Increased activity of phosphate-dependent glutaminase in liver mitochondria as a result of glucagon treatment of rats

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1. Injection of rats with glucagon leads to an increased effective activity of glutaminase in subsequently isolated liver mitochondria. 2. This effect of glucagon is manifested as a decreased requirement of glutaminase for phosphate in the presence of HCO_3^- . The HCO_3^- -concentration-dependence is unchanged. 3. The effect of glucagon is lost on disruption of the mitochondria. 4. In accordance with previous reports, incubation of mitochondria in hypo-osmotic media also increases the effective activity of glutaminase. Glucagon increases glutamine hydrolysis at intermediate osmolarities of the suspending medium, but does not affect glutaminase activity when it is already maximally activated by hypo-osmotic conditions. 5. From this and previous work, it seems that hypo-osmotic incubation conditions, EDTA and glucagon may all activate glutaminase by a common mechanism. It is postulated that this mechanism involves modification of the interaction of glutaminase with the mitochondrial inner membrane.

Glucagon has been shown to cause a 3-fold stimulation of gluconeogenesis from 2 mM-glutamine when added to the incubation of isolated liver cells from starved rats (Joseph & McGivan, 1978a). This effect was accompanied by a decrease in the intracellular concentration of glutamine and an increase in that of glutamate, suggesting that stimulation is effected by increasing the activity of mitochondrial glutaminase (EC 3.5.1.2). This enzyme, in liver, appears to be loosely associated with the inner surface of the mitochondrial inner membrane (McGivan *et al.*, 1980). In disrupted mitochondria, glutaminase was also shown to be activated by the presence of phosphate, HCO_3^- or NH_4^+ ions, and by ATP. In intact mitochondria, the effective activity of glutaminase in the presence of phosphate and either HCO_3^- or NH_4^+ is stimulated by hypo-osmotic swelling and by treatment of the mitochondria with EDTA (Joseph *et al.*, 1980).

Mitochondria isolated from the livers of rats that have been injected with glucagon exhibit increased rates of respiration (Yamazaki & Graetz, 1977; Halestrap, 1978), pyruvate carboxylation (Garrison & Haynes, 1975) and citrulline synthesis (Yamazaki & Graetz, 1977) compared with controls. To elucidate the mechanism by which glucagon stimulates gluconeogenesis from glutamine in the liver, the

effects of the injection of glucagon on glutaminase in subsequently isolated mitochondria have been studied.

Materials and methods

Administration of glucagon and preparation of mitochondria

Female Wistar rats, weighing approx. 220 g, were anaesthetized with Nembutal (60 mg/kg body wt., injected intraperitoneally) and half were injected subsequently with an intraperitoneal dose of 0.1 mg of glucagon (dissolved as a 1 mg/ml solution in 0.5 M-KCl/20 mM-Tris base). After 20 min, the livers were removed and mitochondria were isolated by the method of Chappell & Hansford (1972) in a medium containing 0.25 M-mannitol, 10 mM-Tris/HCl and 2 mM-EGTA at pH 7.3. Each liver was homogenized in a final volume of 50 ml, and the mitochondria were washed once only, since further dilution and washing decreased the persistence of a glucagon effect.

Assays

The rates of O_2 consumption were measured with a Clark oxygen electrode. Citrulline was measured by the method of Archibald (1944). Glutaminase activity in intact mitochondria was determined by measuring the amount of glutamate formed after 10 min incubation of 3–4 mg of mitochondrial protein/ml at 30°C and pH 7.4 in a medium

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containing 10 mM-Tris/HCl, 5 mM-succinate, rotenone (5 µg/ml), 1 mM-EGTA and various concentrations of KCl, glutamine, phosphate and KHCO_3 as indicated. Under these conditions, rotenone completely inhibited the metabolism of glutamate (Joseph & McGivan, 1978b), which was then assayed enzymically, after deproteinization and neutralization of the extracts, by the method of Bernt & Bergmeyer (1965).

P_i was measured by the method of Berenblum & Chain (1938), modified for use with small volumes. Intramitochondrial volume was determined by using $^3\text{H}_2\text{O}$ and D-[1- ^{14}C]mannitol as markers of the total pellet volume and extramitochondrial space respectively (McGivan *et al.*, 1977). Protein was measured by a biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard.

Materials

Glucagon was obtained from Calbiochem, C.P. Laboratories Ltd., Bishops Stortford, Herts., U.K., and $^3\text{H}_2\text{O}$ and D-[1- ^{14}C]mannitol were from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Effect of glucagon on the activity of mitochondrial glutaminase

The rate of oxygen consumption in the presence of succinate and ADP was increased in mitochondria prepared from glucagon-treated rats when compared with controls (Table 1). Similarly, the rate of citrulline synthesis from NH_3 was increased after glucagon treatment, and these two parameters were taken as indicative of effective glucagon administration throughout the experiments reported in this paper. The percentage stimulation of these parameters as a result of glucagon treatment is

consistent with previous reports (Yamazaki & Graetz, 1977; Halestrap, 1978). The activity of glutaminase in intact liver mitochondria, in the presence of 10 mM-potassium phosphate and 10 mM- KHCO_3 , was similarly shown to be consistently increased in mitochondria from glucagon-treated rats compared with controls (Table 1).

Effects of changing the osmolarity of the suspension medium on the activity of mitochondrial glutaminase

Joseph *et al.* (1980) have shown that a decrease in total osmolarity, in both KCl and mannitol media, results in an increase in the effective glutaminase activity of mitochondria. Fig. 1 shows the effects of varying the osmolarity on glutamine hydrolysis in mitochondria pooled from three control or three glucagon-treated rats, in a representative experiment.

Glucagon decreased the sigmoidicity of the glutamine-concentration-dependence curve, and this was also the effect seen with decreasing osmolarity. When the osmolarity of the incubation medium was lowered to 95 mosM, there was no difference between the two types of mitochondria. The greatest effect of glucagon was observed around 200 mosM, and at lower concentrations of glutamine. In four such experiments, the mean values ($\pm$ S.E.M.) for glutaminase activity at 30 mM-glutamine, and a total osmolarity of 215 mosM, were 92.6 ± 6.4 and 142.7 ± 11.3 nmol/mg in 10 min for control and glucagon-treated respectively ($P < 0.01$). The effect of glucagon on glutaminase at higher osmolarity (295 mosM) and 30 mM-glutamine was extremely variable [71.1 ± 12.7 (11) for the control, increasing to 125.2 ± 16.5 (10); $P < 0.05$].

Effect of glucagon on the activation of mitochondrial glutaminase by phosphate and HCO_3^-

Joseph *et al.* (1980) have shown that the addition of EDTA decreases the concentration of phos-

Table 1. *Effect of glucagon injection of rats on the activity of glutaminase and other parameters in isolated liver mitochondria*

Oxygen uptake was measured in mitochondria (2 mg/ml) incubated in a medium containing 100 mM-KCl, 20 mM-Tris/HCl and 5 mM-potassium phosphate at pH 7.4, and 30°C. Rates were measured after the addition of 10 mM-succinate and 1 mM-ADP. Citrulline synthesis was followed by measuring the formation of citrulline after incubation of mitochondria (5–6 mg/ml) for 2–4 min in a medium containing 80 mM-KCl, 20 mM-Tris/HCl, 5 mM-potassium phosphate, 5 mM-succinate, 20 mM- KHCO_3 , 5 mM- NH_4Cl , 20 mM-ornithine and 1 mM-EGTA at pH 7.4 and 30°C. Glutaminase activity was measured, as described in the text, in the presence of 60 mM-KCl, 20 mM-glutamine, 10 mM-potassium phosphate and 10 mM- KHCO_3 (total osmolarity 215 mosM). Values are given as means $\pm$ S.E.M. for the numbers of separate mitochondrial preparations shown in parentheses.

	Controls	+Glucagon	<i>P</i> value	Stimulation (%)
O_2 consumption (ng-atoms of O/min per mg)	58.1 ± 6.2 (11)	96.7 ± 10.3 (10)	<0.01	66
Citrulline synthesis (nmol/min per mg)	8.5 ± 0.8 (11)	13.7 ± 1.0 (10)	<0.001	62
Glutaminase activity (nmol/mg in 10 min)	64.4 ± 2.7 (7)	138.6 ± 6.2 (7)	<0.001	115

Fig. 1. Effect of glucagon treatment of rats and changing osmolarity of the incubation medium on the glutamine-dependence curves of glutaminase in isolated mitochondria

Mitochondria were pooled from three control or three glucagon-treated rats and were incubated, as described in the text, in the presence of 10 mM-KHCO₃, 10 mM-potassium phosphate and various concentrations of KCl and glutamine. (a) 100 mM-KCl (total osmolarity, excluding glutamine, 295 mosM); (b) 70 mM-KCl (235 mosM); (c) 60 mM-KCl (215 mosM); (d) 50 mM-KCl (195 mosM); (e) 20 mM-KCl (135 mosM); (f) no KCl (95 mosM).

●, Mitochondria from glucagon-treated rats; △, control mitochondria.

phate required for half-maximum stimulation of glutaminase activity in intact mitochondria. Fig. 2 shows the phosphate-dependence of glutamine hydrolysis in mitochondria from both control and glucagon-treated rats, and that glucagon also decreases the amount of phosphate required for half-maximum activation. In incubations where the concentration of KHCO₃ was varied, however, there was no difference in the requirement for KHCO₃ (Fig. 3). The effect of glucagon in this case was an overall increase in activity, owing to the change in phosphate-sensitivity, where phosphate was not saturating.

The concentration of phosphate required for half-maximum activation of glutaminase in intact mitochondria was altered by changes in the osmolarity of the medium as well as by treatment of the rats with glucagon, as shown in Table 2. In both cases the effect was considerable and there was no change in $V_{\max}$. In disrupted mitochondria, however, glucagon treatment produced no decrease

in phosphate requirement. This evidence, together with an unchanged K_m for glutamine in disrupted mitochondria from glucagon-treated rats compared with the controls (results not shown), suggests that the intact state of the mitochondrial membrane is important in producing the change in phosphate-sensitivity seen after glucagon treatment.

The change in phosphate-sensitivity could be due either to a change in the affinity of the enzyme itself for phosphate or to an increased accumulation of phosphate across the mitochondrial membrane, if the enzyme were inwardly facing. Such an increased accumulation is unlikely, since the HCO₃⁻-dependence was unchanged by glucagon treatment (Fig. 3), and HCO₃⁻ would be expected to be distributed across the membrane in the same way as phosphate. However, phosphate accumulation was measured in mitochondria from control and glucagon-treated rats, under conditions similar to those described for Fig. 2. With an external phosphate concentration of 3 mM, the

Table 2. *Effect of glucagon treatment of rats, and changes in osmolarity of the suspension medium, on the phosphate requirement of glutaminase in isolated mitochondria*

Glutaminase activity was measured, as described in the legend of Fig. 1, in the presence of 20mM-KHCO₃ and 30mM-glutamine. Mitochondria were also disrupted by freezing and thawing three times, and incubated under the same conditions. Phosphate concentrations were varied between 0 and 30mM, the total osmolarity being kept constant by varying the KCl concentrations accordingly. Mitochondria from three control or glucagon-treated rats were pooled for the experiment.

Total osmolarity	[Phosphate] (mM) required for half-maximum activation of glutaminase		$V_{max.}$ (nmol/mg in 10 min)	
	Control	+Glucagon	Control	+Glucagon
Intact mitochondria				
295 mosM	24.9	12.1	130.5	156.7
215 mosM	6.23	1.15	133.7	148.0
155 mosM	2.11	0.24	130.3	151.4
Disrupted mitochondria	4.18	6.66	95.8	122.5

Fig. 2. *Effect of glucagon treatment of rats on the phosphate-dependence of glutaminase in isolated mitochondria*

The incubation medium contained 20mM-KHCO₃, 30mM-glutamine and other components, as described in the Materials and methods section. The total concentration of KCl plus potassium phosphate was 60mM (total osmolarity 225 mosM). ●, Mitochondria from glucagon-treated rats; Δ, control mitochondria.

Fig. 3. *Effect of glucagon treatment of rats on the HCO₃⁻-dependence of glutaminase in isolated mitochondria*

The incubation medium was similar to that described for Fig. 2, but containing 20mM-potassium phosphate, and with the total concentration of KCl plus KHCO₃ equal to 60mM. ●, Mitochondria from glucagon-treated rats; Δ, control mitochondria.

internal concentrations were 8.78 ± 0.45 mM and 9.01 ± 0.63 mM respectively: these are the mean values $\pm$ S.E.M. for eight control and eight test incubations, with mitochondria pooled from three rats in each case.

From Fig. 2, the rate of glutamine hydrolysis obtained at a concentration of 3 mM external phosphate in mitochondria from glucagon-treated

rats was the same as that at 30 mM external phosphate in the controls. If the actual phosphate-dependence of the enzyme is unchanged by glucagon treatment, this would imply that the internal phos-

phate concentration must be the same in each case, i.e. greater than 30 mM. Such a value is not consistent with the actual measurements cited above. It is concluded that glucagon changes the actual phosphate-dependence of glutaminase rather than affecting the phosphate distribution across the membrane.

Discussion

These experiments show that glucagon treatment of rats, in addition to previously established effects on oxygen consumption, pyruvate carboxylation and citrulline synthesis, causes a stimulation of glutaminase activity which persists into isolated mitochondria. The magnitude of this effect on glutaminase is consistent with the overall stimulation of gluconeogenesis from glutamine observed in the intact liver cell (Joseph & McGivan, 1978a).

In isolated mitochondria, the glucagon effect is manifested primarily by a decrease in the concentration of phosphate required for half-maximum activation, in the presence of HCO_3^- (Fig. 2). As discussed above, it is unlikely that this effect on phosphate requirement is due to a change in phosphate distribution. Glucagon also alters the effective glutamine-concentration-dependence, but it is not possible to deduce from the above data whether this effect is independent of the change in phosphate-sensitivity.

The regulation of glutaminase activity in intact mitochondria is complex. Effective glutaminase activity is increased by incubation of the mitochondria in hypo-osmotic media, and is also stimulated by the treatment of mitochondria with EDTA (Joseph *et al.*, 1980). When glutaminase is fully activated by incubation of mitochondria in a medium of 95 mosM, no further effect of glucagon (Fig. 1) or EDTA (results not shown) is observed. However, effects of glucagon are seen at intermediate osmolarities.

Halestrap *et al.* (1980) have reported that mitochondria from glucagon-treated rats have an internal volume which is increased by 14% compared with controls, in KCl medium. The results depicted in Fig. 1 may indicate that the effects of changes in osmolarity and of glucagon are both mediated by increases in mitochondrial volume. However, it is significant that glucagon decreases the sigmoidicity of the glutamine-dependence curve at all osmolarities, whereas there is little decrease in sigmoidicity as a result of varying the osmolarity of the controls within the range 295–215 mosM.

Since glucagon treatment, hypo-osmotic swelling and EDTA (Joseph *et al.*, 1980) all activate glutaminase in intact mitochondria by changing the phosphate-dependence, it would appear that these

effects are mediated by a common mechanism. It is unlikely that this mechanism involves an obligatory increase in intramitochondrial volume, as discussed above. In addition to this, it has been shown that EDTA activates glutaminase under conditions where swelling does not occur (Joseph *et al.*, 1980). It is postulated that the mechanism by which activation of glutamine hydrolysis occurs involves changes in the nature of the interaction of glutaminase with the mitochondrial inner membrane. The activity of the enzyme has been shown to change on dissociation from the membrane (McGivan *et al.*, 1980). It is possible that the membrane contains a regulatory protein that modifies glutaminase activity, and that glucagon, EDTA and hypo-osmotic swelling influence the interaction of glutaminase with this protein in some way.

It is by no means clear whether the effects of glucagon on various parameters of mitochondrial function are mediated by a single mechanism. It is hoped that a detailed study of the activation of glutaminase by glucagon will shed light on this problem.

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