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Differences in the half-lives of some mitochondrial rat liver enzymes may derive partially from hepatocyte heterogeneity

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The different turnover rates of rat liver mitochondrial enzymes make autophagy unlikely to be the main mechanism for degradation of mitochondria. Although alternatives have been presented, hepatocyte heterogeneity has not been considered. Lighter hepatocytes isolated in a discontinuous Percoll gradient contain more glutamate dehydrogenase (GDH) (half-life 1 day) and a more active autophagic system than heavier hepatocytes. The latter contain more carbamoyl phosphate synthase (CPS) and ornithine carbamoyl transferase (OTC) (half-lives 8 days) but less lysosomal activity. As expected, isolated autophagic vacuoles contain, relative to the mitochondrial content, 3-times less OTC and CPS than GDH, probably reflecting a faster lysosomal engulfment of mitochondria in the light hepatocytes (which contain more GDH). These data may explain some of the half-life differences of the enzymes studied.

Carbamoyl-phosphate synthase; Glutamate dehydrogenase; Ornithine carbamoyltransferase; Hepatocyte heterogeneity; Protein degradation

1. INTRODUCTION

Although proteolytic activity has been described in mitochondria [1–6], the low activity casts doubt on its role in degradation of their mature proteins. In rat liver the major part (70–90%) of protein degradation is carried out by lysosomes [7,8]. Since mitochondria occupy ~20% of the liver cell, lysosomes should be responsible for degradation of most of the mitochondrial proteins. In fact, the presence of mitochondria and proteins thereof within lysosomes has been reported [9]. However, since the half-lives of mitochondrial proteins vary a great deal [10], ranging from 0.5 h for δ-aminolevulinate synthase to 8 days for CPS, the degradation of the bulk of mitochondrial proteins in lysosomes would imply the heterogeneity of lysosomes and/or of hepatocytes.

There is extensive evidence that hepatocytes are functionally, biochemically and morphologically heterogeneous along the liver sinusoid [11,12]. Surprisingly, protein degradation studies have not considered hepatocyte heterogeneity, except for a report [13]. We show in this paper heterogeneity of hepatocytes based on their content of three mitochondrial enzymes and on the activity of the lysosomal system.

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Abbreviations: AAT, alanine aminotransferase; BSA, bovine serum albumin; CPS, carbamoyl phosphate synthase; GDH, glutamate dehydrogenase; LDH, lactic dehydrogenase; PK, pyruvate kinase; OTC, ornithine carbamoyl transferase; $t_{1/2}$, half-life

2. MATERIALS AND METHODS

2.1. *Chemicals*

Collagenase (type I), bovine serum albumin (fraction V) and Hepes were obtained from Sigma.

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Percoll was from Pharmacia and metrizamide from Nyegard. L-[^{14}C]Valine (CFB.75) was from The Radiochemical Centre. The rest of the reagents were of analytical quality.

2.2. *Cell preparation*

Parenchymal cells were isolated from fasted male Wistar rats (200–250 g) by a two-step Ca^{2+}-free/collagenase perfusion [14]. To remove aggregates, the suspension was successively passed through nylon meshes of 200, 75 and 35 μm pore size. Hepatocytes with a viability higher than 95% (measured by trypan blue exclusion) were used. Three cell populations were obtained as described in [15].

2.3. *Biochemical methods*

Cell pellets were resuspended in 10 mM Hepes buffer, pH 7.3, containing 1% Triton X-100 and frozen at −80°C. Hepatocytes were further disrupted by freezing and thawing, three times, and then homogenized with a Super Dispo Tissumizer homogenizer at full speed for 30 s. Portions of the homogenates were analyzed. LDH, AAT, PK, OTC, and GDH were assayed by standard methods [16]. Protein concentrations were determined by a Lowry-deoxycholate method [17].

2.4. *Blotting procedure*

Beef liver GDH was from Boehringer, CPS and OTC were purified from rat liver [18,19]. Polyclonal antibodies were prepared as described [20,21]. Cell pellets were suspended in 0.01 M Tris-HCl, pH 8.2, containing 1 mM EDTA, 1% SDS and 5% β-mercaptoethanol. Proteins from hepatocyte homogenates, mitochondrial and lysosomal extracts were separated by electrophoresis on a 9% polyacrylamide gel containing SDS. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets [22] was performed as described [21]. The sheets were photographed using a transparent acetate paper (Valcaline, Valca) and analyzed in a 2202 ultroscan LKB densitometer.

2.5. *Electron microscopy*

Pelleted hepatocytes were embedded in Vestopal W by standard methods. For each sample, 30 different lead citrate-stained cell sections were randomly selected. Areas were measured in a semi-automatic image analyzer (Kontron MOP/AM-03).

2.6. *Protein degradation*

The cells were suspended in medium S (containing per l: 1 g glucose, 8 g NaCl, 0.4 g KCl, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.59 g $CaCl_2$, 0.06 g $Na_2HPO_3 \cdot 2H_2O$, 0.06 g $KH_2PO_4 \cdot 2H_2O$ and 4.76 g Hepes, pH 7.4) and incubated (0.4 ml, final volume) in rapidly shaking, 15 ml centrifuge tubes at 37°C. Proteins were labeled with [^{14}C]valine (0.5 μM, 250 Ci/mol) for 1 h. The labeled cells were washed and then fractionated in Percoll gradients. The fractions were incubated at 37°C in medium S plus 10 mM cold valine to minimize reutilization. At the end of the chase period cells were precipitated with trichloroacetic acid (5%, w/v, final concentration). Radioactivity in the soluble and precipitable fractions was measured by liquid-scintillation [24].

2.7. *Isolation of subcellular fractions*

Mitochondria were obtained as in [25]. Following chloroquine (5 mg/100 g of rat weight) administration for 1 h, a fraction of secondary lysosomes-autophagic vacuoles was obtained from the liver, by isopycnic centrifugation in a discontinuous metrizamide gradient [26]. The fractions banding at 24%/20% and 20%/15% interphases [26] were pooled and processed for immunoblotting.

3. RESULTS

The discontinuous Percoll gradients contained in the interlayers three populations with a similar

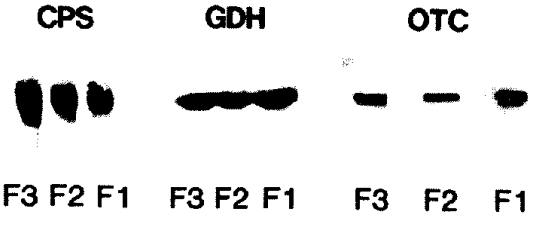

Fig.1. Typical immunoblotting of CPS, GDH and OTC in hepatocyte populations (F1, F2, F3). Wells of SDS-PAGE gels were loaded with 150 μg (GDH, OTC) and 15 μg (CPS) of hepatocyte proteins. The F3/F1 ratios, calculated after densitometric analysis, were 1.4 (CPS), 0.7 (GDH) and 1.6 (OTC).

number of hepatocytes (hereafter called F1, F2 and F3) [15]. F2 and especially F1 hepatocytes have smaller cell volume [15], less protein content and less mitochondrial volume than F3 hepatocytes [15]. To assess the intraacinar origin of the populations, we measured: PK, a perivenous hepatocyte marker, and AAT and LDH, periportal hepatocyte markers [11,12]. The activity of AAT and LDH increases from F1 (light hepatocytes) to F3 (heavy hepatocytes) populations, while that of PK decreases [15].

We selected CPS and OTC as long-lived ($t_{1/2}$ 8 days) and GDH as short-lived ($t_{1/2}$ 1 day) [10] mitochondrial proteins. The content of these mitochondrial enzymes in hepatocyte populations was studied by activity measurements (not shown), which agree well for OTC and GDH with the results from fig.1. However, in isolated hepatocytes CPS activity is rapidly lost; the amounts of the three enzymes were therefore determined by immunoblotting. As shown in fig.1, there was more GDH in light hepatocytes (F1) while OTC and CPS were increased in heavy hepatocytes (F3).

Using morphometric methods, we measured in the three hepatocyte populations the volume density of autophagic vacuoles, lysosomes with recognizable, undegraded cellular content [27], which are believed to be responsible for most of the mitochondrial protein degradation [28]. The volume density of autophagic vacuoles was 2.3-times greater in light hepatocytes.

Hepatocyte proteins were labeled with [^{14}C]valine, hepatocytes were fractionated and protein degradation was tested. Protein degradation in hepatocytes occurs principally by the lysosomal pathway, which is markedly inhibited by NH_4Cl [28]. As shown in table 1, higher degradation rates and inhibition of protein degradation by NH_4Cl were seen in light hepatocytes (F1). These results agree with the morphometric measurements which showed a higher content of autophagic vacuoles in fraction F1.

Table 1

Protein degradation in hepatocyte populations

Fraction	% radioactivity released into the medium/h	% inhibition by NH_4Cl
F1	8.4	57
F2	7.0	38
F3	3.7	34

Hepatocytes were labeled with [^{14}C]valine. The fractions were incubated with or without 10 mM NH_4Cl in medium S containing 10 mM valine and the release of acid-soluble radioactivity was measured (see section 2)

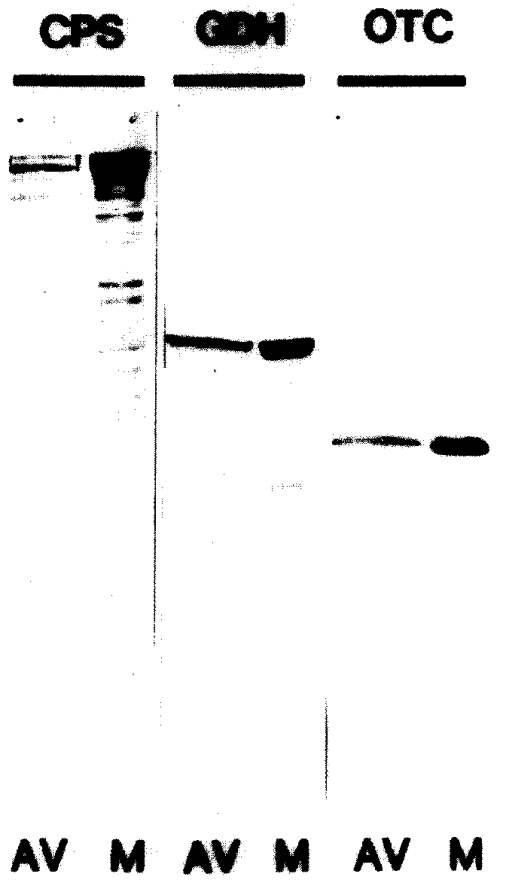

Fig.2. Relative content of GDH, CPS and OTC in autophagic vacuoles (AV) and mitochondria (M). The wells of the polyacrylamide gel (10% acrylamide) were loaded with 50 or 20 μg of lysosomal and mitochondrial proteins, respectively, and treated for immunoblotting. As shown in the figure, the GDH band in lysosomes compared with the band in mitochondria is proportionally higher than the corresponding bands for CPS or OTC. Note the proteolytic polypeptides of CPS as expected from its higher proteolytic susceptibility compared to GDH or OTC.

Table 2

Content of mitochondrial enzymes (CPS, GDH and OTC) in isolated autophagic vacuoles, relative to their content in isolated mitochondria

Enzyme	Mitochondria (*A*)	Autophagic vacuoles (*B*)	$B/A \times 100$
CPS	220 ± 20	13.60 ± 0.24	6.5 ± 2.1
OTC	10 ± 1	0.95 ± 0.11	9.5 ± 1.0
GDH	23 ± 3	5.40 ± 0.63	23.0 ± 3.4

Mitochondrial and lysosomal (autophagic vacuoles and dense bodies) fractions were isolated as described in section 2. CPS, OTC and GDH content of fractions was estimated by densitometric analysis of immunoblottings. Each value represents the mean of four different experiments and duplicated samples

The results described thus far could explain some of the differences in $t_{1/2}$ of mitochondrial proteins; it might be expected, therefore, that early lysosomes (where little or no degradation occurs) would have a higher content of GDH than of CPS or OTC. To test this, we isolated an autophagic vacuole-secondary lysosome fraction, which was assayed by immunoblotting (fig.2). As shown in table 2, autophagic vacuoles contain relatively more GDH than CPS or OTC. Since CPS is more susceptible to proteolysis by lysosomes than GDH or OTC, the CPS content in these fractions (table 2) was calculated by adding the bands due to proteolysis to those corresponding to the CPS peak.

4. DISCUSSION

Several reports have shown functional, morphological and biochemical differences in hepatocytes from different acinar zones of the liver parenchyma [11,12]. However, and surprisingly, very few reports have been focused on the influence of this heterogeneity in intracellular protein degradation. Using electron microscopic immunocytochemical procedures we have previously found that there is homogeneity in the content of CPS and GDH among mitochondria from the same hepatocyte (intracellular homogeneity of mitochondria) [20,21]. Here, we have measured the relative distribution of GDH, CPS and OTC in isolated hepatocyte populations [15]. As shown above, the content of these mitochondrial enzymes is different in the three hepatocyte populations studied. The enzyme with the shorter half-life, GDH, is more abundant in light hepatocytes while the opposite is true for CPS and OTC. These results are in agreement with immunohistochemical and microphotometric activity measurements in liver slices, which have shown a higher content of CPS [29] and OTC [11,12] in periportal hepatocytes and of GDH in perivenous rat liver hepatocytes [11,12].

Both basal and accelerated protein degradation in liver occur mainly by the lysosomal pathway [7,8,28,30], which is inhibited by NH_4Cl [28,30]. Autophagic vacuoles are important sites of mitochondrial protein degradation [28,30]. The quantity of autophagic vacuoles is directly related to increased, basal and decreased protein degradation [30]. Therefore, the differences described here in the content of autophagic vacuoles in hepatocyte populations and in the inhibition by NH_4Cl should reflect their different proteolytic content.

A more direct proof of the importance of heterogeneity in mitochondrial protein degradation is that, as shown here, the GDH/CPS and the GDH/OTC ratios in autophagic vacuoles are, respectively, 3.5- and 2.5-times higher than in mitochondria (table 2). It should be made clear that the presence of mitochondrial proteins in lysosomes was not due to contamination, because the samples used in these studies have a mitochondrial contamination of less than 4%. These results indicate that more GDH is incorporated into autophagic vacuoles and again support the suggested correlation between the heterogeneous distribution of all three enzymes and their half-lives. In other words, heterogeneity can explain ~30% of the differnces in half-lives and possibly more if a better separation of the populations can be achieved. The results described here explain for the first time some of the observed differences in the half-lives of mitochondrial proteins, without invoking new mechanisms for intracellular protein degradation.

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