Inhibition of Protein Degradation in Mouse Hearts by Agents That Cause Lysosomal Dysfunction

Kern Wildenthal, Jacqueline R. Wakeland, Phyllis C. Morton, and Edmond E. Griffin

SUMMARY Although the heart contains lysosomes, it has been uncertain whether these organelles and their proteolytic enzymes play an important role in cardiac protein degradation. Recent studies have demonstrated that fetal mouse hearts in organ culture sustain selective derangements in lysosomal structure and function during exposure to chloroquine or nonmetabolizable sugars. Accordingly, we tested the effects of these agents on cardiac proteolysis under controlled conditions in vitro using two techniques (measurement of loss of radioactivity from trichloroacetic acid-precipitable protein after prelabeling with tritiated phenylalanine and measurement of loss of cold phenylalanine after blockade of protein synthesis with cycloheximide). Chloroquine (0.1 mM) reduced the average rate of protein breakdown in hearts of matched littersmates from 45%/24 hours to 32%/24 hours \( (P < 0.01) \) and decreased the release of cold phenylalanine by 31 ± 5% \( (0.108 \text{ vs. } 0.075 \text{ nmol/mg per hour, } P < 0.01) \). Exposure to 100 mM sucrose for 24-48 hours reduced the rate of breakdown from 44%/24 hours to 33%/24 hours \( (P < 0.01) \) and decreased the release of cold phenylalanine by 35 ± 9% \( (0.092 \text{ vs. } 0.060 \text{ nmol/mg per hour, } P < 0.01) \). The results suggest that interference with lysosomal function in cultured fetal mouse hearts causes a significant reduction in the cardiac capacity to degrade proteins.

ALTHOUGH the myocardium is known to contain lysosomes, it has been uncertain whether these organelles and their proteolytic enzymes play an important role in regulating cardiac protein degradation. One approach which might help to resolve this issue would be to determine whether experimental interference with normal lysosomal properties alters the rate of proteolysis.

One problem in adopting such an approach is to select a suitable experimental model. Agents that cause selective lysosomal derangements, if given in vivo, might cause systemic noncardiac effects which could confuse the interpretation of their specific myocardial actions. Therefore, we chose to use isolated hearts in vitro. Because the effects on lysosomal function might be slow to develop, we chose to use an in vitro model which can function stably for many hours to days, i.e., the fetal mouse heart in organ culture.

A second problem in approaching this question is to choose appropriate agents to interfere selectively with lysosomal function while causing minimal primary abnormalities in other cellular systems. This problem is made more difficult by the fact that many of the agents which might otherwise be suitable, such as some specific inhibitors of individual lysosomal proteolytic enzymes, cannot readily cross cell membranes and gain access to the lysosomes. Recently, however, two types of agents—nonmetabolizable sugars and chloroquine—have been identified which become concentrated in lysosomes of living cells and which produce severe lysosomal dysfunction without causing major abnormalities in other organelles.

Like many other tissues, fetal mouse hearts in organ culture react to these agents by developing striking, selective lysosomal abnormalities. Exposure to chloroquine causes the rapid development of bizarre lysosomes with myelin figures and inclusion bodies; at the same time, the lysosomal pH becomes alkaline (higher than optimal for most lysosomal hydrolases), and the drug produces a direct biochemical inhibition of at least one lysosomal proteinase, cathepsin B. Nonmetabolizable sugars such as sucrose are conventionally viewed as being confined to the extracellular space. This is true only in the short term, however, and over a period of many hours the sugars are slowly taken into cells by endocytosis and progressively stored in lysosomes. During exposure to sucrose for 24-48 hours, lysosomes of fetal mouse hearts in organ culture sequester large amounts of the sugar and swell into giant vacuoles; these vacuoles seem unable to maintain the acid pH required for effective activity of most of the lysosomal enzymes, and many inclusion particles are accumulated. Accordingly, since chloroquine and sucrose provide means to produce selective cardiac lysosomal derangements under precisely controlled conditions in vitro, we undertook studies to test their simultaneous effects on cardiac protein degradation.

Methods

Culture Conditions

As described in detail previously, hearts were removed aseptically from 18- to 20-day fetal mice (Charles River, albino outbred), washed gently, and placed on stainless steel grids at a liquid-oxygen interface in shallow culture dishes. Hearts of this age weigh 2-5 mg and measure 0.5-
2 mm in their major diameters. The hearts beat rhythmically and maintain stable metabolic characteristics for several days under appropriate culture conditions.2, 16

Control hearts were maintained in 50% Earle's salt solution (Grand Island Biological Co.) plus 50% fetal calf serum (Colorado Serum Co.) supplemented with insulin, 50 µg/ml (Schwarz/Mann). Experimental hearts from matched litters were maintained in identical media to which had been added 100 mM sucrose, 100 mM urea, or 0.1 mM chloroquine. Previous studies have established that these concentrations induce major lysosomal alterations without altering the contractile ability of the hearts or producing major abnormalities in other organelles.14, 15

In some experiments, cycloheximide (Sigma) was added to control and experimental media at a concentration of 2.5 µg/ml, a level that, in this system, inhibits incorporation of radioactive amino acid into protein by >90%.17 For studies requiring the use of radioactive amino acids, cultures were exposed to 10 µCi/ml of L-[side chain-2,3-3H]phenylalanine (50 Ci/mmol; Schwarz/Mann) plus non-radioactive carrier, 16 µg/ml. For studies of protein degradation, the hearts were transferred to medium lacking radioactive amino acids and containing excessive concentrations (500 µg/ml) of unlabeled phenylalanine to minimize reutilization of previously incorporated label.

Biochemical Analyses

Analyses were performed on the heart tissue and on the culture medium after cultivation under various conditions for 1–2 days. Total protein content of each heart was determined by the method of Lowry et al.18 after homogenization in a glass Dounce homogenizer.

Measurements of changes in protein degradation were made by two techniques. In the first procedure, hearts were allowed to stabilize in culture for 18–24 hours and then exposed to [3H]phenylalanine for 4 hours. As described in detail previously,19 the hearts were then washed at hourly intervals with nonradioactive medium containing excess unlabeled phenylalanine. After three washes, one-third of each litter was harvested for "zero-time" values, to make allowance for any differences that might be produced in incorporation of label by the prior presence of sucrose. To test for any effects that might be due to the high molarity of the sucrose rather than to its specific effects on lysosomes, similar experiments were made with 100 mM urea, an agent which does not alter lysosomal structure or function significantly.15

Rates of protein degradation for each group of hearts were calculated from measurements of the amount of radioactivity remaining in trichloroacetic acid (TCA)-precipitable protein after the 24-hour degradation period, as compared to the amount in "zero-time" hearts.19 Each heart was homogenized in ice-cold 20 mM NaCl-2 mM phosphate buffer (pH 7.4) in a tightly fitting Dounce homogenizer. Protein was precipitated from a sample of each homogenate with cold 10% TCA, collected on a glass filter (Whatman, GF/C) in a funnel precipitation apparatus (ICN Corp.), washed twice with cold TCA, and then solubilized with NCS solubilizer (Amersham/Searle) in a liquid scintillation counting vial. The radioactivity of each sample was measured in a Packard 2425 tricarb scintillation counter in toluene-2,5-diphenyloxazole-1,4-bis[2-(5-phenyloxazolyl)]benzene.

Table 1 Effects of Chloroquine (0.1 µM) and Sucrose (100 mM) on Protein Degradation as Measured by Release of Tritiated Phenylalanine from TCA-Precipitable Material in Fetal Mouse Hearts

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Control</th>
<th>Experimental</th>
<th>Differences</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine (0- to 24-hr exposure)</td>
<td>45 ± 3.2</td>
<td>32 ± 4.8</td>
<td>13 ± 3.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sucrose (0- to 24-hr exposure)</td>
<td>46 ± 1.0</td>
<td>40 ± 3.9</td>
<td>6 ± 2.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sucrose (24- to 48-hr exposure)</td>
<td>44 ± 2.5</td>
<td>33 ± 3.0</td>
<td>11 ± 2.8</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SEM of 30 hearts from six litters. Differences are calculated from the six sets of matched litters in each experiment.
In other experiments, changes in net amino acid balance in the hearts were determined, as described previously, by assaying the medium with a Durrum analyzer after 1 or 2 days' cultivation for the net gain or loss of 12 individual amino acids. Because phenylalanine is not synthesized or degraded in heart tissue and is involved only in protein synthesis and breakdown, the rate of uptake or release of total phenylalanine was used as an index of net protein balance (synthesis and degradation) as well as of phenylalanine balance per se. When protein synthesis was blocked with cycloheximide, as described above, measurements of phenylalanine release provided an index of the rate of protein degradation alone.

Statistical Analyses

Although results from different litters maintained in organ culture often vary considerably, results from individual hearts within a single litter are highly reproducible (so < 10%). Accordingly, results from hearts exposed to the experimental agents were compared to matched littermate controls using Student’s t-test for paired data. Results are given as the mean ± 1 standard error of the mean for each condition, with n as the number of litters tested.

Results

As reported in previous studies, fetal mouse hearts in organ culture degraded protein at a fairly rapid rate. Thus, as estimated from the loss of radioactive phenylalanine from TCA-precipitable material, 45 ± 3.2% of prelabeled protein was degraded in 24 hours in control hearts (Table 1). In contrast, only 32 ± 4.8% of prelabeled protein was degraded during the 24-hour period that hearts of matched litters were exposed to chloroquine. The difference, which represented a relative retardation in the rate of proteolysis by a factor of almost one-third, was statistically significant (P < 0.01). Experiments in which cycloheximide was present during the degradation period to prevent artifactual differences due to altered reincorporation of label yielded similar results: control hearts degraded protein at a rate of 38 ± 6.5% in the presence of cycloheximide vs. 22 ± 8.2% when chloroquine was added (Fig. 1, P < 0.01).

When sucrose was added to the medium during the degradation period, the rate of protein breakdown was 40 ± 3.9% vs. 46 ± 1.0% in matched controls (Table 1). This fairly small difference during the first 24 hours of exposure to the sugar was of only borderline significance statistically (0.05 < P < 0.10). In experiments in which pre-exposure to sucrose for 24 hours had occurred before the hearts were pulsed and degradation rates were measured, the effects of the sugar were much more pronounced. Thus, hearts degraded protein at a rate of 33 ± 3.0%/24 hours during the second day of exposure to sucrose vs. 44 ± 2.5% in litter-matched controls tested over the same period (Table 1, P < 0.01). In the presence of cycloheximide, the corresponding figures during the second day of sucrose exposure were 26 ± 4.0%/24 hours vs. 39 ± 3.1%/24 hours in matched controls (Fig. 2, P < 0.01). Under identical circumstances, exposure to urea for 2 days caused no changes in the rate of protein degradation (43 ± 4.0%/24 hours in controls vs. 44 ± 4.7%/24 hours).

Measurements of the release into the medium of cold phenylalanine during exposure to cycloheximide provided confirmation of the inhibitory effects of chloroquine and sucrose on proteolysis. As shown in Figure 3, hearts exposed to chloroquine during the first day of culture released phenylalanine at a rate of 0.075 ± 0.0066 mmol/mg heart weight per hour, as compared to 0.108 ± 0.0057 in litter-matched controls. This 31% decrease was statistically significant (P < 0.01). Sucrose reduced phenylalanine release by 23% during the first 24 hours of cultivation in the presence of cycloheximide (0.083 ± 0.0078 mmol/mg per hour vs. 0.106 ± 0.0145, P < 0.01). The sugar’s effect was much more pronounced during the second 24 hours of exposure, when the rate of phenylalanine release was retarded by 35% (0.060 ± 0.0113 vs. 0.092 ± 0.0132, P < 0.01).

When added to media lacking cycloheximide, so that both synthesis and degradation were active, chloroquine and sucrose tended to improve the net balance (i.e.,...
reduce the release or increase the uptake) of all the amino acids measured (Tables 2 and 3).

Discussion

The precise cellular mechanisms by which proteins are broken down to their constituent amino acids are poorly understood. In many tissues, lysosomes appear to play a major role in the process.22-23 In heart and skeletal muscle, because relatively few lysosomal structures can be identified microscopically, because autophagic vacuoles containing myofibrillar elements are seldom if ever encountered, and because nonlysosomal neutral and alkaline proteinases are abundant, considerable scepticism has remained about whether lysosomes and lysosomal enzymes are of major importance in the degradation of proteins in muscular tissues (see refs. 1, 23, and 24 for reviews).

Direct demonstrations of the quantitative importance of specific lysosomal proteinases have been accomplished for liver, using the specific proteinase inhibitor pepstatin encapsulated in liposomes to facilitate entry into the lysosomal compartment.39 Similar studies have been difficult in muscle tissues because myocytes do not readily incorporate liposomes or their contents. Furthermore, some other proteinase inhibitors, such as TLCK or leupeptin, which can penetrate cells more easily than pepstatin, may block nonlysosomal as well as lysosomal proteinases and also may produce nonspecific toxic effects which theoretically might alter proteinolysis secondarily, independent of their actions on lysosomal enzymes (unpublished observations). Accordingly, in the present experiments we sought to test the effects of agents which, in a variety of tissues including fetal mouse hearts in organ culture, readily gain access to the lysosomal compartment and produce their primary effects on lysosomes with little or no actions in other organelles.39-43

Previous studies on isolated fibroblasts have shown that chloroquine inhibits the lysosomal breakdown of endogenous proteins10,11 as well as of certain exogenous proteins that are taken up by endocytosis.12,13 Similarly, our experiments revealed that the breakdown of endogenous cardiac proteins was inhibited by 30% or more during a 24-hour exposure to chloroquine.

Exposure to sucrose causes lysosomal derangements in many tissues, including heart.3-6,15 In fetal mouse hearts, the abnormalities can be observed to appear gradually over a period of 1-3 days.15 In keeping with the rather slow and progressive nature of the development of sucrose-induced lysosomal derangements, changes in protein synthesis would result in a net accumulation of total protein mass of 10-15% per day over control. At this rate, total cardiac size would tend to double within a week. In other words, this degree of change is very significant, not only statistically but also functionally, when viewed in the context of the development of cardiac hypertrophy.

### Table 2: Effect of Chloroquine (0.1 mM) on Net Amino Acid Balance in Fetal Mouse Hearts Maintained in Organ Culture

<table>
<thead>
<tr>
<th></th>
<th>A. Control medium</th>
<th>B. + Chloroquine</th>
<th>C. Difference (B - A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>+0.140 ± 0.0080</td>
<td>+0.055 ± 0.0050</td>
<td>-0.085 ± 0.0077*</td>
</tr>
<tr>
<td>His</td>
<td>+0.038 ± 0.0028</td>
<td>+0.012 ± 0.0035</td>
<td>-0.026 ± 0.0047*</td>
</tr>
<tr>
<td>Arg</td>
<td>+0.068 ± 0.0045</td>
<td>+0.024 ± 0.0037</td>
<td>-0.044 ± 0.0025*</td>
</tr>
<tr>
<td>Pro</td>
<td>+0.020 ± 0.0064</td>
<td>-0.035 ± 0.0090</td>
<td>-0.055 ± 0.0108*</td>
</tr>
<tr>
<td>Gly</td>
<td>+0.401 ± 0.0181</td>
<td>+0.255 ± 0.0175</td>
<td>-0.146 ± 0.0178*</td>
</tr>
<tr>
<td>Ala</td>
<td>+0.713 ± 0.0389</td>
<td>+0.351 ± 0.0224</td>
<td>-0.362 ± 0.0374*</td>
</tr>
<tr>
<td>Val</td>
<td>-0.047 ± 0.0102</td>
<td>-0.088 ± 0.0134</td>
<td>-0.041 ± 0.0097*</td>
</tr>
<tr>
<td>Met</td>
<td>+0.033 ± 0.0054</td>
<td>+0.003 ± 0.0039</td>
<td>-0.030 ± 0.0053*</td>
</tr>
<tr>
<td>Ile</td>
<td>-0.034 ± 0.0032</td>
<td>-0.060 ± 0.0045</td>
<td>-0.026 ± 0.0032*</td>
</tr>
<tr>
<td>Leu</td>
<td>-0.035 ± 0.0084</td>
<td>-0.089 ± 0.0092</td>
<td>-0.054 ± 0.0056*</td>
</tr>
<tr>
<td>Tyr</td>
<td>+0.052 ± 0.0076</td>
<td>+0.013 ± 0.0047</td>
<td>-0.039 ± 0.0063*</td>
</tr>
<tr>
<td>Phe</td>
<td>+0.062 ± 0.0068</td>
<td>+0.012 ± 0.0073</td>
<td>-0.050 ± 0.0066*</td>
</tr>
</tbody>
</table>

Results in columns A and B represent the net release (positive numbers) or uptake (negative numbers) of each amino acid (in nmol/mg heart weight per hour) from the heart into the medium during the first day of cultivation; each value represents the mean ± 1 SEM for 24 hearts from eight litters. Column C gives the mean difference ± 1 SEM between the eight matched litters; negative numbers in column C indicate either increased net uptake or reduced net release.

* P < 0.05 by Student's paired t-test.
With any pharmacological agent that is used to produce selective effects on a single type of organelle or on a single aspect of cellular function, it is probable that other organelles and other functional characteristics will also be altered to some extent. Indeed, this is intrinsically inevitable if the primary disturbance is on an important aspect of cell function, since the complex integration of all the cell's processes will secondarily become upset when a vital link is perturbed. Thus, in the present studies, as in all others in which efforts are made to produce selective perturbations of particular aspects of cell function, one must be concerned whether the final observations represent predominantly the results of the primary perturbation in question or whether secondary, nonspecific toxic effects might not be the cause. Specifically, do the major lysosomal abnormalities known to be produced by sucrose and chloroquine in fetal mouse hearts account for the subsequent inhibition of proteolytic capacity observed in the present experiments, or is some other factor more important?

Ultimately, of course, this question can never be answered with certainty. Nevertheless, in the case of sucrose, there is no evidence of apparent abnormalities in any other organelles, cardiac beating (which is an exquisitely sensitive indicator of even mild toxicity or damage in hearts in organ culture) is not depressed over the interval of time observed, and there is a close concurrence of the time-course of the observed effects on lysosomes and on proteolysis. These facts lead one to conclude with some confidence that sucrose-induced inhibition of protein breakdown probably is caused by specific lysosomal dysfunction rather than by nonspecific toxicity. In the case of chloroquine, evidence of some mild, nonspecific toxicity is a bit more disturbing. Although beating remains strong over the first day, exposure to chloroquine leads ultimately to contractile depression (unpublished results). Furthermore, some mitochondrial abnormalities develop after 1–2 days, which might lead to inadequate oxidative metabolism. Because interference with normal oxidative metabolism can itself produce inhibition of cardiac proteolysis, one must consider the possibility that the observed results might result primarily from mitochondrial damage and inadequate oxidative energy production rather than from lysosomal dysfunction. If toxic damage were severe enough to inhibit oxidative metabolism sufficiently to depress proteolysis, however, alanine production and release from the heart would be expected to increase markedly. Since no such increase occurred (Table 2), it seems unlikely that the observed inhibition of proteolysis can be explained by nonspecific cellular toxicity and inadequate oxidative energy production.

In summary, considering all the experiments involved both sucrose and chloroquine, we believe that the most likely explanation of the results is that the observed reductions in protein breakdown are caused primarily by lysosomal derangements. Thus it appears that, in this experimental model, cardiac lysosomes can play a major role in the degradation of myocardial protein and that interference with lysosomal function retards proteolysis.

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References

Velocity Distribution and Intimal Proliferation in Autologous Vein Grafts in Dogs

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SUMMARY Autologous femoral veins grafted between the external iliac arteries in 18 dogs provided a model for studying the hemodynamics and histopathology of vein graft bypasses. The angle of proximal anastomosis was varied by groups (<90°, 90°, >90°) to produce a wide range of flow conditions within the grafts. Four months after implantation, point velocity measurements of blood flow and histological examination of the superior and inferior walls were made at proximal, middle, and distal locations in each graft. Hot-film velocity measurements revealed outwardly skewed velocity profiles in the proximal locations in all grafts, and flow visualization models showed secondary fluid motions and separation zones at those regions. Velocity profiles in the middle and distal regions of the grafts were more symmetrical and showed no flow separation. Histological examination of wall sections along the graft length showed that intimal proliferation occurred focally and ranged from 1 to 100 µm in thickness. No significant correlation between graft angle and degree of intimal proliferation was found. However, there was a weak inverse correlation between the apparent fluid shear rate and the corresponding degree of intimal proliferation, with the greatest proliferation occurring in the regions experiencing the lowest shear stress. Regions of low shear rate should be avoided to maintain adequate flow rates through the grafts and to minimize losses of patency due to both thrombus formation and intimal proliferation.

HUMAN AORTOCORONARY saphenous vein bypass procedures are widely employed in the treatment of severe coronary artery disease. However, loss of graft patency has led to concern about the overall benefit of this procedure. Bypass failure rates of up to 23% during the first year have been attributed primarily to early occlusion (up to 1 month) by thrombosis or late occlusion (after 1 month) by intimal or subendothelial proliferation. Although its exact etiology is unknown, many investigators believe intimal proliferation to be a series of repairative

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