# GLUCAGON-INDUCED STIMULATION OF 2-OXOGLUTARATE METABOLISM IN MITOCHONDRIA FROM RAT LIVER

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Received 10 July 1978

## 1. Introduction

Since the discovery [1] that liver mitochondria isolated from glucagon-treated rats show an increased rate of pyruvate carboxylation, evidence for mitochondrial action(s) of this hormone is growing. In addition to the effects on pyruvate metabolism [1–7] glucagon administration has been demonstrated to increase mitochondrial respiration [5,7–11], ATP formation [7,10,12], ATPase activity and $K^+$ uptake [13], citrulline formation [6], hydroxyl ion concentration [7,9] and membrane potential [7]. With respect to the regulation of gluconeogenesis the observation is of special interest that glucagon markedly lowered the levels of 2-oxoglutarate and glutamate in the perfused liver [14,15], and in the cytosolic and mitochondrial compartments of isolated liver cells [12]. Therefore the present study examined whether glucagon treatment of the intact rat might lead to changes in the hepatic metabolism of 2-oxoglutarate persisting after isolation of the mitochondria.

## 2. Materials and methods

### 2.1. *Animals*

Normal fed male Sprague-Dawley rats (E. Jautz, Kisslegg) 200–300 g, were anaesthetized by intraperitoneal injection of 0.1 ml/kg nembutal® (Abbott, Ingelheim). They were allowed to sleep for 20–30 min prior to a tail vein injection of 20 μg glucagon in 0.1 ml solvent [8]. Controls received the solvent only.

### 2.2. *Chemicals*

Glucagon was kindly donated by Hoechst, Frankfurt-Hoechst. Enzymes and coenzymes were purchased from Boehringer, Mannheim. Sodium 2-oxo[1-$^{14}$C]glutarate was obtained from New England Nuclear, Dreieichenhain. Sucrose p.a., phenazine methosulfate and phenethylamine were products of Roth, Karlsruhe. Ethyleneglycol bis (β-aminoethylether)-*N,N'*-tetraacetic acid and morpholinopropane sulfonic acid came from Serva, Heidelberg. (*N*-Tris (hydroxymethyl) methyl 2-aminoethane sulfonic acid, rotenone and cysteinesulfinate were bought from Sigma, Munich. Lubrol WX was a gift of ICI, Frankfurt. Bovine serum albumin was supplied by Behringwerke, Marburg. The source of 2,6-dichlorophenolindophenol and all other reagents was Merck, Darmstadt.

### 2.3. *Methods*

Mitochondria from livers removed 20 min after glucagon injection were prepared as in [6]. Mitochondria from heart muscle were prepared as in [16]. Hepatic metabolite contents were determined in perchloric acid extracts of freeze-clamped liver samples prepared and analyzed by the methods in [12,17].

Succinate dehydrogenase (EC 1.3.99.1) was assayed at 16°C spectrophotometrically as in [18] using about 150 ng protein/ml reaction mixture. The activity of glutamate dehydrogenase (EC 1.4.1.3) was measured at 25°C by the method in [19] after appropriate dilution of the mitochondrial suspension with 20 mM potassium phosphate buffer, pH 7.0 containing 1% Lubrol. The same buffer mixture fortified with 5% (v/v) normal rat serum was used for extrac-

tion of 2-oxoglutarate dehydrogenase (EC 1.2.4.2) just prior to the assay with the system in [20] except that pyruvate was replaced by 2-oxoglutarate.

Oxygen consumption was measured polarographically at 28°C using a Clark-type electrode under the conditions in [8] except that succinate was 2 mM and rotenone was omitted if not stated otherwise. The respiratory control ratios ranged from 4–10 and 3–5 for liver and heart mitochondria, respectively. Mitochondria were stored at 30–40 mg protein/ml isolation medium, the protein being determined by the biuret method [21] employing KCN to correct for turbidity [22].

Tabulated data are given as the means ± SEM for the numbers of different mitochondrial preparations in parentheses. Statistical significance $P$ of the mean values was calculated according to Student's $t$-test and is indicated in the tables for values below 0.05.

## 3. Results and discussion

In agreement with [8–10] we found [11] that glucagon treatment of Wistar rats increases the ADP-stimulated respiration of the subsequently isolated liver mitochondria in the presence of a variety of substrates. In the present study using Sprague-Dawley rats state 3 respiration with succinate as the substrate was likewise enhanced, the stimulatory effect lasting at least for 60 min (table 1). In the presence of 2-oxoglutarate state 3 respiration was increased from 65.5 ± 3.1 to 79.1 ± 3.3 nmol O/min/mg protein ($n = 29$; $P < 0.0025$) 20 min after glucagon injection, whereas no change in state 4 respiration was detectable (data not shown). In contrast to liver, heart muscle mitochondria obtained from the same animals failed to display an increased rate of respiration after glucagon administration (table 2).

In order to get information on the stability of the glucagon effect we measured the oxygen consumption of liver mitochondria kept on ice for various times. The results summarized in table 3 indicate that the property of increased state 3 respiration is retained up to 17 h.

So far no information is available concerning the question whether glucagon administered to the intact rat acts directly on liver mitochondria or by involvement of extrahepatic factor(s). Therefore, we have performed liver perfusion experiments which revealed that undiminished stimulation of state 3 respiration

Table 1
Increased respiration of liver mitochondria as a function of time after glucagon injection

| Time (min) | State 3 respiration (nmol O/min/mg protein) Control | Glucagon | $P$ |
|---|---|---|---|
| 20 | 137.4 ± 5.4 (35) | 178.2 ± 5.7 (35) | <0.0005 |
| 60 | 135.7 ± 4.3 (6) | 180.7 ± 11.4 (6) | <0.0025 |
| 120 | 152.5 ± 10.5 (5) | 159.4 ± 10.2 (5) | |

Rats were injected with glucagon as described in section 2 and livers were removed at the times indicated. Anaesthesia was maintained by injecting 50% first pentobarbital dose every 50 min. Oxygen consumption was measured at 29°C in the presence of 2 mM succinate. State 4 respiration after 20 min was increased by glucagon from 31.0 ± 1.7 to 35.5 ± 1.8 nmol O/min/mg protein ($n = 32$; $P < 0.05$)

Table 2
Effect of glucagon treatment of intact rats on ADP-stimulated respiration of mitochondria isolated from liver and heart muscle

| Organ | State 3 respiration (nmol 0/min/mg protein) | | P |
|---|---|---|---|
| | Control | Glucagon | |
| Liver | 120.1 ± 7.7 | 175.8 ± 8.3 | <0.0005 |
| Heart muscle | 291.9 ± 25.4 | 312.0 ± 23.6 | |

The organs were removed 20 min after the injection of glucagon (see section 2). Respiration at 2 mM succinate was measured in the presence of 1 μM rotenone. Mean values ± SEM of 8 control rats and 8 glucagon-treated rats are given

Table 3
Stability of the effect of glucagon treatment on the ADP-stimulated respiration of rat liver mitochondria

| Time (h) | State 3 respiration (nmol 0/ming/mg protein) | | P |
|---|---|---|---|
| | Control | Glucagon | |
| 0 | 117.1 ± 7.1 (8) | 158.2 ± 6.3 (8) | <0.0005 |
| 2 | 108.6 ± 6.1 (8) | 143.3 ± 7.5 (8) | < 0.0025 |
| 4 | 97.9 ± 5.2 (8) | 143.8 ± 5.1 (8) | < 0.0005 |
| 8 | 78.9 ± 4.3 (8) | 112.7 ± 10.4 (8) | <0.005 |
| 17 | 66.2 ± 4.4 (8) | 90.1 ± 6.9 (8) | <0.01 |
| 22 | 47.2 ± 7.1 (6) | 60.6 ± 3.2 (6) | |

Mitochondria from the livers removed 20 min after glucagon injection were kept on ice for the times indicated prior to the measurement of oxygen consumption with succinate (2 mM) as the substrate

Table 4
ADP-stimulated respiration of mitochondria isolated from livers perfused with glucagon or 3′:5′-cyclic AMP

| Addition to perfusate | Final concentration | State 3 respiration (nmol O/min/mg protein) | P |
|---|---|---|---|
| Solvent | – | 98.7 ± 6.5 (7) | |
| Glucagon | 2 μg/ml | 131.5 ± 4.6 (7) | <0.0025 |
| 5′-AMP | 0.2 mM | 106.4 ± 8.1 (8) | |
| 3′:5′-cAMP | 0.2 mM | 132.3 ± 5.9 (8) | <0.0125 |

Rat livers were perfused at 34°C in a standard recirculating system with $Ca^{2+}$-free Krebs-Henseleit bicarbonate buffer maintained at pH 7.4 by gassing with 95% $O_2$–5% $CO_2$. The effectors were added as a single dose (0.2–1.0 ml) to the perfusate (100 ml) after 5 min equilibration to give the final concentrations indicated and perfusion was continued for 25 min. Respiratory substrate was 2 mM succinate

by glucagon and also by 3′:5′-cyclic AMP (table 4) occurs in vitro.

As to the physiological meaning of the increase of the respiration rate, it is of interest that it was accompanied by marked changes in hepatic metabolite contents as summarized in table 5. In addition to these results, which are qualitatively similar to [23], we found that 60 min after glucagon injection the amount of glutamate was still decreased ($P < 0.0125$) while the levels of 2-oxoglutarate, malate and citrate were no longer significantly different from the controls. In order to see whether the increase in hepatic malate could be related to the mitochondrial action(s) of glucagon, the rate of malate production from 2-oxoglutarate and succinate of mitochondria isolated from control and glucagon-treated rats was determined. From the data of table 6 it appears conceivable that the increase of the malate levels and citrate occurring in the liver after glucagon (table 5) resulted from an enhanced flux of 2-oxoglutarate through the citric acid cycle.

Table 5
Effect of glucagon treatment of rats on hepatic metabolite contents

| Metabolite | Amount (nmol/g fresh wt) | | |
|---|---|---|---|
| | Control | Glucagon | P |
| Malate | 319 ± 22 | 706 ± 108 | <0.0025 |
| Glutamate | 2087 ± 132 | 1539 ± 162 | <0.0125 |
| 2-Oxoglutarate | 145 ± 10 | 110 ± 10 | <0.01 |
| Citrate | 362 ± 31 | 522 ± 54 | <0.01 |

Livers were freeze clamped 20 min after glucagon application and extracted with $HClO_4$ as described in section 2. Means ± SEM for 14 different rats in each group are given

Table 6
Effect of glucagon treatment of rats on malate production from 2-oxoglutarate and succinate by isolated liver mitochondria

| Substrate | [mM] | Malate production (nmol/min/mg protein) | | |
|---|---|---|---|---|
| | | Control | Glucagon | *P* |
| 2-Oxoglutarate | 18 | 1.74 ± 0.17 (14) | 2.16 ± 0.18 (14) | <0.05 |
| Succinate | 9 | 6.44 ± 0.29 (13) | 7.62 ± 0.48 (13) | <0.025 |

The rate of malate production at 25°C was determined in a reaction mixture containing 0.2 ml medium [8] for respiration measurements, 0.05 ml 0.2 M glucose, 0.01 ml 0.6 M 2-oxoglutarate or 0.3 M succinate, 0.01 ml 0.1 M ADP and 0.01 ml hexokinase, corresponding to 14 U. Incubation began with addition of 0.05 ml mitochondria, corresponding to about 2 mg protein and was terminated with 0.05 ml 70% (w/v) of $HClO_4$. Malate production was linear with time up to 10 min

To assess the possible site(s) of glucagon action we measured the activities of 2-oxoglutarate dehydrogenase, glutamate dehydrogenase and succinate dehydrogenase in crude mitochondrial extracts. No effect of hormone treatment was discernible with regard to the activities of glutamate dehydrogenase and 2-oxoglutarate dehydrogenase which amounted to 4961 ± 274 ($n$ = 18) and 35.7 ± 2.2 ($n$ = 29) mU/mg protein, respectively. The respective values of the controls were 4828 ± 298 ($n$ = 18) and 35.8 ± 1.7 ($n$ = 31) mU/mg protein. In the case of succinate dehydrogenase, however, the enzyme activity was found to be significantly ($P$ <0.0005) increased in liver mitochondria from animals that had received glucagon. At 16°C the rate of succinate oxidation shown by control mitochondria was 81.3 ± 6.9 ($n$ = 27) nmol/min/mg protein, a value 50% lower than that of mitochondria from glucagon-injected rats, which was determined to be 126.5 ± 9.6 ($n$ = 27) nmol/min/mg protein. This stimulatory effect was neither abolished by freezing and thawing of the mitochondrial suspension nor by treatment with 0.2% Lubrol. Thus the hormone effect was attributable rather to a persistent change in the catalytical function of succinate dehydrogenase than to an improved permeability of the inner membrane for the assay components. In view of the fact that the specific activity of succinate dehydrogenase was about 4-fold

Table 7
Effect of glucagon treatment of intact rats on the activity of 2-oxoglutarate dehydrogenase activity in intact mitochondria and mitochondrial extracts at 25°C

| Preparation | 2-Oxoglutarate decarboxylation (nmol/min/mg protein) | |
|---|---|---|
| | Control | Glucagon |
| Intact mitochondria | 12.4 ± 0.9 | 20.3 ± 0.9[a] |
| Lysed mitochondria | 29.7 ± 3.3 | 25.6 ± 2.7 |

[a] $P$ <0.0005

Mean values ± SEM for 10 different preparations in each group are given. Further details are described in section 2. The rate of 2-oxo-[1-$^{14}$C]glutarate decarboxylation by intact mitochondria was determined as follows: to a prewarmed plastic vial containing 0.2 ml medium [8] for respiration measurements and 0.01 ml 0.1 M ADP, at 25°C, 0.02 ml mitochondrial suspension, corresponding to about 250 μg protein, was added and mixed prior to the addition of 0.02 ml 80 mM 2-oxo-[1-$^{14}$C]glutarate corresponding to 0.2 μCi. Immediately thereafter the vials were closed with a rubber cap equipped with a center well containing 0.1 ml phenethylamine. Incubation at 25°C was terminated at 2 min intervals by injection of 0.2 ml 2 N $H_2SO_4$. The velocity of $^{14}CO_2$ formation in the first 2–4 min was somewhat lower than the linear rate between 4–10 min

that of extractable 2-oxoglutarate dehydrogenase it appears difficult to appraise the physiological relevance of the stimulation of succinate dehydrogenase following glucagon administration. Nevertheless, we could show that malate formation from succinate (like that from 2-oxoglutarate) was stimulated by glucagon treatment (table 6). The rate-limiting role of 2-oxoglutarate dehydrogenase became even more apparent, when the rate of 2-oxoglutarate decarboxylation by mitochondrial extracts was compared with that realized in the intact organelle. The data of table 7 indicate that under control conditions the flux through 2-oxoglutarate dehydrogenase corresponds to only 40% total extractable enzyme activity. This restraint was found to be remarkably relieved (table 7) in the mitochondria from glucagon-treated animals. (Identical results were obtained with mitochondria preincubated for substrate depletion for 5 min with ADP prior to the addition of 2-oxo-[1-$^{14}$C]glutarate, thereby ruling out the possibility of isotope dilution by endogenous substrate.) Compared with the normal and hormone-stimulated rates of state 3 oxygen consumption of 65.6 ± 2.6 and 76.0 ± 2.5 nmol 0/min/mg protein ($n$ = 10; $P$ <0.005), respectively, one might conclude from the flux measurements performed with these mitochondria (table 7) that glucagon administration caused 2-oxoglutarate to become the preferred substrate of mitochondrial respiration.

**Acknowledgements**

The expert technical assistance of Miss H. Wenzel and Miss M. Engel is greatfully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft, Bad Godesberg, FRG.

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