PROTEOLYSIS IN HOMOGENATES OF PERFUSED RAT LIVER: RESPONSES TO INSULIN, GLUCAGON AND AMINO ACIDS*

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SUMMARY

The proteolytic release of leucine and isoleucine was assessed in homogenates of rat livers perfused under conditions known to influence protein degradation in the intact liver. Release was increased by perfusion alone and by additions of glucagon and was inhibited by insulin and amino acids. These responses correlated both with rates of proteolysis during perfusion and with physical alterations of the lysosomal system, reported earlier. Homogenate proteolysis appeared to comprise two components: the release of free amino acids from the total particulate fraction and from peptides in the cytosol. Both components are believed to be generated by elements of the lysosomal system.

INTRODUCTION

We reported recently that lysosomes in rat liver after perfusion become larger, more sensitive to osmotic shock, and exhibit greater density on equilibrium centrifugation (1,2). These alterations can be reversed by additions of insulin or amino acids and thus they probably appear as a consequence of the loss of inhibitors normally operating in the intact animal. The fact that these spontaneous physical alterations resemble lysosomal changes induced by glucagon (3,4), suggests that they represent a form of cellular autophagy. They differ from glucagon-induced changes, however, in that they become manifest in the absence of an increase in the tissue level of adenosine 3',5'-cyclic monophosphate (5).

General or bulk protein degradation increases during control liver perfusion (6) and after glucagon (7) and is directly inhibited by insulin (6) and amino acid

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additions (8). In view of the association with the above lysosomal alterations, we have considered the notion that the lysosomal system directly participates in these degradative effects by the sequestration and exergonic hydrolysis of cellular proteins. This possibility was investigated in the present study by assessing the effects of insulin, glucagon and amino acid additions during liver perfusion on proteolysis in tissue homogenates.

METHODS

Liver donors were male rats of the Lewis strain (Microbiological Associates); they were fed ad libitum and weighed from 125 to 135 g at the time of experiment. Livers were cyclically perfused in situ by a technic detailed earlier (9-11). The perfusing medium consisted of a suspension of washed sheep cells (0.27, v/v) in a solution of Krebs-Ringer bicarbonate buffer and 4% bovine albumin (fraction V, Pentex) (12). At the termination of perfusion, livers were quickly excised and homogenized in a cold solution of 0.25M sucrose and 10^{-3}M disodium ethylenediamine tetraacetate with an all-glass Dounce tissue grinder (Kontes). Previous experience with this method has shown that reasonably complete cell disruption can be obtained with minimal lysosomal damage (1,2).

Whole homogenates or supernatant fractions (see Figures 3 and 4) were incubated in a shaking water bath at 37°. Samples were removed at intervals and cold CCl_3COOH added to a concentration of 5%. The clear filtrates then were passed through small columns of Dowex-50 (H^+) (6). The columns were washed with water, eluted with 5N NH_4OH, and leucine and isoleucine measured together by a chromatographic method described earlier for valine (6). Control experiments showed that the combined amounts of the two leucines recovered under conditions of this study were proportional to valine by a factor of approximately 2.

The stability of branched-chain amino acids released during incubation was assessed by methods previously described (6). L-[1-^{14}C] valine was added to whole homogenates in stoppered center-well flasks containing Hyamine. In two experiments, 0.4% (0.34 - 0.46) of the total label added was recovered as [^{14}C]O_2 after 30 min of incubation at 37°; 0.02% was incorporated into protein. Thus rates of amino acid decarboxylation and protein synthesis were negligible. The pH of the homogenate ranged from 6.9 to 7.0 over 90 min of incubation; aging of the homogenate for 60 min at 0° had no discernable effect on leucine release during subsequent 37° incubations.

RESULTS

Our rationale for the use of leucine + isoleucine as a measure of homogenate proteolysis was based on the wide distribution of leucines in protein, precision

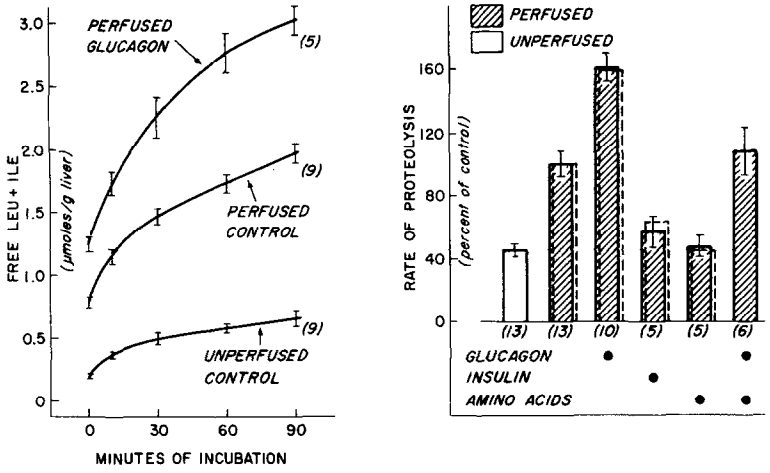

Figure 1 (left). Release of free leucine + isoleucine during incubation of unfractionated homogenates of unperfused rat liver and of liver perfused 60 min with and without glucagon. Glucagon (Lot No. 258-234 B-167-1, Lilly Research Laboratories) was dissolved in dilute HCl, added to a solution of 0.5% bovine albumin and 0.85% NaCl, and then infused into the perfusion medium at rates of 1 or 10 μg/hr (in 0.22 ml) over the 60-min perfusion period. No differences in responses were noted between the two infusion rates. The vertical bars depict means ± one S.E.; numbers of experiments are in parentheses.

Figure 2 (right). Relative rates of proteolysis in unfractionated liver homogenates compared with rates of proteolysis measured during liver perfusion. Homogenate proteolysis (solid lines) was assessed from the increase in leucine plus isoleucine between 0 and 30 min of incubation, expressed as percent of the mean value for control perfused livers. Livers were perfused for 60 min in the presence or absence of the following additions: Glucagon--infused as in Figure 1; insulin (Lot No. 499667, Lilly Research Laboratories)--infused at 2.4 μg/hr as previously detailed (6); amino acids--a mixture of 20 amino acids simulating the composition of plasma amino acids at 10x normal concentrations was added initially and after 30 min of perfusion as described previously (8). Vertical lines are means ± one S.E.; numbers of experiments are in parentheses.

The offset broken lines depict average rates of proteolysis measured by the release of valine during perfusion in separately reported experiments (6-8). Each group was normalized to its respective mean perfused control value, set to equal 100. Additions of glucagon, insulin and amino acids were comparable to those used in the present experiments.

of measurement (6) and the stability of the free residues as shown above with [^{14}C]labeled valine. Preliminary determinations of total ninhydrin reactive material were not satisfactory owing to rather high and variable initial values which tended to obscure differences between groups. These high values were probably related to the presence of peptides in the extracts (see Figure 4).

It is evident from Figures 1 and 2 that control perfusion per se is a significant stimulus to proteolysis in unfractionated homogenates. In both perfused and unperfused control groups, the initial accumulation of leucine was rapid,

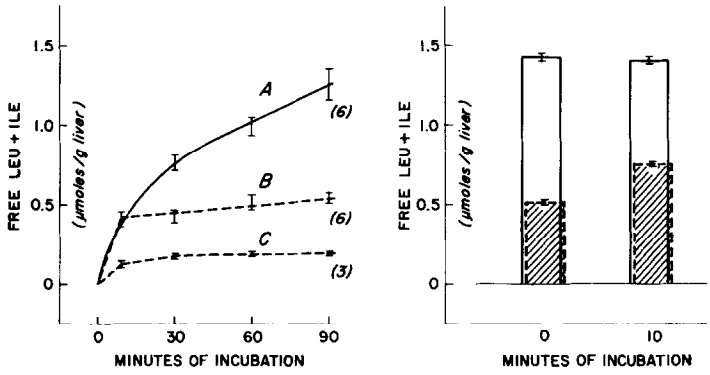

Figure 3 (left). Cumulative release of leucine + isoleucine during incubation of whole and supernatant rat liver homogenate fractions. Solid line (A), whole (unfractionated) homogenate. Broken lines, supernatant fractions from perfused control livers (B) and unperfused control livers (C). The latter fractions were prepared by centrifuging whole homogenates at 150,000 x g for 20 min. Vertical lines depict means ± one S.E.; numbers of experiments are in parentheses.

Figure 4 (right). Free leucine + isoleucine in CCl_3 COOH extracts of liver supernatant fractions before and after acid hydrolysis. Supernatant fractions (3.0×10^6 x g • min) from 4 control perfused rat livers were pooled and extracted with 5% CCl_3 COOH before and after incubation for 10 min at 37°. The extracted cationic material, which included free amino acids and small peptides, was taken up on columns of Dowex-50 for the determination of leucine plus isoleucine as described in Methods. A portion of the eluted material was hydrolyzed in 6N HCl *in vacuo* at 110° for 24 hr before the leucine determinations. Broken lines, free leucine + isoleucine before acid hydrolysis; solid lines, free leucine after acid hydrolysis; the clear space between bar heights represents the acid-hydrolyzable fraction. Vertical lines denote duplicate determinations.

and linear rates were observed between 30 and 90 min. The average accumulation of leucine + isoleucine in the unperfused control group from 30 to 90 min was 0.158 μmoles/g; the corresponding value for the control perfused group was 0.502 μmoles/g. The administration of glucagon during perfusion increased proteolysis even further. Most of this increase occurred in the first 60 min; thereafter the time-course tended to parallel the perfused control curve.

Additions of insulin or a complete mixture of amino acids during perfusion inhibited proteolysis and virtually abolished the increase by perfusion (Figure 2). Of particular interest to us was close association in relative rates between homogenate proteolysis and proteolysis measured during perfusion by the valine turnover method, the latter reported separately (6-8). Since the mechanism of proteolysis and its requirements are still largely undefined, we did not attempt to compare absolute rates.

The effect on proteolysis of removing lysosomal elements and the majority of subcellular particles from the cytosol is shown in Figure 3. The initial rates of release of leucine in supernatant fractions from perfused and unperfused liver were about the same as their respective values in the unfractionated parent homogenates (Figure 1), but little increase was noted after 10 min. Owing to the rapidity of appearance of free leucine and its self-limited course, we felt that a likely source of the amino acids was the degradation of small peptides by peptidases known to exist in the supernatant fraction (13-15). As shown in Figure 4, the supernatant fraction of perfused liver contains a sizeable pool of trichloracetic acid-soluble material which releases free leucine and isoleucine after hydrolysis with 6N HCl. Since close quantitative agreement was obtained between the spontaneous increase in free leucines and the decrease in the acid-hydrolyzable fraction after 10 min of incubation, it seems likely that the free leucines were derived in part from this fraction. The smaller degree of spontaneous hydrolysis that is seen in Figure 4 when compared with Figure 3 relates simply to variability between groups of livers. Although the measurements reported here were limited to leucine and isoleucine, all other amino acids that could be identified on the chromatograms appeared to increase proportionately after acid hydrolysis.

Studies now in progress indicate that the acid-hydrolyzable leucine pool increases about 75% with perfusion. We have not identified specifically those components which are degraded spontaneously during the 37^{o} incubations, but we believe they represent peptides of low molecular weight, perhaps dipeptides. The latter possibility is of interest since dipeptides were thought by Coffey and deDuve to accumulate as one of the major products of proteolysis when denatured human globin was exhaustively digested by lysosomal enzymes (13).

DISCUSSION

The notion that the lysosome participates in the general regulation of hepatic proteolysis is supported by the relationship among (a) measurements of proteolysis in the intact liver (6-8), (b) physical alterations of the lysosomal system (1,2),

and (c) proteolysis in liver homogenates. A closer look at characteristics of the last group, however, indicates that the participation by lysosomes, if real, is not limited simply to intralysosomal events. A comparison of data in Figures 1 and 3 suggests that more than one-half of the free leucine generated during the 30-min incubations in Figure 2 was derived by proteolysis in the supernatant fractions. Assuming that the released amino acids arose from the hydrolysis of small peptides by soluble peptidases, as our present findings indicate, it is possible, considering the findings of Coffey and de Duve, that the lysosome was the ultimate source of these peptides. The enzyme, dipeptidyl aminopeptidase (cathepsin C), which cleaves dipeptide fragments from proteins or larger peptides (16), is thought to be exclusively lysosomal in localization (16). If future studies show the existence of a regulated dipeptide pool in the cytosol of liver, then it may be possible to establish a firm link between the lysosome and certain phases of hepatic proteolysis.

The uptake and sequestration of cellular proteins by the lysosomal system (cellular autophagy) is the one mechanism which we believe is consistent both with the physical alterations in the lysosomal system, cited in the introduction, and the enhanced proteolysis observed with perfusion alone and after glucagon administration. In general terms, this view presupposes that an increase in protein substrate is largely responsible for the enhanced production of free amino acids observed in both fractionated and unfractionated homogenates, but it would not eliminate the possibility of an associated elevation of protease activity.

An alternative explanation for the observed stimulation of proteolysis by perfusion alone or glucagon is an alteration of the lysosomal membrane which would permit the passage of proteins between the intralysosomal space and the cytosol. This would be expected to lead to an increase of free enzyme activity in the initial, isotonic homogenates. Such an increase has been reported after glucagon treatment and was attributed to an enhanced sensitivity of autophagic vacuoles to mechanical stress (13,17,18). We have not observed any significant elevation of

free lysosomal enzyme activity in isotonic homogenates, either after glucagon treatment or perfusion (1,2). This apparent discrepancy might be related in part to differences in methods of tissue homogenization.

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