PROTEIN DEGRADATION IN ISOLATED RAT HEPATOCYTES IS INHIBITED BY AMMONIA

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SUMMARY

Freshly isolated rat hepatocytes, prepared by collagenase perfusion, exhibit a high rate of endogenous proteolysis which is unaffected by amino acids (added as a physiological mixture). However, ammonia, a natural product of amino acid catabolism, inhibits protein degradation as measured by the release from protein of either acid-soluble radioactivity or nitrogen (amino acids + urea). Incorporation of radioactive amino acids into protein is not affected. Thus, ammonia appears to inhibit endogenous hepatic protein degradation in a selective manner, providing a regulatory circuit of end-product inhibition.

INTRODUCTION

Liver protein turns over at a rapid rate, estimated in the rat to be about 1 % per h in vivo (1,2) and 3-4 % per h in the perfused liver (3,4). In the latter system, protein degradation as well as protein synthesis has been shown to be controlled by amino acids, with a potentiating effect of insulin (3-6).

By the method of collagenase perfusion (7,8) it is now possible to prepare intact, purified rat hepatocytes in large quantities. This system offers considerable experimental advantages as compared to the perfused liver, and makes it possible to study protein metabolism in a homogeneous cell population under carefully controlled conditions. Isolated rat liver cells have been shown capable of synthesizing a variety of proteins (9-12), although they are in a strongly negative nitrogen balance (P.O.

Seglen, in preparation). In a search for conditions which would make the nitrogen balance less negative, it was found that protein degradation in these cells is subject to end-product inhibition by ammonia rather than by amino acids.

MATERIALS AND METHODS

Male Wistar rats, 270-290 g, were maintained on a controlled feeding and illumination schedule, and isolated liver cells were prepared by collagenase perfusion (8) by the end of a 16 h fasting period. The purified hepatocytes were incubated at 37°C in suspension buffer under an atmosphere of air as previously described (13), and analyses were made on perchloric acid extracts of the complete system (cells + medium).

Urea and ammonia were measured with the Sigma urea nitrogen kit no. 14 (determination of ammonia with the Nessler reagent, with or without preincubation with urease, respectively), and amino acids were determined by the ninhydrin method (14). The total nitrogen balance was calculated as the sum of amino acids and urea formed, corrected for the metabolism and analytical interference by ammonia. A net nitrogen loss signifies that the formation of urea plus amino acids exceeds the consumption of amino acids plus ammonia.

The balanced amino acid mixture used in these experiments was a mixture of the 20 amino acids found in protein, plus ornithine and citrulline. The amino acid concentrations defined here as "normal" were intermediate between those found in rat plasma and the equilibrium concentrations reached in the perfused liver (15), except for some rapidly convertible (alanine, glutamine, glutamate) or non-equilibrating (leucine, isoleucine, valine) amino acids which were used at lower concentrations. In the incubations, the amino acid mixture was used at 4-5 x the "normal" concentration (the exact composition will be given elsewhere).

In the isotopic labelling experiments, the cells were incubated with ^{14}C-labelled protein hydrolysate (Amersham CFB.25). For measurement of proteolysis, cells were pre-labelled by incubation for 1 h in reciprocally shaken Petri dishes at high isotope and cell concentration (5 µCi and 70 mg cells/ml), then washed 7 x in warm (37°C) washing buffer (8) before the final incubation in isotope-free buffer. Radioactivity in perchloric acid extracts or precipitates (solubilized in NaOH before counting) was measured by liquid scintillation counting in Unisolve 1 (Koch-Light).

RESULTS

The isolated hepatocytes as incubated in buffered saline are in a highly negative nitrogen balance, with a nitrogen loss amounting to 60 µatoms nitrogen/g per h (table 1). On the basis of an average molecular weight for amino acids (in a balanced mixture) of 131, and a hepatocytic protein content of 228 mg/g (8), this nitrogen loss is equivalent to 3.5 % of the cellular

Table 1. Effects of amino acids, ammonia and cycloheximide on nitrogen balance in isolated rat hepatocytes.

	Nitrogen loss (μatoms/g per h)	
	No cycloheximide	Cycloheximide, 1 mM
Experiment 1		
Control	60.6 ± 2.1 (4)	59.3 ± 2.0 (4)
Amino acids, 5 x normal concentration	58.5 ± 1.8 (4)	58.8 ± 2.5 (4)
Experiment 2		
Control	60.6 (2)	62.3 (2)
NH_4Cl, 5 mM	27.8 (2)	24.4 (2)

Isolated rat hepatocytes were incubated for 60 min. at 37°C with the additions indicated, and the nitrogen balance was calculated by measuring the changes in amino acids, urea and NH_3 in the system (cells + medium). A net nitrogen loss means an increase in total perchloric acid-soluble nitrogen.

protein per h, which is similar to the proteolytic rate found in the perfused liver (3,4). Cycloheximide, a potent inhibitor of protein synthesis in these cells, surprisingly does not affect the nitrogen balance significantly, indicating that the rate of protein synthesis is negligible in comparison with the rate of protein degradation.

Amino acids (complete, balanced mixture) do not change the nitrogen balance in the isolated cells (table 1), in contrast to the situation found in the perfused liver, where amino acids inhibit proteolysis markedly (4). In order to find conditions which could improve the nitrogen balance of isolated hepatocytes (i.e. reduce the rate of protein degradation), an extensive search was undertaken, the details of which will be published elsewhere (P.O. Seglen, in preparation). Eventually it was found that ammonia, either formed endogenously under hypoxic conditions, or administered as NH_4Cl, was capable of reducing the nitrogen loss. This effect of NH_4Cl is shown in table 1 (expt.

2). The extent of inhibition of nitrogen loss is the same in the presence or absence of cycloheximide (table 1), indicating that ammonia exerts its effect on protein degradation rather than on protein synthesis.

The effect of NH_4Cl is dose-dependent, with maximal inhibition of nitrogen loss (for 1 h incubations) at 10 mM (fig. 1). This concentration of ammonia did not affect the viability of the cells (as measured by trypan blue exclusion).

When cells were incubated with 5 mM NH_4Cl, an inhibition of nitrogen loss could be observed during the first 30 min. However, as the added ammonia was metabolized (mostly to urea), the rate of nitrogen loss gradually increased, and had returned to normal within one hour of incubation (fig. 2). The inhibition of protein degradation by ammonia is therefore apparently fully reversible.

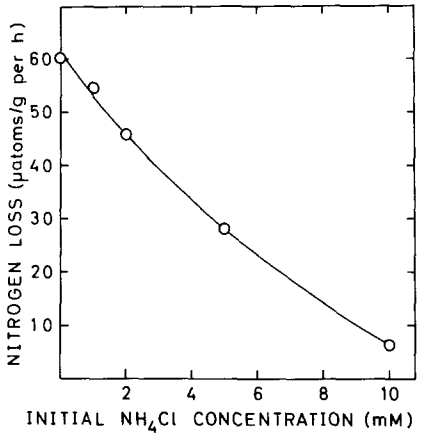

Fig. 1. Dose-dependent inhibition of nitrogen loss from isolated rat hepatocytes by ammonia. Isolated hepatocytes were incubated at 37°C for 60 min. at the initial concentration of NH_4Cl indicated, and the nitrogen loss (increase in perchloric acid-soluble nitrogen in the complete system of cells + medium) was calculated on the basis of changes in amino acids, urea and NH_3.

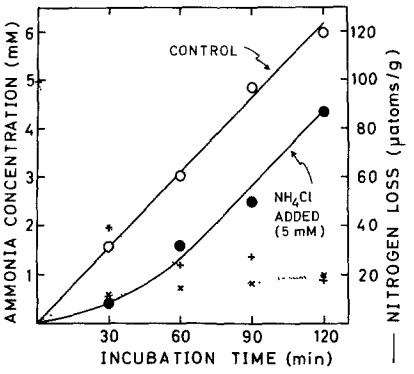

Fig. 2. Time course of nitrogen loss and ammonia metabolism in isolated rat hepatocytes incubated at 37°C with or without NH_4Cl. At various time points samples were analysed for total nitrogen loss (increase in perchloric acid-soluble nitrogen, whole lines) with (●——●) or without (o——o) 5 mM NH_4Cl added initially. The dotted lines show the levels of ammonia with (+···+) or without (x···x) NH_4Cl addition.

In order to test the ammonia effect by an alternative approach, cellular protein was pre-labelled by incubating the cells with a radioactive amino acid mixture, and the net increase in perchloric acid-soluble radioactivity during a subsequent incubation of the washed cells was measured at various concentrations of NH_4Cl. As shown in fig. 3, ammonia inhibited the release of radioactivity from labelled protein in a dose-dependent fashion. The dose-response curve resembles that found for inhibition of total nitrogen loss, and supports the contention that ammonia inhibits proteolysis.

The basal proteolytic rate appears to be higher (6 % per h) in isotope release experiments than in nitrogen balance calculations. The discrepancy may be due to radioactive, acid-soluble degradation products with a lower ninhydrin-reactivity than amino acids, e.g. small peptides (16).

The inhibition of proteolysis could conceivably have been a non-specific toxic effect of ammonia on cellular metabolism, and

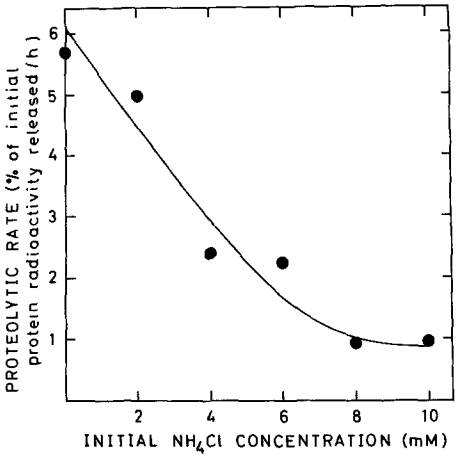

Fig. 3. Effect of ammonia on the release of acid-soluble radioactivity from pre-labelled protein. Isolated rat hepatocytes were pre-labelled by incubation with a ^{14}C-amino acid mixture, and the increase in perchloric acid-soluble radioactivity during a subsequent 60 min. incubation (in the presence of 1 mM cycloheximide to block protein synthesis) was measured at various initial concentrations of NH_4Cl. The increase in acid-soluble radioactivity is expressed in per cent of the total acid-insoluble radioactivity present initially.

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as a control experiment its effect on protein synthesis was tested. When a tracer dose of radioactive amino acids was used (fig. 4, "low amino acid concentration"), an apparent stimulation of protein labelling was observed with increasing concentrations of ammonia. Presumably the inhibition of proteolysis by ammonia reduced the dilution of isotope by non-radioactive amino acids, so that the specific radioactivity in the protein precursor pool became higher. In accordance with this assumption, when large amounts of unlabelled amino acids were added in order to keep the specific activity of the amino acid pool constant (fig. 4, "high amino acid concentration"), no effect of ammonia on protein synthesis was seen. It would thus seem reasonable to conclude that ammonia inhibits proteolysis in a selective fashion.

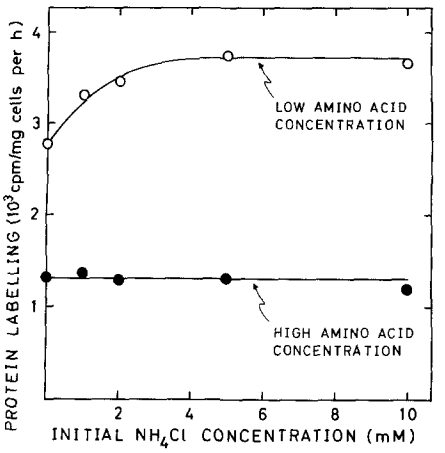

Fig. 4. Effect of ammonia on protein synthesis. Isolated rat hepatocytes were incubated at 37°C with a tracer dose (0.8 µCi/ml) of ^{14}C-amino acids (o——o, "low amino acid concentration"), or with an amino acid mixture at 4 x normal concentration containing 1.6 µCi/ml of ^{14}C-label (●——●, "high amino acid concentration"). The incorporation of radioactivity into perchloric acid-insoluble material during 60 min. was measured at various initial concentrations of NH_4Cl.

DISCUSSION

Mortimore et al. (16) observed that protein degradation proceeded at a higher rate in the perfused liver than in vivo, and attributed this to an increased activity of the phagosome/lysosome system. We have previously found that isolated hepatocytes contain more phagosomes than liver tissue in situ (17), so it seems very likely that the separation of liver cells (or tissue) from the rest of the body somehow stimulates hepatic protein degradation. The presence of 5-10 % damaged cells does not account for the high rate of proteolysis; on the contrary, the proteolytic rate declines with increasing cell damage (P.O. Seglen, in preparation).

The inhibition of protein degradation by ammonia could be due to interference with any of the steps from autophagosome forma-

tion to lysosomal enzyme action, or any non-lysosomal protein degradation system. As yet we do not know whether the inhibition is due to ammonia itself, or to some of the metabolic perturbations caused by ammonia in isolated liver cells (18,19). The insensitivity of hepatocyte proteolysis to a complete amino acid mixture makes it unlikely that amino acids mediate the ammonia effect, unless the individual amino acid ratios are of critical importance. It would seem more plausible that the anti-proteolytic effects of amino acids in the perfused liver (4) could be mediated by ammonia, e.g. if the perfusion conditions were slightly hypoxic.

Although it cannot be excluded that inhibition of hepatic proteolysis is an aspect of ammonia toxicity (20), it is attractive to think of it as a physiologically important end-product inhibition, which may play a role in the nitrogen economy of the body and/or in the regulation of liver cell growth. It is noteworthy that growth of the regenerating liver reflects a reduced protein degradation rather than an increased protein synthesis (21,22), possibly related to the impaired ability of the regenerating liver to convert ammonia to urea (23).

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