GLUCAGON TREATMENT STIMULATES THE OXIDATION OF DUROHYDROQUINONE BY RAT LIVER MITOCHONDRIA

Michael A. TITHERADGE[+] and Robert C. HAYNES, jr*
Department of Pharmacology, University of Virginia School of Medicine, Charlottesville, VA 22908, USA

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1. Introduction

Recent work has indicated that brief treatment of intact rats and isolated hepatocytes with glucagon results in a stimulation of state 3 and uncoupled rates of respiration in subsequently isolated liver mitochondria oxidizing either NAD-linked substrates or succinate [1–6]. Yamazaki [1] found no stimulation of malate dehydrogenase and concluded that the effect of glucagon was mediated either through a stimulation of the respiratory chain itself or through a stimulation of substrate transport rather than through an effect on the respective dehydrogenase enzymes. In addition it has been found that when ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) are used as substrates no stimulation by glucagon is evident [1–3]. The combination of ascorbate and TMPD requires neither dehydrogenase activity nor membrane transport because the artificial electron donor TMPD is oxidized at the outer surface of the inner mitochondria membrane directly by cytochrome *c*. The absence of a stimulatory effect on oxidation of ascorbate-TMPD left open the possibilities that the hormone acts on substrate transport or at a site before complex IV in the electron transport chain. Of these two possibilities, transport has been eliminated as a unique locus of glucagon action by the finding that the effects of the hormone were persistent in submitochondrial particles in which the membrane is inverted and the dehydrogenase enzymes have direct access to substrates [5]. Thus by a process of elimination it has become increasingly likely that a major site of action of glucagon is the electron transport chain itself. Recently, however, Siess et al. [4,6] have demonstrated that glucagon treatment results in a stimulation of the activity of the succinate dehydrogenase complex in intact mitochondria. This might explain the enhanced rate of respiration with succinate as substrate without any further requirement for increased respiratory chain activity. The aim of this work was to confirm the stimulation of succinate dehydrogenase activity and to determine whether this is indeed the sole site of action of glucagon in the segments of the respiratory chain utilized when succinate is oxidized. We find that there are at least two sites of stimulation in the chain.

2. Experimental

Fed, male Wistar rats (250–350 g) were anesthetized by i.p. injection of sodium pentobarbital (60 mg/kg) and then treated with glucagon (100 μg s.c.) or vehicle (0.9% saline–0.05% bovine serum albumin). After 20 min the livers were rapidly removed, chilled and homogenized in 0.3 M sucrose. The homogenate was centrifuged at 700 × *g* for 10 min, and the supernatant centrifuged at 7000 × *g* for 10 min to obtain the mitochondrial pellet. The mitochondria were then washed 2 times before resuspending in 0.3 M sucrose to ~30 mg protein/ml final conc.

[+] Present address: Department of Biochemistry, School of Biological Sciences, University of Sussex, Falmer, Brighton BN19QG, Sussex, England

* To whom correspondence should be addressed

Enzyme measurements were made at 20°C using mitochondria solubilized with 0.3 mg lubrol/mg mitochondrial protein. Three freeze–thaw cycles were insufficient to allow maximum expression of enzyme activities under our conditions. Succinate dehydrogenase (EC 1.3.99.1) was assayed as in [7]. Glutamate dehydrogenase (EC 1.4.1.3) was measured as in [8] and malate dehydrogenase (EC 1.1.1.37) as in [9].

Mitochondrial respiration was measured polarographically at 26°C using a Clark-type oxygen electrode in the following medium: 125 mM KCl; 50 mM morpholinopropane sulfonic acid (pH 7.0); 1 mM ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; 5 mM K_2HPO_4; 0.2 mM $MgCl_2$. State 3 respiration was initiated by the addition of 0.3 μmol ADP and uncoupled respiration by the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to 0.25 μM final conc. Durohydroquinone was prepared by reducing duroquinone with sodium borohydride and was added in methanolic solution.

Protein was determined by the Lowry technique [10] using crystalline bovine serum albumin as standard.

Results are expressed as means ± estimates of the standard errors of the means. Analysis of data was performed using a 2-tailed Student's *t*-test.

Glucagon, phenazine methosulfate, 2,6-dichlorophenolindophenol, cysteine sulfinate, ADP, and duroquinone were from Sigma; thenoyl trifluoroacetone (TTFA) was obtained from Aldrich Chem. Co.

3. Results and discussion

Table 1 shows the effect of glucagon treatment on the activities of succinate dehydrogenase, malate dehydrogenase and glutamate dehydrogenase. There was a significant stimulation of succinate dehydrogenase activity but no effect of the hormone on either malate dehydrogenase or glutamate dehydrogenase activity in agreement with the data in [1,4,6]. The results imply that the stimulation of respiration with NAD-linked substrates is not mediated through alterations in the activity of the respective dehydrogenase enzymes, but is possibly the result of a stimulation of one or more sites in the respiratory chain. Further support for this concept is evident from our earlier finding that in submitochondrial particles prepared from glucagon-treated rats there is an enhanced rate of oxidation of exogenous NADH [5]. Figure 1 shows an abbreviated representation of the enzyme complexes of the electron transport system as proposed by Hatefi [11] who summarized data indicating that the rate of electron flux through complex II of the respiratory chain is limited by the activity of the succinate dehydrogenase enzyme

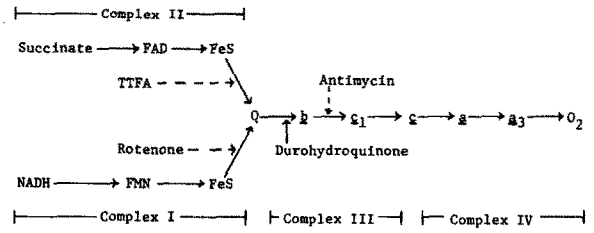

Fig.1. Sequence of electron carriers of the respiratory chain. This figure is modified from Hatefi [11] principally by not designating separate iron–sulfur centers. Broken arrows indicate the apparent sites of action of inhibitors. Solid arrows indicate paths of electron flow.

Table 1
Effect of glucagon treatment of rats on succinate dehydrogenase, glutamate dehydrogenase and malate dehydrogenase activity

Treatment	Succinate dehydrogenase (U/mg protein)	Malate dehydrogenase (U/mg protein)	Glutamate dehydrogenase (U/mg protein)
Control	0.19 ± 0.01	2.9 ± 0.4	4.5 ± 0.2
Glucagon	0.27 ± 0.01[a]	2.7 ± 0.4	4.7 ± 0.2

[a] $p < 0.001$

Enzyme assays were carried out as described in section 2 with solubilized mitochondrial fractions. Results shown are means ± SEM for 8 different preparations

Table 2
Effect of glucagon treatment on oxidative phosphorylation in isolated rat liver mitochondria

Substrate	Treatment	State 3	State 4	Uncoupled	RCR
Durohydroquinone	Control	152 ± 8	51 ± 4	214 ± 8	3.3 ± 0.2
	Glucagon	223 ± 11[a]	46 ± 1	342 ± 14[a]	4.9 ± 0.3
Succinate	Control	138 ± 4	29 ± 1	153 ± 5	4.9 ± 0.2
	Glucagon	203 ± 6[a]	34 ± 2	211 ± 9[a]	5.8 ± 0.2
Malate-L-glutamate	Control	123 ± 5	14 ± 1	131 ± 6	8.9 ± 0.6
	Glucagon	165 ± 8[a]	16 ± 1	164 ± 8[a]	10.5 ± 1.6

[a] $p < 0.001$

Oxygen uptake (ng atoms O .min^{-1} .mg protein^{-1}) was measured polarographically as described in section 2 using 0.75 mg/ml mitochondrial protein. Substrates were added to the final concentrations as follows: durohydroquinone, 0.75 mM; succinate, 5 mM; malate, 0.5 mM; glutamate, 5 mM. Results are means ± SEM for 8 different mitochondrial preparations

complex. It was therefore important to determine whether there is a further site of stimulation in complex III or complex IV of the respiratory chain which could complement the effect on succinate dehydrogenase and could also be responsible for the observed effects on substrates feeding into complex I.

To examine this possibility we employed the artificial electron and proton donor, durohydroquinone, to feed into the respiratory chain at complex III [12]. The results are shown in table 2. It can be seen that glucagon treatment resulted in a highly significant stimulation of both state 3 and uncoupled rates of respiration with no apparent effect on the state 4 rate. The uncoupled rate of respiration with durohydroquinone was significantly higher than that of the state 3 rate ($p < 0.001$) although the hormone effect was still persistent. The state 3 rates of respiration and percentage hormone effects with durohydroquinone and succinate as the substrates were not significantly different ($p < 0.4$) which may indicate that the rate-limiting step under these conditions is the same and not due to the enhanced succinate dehydrogenase activity. The rates of respiration with glutamate–malate are included to emphasize that, despite the lack of hormonal effect on malate dehydrogenase and glutamate dehydrogenase, the oxidation of these substrates was significantly enhanced by glucagon.

To make certain that the observed effects on durohydroquinone oxidation were not due to contributions of electrons from either complex I or II, rotenone was added to block complex I and TTFA to inhibit complex II. The results of such an experiment are shown in fig.2. Succinate was added as the original substrate in the presence of rotenone and then FCCP was added to obtain an uncoupled rate. The addition of 1 mM TTFA completely blocked succinate oxidation; however oxygen consumption was restored by the addition of durohydroquinone. The oxidation of durohydroquinone was in turn inhibited by antimycin. The hormonal stimulation was apparent on the uncoupled rates of respiration when either succinate alone or durohydroquinone in the presence of TTFA was the substrate. An incidental finding was that TTFA, independent of its inhibitory action on complex II, also behaved as an uncoupler of oxidative phosphorylation with durohydroquinone as the substrate, the degree of uncoupling being dependent upon the concentration of TTFA up to 1 mM (data not shown). Taking the data that show an absence of hormone effect on the respiratory chain in complex IV [1–3] together with the findings reported here it appears that there is a second locus of glucagon action in the region of the respiratory chain designated complex III. This is in agreement with the crossover observed in the respiratory chain spectra [3]. The negative experiments [1–3] with ascorbate and TMPD must be considered with a caveat however. The rate of respiration with ascorbate-TMPD may in fact be limited by the concentration of TMPD and the velocity with which it reduces cytochrome *c*. Until this has been clarified it is necessary to keep alive

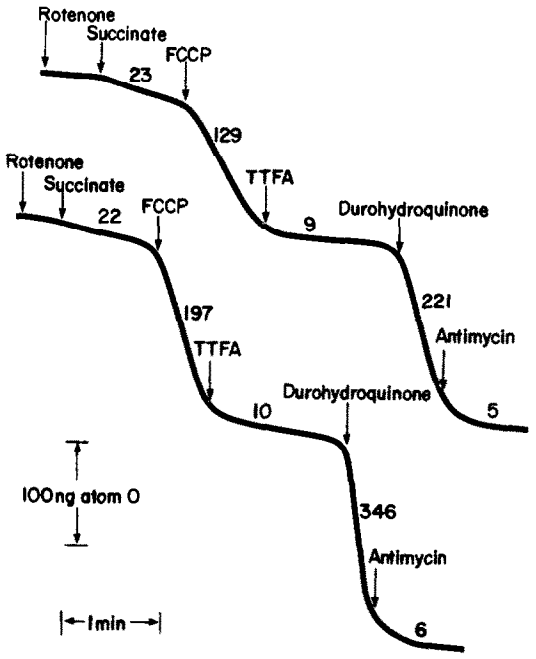

Fig.2. Polarographic recording of effect of inhibitors on durohydroquinone-dependent respiration by mitochondria isolated from control (upper curve) and glucagon-treated rats (lower curve). Mitochondrial oxygen uptake was measured as described in section 2. Mitochondria were present at 0.75 mg/ml, succinate at 5 mM and durohydroquinone at 0.75 mM. Rotenone was added to 0.7 μg/ml final conc., antimycin 0.25 μg/ml and TTFA 1 mM. Numbers beside the curves represent rates of oxygen uptake expressed as ng atoms O $.min^{-1}.mg\ protein^{-1}$.

the possibility that glucagon may stimulate a site in complex IV and thereby account for the findings reported here with durohydroquinone.

In conclusion, there are at least two sites in the respiratory chain concerned with succinate oxidation where glucagon can exert its influence:

1. Succinate dehydrogenase in complex II;
2. Complex III or less likely complex IV.

Whether there is also an effect at complex I has not been ascertained at this time, as the stimulation of oxidation of NAD-linked substrates may be the result of a stimulation of complex III or IV. The finding that the state 3 rate of respiration with durohydroquinone was significantly less than the uncoupled rate suggests that in complex III at least, the rate of respiration under state 3 conditions is not limited by the activity of the electron transport chain but by another factor such as the rate of adenine nucleotide translocation. There are several reports in the literature which indicate adenine nucleotide transport is rate limiting for oxidative phosphorylation [13–17], and we have recently shown that the rate of adenine nucleotide uptake by mitochondria is stimulated by glucagon [18]. This has also been shown [19] and appears to be the mechanism whereby glucagon stimulates the mitochondrial ATPase reaction [18]. This may explain why the rates of oxidation of durohydroquinone and succinate are identical under state 3 conditions but not under conditions where adenine nucleotide transport is obviated by the presence of uncoupler.

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