Distinctive Effects of Glucagon on Gluconeogenesis and Ketogenesis in Hepatocytes Isolated from Normal and Biotin-Deficient Rats

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In hepatocytes from 48h-starved rats identical glucagon dose-response curves were obtained for the stimulation of gluconeogenesis from lactate, for ketogenesis and for the decreasing of the C₅-dicarboxylate pool. Glucagon (20nM) caused a 5-fold increase in 3-hydroxybutyrate formation, but decreased acetoacetate production to 50% of that of the control. In hepatocytes from biotin-deficient rats glucagon no longer stimulated gluconeogenesis from lactate, but still produced its effects on the mitochondrial redox state and the C₅-dicarboxylate pool. The results suggest that the primary site of the hormone action on gluconeogenesis is located within the mitochondria rather than in the cytosol.

Rat liver perfusion studies with glucagon have established that the stimulation of gluconeogenesis is accompanied by an increased rate of ketone-body production (Struck *et al.*, 1965; Williamson *et al.*, 1966*a,b,c*). From this and the fact that oleate likewise increases both glucose formation from lactate and pyruvate, and ketogenesis (Struck *et al.*, 1966; Ross *et al.*, 1967*a*; Teufel *et al.*, 1967; Söling *et al.*, 1968; Williamson *et al.*, 1969*a*; Johnson *et al.*, 1972; Krebs *et al.*, 1974; Siess & Wieland, 1975), it was attractive to explain the gluconeogenic action of the hormone by increasing fatty acid supply (Struck *et al.*, 1965; Williamson *et al.*, 1966*a,b*; Struck *et al.*, 1966; Ross *et al.*, 1967*b*; Söling *et al.*, 1968). Evidence has accumulated, however, that glucagon and long-chain fatty acids act by different mechanisms, as glucagon stimulates gluconeogenesis even at maximally effective concentrations of fatty acids (Ross *et al.*, 1967*b*; Exton *et al.*, 1969; Fröhlich & Wieland, 1971), and also when lipolysis in liver is blocked (Fröhlich & Wieland, 1971). Further, the hormone causes large changes in cellular amounts of 2-oxoglutarate, glutamate and phosphoenolpyruvate in contrast with oleate (Ui *et al.*, 1973*b*; Siess *et al.*, 1977; Cook *et al.*, 1977). From perfusion experiments with livers from fed rats, which showed much greater sensitivity of gluconeogenesis than of ketogenesis to glucagon, the physiological significance of the hormone for the latter process was questioned (Exton *et al.*, 1971). No information is available on that point under gluconeogenic conditions, e.g. starvation. We have therefore studied the effect of glucagon on gluconeogenesis and ketogenesis in liver cells from starved rats. Moreover, we have used hepatocytes from biotin-deficient rats as a possible model for investigating

the influence of glucagon on liver metabolism independent of its action on gluconeogenesis. Part of this work has been presented elsewhere (Wieland *et al.*, 1978).

Materials and Methods

Liver cells were prepared as described (Siess *et al.*, 1976) from male Sprague-Dawley rats (E. Jautz, Kisslegg, Germany) kept on laboratory chow (Herilan R/M 204, E. Eggersmann, Rinteln, Germany) or on an egg-white diet (Patel & Mistry, 1968) for 3-4 weeks to induce biotin deficiency. Before use, normal rats (180-220g) were starved for 48h. Biotin-deficient rats (160-200g) were starved for 24h only to avoid fatal hypoglycaemia (Bhagavan *et al.*, 1969). Viability of normal and biotin-deficient cells was about 95%, as judged by exclusion of 0.2% Trypan Blue. Hepatocytes corresponding to about 15mg dry wt. were shaken at 37°C in a medium (Siess *et al.*, 1977) containing undialysed blood serum from normal fed rats to improve the glucagon response (Siess & Wieland, 1975) and lactate or glutamine as gluconeogenic substrate in concentrations indicated in the Table legends. After 30min of incubation 5µl of glucagon dissolved in 1.3% (w/v) NaHCO₃ or 5µl of 1.3% NaHCO₃ was added to the incubation mixture (1.36ml) and incubation was continued for 10min. For glucose and ketone-body measurements, samples (100µl) of the incubation mixture were removed and mixed with 20µl of 70% (w/v) HClO₄.

For determination of intracellular amounts of metabolites, the hepatocytes were separated from the incubation medium by centrifugation through silicone oil as described by Siess *et al.* (1976). The sources of

chemicals and the analytical procedures were those described earlier (Siess *et al.*, 1977), if not stated otherwise. Glutamine was purchased from Serva, Heidelberg, Germany. Pyruvate carboxylase (EC 6.4.1.1) was determined as described by Deodhar & Mistry (1969). Deep-frozen ground liver tissue or frozen cells were extracted with 11 ml of 0.1 M-potassium phosphate buffer, pH 7.2, containing 1 mM-reduced glutathione (Boehringer, Mannheim, Germany) and 0.5% Lubrol WX (ICI, Frankfurt, Germany) per g fresh wt. by stirring with a glass rod between two freezing-thawing cycles. After centrifugation for 4 min at 10000g (Eppendorf centrifuge, model 3200), the supernatant was used for pyruvate carboxylase assay. Controls were run in the presence of 1 mg of avidin (Serva)/ml as described by Böttger *et al.* (1969) to correct for unspecific [14 C]bicarbonate fixation. As identical values were obtained when liver tissue was disintegrated by either sonication (Böttger *et al.*, 1969) or Lubrol, the latter was used for enzyme extraction from the isolated hepatocytes. Tabulated data are given as mean values $\pm$ S.E.M. Statistical significance was calculated by Student's *t* test for paired data.

Results and Discussion

Fig. 1 illustrates dose-response curves for glucagon on gluconeogenesis, ketogenesis and on the amounts of 2-oxoglutarate and glutamate in isolated rat hepatocytes incubated in the presence of lactate. Obviously, the curves obtained for all metabolites tested are within the same range of glucagon concentrations. Nearly maximal hormone effects were obtained at 20 nM, the lowest glucagon concentration yielding a significant effect ($P < 0.05$ compared with hormone-free control) being around 2 nM. Thus it appears that gluconeogenesis from lactate and ketogenesis in liver cells from starved rats are equally sensitive to glucagon. This is in contrast with the situation in the perfused liver from fed rats, where the glucagon concentrations for stimulation of gluconeogenesis and ketogenesis differ by two orders of magnitude (Exton *et al.*, 1971). Moreover, Fig. 1 demonstrates that glutamate and 2-oxoglutarate decrease in parallel with the increase in the rates of production of glucose and 3-hydroxybutyrate during glucagon stimulation. Evidently glucagon does not affect preferentially a single metabolic pathway, but rather causes a complex sequence of events. This is further illustrated by the fact that the same glucagon-dependence as shown in Fig. 1 was displayed by the mitochondrial redox state (Williamson *et al.*, 1967), as indicated by the [3-hydroxybutyrate]/[acetoacetate] ratio (Fig. 2). Although it is known that glucagon stimulates lipolysis in liver (Struck *et al.*, 1965; Williamson *et al.*, 1966a,b,c; Bewsher & Ashmore, 1966; Menahan &

Fig. 1. Amounts of 2-oxoglutarate ($\circ$) and glutamate ($\bullet$), and stimulation of gluconeogenesis (Δ) and ketogenesis ($\blacktriangle$), in isolated hepatocytes from 48 h-starved rats incubated with 12 mM-lactate as a function of glucagon concentration. Mean values of six different cell preparations as a percentage of that of hormone-free control are given. The S.E.M. values, not shown for the sake of clarity, were about 10% of the mean values. The control values for 2-oxoglutarate and glutamate were 1.46 ± 0.18 and $13.6 \pm 1.6 \mu\text{mol/g dry wt.}$ respectively. The control values for glucose production, 3-hydroxybutyrate and acetoacetate formation were 54.8 ± 4.5 , 1.6 ± 0.3 and $4.5 \pm 0.4 \mu\text{mol/10 min per g dry wt.}$ respectively. For further details see the Materials and Methods section.

Fig. 2. Effect of different glucagon concentrations on the formation of 3-hydroxybutyrate ($\bullet$) and acetoacetate ($\circ$) and on the [3-hydroxybutyrate]/[acetoacetate] ratio ($\blacksquare$) in isolated liver cells from 48 h-starved rats. Means $\pm$ S.E.M. for six different experiments are shown. With respect to ketone-body production the ordinate refers to $\mu\text{mol/g dry wt.}$ for the incubation period between 30 and 40 min.

Wieland, 1969; Claycomb & Kilsheimer, 1969), the results shown in Fig. 2 can hardly be explained merely by an increase in fatty acid supply and oxi-

Table 1. Additive effect of glucagon on the [3-hydroxybutyrate]/[acetoacetate] ratio of hepatocytes isolated from 48 h-starved rats incubated in the presence of oleate

Mean values $\pm$ S.E.M. for five experiments are given. The cells were incubated with 12 mM-lactate for 30 min before glucagon (7 μ g/ml) and the other substrate(s) indicated were added and incubation was continued for 10 min. The rate of ketone-body formation for the 10 min period between 30 and 40 min of incubation is expressed in μ mol/10 min per g dry wt. * P < 0.025, ** P < 0.0025, *** P < 0.005.

Substrates added ...	Lactate (12 mM)+albumin-oleate (0.66 mM)		Lactate (12 mM)+albumin-oleate (0.66 mM)+alanine (3.3 mM)	
	Control	Glucagon	Control	Glucagon
3-Hydroxybutyrate	46.4 $\pm$ 3.2	57.6 $\pm$ 3.6***	27.4 $\pm$ 2.2	49.2 $\pm$ 3.1**
Acetoacetate	11.9 $\pm$ 0.3	5.3 $\pm$ 1.3***	10.7 $\pm$ 0.9	7.6 $\pm$ 0.2*
3-Hydroxybutyrate+acetoacetate	58.3	62.9	38.1	56.8
[3-Hydroxybutyrate]/[acetoacetate]	3.9	10.8	2.5	6.4

Table 2. Effect of glucagon on gluconeogenesis from 8 mM-lactate or 8 mM-glutamine in hepatocytes from biotin-deficient and normal rats

Rate measurements for the 10 min period between 30 and 40 min of incubation are given for the numbers of different cell preparations in parentheses. For further details see the Materials and Methods section. † P < 0.05, †† P < 0.01, ** P < 0.0025.

Treatment of donor animal	Glucose formation (μ mol/10 min per g dry wt.)			
	Lactate		Glutamine	
	Control	+Glucagon (0.5 μ g/ml)	Control	+Glucagon (0.5 μ g/ml)
Biotin-deficient diet (0.55 $\pm$ 0.09; n = 13) †	24.3 $\pm$ 2.5 (12)	21.4 $\pm$ 2.1 (12)	27.7 $\pm$ 4.1 (8)	49.7 $\pm$ 11.2† (8)
Biotin-deficient diet+200 μ g of biotin 14 h before use	33.1 $\pm$ 7.2 (6)	95.1 $\pm$ 8.4** (6)	25.9 $\pm$ 5.8 (5)	60.2 $\pm$ 7.1†† (5)
Normal diet (5.53 $\pm$ 0.7; n = 6) †	54.8 $\pm$ 5.9 (5)	108.7 $\pm$ 8.3** (5)	20.8 $\pm$ 5.3 (6)	42.2 $\pm$ 7.6** (6)

† Pyruvate carboxylase activity of freeze-clamped livers from unstarved rats, in μ mol of CO₂ fixed/min per g fresh wt.; n = number of animals. Pyruvate carboxylase activity of the cells used in these studies was 0.68 $\pm$ 0.08 μ mol of CO₂ fixed/min per g fresh wt. (n = 11).

dation. Glucagon increases 3-hydroxybutyrate production and simultaneously decreases acetoacetate formation (Fig. 2), whereas oleate in perfused liver and in isolated liver cells leads to an increase in synthesis of both acetoacetate and 3-hydroxybutyrate, the latter being more pronounced (Krebs *et al.*, 1974; Siess *et al.*, 1976; Williamson *et al.*, 1969b). Moreover, glucagon increases the [3-hydroxybutyrate]/[acetoacetate] ratio at a much lower rate of ketone-body formation than does oleate (Siess *et al.*, 1977). The profound changes in the mitochondrial redox state induced by glucagon point to a specific mechanism at the mitochondrial level, which cannot be explained by mere accumulation of reducing equivalents from increased fatty acid supply. This is further documented by the results in Table 1, showing the ketogenic effect of glucagon in the presence of oleate.

The results summarized in Figs. 1 and 2 do

not answer the question whether the effects of glucagon on ketogenesis and 2-oxoglutarate metabolism are secondary to its action on the gluconeogenic pathway. Therefore experiments were conducted with hepatocytes isolated from biotin-deficient rats, taking advantage of the fact that among the gluconeogenic key enzymes only the activity of pyruvate carboxylase is decreased by biotin deprivation (Deodhar & Mistry, 1969). As shown in Table 2, glucagon no longer stimulated gluconeogenesis in the presence of lactate when pyruvate carboxylase activity was deficient. The gluconeogenic response to glucagon, however, was that of normal rats, or even greater, when the biotin-deficient animals had received 200 μ g of D(+)-biotin (Merck, Darmstadt, Germany) intraperitoneally 14 h before liver-cell preparation (Table 2). The diminution of basal and the abolition of glucagon-stimulated glucose production with lactate (Table 2) point to the key role

of pyruvate carboxylase in gluconeogenesis from this substrate. This is further substantiated by the finding that gluconeogenesis from glutamine (Table 2), which circumvents the pyruvate carboxylase step, was unaffected by biotin deficiency both in the absence and in the presence of glucagon. On the basis of a pyruvate carboxylase activity of $0.68 \mu\text{mol}$ of CO_2 fixed/min per g fresh wt. one can estimate that the biotin-deficient hepatocytes synthesize quite an appreciable amount of glucose without apparent involvement of pyruvate carboxylase, possibly via 'malic' enzyme (EC 1.1.1.40). This interpretation is consistent with the fact that the activities of phosphoenolpyruvate carboxykinase (EC 4.1.1.32), fructose 1,6-bisphosphatase (EC 3.1.3.11) and glucose 6-phosphatase (EC 3.1.3.9) in rat liver remain unaltered when biotin is lacking (Deodhar & Mistry, 1969), in agreement with our data on gluconeogenesis from glutamine (Table 2). As to the mechanism of glucagon action on gluconeogenesis, one might therefore conclude from the data of Table 2 that the hormone does not act primarily on a key gluconeogenic enzyme reaction or reactions in the cytosol.

The effect of glucagon on ketogenesis in biotin-deficient hepatocytes is shown in Table 3. Although the basal rate of ketone-body production by biotin-deficient cells is about 3–5 times that of cells from biotin-substituted ($2.7 \pm 0.5 \mu\text{mol}/10 \text{ min per g dry wt.}$,

$n = 3$) or normal rats (Siess *et al.*, 1977), glucagon still exerts a considerable ketogenic effect. Further, glucagon increases the $[3\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ ratio from 10 to 32. These results clearly show that the effect of glucagon on ketone-body formation and mitochondrial redox state persists in biotin deficiency and that it can be differentiated from that on gluconeogenesis. The same seems to be true with respect to the glucagon-dependent fluctuations of 2-oxoglutarate and glutamate. The concentrations of these and other metabolites gradually diminish in liver cells when biotin deficiency develops (results not shown). In the hepatocytes used in this study the concentrations of 2-oxoglutarate, glutamate, malate, citrate and phosphoenolpyruvate were 37, 40, 37, 30 and 32% respectively of the values found in normal liver cells (Siess *et al.*, 1977). As shown in Table 4, glucagon markedly decreased the amounts of 2-oxoglutarate and glutamate, as was shown for the isolated perfused liver (Ui *et al.*, 1973b; Cook *et al.*, 1977) and normal liver cells (Siess *et al.*, 1977). These changes were not accompanied by an increase in malate concentration (Table 4), whereas hepatocytes with an intact pyruvate carboxylase show an increase in malate after glucagon treatment (Siess *et al.*, 1977). This hormone effect appears therefore to be attributable to an activation of pyruvate carboxylation rather than to an increased formation of malate from the C_5 -dicarboxylate pool. With respect to phosphoenolpyruvate synthesis, the increase by glucagon found normally (Ui *et al.*, 1973a; Siess *et al.*, 1977) contrasts with the lowering found in biotin-deficient cells (Table 4). Studies on metabolite compartmentation in these cells revealed that the mitochondrial phosphoenolpyruvate content, amounting to $0.1 \pm 0.04 \mu\text{mol/g dry wt.}$ ($n = 4$), remained unchanged by glucagon, whereas the cytosolic portion was significantly decreased. This might indicate that the mitochondrial phosphoenolpyruvate carboxykinase is of importance not only in normal (Siess *et al.*, 1977) but also in biotin-deficient rats.

Taken together, the results of the present study are consistent with the view that glucagon exerts its concomitant effects on different metabolic pathways by changing mitochondrial function(s)

Table 3. *Effect of glucagon on ketogenesis in hepatocytes isolated from biotin-deficient rats starved for 24 h*
Rate measurements for the 10 min period between 30 and 40 min of incubation are given for five different cell preparations incubated initially with 12 mm-lactate. Pyruvate carboxylase activity of the liver cells was determined to be $0.55 \pm 0.09 \mu\text{mol}$ of CO_2 fixed/min per g fresh wt. ($n = 4$). $\dagger P < 0.05$, $\dagger\dagger P < 0.01$.

Condition	Metabolite change ($\mu\text{mol}/10 \text{ min per g dry wt.}$)	
	Acetoacetate	3-Hydroxybutyrate
Control	1.4 ± 0.27	14.7 ± 4.4
Glucagon ($0.5 \mu\text{g/ml}$)	$0.7 \pm 0.2\dagger$	$23.4 \pm 4.6\dagger\dagger$

Table 4. *Effect of glucagon on the amounts of various metabolites in liver cells isolated from biotin-deficient rats starved for 24 h*

The cell preparations and the conditions of incubation were identical with those described in the legend to Table 3. Numbers of different cell preparations are given in parentheses. $*P < 0.025$, $\dagger\dagger P < 0.01$, $**P < 0.0025$.

Condition	Metabolites ($\mu\text{mol/g dry wt.}$)				
	2-Oxoglutarate	Glutamate	Malate	Citrate	Phosphoenolpyruvate
Control	0.82 ± 0.12 (5)	5.4 ± 0.9 (5)	0.37 ± 0.04 (5)	0.55 ± 0.04 (4)	0.25 ± 0.03 (5)
Glucagon ($0.5 \mu\text{g/ml}$)	$0.31 \pm 0.03^{**}$ (5)	$2.5 \pm 0.5\dagger\dagger$ (5)	0.36 ± 0.06 (5)	0.44 ± 0.11 (4)	$0.15 \pm 0.02^*$ (5)

(Adam & Haynes, 1969; Yamazaki, 1975; Titheradge & Coore, 1976; Yamazaki *et al.*, 1977; Bryła *et al.*, 1977; Wieland *et al.*, 1978). Further, they point to the fundamental role of the interplay between pyruvate carboxylase and metabolite transport processes in the promotion of gluconeogenesis by glucagon.

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