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Control *in vivo* of Rat Liver Phosphofructokinase by Glucagon and Nutritional Changes

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Glucagon (250μg/kg body wt.) intravenously injected into normal fed rats produces within 5 min a marked inactivation of liver phosphofructokinase, only observed when the enzyme activity is measured at subsaturating concentrations of fructose 6-phosphate. Since half-maximal inactivation is observed at a dose of glucagon of 0.02μg/body wt., a dose within the range of the physiological concentrations of the hormone, the inactivation of phosphofructokinase can occur *in vivo* in response to physiological changes in the concentration of glucagon. In gluconeogenic conditions (starved rats or high-protein-diet-fed rats), there is a marked inactivation of liver phosphofructokinase at subsaturating concentrations of fructose 6-phosphate similar to that found in normal fed rats after glucagon treatment. In these gluconeogenic conditions a 50% decrease in the $V_{max.}$ of the enzyme is also observed. No significant changes in phosphofructokinase activity either at subsaturating concentrations of fructose 6-phosphate or in the $V_{max.}$ of the enzyme are observed when rats are fed on a high-carbohydrate diet. In the last dietary condition, glucagon treatment produces similar effects to that described in the normal fed rats. Similar results have been obtained in the above conditions for pyruvate kinase L activity when measured at subsaturating concentrations of phosphoenolpyruvate.

It has been shown that glucagon added to isolated rat hepatocytes produces a stable cyclic AMP-dependent inactivation of phosphofructokinase, only observed when the enzyme activity is measured at subsaturating concentrations of fructose 6-phosphate (Castaño *et al.*, 1979); similar results have been reported by Pilkis *et al.* (1979). Kagimoto & Uyeda (1979) have shown that this stable modification is a cyclic AMP-dependent phosphorylation of the enzyme.

The purpose of the present work is to show that the short-term control of liver phosphofructokinase occurs *in vivo* at the physiological range of concentrations of glucagon and that the long-term control of the enzyme activity by different dietary conditions operates, although not exclusively, by changing the proportion of active phosphofructokinase in liver. These results *in vivo* suggest that the regulation of the proportion of active form of liver phosphofructokinase, as well as of pyruvate kinase L (Felíu *et al.*, 1977; the present paper), is one of the mechanisms by which glucagon and different dietary conditions control the glycolytic–gluconeogenic flux in liver.

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Materials and Methods

Male Wistar rats weighing 250–300 g were used. The animals were fed with different diets supplied *ad libitum* or starved for 24 or 48 h. Animals fed on stock diets are referred to as normal fed. Animals fed with 90%- casein or 90%-glucose (w/w) diets, supplied for 4 days and prepared as described by Pitot *et al.* (1961), are referred to as high-protein- or high-carbohydrate-fed animals respectively. All animals were housed under controlled conditions providing light from 07:00 to 19:00 h.

To determine the different liver enzyme activities, the animals were anaesthetized by intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt.). The abdomen was opened 5 min after the injection of the anaesthetic. Saline (0.9% NaCl) or glucagon (250μg/kg body wt., unless otherwise stated) were injected in the femoral vein at 8 min. A liver biopsy was taken 5 min later and quickly

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freeze-clamped in liquid N_2. All these manipulations were carried out between 07:00 and 08:00h. The samples were stored in liquid N_2 until homogenization.

Homogenization was in 3vol. of homogenization buffer (50mM-Hepes, pH7.4, 15mM-EGTA, 0.1M-KF) in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 30000*g* for 30min at 4°C and a part of the supernatant was used to determine L-serine dehydratase (EC 4.2.1.13) and glucose 6-phosphate dehydrogenase activities, as described by Pestaña (1969) and Sapag-Hagar *et al.* (1973) respectively.

In order to remove metabolites that could interfere with the assay of phosphofructokinase, 0.2ml of the above supernatant was filtered through a Sephadex G-25 column (1cm x 20cm) equilibrated with homogenization buffer. The main protein fraction was collected and used for the determination of phosphofructokinase activity, as described by Castaño *et al.* (1979). Recovery of phosphofructokinase activity from the gel filtration was in all cases at least 95%. As has been shown for phosphofructokinase from isolated rat hepatocytes (Castaño *et al.*, 1979), further purification of the enzyme by affinity chromatography on agarose–ATP does not change the results obtained with the enzyme from gel-filtered crude extracts. The enzyme activity was measured at 0.25mM ($v_{0.25}$) and 5mM ($V_{max.}$) concentrations of fructose 6-phosphate and expressed as $V_{max.}$ or as $(v_{0.25}/V_{max.}) \times 100$. In kinetic experiments the following terms are used; $s_{0.5}$, defined as [S] when $v = V_{max.}/2$ for sigmoidal kinetics, and h, defined as the maximum slope of a Hill plot, of $\log[v/(V_{max.}-v)]$ against log[S], where [S] is [fructose 6-phosphate].

Processing of liver samples, assay and expression of pyruvate kinase L activity and glycogen phosphorylase *a* activity were as described by Felíu *et al.* (1977) and Stalmans & Hers (1975) respectively.

Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard. Specific activity of the enzymes is expressed as nmol of substrate transformed/min per mg of protein at 25°C.

Biochemicals were from Sigma or Boehringer. Other reagents were of analytical-reagent grade.

Results and Discussion

Short-term control of liver phosphofructokinase by glucagon

Table 1 shows in the first line that a marked inactivation of liver phosphofructokinase occurs 5min after the intravenous injection of a saturating dose of glucagon to normal fed rats. This inactivation appears when the enzyme activity is measured at subsaturating concentrations of fructose 6-phosphate (0.25mM), as it is clearly reflected by the decrease in the ratio $(v_{0.25}/V_{max.}) \times 100$. No change in $V_{max.}$ is produced by glucagon treatment. *In vivo*, phosphofructokinase inactivation parallels the known inactivation of pyruvate kinase L (Table 1) and activation of glycogen phosphorylase *a* from 70 ± 6 (saline) to 115 ± 10 (glucagon) nmol/min per mg of protein. Fig. 1 shows the saturation curves of phosphofructokinase for fructose 6-phosphate, from saline-treated (control) and glucagon-treated animals. Glucagon produces a marked increase in both $s_{0.5}$ (from 0.3 to 0.5mM) and h (from 3 to 5) of liver phosphofructokinase for fructose 6-phosphate. These results confirm *in vivo* our previous observations *in vitro* with phosphofructokinase from isolated rat hepatocytes (Castaño *et al.*, 1979).

Table 1. *Effects of different dietary conditions and their influence on the action of a saturating dose of glucagon on liver phosphofructokinase and pyruvate kinase L activities*

Liver biopsies were taken 5min after the injection of saline or glucagon (250μg/g body wt.). $V_{max.}$ is expressed as nmol/min per mg of protein. Processing of liver samples and assays of enzyme activities were carried out as described in the Materials and Methods section. Values are means ± S.E.M. from four different animals. Significance levels of the differences versus normal fed rats are indicated: *$0.10 < P < 0.15$; **$P < 0.02$; ***$P < 0.01$.

	Phosphofructokinase				Pyruvate kinase L			
	Saline		Glucagon		Saline		Glucagon	
Nutritional state	$V_{max.}$	$\frac{v_{0.25}}{V_{max.}} \times 100$	$V_{max.}$	$\frac{v_{0.25}}{V_{max.}} \times 100$	$V_{max.}$	$\frac{v_{0.15}}{V_{max.}} \times 100$	$V_{max.}$	$\frac{v_{0.15}}{V_{max.}} \times 100$
Normal fed	10 ± 1	30 ± 5	10 ± 2	5 ± 2	520 ± 40	33 ± 4	510 ± 30	10 ± 3
24h starved	5 ± 1	12 ± 4***	5 ± 1	12 ± 1	300 ± 30	25 ± 4*	350 ± 35	15 ± 1
48h starved	5 ± 1	12 ± 2***	5 ± 2	12 ± 3	310 ± 20	24 ± 5*	320 ± 20	13 ± 2
90%-casein diet	6 ± 1	10 ± 2***	7 ± 1	7 ± 1	170 ± 30	20 ± 3**	190 ± 40	12 ± 2
90%-glucose diet	8 ± 2	32 ± 7	8 ± 2	10 ± 1	1400 ± 90	36 ± 4	1300 ± 200	13 ± 5

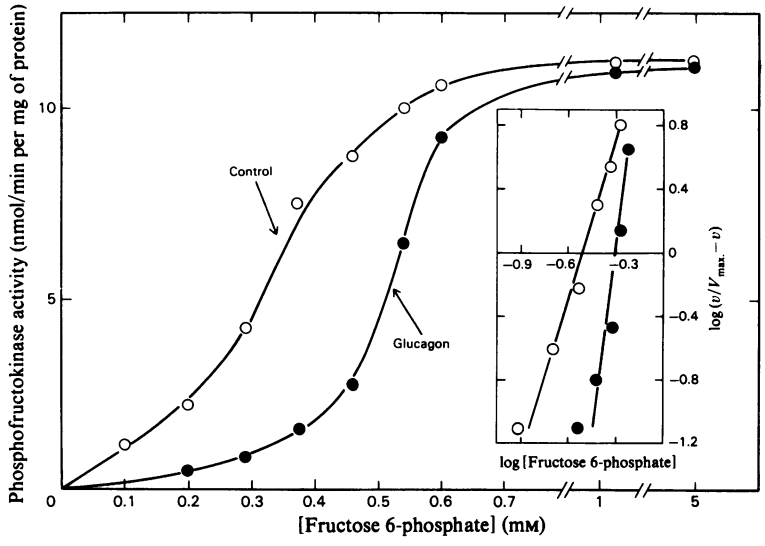

Fig. 1. *Saturation curves of liver phosphofructokinase for fructose 6-phosphate from normal fed rats treated with saline (control) or glucagon*

O, Saline-treated animals; ●, glucagon (250 μg/kg body wt.)-treated animals. Liver biopsies were taken 5 min after the intravenous injection of saline or glucagon. Processing of liver samples and assay of phosphofructokinase activity were carried out as described in the Materials and Methods section. Insert shows Hill plots of the saturation curves. Data are from a representative experiment.

The effect of different intravenously injected doses of glucagon on phosphofructokinase and pyruvate kinase L activities is shown in Fig. 2. Half-maximal inactivation of phosphofructokinase is achieved at a dose of glucagon of 0.02 μg/kg body wt. Taking in account that the plasma volume of the rat is about 40 ml/kg body wt. (Altman & Dittmer, 1964), this half-maximal inactivating dose corresponds approximately to a concentration of 500 pg of glucagon/ml of plasma. As the physiological concentrations of glucagon are within the above values (Unger & Orci, 1976), the inactivation of liver phosphofructokinase can occur in response to physiological changes of glucagon in the plasma. The half-maximal effect for pyruvate kinase L is attained at a slightly higher dose of glucagon (0.04 μg/kg body wt.), but also within the physiological range of concentrations of the hormone. These results suggest a sequential inactivation of phosphofructokinase and pyruvate kinase L mediated by glucagon. Indeed, at a subsaturating dose of glucagon such as 0.015 μg/kg body wt., there is no significant inactivation of pyruvate kinase L, whereas phosphofructokinase is inactivated by about 40%. The fact that fructose 1,6-bisphosphate is a potent activator of pyruvate kinase L and can prevent the cyclic AMP-dependent inactivation of the enzyme in crude extracts from isolated rat hepatocytes (Feliu *et al.*, 1977) makes it likely that

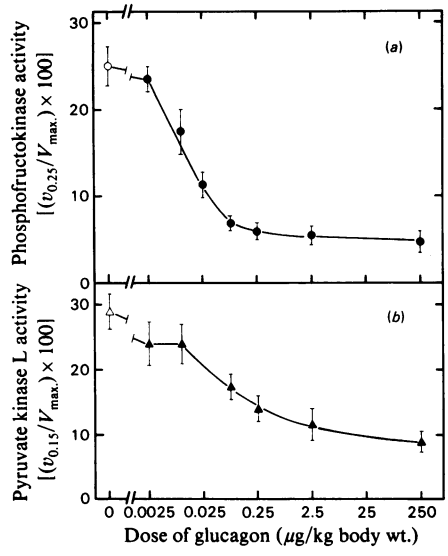

Fig. 2. *Effect of different intravenously injected doses of glucagon on liver phosphofructokinase (a) and pyruvate kinase L (b) activities from normal fed rats*

Open symbols are control values from animals injected with saline. Liver biopsies were taken 5 min after the intravenous injection of saline or glucagon. Processing of liver samples and assay of enzyme activities were carried out as described in the Materials and Methods section. Values are means ± S.E.M. from four different animals.

the inactivation of pyruvate kinase L by glucagon would occur only after a fall in the fructose 1,6-bisphosphate concentration caused by the previous inactivation of phosphofructokinase. Further knowledge of the influence of allosteric effectors of phosphofructokinase on the mechanism of inactivation of the enzyme by glucagon is required to ascertain the above explanation. The preceding considerations point to the existence of a complex interaction between interconversion and allosteric regulation of the two key regulatory enzymes, phosphofructokinase and pyruvate kinase L, in the control by glucagon of the glycolytic and gluconeogenic flux in liver.

Long-term control of phosphofructokinase activity by different dietary conditions

The effect of different dietary conditions on the activity of liver phosphofructokinase and their influence on the short-term control of the enzyme activity by a saturating dose of glucagon have also been studied. The changes of pyruvate kinase L, serine dehydratase and glucose 6-phosphate dehydrogenase activities under the same dietary conditions are also presented.

Changes in the $V_{max.}$ of serine dehydratase and glucose 6-phosphate dehydrogenase activities were measured as control enzymes to ascertain the effectiveness of the diets. An increase of serine dehydratase and a decrease of glucose 6-phosphate dehydrogenase activities in gluconeogenic conditions (starvation and high-protein diet) are observed, whereas the opposite is found with the high-carbohydrate diet. These results are in accordance with those previously reported by others (Pestaña, 1969; Sapag-Hagar *et al.*, 1973). Glucagon treatment has no effect on the $V_{max.}$ of these enzymes. Results are summarized in Table 2.

Data for phosphofructokinase and pyruvate kinase L activities are presented in Table 1. In gluconeogenic conditions (starvation and high-protein diet) there is a marked decrease in the proportion of active phosphofructokinase, measured by the ratio $(v_{0.25}/V_{max.}) \times 100$. This extent of inactivation is not further increased after glucagon treatment, and it is similar to the extent of inactivation found in liver from normal fed rats after injection of glucagon. These results are in accordance with the high glucagon/insulin ratio characteristic of these experimental conditions (Unger & Orci, 1976). There is also in these conditions a 50% decrease in the $V_{max.}$ of the enzyme that is not affected by glucagon treatment. In all dietary conditions yet tested, no effect of glucagon on the $V_{max.}$ of the enzyme has been observed. We have not further investigated this change in the $V_{max.}$ of the enzyme, but currently two mechanisms to account for the decrease of the $V_{max.}$ of liver phosphofructokinase during starvation have been proposed: a cyclic AMP-independent interconversion by Brand *et al.* (1976) and a proteolytic degradation by Dunaway & Weber (1974). High-carbohydrate diet has no significant effect either on the proportion of active form or on the $V_{max.}$ of the enzyme. Glucagon treatment produces a decrease in the $(v_{0.25}/V_{max.}) \times 100$ ratio similar to that found in the normal fed rats, and also without change in $V_{max.}$. The results with the high-carbohydrate diet are in accordance with essentially identical changes in the phosphorylation of the enzyme produced by glucagon in this condition and in the normal fed rats (Kagimoto & Uyeda, 1979).

With respect to pyruvate kinase L activity, we confirm previous results of Felíu *et al.* (1977) on the effect of starvation and high-carbohydrate diet. With high-protein diet the results are similar to that found in starvation, but a further decrease of the $V_{max.}$ of the enzyme is observed, perhaps in relation to the longer time of diet treatment involved.

The proportions of active forms of phospho-

Table 2. *Effects of different dietary conditions and their influence on the action of a saturating dose of glucagon on liver serine dehydratase and glucose 6-phosphate dehydrogenase activities*

Liver biopsies were taken 5 min after the injection of saline or glucagon (250 μg/kg body wt.). Processing of liver samples and assay of enzyme activities were carried out as described in the Materials and Methods section. Values are means ± S.E.M. from four different animals.

Nutritional state	Activity (nmol/min per mg of protein)			
	Serine dehydratase		Glucose 6-phosphate dehydrogenase	
	Saline	Glucagon	Saline	Glucagon
Normal fed	4 ± 1	4 ± 1	47 ± 5	53 ± 6
24 h starved	15 ± 3	13 ± 4	18 ± 2	18 ± 3
48 h starved	17 ± 2	18 ± 3	18 ± 2	19 ± 3
90%-casein diet	16 ± 4	16 ± 4	12 ± 2	13 ± 1
90%-glucose diet	0.6 ± 0.2	0.6 ± 0.3	94 ± 7	86 ± 6

fructokinase and pyruvate kinase L, measured by their respective ratios of velocities at subsaturating concentrations of substrates relative to $V_{max.}$, are in parallel in several dietary conditions. In gluconeogenic conditions there is a decrease in the proportion of active form, more significant for phosphofructokinase than for pyruvate kinase L, but no significant changes are observed with the high-carbohydrate diet. The absence of change in the proportion of active forms of both enzymes in the high-carbohydrate-diet condition could be attributed to the unexpectedly low concentrations of insulin in the blood of these animals, similar to that found in starvation (Seitz *et al.*, 1977). Not such a good correlation exists between the $V_{max.}$ activities of both enzymes: although both $V_{max.}$ values are decreased in gluconeogenic conditions, there is an increase in the $V_{max.}$ of pyruvate kinase L in the high-carbohydrate-diet condition and no change in the $V_{max.}$ of phosphofructokinase.

From the results discussed, it seems clear that changes both in the proportion of active form and in the maximal rate of liver phosphofructokinase and pyruvate kinase L are implicated in the adaptive response of liver to the different nutritional states.

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