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Intracellular disruption of rat heart lysosomes by leucine methyl ester: Effects on protein degradation

(protein turnover/lysosomal enzymes/lysosomal membrane/amino acid accumulation)

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ABSTRACT **Perfusion of rat hearts with Krebs–Henseleit medium containing 10 mM L-leucine methyl ester leads to swelling of lysosomes and loss of lysosmal integrity within 30–60 min. No morphological changes can be detected in the nuclei, mitochondria, sarcoplasmic reticulum, or Golgi complex as a result of the treatment with leucine methyl ester, and the hearts continue to beat normally during the treatment period. Homogenates of rat hearts perfused with the methyl ester exhibit a decrease in the sedimentability of cathepsin D activity compared to controls, thus providing additional evidence for a loss of lysosomal integrity. Swelling and disruption of the lysosomes presumably occurs because of the extensive accumulation of leucine within the organelles resulting from the intralysosomal hydrolysis of the freely permeating methyl ester. The lysosomal dysfunction that occurs with exposure to leucine methyl ester produces a 30% decrease in cardiac protein degradation. These results provide an estimate of the contribution of lysosomes to total protein degradation in the rat heart, and they also suggest that the enzymes released as a result of lysosomal disruption are relatively inactive in hydrolyzing cellular constituents under the perfusion conditions used here. The use of amino acid methyl esters to produce rapid, specific loss of lysosomal integrity *in situ* provides an approach to the study of lysosomal function in intact cells.**

Goldman and her colleagues (1–4) demonstrated that the methyl esters of certain amino acids at concentrations of 0.1–10 mM cause a loss of latency and sedimentability of the enzyme activities of isolated lysosomes. This was assumed to be due to the extensive accumulation of amino acids within the lysosome, resulting from the intralysosomal hydrolysis of the readily permeating methyl ester. At sufficiently high concentrations, the osmotic effects of the accumulated amino acid would lead to swelling and disruption of the lysosomes. Reeves (5) showed recently that amino acids do in fact accumulate against a high concentration gradient when lysosomes are incubated with sublytic concentrations of ^{3}H-labeled amino acid methyl esters. Amino acid accumulation under these conditions was shown to be a specific property of lysosomes (5).

The high permeability of biological membranes for amino acid methyl esters and the specificity of amino acid accumulation for lysosomes suggest that lysosomes in intact cells might become swollen and disrupted if the cells were exposed to millimolar concentrations of an amino acid methyl ester. We chose heart tissue to test this hypothesis because of our long-standing interest in delineating the contributions of lysosomes to protein degradation and to the effects of ischemia in this tissue. In this report, we show that perfusion of rat hearts with 10 mM L-leucine methyl ester (Leu-OMe) causes loss of lysosomal integrity without damage to other cellular organelles. The lysosomal dysfunction is accompanied by a 30% decline in the rate of protein degradation, as measured by phenylalanine release in the presence of cycloheximide. Some of these results have been presented in abstract form (6).

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METHODS

Heart Perfusions. Male albino rats (8–10 weeks old, Charles River Breeding Laboratories) were used. The animals were heparinized (2.5 mg intraperitoneally) and anesthesized with ether. The hearts were excised and mounted on a modified Langendorff perfusion apparatus as described (7). They were perfused with Krebs–Henseleit buffer containing 15 mM glucose and bovine insulin (Sigma) at 50 μg/ml, with or without 10 mM Leu-OMe hydrochloride (pH adjusted to 7.4).

Distribution of Lysosomal Enzyme Activity. After the perfusion period, the hearts were placed in ice-cold 0.25 M KCl buffered with 0.1 mM EDTA and Tris·HCl (pH 7.2), minced, and gently homogenized, as described (8). After a preliminary centrifugation at 350 × *g* (5 min) to remove nuclei and cellular debris, the supernatant was centrifuged at 40,000 × *g* for 20 min. The activity of cathepsin D in the pellet from this second centrifugation was termed the "sedimentable" fraction; the activity remaining in the supernatant was termed the "nonsedimentable" fraction. The ratio of the nonsedimentable activity to the total activity (nonsedimentable plus sedimentable) was used as an index of lysosomal fragility or disruption. Cathepsin D activity was measured as described (8).

Ultrastructural and Cytochemical Procedures. Portions of the perfused left ventricular myocardium were preserved in 2% (wt/vol) glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 90 min at 4°C. The tissue was then washed extensively in 0.1 M cacodylate buffer, pH 7.4, and 20- to 40-μm nonfrozen sections were prepared on a Smith–Farquhar tissue chopper. Sections were then incubated in a modified Barka and Anderson (9) medium to reveal the localization of lysosomal acid phosphatase reaction product. Other sections were incubated in Novikoff and Goldfischer's (10) inosine nucleoside diphosphatase medium to label the Golgi complex and elements of the endoplasmic reticulum. After a 1-hr incubation, sections were rinsed, postfixed in 1% osmium tetroxide, and stained *en bloc* with 0.5% uranyl acetate. After dehydration in ethanol and propylene oxide, tissues were embedded in Epon 812. Thin sections were cut on a Porter–Blum MT-2B ultramicrotome and viewed (unstained or treated with uranyl acetate and lead citrate) in a Philips 200 electron microscope.

Protein Degradation. Hearts were perfused as above with the addition to the medium of 10 μM cycloheximide. The amino acid concentrations in the medium after 60 min of recirculating perfusion were measured by using a Durrum amino acid ana-

Abbreviation: Leu-OMe, leucine methyl ester.

lyzer. The rate of phenylalanine accumulation in the perfusate under these conditions was taken as a measure of the rate of protein degradation. For a discussion of the advantages and limitations of this method of assessing protein degradation, see ref. 11–13.

Statistical Treatment. Analyses of the statistical significance of differences in phenylalanine release and in redistribution of lysosomal enzyme activities were performed by using Student's two-tailed *t* test for unpaired samples.

RESULTS

As shown in Table 1, homogenates of rat hearts perfused for 30 or 60 min with 10 mM Leu-OMe exhibited an increase in the proportion of cathepsin D activity that was nonsedimentable. The effects of Leu-OMe were not reversed when the Leu-OMe perfusion period was followed by an additional 30-min perfusion with Krebs–Henseleit buffer without Leu-OMe (data not shown). These results suggest that Leu-OMe treatment produces lysosomal alterations that either lead to disruption *in situ* or predispose the lysosomes to disruption during homogenization.

In an attempt to correlate the biochemical changes with structural alterations, and to assess whether or not the effects of Leu-OMe are specific for lysosomes, the ultrastructural appearance of hearts perfused for 30–60 min with 10 mM Leu-OMe was examined. As shown in Fig. 1*A*, secondary lysosomes were normally present in the paranuclear regions of most cardiac myocytes of normally perfused hearts (14). These granules usually displayed dense deposits of reaction product for acid phosphatase (Fig. 1*A*). Elements of the Golgi complex and the GERL (Golgi–endoplasmic reticulum–lysosome complex) also revealed acid phosphatase staining (Fig. 1*A*). Other intramembranous compartments, including the rough endoplasmic reticulum and the sarcoplasmic reticulum, failed to disclose evidence of acid phosphatase activity.

Perfusion of hearts with medium containing 10 mM Leu-OMe rapidly and selectively produced swelling of secondary lysosomes. A 30-min perfusion with Leu-OMe induced profound enlargement and vacuolization of cardiac lysosomes (Fig. 1*B*). Dense bodies and autophagic vacuoles were swollen, and their contents were partitioned into amorphous clumps of granular material and membranous arrays that resemble myelin figures. The granular components of the dilated lysosomes continued to exhibit intense deposits of acid phosphatase reaction product. In contrast, other aspects of cell structure appeared unaltered during the perfusion, with mitochondria, sarcoplasmic reticulum, and the Golgi complex revealing no adverse changes in response to the amino acid methyl ester.

After 1 hr of exposure to Leu-OMe, secondary lysosomes became enormously dilated (Fig. 1 *C* and *D*). As the granules enlarged, they assumed the configuration of the surrounding organelles that encompassed them. The vacuoles were so abnormal that they were not recognizable as lysosomes *per se*, except in a few instances in which some acid phosphatase reaction product could be observed (Fig. 1*C*). In most vacuoles the enzyme had apparently leaked out of the organelles through disruptions in the vacuolar membranes. The vacuoles usually retained a variety of membranous profiles of unknown origin (Fig. 1 *C* and *D*). It should be emphasized that the Golgi complex, which houses considerable acid phosphatase reaction product (Fig. 1*C*), and endoplasmic reticulum, which displays nucleoside diphosphatase activity (Fig. 1*D*), were not altered by Leu-OMe treatment. The observations indicate that Leu-OMe alters secondary lysosomal structures rapidly, selectively, and profoundly. We were unable to identify any cells that contained normal secondary lysosomes.

Table 1. Perfusion of rat hearts with Leu-OMe: Effect on sedimentability of cathepsin D activity

Perfusion time, min	Nonsedimentable cathepsin D, % of total: Control	With 10 mM Leu-OMe	*P*
30	49 ± 1.6	57 ± 1.7	<0.01
60	47 ± 2.9	59 ± 0.9	<0.01

Rat hearts were perfused for 30 min ($n = 12$) or 60 min ($n = 6$) with or without 10 mM Leu-OMe. Nonsedimentable cathepsin D activity is expressed as a percentage (±SEM) of the total activity (sedimentable plus nonsedimentable).

Lysosomes have been suggested to play an important role in the turnover of cellular protein in the heart (15). Accordingly, in view of the above results showing that Leu-OMe rapidly produces loss of myocardial lysosomal integrity, it was of interest to assess the effects of this agent on the rate of cardiac protein degradation. Hearts were perfused for 60 min in the presence of insulin and cycloheximide with or without 10 mM Leu-OMe, and the extent of phenylalanine release in the perfusate over this period was measured. Because phenylalanine is neither synthesized nor degraded by heart tissue (16) and because the presence of cycloheximide prevents the reincorporation of phenylalanine into newly synthesized proteins, the appearance of phenylalanine in the perfusate provides a measure of the breakdown of intracellular protein. As shown in Fig. 2, hearts perfused with 10 mM Leu-OMe exhibited lower rates of phenylalanine release (mean ± SEM = 0.080 ± 0.003 nmol per mg wet weight per hr) than hearts perfused with control medium (0.115 ± 0.005 nmol per mg wet weight per hr). Because Leu-OMe did not affect the intracellular content of phenylalanine (data not shown), the reduced rate of phenylalanine release cannot be ascribed to an inhibition of the efflux of free phenylalanine from the cell. Thus, swelling and disruption of lysosomes by treatment with Leu-OMe reduced the rate of protein degradation in the heart by 30% ($P < 0.001$).

The branched chain amino acids, and specifically leucine, have been found to inhibit protein degradation in heart and skeletal muscle under some conditions (17–19). Thus, one must consider the possibility that the observed decrease in protein degradation caused by Leu-OMe might be mediated by an increased supply of leucine rather than by primary abnormalities in lysosomes. Accordingly, in parallel experiments hearts were perfused with medium containing 10 mM leucine rather than Leu-OMe. The rate of phenylalanine release from leucine-perfused hearts was 0.100 ± 0.006 nmol per mg per hour, compared to 0.105 ± 0.007 for the control group ($P > 0.20$). These results confirm the findings of Chua *et al.* (18), who reported that in hearts perfused in the presence of insulin, leucine had no effect on protein degradation.

DISCUSSION

The results presented here demonstrate that 10 mM Leu-OMe causes a rapid loss of lysosomal integrity in intact perfused rat hearts. This presumably reflects the following sequence of events: The methyl ester, due to the low pK_a (7.6) of the amino group, readily crosses the cellular plasma membrane and enters intracellular lysosomes, where it is converted to the free amino acid by lysosomal hydrolases. The free amino acid, because of its high polarity, cannot easily diffuse back across the lysosomal membrane and so it accumulates to high concentrations, leading eventually to osmotic disruption of the lysosomes.

The effects of Leu-OMe are rapid, with changes in lysosomal morphology becoming evident as early as 15 min of perfusion (data not shown). More extensive changes are observed after

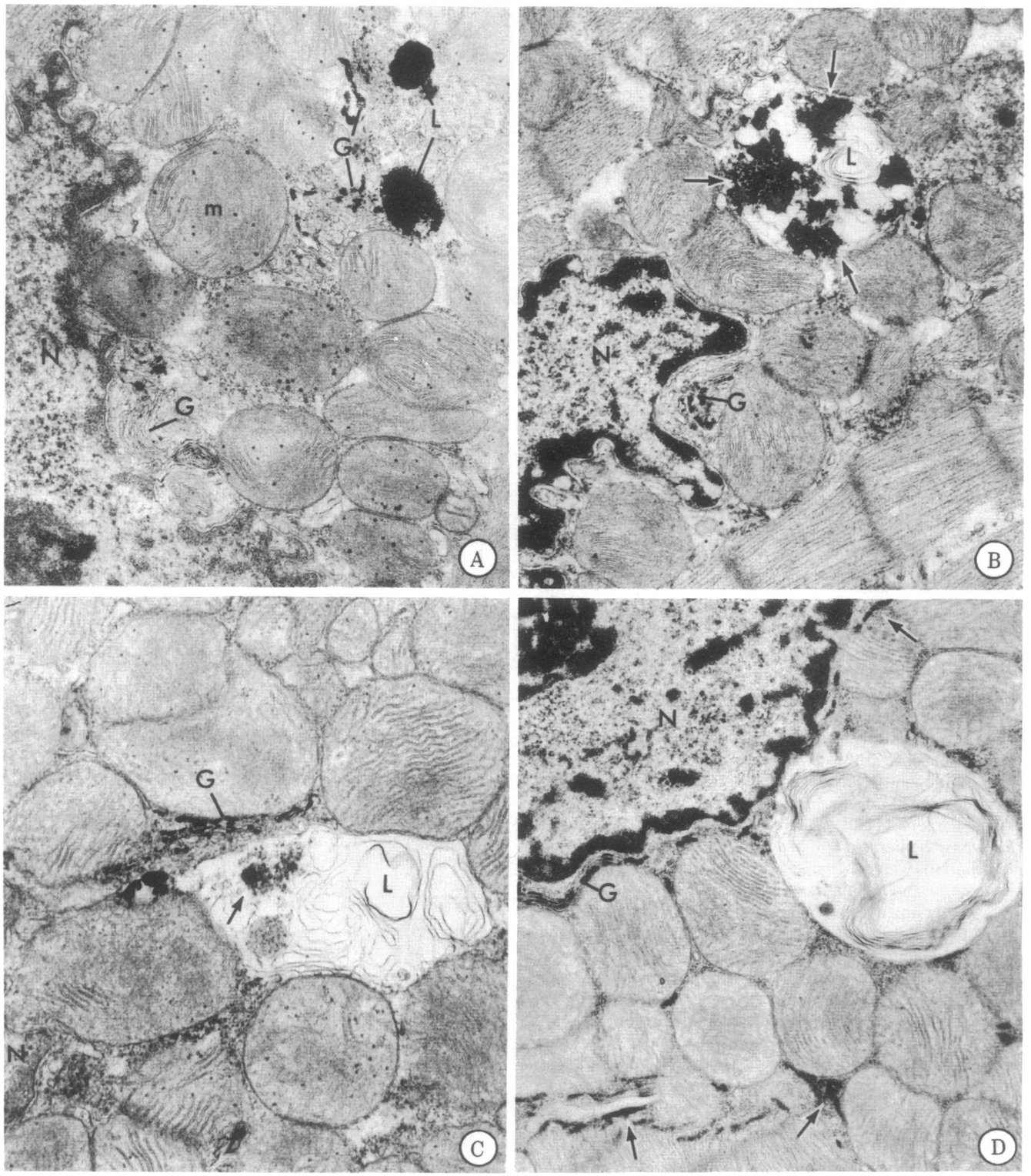

FIG. 1. Effects of 10 mM Leu-OMe on the ultrastructure and cytochemical reactivity of cardiac lysosomes. Acid phosphatase-positive secondary lysosomes (L) and Golgi complexes (G) reside in the paranuclear (N) regions of control myocytes (*A*). A 30-min perfusion with Leu-OMe (*B*) induces pronounced swelling of lysosomes (L) but fails to alter the Golgi complex or neighboring mitochondria (m). After a 1-hr perfusion (*C* and *D*), lysosomes appear maximally dilated and lose most of their acid hydrolase-positive matrix (arrows), leaving numerous myelin figures. Again, neither the Golgi complex (*C* and *D*) nor the nucleoside diphosphatase-positive endoplasmic reticulum (*D*, arrows) is influenced by Leu-OMe. (×19,400.)

30 min of perfusion (Table 1, Fig. 1*B*), and by 60 min maximal redistribution of enzyme sedimentability has occurred and the morphological appearance of the lysosomes suggests that disruption of organellar membranes and leakage of lysosomal enzymes into the cytoplasm have developed. The effects of Leu-OMe are not readily reversible after 30 min of perfusion, because a subsequent 30 min of perfusion in Krebs–Henseleit without Leu-OMe does not restore normal lysosomal morphology or enzyme sedimentability profiles.

Of special interest is the observation that the effects of the methyl ester appear to be restricted to lysosomes. No morphological changes could be detected in the nuclei, mitochondria, sarcoplasmic reticulum, or Golgi complex as a result of Leu-OMe treatment. Another indication of the innocuous nature of

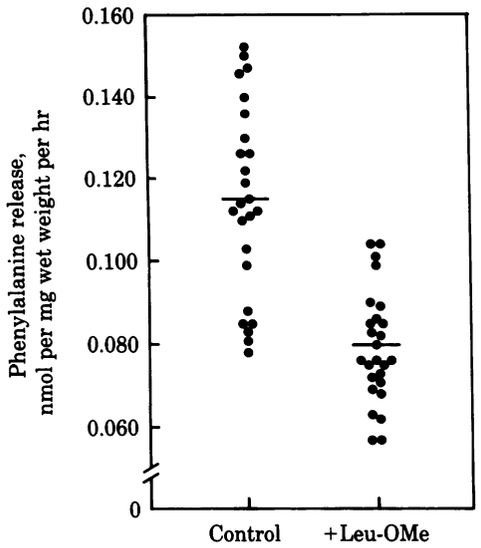

FIG. 2. Effect of Leu-OMe on cardiac proteolysis. Each point represents the rate of release of phenylalanine from a single heart over a 60-min perfusion period in the presence or absence of Leu-OMe. The bar represents the mean value.

Leu-OMe treatment with respect to nonlysosomal functions of rat hearts is that the hearts continue to beat normally during the perfusion with Leu-OMe. This remarkable tolerance to Leu-OMe appears to be species specific, however, because the methyl ester was found to be highly toxic to rabbit hearts when they were perfused under essentially the same conditions as described above for rat hearts. The basis for this difference between the two species is unknown.

The rapidity of the effects of Leu-OMe and its specificity for lysosomes (at least in rat tissue) suggest that the use of amino acid methyl esters could provide an approach to the study of lysosomal function in intact tissue. As an illustration of this approach, we measured the rates of protein degradation in hearts perfused for 60 min with or without Leu-OMe and found that the methyl ester produced a 30% decrease in the rate of protein degradation. Leucine alone had no effect on proteolysis under identical conditions, indicating that this action was a specific property of the amino acid methyl ester. Previous studies have indicated that leupeptin, an inhibitor of several lysosomal proteinases (20), decreases cardiac proteolysis by 20–40% (15, 21, 22). Leupeptin also inhibits some nonlysosomal proteinases, however, and it is not possible with certainty to ascribe its effects to its lysosomal actions. More specific lysosomotropic agents, including chloroquine and sucrose, also reduce cardiac proteolysis by 20–40% (23). The effects of these agents are rather slow to develop (24, 25), however, and the possibility exists that secondary responses of the cells might have either exacerbated or mitigated the primary lysosomal actions and obscured the true magnitude of the effects of lysosomal dysfunction on proteolysis (26). The rapid and selective lysosomal effects of Leu-OMe, along with the simultaneously developing reduction in protein degradation by 30%, indicate that lysosomal processes do indeed play an important role [but not an exclusive one (27)] in cardiac proteolysis.

The decline in protein degradation under conditions in which lysosomal hydrolases have been released from the organelles into the cytoplasm suggests that the translocated lysosomal enzymes are not active in hydrolyzing cytoplasmic cellular constituents to an important extent under the perfusion conditions used here. Thus, if the hydrolases were active, one might have expected an increase in the rate of protein degradation, and possibly morphological evidence of abnormal tissue breakdown as well; the absence of such a response suggests that the cell cytoplasm is an unfavorable environment for the activity of lysosomal acid hydrolases, due presumably to the increased pH in comparison to that found within the lysosome.

In summary, our results demonstrate that Leu-OMe induces a rapid and specific loss of integrity of intracellular lysosomes in rat heart tissue, and this in turn reduces the rate of myocardial protein degradation by approximately 30%. The intracellular disruption of lysosomes by using amino acid methyl esters may prove to be a method of general utility for studying lysosomal functions in intact cells.

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