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The mitochondrial probe rhodamine 123 inhibits in isolated hepatocytes the degradation of short-lived proteins

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The fluorescent dye rhodamine 123 (R123) decreases the intracellular ATP levels and also inhibits the degradation of short-lived proteins in isolated hepatocytes. This inhibition affects lysosomal and, to some extent, non-lysosomal mechanisms. The degradation of short-lived proteins decreases more when ATP levels are less than 40% of those in control cells, in contrast to the reported linear correlation between ATP levels and degradation of long-lived proteins. R123 provides a powerful probe for clarifying the proteolytic mechanisms involved in degradation of short-lived proteins and the ATP requirements in protein degradation. Indeed, as illustrated, the results suggest different mechanisms for the degradation of short- and long-lived proteins. Moreover, they provide a warning for the clinical use of this reagent.

Rhodamine 123; Isolated hepatocyte; Short-lived protein; Protein degradation

1. INTRODUCTION

Intracellular proteins have been classified into two main groups [1–3]: (i) long-lived proteins, with relatively slow turnover rates, which constitute the bulk of cellular proteins; and (ii) short-lived proteins, with an exceptionally high turnover rate, which in liver represent less than 1% of the protein content, but 15–20% of protein synthesis [4,5]. The degradative mechanisms of short-lived proteins remain to be clarified, mainly due to the lack of specific methods for modifying their degradation rate [1–3].

It has been hypothesized that part of the short-lived proteins correspond to the cleavage and proteolysis of signal sequences from mitochondrial and other proteins [6]. Since the fluorescent dye rhodamine 123 (R123) in isolated hepatocytes inhibits the transport and processing of mitochondrial protein precursors [7], it seemed useful for the study of degradation of short-lived proteins. We show here that R123 strongly inhibits the degradation of short-lived proteins in isolated hepatocytes.

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2. MATERIALS AND METHODS

2.1. *Cells and culture conditions*

Hepatocytes were prepared from livers of male Wistar rats (200–300 g body wt) by the collagenase perfusion method [8] and incubated in a rapidly shaking water bath at 37°C under an atmosphere of 10% CO_2/90% air, in Hanks' medium supplemented with penicillin (30 μg/ml), streptomycin sulphate (250 μg/ml), a mixture of 20 amino acids (at concentrations approx. 1.5-times their physiological levels) and 20 mM Hepes (pH 7.3) [9]. Cell viability, measured by the trypan blue exclusion test, was greater than 90% at the end of incubations.

2.2. *Protein degradation*

Protein degradation was measured in isolated hepatocytes (10^7 cells/ml) based on the release of [^{14}C]valine from prelabelled proteins [10]. Short-lived proteins were labelled by incubation of 25 ml cell suspension (10^7 cells/ml) in 100-ml conical flasks for 1 h [2], with 250 μCi [^{14}C]valine (280 Ci/mol, 50 mCi/l). The bulk of the non-incorporated [^{14}C]valine was removed by washing cells twice in fresh medium containing 2 mM valine ('cold' medium). The cells were then incubated for 10 min in 500 ml conical flasks with 50 ml cold medium and, finally, washed twice.

Experiments were carried out in cold medium with the addi-

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tions indicated in the figures. Portions of the cell suspension (1 ml) were taken at different times during the chase period, and assayed for trichloroacetic acid-soluble and -precipitable radioactivity by liquid scintillation counting [2,10]. The net release of trichloroacetic acid-soluble radioactivity during the incubation period was expressed as % of the initial radioactivity incorporated into the protein [2,10].

2.3. *Measurements of ATP and protein*

ATP was measured by the firefly luciferin-luciferase reaction [11]. 1-ml aliquots of the suspension of hepatocytes (10^7 cells/ml) were centrifuged at 600 × *g*. The cell pellets were mixed with 1 ml of 1 M $HClO_4$. After 30 min at 0–4°C they were sonicated (200 W, 1 min, at 0–4°C) and then centrifuged at 10000 × *g* for 10 min. Supernatants were neutralized. The assay was linear from about 6×10^{-10} to 10^{-8} M ATP. Biuret [12] and Lowry [13] methods were used for protein measurements.

2.4. *Chemicals*

R123 (Eastman-Kodak, Rochester, NY) was dissolved in ethanol (20 mg/ml). [^{14}C]Valine was purchased from Amersham (England). Leupeptin was obtained from Sigma (St. Louis, MO) and ammonium chloride from Merck (Darmstadt, FRG). The ATP-bioluminescence test was from Boehringer (Mannheim). The rest of the chemicals were of the highest purity available.

3. RESULTS AND DISCUSSION

R123 produced a dose-dependent decrease in degradation rate of short-lived proteins (fig.1), with the maximum inhibitory effect (~65%) at 100 μg/ml (higher concentrations reduced cell viability below 85% and were therefore not used). Similar effects were also obtained when R123 was added during the 'pulse' period (not shown). Moreover, under these conditions, there was 50–60% inhibition of protein synthesis. In hepatocytes, mitochondrial proteins represent 20% of the total; more than 90% of these proteins are synthesized in the cytosol as precursors [14]. However, the observed inhibition appears greater than that expected from only an impairment of transport and processing of mitochondrial protein precursors. Therefore, we next explored the mechanism(s) by which R123 affects the degradation of short-lived proteins.

R123 directly and selectively stains mitochondria in fibroblasts [15] and hepatocytes [16]. It has been shown that in isolated mitochondria R123 inhibits oxidative phosphorylation [17] and that its uptake depends on the mitochondrial membrane potential [18]. Since ATP is often required for protein degradation [3,19], the inhibitory effect of R123 could be due to a decrease in intracellular levels of ATP. As shown in fig.2, at all R123 concentrations used, there was a sharp and rapid decrease in ATP content. When the rates of degradation of short-lived proteins in the presence of different concentrations of R123 were plotted vs intracellular ATP levels (fig.3), no linear correlation between ATP content and degradation rate of short-lived proteins was found. The initial rapid fall in ATP (down to about 35% of control values) produced by the lowest concentration of R123 used decreased proteolysis of short-lived proteins by only 20% (fig.3), but as the level of ATP decreased to about 20% of control values, the proteolysis was reduced much more (~65%). These results contrast with the linear relationship found in isolated hepatocytes between the inhibition in degradation rate of long-lived proteins by autophagic processes and decrease in ATP content [20]. They are, however, similar to those reported in growing fibroblasts with both short- and long-lived proteins [21]. Therefore, the findings described here support the existence of different mechanisms for the degradation of short- and long-lived proteins in hepatocytes.

In growing fibroblasts, short- and long-lived proteins appear to be degraded via non-lysosomal pathways [19,21]. In hepatocytes most degradation of long-lived proteins is lysosomal [22–24], whereas only about one-half of the short-lived pro-

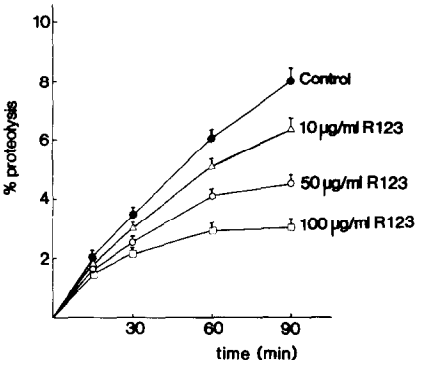

Fig.1. Inhibition by R123 of the degradation of short-lived proteins in isolated hepatocytes. Hepatocytes labeled as described in section 2 were washed and incubated in the absence (●) or presence of 10 (△), 50 (○) and 100 (□) μg/ml R123. Each point is the mean of 4 separate experiments with duplicated samples. Bars represent standard errors.

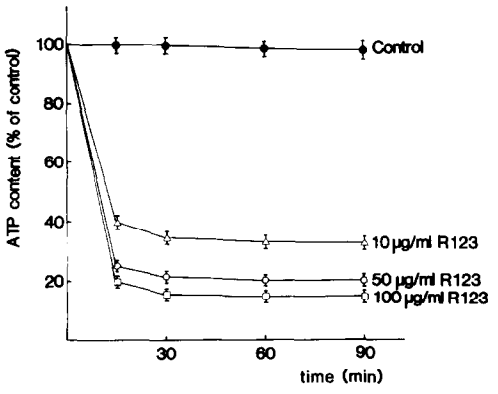

Fig.2. ATP content in control and R123-treated hepatocytes. Hepatocytes were incubated in the absence (●) or presence of 10 (△), 50 (○) or 100 (□) µg/ml R123 as described in section 2. Data are expressed as percentages of the ATP content at time zero. The average ATP content of control cells, in three separate experiments, was 4.3 nmol/mg protein. Each point is the mean of 4 separate experiments with duplicated samples. Bars represent standard errors.

teins are degraded via the lysosomal pathway [23]. To clarify which pathway (i.e. lysosomal or non-lysosomal) of short-lived proteins in hepatocytes is inhibited by R123, we used leupeptin and NH_4Cl, which suppress almost all lysosomal degradation in hepatocytes [23] (table 1). The inhibitory effects of R123 and leupeptin and NH_4Cl were not additive and it can therefore be concluded that R123 suppresses most of the lysosomal degradation. Moreover, since R123 also inhibited ~35% of the degradation not affected by leupeptin and NH_4Cl, it appears that R123 also affects non-lysosomal degradation of short-lived proteins.

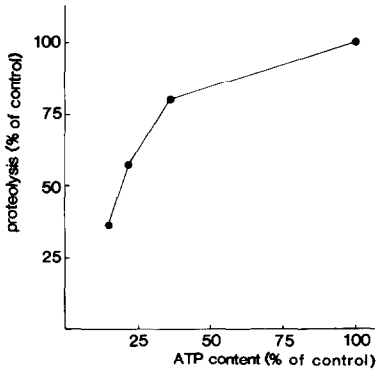

Fig.3. Relationship between inhibition of the degradation of short-lived proteins and cellular ATP content. The data were calculated from figs 1,2 for 60 min incubation. Both short-lived protein degradation and ATP content are referred as % of control. Each point corresponds to a different R123 concentration.

Table 1

Effect of R123 and lysosomal inhibitors on the degradation of short-lived proteins in isolated hepatocytes

Additions	Protein degradation (%)	Inhibition (%)
None	17.0 ± 1.4	0
Leupeptin + NH_4Cl	8.2 ± 0.5	50
R123	6.0 ± 0.3	65
Leupeptin + NH_4Cl + R123	5.4 ± 0.4	68

Hepatocytes were labelled as described in section 2 and incubated for 2 h with the indicated additions: leupeptin (0.25 mM) and ammonium chloride (10 mM) and/or R123 (100 µg/ml). Values are means ± SD from 3 different experiments

In conclusion, it appears that in hepatocytes there are at least three different mechanisms operating in the degradation of short-lived proteins: a lysosomal mechanism (which requires ATP, as shown in [20], but which is different, in terms of quantitative requirements for ATP, from those operating in the degradation of long-lived proteins) and two non-lysosomal mechanisms, one requiring and the other not requiring ATP. These two non-lysosomal mechanisms of protein degradation have already been proposed in growing fibroblasts [21]. Further, it has been reported that the lysosomal mechanisms in the degradation of short-lived proteins in hepatocytes (which could represent crinophagy or microautophagy) are not necessarily identical to those involved in the degradation of long-lived proteins (which could represent mainly macroautophagy) [23].

At any rate, since R123, in contrast to other metabolic inhibitors which affect many cellular functions, selectively affects mitochondrial processes [15–18], it provides an excellent tool for investigating the relationship between energy needs and protein degradation under a variety of conditions. Finally, our results showing depletion of ATP content in normal hepatocytes suggest caution in the clinical use of R123 in carcinoma chemotherapy [25].

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