochondria and the sarcolemma.

The mean collateral blood flow to the ischemic risk region was $0.08 \pm 0.01$ ml·min$^{-1}$·g$^{-1}$ in the 111 samples from myocardium that was not reperfused and $0.10 \pm 0.12$ ml·min$^{-1}$·g$^{-1}$ in 136 samples from myocardium reperfused for 180 min ($P = \text{not significant}$). Of the 42 samples reperfused for 5 min, mean collateral flow was $0.08 \pm 0.09$ ml·min$^{-1}$·g$^{-1}$, and of the 54 samples reperfused for 90 min, mean collateral flow was $0.12 \pm 0.13$ ml·min$^{-1}$·g$^{-1}$. There were no significant differences in mean collateral flow to the ischemic risk region between the samples without reperfusion and those samples with 5-, 90-, or 180-min reperfusion.

The frequency of irreversible injury is compared for samples from myocardium without reperfusion and those with 180-min reperfusion in Table 2. Data are
shown for samples from 12 dogs in each group, and samples are grouped according to transmural location and the presence or absence of detectable collateral flow. Nearly all samples without detectable collateral flow had irreversible injury, whether or not the myocardium was reperfused. Among samples with detectable collateral flow, 24 of 74 samples from myocardium without reperfusion had irreversible injury. In contrast, 55 of 106 samples from myocardium reperfused for 180 min had irreversible injury. There was significantly more irreversible injury after 180-min reperfusion in both the endocardium and the midwall, despite similar collateral blood flows in the groups with and without reperfusion. The relationship between collateral blood flow and occurrence of irreversible injury in reperfused myocardium is further illustrated in Fig. 3. In the endocardial region, samples with collateral flow of 0.01–0.10 ml·min\(^{-1}\) had significantly more irreversible injury after 180-min reperfusion than did samples without reperfusion. Only a few samples with collateral flow >0.10 ml·min\(^{-1}\) had irreversible injury, even after 180-min reperfusion. The same pattern was evident in the midwall region, but irreversible injury was less frequent in the epicardial region. These grouped data, with samples matched for transmural location and collateral flow, indicate that irreversible injury is more frequent after 180-min reperfusion than in the absence of reperfusion.

The probability of irreversible injury occurring in individual samples was then examined by multiple logistic regression. A regression model (Table 3) was developed for 111 samples (n = 12 dogs) taken from ischemic myocardium in the absence of reperfusion, to describe the risk of irreversible injury occurring during ischemia alone. The predictive accuracy of the model was 75%, and collateral flow was identified as the main predictive variable. This model was then applied to the 136 individual samples from myocardium reperfused for 180 min. The comparison between the number of samples predicted to be necrotic from the regression model for ischemic injury and the number of samples observed to be necrotic after 180 min is shown in Table 4. For samples without detectable collateral flow, the model predicted that nearly all samples would have irreversible injury, and this was the observed result.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B ± Error</th>
<th>Wald</th>
<th>Significance</th>
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<tbody>
<tr>
<td>(Q_{coll})</td>
<td>24.94 ± 5.93</td>
<td>17.79</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Layer</td>
<td>-0.41 ± 0.92</td>
<td>0.20</td>
<td>0.66</td>
</tr>
<tr>
<td>Constant</td>
<td>-1.11 ± 0.51</td>
<td>4.70</td>
<td>0.03</td>
</tr>
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</table>

Multiple logistic regression with dependent variable of probability of sample viability for 111 samples from 12 dogs without reperfusion. \(Q_{coll}\), collateral flow; B, parameter estimate. Model \(\chi^2\)-square = 50.6; P < 0.00001; predictive accuracy = 75%.
was examined by logistic regression for all 343 samples of risk of irreversible injury in individual samples, the duration of reperfusion, as a determinant of irreversible injury in individual samples, was examined by logistic regression for all 343 samples.

The data in Table 4 were calculated using a 50% probability as the threshold for prediction of irreversible injury or otherwise in an individual sample. Because there is some error associated with the variable coefficients of the model (see Table 3), the use of a single threshold value for prediction of irreversible injury might be misleading. Therefore, the frequency of irreversible injury predicted by the model was compared with the observed frequency of irreversible injury for a wide range of probability thresholds in Fig. 4. As the required probability for definition of irreversible injury increased, fewer myocardial samples were predicted to be injured (i.e., specificity increases but sensitivity decreases; Fig. 4A). There was, however, no difference in the pattern of prediction of irreversible injury between samples from myocardium without reperfusion and samples with 180-min reperfusion. In contrast, the observed data show an excess proportion of irreversible injury after 180-min reperfusion. In samples predicted to be viable (absence of irreversible injury), an excess of irreversible injury (Fig. 4B) was evident across the whole range of probability thresholds for definition of irreversible injury.

The effect of the duration of ischemia on the frequency of irreversible injury was then examined. As shown previously, samples without detectable collateral flow nearly all had irreversible injury (10 of 11 samples with 5-min reperfusion, 6 of 6 samples with 90-min reperfusion, and 25 of 30 samples with 180-min reperfusion). Among samples with detectable collateral flow, irreversible injury was less frequent after 5-min reperfusion than after 90- or 180-min reperfusion (Table 5). This difference was evident in the endocardial, midwall, and epicardial regions of the myocardium. The role of the duration of reperfusion, as a determinant of risk of irreversible injury in individual samples, was examined by logistic regression for all 343 samples from ischemic and reperfused myocardium (Table 6, model A). The overall predictive accuracy of the model was 73%, and duration of reperfusion remained a significant and independent predictor when collateral flow and transmural position were included. The logistic regression was then repeated for the 186 samples from reperfused myocardium, which had detectable collateral flow (Table 6, model B), and the duration of reperfusion was again found to be a significant predictor of irreversible injury. These findings show that reperfusion is associated with a greater frequency of irreversible injury than is ischemia without reperfusion, and the risk of irreversible injury appears to increase during the first 180 min of reperfusion.

**DISCUSSION**

The concept of myocardial reperfusion injury continues to be a source of controversy. The present study, which represents the first electron microscopic study to

<table>
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<tr>
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<th>Predicted</th>
<th>Observed</th>
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<tr>
<td>Endo</td>
<td>Q̇coll ≥ 0.0 ml·min⁻¹·g⁻¹</td>
<td>18 (100%)</td>
</tr>
<tr>
<td>Midwall</td>
<td>Q̇coll ≥ 0.0 ml·min⁻¹·g⁻¹</td>
<td>20 (20%)</td>
</tr>
<tr>
<td>Epi</td>
<td>Q̇coll ≥ 0.0 ml·min⁻¹·g⁻¹</td>
<td>10 (100%)</td>
</tr>
<tr>
<td></td>
<td>Q̇coll &gt; 0.0 ml·min⁻¹·g⁻¹</td>
<td>45 (42%)</td>
</tr>
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n, No. of samples. *P = 0.002, †P = 0.035 vs. predicted.

![Fig. 4](http://ajpheart.physiology.org/)

Fig. 4. A: proportion of samples predicted to have irreversible injury according to different probability thresholds for logistic regression in Table 3. Data are shown for samples with collateral blood flow > 0.0 ml·min⁻¹·g⁻¹ without reperfusion (T<sub>rep</sub> = 0, n = 74) or after 180-min reperfusion (T<sub>rep</sub> = 180, n = 106). There was no significant difference in predicted proportions of irreversible injury between the 2 groups. B: frequency of irreversible injury actually observed among samples predicted to be viable according to regression model. At all levels of probability threshold there is an excess of irreversible injury observed in samples reperfused for 180 min. *P < 0.05, **P < 0.01, ***P < 0.005 vs. T<sub>rep</sub> = 0 min.
myocardial necrosis during the ischemic period be of this approach is that the major determinants of reperfusion in different animals. A critical requirement to compare multiple samples after different durations of ultrastructural studies on the same myocardial sample and reperfused myocardium.

Prediction of irreversible injury in ischemic Table 6.

<table>
<thead>
<tr>
<th>Variable</th>
<th>B ± Error</th>
<th>Wald</th>
<th>Significance</th>
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<tbody>
<tr>
<td>(Q_{\text{coll}})</td>
<td>11.53 ± 1.81</td>
<td>40.7</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Layer</td>
<td>1.59 ± 0.52</td>
<td>9.30</td>
<td>0.0023</td>
</tr>
<tr>
<td>(T_{\text{rep}})</td>
<td>−0.0044 ± 0.0016</td>
<td>7.6</td>
<td>0.0057</td>
</tr>
<tr>
<td>Constant</td>
<td>−1.48 ± 0.32</td>
<td>21.10</td>
<td>0.0001</td>
</tr>
<tr>
<td>Model B: reperfused samples with collateral flow (&gt;0.0 \text{ ml·min}^{-1} \cdot \text{g}^{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Q_{\text{coll}})</td>
<td>6.46 ± 1.98</td>
<td>10.7</td>
<td>0.0011</td>
</tr>
<tr>
<td>Layer</td>
<td>2.42 ± 0.75</td>
<td>10.5</td>
<td>0.0012</td>
</tr>
<tr>
<td>(T_{\text{rep}})</td>
<td>−0.011 ± 0.003</td>
<td>13.2</td>
<td>0.0003</td>
</tr>
<tr>
<td>Constant</td>
<td>−0.39 ± 0.56</td>
<td>0.5</td>
<td>0.49</td>
</tr>
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</table>

Multiple logistic regression with dependent variable of probability of sample viability for 343 samples from dogs with and without reperfusion (model A: samples with \(Q_{\text{coll}}\) = 0.0 and \(>0.0 \text{ ml·min}^{-1} \cdot \text{g}^{-1}\)) and 186 samples from reperfused myocardium with \(Q_{\text{coll}}\) > 0.0 ml·min\(^{-1}\)·g\(^{-1}\) (model B). Model A: \(\chi^2\)-square = 110, \(P < 0.000001\), predictive accuracy = 73%; model B: \(\chi^2\)-square = 54.7, \(P < 0.000001\), predictive accuracy = 72%.

This study required measurement of regional blood flow in small myocardial samples. The degree of spatial heterogeneity between small samples is such that extrapolation of flow measurements from large samples may not accurately reflect the level of collateral flow in myocardial blood flow, and transmural location of, each myocardial sample was included in the multivariate analysis. After these principal determinants of ischemic necrosis were controlled for, the duration of reperfusion remained as a significant and independent predictor of irreversible injury.

Electron microscopy was used to identify the changes of irreversible myocardial injury in this study because the method is equally applicable to reperfused and nonreperfused myocardium and the ultrastructural criteria of irreversible injury are not difficult to identify and are well established in the literature (22, 23, 35, 39–41). Although it is quite impractical to examine the entire ischemic risk region by electron microscopy, numerous samples were taken from the ischemic-reperfused region in each dog and multiple micrographs (n = 30–40) were examined for each sample before classification of injury. Functional criteria for loss of viability might appear preferable to morphological ones, but many criticisms can be offered against contractile function, metabolic activity, and perfusion, for example, as reliable indicators of viability, and practical issues limit the nondestructive serial application of most of these approaches to small samples of myocardium.

Schaper et al. (40) validated the ultrastructural criteria for irreversible injury in ischemic-reperfused canine hearts. Hearts undergoing ischemic arrest for 90 min developed marked contracture on reperfusion, associated with severely reduced myocardial ATP content (<0.5 µmol/g wet wt) and no subsequent return of contractile function or increase in myocardial ATP content. Serial biopsies from these irreversibly injured hearts consistently demonstrated certain ultrastructural features before reperfusion, including mitochondrial clearing and almost complete disappearance of the cristae, the presence of large, amorphous densities within the mitochondria, and severe nuclear swelling or pyknosis. After reperfusion, these morphological abnormalities became even more pronounced, with the appearance of sarcomere disruption, prominent contraction bands, marked edema, sarcolemmal breaks, and mitochondrial dense granules (22, 23, 35, 41). Shorter periods of ischemic arrest were associated with less severe ultrastructural abnormalities that often disappeared during reperfusion, and systolic function and myocardial ATP content recovered during the reperfusion period (39, 40). The point of transition from reversible to irreversible injury was marked by the appearance of mitochondrial amorphous densities, which probably represent complexes of insoluble proteins and lipids resulting from destruction of mitochondrial membranes (21).
individual samples (4, 11). Because the number of microspheres is reduced with small myocardial samples, we injected 20 million microspheres, which is ~10 times the usual quantity for regional blood flow measurements. It is unlikely, however, that this large number of microspheres would contribute to ischemic myocardial injury, because it is estimated that <5% of capillaries would be occluded by these microspheres (47). Nonetheless, the stochastic error of microsphere flow measurements is increased in very small samples. If a 50-mg sample received only 50 microspheres at a flow of 0.1 ml·min\(^{-1}·g\(^{-1}\), the relative error in flow measurement would be 14% (3, 10). When counting and weighing errors are included, the relative error in flow measurement for such a sample is probably 25%. As a result of these errors in flow measurement, some samples may have been incorrectly classified as having severe ischemia or vice versa. However, inaccuracies in blood flow measurement in small individual samples cannot explain our finding of greater irreversible injury with reperfusion, because stochastic or weighing errors would apply equally to samples from all groups. Tissue edema could increase during reperfusion, but we calculated corrected flows for each sample from reperfused myocardium, based on an estimated 25% increase in tissue wet weight during reperfusion (34). Statistical analyses of raw and corrected flow data revealed the same results.

Comparison to previous studies. Several other groups have addressed the issue of reperfusion injury from a histopathological perspective. In a canine study, Hofmann et al. (20) occluded two coronary artery branches of approximately the same size in the same heart for either 3 or 6 h. Because one artery was reperfused for 60–90 min and the other was not, the duration of occlusion was made equal by staggering the onset of occlusion in the two arteries. These workers found no difference in infarct size between reperfused and nonreperfused territories, but collateral flow was not measured, and 6-h coronary occlusion usually results in extensive ischemic necrosis and large infarcts (33), precluding the possibility of further injury occurring during reperfusion.

Subsequently, Ganz and co-workers (16) were also unable to detect reperfusion injury in the canine heart. After occlusion of the left anterior descending artery for 90–240 min, the distal half of the risk region remained ischemic while the proximal half was reperfused for 5 min. The transmural extent of necrosis was similar in nonreperfused and reperfused regions. In a recent study by the same group (48), a similar result was found when the reperfusion period was extended to 3 h. In neither study, however, was collateral blood flow to the reperfused and nonreperfused regions measured in the hearts used for anatomic study. The relative severity of the ischemic episode in each region thus remains open to question. In both studies the extent of infarction was indexed by measuring the transmural extent of infarction on 2,3,5-triphenyltetrazolium chloride (TTC)-stained myocardial slices, but infarct edges in this model are very irregular, and therefore the measurement of transmural extent is subject to considerable variability. In addition, definition of necrotic myocardium by TTC in nonreperfused myocardium is less reliable than in reperfused myocardium (39, 40).

Three other studies have reported findings consistent with the occurrence of further necrosis during reperfusion. In rabbit hearts subjected to 30-min ischemia and up to 180-min reperfusion, Farb et al. (14) showed that infarct size, measured by tissue staining, increased from 45 ± 3% of the risk region at the onset of reperfusion to 60 ± 3% after 3 h (P = 0.0002). Infarct size immediately after reperfusion was measured using horseradish peroxidase, a protein that will only cross the sarcolemma of irreversibly injured myocytes. After 3-h reperfusion, infarct size was measured by TTC staining. In a canine study, Frame et al. (15) also found evidence of extension of infarction during reperfusion of ischemic myocardium. These workers used anti-myosin F(ab\(^{-2}\) fragments to identify irreversibly injured myocytes. After 60-min coronary occlusion, radionuclide-labeled anti-myosin antibodies were injected into the coronary circulation at the onset of reperfusion, and 45 min later, additional anti-myosin antibodies with a different radionuclide label were administered. After each injection of antibodies, coronary sinus blood flow was temporarily diverted to prevent recirculation of the antibody. The binding of the antibody to the reperfused myocardium increased by 170% over the 45-min interval, suggesting progressive membrane damage and further myocyte necrosis.

Most recently, Matsumura et al. (27) demonstrated that, after 90-min ischemia in a canine model, as many as 65% of myocardial samples taken from the infarcted region (defined by TTC 4 h after reperfusion) were actually viable early after reperfusion. Viability in this study was defined metabolically by tissue 2-deoxy-D-glucose uptake. The amount of reperfusion-related necrosis was greatest for animals with moderate reductions of blood flow during ischemia and least for animals with very severe ischemia, similar to the results found in the current study.

Interpretation of findings. Two different arguments can be advanced to account for our findings without invoking the concept of reperfusion-related necrosis. In the first instance, reperfusion might simply exacerbate the structural deterioration of myocytes that were already irreversibly injured. This process is known to occur within a few minutes of reperfusion and would already be manifest in samples taken after 5-min reperfusion. The time course of this explosive damage is too short to account for the observed differences in the extent of necrosis between myocardium reperfused for 5 and 90 or 180 min. This interpretation also implies that samples were misclassified by electron microscopy as reversibly injured during early reperfusion when the samples were actually already irreversibly damaged. This error is unlikely, in view of the previously established clear ultrastructural criteria of irreversible injury (22, 23, 35, 39–41) that were used in this study. In addition, experimental studies show that myocardium with a pattern of reversible injury exhibits structural,
metabolic, and functional recovery during reperfusion (23, 39, 41) rather than further deterioration. It is unlikely, therefore, that there was a population of nonviable myocytes, with the ultrastructural appearance of irreversible injury, that survived ischemia and early reperfusion only to suffer major structural damage later during reperfusion.

Another interpretation of the present findings is that there is a population of myocytes that survive the ischemic period but are “programmed” by events during ischemia to undergo necrosis, irrespective of subsequent reperfusion. This is not reperfusion injury per se but, rather, a failure of reperfusion to salvage these myocytes. Although this interpretation may be consistent with the present findings, it is not consistent with the results of many intervention studies showing a reduction in infarct size by treatments given at the time of reperfusion. Studies from Buckberg’s laboratory, for example, in canine surgical models, have reported that mitochondria remain surprisingly intact, both ultrastructurally and functionally, even after 6 h of ischemia (7) and that postischemic recovery can be enhanced by controlled reperfusion with substrate-enriched blood cardioplegic solution and left ventricular venting (1). These types of treatment studies suggest that cell death occurring during reperfusion is not inevitable but is amenable to therapeutic intervention, consistent with the hypothesis that reperfusion does cause necrosis of some myocytes.

Mechanism of reperfusion injury. Although the present findings are consistent with the occurrence of necrosis during reperfusion, they do not provide direct information about the mechanism(s) responsible. Myocyte calcium overload, oxygen free radicals, microvascular obstruction, and neutrophil leukocytes have all been implicated. One feature of the present findings is that morphological changes of irreversible injury appear to increase in frequency between 5 and 90 or 180 min of reperfusion rather than occurring as a single event immediately on reperfusion. Neutrophils have been observed to accumulate progressively during the several hours after reperfusion (12, 17, 44), which would be consistent with the apparent time course of irreversible injury found in the present study.

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