Extent of damage in ischemic, nonreperfused, and reperfused myocardium of anesthetized rats

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Vetterlein, Friedrich, Christina Schrader, Rolf Volkmann, Marion Neckel, Matthias Ochs, Gerhard Schmidt, and Gerhard Hellige. Extent of damage in ischemic, nonreperfused, and reperfused myocardium of anesthetized rats. Am J Physiol Heart Circ Physiol 285: H755–H765, 2003; 10.1152/ajpheart.00269.2002.—To investigate the localization of the earliest damage in ischemic and ischemic-reperfused myocardium, anesthetized rats were subjected to coronary occlusion for 15, 30, 45, or 90 min. One-half of the animals in each group had no reperfusion, whereas the other half was reperfused for 14 min. With the use of histological methods, preferentially in the periphery of the area at risk, localized zones were detected that lacked the hypoxia-specific increase in NADH fluorescence. The extent of these areas displaying injured tissue was found to be significantly smaller in the ischemic-nonreperfused hearts than in the ischemic-reperfused organs (15-min ischemia: 0.22 ± 0.12% vs. 43.0 ± 5.0%; 30-min ischemia: 5.7 ± 2.7% vs. 64.6 ± 2.9%; 45-min ischemia: 5.6 ± 1.2% vs. 66.0 ± 7.5%; 90-min ischemia: 39.3 ± 5.5% vs. 86.7 ± 1.8% of the area at risk). The results point to a localized initiation of the damage close to the surrounding oxygen-supplied tissue during ischemia and an expansion of this injury by intercellular actions into yet-intact areas upon reperfusion.

AFTER LONGER PERIODS OF CORONARY OCCLUSION, reperfusion may not only affect the heart beneficially but may also cause functional disturbances such as contractile dysfunction and arrhythmias. Although these effects are generally acknowledged, another question remains unanswered and continues to be a source of dispute: Can reperfusion lead to the death of those myocytes that remain alive through ischemia (e.g., Refs. 2, 18, 25, 28, and 35)?

As studies on isolated cells have shown, there is no doubt that resupplying substrates imposes enormous stress on a myocyte that has been subjected to simulated ischemia (e.g., Ref. 29). During energy depletion, sodium and calcium accumulate in the cell to abnormally high levels, the intracellular osmotic load causes explosive swelling of the cell (29). Whether the injury remains reversible and the cell ultimately recovers or whether membrane disruptions occur, resulting in irreversible injury and death of the cell, depends on the intensity of these events (18, 31).

Although these event sequences have been relatively well characterized in isolated myocytes, there are a large number of uncertainties when the aforementioned processes are studied under in vivo conditions. Interactions between adjacent myocytes have to be taken into account necessitating an analysis of intact systems. This is further complicated by the fact that a part of the reperfusion-induced effects may arise instantaneously upon reflow, whereas additional damage may develop during continued reperfusion due to systemic inflammatory reactions (28). The present study deals exclusively with processes of the acute reflow phase.

With respect to this initial reflow state, some authors have even denied the occurrence of reperfusion injury in vivo. Their conclusion is that reperfusion injury simply discloses the insult that was incurred during energy withdrawal (13, 45). This assumption is based on observations of canine myocardium subjected to ischemia and ischemia/reflow whereby similar extents of infarction were found under both conditions. The tetrazolium method used in these studies is, however, limited by the fact that it is less reliable in ischemic than ischemic-reperfused myocardium due to the lack of substrate washout in the ischemic state. In addition, the rather long periods of ischemia in these studies (90 min and longer) may have induced so much ischemic damage that already irreversibly injured myocytes were then subjected to reflow-induced stress.

Electron-optic techniques are not dependent on reflow conditions and have been applied under similar, although less extreme, conditions of ischemia (2, 23). Irreversible damage has been found significantly more...
often in ischemic-reperfused tissue than in ischemic-nondeprived tissue; this has led to the conclusion that even though the ischemic injury may appear reversible, a certain fraction of myocytes may, nevertheless, develop necrosis upon reperfusion, thus clearly supporting the existence of specific reperfusion-induced damage in vivo.

One might argue that those cells that were classified as having incurred reversible injury had, in fact, already suffered irreversible damage that could simply not be detected because of a marginal intensity. As already discussed in Ref. 2, such a condition appears, however, to be quite unlikely in view of the well-established criteria for the electron-optic diagnosis of reversible/irreversible injury in myocytes. Moreover, the numerous positive interventional studies of similar infarction models (20) support the view that significant fractions of myocytes may indeed remain viable during ischemia but that they undergo irreversible damage upon reflow unless specific measures are taken.

If the assumption is correct that a fraction of myocytes does not suffer reversible injury during ischemia but becomes necrotic upon reflow, then this does not concur with results obtained in isolated cells. In isolated systems, a myocyte with reversible damage, by definition, recovers when substrates are resupplied.

What processes lead to such a discrepancy? It has been hypothesized (14, 29) that in vivo only a certain fraction of tissue initially suffers irreversible injury followed by hypercontraction upon reflow, as has been observed in isolated cells. Such myocytes may then cause mechanical derangement of the plasma membrane of neighboring cells, either by direct mechanical interactions and/or by diffusion of sodium via gap junctions, thereby inactivating hypercontraction in the adjacent myocytes (33). Nontypical structures of hypercontracted cells observed in reperfused myocardium have been viewed as an indication of such a spreading necrosis.

A proof of this view is quite difficult to obtain because it necessitates the detection of the pattern of injury within the entire area at risk during ischemia and after reflow. Electron-optic studies may only be performed on a limited number of samples. The tetrazolium technique allows an overview of the entire infarct but may go undetected when substrates are resupplied. However, to be quite unlikely in view of the well-established criteria for the electron-optic diagnosis of reversible/irreversible injury in myocytes. Moreover, the numerous positive interventional studies of similar infarction models support the view that significant fractions of myocytes may indeed remain viable during ischemia but that they undergo irreversible damage upon reflow unless specific measures are taken.

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The latter precondition does not imply that cellular losses of NADH have not yet occurred during ischemia. The changes are simply too discrete to be detected by the macroscopic tetrazolium method. In a previous microscopic study (4) on changes of tissue NADH fluorescence, it was clearly shown that myocytes may have already lost this signal in the ischemic state.

In the present investigation, we applied the aforementioned technique (4, 44) to study the microscopic pattern of injury within the entire area at risk during ischemia and ischemia/reflow. First, we validated the results by comparing NADH fluorescence to that of the permeability stain propidium iodide and to the intensity of tetrazolium staining. We then used an electron microscopic analysis. After substantiating the methodological approach (group I), we studied the changes in NADH fluorescence in myocardium exposed to different periods of flow interruption with and without reflow being established (group II).

With this approach, we wanted to determine whether there is any regional preference of injury within the affected areas of the heart; any pattern that arises could contribute insights into the primary processes of ischemic damage in vivo.

METHODS

General Experimental Procedures

The experiments were performed on female Wistar rats (200–220 g body wt) anesthetized with thiobutabarbital (120 mg/kg ip) and continuously ventilated with a gas mixture of 70% N2O and 30% O2. Catheters were placed into the left femoral artery to monitor blood pressure (Statham P23Db and SP 1200) and heart rate (beat-to-beat counter) and into the left femoral vein for drug applications. The trachea was carefully accessed, and a tube was inserted for artificial ventilation (type 681, Harvard respirator; tidal volume 3.5 ml, respiration rate 40 breaths/min). The thoracic cavity was opened, and a catheter for monitoring left atrial pressure (Statham P23Db and SP1200) was inserted into the left atrium via the left atrial appendage.

A 7-0 thread was placed around the descending branch of the left coronary artery, and the free ends of the thread were introduced into a polyethylene tube. A careful shifting of the thread against the tube (protected against lesions by a small plastic rod) caused a reversible occlusion of the coronary artery. At regular intervals, the blood gases were monitored (AVL 990, automatic blood gas system) and the respiration volume was readjusted, if necessary. Occlusion of the coronary artery was then performed.

Experimental Group I

In the first experimental group, NADH fluorescence was compared with established parameters indicating losses of cellular viability. The tetrazolium method was applied in reperfusion experiments; vital staining with propidium iodide was used in ischemia and ischemia-reperfusion experiments (n = 4 in each subgroup). In further experiments, ischemic and ischemic-reperfused myocardium was studied by electron microscopy.

Comparison of NADH fluorescence and nitroblue tetrazolium staining in ischemic-reperfused hearts. Coronary occlusion was maintained for 45 min and reperfusion for 14 min. The heart was excised, and a slice ~4 mm thick was cut perpendicular to the cardiac axis on the same plane as the infarct center. The exposed surface was immediately covered with a microscopic coverglass. The slice was then placed under the fluorescent microscope to observe NADH fluorescence. To make it possible to view the entire section, the objective of the microscope was removed and a charge-coupled device (CCD) camera (CF8/1DXC, Kappa; Gleich, Germany) equipped with a standard variable macro lens (focal length 18–105 mm, working distance 10–30 cm) was mounted on the microscope above the fluorescence filters. The image obtained under ultraviolet light excitation (365/395/420-nm filter system) was then stored. The slice was then incubated in p-nitroblue tetrazolium [NBT; 0.25 g/l in phosphate buffer (pH 7.4)] for 30 min and, after being
washed, was retransferred to the microscopic stage. With the use of white incident light, the image was again stored as described above. Larger vessels and natural margins of the tissue made it possible to compare the extent of NADH-deficient and NBT-negative areas within the same tissue.

Comparison of fluorescence of NADH and propidium iodide in ischemic and ischemic-reperfused myocardium. In the ischemia experiments, propidium iodide (Sigma) was injected intravenously at a dosage of 1.0 mg/kg (1.0 mg/ml) approximately one-half hour before the coronary vessel was occluded. Coronary occlusion was maintained for 45 min; FITC-albumin was then injected, and the heart removed, frozen, and freeze sectioned along the same plane as described above. In the reperfusion experiments, propidium iodide was injected intravenously at a dosage of 0.5 mg/kg before occlusion, which again lasted for 45 min. The same dose was injected 1 min after initiation of reflow. At the 14th min of reperfusion, the coronary artery was reoccluded; FITC was injected, and the heart was frozen as described above.

Microscopic images, each covering an area of 310 × 230 μm, were recorded by the CCD camera and stored electronically for evaluation. In the ischemic zone, 10 areas were selected, which showed reductions in NADH fluorescence. The same areas were revisited, and the corresponding images were recorded using the 546/590 nm filter system for detection of propidium iodide fluorescence. To avoid overlooking any myocytes that had changed their fluorescence in only one of the dyes, the fluorescence of propidium iodide was registered first and then that of NADH was registered in 10 additional areas. Intracellular cytoplasmatic intensities of both types of fluorescent light were compared cell by cell through the use of an image-analysis system (SigmaScan Image, Jandel Scientific).

Comparison of NADH fluorescence and electron microscopic changes in ischemic and ischemic-reperfused myocardium. In an attempt to validate the changes observed through fluorescence microscopy, an independent method, transmission electron microscopy, was applied in experiments in rats subjected to coronary occlusion for 45 min (n = 3) and to 45 min occlusion followed by 60 min reflow (n = 3). Evans blue (1%, 0.5 ml iv) was used to label the nonischemic area. In the ischemia experiments, the dye was injected at the onset of the last minute of coronary occlusion; in the ischemia-reperfusion experiments, this was done at the onset of the last minute of reflow after the vessel had been reocluded. The heart was rapidly excised and cut perpendicularly through the infarct zone; pure nitrogen was passed over the tissue to avoid reoxygenation of the superficial cells during processing. The cut surface was cleaned with nitrogen-gassed Ringer solution, and a coverglass was placed onto the cut surface. A digital image of NADH fluorescence of the entire surface was stored together with a second image of the same area illuminated by incident white light to observe the distribution of Evans blue. The sample was transferred into a buffered fixation fluid (1.5% glutaraldehyde and 1.5% formaldehyde at 4°C) for 2 wk. A wedge-shaped sample (2 × 2 × 4 mm) was then cut from the fixed tissue to provide a transmural portion of the tissue in the area at risk. A second sample of similar size was prepared from the perfused noninfarcted zone.

Further processing (osmication, uranyl acetate bloc staining, dehydration, embedding, sectioning, and lead citrate staining) was performed as described in Ref. 9.

With the use of the results of previous fluorescence microscopy as a basis, the following three zones were analyzed: a subepicardial, outermost zone (zone I); an intermediate zone, 17–20 cell layers (400–450 μm) apart from the outer surface containing the ischemic area of reduced NADH fluorescence (zone II); and the adjacent innermost zone (zone III).

To evaluate the degree of ultrastructural damage, semi-quantitative scoring systems were used for cardiomyocyte mitochondria, for the contraction state of sarcomeres, and for capillary endothelium. Ten test fields per zone were analyzed at a primary magnification of ×3,000–20,000. Within each zone, test fields were selected by systematic uniform random sampling. The person performing the ultrastructural analysis was not informed as to which zone the test fields belonged to.

Ultrastructural damage of cardiomyocyte mitochondria was assessed according to a modified scoring system described previously (22, 36): grade 1, unchanged with an electron-dense matrix with numerous matrix granules and densely packed cristae; grade 2, matrix locally electron lucent and loss of granules; grade 3, matrix electron lucent, local loss of matrix, and fragmentation of cristae; and grade 4, loss of matrix and lysis of cristae. The following classification of the contraction state of myofibrillar sarcomeres was used (37): grade 1 (relaxation), I-bands always visible; grade 2 (contraction), I-bands generally nonvisible and Z-band spacing slightly wider than the A-band; grade 3 (relaxation/contraction), relaxed and contracted sarcomeres in the same test field; grade 4 (overcontraction), submaximal contraction with A-band compression and Z-band thickening; grade 5 (hypercontraction), submaximal to maximal contraction with additional myofilament clumping; and grade 6 (contraction bands), maximal contraction with additional distortion of contractile system and membrane rupture. The first three contraction states are considered to be physiological forms; the last three states are considered to be pathological forms of contraction (37). Ultrastructural preservation of the myocardial capillary endothelium was evaluated according to a score involving the nucleus, basal lamina, mitochondria (as described for cardiomyocytes above), and cytoplasm (22): grade 1, homogenous chromatin, thin and continuous basal lamina, and homogenous cytoplasm; grade 2, condensed chromatin, locally widened and electron lucent basal lamina, and small cytoplasmic blebs; grade 3, clumping of chromatin, basal lamina of increased width over a long distance, and cytoplasmic blebs; and grade 4, rupture of the nuclear membrane and caryolysis, basal lamina of constantly increased width, large cytoplasmic blebs, and rupture of the cell membrane.

Experimental Group II

The question of spatial and temporal changes in ischemia-induced myocardial damage was studied in the second experimental group. Coronary occlusion was applied for 15, 30, 45, and 90 min, respectively. In one-half of these experiments, reperfusion was induced for 14 min. In the nonreperfusion experiments, the tissue outside the area at risk was labeled with FITC; in the reperfusion experiments, FITC was injected 1 min before the heart was frozen after the artery had been reoocluded. Further processing was performed as described above. The investigation of four periods of ischemia with and without reflow resulted in eight subgroups (n = 6 in each subgroup). In an additional group, the artery was also occluded for 15 min; however, reperfusion was maintained for 60 min (n = 4).

Frozen heart sections (5 μm thick) taken on the same level as the center of the infarct were freeze dried; the distribution of FITC and NADH was observed through fluorescence microscopy. The following filter combinations were used: 460/
510/528 nm (FITC) and 365/395/420 nm with a short-pass filter of 500 nm added to the secondary filter system (NADH).

To obtain an overview of changes in dye fluorescence, the complete transection of the heart was evaluated in a semi-quantitative manner. The section was scanned automatically step by step (H11001, 1,500 measuring points), and in each microscopic field it was established 1) whether the central, circular field of 60 µm diameter contained the intravascular label FITC in zero to one or more than one capillary and 2) whether the cell located at or closest to the center point of the viewing field had changed its autofluorescence (365/395/420-nm filter system) from bright blue to a dark brown tone.

For comparison of mean values, the Mann-Whitney U-test was used. Paired data were tested by the exact sign-rank test. Unless otherwise stated, values are given as means ± SE.

All experiments were approved by the federal authority and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996).

RESULTS

Experimental Group I

Comparison of NADH fluorescence and NBT staining in ischemic-reperfused myocardium. When the changes of NADH fluorescence in reperfused hearts were directly compared with changes in NBT stainability, a close correlation was found between the two sets of results. The areas not stained with NBT were considered to be 100% [24.8 ± 7.2% (mean ± SD) of the cross-sectional area of the heart]; a loss of NADH was found to cover 100.5 ± 3.9% of that area. A representative example is shown in Fig. 1.

Comparison of fluorescences of NADH and propidium iodide in ischemic and ischemic-reperfused myocardium. ISCHEMIA EXPERIMENTS. The dye did not induce any apparent changes in blood pressure or heart rate. In the nonischemic zones of the heart, it was only slightly visible as a faint red fluorescent coloring in the extracellular space. In the area at risk, however, distinct myocytes became discernable, which showed brilliant fluorescence in their nuclei and a slightly less, although still discernable, fluorescence in the remaining cell bodies (Fig. 2). A comparison of changes in NADH and propidium iodide fluorescence in the cytoplasm showed that myocytes that had lost NADH fluorescence to a certain degree developed an intracellular accumulation of propidium iodide (Fig. 3A). The correlation shows that accumulation of propidium iodide was especially pronounced in those cells that had, despite hypoxic conditions, lost most of their NADH fluorescence.

ISCHEMIA-REPERFUSION EXPERIMENTS. When reflow was allowed to occur under the latter condition, not only did a much larger number of NADH-deficient myocytes appear, but the latter fraction of myocytes also displayed a much greater accumulation of propidium iodide than observed in the former group. Whereas the majority of myocytes in the ischemia experiments still

Fig. 1. Thick slice of a heart that was exposed to ischemia (45 min) and reflow (14 min). Immediately after the heart was sectioned, tissue NADH fluorescence was photographed (A), and the slice was then incubated with nitroblue tetrazolium and photographed again (B).

Fig. 2. Photomicrograph of ischemic myocardium showing fluorescence of NADH (A) and propidium iodide (B). The heart was subjected to regional ischemia (45 min) without reflow.
revealed a high NADH fluorescence and a lack of propidium iodide accumulation, the vast majority of myocytes in the reperfused state had lost NADH fluorescence and accumulated propidium iodide (Fig. 3B).

Comparison of NADH fluorescence and electron microscopic changes in ischemic myocardium. In the three zones of the ischemic myocardium as well as the ischemic-reperfused myocardium, ultrastructural damage was evident but not to an equal extent. In the outermost zone (zone I) in the ischemia experiments, only slight to moderate alterations in cardiomyocytes and capillary endothelial cells were noted (Fig. 4A). The most severe ultrastructural damage was observed in the intermediate zone (zone II, Fig. 4B), whereas only moderate alterations were detected in the innermost zone (zone III, Fig. 4C). The degree of severity ranged between that observed in zones I and II. The pooled data of the semiquantitative evaluation of ultrastructural damage are shown in Table 1. All parameters studied (the state of cardiomyocyte mitochondria and sarcomeres and that of the capillary endothelia) revealed damage that increased in severity as one moved from zone I via zone III toward zone II. Pooled data from reperfusion experiments showed a higher degree of ultrastructural damage in all three zones compared with values from the ischemia experiments. The gradient of damage when moving from zone I via zone III toward zone II, however, remained the same (Table 1).

Experimental Group II

Ischemia and ischemic-reperfusion experiments. In the following experiments, temporal and spatial changes in NADH fluorescence were studied in hearts exposed to ischemia for different periods of time; in one-half of the experiments, ischemia was followed by reflow. With respect to the general hemodynamic data, no significant differences between the experimental groups were found in the preocclusion control period. In all experiments, the initial blood pressure was above 120/80 mmHg, the heart rate was below 400 beats/min, and the left atrial pressure was below 5.0 mmHg. As the duration of ischemia increased, a decrease in arterial blood pressure and heart rate and an increase in left atrial pressure became apparent. This reached a significant level in a part of those groups exposed to ischemia for 15, 30, and 90 min, respectively (Table 2).

In sharply demarcated, scattered areas in the histological sections of the hearts, a loss of the hypoxia-specific fluorescence of NADH was found. This change occurred to a much smaller degree in the hearts exposed only to ischemia than in the reperfused organs. In the latter, the loss of fluorescence covered a much larger fraction of the area at risk (Fig. 5). The point-counting technique revealed the loss of NADH fluorescence in 0.22 ± 0.12% of the area at risk in the non-reperfused hearts, which had been exposed to ischemia for 15 min. This fraction was 43.0 ± 5.0% in the organs reperfused for 14 min after an ischemic period of the same duration. When reflow was extended to 60 min, the latter fraction was found to be even larger (55.9 ± 5.0% of the area at risk). The data in the hearts exposed to 30, 45, and 90 min of ischemia, with and without 14 min of reflow, were 5.7 ± 2.7% vs. 64.6 ± 2.9%, 5.6 ± 1.2% vs. 66.0 ± 7.5%, and 39.3 ± 5.5% vs. 86.7 ± 1.8%, respectively (Fig. 6).

The latter data revealed not only a highly significant difference between nonreperfused and reperfused hearts but also showed that the differences between these states were most evident for periods of ischemia ≤45 min. These differences became less obvious but did not disappear completely when this period was prolonged. The ratios of affected tissue in the ischemic versus ischemic-reperfused organs were 0.005, 0.088,
0.085, and 0.419 in the hearts subjected to ischemia for 15, 30, 45, and 90 min, respectively.

When we examined the localization of those myocytes that had lost the hypoxia-specific increase in NADH fluorescence, especially in the hearts exposed to 30 or 45 min of ischemia without reflow, a phenomenon became evident that was unexpected based on prior theoretical considerations with regard to supply conditions. Reductions in NADH fluorescence appeared to concentrate at the periphery of the infarct zone. In close proximity to the endocardial as well as epicardial surface of the heart, NADH-reduced cells often formed seemingly long columns of affected cells that were aligned parallel to the myocardial wall. In the lateral border zones such an increase also became evident. In other words, the effect described apparently developed least often in the central region of the area at risk (Figs. 5 and 7).

For quantification of this phenomenon, the myocardial wall was divided into three layers of equal width: subepicardial, midwall, and subendocardial. The two regions in direct proximity to the nonischemic myocardial wall (the lateral border zones) were considered to be separate areas. This approach revealed that the observed effect was indeed most pronounced in the 45-min ischemic-nonreperfused hearts (Fig. 8). In this subgroup, the fraction of cells lacking an increase in NADH fluorescence amounted to $0.76 \pm 0.35\%$ in the midwall area. In contrast, the remaining zones consistently displayed higher fractions: subendocardium, $7.3 \pm 1.8\%$; subepicardium, $6.9 \pm 1.7\%$; and anterior and posterior lateral border zones, $8.0 \pm 2.1\%$ and $7.9 \pm 2.2\%$, respectively.

Apparently, because the chance of hitting upon a NADH-reduced cell during the early state of ischemia was simply too low in the 15-min experiments, no spatial preference was detected in this group. On the other hand, when the period of ischemia was extended to 90 min, localized reductions in NADH fluorescence were quite often observed in all layers of the heart, thus revealing a nonsignificant tendency toward a preference for the marginal regions of the infarct (Figs. 7 and 8).

When the ischemic zone was reperfused, no corresponding preference of damage was observed. There was a slightly larger amount of tissue showing a loss of NADH in the midzones as a result of the fact that the small layers of tissue beneath the epicardium and endocardium were constantly excluded from injury be-

![Fig. 4. Electron micrographs demonstrating the degree of ultrastructural damage in three zones of ischemic-nonreperfused myocardium. A: zone I. The contraction state of myofibrillar sarcomeres shows overcontraction with compression of A-bands and thickening of Z-bands (arrows). B: zone II. Severe distortion of sarcomeres with local rupture of myofilaments (arrows) is shown. C: zone III. Hypercontraction of sarcomeres with local clumping of myofilaments (arrows) is shown.](http://ajpheart.physiology.org/)

### Table 1. Assessment of ultrastructural damage of ischemia- and ischemia-reperfusion-exposed hearts

<table>
<thead>
<tr>
<th></th>
<th>Mitochondria</th>
<th>Contraction State</th>
<th>Endothelium</th>
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<tbody>
<tr>
<td>45-min Ischemia</td>
<td></td>
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</tr>
<tr>
<td>Zone I</td>
<td>2.13 ± 0.15</td>
<td>4.10 ± 0.17</td>
<td>2.17 ± 0.21</td>
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<tr>
<td>Zone II</td>
<td>3.47 ± 0.23</td>
<td>5.87 ± 0.23</td>
<td>3.57 ± 0.21</td>
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<tr>
<td>Zone III</td>
<td>2.67 ± 0.57</td>
<td>5.13 ± 0.51</td>
<td>2.80 ± 0.00</td>
</tr>
<tr>
<td>45-min Ischemia and 60-min reperfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone I</td>
<td>3.33 ± 0.21</td>
<td>4.97 ± 0.21</td>
<td>3.17 ± 0.25</td>
</tr>
<tr>
<td>Zone II</td>
<td>4.00 ± 0.00</td>
<td>5.93 ± 0.12</td>
<td>3.87 ± 0.23</td>
</tr>
<tr>
<td>Zone III</td>
<td>3.60 ± 0.53</td>
<td>5.53 ± 0.38</td>
<td>3.67 ± 0.32</td>
</tr>
</tbody>
</table>

Data are given as means ± SD; 10 test fields were investigated per zone; each group underwent 3 experiments. See METHODS for details of the scoring system.
cause of the supply of oxygen from environmental air
and left ventricular blood, respectively.

**DISCUSSION**

The aim of the present investigation was to improve
our understanding of processes that initiate damage
during regional myocardial ischemia. As a basic indicator
of altered cellular viability, losses of NADH fluorescence were studied in histological sections of the heart.

**What Does a Lack of NADH Fluorescence in Ischemic Myocytes Imply?**

Hypoxic, but still vital, myocytes develop blue autofluorescence because the lack of oxygen causes an interruption of oxidative phosphorylation; this in turn leads to almost complete conversion of the nonfluorescent NAD to its reduced, intensely blue fluorescent form: NADH. The absence of this effect may be caused by the following mechanism. An ischemia-induced decrease in ATP production causes a rise in intracellular calcium, which then leads to the opening of large permeability transition pores in the mitochondria (e.g., Refs. 7 and 15). Via these nonspecific pores, mitochondria not only lose small ions, and thereby the ability to separate charges and to maintain membrane polarizations, but, in addition, they also lose essential substrates, e.g., NAD/NADH. These pyridine nucleotides are hydrolyzed by glycohydrolases outside the matrix space. This occurrence causes a decrease in cellular blue autofluorescence. Because these changes have been observed during the transitional period before irreversible damage, a lack of NADH fluorescence in hypoxic myocytes has been viewed as a characteristic sign of severe cellular injury (7).

**How Do the Missing NADH Fluorescence and Lack of Tetrazolium Staining Correlate With Each Other?**

It is interesting to note that the tetrazolium technique, the most commonly used method of detection of myocardial infarction, is also based on the loss of, for the most part, intracellular NAD/NADH. Cells become unable to generate the tetrazolium dye when depleted of these substrates. They serve as essential coenzymes in the aforementioned reaction. In the reperfused state, in which the substrates have been completely washed out, decreased tetrazolium staining and NADH fluorescence would be expected in identical areas of the myocardium. In the present study, the extent of the respective zones was indeed parallel in hearts

**Table 2. General hemodynamic data in the experiments on myocardial ischemia of various periods of duration with or without reperfusion**

<table>
<thead>
<tr>
<th>Time 1</th>
<th>Time 2</th>
<th>Time 3</th>
</tr>
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<tbody>
<tr>
<td>15-min Ischemia</td>
<td>15-min Ischemia and 14-min reperfusion</td>
<td>15-min Ischemia and 60-min reperfusion (n = 4)</td>
</tr>
<tr>
<td>Arterial Blood Pressure, mmHg</td>
<td>Heart Rate, beats/min</td>
<td>Left Arterial Pressure, mmHg</td>
</tr>
<tr>
<td>15-min Ischemia</td>
<td>15-min Ischemia and 14-min reperfusion</td>
<td>15-min Ischemia and 60-min reperfusion (n = 4)</td>
</tr>
<tr>
<td>Time 1</td>
<td>Time 2</td>
<td>Time 3</td>
</tr>
<tr>
<td>124 ± 7/82 ± 8</td>
<td>367 ± 7</td>
<td>349 ± 9</td>
</tr>
<tr>
<td>132 ± 10/85 ± 10</td>
<td>371 ± 14</td>
<td>6.6 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; unless otherwise stated, n = 6 per group. Data refer to the time directly before the induction of ischemia (time 1), at the end of ischemia (time 2), and at the end of reperfusion (time 3). *Significantly different (U-test) versus the preconditioning value (time 1).
exposed to longer periods of ischemia followed by reperfusion. A discrepancy seems to exist only with respect to shorter periods of flow interruption. In hearts subjected to ischemia/reflow for periods as short as 15 min/14 min, relatively large areas of lower NADH fluorescence were observed; however, whenever the tetrazolium method has been used, most investigators have not found signs of damage after these short periods of ischemia (e.g., Ref. 16). The latter observations mostly refer to studies performed in larger species, which have a lower metabolic rate and in which any tissue damage thus occurs more slowly. Other authors who have also used rats (41) have also observed staining defects even after 20 min of ischemia followed by reflow. It should be noted that they found a lower degree of defects than we established under corresponding conditions in NADH fluorescence. In a pilot experiment, we were able to confirm that tetrazolium-negative areas also arose after such short periods of ischemia/reflow.

Another factor might be playing a role here as well. Tetrazolium staining only works when the activity of specific dehydrogenases is intact (e.g., Refs. 1 and 10).

It has been observed that the decline in NAD/NADH content precedes the functional failure of the enzyme (19); this effect might contribute to a higher sensitivity of NADH fluorescence detection. Application of the latter method in histological sections further accentuates the difference between it and the macroscopic tetrazolium technique.

There is general agreement that the tetrazolium method is unsuited for the detection of damage in nonreperfused myocardium except under vascular occlusion of extreme duration (e.g., Refs. 3 and 10). For this reason, a comparison of both methods has not been performed in the present study under such conditions.

**What Level of Injury Is Indicated by a Loss of NADH Fluorescence?**

The current electron microscopic observations support the view that tissue damage was highest in the areas in which losses of NADH fluorescence were most pronounced. Disruptions of mitochondrial cristae and sarcomere hypercontractions, typical signs of ischemic damage (22, 36, 37), were most often found by our “blind” observer close to the outermost layers of the area at risk. These were the same areas in which reductions in NADH fluorescence were most often observed. A more differentiated evaluation, e.g., a cell-by-cell comparison was, however, not possible due to limitations in tissue fixation. In order not to induce artifacts as a result of unwanted reoxygenation of the tissue, it was not possible to apply perfusion-fixation, the best procedure for preserving the in vivo state. Block fixation, as currently used, induces an inevitable delay in fixation; this also causes mild signs of disturbance in basal tissue integrity in the nonoccluded areas. Still, the most important parameter, the regional differences in tissue injury, was not affected by the aforementioned limitations, thus supporting the conclusion that a specific regional injury must have occurred.

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**Fig. 5.** Photomicrograph of myocardium subjected to regional ischemia for 45 min without reflow (A) and to 45 min of ischemia and 14 min of reflow (B). Blue fluorescence is due to myocyte NADH; green fluorescence is due to FITC injected intravenously.

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**Fig. 6.** Extension of NADH-reduced cells in sections of the heart subjected to regional ischemia for different periods of time. In one-half of the cases, ischemia was followed by reflow for 14 min. Values are expressed as a percentage of the area at risk.
The nonelectron-optic methods for quantifying tissue injury are generally based on the detection of pathological plasmalemmal permeability, indicated by the intracellular uptake of tracers that are normally cell impermeant. The penetration of horseradish peroxidase (e.g., Refs. 8 and 41) or propidium iodide into the intracellular space is considered to be an indication of cellular injury and death (6, 8, 16, 17). Against this background, the uptake of propidium iodide, combined with the loss of NADH fluorescence, points to severe membrane disturbances and irreversible cellular damage in those cells.

It should be emphasized that, in the nonreperfused myocardium, only part of those cells that developed a lower NADH fluorescence also revealed a permeabilization to propidium iodide. A similar finding was reported by Takashi and Ashraf (41) in that only a fraction of tetrazolium-negative cells were found to have also taken up the horseradish peroxidase marker. These differences may be explained by disturbances in mitochondrial function and a loss of NADH that preceded changes in plasmamembrane permeability. Through the use of distribution dyes (e.g., propidium iodide) in studies (5, 26) on isolated myocytes and hepatocytes, a breakdown of the mitochondrial membrane potential and a loss of NADH fluorescence have distinctly preceded the loss of sarcolemmal barrier function during chemically simulated hypoxia.

Does Reperfusion Induce an Expansion of Damage?

Studies on isolated myocytes have clearly shown that energy deprivation causes increasing deterioration of the mitochondrial and plasmalemmal integrity of the cell and that resupplying energy induces great stress to such a cell. Calcium and sodium overload lead to hypercontraction and membrane disruption when the injury due to energy failure has surpassed the level at which reversibility is still possible.

On the basis of these data, the results of the numerous positive interventional studies might be explained by the mitigation of reenergization-induced stress, which thus ensured the survival of cells that had already incurred damage, which would otherwise have caused necrosis. The fact, however, that tissue that only displayed signs of reversible damage but that nevertheless became necrotic upon reflow apparently requires a different explanation.

The concept of spreading necrosis offers an interpretation of a reperfusion-induced transfer from an intact state to irreversible injury (14). It has been hypothesized that localized irreversible damage arises during ischemia and that injured hypercontracting myocytes transfer their membrane disturbances to neighboring cells. The question is whether the present findings support this hypothesis. In the ischemic hearts, uptake of propidium iodide has only been observed in part of the areas of lower NADH fluorescence, which means that propidium-negative cells with lower NADH fluorescence have been only moderately affected. An argument against the possibility that necrosis may have...
spread might be found in the assumption that only the areas of slightly injured cells expanded, whereas severe damage, indicated by the uptake of propidium iodide, did not, in fact, increase upon reflow. Just the opposite proved, however, to be the case. In the reperfused state not only did the losses of NADH expand, but also almost every cell that lost NADH also took up propidium iodide. (The especially high fluorescence intensity of these cells may simply be the result of the re instituted exchange of the extracellular fluid, which not only completed the removal of NADH, but also provided a practically unlimited supply of propidium iodide that could enter into these cells.) These findings clearly support the view that a real expansion of injury occurred upon transition to reperfusion. This clearly supports the possibility of an expanding necrosis.

What Could Be the Cause of the Obvious Localizations in Low NADH Fluorescence?

In the present study, it was most striking to find that the earliest signs of ischemic damage were concentrated at the periphery of the areas at risk. Apparently, despite the fact that each myocyte had been exposed to an interruption in its perfusion, effects of quite a different dimension had occurred.

Few observations have been reported in the literature that also point to such an obvious localization of ischemia-induced lesions. Certain activated complement factors (C3, C4, and C5), proteins that initiate nonspecific inflammation and ultimately cellular destruction, have been found to preferentially bind to the subepicardium and subendocardium in experimental coronary occlusion. The concentrations tend to decrease progressively toward the midwall region of a transmural infarction (24). In parallel, the highest concentrations of the cytolytic terminal complement complex have been observed in the marginal zones of human infarctions (34).

Interconnected netlike structures of contraction-band necrosis (29, 30, 39), which led to the hypothesis of an expanding necrosis, were not found to be randomly distributed within the areas at risk but rather concentrated in the border zones of reperfused hearts. It can be speculated that these effects, although observed in reperfused hearts, might have originated from areas of decreased NADH fluorescence as presently described for the nonreperfused state.

What Mechanism Could Have Initiated the Localized Damage?

With respect to the mechanism and significance of the pointal localization of cellular injury within infarctions, the following working hypothesis is proposed. The failure of regional perfusion leads to a drastic decline in oxygen concentrations, but traces of oxygen still diffuse from the surroundings of the area at risk toward its interior. These quantities are not sufficient enough to sustain oxidative phosphorylation, but in this state a cyanide-insensitive NADH oxidase may still be active (43), and some oxidation may still take place independently of the respiratory chain (32). In contrast to regular supply conditions, however, the activity of this enzyme is linked to the generation of superoxide radicals and hydrogen peroxide (27, 43). These toxic metabolites, detected in ischemic myocytes (42), persist in the tissue because the activity of radical scavenging systems, e.g., glutathione peroxidase, is depressed in this state (38). Because oxygen from the surroundings of the infarct is considered to be the source of these radicals, effects on myocyte viability are expected to be greater in the border zones than at the center of the ischemic area. The activation of NADH oxidase has indeed been found to occur most notably in the tissue surrounding the infarct zone (43); this finding also appears logical in light of the losses of NADH fluorescence observed mostly in the outer regions of the ischemic zone. Interestingly, it is in line with this view that isolated myocytes exposed to traces of oxygen were found to lose their mitochondrial membrane polarization and overall viability more rapidly than cells subjected to complete anoxia (21).

In conclusion, the changes observed in NADH fluorescence within the ischemic myocardium support the view that a large fraction of tissue initially remains intact during ischemia, whereas only a small number of cells develop severe, apparently irreversible, damage during this phase. It appears that such damage is first greatly expanded as a result of reperfusion. The initial lesions observed in the periphery of the infarct are assumedly initiated by traces of oxygen from the surrounding tissue and act as sources of necrosis, which spreads into viable myocardium upon reperfusion. This mechanism would seem to be of significance for interventional studies whose aim is to limit the size of myocardial infarctions.

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REFERENCES

6. Communal C, Verdetti J, Estrade C, Humbert T, and Demenge P. Heterogeneous distribution of a fatty acid analogue...