Glucagon administration *in vivo* stimulates hepatic RNA and protein breakdown in fed and fasted rats

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Liver RNA and protein breakdown rates were measured simultaneously in fed and in 24 h-fasted rats during a short-term cyclic perfusion, 1 h after an intraperitoneal injection of glucagon or of saline. RNA was labelled *in vivo* by an intraperitoneal injection of [6-¹⁴C]orotic acid, 60 h before the start of the perfusion. The accumulation of radioactive cytidine and valine in the perfusion medium for 15 min was used to determine RNA breakdown and proteolysis respectively. The portal glucagon/insulin ratio was significantly higher in the fasted glucagon-treated rats than in their fed counterparts. Although glucagon administration significantly increased RNA and protein degradation rates in the fasted and in the fed groups, the effect was

greater after 24 h of starvation. The relationship between these biochemical changes and the alterations of the hepatocyte lysosomal system was investigated by determining the fractional cytoplasmic volume of lysosomal structures (autophagic vacuoles and dense bodies) by morphometry in the fasted glucagon-treated rats and in their controls. Hyperglucagonaemia significantly enhanced the relative volume of autophagic vacuoles without affecting that of dense bodies. The results showed that hyperglucagonaemia induced *in vivo* stimulated both liver RNA and protein breakdown and that this effect was modulated by the nutritional status of the rats.

INTRODUCTION

Degradation of liver RNA and protein mainly occurs in the lysosomal system, through macroautophagy [1,2]. This mechanism is strongly regulated by nutritional and hormonal factors. In particular, macroautophagy is inhibited by insulin and amino acids and stimulated by glucagon or by amino acid deprivation [1,2]. Glucagon is known to increase hepatic lysosomal activity [3–7] and to stimulate proteolysis in isolated or cultured hepatocytes [8,9] and in the perfused liver [10,11]. Its effects on RNA breakdown are much less documented. In the perfused liver from fed rats, portal administration of glucagon, together with a normal plasma amino acid concentration, did not affect RNA degradation, whereas proteolysis was significantly increased [12]. The response of RNA degradation to glucagon has been studied only after hormonal administration *in vitro* and in rats fed *ad libitum*. However, the lack of glucagon effect on RNA breakdown might be ascribed to the nutritional status of the rats, as different studies have shown that the efficiency of glucagon action on lysosomal activity was improved in a fasting state [6,13]. Besides this aspect, the results mentioned above cannot be extrapolated to hyperglucagonaemia induced *in vivo*, because other hormones, and especially insulin, can modify the macroautophagic process.

The present study was designed to determine whether hyperglucagonaemia induced *in vivo* can modulate RNA breakdown together with proteolysis in both fed and fasted rats. The second aim was to correlate protein and RNA degradation rates with morphometric changes in lysosomal structures.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (Charles River, Saint Aubin-Les-Elbeuf, France) weighing 170–200 g were used. The animals were maintained under controlled lighting (off 19:00 h, on 07:00 h)

on a commercial diet (UAR, Villemoisson-sur-Orge, France) and were allowed water *ad libitum*. RNA was labelled *in vivo* by an intraperitoneal injection of 20 μ Ci of [6-¹⁴C]orotic acid in 0.85 % NaCl. RNA and protein degradation rates were determined during short-term cyclic liver perfusions in rats either fed *ad libitum* or fasted for 24 h, at 1 h after an intraperitoneal injection of 45 μ g of glucagon/100 g body wt. or of saline. Before liver perfusion, the animals were anaesthetized with Nembutal (100 μ l/100 g body wt.); a 1.5 ml portal blood sample was collected, transferred in an Eppendorf tube containing Trasylol (2000 kallikrein-inhibitory units/ml) and EDTA (6 mg/ml), and rapidly centrifuged. Plasma was immediately frozen at –20 °C. Livers used for ultrastructural morphometry studies were obtained from 24 h-fasted rats subjected to the same treatments as described above.

Measurements of RNA and protein breakdown

Liver perfusion

Perfusions were started at 11:00 h, 60 h after [6-¹⁴C]orotic acid injection. Livers were cyclically perfused *in situ* at a flow rate of 7 ml/min as described by Mortimore and Mondon [14]. The perfusion medium was composed of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 3 % (w/v) BSA (fraction V) and 27 % (v/v) washed bovine erythrocytes. Other compounds were added as indicated below. After wash-out for 45–60 s, the perfusate was recycled and five 3 ml samples were collected between 5 and 15 min for the measurement of plasma [¹⁴C]cytidine and valine accumulation. At the end of the perfusion, livers were rapidly excised, weighed and frozen in liquid N₂.

Determination of RNA and protein fractional degradation rates

The degradation rate of both macromolecules was determined simultaneously between 5 and 15 min of perfusion. RNA break-

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Figure 1 Electron micrographs showing the main lysosomal structures in rat hepatocytes

Lysosomes are classified in accordance with Schworer et al. [24] into autophagic vacuoles and dense bodies. Panels: (a) and (b), initial autophagic vacuoles (arrows); (c) and (d), digestive vacuoles (arrow-heads) with sequestered material in various stages of degradation. Dense bodies also comprise two subgroups: types A (---A) and R (---R) dense bodies (d). Abbreviations: GA, Golgi apparatus; BC, bile canaliculus; N, nucleus. Magnification $\times 34\,000$.

down was calculated from the accumulation of radioactive cytidine in the perfusate plasma plus liver extracellular water space in the presence of unlabelled cytidine (0.5 mM) to prevent the reutilization of radioactive cytidine [15]. Rates of release (d.p.m./min) were computed by least-squares regression. Cytidine accumulation, expressed as $\mu\text{mol}/\text{min}$ per liver, was then computed by dividing the rate of label release by the specific radioactivity of 3'-CMP in liver RNA [16]. Absolute rates of RNA degradation were determined by dividing the rate of cytidine release by the proportion of CMP residues in RNA, $0.87 \mu\text{mol}/\text{mg}$ of RNA [15]. Fractional rates of RNA degradation were obtained by dividing rates of RNA degradation by the hepatic RNA content at the end of the perfusion.

Proteolysis rates were determined from the accumulation of valine in the perfusate volume (plasma plus 60% of red-cell volume) and liver extracellular water space in the presence of cycloheximide ($18 \mu\text{M}$) to prevent valine reutilization [17]. Proteolytic rates were computed by least-squares regression from changes in total valine release. Valine accumulation was then converted into protein equivalent (1 mg of protein containing

approx. $0.591 \mu\text{mol}$ of valine [18]). Fractional rates of proteolysis were computed by dividing rates of protein breakdown by the hepatic protein content at the end of the perfusion.

Analytical procedures

Radioactive cytidine was extracted from perfusate plasma as described by Lardeux et al. [15].

For valine determination, a constant amount of nitrotyrosine, used as an internal standard, was added to perfusate plasma samples. The samples were deproteinized with trichloroacetic acid (20% final concn.) and the amino acids were extracted by Dowex 50W-X4 chromatography (Mortimore and Mondon [14]). The eluates were air-dried and the dried amino acid extracts were dissolved in distilled water. Dabsyl (4-dimethylaminoazobenzene-4'-sulphonyl) chloride derivative formation was then carried out as described by Vendrell and Aviles [19] on $20 \mu\text{l}$ samples and standards. These standards contained different valine concentrations and a constant amount of nitrotyrosine, in order to obtain the same final amount of nitrotyrosine as in the

samples. Reverse-phase chromatography was then performed in a Beckman h.p.l.c. system using C-18 Ultrasphere columns (4.6 mm $\times$ 250 mm; 5 μ m particles). The h.p.l.c. eluents were as follows: eluent A consisted of 10 mM sodium citrate (pH 6.5) containing 4% dimethylformamide, and eluent B was made of 30% eluent A and 70% of acetonitrile containing 4% dimethylformamide. After sample injection, the column was eluted at room temperature, at a flow rate of 1.4 ml/min, with the following gradient: 75% A and 25% B at 0 min, 40% B at 2 min, 56% B at 7.20 min, 86% B at 11 min, 100% B at 12 min; solvent B was maintained at 100% for 9 min, followed by a linear decrease to 25% B in 0.25 min. The run was completed within 27.5 min. The A_{436} was recorded. Valine concentrations were computed by linear regression from the valine/nitrotyrosine ratios in the standard curve and in the samples.

Liver protein was measured by the method of Lowry et al. [20], with BSA as a standard, and liver RNA content was assessed by the method of Munro and Fleck [21] modified by Ashford and Pain [22], except for the time of hydrolysis [15].

Portal glucagon concentrations were measured by radioimmunoassay using a monospecific pig anti-glucagon serum which does not cross-react with enteroglucagon, and with pig glucagon as a standard. Portal insulin concentrations were determined by radioimmunoassay using a monospecific pig anti-insulin serum and with rat insulin as a standard.

Electron microscopy

The livers from three fasted glucagon-treated rats and their three respective controls were fixed by perfusion through the portal vein with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature for 4 min. Small pieces of liver were then left in the fixative buffer for 2 h at 4°C. After washing with the phosphate buffer, they were post-fixed with 1.5% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature for 1 h. The pieces were then dehydrated in an ethanol series and embedded in Epon 812 after treatment with propylene oxide. Semi-thin sections were cut from three randomly selected blocks per animal. Ultrathin sections cut from the same blocks were stained with uranyl acetate and lead citrate before examination with a Phillips EM 200 electron microscope.

Morphometry

Twelve micrographs per block were taken at random in the periportal area of the hepatic lobule at a primary magnification of $\times 10000$ and were enlarged to a final magnification of $\times 24000$. A calibrated grid was used to monitor possible variations of magnification. The fractional cytoplasmic volume of lysosomal components was determined by the stereological method of Weibel et al. [23], with the use of a test grid containing 3520 intersections projected on an area of 342 cm². Hepatocyte cytoplasmic area was defined by subtracting the number of intersections overlying nuclei and extracellular spaces from the total number of intersections of the test grid. The criteria used to discriminate among the different lysosomal structures were those defined by Schworer et al. [24]. The structures examined were initial and digestive autophagic vacuoles and all dense bodies, including types R and A. These structures are illustrated in Figure 1. Although glucagon has been reported to induce cell shrinkage [25], no correction was made for possible changes in the cytoplasmic volume between both groups, for the following reasons: firstly, no significant difference in liver weight was observed in the glucagon-treated rats and in their controls

(5.27 ± 0.14 and 5.25 ± 0.12 g respectively) and secondly, the hepatocyte cytoplasmic area examined was similar in both groups (3363 ± 19.6 and 3319 ± 22.2 intersections in the control and in the glucagon-treated rats respectively).

Statistical analysis

Results are means $\pm$ S.E.M. Comparisons of RNA and protein degradation rates and of hormone concentrations between groups were performed by using two-way variance analysis followed by the least-significant-difference test [26]. Log transformations of the data were made, except for insulin and glucagon concentrations. Comparisons of the fractional cytoplasmic volumes in the control and in the glucagon-treated rats were carried out by Student's *t*-test.

Chemicals

[6-¹⁴C]Orotic acid (40–60 mCi/mmol) was purchased from Dositek, Orsay, France. Pig glucagon, pig anti-glucagon and anti-insulin antisera and rat insulin were from Novo Nordisk, Bagsvaerd, Denmark. Trasylol was provided by Bayer, Puteaux, France. Dimethylformamide was from Aldrich Chemical Co, Milwaukee, WI, U.S.A. Acetonitrile was from Merck, Darmstadt, Germany. Cycloheximide, cytidine, nitrotyrosine and BSA were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Dabsyl chloride was from Pierce, Rockford, IL, U.S.A.

RESULTS

Hormonal status after glucagon administration *in vivo*

Basal glucagon levels were similar in the fed and fasted rats (Table 1). After glucagon administration, the portal concentrations were dramatically enhanced and were not significantly different in both groups. Basal insulinaemia was significantly lower in the fasted than in the fed rats ($P < 0.05$). After glucagon administration, portal insulin levels increased significantly only in the fed group. The molar glucagon/insulin ratio was significantly higher in both groups of glucagon-treated rats than in their respective controls. This ratio was considerably higher in the glucagon-injected fasted rats than in their fed counterparts.

Effects of hyperglucagonaemia *in vivo* on liver RNA and protein degradation rates

RNA and protein degradation rates were not significantly different in the control fed and fasted rats (Table 2). Glucagon

Table 1 Portal insulin and glucagon levels in fed and 24 h-fasted rats after glucagon administration *in vivo*

Portal blood was collected from anaesthetized rats 1 h after an intraperitoneal injection of glucagon and just before liver perfusion. The results are means $\pm$ S.E.M. of five rats per group, except in the glucagon-treated fed group, which included seven animals: ^a $P < 0.05$, ^b $P < 0.01$ versus the respective controls (least-significant-difference test). The glucagon/insulin molar ratio was computed by using molecular masses of 3550 and 5734 Da for glucagon and insulin respectively.

Rats	Insulin (ng/ml)	Glucagon (ng/ml)	Glucagon/insulin molar ratio
Fasted	0.13 ± 0.02	0.23 ± 0.02	3.1 ± 0.47
Fasted + glucagon	0.22 ± 0.04	5.60 ± 1.30^b	51.4 ± 22.3^b
Fed	1.26 ± 0.24	0.26 ± 0.01	0.4 ± 0.05
Fed + glucagon	2.37 ± 0.45^a	4.78 ± 0.77^b	3.6 ± 0.81^b

Table 2 Liver RNA and protein degradation rates in glucagon-treated fasted and fed rats

RNA and protein degradation rates were measured in fed and 24 h-fasted rats during a 15 min cyclic perfusion and 1 h after an intraperitoneal injection of saline or of glucagon. RNA was labelled *in vivo* by intraperitoneal injection of [6-¹⁴C]orotic acid 60 h before liver perfusion. Each value is the mean $\pm$ S.E.M. (the number of rats in each group was between 10 and 12): ^a*P* < 0.05, ^b*P* < 0.01 versus the respective controls (least-significant-difference test).

Rats	RNA degradation rate (%/h)	Proteolysis rate (%/h)
Fasted	0.63 $\pm$ 0.07	2.16 $\pm$ 0.20
Fasted + glucagon	1.66 $\pm$ 0.22 ^b	3.74 $\pm$ 0.37 ^b
Fed	0.82 $\pm$ 0.07	1.83 $\pm$ 0.13
Fed + glucagon	1.13 $\pm$ 0.07 ^a	2.47 $\pm$ 0.17 ^a

Table 3 Changes in the fractional cytoplasmic volume of lysosomal structures (%) after glucagon administration in the fasted rats

Livers from two groups of fasted rats were fixed by perfusion 1 h after an intraperitoneal injection of glucagon or of saline. The fractional cytoplasmic volume of lysosomal structures was determined by point counting on micrographs taken from ultrathin sections. The structures examined were: initial (AVi) and digestive (AVd) autophagic vacuoles and total (types R and A) dense bodies (DB). The data are the means $\pm$ S.E.M. of three rats in each group: ^a*P* < 0.05 versus the control group (Student's *t* test).

Rats	AVi	AVd	Total AV	Total DB
Control	0.47 $\pm$ 0.06	0.97 $\pm$ 0.16	1.44 $\pm$ 0.11	0.51 $\pm$ 0.13
Glucagon-treated	0.68 $\pm$ 0.10	1.25 $\pm$ 0.10	1.93 $\pm$ 0.06 ^a	0.56 $\pm$ 0.08

administration stimulated both RNA breakdown and proteolysis in the fasted and fed rats. However, starvation for 24 h enhanced the effect of glucagon: RNA degradation rates increased by 164 and 38% in the fasted and fed rats respectively, whereas proteolysis rates increased by 73 and 35% in the same groups respectively. The fraction of degradation rates induced by glucagon (difference between the fractional degradation rates in the glucagon-treated rats and in their respective controls) was 1.03 for RNA and 1.58 for protein in the fasted rats, and the corresponding values were 0.31 and 0.64 in the fed animals.

Effect of hyperglucagonaemia *in vivo* on the fractional cytoplasmic volume of lysosomal structures

This effect was quantified only in the fasted rats, as they showed the highest increase in RNA and protein degradation rates. The occurrence of hyperglucagonaemia in the glucagon-treated rats was confirmed by high portal glucagon levels (exceeding 5 ng/ml for each rat). As shown in Table 3, the sum of the relative volumes of initial and digestive autophagic vacuoles was significantly higher after glucagon administration. This increase was mainly due to the enhancement of initial vacuole volume. The relative volume of total dense bodies was significantly different in both groups.

DISCUSSION

The perfused liver is the only technique allowing direct measurements of short-term changes in RNA or protein breakdown *in vivo* [27–29]. As liver macromolecule degradation is insensitive to any regulatory factor (amino acids, hormones) for about 20 min after the start of the perfusion [27,29], the measurement of RNA

or protein degradation rates during a cyclic 15 min perfusion provides an instantaneous picture of the rates occurring in the liver *in vivo*. The measurement of radioactive cytidine and of valine during a short cyclic perfusion allowed the simultaneous determination of RNA and protein breakdown rates. The accumulation of radioactive cytidine in the perfusion medium, 60 h after RNA labelling *in vivo* has been shown to reflect mainly the breakdown of long-lived RNA, in particular of rRNA and tRNA, which have similar turnover rates and make up over 90% of the cytoplasmic RNA [27,30]. Inasmuch as rRNA amounts to 85–90% of the cytoplasmic RNA [30], the technique used provides a good estimate of rRNA degradation. The measurement of valine accumulation in the perfusate without previous protein labelling reflects total hepatic proteolysis corresponding to the combined degradation of long-lived, short-lived and endocytosed proteins [31]. However, as the short-lived protein breakdown is not influenced by nutritional and hormonal factors [1], the difference between the proteolysis rates in the glucagon-injected rats and their controls is the sum of the fractions of long-lived and endocytosed protein degraded in response to hormonal administration.

RNA and protein breakdown rates were not significantly different in the control fed and 24 h-fasted rats. A similar finding has been reported in mice for total proteolysis or for total proteolysis minus the short-lived fraction [31]. Nevertheless, as in that study and the present one the animals fed *ad libitum* at 11:00 h were already in post-absorptive conditions, these results cannot be interpreted as the absence of any effect of fasting on RNA and protein breakdown. Indeed, Lardeux et al. [27] showed that in meal-fed rats, perfused immediately after the meal (absorptive period), RNA degradation was 7 times lower than in rats fed *ad libitum* perfused in a post-absorptive period (10:00 h) or in meal-fed rats perfused 14 h after the meal. Similarly, Millward [32] reported that, in meal-fed rats, the increase in proteolysis started 2 h after the end of feeding.

This study clearly shows, on the one hand, that glucagon can stimulate proteolysis as well as RNA degradation *in vivo* and, on the other hand, that this effect is modulated by the nutritional status of the rats. Indeed, the glucagon effect is greater in the fasted than in the fed rats. In this study, one of the main differences between the two groups of glucagon-treated rats is their hormonal status. Besides amino acids, glucagon and insulin are considered as important regulatory and antagonistic factors controlling liver RNA and protein degradation. When glucagon is injected *in vivo*, the plasma insulin concentration increases [33]. It therefore seemed important to determine whether the portal concentration of glucagon or the value of the glucagon/insulin ratio could explain the increased glucagon effect in the fasted rats. The data showed that the portal glucagon levels were not directly related to RNA and protein degradation rates. Parilla et al. [34] have shown that in the perfused liver, several metabolic processes, e.g. proteolysis, depended on the glucagon/insulin ratio rather than on the absolute concentration of either hormone. They measured valine production in livers perfused with a constant glucagon level and decreasing insulin concentrations, so that the glucagon/insulin ratio ranged from 0.026 to 2600. Valine production started to increase when the ratio exceeded 2.6. In our study, the glucagon/insulin ratio was significantly higher in the hyperglucagonaemic rats than in their respective controls, and in the fasted glucagon-treated rats than in the fed counterparts. This suggests that the difference in the glucagon/insulin ratios between the fed and the fasted glucagon-injected rats could partly explain the greater glucagon effect after 24 h of starvation.

Among the different components of liver RNA and protein,

the long-lived fraction of RNA and of intracellular protein is mainly degraded by macroautophagy [18,35], one of the main mechanisms of the lysosomal pathway [1,2]. Several studies have reported that glucagon administration *in vivo* and *in vitro* resulted in an increase of lysosomal structures and particularly of newly formed autophagic vacuoles [3–7]. However, the fractional cytoplasmic volume of initial and digestive autophagic vacuoles and of dense bodies has not been quantified after glucagon injection *in vivo*. We have only observed an increase in total autophagic vacuoles, which mainly resulted from an enhancement of initial autophagic vacuoles. This result corroborates the fact that glucagon exerts its effect at the sequestration step of macroautophagy, most probably through its action on amino acids [1,9].

Besides its stimulating effect on the formation of autophagic vacuoles, glucagon might also have a more specific action on the sequestration step of macroautophagy. Indeed, Lardeux and Mortimore [12] have shown that after glucagon administration *in vitro* in fed rats, proteolysis was enhanced much more than RNA degradation. These authors argued that this result was due to a low rate of sequestration of rough endoplasmic reticulum into autophagosomes. Considering the fraction of degradation rates induced by glucagon for RNA (0.31) and for protein (0.64) in the fed animals, it appears that twice as much protein as RNA was degraded. This difference tended to diminish in the fasted rats. Therefore, at least in fed animals, our results are in favour of a selective effect of glucagon on macroautophagy.

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