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ALTERATION OF LYSOSOMAL DENSITY BY SEQUESTERED GLYCOGEN DURING DEPRIVATION-INDUCED AUTOPHAGY IN RAT LIVER

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SUMMARY

Livers from fed rats were perfused in the single-pass mode with and without 10 mM glucose; autophagy then was induced by deleting amino acids. The decrease in glycogen which occurred in the absence of glucose did not influence the magnitude of the autophagic response, but it did affect the composition of autophagic vacuoles and the distribution of lysosomal marker on isopycnic centrifugation. In livers undepleted of glycogen, amino acid omission shifted a substantial portion of the β-acetylglucosaminidase peak into heavier gradient fractions. This shift was reduced 50% in partially depleted livers and was accompanied by a 40% decrease in glycogen-containing particles. These findings support the notion that glycogen sequestered during autophagy is responsible for the enhanced lysosomal density.

INTRODUCTION

We have previously shown that when fed rat livers are perfused with an unsupplemented medium, cellular autophagy and intracellular protein degradation are substantially greater in magnitude than are seen after additions of insulin or amino acids (1-4). Similar responses to deprivation have been noted in other isolated cell and tissue preparations (5-10). Although little yet is known of the nature of the cytoplasmic sequestrational process, we have observed that glycogen and smooth endoplasmic reticulum may be internalized when deprivation autophagy is induced in perfused livers from fed rats (1,2,11). The sequestration of a substance of high specific gravity, such as glycogen (12,13), could explain the alterations of lysosomal density which were reported under similar conditions (1,11).

The purpose of this study is to substantiate this putative, density-enhancing role of glycogen by inducing autophagy in perfused livers of fed rats in which the glycogen content is varied by prior perfusion with and without 10 mM glucose. The results provide further support for the view that internalized glycogen can increase lysosomal density, a finding which could be of importance in further elucidating autophagic function.

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MATERIALS AND METHODS

Liver Perfusion--Male rats of the Lewis strain (Microbiological Associates) were used as liver donors; they were fed ad libitum and weighed 125-140 g at the time of perfusion. Livers were perfused in situ in the single-pass mode (2,14); the medium consisted of Krebs-Ringer bicarbonate buffer containing 3% bovine plasma albumin (Pentex, fraction V, Miles Laboratories) and washed bovine erythrocytes, 0.27 (v/v). Before use, the dissolved albumin was filtered through 3 and 0.3 μm Millipore filters to remove bacteria and other particulate matter.

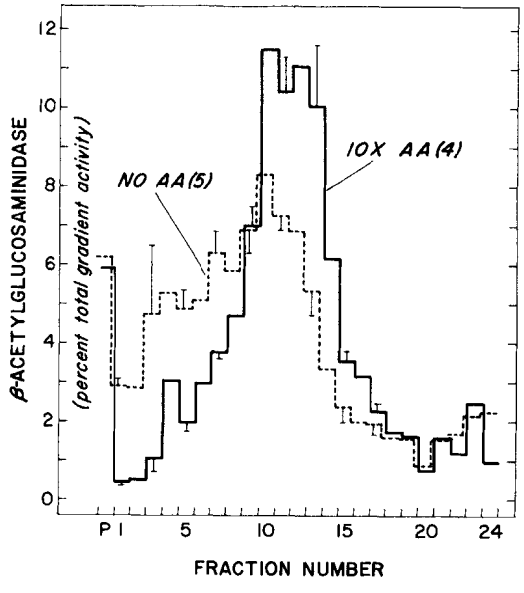
All perfusions were carried out for 90 min with or without 10 mM glucose. Hutson et al. (15) have shown that glycogen in isolated hepatocyte suspensions could be reduced 50% over a 90-min period by maintaining low glucose levels in the medium. In a series of 8 90-min single-pass perfusions, glucose omission caused an average glycogen reduction of 8.8 ± 2.2 mg/g liver, a value equivalent to 25% of the initial content; with 10 mM glucose, the corresponding loss was 1.2 ± 1.2. Although glycogenolysis could have been augmented by additions of glucagon or epinephrine, data interpretation would have been complicated by parallel stimulatory effects on autophagy (16). Initial liver glycogen was estimated from its content in the excised caudate lobe; both initial and final values were determined by the method of Carroll et al. (17). During the first 60 min of all experiments, livers were perfused with medium containing a 20-amino acid mixture at 10x plasma concentrations (4), an addition known to suppress autophagy effectively (2). In the 60 to 90-min period, perfusion was continued with the same amino acid-containing medium or, when autophagy was to be induced, perfusion was switched to a medium virtually devoid of amino acids (2). Maximal autophagy has been shown to be attained by 20 min (2).

Density Gradient Centrifugation--At the end of perfusion, livers were homogenized in 0.25 M sucrose-1 mM EDTA (18), and a mitochondrial-lysosomal (M + L) fraction prepared (19). The M + L pellet was resuspended in sucrose-EDTA, and 1 ml layered on a linear sucrose gradient as described (1). Centrifugation was accomplished in a SW 41 rotor spun at 41,000 rpm for 75 min (1). Gradient fractions, ranging in density from 1.116 to 1.267, were collected from the bottom of the tubes (1) and N-acetyl-β-D-glucosaminidase activity determined (20-21). The values for each fraction or group of fractions were expressed as a percentage of total activity on the gradient.

Electron Microscopy--In parallel experiments, livers were fixed through the vascular bed using a paraformaldehyde-glutaraldehyde fixative (22). The tissue then was prepared for electron microscopy by methods detailed earlier (2). The fine structure of hepatocytes and other cellular elements was examined and components of the lysosomal system in the former were evaluated by standard stereologic techniques (23-24). Two blocks of tissue were examined from each liver, one from the left lobe and one from the median lobe. Six to 8 micrographs from each block were evaluated.

RESULTS AND DISCUSSION

Fig. 1 shows that the complete omission of amino acids during single-pass perfusions causes a pronounced shift in the density distribution of a typical lysosomal marker, β-acetylglucosaminidase. This finding is similar, although not identical, with results from earlier cyclic perfusions (1). In the present runs, amino acid omission was associated with a broad spread of enzyme marker activity into the heavier parts of the gradient; a narrower, bimodal distribu-



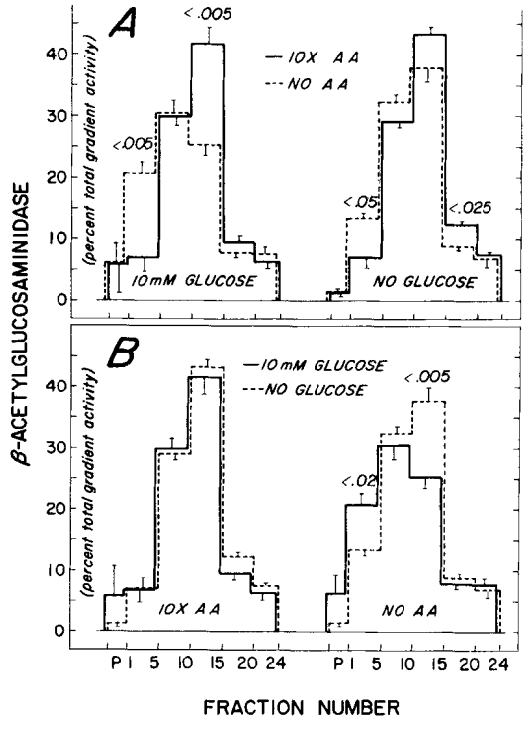


Figure 1 (left). Effect of amino acids on the density distribution of lysosomes in perfused rat livers. Livers were perfused for 90 min in the single-pass mode with and without 10x levels of a plasma amino acid mixture; 10mM glucose was present in all experiments. β-acetylglucosaminidase activities in fractions from highest (1.267) to lowest (1.116) densities of the linear gradients are plotted from left to right, respectively. Fraction P denotes the variable amount of material which pelleted in the tubes. Numbers of experiments are shown in parentheses.

Figure 2 (right). Density distributions of lysosomes from livers perfused in the presence and absence of glucose and amino acids. Livers were perfused for 90 min in the single-pass mode. Glucose (10 mM) was either present or absent for the entire period. The above 10x amino acid mixture was present for the first 60 min in all runs; it was continued through 90 min in experiments labeled, 10x AA, and omitted from 60 to 90 min in the rest (NO AA). β-acetylglucosaminidase activities from pooled fractions are depicted for the various groups in panels A and B. Each profile represents the mean ± S.E. of 4 or 5 perfusions. Gradient densities are the same as those in Figure 1.

tion was observed earlier in livers cyclically perfused with an unsupplemented medium (1). This difference can probably be explained by the greater diversity of autophagic elements that are generated with stringent amino acid depletion as compared to cyclic perfusion where the depletion is less severe (1,2).

To facilitate comparison among the different experiments, the gradients were divided into groups of 4 or 5 fractions (Figs. 2A and B). Perfusions carried out in the absence of added glucose (Figs. 2A, right and 2B, right) revealed a shift in enzyme distribution with amino acid lack similar to that in Fig. 1, but its magnitude was substantially attenuated (about 50%). No effect of glucose was observed when autophagy was fully suppressed by the addition of the 10x mixture (Fig. 2B, left).

Some unexpected pelleting was noted during the gradient runs. The nature of the material has not been established, but owing to the high specific gravity of glycogen (12,13), this substance would be a likely possibility. The fact that the pellets were larger in livers perfused with glucose supports this contention. The association of β-acetylglucosaminidase with the pellet suggests that some of the putative glycogen was sequestered within lysosomes (see below). We did not, however, observe a reduction in the amount of pelleted enzyme after amino acid additions. Individual values were extremely variable, and such an effect, if it existed, might be difficult to show.

Fig. 3 confirms previous findings in showing that the hepatic lysosomal-vacuolar system responds rather dramatically to amino acid deprivation (2). The alterations appeared to be limited to hepatocytes; Kupffer cells were not involved. The most striking changes were increases in the fractional cytoplasmic volumes of the larger autophagic vacuoles (types B and C), although all types were enhanced. Of particular relevance to the present study, however, is the fact that the autophagic responses were quantitatively the same in the presence and absence of glucose.

Assuming that less glycogen would be internalized when autophagic vacuoles are formed in an environment containing less glycogen, we determined the effect of glucose omission on the proportion of autophagic vacuoles containing visible glycogen. The results of this evaluation are presented in Table I as ratios of the fractional volumes of glycogen-containing elements to total fractional volumes. Perfusion with medium lacking glucose resulted in significant reductions of glycogen-containing particles in the three pools of autophagic vacuoles and a nearly 40% overall decrease in these particles, an effect which closely paralleled the aforementioned density attenuation. While the effects of glucose omission on lysosomal density and composition were larger than that on total glycogen depletion (see MATERIALS AND METHODS),

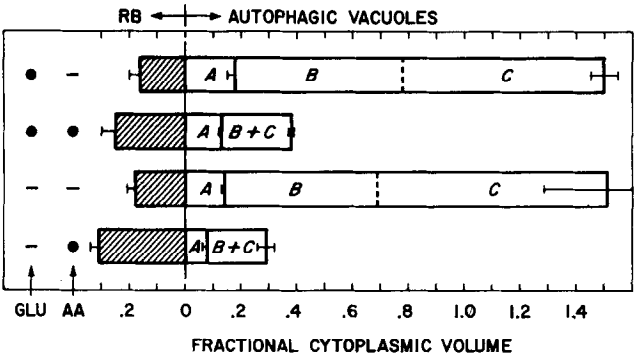


Figure 3. Effects of glucose and amino acids on the fractional cytoplasmic volume of lysosomal components in perfused rat livers. Experimental conditions correspond to those in Figures 1 and 2. Fractional cytoplasmic volumes were determined by standard stereologic methods (see MATERIALS AND METHODS). The classification of lysosomal components we used has been described (2) and is summarized as follows: RB, residual bodies, are electron-dense elements usually found in the vicinity of bile canaliculi. The remaining vacuoles were divided into three categories, A, B and C, according to their apparent contents (2). While these are labeled autophagic vacuoles at the top of the figure, we recognize that some of the A and B vacuoles may have been formed by fusion with endocytotic vesicles. Type A elements contain an electron-dense zone similar in appearance to RB substance and, in addition, a sharply demarcated, electron-lucent area indicating the presence of glycogen, smooth endoplasmic reticulum (SER), or both. B components appear to contain only SER and/or glycogen and are devoid of electron-dense zones. C vacuoles are distinguished by the presence of smooth and rough ER, glycogen, free ribosomes and mitochondria, all in various combinations. A significant proportion also contain unrecognizable membrane remnants and amorphous material which may represent cytoplasmic constituents in various stages of digestion. The horizontal bars depict means ± S.E.; errors at the ends of the bars (extreme right) are for B and C combined. Each group comprised 3 to 4 livers.

localized losses were probably highly variable and possibly greater at sites of autophagic vacuole formation (1,2).

Glycogen has been demonstrated within hepatic lysosomes in a variety of physiologic, pathologic and experimental situations (1,25-31), but to our knowledge no one has called attention to its density-altering potential. Surveys of autophagic vacuole profiles on electron micrographs indicate that our A and B-type particles (Fig. 3, Table I) probably have the largest amounts of glycogen per vacuole with estimates ranging upward to more than half. Profiles strikingly similar to these have been described in livers of rats made insulin deficient by streptozotocin (30) or anti-insulin serum (N. B. Ruderman and A. L. Jones, personal communication). Assuming that the density of glycogen in our preparations is of the order of 1.5 (12,13) and appreciably higher than that of residual body substance (~ 1.2), vacuoles containing as little as 20% glycogen would have an overall density close to the lower limit

Table I. Effect of perfusate glucose on the distribution of visible glycogen in lysosomal elements.

Vacuole type	FCV* of vacuoles containing visible glycogen / FCV of total X 100		p
	Glucose (4)	No glucose (3)	
A	89 ± 1.4	73 ± 4.7	<.025
B	39 ± 6.3	21 ± 1.4	<.05
C	43 ± 3.4	27 ± 1.6	<.01
A + B + C	47 ± 4.4	29 ± 1.2	<.02

*Fractional cytoplasmic volume

Livers were perfused for 90 min in the single-pass mode with and without 10 mM glucose. The 10x plasma amino acid mixture, which was present for the first 60 min, was omitted from 60 to 90 min. See legend to Figure 3 for classification of vacuoles; glycogen was identified by its characteristic granular pattern (1,2). The values are means ± S.E. of 3 to 4 perfusions (numbers in parentheses).

of the gradient. While we have no knowledge of the actual specific gravities of the major constituents, nevertheless, this prediction is not out of line with our findings. It obviously will be important in future studies to measure the glycogen content of purified, intact lysosomes. Although this is not practicable with the hyperosmolar gradients used here, there are newer gradient materials such as Ludox (colloidal silica) which hold considerable promise (31).

Glycogen sequestration is, of course, not the only factor which can affect lysosomal density (see reference 1 for a more complete discussion), but it is the most likely explanation for the selective density alterations, such as those in Fig. 1 and in earlier studies (1,11). Although the density alterations *per se* have no known physiological relevance, the indication that glycogen is internalized by some elements which traditionally have been considered part of the normal population of heterolysosomes compels us to reexamine the morphologic and functional limits of autophagy. Geddes and Stratton have reported that in normal, fed rats 10% of liver glycogen is intralysosomal and releasable by osmotic shock (32). This finding implies that some glycogen as well as cellular constituents associated with it could be processed by lysosomes under basal or steady state conditions. In view of the close relationship between glycogen and the smooth endoplasmic reticulum (33), it is not unreasonable to suppose that the latter is sequestered along with glycogen (1,2,11). A functionally important aspect of autophagy is that

it is not completely suppressible by amino acids and insulin. These findings offer one experimental approach to the general problem of basal protein degradation.

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