Altered Distribution of Lysosomal Cathepsin D in Ischemic Myocardium

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ABSTRACT To determine the influence of cardiac ischemia on the activity and subcellular localization of lysosomal cathepsin D, anesthetized rabbits were subjected to ligation of the circumflex coronary artery. Total enzyme activity remained unchanged throughout the 2-h ischemic period, but the subcellular distribution of cathepsin D, as analyzed by biochemical and immunohistochemical techniques, was altered dramatically. A marked increase in nonsedimentable (i.e., 40,000-g supernate) activity developed by 30–45 min and increased further by 2 h. Simultaneously, the immunofluorescent localization of cathepsin D was also changed significantly. Within 30–60 min after occlusion, the fine, particulate staining observed in control myocytes was replaced by bright fluorescent patches composed of large granules. Many of these structures displayed prominent halos of diffuse fluorescent staining in the neighboring cytoplasm, apparently outside lysosomes per se. After 2 h, when nonsedimentable activity was maximally elevated, most of the fluorescent particles had disappeared completely. During this same interim there was no detectable change in the distribution of lysosomal cathepsin D within interstitial cells. These results are consistent with the hypothesis that an early feature of cardiac ischemia is the release of cathepsin D from myocytic lysosomes into the cytosol of damaged cells.

INTRODUCTION

The early biochemical observation that liver ischemia is accompanied by alterations in hepatic lysosomal integrity generated the notion that the release of acid hydrolases into the cytoplasm of acidic and hypoxic cells may precipitate intracellular damage and, ultimately, cell death (1–3). Myocardial ischemia has, likewise, been shown to induce analogous alterations in cardiac lysosomes in some (but not all) studies (see 4 for review). Homogenates of ischemic myocardial tissue have revealed a significant redistribution of hydrolytic enzyme activity from the particulate (or sedimentable) state to the soluble (or nonsedimentable) state in several experiments (5–10), while depletion of membrane-bound acid phosphatase and aryl sulfatase activity has been demonstrated cytochemically (9–11). Based on these changes, it has been postulated that the intracellular release of lysosomal enzymes and their subsequent extralysosomal activity may exercise a pivotal role in the progressive modifications that lead from reversible myocardial ischemia to irreversible infarction.

Although support for the lysosomal hypothesis is attractive, definitive corroboration has been lacking (see 4 for a detailed discussion). Briefly, resolution of the question has been hindered by several factors: (a) traditional methods of homogenizing tissues for subsequent analysis of compartmentalization of lysosomal enzymes, which were originally developed for liver, are not optimal for muscular tissues (12, 13); (b) variations in the distribution of lysosomal enzymes may simply reflect changes in the susceptibility of lysosomes to damage during homogenization and

not a true in vivo release (14); (c) biochemical analyses cannot differentiate changes in myocytes (the cells of primary interest) from changes in interstitial cells; (d) cytochemical observations on the distribution of acid phosphatase in ischemic heart, although of considerable interest, are inevitably somewhat limited in scope, since acid phosphatase is unlikely to be involved importantly in cellular breakdown, since only a fraction of myocytic lysosomes contain demonstrable acid phosphatase (15), and since much acid phosphatase activity as assayed biochemically and histochemically resides outside typical myocytic lysosomes (4, 10, 11, 16-18); and (e) most importantly, until now, published studies have concentrated on changes that appear fairly late after coronary occlusion (i.e., 1 h or more), at a time when irreversible necrosis can be expected already to have appeared in most cells (19-21) and, thus, at a time when it is impossible to differentiate lysosomal changes that are the result of necrosis from changes that might mediate necrosis.

The present study was initiated to reinvestigate the question of how and when cardiac ischemia can alter myocytic lysosomes by taking advantage of two newly developed techniques for studying the distribution of lysosomal cathepsin D, the major acid proteinase of the heart. First, an improved method for homogenizing myocardium was employed to determine bio-chemically the distribution of enzyme activity between particulate and supernatant fractions (13). Second, an immunohistochemical method for localizing cathepsin D in cells and tissues (22-24) was utilized to identify the morphological distribution of the enzyme.

METHODS

Animal care. New Zealand white male rabbits, aged 6-12 wk (body wt = 1.5-2.0 kg), were fed Wayne rabbit chow (Wayne Lab-Blox, Allied Mills, Inc., Chicago, Ill.) ad libitum. The rabbits were anesthetized with sodium pentobarbital (30 mg/kg) and ventilated with a Harvard respirator (Harvard Apparatus Co., Inc., Millis, Mass.). Blood gas partial pressures and pH were maintained within normal ranges throughout the experiments. The chest was opened to expose the left atrium and ventricle, and a 3-0 silk ligature was tied around the circumflex branch of the left coronary artery. The artery was ligated for up to 2 h, and the ischemic area was located visually.

At 0, 30, 45, 60, and 120 min after arterial occlusion, the heart was excised and ischemic tissue displaying cyanosis was removed from the center of the area supplied by the ligated artery. A sample of distant, presumably nonischemic, left ventricle that appeared grossly normal was removed simultaneously, as was a portion of the right ventricle. Left ventricular tissue in the area of circumflex artery perfusion was also obtained under identical conditions from sham-operated control rabbits which had not been ligated. The tissue samples were immediately placed in ice-cold 0.25 M KCl and half of each sample was homogenized and assayed for cathepsin D and protein, while the other half was used for immunofluorescent and electron microscopy.

Fractionation of heart. Ischemic and control tissues were placed in 10 vol of 0.25 M KCl and 1 mM EDTA buffered with 0.05 M Tris (pH 7.4) and homogenized for 30 s at 4°C in a Wilems Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.) set to rotate at a slow speed (13). The homogenate was centrifuged at 350 g for 5 min and the pellet, which contained nuclei, tissue fragments, and cell debris, was discarded. The supernate was resuspended at 40,000 g for 20 min. The second pellet, which contained intact lysosomes, mitochondria, and other organelles, was resuspended in 0.1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) in 0.25 M KCl and vigorously homogenized to release lysosomal enzyme activity. This fraction, termed the “sedimentable” fraction, was assayed for cathepsin D activity as described below. The 40,000 g supernate, termed the “nonsedimentable” fraction, was assayed similarly. Results were expressed as the proportion of the 350-g supernate that remained in the 40,000-g supernate (i.e., the ratio of the nonsedimentable to the nonsedimentable-plus-sedimentable activity).

Assay of cathepsin D. Cathepsin D activity was assayed by the method described by Barrett (25), which is a measure of PCA-soluble, Poli-reactive products of hemoglobin digestion at pH 3.0. Protein was determined by the method of Lowry et al. (26). Analyses of statistical significance for differences between ischemic and control samples were made by Student’s two-tailed t test for unpaired samples.

Immunofluorescent localization of cathepsin D. Trans-}

mural blocks of ischemic and control tissue were immersed in a mixture of 7% (wt/vol) gelatin and 150 mM NaCl at 30°C and immediately frozen for 30 s with liquid nitrogen. Frozen sections (4-6 mm thick) were cut parallel to the outer surface of the wall of the ventricle and fixed for 5 min in 4% (wt/vol) formaldehyde in phosphate-buffered saline (PBS), pH 7.4, freshly prepared from paraformaldehyde. Sections were washed in PBS containing 5 mM cysteine for at least 1 h before use.

Cathepsin D was localized by either direct or indirect immunofluorescence methods, as described in detail previously (23), with a specific sheep antiserum for cathepsin D and Fab’ antibody subunits to ensure penetration of fixed cell membranes and to minimize nonspecific binding (22, 24). In the direct method, sections were first treated with sheep anti-(rabbit cathepsin D) Fab’ (SaRD Fab’) or nonimmune sheep Fab’ (Ns Fab’) at 5 mg/ml and subse-}

quently stained after washing with fluoresceinated SaRD Fab’ at 1 mg/ml. Prior treatment with SaRD Fab’ (but not Ns Fab’) produced a saturation of antigenic sites on cathepsin D with nonfluoresceinated antibody Fab’, which served to prohibit subsequent specific binding of fluorescent antibody to the enzyme; thus, these sections served as controls for excess nonspecific staining. In test sections, which were initially treated with Ns Fab’, fluoresceinated SaRD Fab’ was able to attach specifically to cathepsin D in the tissue and, thus, to allow identification of its precise subcellular location. With the indirect method, sections were initially exposed to SaRD Fab’ (test sections), or Ns Fab’ (control for nonspecific staining). Subsequently, the sections were treated with fluoresceinated pig anti-(sheep Fab’) Fab’ at 0.2 mg/ml to identify specific sites revealing cathepsin D.

1 Abbreviations used in this paper: Ns Fab’, nonimmune sheep Fab’; PBS, phosphate-buffered saline; SaRD Fab’, sheep anti-(rabbit cathepsin D) Fab’.

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With both techniques, sections were exposed to reagents for 45 min at room temperature and immediately washed with PBS containing 5 mM cysteine which was present throughout staining and washing to maintain the monovalency of Fab' antibodies. Finally, sections were washed, mounted, and examined with a Leitz Orthoplan fluorescent microscope (E. Leitz, Inc., Rocheligh, N. J.) linked with a Ploem incident illumination and a 200-W mercury vapor lamp.

Electron microscopy. Small samples of tissue were immediately fixed in 3% glutaraldehyde buffered in 0.1 M cacodylate (pH 7.4) for 3 h and then washed overnight at 4°C in 0.1 M buffer with 7% (wt/vol) sucrose. Sections were postfixed in 1% OsO4 and stained “in block” with uranyl acetate. The tissue was then dehydrated and embedded in Epon 812 (Shell Chemical Co., New York). Ultrathin sections were cut and stained with aqueous uranyl acetate and lead citrate and viewed in a Philips 200 electron microscope (Philips Electronic Instruments, Inc., Mt. Vernon, N. Y.).

RESULTS

Biochemical analyses of cathepsin D activity. Total (i.e., sedimentable-plus-nonsedimentable) specific activity of cardiac cathepsin D remained unchanged throughout the 2-h experimental period, both in ischemic and control tissues, but marked modifications developed in the distribution of activity within the ischemic homogenates (Table I). In the left ventricular tissue from sham-operated controls, the ratio of nonsedimentable to total activity (activation ratio) averaged 0.25 (range 0.17–0.33); that is, only 25% of the cathepsin D activity was nonsedimentable. In contrast, in ischemic tissue, the ratio rose to 0.35–0.40 after 30–45 min, and by 2 h over 50% of the cathepsin D activity was nonsedimentable (Table I).

The differences in activation ratios between ischemic tissue and matched control tissues (from “nonischemic” left ventricle, right ventricle, or sham-operated animals) at equivalent times were statistically significant in all instances.

Immunofluorescent distribution of cathepsin D. Sections of sham-operated left ventricle treated with nonimmune sheep Fab' exhibited only a weak, diffuse staining after subsequent incubation with fluoresceinated pig anti-(sheep Fab') Fab' (Fig. 1), demonstrating no obvious nonspecific staining. In contrast, when an adjacent section was reacted with sheep anti-(rabbit cathepsin D) Fab' instead of Ns Fab', green particulate cytoplasmic fluorescence was revealed, primarily in the paranuclear regions of myocardial cells (Fig. 2). The diffuse cathepsin D staining which radiates away from the nuclear poles (Fig. 2) may reflect enzyme that is housed in the sarcoplasmic reticulum and the Golgi complex (9-11, 15); however, definitive conclusions must await the adaptation of our technique for the electron microscope. Interstitial cells displayed similar intense particulate staining but exhibited no diffuse cytoplasmic fluorescence. This staining pattern, which represented the sites of lysosomal cathepsin D (22-24), was the predominant characteristic of all nonischemic tissues (left ventricle, right ventricle, and sham-operated controls) throughout the experimental period.

By 30–45 min after circumflex artery ligation, the intensity and distribution of cathepsin D fluorescence in the ischemic area was graphically altered in all areas, from endocardium to epicardium. Intense patches of fluorescence were visualized in the paranuclear region of most myocytes (Fig. 3). Examination of such regions at somewhat higher magnification disclosed numerous granules, many of which were markedly enlarged compared to control tissues. Around the larger stained lysosomes, intense halos of diffuse fluorescent material frequently could be detected (Fig. 4); such halos, which suggest the presence of enzyme outside secondary lysosomal particles, were never observed in sham-operated control hearts or in right ventricular tissue from the experimental animals. Such observations were made only rarely in the nonischemic left ventricular sections from hearts with coronary ligation.

Unlike myocytes, interstitial cells displayed no visible alteration in the pattern of staining for cathepsin D during early ischemia. The fluorescent halos that were so prominent in ischemic muscle cells were never detected in interstitial cells, even in the same sections of ischemic tissue (Fig. 5). After 2 h, some depletion in the number of organelles which stained for cathepsin D in interstitial cells had occurred, but the change was not as severe as in myocytes.

TABLE I

<table>
<thead>
<tr>
<th>Non sedimentable cathepsin D activity</th>
<th>%</th>
<th>P</th>
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<tr>
<td>Sham-operated controls</td>
<td>19</td>
<td>25±1.6</td>
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<tr>
<td>Ischemia</td>
<td></td>
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<tr>
<td>30 min</td>
<td>7</td>
<td>35±2.9</td>
</tr>
<tr>
<td>45 min</td>
<td>7</td>
<td>40±1.9</td>
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<td>120 min</td>
<td>5</td>
<td>51±5.3</td>
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Control left ventricular tissues were obtained 30–120 min after completion of surgery in open-chest, anesthetized rabbits that did not undergo coronary ligation; control values were pooled since there were no significant changes with time. Values are expressed as means±1 SEM.
most completely devoid of immunofluorescent cathepsin-D-containing particles, with only a few brightly stained lysosomes discernible (Fig. 6). Some cells displayed increased background staining for cathepsin D, as if enzyme had been released from lysosomes and was now diffusely distributed among cellular elements; however, it is inevitably difficult with our present technology to distinguish this feature from inherent tissue autofluorescence.

It could be argued that the diffuse extralysosomal staining in the paranuclear regions of ischemic myocytes might have resulted from an artifactual release of cathepsin D through damaged lysosomal membranes during tissue preparation in vitro rather than from translocation of the enzyme in vivo. Accordingly, several alternative freezing procedures were employed to test the extent to which the observed changes might have been influenced by freeze-related factors. First, tiny pieces of myocardium (2 mm in size) were frozen in Freon 22 (Virginia Chemicals, Inc., Portsmouth, Va.) cooled at liquid nitrogen temperatures. This procedure is known to quench tissue much more rapidly and uniformly than liquid nitrogen (27). Second, myocardium was immersed in either 10% or 20% (vol/vol) glycerol or 10% (vol/vol) dimethylsulfoxide in PBS for 30 min and then frozen in Freon 22 in liquid nitrogen. Since both these cryoprotectants inhibit ice crystal formation during freezing, they should theoretically prevent damage to cell membranes and thereby minimize freezing artifacts (27). In each instance, the picture of cathepsin D staining remained unaltered; i.e., perilyosomal halos were still prominent in ischemic myocytes, whereas control myocardial cells exhibited particulate staining for the enzyme. Finally, small pieces of tissue were fixed for 30 min in 4% formaldehyde with 10% dimethylsulfoxide buffered in PBS and frozen in liquid nitrogen or Freon 22. Although this 30-min fixation reduced the intensity of the fluorescent staining, presumably because of denaturation of the cathepsin D antigenic binding sites, the distribution of cathepsin D appeared identical to that described previously. Because of the consistency of the observed changes, no matter what preparative techniques were employed, we believe that the observed redistribution of cathepsin D during ischemia had transpired in vivo and was not artifactual.

**Morphology of ischemic myocytes.** To establish the extent of damage that had been suffered by tissue that displayed translocation of cathepsin D, adjacent sections were prepared for routine electron microscopy. Although characteristic ultrastructural signs of irreversible necrosis, as described by Jennings and his collaborators (19-21), were present after 1-2 h, sections taken at 30-45 min revealed changes indicative only of reversible injury when compared to

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**FIGURE 1** A section from a sham-operated left ventricle treated with nonimmune sheep serum Fab' followed by pig anti-(sheep Fab') Fab' labeled with fluorescein isothiocyanate does not reveal cathepsin D-containing granules. Magnification ×400.

**FIGURE 2** A sham-operated left ventricular section incubated in sheep anti-(rabbit cathepsin D) Fab' followed by pig anti-(sheep Fab') Fab' labeled with fluorescein isothiocyanate. Particulate fluorescence is prominent in the paranuclear (N) regions of the myocytes, revealing cathepsin D in secondary lysosomes. Magnification ×600.
sham-operated animals (Figs. 7 and 8). Thus, as at equivalent times in dogs (19-21), cells that had been ischemic for 30-45 min had swollen mitochondria and some glycogen depletion, but definite signs of necrosis such as osmiophilic densities in mitochondria, near-complete glycogen depletion, and a leaching out of cytoplasmic matrix were not yet apparent (Fig. 8), suggesting that the damage was not yet irreversible (19-21). Lysosomal structures were enlarged, but their membranes appeared intact. (Fig. 8). After 1 h and, especially, 2 h of ischemia, evidence of severe injury and necrosis was apparent in many cells. The cytoplasmic matrix was poorly resolved, glycogen stores were nearly exhausted, nuclei were pyknotic, and mitochondria were swollen.

FIGURE 3 A section from a 45-min ischemic left ventricle incubated for staining of cathepsin D as in Fig. 2. Bright patches of fluorescence are readily apparent in the paranuclear (N) cytoplasm. Magnification x600.

FIGURE 4 Two ischemic myocytes, stained as in Fig. 2, are depicted here 45 min after coronary artery ligation. Several large granules are encompassed by brilliant fluorescent halos (arrows). Magnification x1,000.

FIGURE 5 After staining for cathepsin D as in Fig. 2, interstitial cells (arrow) can be seen in the ischemic heart (60 min), but their fluorescent granules appear normal, without halos. Two nearby myocytes (M) exhibit extensive fluorescent staining, with halo formation. Magnification x600.

FIGURE 6 Few fluorescent granules can be demonstrated in 2-h ischemically damaged myocytes. Only a diffuse fluorescence is observed around the nuclei (N). Magnification x600.

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and displayed osmiophilic densities (Fig. 9). There was a decrease in the number of visible lysosomal structures and by 2 h they were infrequently observed.

DISCUSSION
Until now, significant and consistent lysosomal alterations during myocardial ischemia in vivo have

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been documented only 1 h or more after the onset of coronary occlusion, at a time when irreversible injury and necrosis have already developed in many of the affected cells (19–21). Consequently it has been impossible to ascertain whether the lysosomal changes participate in causing the progression of potentially irreversible injury to necrosis, or whether lysosomal abnormalities are merely the passive result of lethal damage that has already transpired. Elucidation of the precise role of lysosomal alterations in the progression

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2 h after occluding the circumflex coronary artery mitochondria (m) become severely dilated, with most exhibiting osmiophilic densities and little matrix. Cytoplasmic ground substance is reduced significantly, little glycogen is apparent, and nuclei (N) are pyknotic. Sarcomeres (S) appear contorted at this time and many reveal Z bands that are extremely electron dense. Lysosomal images are rare after 2 h of ischemia. Magnification ×20,000.

of infarction has been further hindered because evidence obtained solely by biochemical means (5–9) has failed to differentiate changes that might have occurred in myocytes per se from changes in interstitial cells. The latter, although of interest in their own right, are of less immediate relevance to the critical question of what factors influence the development of myocellular injury and death after coronary
The precise identification of the anatomical location of occlusion. A fuller understanding of the nature and implications of the lysosomal changes demands a precise identification of the anatomical location of the changes at the cellular level. Previous investigations have focused primarily on electron microscopic identification of acid phosphatase and aryl sulfatase (9-11). These studies have provided interesting supportive results, but the selection of these enzymes are in some ways less than optimal for the reasons cited earlier.

In the present study, newly developed biochemical and immunohistochemical techniques were employed to obviate some of these problems and to focus on changes involving cathepsin D, a lysosomal proteolytic enzyme that seems likely to be involved (if any lysosomal enzymes are) in tissue destruction under ischemic severely acidic conditions. First, an improved method for homogenizing cardiac tissue to yield maximum numbers of intact lysosomes (13) was utilized. This technique, which uses KCl rather than the conventional sucrose as the homogenizing solution, provides lower values for nonsedimentable cathepsin D activity than can be obtained otherwise (13) and, thus, makes it easier to observe small changes early in the course of ischemia. Second, a mono-specific antiserum to cathepsin D was employed to distinguish changes occurring in ischemic myocytes from those in interstitial cells (23).

Although the specific activity of total cathepsin D does not fluctuate meaningfully after ischemia, marked changes are observed in its subcellular distribution. By 30-45 min after ligation of the circumflex artery, a significant decrease in particulate enzyme activity is detectable biochemically. At the same time, enlarged granules with diffuse fluorescent halos populate ischemic myocytes. It seems plausible that these halos, which become increasingly prominent between 30 and 60 min after occlusion, may represent a morphological depiction of lysosomal damage. As has also been observed by others (21), electron micrographs of tissue prepared at the same interval only rarely reveal broken lysosomal membranes; therefore, it seems probable that, as a result of changes in membrane permeability, the loss of enzyme from lysosomes may precede the detectable morphological loss of lysosomal membrane integrity (28). The biochemically detectable redistribution of cathepsin D activity from the particulate to the nonsedimentable fraction of the tissue homogenates may be due both to a release of soluble enzyme in vivo and to the lysosomal population harboring this enzyme becoming more fragile and more readily damaged by homogenization (14).

Others previously have described changes after 1 or more h of cardiac ischemia in lysosomal enzyme availability, as reflected by alterations in the proportion of enzyme present in nonsedimentable fractions (5-10), as well as depletion of acid phosphatase stores as demonstrated histochemically (9-11). Our results support these findings and extend them to illustrate that significant changes in cathepsin D localization, as demonstrated both biochemically and immunohistochemically, develop earlier than has previously been demonstrated, i.e., by 30-45 min after coronary occlusion, at or before the time when irreversible necrosis develops (19-21). Our results thus strongly support the hypothesis that at least one lysosomal proteinase is released into the cytoplasm of ischemic myocytes early in the course of infarction, and suggest that such release may occur at a time when cellular damage may still be reversible. Subsequently the intracellular activity of that and other proteinases (e.g., cathepsin B1 and neutral or alkaline proteinases) may lead to irreversible injury and ultimately myocardial death. It remains unclear whether nonlysosomal neutral and alkaline proteinases that are present in heart and skeletal muscle (29-32) play an important role in physiological or pathological phenomenon, but because of their pH optima it seems probable that their actions might be blunted, rather than enhanced, by ischemia with its attendant cellular acidosis.

It should be emphasized that our studies do not yet prove a causal role for lysosomal enzyme release in mediating cardiac cell death. It remains possible that an unobserved pivotal event has already occurred before the time lysosomal changes appear. Alterations in ATP levels and in the structure and function of other cell components also develop early in the course of infarction (19-21, 33), and it has been suggested, for example, that irreversible damage to mitochondria or to cell membrane integrity may constitute the critical “point of no return.” Even if lysosomal activation does indeed precede the point of irreversibility, such a phenomenon could be coincidental rather than causal. Certainly, inasmuch as prolonged ischemia and anoxia fail to accelerate total proteolysis (indeed, the reverse seems to be true [34]), it seems unlikely that the relation between lysosomal enzymes and cell death can be a simplistic one of increased hydrolytic enzyme release leading to massive and fatal breakdown of the cell. This, of course, is not to say that a more subtle, incomplete hydrolysis by lysosomal enzymes of a few key substances and resultant selective damage to key structures (e.g., mitochondria or cell membranes) could not be fatal to the cell without the need for complete breakdown of all or most of its constituents. Finally, experiments in vitro suggest that lysosomal activation is more closely associated with cardiac reparative processes after an anoxic insult than with the injury process itself (35), and changes observed in vivo after 30-60 min might conceivably reflect the initiation of the cells’

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reparative efforts and not be directly related to the damage being inflicted.

Thus, even though the results of this study indicate that major lysosomal changes occur early in the course of developing infarction, at or before the conventionally accepted time of “irreversibility,” definitive statements concerning the exact role of these changes cannot be tendered at this juncture. It would be profitable to examine still more closely whether some myocytes may already be irreversibly damaged before enzyme release, or whether the lysosomal changes are independent of and dissociable from the necrotic process. Alterations in other lysosomal proteinases such as cathepsin B1 will be examined in the future. Particular interest will revolve around the issue of whether pathological or therapeutic interventions known to alter lysosomes of normal hearts in vivo (4, 23, 36) can influence the lysosomal responses that accompany ischemia and, if so, whether injury to the cell as a whole is modified as well.

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