Hormonal and Nutritional Substrate Control of Cardiac Lysosomal Enzyme Activities

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SUMMARY Prolonged starvation is known to induce significant alterations in several cardiac lysosomal enzymes, particularly the acid proteinase cathepsin D. To determine what specific factors might mediate these changes, fetal mouse hearts in organ culture were maintained in media designed to simulate selected hormonal or nutritional substrate changes that accompany starvation. Reduced concentrations of glucose caused an increase in the activity of cardiac lysosomal enzymes. In addition, insulin deprivation caused significant increases (7-25%) in the activities of all three enzymes. Insu- lin deprivation and excess ketones, but not the other interventions, increased the proportion of enzyme activity which was non-sedimentable (i.e., non-particulate-bound) and the influence of calcium.

PROLONGED starvation is accompanied by characteristic alterations in the activities and distribution of several cardiac lysosomal enzymes. The specific activity of cathepsin D, the major acid proteinase in the heart, is increased by 20-40% after 3-6 days of food deprivation in mice, rats, and rabbits, and the proportion of activity that is present in the non-sedimentable (i.e., non-particulate-bound) fraction of the tissue homogenate becomes much greater.

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than in hearts of fed animals. Immunohistochemical localization studies have revealed the changes to be confined specifically to cardiac myocytes rather than interstitial or endothelial cells. The myocytes display not only increased amounts of the enzyme but also alterations in its subcellular distribution that suggest an increased presence of cathepsin D outside secondary lysosomes. Some other enzymes of lysosomal origin are affected differently by starvation: acid phosphatase activity is increased minimally, whereas the activity of β-acetylglycosaminidase remains constant or declines.1,2

Prolonged starvation is accompanied by a number of chronic metabolic changes that might play a role in causing the alterations in cardiac lysosomal function. Concentrations of fatty acids and ketones become elevated, while glucose is reduced. Levels of growth hormone and glucagon rise, and insulin falls. The present study was undertaken to test under controlled conditions which of these factors might possess the potential to play a role in mediating the effects of prolonged starvation on the heart. As a practical matter it would be impossible to test the chronic effects of these agents using conventional experimental models, in which the period of relative stability is limited to a few hours. Accordingly, an organ culture system was employed, in which intact hearts from fetal mice can be maintained in a reasonably stable functional state for several days under precisely controlled conditions in vitro.3 The use of any in vitro model involves, of course, the dilemma of knowing to what extent it is legitimate to extrapolate results to related situations in vivo, and hearts in organ culture are no less subject to this problem than are traditional systems. The use of fetal hearts may compound the problem when attempts are made to relate results to adult hearts. Nevertheless, results in vitro may provide valuable hints about the potential ability of specific interventions to elicit analogous responses in vivo, so long as caution is used in drawing extrapolations.

Methods

Intact hearts of 18- to 20-day fetal mice were maintained in organ culture as described in detail previously.3 Briefly, the hearts, which weighed 2–4 mg and measured <1 mm in thickness, were isolated under sterile conditions and explanted on stainless steel grids at the liquid-air interphase of shallow culture chambers. They were maintained at 37.5°C and exposed to an atmosphere of 95% O2 -1- 5% CO2. The basic nutritive solution was medium 199 or Earle’s salt solution (Grand Island Biologicals); additions to or deletions from the basic medium were employed as required by the experimental protocol. Glucose was used in concentrations ranging from 50 to 400 mg/100 ml of solution. Other substrates added to the basic medium included sodium octanoate (2 mm), sodium acetocetate (5 mm), and sodium β-OH-butyrate (10 mm). Studies on effects of variations in glucose concentration were made with medium 199 supplemented with 2 mm octanoate to ensure that changes induced by low glucose could not be due to an inadequate energy supply per se. For tests of hormonal effects, crystalline bovine insulin (50 μg/ml; Sigma), glucagon (0.5–50 μg/ml; Lilly), and growth hormone (20–100 μg/ml; Miles) were added to the medium. For studies of effects of long chain fatty acid, hearts were cultured in medium 199 supplemented with 35% fetal calf serum (Colorado Serum Co.); oleic acid (final concentration = 1.7–2.0 mm) was saponified with NaOH and then combined slowly at 45°C with undiluted serum that had previously been chilled to 4°C, after which the serum was added to the chemically defined medium.

After cultivation for 3 days, hearts were assayed for protein and for activities of creatine phosphokinase (a nonlysosomal enzyme which served as a general marker of cellular viability) and cathepsin D, β-acetylglycosaminidase, and acid phosphatase (all of which are localized at least partially in lysosomes in heart). Preliminary experiments revealed that specific activities of the lysosomal enzymes rose gradually during the 3-day period of cultivation; thus, an agent which caused an increase in activity over matched controls would do so by stimulating enzyme activity over and above the normal effects of cultivation per se.

For measurements of total enzyme activities, the hearts were homogenized vigorously in a 0.1% solution of Triton X-100 with a Polytron homogenizer to disrupt cells and organelles maximally. Cellular debris was sedimented at 350 g for 5 minutes, and assays were performed on the supernatant fluid. Protein was measured by the technique of Lowry et al.4 and creatine phosphokinase (CPK) by the technique of Szasz et al.5 The lysosomal enzymes were assayed by modifications of the methods of Barrett as described previously.3 Briefly, cathepsin D activity was determined from formation of Folin-reactive products from purified hemoglobin at pH 3.2 and 45°C (Barrett’s method II); glucosaminidase, from cleavage of nitrophenol from p-nitrophenyl-β-acetylglycosaminitide at pH 4.3 and 37°C; and acid phosphatase, from cleavage of nitrophenol from p-nitrophenyl phosphate at pH 4.5 and 37°C after inhibition of nonlysosomal phosphatases with sodium acetate.6

For measurements of sedimentable vs. nonsedimentable enzyme activity, the tissue was homogenized gently for 20 seconds in a Potter-Elvehjem homogenizer, using a buffered solution (pH 7.4) of 0.25 m KCl, to retain intact lysosomes insofar as possible.7 After initial centrifugation at 350 g to remove undisrupted cells, nuclei, and debris, the supernatant fluid was recentrifuged at 40,000 g for 20 minutes. The supernatant fluid from this second centrifugation was assayed for enzyme activity (termed “sedimentable” activity); the pellet was rehomogenized vigorously in 0.1% Triton X-100 and also assayed for enzyme activity (termed “sedimentable” activity). The ratio of nonsedimentable to total (i.e., “sedimentable” plus “nonsedimentable”) activity was calculated as an index of lysosomal fragility or of the presence of enzyme outside lysosomes, or both.

Samples were maintained at 4°C at all times until assay. Three to eight hearts from a single litter were pooled to provide enough material for the assays. In all studies, results from hearts exposed to the experimental interventions were compared with results from control hearts from matched littersmates that had been cultured and assayed at the same times under similar conditions. Statistical comparisons were made using Student’s t-test for paired observations. Values
are expressed throughout the paper as the mean ± 1 SEM of each group of measurements.

Results

Spontaneous and rhythmic beating persisted throughout the 3-day culture periods in all hearts in all media. Except for hearts exposed to glucose, which causes tachycardia at the higher concentrations used, beating rates were similar in all media.

Variations in substrate composition failed to alter the specific activity of CPK but did exert selective effects on the three lysosomal enzymes that were assayed. Summary results are given in Table 1.

Reductions in the concentration of glucose over a physiological range had no effect on cathepsin D or acid phosphatase activities, but produced a significant increase in the activity of β-Acetylglucosaminidase. This effect, which was progressive over a wide range of glucose concentrations (Table 2), was opposite to that which would have been expected if hypoglycemia participated in starvation-induced alterations in cardiac glucosaminidase activity in vivo. 2

Addition of high concentrations of ketones to the culture medium of glucosaminidase in hearts maintained for 3 days in media using media containing oleate-enriched serum. The activity of glucosaminidase was unaltered in the same

Despite their inability to be transported into mitochondria and oxidized by fetal heart cells, however, long chain fatty acids can be taken up and incorporated into triglycerides. Accordingly, to establish whether the effect of fatty acids was dependent on their oxidation or whether their uptake alone would suffice to produce the changes, studies were repeated using media containing oleate-enriched serum. The activity of glucosaminidase in hearts maintained for 3 days in media containing unenriched serum (FFA = 260 μg/ml) was 784 ± 55

Addition to the medium of a soluble free fatty acid (FFA), octanoate, produced no change in cathepsin D or acid phosphatase activity. However, it did induce a small, consistent decrease (7%) in glucosaminidase activity similar to that observed in vivo after starvation or high fat diets. 3 A medium chain fatty acid such as octanoate is especially useful in studies of fetal hearts, which in many species, including mice, have not yet developed the capacity to utilize long chain fatty acids, presumably because of inadequate cardiac levels of carnitine and acylcarnitine transferase. 3 4

Results

Phosphokinase in Fetal Mouse Hearts Maintained in Organ Culture for 3 Days

<table>
<thead>
<tr>
<th>Glucose concentration</th>
<th>400 mg/100 ml</th>
<th>200 mg/100 ml</th>
<th>100 mg/100 ml</th>
<th>50 mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Acetylglucosaminidase activity (nmol nitrophenol/hr x mg protein)</td>
<td>668 ± 35</td>
<td>752 ± 29*</td>
<td>821 ± 33*</td>
<td>838 ± 36*</td>
</tr>
<tr>
<td>% of activity at 400 mg/100 ml</td>
<td>113%</td>
<td>123%</td>
<td>125%</td>
<td></td>
</tr>
</tbody>
</table>

Hearts of littersmates were compared by Student's t-test at four concentrations of glucose. Each value represents the mean ± 1 SEM of five matched samples (15 hearts).

* P < 0.05, compared to preceding concentration.

P < 0.05, compared to value at 400 mg/100 ml.
nmol nitrophenol/hour × mg protein, and the activity in hearts of matched littermates exposed to increased oleate (FFA = 1840 μM) was 769 ± 39 (−2% ± 4%, P > 0.30), indicating that long chain fatty acids could not mimic the effect of octanoate in fetal mouse hearts.

The effect of combined addition to the medium of fatty acids and ketones is shown in Figure 1. The individual effects of each alone were additive, and together they produced an 18 ± 6% increase in cathepsin D activity (from 88 μg tyrosine/hour × mg protein in controls to 104 in hearts receiving the fatty substrates) and a simultaneous 19 ± 3% decrease in glucosaminidase activity (from 765 nmol nitrophenol/hour × mg protein to 619), while acid phosphatase was increased only minimally—i.e., changes analogous to those induced in vivo by starvation.

Hormonal influences on cardiac lysosomal enzymes are also shown in Table 1. Glucagon in concentrations of 0.5–50 μg/ml produced no changes in cathepsin D, acid phosphatase, or glucosaminidase. Growth hormone had no effect on the latter two enzymes, but caused a small (6%) increase in the specific activity of cathepsin D. In contrast, insulin deprivation produced changes in the activities of all enzymes. Cathepsin D and glucosaminidase were affected similarly (+25% for both, P < 0.01), while acid phosphatase activity was increased less markedly (Fig. 2).

In addition to its effects on total enzyme activities, each agent was tested for its effect on the distribution of enzyme activity between nonsedimentable and sedimentable fractions of the tissue homogenate. The proportion of activity which was nonsedimentable was unaltered by changes in glucose concentration or by the addition to the medium of glucagon or growth hormone. Oleic acid had no effect, but octanoate caused small decreases in the nonsedimentable fraction (i.e., changes opposite to those induced by starvation). As shown in Table 3, both insulin deficiency and excess ketones produced increases in the nonsedimentable fraction. The changes were slightly more pronounced for cathepsin D than for glucosaminidase (as is true for hearts of starved animals), but the changes were small compared to those encountered in vivo.1, 2

**Discussion**

Starvation-induced alterations in lysosomal enzymes have been demonstrated in heart,1, 2 skeletal muscle,12 and liver.13–14 Unlike liver, changes in heart and skeletal muscle are not "pan-lysosomal" but rather are quite distinct for different enzymes.1, 2, 12 The specific activities of some enzymes of lysosomal origin increase during prolonged starvation (e.g., cathepsin D and, to a lesser extent, acid phosphatase), while the activities of others may remain constant or even decrease (e.g., β-acetylglucosaminidase). These alterations, along with simultaneous changes in the lability of the enzymes, develop gradually, and significant changes are not demonstrable for a day or more after starvation has begun.1, 2, 13 Interestingly, starvation-induced increases in the degradation rates of cardiac and skeletal muscle proteins also appear only after a lag period of a day or more, despite earlier alterations in rates of protein synthesis,14 which suggests the possibility that increases in lysosomal proteolytic activity or enzyme "availability" or both may be causally linked with increases in muscle protein degradation during starvation, possibly in concert with increased activities of nonlysosomal proteinases.19, 27–29

The metabolic basis for starvation-induced lysosomal changes has been uncertain. Both insulin deprivation and glucagon excess are known to alter lysosomal function and protein degradation in perfused liver,29 and the hypoinsulinemia and hyperglucagonemia of starvation have therefore seemed likely candidates for mediating hepatic changes.
Lack of insulin has been shown in cultured hearts to increase the activity of cathepsin D, particularly in the nonsedimentable fraction of the tissue homogenate, and this change can be correlated with concomitant increases in the rate of protein degradation. On the other hand, short-term studies of perfused hearts have suggested that insulin-dependent alterations in lysosomal function and protein degradation develop rapidly, unlike the delayed appearance of starvation-induced changes. Furthermore, in short-term studies of perfused hearts glucosaminidase is affected in a similar way and to a similar degree as cathepsin D.

The present experiments confirm that insulin deficiency in vitro is a potent stimulator of lysosomal enzyme activity and demonstrate that in cultured hearts, as in perfused hearts, glucosaminidase is altered in the same way as cathepsin D, even after several days. Thus, cardiac lysosomal alterations produced by insulin deprivation in vitro do not mimic exactly those observed after prolonged starvation. Therefore, although hypoinsulinemia may well contribute to some of the delayed effects of starvation, it seems unlikely to be the sole explanation for the complex lysosomal alterations that are observed in vivo.

On the basis of the present experiments, glucagon seems unlikely to play a vital role in lysosomal function in fetal mouse hearts, in contrast to its probable importance for lysosomal function in liver. It is impossible from these data alone to know whether this is a universal distinction between cardiac and hepatic responses or whether it is species-specific. Like guinea pig hearts but unlike liver and unlike hearts of many other species, mouse hearts apparently lack the capacity to respond to glucagon with increases in adenylyl cyclase activity and cyclic AMP. Thus, if glucagon-induced lysosomal alterations are mediated through the adenylyl cyclase-cyclic AMP system, it might be expected that mouse hearts (and, presumably, guinea pig hearts as well) would fail to be affected, even though hearts of other, responsive species could react the way liver does. Nevertheless, since starvation alters cardiac lysosomes similarly in the mouse as in other species, it seems doubtful that glucagon is of primary importance in causing cardiac changes in any species during starvation.

Growth hormone in very large concentrations causes only a minimal increase in cardiac cathepsin D activity, with no change in the distribution of the enzyme. Thus, on the basis of its effects in fetal hearts in vitro, its role in mediating lysosomal changes in vivo therefore seems likely to be negligible.

Substrate-dependent lysosomal changes are more complex. The effects of high concentrations of ketones and free fatty acids result in directional changes in the activities of cathepsin D, acid phosphatase, and glucosaminidase that resemble those during starvation. Increases in cathepsin D and, to a lesser extent, acid phosphatase are induced by ketones, whereas metabolizable fatty acids produce a decrease in glucosaminidase. Since these substrates cause a reduction in the uptake and utilization of glucose and, since reciprocal decreases in circulating levels of glucose often occur simultaneously with increases in fatty acids and ketones in vivo, it might have been expected that reduced concentrations of glucose in vitro would cause effects similar to those of ketones or fatty acids, or both. Paradoxically, this was not the case, and decreased glucose availability was accompanied by increased glucosaminidase activity over a wide range of concentrations; thus, hypoglycemia seems unlikely to play a direct role in causing the lysosomal alterations that accompany starvation.

The demonstration that several of the interventions can influence individual lysosomal enzymes in different ways confirms in vitro the observation in several tissues in vivo, including heart, that lysosomal enzyme respond to some stimuli in a heterogeneous manner. In at least some instances in vivo this could be due to an influx of migratory cells and a resultant alteration in the cellular origin of the lysosomes. Such an influx is clearly impossible in an organ culture system. One possible explanation is that there are separate effects on the several subsets of distinct lysosomal populations that are known to exist in heart. Alternatively, it remains possible that the synthesis or degradation

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**Table 3** Effects of Insulin Deprivation and of Acetoacetate (5 mM) and β-OH-butyrate (10 mM) on the Proportion of Lysosomal Enzyme Activities Present in the Nonsedimentable Fraction of Cardiac Homogenates

<table>
<thead>
<tr>
<th></th>
<th>% nonsedimentable cathepsin D activity (100 x nonsedimentable/total activity)</th>
<th>% nonsedimentable β-acyt/β-glucosaminidase activity (100 x nonsedimentable/total activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Medium 199 (n = 8)</td>
<td>46 ± 1.9</td>
<td>50 ± 1.7</td>
</tr>
<tr>
<td>Medium 199 + ketones (n = 8)</td>
<td>49 ± 1.7</td>
<td>51 ± 1.6</td>
</tr>
<tr>
<td>Difference</td>
<td>+3 ± 0.8</td>
<td>+1 ± 0.5</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.01</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>B. Medium 199 with insulin (n = 12)</td>
<td>41 ± 1.6</td>
<td>47 ± 1.3</td>
</tr>
<tr>
<td>Medium 199 without insulin (n = 12)</td>
<td>45 ± 1.7</td>
<td>50 ± 1.4</td>
</tr>
<tr>
<td>Difference</td>
<td>+4 ± 1.4</td>
<td>+3 ± 1.1</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.01</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

For each intervention, hearts of matched littermates (eight hearts per sample) were compared by Student's t-test for paired observations. Each value represents the mean ± 1 SEM.
of certain enzymes within a given lysosomal population may be altered independently from other enzymes in the same lysosomes.

Finally, it should be reiterated that changes observed in a relatively unphysiological system such as organ culture should be extrapolated to hearts in vivo only with caution; factors important in this highly catabolic model may be different in some regards from those important in adult hearts in vivo, and confirmatory studies in intact animals would be valuable. It also should be noted that the relatively small changes in hydrolytic enzyme activities and distribution noted here do not necessarily imply that concomitant alterations in cellular catabolism must occur in all instances, and actual measurements of the rate of proteolysis are required before postulations about the ultimate metabolic effect of the observed lysosomal changes should be entertained.

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