Vol. 117, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1983 Pages 509-516

PARADOXICAL STIMULATION BY AMINO ACIDS OF THE DEGRADATION OF [^{35}S]METHIONINE-LABELLED, SHORT-LIVED PROTEIN IN ISOLATED RAT HEPATOCYTES

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Received October 24, 1983

A complete amino acid mixture inhibited the degradation of long-lived and [^{14}C]valine-labelled short-lived protein in isolated rat hepatocytes, but paradoxically stimulated the degradation of [^{35}S]methionine-labelled short-lived protein. The stimulation persisted in the presence of autophagic-lysosomal pathway inhibitors like 3-methyladenine and propylamine, indicating the existence of an hitherto unrecognized non-lysosomal degradation mechanism with selectivity towards methionine-rich proteins or peptide regions.

Cellular proteins are degraded by lysosomal as well as non-lysosomal mechanisms, the former showing preference for long-lived and the latter for short-lived proteins (1-3). Previous studies of isolated rat hepatocytes, using [^{14}C]valine as a protein labelling agent, indicated that about 75 % of the long-lived (24-h labelled) and about 50 % of the short-lived (1-h labelled) proteins could be degraded lysosomally (3,4). The degradation of long-lived protein was inhibited to almost the same extent by autophagy inhibitors like amino acids and 3-methyladenine as by lysosome inhibitors like propylamine and leupeptin (5,6), whereas amino acids had much less effect than propylamine on the degradation of short-lived protein (5), indicating a greater heterogeneity in degradation mechanisms for this protein class.

In the present study we have, by using [^{35}S]methionine to label cellular protein, obtained evidence for the existence of a non-lysosomal mechanism of degradation of short-lived proteins which is stimulated rather than inhibited by amino acids.

0006-291X/83 $1.50

MATERIALS AND METHODS

Isolation and incubation of cells. Hepatocytes were isolated from the liver of 16-h starved male Wistar rats (250-300 g) by the two-step collagenase perfusion technique (7). The cells were incubated as shaking suspensions in suspension buffer (7) fortified with pyruvate (20 mM) and $MgCl_2$ (2 mM). The mixture of amino acids used in the experiments was the same as in Seglen's hepatocyte medium SM-1 (8), except for the concentrations of asparagine and glutamine which were lowered to 0.3 mM.

Measurement of protein degradation. Protein degradation was measured as the release of acid-soluble [^{14}C]valine from prelabelled protein or as the loss of radioactivity from acid-precipitable material prelabelled with [^{35}S]methionine or [^{14}C]valine. For the labelling of predominantly short-lived protein, hepatocytes were incubated for 1 h at 37°C in fortified suspension buffer in the presence of 0.025 µCi/ml [^{14}C]valine or 5 µCi/ml [^{35}S]methionine. After extraction of as much acid-soluble radioactivity as possible (3x incubation for 10 min at 37°C), and washing of the cells, protein degradation was measured at 37°C in the presence of unlabelled amino acid (10 mM valine or 12 mM methionine, to prevent reincorporation). Incubations (0.4 ml cells at a concentration of 70-80 mg wet wt./ml) were terminated by the addition of 100 µl 10 % (w/v) cold perchloric acid (for measurement of acid-soluble radioactivity) or 4 ml 2 % (w/v) cold perchloric acid (for measurement of acid-insoluble radioactivity). The precipitates were solubilized in 0.5 ml 0.1 N NaOH/0.4 % deoxycholate. 8 ml scintillation fluid was added, and the sample radioactivity measured by liquid scintillation counting. For the labelling of predominantly long-lived protein, rats were injected intravenously with 1 ml [^{35}S]methionine (10.5 mCi/ml) or 1 ml [^{14}C]valine (50 µCi/ml) 25 h before the isolation of the cells. Degradation was then measured as above.

Attempts were also made to measure protein degradation as the release of acid-soluble radioactivity after [^{35}S]methionine prelabelling (data not shown). For the degradation of long-lived protein, this method gave the same result as the measurement of acid-insoluble radioactivity. However, the results obtained with short-lived protein were too variable to be meaningful, because the intracellular pool of [^{35}S]methionine, an actively transported amino acid (9), could not (in contrast to [^{14}C]valine) be sufficiently depleted by serial extraction to provide an acceptably low background level.

Materials. [^{35}S]methionine (1300 Ci/mmol; 12.5 µCi/ml; SJ 204) and [^{14}C]-valine (275 Ci/mol; 50 µCi/ml; CFB 75) were purchased from the Radiochemical Centre, Amersham, Bucks, England. 3-Methyladenine (6-amino-3-methyl-purine) was from Fluka AG, Buchs, Switzerland, and propylamine from Koch-Light Laboratories, Colnbrook, England. All other biochemicals were from Sigma Chemical Co., St.Louis, Mo., USA.

RESULTS

Degradation of long-lived protein. Previous studies have shown that the degradation of long-lived protein in isolated rat hepatocytes, measured as the release of [^{14}C]valine from 24-h labelled protein, takes place at a rate of 4-5 %/h in an amino acid-free medium (3-6). A corresponding value was found when degradation was measured as the net loss of protein radioactivity (Fig. 1B). [^{35}S]Methionine-labelled cells (Fig. 1A) lost protein-

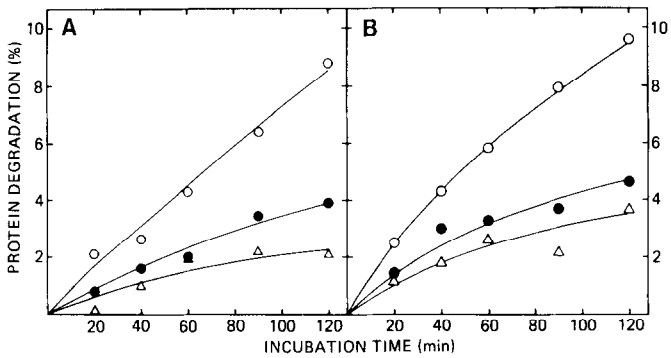

Fig. 1. Degradation of long-lived protein: inhibition by amino acids and propylamine. Hepatocytes, labelled for 24 h *in vivo* with [^{35}S]methionine (A) or [^{14}C]valine (B) were incubated at 37°C in the presence of non-radioactive methionine (12 mM) or valine (10 mM), respectively. Protein degradation was measured as the loss of acid-precipitable radioactivity from the system (cells + medium), expressed in per cent of the initial value. o, unsupplemented medium; •, amino acid mixture (1x); Δ, propylamine (10 mM). Each point is the mean of 3-5 cell samples from one experiment (A) or the mean of 3 experiments (B); standard errors were within the range ± 0.5 % (absolute value).

radioactivity at a similar rate, indicating that methionine and valine are equally valid as labelling agents for long-lived protein.

The degradation of [^{35}S]methionine-labelled and [^{14}C]valine-labelled long-lived protein was similarly inhibited, both by the lysosome inhibitor propylamine and by a complete amino acid mixture (Fig. 1). The effect of the amino acids was not as strong as in previous experiments (4-6), probably reflecting the fact that two of the most active inhibitors, asparagine and glutamine, were present at reduced concentrations (0.3 mM as compared to 5 mM in the mixture given in ref. 8), a modification introduced to prevent the recently described suppression of DNA synthesis by these amino acids (11).

Degradation of short-lived protein. A short (e.g. 1 h) isotope pulse selectively labels the short-lived protein of the cells, a protein class which constitutes only a very small part of the total cellular protein, but which may contribute significantly to over-all protein turnover (12). As shown in Fig. 2, [^{35}S]methionine-labelled (Fig. 2A) and [^{14}C]valine-labelled (Fig. 2B) short-lived protein was degraded at the same high rate,

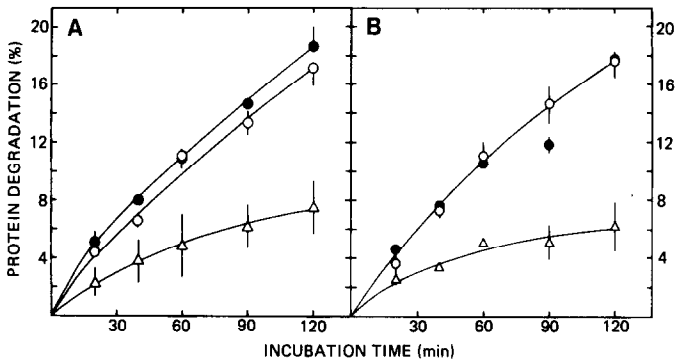

Fig. 2. Degradation of short-lived protein: effect of amino acids and propylamine. Hepatocytes, labelled for 1 h in vitro with [^{35}S]methionine (A) or [^{14}C]valine (B) were extracted 3 x 10 min. at 37°C and then incubated at 37°C for measurement of protein degradation as described in the legend to Fig. 1. Symbols as in Fig. 1; each point is the mean ± S.E. of 3 experiments (some of the S.E.'s are hidden by the symbols).

the degradation being 50-60 % inhibited by propylamine. However, the amino acid mixture, which inhibited the degradation of long-lived protein strongly, had little or no effect on the degradation of [^{14}C]valine-labelled short-lived protein, and in fact stimulated the degradation of [^{35}S]methionine-labelled short-lived protein slightly (but significantly). The results confirm the notion that part of the lysosomal degradation of short-lived protein occurs by a distinct, amino acid-resistant mechanism (5). Much of the amino acid inhibition of short-lived protein degradation previously observed could probably be ascribed to the high concentrations of asparagine and glutamine then used, possibly reflecting a post-sequestrational effect of those two amino acids (5,13).

Paradoxical effect of amino acids. The paradoxical stimulation of the degradation of short-lived [^{35}S]methionine-labelled protein by the amino acid mixture was further examined, including the combination of amino acids with other inhibitors. 3-Methyladenine (3MA), a specific suppressor of autophagic sequestration (6), inhibited the degradation of [^{35}S]methionine-labelled, short-lived protein 50 % (Fig. 3), indicating the partial involvement of an autophagic mechanism in the degradation of this protein class. A mixture of leucine and histidine (10 mM of each), likewise found to

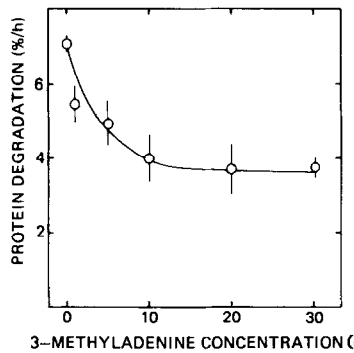

Fig. 3. Effect of 3-methyladenine on the degradation of short-lived protein. Hepatocytes were labelled for 1 h in vitro with [^{35}S]methionine, and protein degradation measured between 30 and 90 min of incubation (in the presence of various concentrations of 3MA) as described in the legend to fig. 1. Each point is the mean ± S.E. of 3 experiments.

suppress autophagic sequestration specifically (P.O. Seglen and P.B.Gordon, unpublished experiments), inhibited short-lived protein degradation some 30 % (Tables I and II).

Table I. Effects of different compounds on the degradation of [^{35}S]methionine-labelled, short-lived protein.

Additions	Effect on protein degradation (% change relative to inhibitor-free control)			
	Control	+ Amino acids (1x)	+ Cycloheximide (1 mM)	+ Amino acids + Cycloheximide
None	0	+ 15.5 ± 3.5 (13)**	- 12.7 ± 4.5 (12)	- 16.3 ± 7.7 (12)
Propylamine (20 mM)	- 68.6 ± 6.2 (6)	- 25.0 ± 10.4 (5)*	- 65.5 ± 10.0 (5)	- 47.3 ± 16.3 (5)
3-Methyladenine (20 mM)	- 49.0 ± 3.8 (12)	- 12.4 ± 5.5 (12)**	- 40.9 ± 7.2 (4)	- 17.0 ± 11.6 (5)
Histidine + Leucine (10 mM + 10 mM)	- 29.4 ± 4.2 (13)	- 8.5 ± 9.1 (8)	- 27.2 ± 4.0 (5)	- 27.1 ± 16.6 (5)

Hepatocytes were labelled with [^{35}S]methionine for 1 h in vitro. After extraction (3 x 10 min at 37°C) of as much intracellular acid-soluble [^{35}S]radioactivity as possible, the cells were incubated in isotope-free medium at 37°C in the presence of the compounds indicated, and protein degradation was measured as the loss of acid-precipitable [^{35}S]radioactivity from cellular protein between 30 and 90 min of incubation. The control rate of degradation (% isotope released) averaged 7.9 ± 0.2 %/h (mean of 13 expts.); the effect of inhibitor combinations are expressed as % difference from control (each value is the mean ± S.E. of the number of expts. given in parentheses). Significance of amino acid effect vs. the respective control according to the t-test:

*P < 0.01; **P < 0.001.

Table II. Effects of different compounds on the degradation of [^{14}C]-labelled, short-lived protein.

Additions	Inhibition of protein degradation (%)			
	Control	+ Amino acids	+ Cycloheximide	+ Amino acids + Cycloheximide
None	0	- 11.5 ± 1.3	- 20.3 ± 2.6	- 35.0 ± 1.3
Propylamine (20 mM)	- 57.6 ± 2.2	- 54.0 ± 2.2	- 60.2 ± 1.4	- 60.1 ± 2.5
3-Methyladenine (20 mM)	- 37.0 ± 2.0	- 31.4 ± 6.3	- 44.6 ± 2.7	- 54.5 ± 2.3
Histidine + Leucine (10 mM + 10 mM)	- 33.0 ± 5.6	- 31.8 ± 6.6	- 38.3 ± 6.7	- 49.5 ± 5.7

Hepatocytes were labelled with [^{14}C]valine for 1 h in vitro. After extraction (3 x 10 min at 37°C) of intracellular acid-soluble [^{14}C]radioactivity, the cells were incubated in isotope-free medium at 37°C in the presence of the compounds indicated, and protein degradation was measured as the release of acid-soluble [^{14}C]radioactivity from the cells between 30 and 90 min of incubation. The control rate of degradation (% isotope released) averaged 9.4 ± 0.2 %/h (mean of 5 expts.); the effects of inhibitor combinations are expressed as % inhibition relative to control (each value is the mean ± S.E. of 5 experiments).

A significant stimulation by the amino acid mixture of the degradation of [^{35}S]methionine-labelled short-lived protein was observed in the presence of propylamine as well as with 3MA, the results with His+Leu being more variable (Table I). With [^{14}C]valine-labelled protein, the amino acid effects were, if anything, slightly inhibitory (Table II). Cycloheximide, which markedly inhibits the degradation of long-lived protein (14,15), inhibited the degradation of short-lived protein moderately (Tables I and II), but in the presence of amino acids or other inhibitors the results became too variable to be meaningful (Table I).

DISCUSSION

Paradoxical protein degradation-stimulatory effects of amino acid mixtures have previously been observed both in the perfused liver (leucine antagonism, ref. 16) and in isolated hepatocytes (glutamine antagonism, our unpublished experiments), but they are not necessarily related to the present stimulation which is confined to [^{35}S]methionine-labelled short-

lived protein. One candidate process capable of accounting for such selectivity may be the proteolytic processing of N-terminal peptide regions, which would be expected to be enriched in methionine both by virtue of the initiator function of Met-tRNA (17) and due to the relative abundance of methionine in the signal peptide region of secretory protein precursors (18). Processed regions would be included in the short-lived protein category; N-terminal methionine has in fact been demonstrated to turn over more rapidly than the bulk of protein in L cells (19). Since proteolytic processing is involved in the formation of both secretory and mitochondrial protein (20), each of which accounts for 25-30 % of the protein synthesized by hepatocytes (21, 22 and our unpublished experiments), the contribution of signal peptide degradation (the ultimate site of which is unknown) to the degradation of [^{35}S]methionine-labelled short-lived protein could be considerable.

The ability of amino acids to stimulate degradation of this particular protein class even in the presence of autophagic-lysosomal inhibitors like 3MA and propylamine clearly indicates that the proteolytic process affected is non-lysosomal. Non-lysosomal mechanisms account for 40-50 % of the degradation of short-lived protein in hepatocytes (3-5, and e.g. mitochondria are well equipped with proteolytic enzymes which might conceivably participate in the degradation of signal peptides or other short-lived protein material (23-25). Although the well-established stimulation of peptide chain initiation by amino acids (26,27) would not affect the present measurements because pre-labelled protein was used, any effect of amino acids on chain elongation, attachment of signal peptide regions to membranes (28, transmembrane peptide transfer or other uptake processes, or proteolysis itself, might be detected as a paradoxical stimulation of the degradation of [^{35}S]methionine-labelled short-lived protein.

ACKNOWLEDGEMENTS

We wish to thank Indrejit Dybsjord for excellent technical assistance, and The Norwegian Cancer Society for generous support.

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