ISOLATED HEPATOCYTES AS A MODEL FOR THE STUDY OF STABLE GLUCAGON EFFECTS ON MITOCHONDRIAL RESPIRATORY FUNCTIONS

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1. Introduction

Suzuki was the first to report that the intravenous injection of glucagon to rats increased the oxygen consumption of liver slices prepared 15 min after the injection [1]. Further work by Yamazaki [2] and others [3–8] revealed that mitochondria isolated from glucagon-treated rats show an increase of ~40% in state 3 respiration with succinate as the substrate. Efforts to elucidate the mechanism of this hormone effect are hampered by the fact that individual rats vary considerably in their response to the hormonal treatment and in their mitochondrial metabolic activity. Further, the administration of a hormone to the intact rat does not allow discrimination between direct and indirect effects on the hepatic parenchymal cell. Therefore, it was desirable to establish, whether isolated liver cells can serve as a model for the investigation of glucagon action(s) on mitochondria persisting after isolation of the organelle. This study provides evidence that glucagon influences respiration, succinate dehydrogenase activity and Ca^{2+} metabolism of mitochondria prepared from isolated liver cells after incubation with the hormone. Moreover, we could show that in the hepatocyte the effect of puromycin on the action of glucagon is different from that observed in the intact animal [8,9].

2. Materials and methods

2.1. *Liver cell preparation*

Male Sprague-Dawley rats (A. Ivanovas, Kisslegg) 180–220 g after starvation for 48 h, were anesthetized by intraperitoneal injection of 1 ml/kg nembutal (Abbott, Ingelheim) or thiogenal (Merck, Darmstadt) and liver cells were prepared in principle according to [10] as described [11].

2.2. *Chemicals*

Digitonin (Merck, Darmstadt) was recrystallized from hot ethanol. Puromycin was purchased from Sigma (Munich). Dibutyryl cyclic AMP was produced by Boehringer (Mannheim). L-[4,5-^{3}H]leucine and $^{32}P_i$ came from the Radiochemical Centre (Amersham). The NCS tissue solubilizer was supplied by Searle (Amersham). The other chemicals used in this study were those detailed in [7].

2.3. *Methods*

Mitochondria from liver cells incubated with 8 mM lactate as in [12] were prepared by the method in [13], except that a glass–teflon homogenizer was used and that the 500 × *g* pellet was re-extracted once to improve the mitochondrial yield. Mitochondria were stored on ice at ~30 mg protein/ml. In some experiments mitochondria were subjected to the digitonin treatment in [14] to diminish lysosomal contamination. The methods for the measurement of oxygen consumption, succinate dehydrogenase (EC 1.3.99.1) activity and protein were those in [7]. The effect of Ca^{2+} on succinate respiration was determined using the incubation medium in [9]. The preparation of inner and outer mitochondrial membrane fractions was accomplished by the method in [15]. Electrophoresis on polyacrylamide slab-gels was performed according to [16]. The gels were stained and destained by the method in [17] prior to autoradiography on

Kodak X-omat R film. Tabulated data are given as the means ± SEM for the numbers of different liver cell preparations in parentheses. Statistical significance P was calculated according to Student's t-test for paired data.

3. Results and discussion

Mitochondria isolated from hepatocytes after incubation for 10 min with glucagon, display an enhanced rate of state 3 respiration (table 1), comparable to that found with liver mitochondria from the intact rat [2–8] or the perfused organ [7,8], while state 4 respiration remained unchanged (data not shown). A similar effect (table 1) was seen after further purification of the mitochondrial fraction by digitonin treatment which, in agreement with [14] removed ~90% of contaminating enzyme activities such as acid phosphatase (EC 3.1.3.2) and glucose-6-phosphatase (EC 3.1.3.9) and ~50% of 5′-nucleotidase (EC 3.1.3.5) and catalase (EC 1.11.1.6) but no adenylate kinase (EC 2.7.4.3) and glutamate dehydrogenase (EC 1.4.1.3) activity (D. G. Brocks, E.A.S., O.H.W., unpublished).

In [7] succinate dehydrogenase activity was found to be increased after glucagon injection into intact rats. The data of table 1 indicate that a similar effect of the hormone is demonstrable also in vitro with the isolated hepatocytes. Moreover, dibutyryl cyclic AMP could be shown to produce the same effect as glucagon with respect to both the stimulation of state 3 respiration and succinate dehydrogenase activation (table 1).

Recently, Hughes and Barritt [8] and Prpić et al. [9] working with the intact rat have reported that the

Table 1
Effect of incubation of isolated liver cells with glucagon or dibutyryl cyclic AMP on state 3 respiration and succinate dehydrogenase activity of normal and digitonin-treated mitochondria

Condition	State 3 respiration (nmol O/min/mg prot.)	Increase P (%)	Succinate dehydrogenase (mU/mg prot.)	Increase P (%)
I. Untreated mitochondria				
Control	76.9 ± 4.8 (11)		69.8 ± 8.9 (13)	
Glucagon	114.4 ± 4.7 (11)	49 < 0.0005	114.0 ± 11.5 (13)	63 < 0.0005
Control	86.8 ± 4.1 (5)		46.4 ± 5.3 (3)	
Dibutyryl cyclic AMP (0.1 mM)	120.6 ± 8.8 (5)	39 < 0.0025	99.0 ± 3.1 (3)	114 < 0.0125
II. Digitonin-treated[a] mitochondria				
Control	57.0 ± 5.8 (5)		64.6 ± 6.1 (7)	
Glucagon	90.4 ± 6.4 (5)	58 < 0.01	96.9 ± 10.2 (7)	50 < 0.0005

[a] Digitonin treatment was performed at 2–4°C as follows: mitochondria were shaken (Eppendorf shaker model 3300) for 2 min at 30 μg digitonin/mg protein prior to a 5-fold dilution with the isolation medium in [13] and centrifugation for 5 min at 12 300 rev./min in the Sorvall SS34 rotor. The pellet was resuspended in the same volume of medium and centrifuged as before. This washing step was repeated once. The final pellet was homogenized by hand in a glass–teflon potter with isolation medium to yield 20–25 mg protein/ml. For further experimental details see section 2

effect of glucagon on mitochondrial Ca^{2+} metabolism was abolished when cycloheximide or puromycin was administered to the animals together with glucagon. This observation prompted us to examine the question as to whether the action of glucagon on mitochondrial functions depends on the unimpaired rate of protein synthesis. In our liver cells 472 μg puromycin/ml inhibited leucine uptake into CCl_3COOH-stable products by ~90%, in agreement with [18] (data not shown). At this saturating concentration of puromycin, leucine incorporation ceased after 15–30 min of incubation (fig.1). It was therefore of interest to compare the effects of glucagon on mitochondrial Ca^{2+} metabolism, state 3 respiration and succinate dehydrogenase activity when the hormone was added after preincubation of the hepatocytes for 30 min in the absence or presence of puromycin. In agreement with the results in [9] obtained with liver mitochondria from glucagon-treated rats we find that the mitochondria from hepatocytes incubated with glucagon are rather refractory against stimulation of respiration by Ca^{2+}. In contrast with the situation in the intact rat, however, puromycin failed to diminish this effect of glucagon (table 2). Moreover, puromycin was not able to abolish the action of glucagon on both the state 3 respiration and succinate dehydrogenase activity, although a significant decrease of the hormone effect was caused (table 3). It should be mentioned that puromycin did not affect the mitochondrial functions of liver cells incubated in the absence of glucagon (data not shown). Thus it appears that glucagon still produces significant changes in mitochondrial activities even when protein synthesis is arrested.

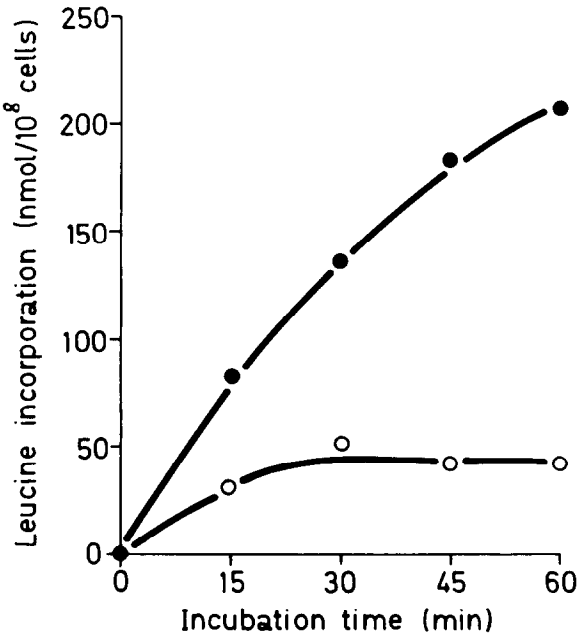

Fig.1. Incorporation of leucine into hepatocytes as a function of incubation time. Liver cells (53 mg wet wt) were incubated in a mixture containing 0.48 mM of L-[4,5-^{3}H]leucine for 30 min as in section 2 in the absence (•) or presence (○) of 472 μg puromycin/ml before 5 μl of glucagon was added to yield 5 μg/ml. At the times indicated aliquots (0.1 ml each) were removed from the incubation mixture and centrifuged (Eppendorf centrifuge model 3200) through silicone oil into 12% (w/v) $HClO_4$ as in [22]. The protein pellet was washed 3 times with 1 ml 6% (w/v) $HClO_4$ and dissolved in 0.5 ml NCS tissue solubilizer. Radioactivity was measured in a Packard scintillation counter. Very similar results were obtained in 3 additional experiments.

As to the mechanism by which glucagon or cyclic AMP exert their action(s) on mitochondrial functions it is tempting to speculate that mitochondrial protein phosphorylation might be involved. This view is supported by the report of Zahlten et al. [19] that glucagon administration to intact rats increases the ^{32}P content of liver mitochondrial membranes. More recently Garrison [20] and Avruch et al. [21] have shown that the pattern of cytosolic phosphoproteins as revealed by autoradiography of polyacrylamide gels was changed when isolated liver cells had been incubated in the presence of glucagon. Our finding that in a homogeneous population of cells glucagon induces stable changes in mitochondrial functions raised the question of what changes in the pattern of mitochondrial phosphoproteins might occur after glucagon. For this purpose liver cells were preincubated for 30 min in the presence of $^{32}P_i$ prior to the incubation for 10 min, both in the presence and absence of glucagon. In agreement with [20,21] glucagon caused the appearance of additional ^{32}P-labelled protein bands in the autoradiographs when the 500 × *g* supernatant of the cell homogenate was analyzed (fig.2). However, no difference between hormone-stimulated and control cells was detectable with respect to the distribution of ^{32}P between the proteins of mitochondria or the inner and outer membranes thereof (fig.2). Thus it appears possible that the mitochondrial effects of glucagon and

Table 2
Effect of Ca^{2+} on succinate respiration of mitochondria isolated from rat liver cells

Condition		Increase[e] in oxygen consumption due to Ca^{2+} (nmol O/min/mg prot.)		
		44 μM	67 μM	89 μM
I	Control	Δ+9.6 ± 1.5 (6)	Δ+29.3 ± 7.1 (7)	Δ+51.2 ± 7.1 (5)
II	Glucagon	0[a] (6)	Δ+9.0 ± 3.6[a] (7)	Δ+18.3 ± 1.5[d] (5)
III	Glucagon + puromycin	0[a] (6)	Δ+7.2 ± 1.3[c] (7)	Δ+24.4 ± 7.1[d] (5)

[a] $P < 0.0025$; [b] $P < 0.005$; [c] $P < 0.01$; [d] $P < 0.025$; [e] Prior to the addition of $CaCl_2$ the respiration rate for condition I, II and III was 34.5 ± 2.4, 37.4 ± 2.6 and 37.3 ± 2.4 nmol O/min/mg prot., respectively (n = 11)

Hepatocytes were incubated for 30 min in the absence or presence of puromycin (472 μg/ml) as stated in section 2 before glucagon (5 μg/ml) or solvent was added and incubation was continued for 10 min prior to the isolation of the mitochondria according to [13]. Oxygen consumption at 2.2 mM of D,L-succinate was measured polarographically under the conditions in [9]. Three single doses of $CaCl_2$ corresponding to 80, 40 and 40 nmol, respectively, were added sequentially to the respiration chamber to give the final concentrations indicated

cyclic AMP are mediated indirectly by the modification of extramitochondrial components rather than by direct phosphorylation of mitochondrial protein(s). This view is consistent with the observation that glucagon, 3′,5′-cyclic AMP or its dibutyryl derivative were ineffectual on succinate respiration of liver mitochondria isolated from fed (n = 6) or 48 h fasted (n = 4) rats, when added directly to the respiration chamber to give final concentrations of 10 μg/ml and 0.5 mM, respectively (E.A.S., O.H.W., unpublished). Further work employing subcellular fractions from hormone stimulated and control cells could help to clarify this functional, as yet hypothetical, interrelationship.

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Table 3
Effect of puromycin on the action of glucagon on state 3 respiration and succinate dehydrogenase activation

Condition		State 3 respiration (nmol O/min/mg prot.)		Succinate dehydrogenase (mU/mg prot.)	
I	Control	103.8 ± 8.1		60.8 ± 4.9	
II	Glucagon	136.4 ± 7.6	$P < 0.005$	101.9 ± 7.8	$P < 0.0005$
III	Glucagon + puromycin	119.1 ± 7.6		85.0 ± 7.2	
		III versus I	$P < 0.005$	III versus I	$P < 0.0005$
		III versus II	$P < 0.0025$	III versus II	$P < 0.0025$

The hepatocytes were incubated as given in the legend to table 2. For further experimental details see section 2. Results of 7 different cell preparations are summarized

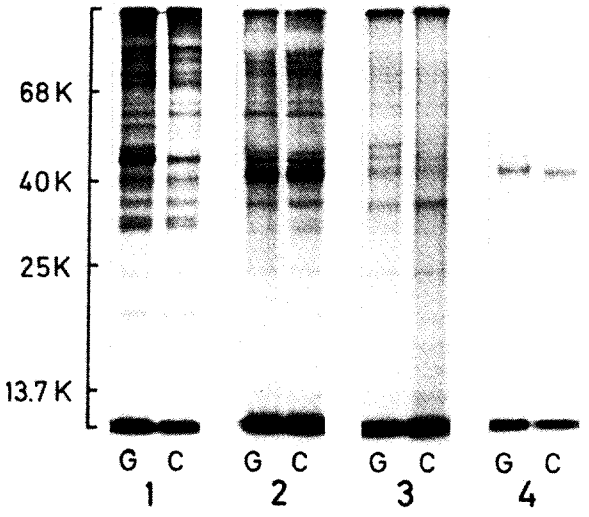

Fig.2. Effect of glucagon on the pattern of [^{32}P]phosphoproteins in liver cell fractions. Liver cells were incubated as stated in section 2 except that the P_i concentration of the medium was reduced to 10% of the normal value and that ~0.3 mCi $^{32}P_i$/100 mg wet cells was added. After preincubation for 30 min, glucagon (5 μg/ml) or solvent only was added to the incubation mixture which was further incubated for 10 min before it was diluted (~1:3) with ice-cold 0.31 M sucrose containing 1 mM EGTA (pH 7.4) and processed according to [13]. Prior to electrophoresis [16] lipids were extracted from the mitochondria and the mitochondrial fractions [15] as in [19]. Autoradiographs of: (1) 500 × *g* supernatant, (2) mitochondria, (3) outer mitochondrial membrane, (4) inner mitochondrial membrane, from liver cells incubated in the presence (G) or absence (C) of glucagon. The positions of marker proteins such as ribonuclease (13.7 k), chymotrypsinogen (25 k), aldolase (40 k) and bovine serum albumin (68 k) on the Coomassie blue-stained gel and their molecular weights are indicated. It was ascertained that $^{32}P_i$ added in the mixture of marker proteins was completely removed under the conditions employed. For further experimental details see section 2. The results are typical of 5 experiments.

References

[1] Suzuki, Y. (1964) Chem. Abstr. 61, 12280 h.
[2] Yamazaki, R. K. (1975) J. Biol. Chem. 250, 7924–7930.
[3] Titheradge, M. A. and Coore, H. G. (1976) FEBS Lett. 71, 73–78.
[4] Wakat, D. K. and Haynes, R. C., jr (1977) Arch. Biochem. Biophys. 184, 561–571.
[5] Bryła, J., Harris, E. J. and Plumb, J. A. (1977) FEBS Lett. 80, 443–448.
[6] Halestrap, A. P. (1978) Biochem. J. 172, 389–398.
[7] Siess, E. A. and Wieland, O. H. (1978) FEBS Lett. 93, 301–306.
[8] Hughes, B. P. and Barritt, G. J. (1978) Biochem. J. 176, 295–304.
[9] Prpić, V., Spencer, T. L. and Bygrave, F. L. (1978) Biochem. J. 176, 705–714.
[10] Berry, M. N. and Friend, D. S. (1969) J. Cell Biol. 43, 506–520.
[11] Siess, E. A., Brocks, D. G. and Wieland, O. H. (1976) FEBS Lett. 69, 265–271.
[12] Siess, E. A., Brocks, D. G., Lattke, H. K. and Wieland, O. H. (1977) Biochem. J. 166, 225–235.
[13] Garrison, J. C. and Haynes, R. C., jr (1975) J. Biol. Chem. 250, 2769–2777.
[14] Loewenstein, J., Scholte, H. R. and Wit-Peeters, E. M. (1970) Biochim. Biophys. Acta 223, 432–436.
[15] Schnaitman, C. and Greenawalt, J. W. (1968) J. Cell Biol. 38, 158–175.
[16] Laemmli, U. K. (1970) Nature 227, 680–685.
[17] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[18] Schreiber, G. and Schreiber, M. (1972) J. Biol. Chem. 247, 6340–6346.
[19] Zahlten, R. N., Hochberg, A. A., Stratman, F. W. and Lardy, H. A. (1972) Proc. Natl. Acad. Sci. USA 69, 800–804.
[20] Garrison, J. C. (1978) J. Biol. Chem. 253, 7091–7100.
[21] Avruch, J., Witters, L. A., Alexander, M. C. and Bush, M. A. (1978) J. Biol. Chem. 253, 4754–4761.
[22] Siess, E. A. and Wieland, O. H. (1976) Biochem. J. 156, 91–102.