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Control of proteolysis in perifused rat hepatocytes

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The mechanism by means of which amino acids inhibit intrahepatic protein degradation has been studied in perifused rat hepatocytes. Proteolysis was extremely sensitive to inhibition by low concentrations of amino acids. A mixture of 0.5 mM leucine and 1–2 mM alanine, concentrations found in the portal vein of the rat after feeding, inhibited proteolysis to the same extent as a complete physiological mixture of amino acids. Inhibition by these two amino acids was accompanied by a rise in the intracellular concentrations of glutamate and aspartate, and was largely prevented by addition of glucagon, by addition of the transaminase inhibitor aminooxyacetate, or by omission of K^+. Acceleration of proteolysis by K^+ depletion was accompanied by a fall in intracellular glutamate caused by an increased rate of transport of this amino acid to the extracellular fluid. It is concluded that intracellular leucine, glutamate and aspartate are important elements in the control of hepatic protein degradation.

Proteolysis; Hepatocyte

1. INTRODUCTION

Intrahepatic protein degradation is an important source of amino acids for gluconeogenesis in the starved rat. Proteolysis under these conditions mainly occurs via macroautophagy (for reviews see [1,2]). Little is known about the mechanisms by means of which this process is controlled, except that amino acids and a low glucagon/insulin ratio are inhibitory. The nature of the amino acids responsible for inhibition of macroautophagy has been subject of several studies [1,2]. In such studies the perfused liver (see e.g. [3,4]) and suspensions of isolated hepatocytes in flask incubations [5,6] have been used as the experimental material. The results obtained with these preparations are conflicting. Whereas in the perfused liver amino acids at physiologically low concentrations inhibit protein breakdown [3,4], in isolated hepatocytes unphysiologically high concentrations of amino acids are required to obtain substantial inhibition [5,6].

In order to study protein breakdown under steady-state conditions, we have used perifused hepatocytes as the experimental material. Proteolysis in this preparation was extremely sensitive to inhibition by low concentrations of amino acids.

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Abbreviation: AOA, aminooxyacetate

2. MATERIALS AND METHODS

Hepatocytes from 24-h starved male Wistar rats (body wt 200 g) were isolated as in [7]. Perifusion was carried out as described in [7]. The perifusion medium was Krebs-Henseleit bicarbonate buffer, fortified with 10 mM glucose, 0.2 mM octanoate and 0.2 mM ornithine; the medium was saturated with 95% O_2 and 5% CO_2. Other additions are indicated in the legends to the tables and the figure.

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The volume of the perifusion chamber was 12 ml, to which 150–200 mg dry wt of cells were added. Perifusion flow was 5 ml per min; the temperature 37°C. Under each condition in the various experiments, a steady state was reached after 35 min of perifusion. Samples of the perifusate were always taken after 40 to 45 min.

Incubation of hepatocytes (5–10 mg dry wt per ml) in closed flasks was carried out in the same medium as mentioned above, except that octanoate and ornithine were present at 2 mM.

Samples of the perifusate or of the cell incubations were quenched with sulphosalicylic acid (final concentration 4%, w/v) and neutralised to pH 2.2 with LiOH after removal of precipitated protein by centrifugation. The amino acid analysis was carried out on an LKB alpha plus amino acid analyser, using a lithium citrate buffer system as specified by the manufacturer. Intracellular amino acid levels were obtained after centrifugation of the cells through a layer of silicone oil (wacker AR 20:200 = 2:3) into a layer of sulphosalicylic acid (20%, w/v). In order to minimise correction for amino acids present in the adherent fluid, the suspension was diluted five-fold with ice-cold Krebs-Henseleit bicarbonate buffer 10 s before centrifugation.

Statistical significance of the difference between groups of observations was tested with Student's *t*-test.

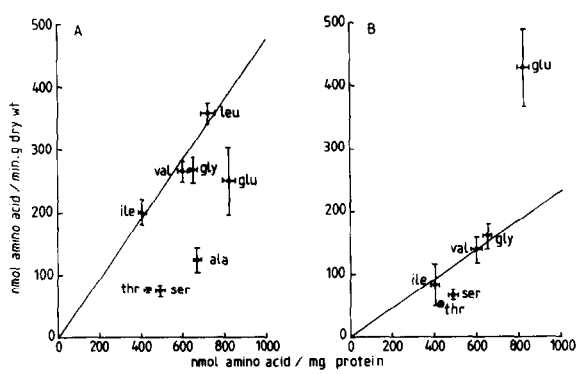

Fig.1. Proteolysis in perifused hepatocytes. Hepatocytes were perifused in the absence (A) or presence (B) of 0.5 mM leucine plus 1-2 mM alanine. Amino acids were determined in the perifusate. Data (means ± SE) are from 12 different hepatocyte preparations. The amino acid composition from liver protein was derived from [8].

3. RESULTS

When hepatocytes were perifused in the absence of added amino acids, the rates of production of histidine, isoleucine, valine, glycine and leucine were proportional to their quantitative occurrence in liver protein (fig.1A). Amino acids with data points below the line apparently were catabolised further. These results and those to be discussed below were not significantly affected by low concentrations of cycloheximide (20 μM) which inhibited protein synthesis by 90–95% (not shown). On the basis of these observations we have expressed proteolytic rates as the production rate of valine, although in principle each of the other

Table 1

Inhibition of proteolysis by amino acids

Experimental system	Additions	Valine release (nmol · min^{-1} · g dry wt^{-1})	
Perifusion	None	263 ± 16	(18)
	Amino acid mixture	143 ± 33	(4)[a]
	Amino acid mixture + 10^{-7} M glucagon	222	(1)
	Alanine, 2 mM	230 ± 16	(3)
	Leucine, 0.5 mM	253 ± 10	(3)
	Alanine + leucine	136 ± 12	(9)[a]
	Alanine + leucine + glucagon	212 ± 21	(5)[b]
Incubation of cells	None	177 ± 7	(7)
	Alanine, 5 mM	148 ± 10	(6)[a]*
	Leucine, 1 mM	166 ± 14	(5)
	Alanine + leucine	105 ± 7	(7)[a]
	AOA, 0.5 mM	179 ± 18	(3)
	AOA + alanine + leucine	145 ± 5	(3)[c]

[a] Smaller than the control value without addition ($P<0.01$; a*, $P<0.05$)

[b] Higher than the corresponding value in the absence of glucagon ($P<0.01$)

[c] Higher than the corresponding value in the absence of AOA ($P<0.01$)

Hepatocytes were either perifused or incubated in closed vessels under the conditions indicated. The composition of the amino acid mixture resembled that in the portal vein of the starved rat and is described in [9]. Data are the means ± SE with the number of different hepatocyte preparations in parentheses

amino acids mentioned can be used for this purpose.

Addition of a physiological mixture of all amino acids, each of which was present at its concentration in the portal vein of the starved rat, decreased the rate of proteolysis by 45%; this inhibition was relieved upon infusion of glucagon (table 1). These data are in agreement with those obtained with the perfused liver [3]. The same result was obtained when the entire amino acid mixture was replaced by a combination of 0.5 mM leucine plus 1–2 mM alanine (fig.1B, table 1). We selected these amino acids on the basis of work in [4] and [6] showing that leucine and alanine in particular contribute to inhibition of hepatic proteolysis by physiological mixtures of amino acids. Inhibition by leucine and alanine was also observed in hepatocytes incubated in closed vessels, although slightly higher concentrations of the amino acids were required (table 1). In both experimental systems, leucine and alanine acted synergistically (table 1). Inhibition by leucine and alanine was largely overcome by the transaminase inhibitor AOA (table 1). Since leucine, like the other branched-chain amino acids, is not transaminated in rat liver [10], this indicates that it is alanine that must be metabolised in order to exert its effect.

When leucine plus alanine were added to perifused hepatocytes, intracellular concentrations of glutamate and aspartate increased; by contrast, the concentration of other amino acids decreased (table 2).

Inhibition of proteolysis by leucine and alanine was not observed in hepatocytes perifused in the absence of K^+ (table 3). Omission of K^+ in the presence of alanine plus leucine caused a decrease in the intracellular concentration of glutamate (table 2) and the transport of glutamate to the extracellular fluid was greatly stimulated (table 3).

Table 2

Effect of alanine plus leucine on intracellular concentrations of amino acids in perifused hepatocytes

Amino acid	Intracellular amino acid concentration (mM)		
	Control	+ Alanine + Leucine	+ Alanine + Leucine − K^+
Aspartate	0.14 ± 0.02	1.39 ± 0.31[a]	0.37 ± 0.10[b]
Threonine	0.81 ± 0.12	0.20 ± 0.03[a]	0.22 ± 0.02
Serine	0.72 ± 0.12	0.36 ± 0.08[a]*	0.21 ± 0.04
Glutamate	2.60 ± 0.47	6.09 ± 0.88[a]	1.67 ± 0.22[b]
Glycine	1.72 ± 0.18	0.71 ± 0.04[a]	0.39 ± 0.04[b]
Alanine	0.23 ± 0.02	1.02 ± 0.19[a]	0.62 ± 0.06
Valine	0.19 ± 0.04	0.06 ± 0.01[a]	0.07 ± 0.01
Leucine	0.25 ± 0.06	0.68 ± 0.09[a]	0.68 ± 0.03

[a] Different from the control values ($P<0.01$; a*, $P<0.05$)

[b] Different from the corresponding values in the presence of K^+ ($P<0.01$)

Hepatocytes were perifused in the absence or presence of 0.5 mM leucine plus 1 mM alanine. After 45 min, the cells were removed from the perifusion chamber and subjected to silicone oil centrifugation. When K^+ was omitted, all K^+ salts in the Krebs–Henseleit medium were replaced by the corresponding Na^+ salts. The data (means ± SE) are from experiments carried out with 5 different hepatocyte preparations

Table 3

Effect of K^+ depletion on the inhibition of proteolysis by alanine plus leucine in perifused hepatocytes

Conditions	Release of (nmol · min^{-1} · g dry wt^{-1})	
	Valine	Glutamate
Control	260 ± 13 (4)	279 ± 53 (4)
Alanine + leucine	140 ± 20 (5)[a]*	426 ± 67 (5)[a]
− K^+	242 ± 13 (4)	421 ± 80 (4)[a]
− K^+ + alanine + leucine	246 ± 14 (5)	797 ± 98 (5)[b]

[a] Different from the control values ($P<0.05$; a*, $P<0.01$)

[b] Different from the corresponding value in the absence of alanine and leucine ($P<0.05$)

Hepatocytes were perifused in the absence or presence of 0.5 mM leucine plus 1 mM alanine, in the presence or absence of K^+. The data are the mean values (± SE) obtained with different hepatocyte preparations, the number of which is given in parentheses

4. DISCUSSION

Perifused hepatocytes resemble the perfused liver in that protelysis is highly sensitive to inhibition by mixtures of amino acids at physiological concentrations (cf. [3,4]). The absolute rates of protein breakdown in the absence of amino acids in both preparations are also comparable (cf. the

value of 0.263 μmol valine released per min per g dry wt, table 1, with a similar value reported in [3,4]).

Pösö and Mortimore [4] have shown that, although alanine alone does not inhibit proteolysis, omission of alanine from a physiological mixture of amino acids prevented the inhibition by the entire amino acid mixture; because of the presence of so many other amino acids a mechanism for the inhibition of proteolysis by alanine under these conditions could not be given [4]. Using perifused hepatocytes we have now been able to show that the entire amino acid mixture can be effectively replaced by a mixture of alanine and leucine alone; this considerably simplifies interpretation of the results. The concentrations of alanine (1–2 mM) and leucine (0.5 mM) are entirely physiological since they are similar to those found in the portal vein of the rat after feeding [11].

The fact that the intracellular concentrations of many amino acids decreased upon addition of alanine and leucine (table 2) was clearly a reflection of their diminished production via proteolysis (cf. fig.1). Only the concentrations of intracellular glutamate and aspartate increased. Because the effect of alanine on proteolysis could be blocked by AOA we conclude that, apparently, intracellular glutamate and/or aspartate, in addition to leucine, are crucial elements in the control of hepatic proteolysis. Further support was obtained in experiments (not shown) in which the effect of alanine could be mimicked by low concentrations of proline, asparagine or glutamine, amino acids that rapidly produce glutamate and aspartate in the course of their metabolism. Also consistent with this view is the fall in intracellular glutamate that accompanied the increase in proteolytic rate after K^+ depletion in hepatocytes perifused with alanine and leucine (table 2). The enhanced efflux of glutamate from the hepatocytes under these conditions is probably mediated by the Na^+-dependent glutamate transport system in the plasma membrane [12]. The acceleration of proteolysis by K^+ depletion is strikingly analogous to that reported for muscle in which a rise in intramuscular Na^+ is accompanied by a large efflux of (in this case) glutamine as the amino acid that is believed to play an important role in the regulation of muscle proteolysis [13].

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