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ADENINE NUCLEOTIDE CONTENT OF LIVER MITOCHONDRIA INCREASES AFTER GLUCAGON TREATMENT OF RATS OR ISOLATED HEPATOCYTES

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Summary. Glucagon injected into rats via tail vein or incubated with isolated hepatocytes in vitro caused a 22-35% increase in the intramitochondrial ATP+ ADP+AMP content. There was no statistically significant change in state 3 or uncoupled respiratory rates in these mitochondria. Similar glucagon effects were noted irrespective of the medium used or time required for isolation of mitochondria. There has been recent controversy over possible artifacts in assessing the effects of glucagon on mitochondrial function. The present results suggest that the glucagon-induced increase in the matrix adenine nucleotide content is a reproducible phenomenon with probable physiological significance.

INTRODUCTION

Glucagon injected in vivo or administered to hepatocytes in vitro has been reported to have an effect on the functional capabilities of subsequently isolated rat liver mitochondria. Recently, it was shown that many of these altered functions are attributable to a stabilizing effect of glucagon on the mitochondrial membrane (1). Thus, it was proposed that mitochondria from glucagon-treated tissue may not necessarily function differently in situ, but because they are more resistant to damage during isolation, they only appear to have stimulated functions in vitro. This explanation does pertain to some, but not all the effects of glucagon, e.g., stimulated citrulline synthesis (2). A reevaluation of many previous observations seems to be in order.

It has been shown that glucagon treatment seems to result in an increase in the adenine nucleotide pool size of the mitochondria (3, 4, 5). A real increase in the adenine nucleotide content of the mitochondria could contribute to increased organelle stability during manipulations in vitro, and thus account for some of the proposed (1) secondary effects of glucagon. Furthermore, alterations in the matrix ATP+ADP+AMP pool size might actually play a direct

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role in the regulation of adenine nucleotide-dependent metabolic functions in the matrix compartment, e.g., oxidative phosphorylation, citrulline synthesis (6), and pyruvate carboxylation (7). The purpose of this study was to investigate the potential significance of an increased matrix adenine nucleotide pool size in the context of current controversy about possible artifacts (1, 2).

METHODS

Tail vein injections. Male Sprague-Dawley rats (150-250 g) fed ad libitum were anesthetized with pentobarbitol given i.p. at a dose of 60 mg/kg. After 30 min, the tail vein was injected with either vehicle (0.9% NaCl, 0.005% BSA) or 20 µg of glucagon (0.1 mg/ml of vehicle). The rats were killed by decapitation after 6 min and livers were removed to ice-cold medium for the isolation of mitochondria.

Isolation and incubation of hepatocytes. Hepatocytes were prepared as described by Seglen (8), but with an initial 10 min in situ perfusion of the liver using Ca^{2+}-free Krebs bicarbonate buffer (pH 7.6) gassed with 95% O_2, 5% CO_2 prior to use. Collagenase (Worthington Type II , dissolved in Krebs bicarbonate buffer containing 1 mM $CaCl_2$, was then perfused for 5-6 min. Yields ranged from 4-6 x 10^8 cells/liver, and at least 90% of the cells excluded 0.2% Trypan Blue. About 0.8-1.2 x 10^8 washed cells in 5 ml were preincubated in Krebs bicarbonate buffer, 1 mM $CaCl_2$, 2 mM glucose, and 4 mg/ml defatted BSA, pH 7.6, for 20 min at 37°C with shaking in gassed (95% O_2, 5% CO_2) and sealed 10 ml flasks. At the end of preincubation, glucagon was added to a final concentration of 5 x 10^{-7} M. Controls received vehicle only. The flasks were regassed, sealed, and incubated for 10 min with shaking at 37°C.

Isolation of mitochondria. Mitochondria were isolated from intact liver tissue by differential centrifugation as previously described (9), in one of two different media. In some experiments the medium used was 0.25 M sucrose, 1 mM Tris, 1 mM EDTA, pH 7.4, with EDTA omitted for the third and final wash of the 8000 g pellet and for the final suspension. In other experiments, a different isolation medium (6) consisting of 0.225 M mannitol, 0.075 M sucrose, 10 mM Tris, 0.1 mM EDTA, pH 7.4, was used throughout. The final mitochondrial pellet was suspended to approximately 25 mg protein/ml and held on ice. The isolation procedure required about 1½ hours.

Mitochondria were isolated from hepatocytes as follows. After incubation, cells were transferred to 25 ml ice-cold isolation medium (one of the two described above) and pelleted by centrifugation. The cells were resuspended in isolation medium and fractionated by the rapid method of Lodola, et al. (10), except that a single low-speed (3000 g) spin was included after the second homogenization to remove nuclei and unbroken cells. The mitochondrial pellet was washed twice and finally suspended to 14-16 mg protein/ml for use in subsequent assays. The entire procedure required only 12-15 min.

Assays. Adenine nucleotides were determined enzymatically in neutralized acid extracts. Respiration was assayed polarographically with glutamate plus malate as a substrate. Cytochrome oxidase was assayed spectrophotometrically. Protein was determined by the biuret reaction or the Lowry procedure using BSA as a standard. Details for these procedures were essentially as described previously (11).

RESULTS

Liver mitochondria isolated 6 min after glucagon injection in vivo had higher adenine nucleotide contents than did mitochondria from animals injected with vehicle only (Table 1). In each experiment, an increase in both state 3

Table 1. Adenine nucleotides (ATP+ADP+AMP), respiration, and cytochrome oxidase activity in rat liver mitochondria after glucagon injection by tail vein. Mitochrondria were isolated in the sucrose-Tris-EDTA medium in Methods. All assays were performed exactly as described under Methods. Values are means ± S.E. for n = 4 (adenine nucleotides), n = 3 (respiration), or n = 4 (cytochrome oxidase).

	Adenine nucleotides (nmol/mg protein)	Respiration (nmol $\frac{1}{2}O_2$ /min/mg protein)		Cytochrome oxidase (nmol/min/mg protein)
		State 3	Uncoupled	
Vehicle	13.90 ± 0.39	114.6 ± 12.9	149.0 ± 13.1	1818 ± 108
Glucagon	16.89 ± 0.82[a]	124.4 ± 8.3[b]	167.0 ± 19.2[b]	1799 ± 132[b]

[a] $p < .05$, or [b] n.s. for glucagon compared to vehicle using unpaired t test (two-tailed).

and uncoupled respiratory rates was noted as a result of glucagon injection, but with n = 3 these effects on respiration did not reach statistical significance (Table 1). There was no difference in the purity of the preparations, based on the specific activity of a marker enzyme, cytochrome oxidase, which was similar in mitochondria from glucagon and vehicle injected animals (Table 1).

The same relative increase in mitochondrial adenine nucleotide content was observed with either of two different isolation media (Table 2).

A similar effect of glucagon on mitochondrial adenine nucleotides was observed in isolated hepatocytes incubated with 5×10^{-7} M glucagon for 10 min (Table 3). As has been seen for *in vivo* glucagon injection (Table 1), there was a tendency toward higher respiratory rates that was not statistically significant (Table 3).

Table 2. Adenine nucleotides (ATP+ADP+AMP) in rat liver mitochondria isolated in different media after glucagon injection via tail vein. Control animals received no treatment at all. Injected animals were anesthetized and received either vehicle or glucagon by tail vein. Each lobe of the liver was bissected. One half was used for isolation of mitochondria in the sucrose-Tris-EDTA (Medium A) described under Methods. The other half was used for isolation of mitochondria in mannitol-sucrose-Tris-EDTA (Medium B). Assays of respiration and adenine nucleotide content were performed as under Methods. Values for controls are means ± S.E. for 3 or 4 separate experiments. Glucagon and vehicle injected animals were paired in two separate experiments.

		Adenine nucleotides (nmol/mg protein)		State 3 respiration (nmol $\frac{1}{2}O_2$/min/mg protein)	
		Medium A	Medium B	Medium A	Medium B
Control		14.01 ± 0.28	12.68 ± 0.89	106.2 ± 17.4	78.9 ± 15.4
Injected:	Vehicle	14.94 ± 0.12	11.56 ± 0.61	100.0 ± 18.6	74.6 ± 17.3
	Glucagon	18.43 ± 1.76	15.86 ± 0.99	106.6 ± 15.7	93.1 ± 24.1

Table 3. Adenine nucleotides (ATP+ADP+AMP), respiration, and cytochrome oxidase activity in mitochondria from rat hepatocytes after incubation with 5×10^{-7} M glucagon for 10 min. Mitochondria were isolated in the mannitol-sucrose-Tris-EDTA medium here, but similar results were obtained with the sucrose-Tris-EDTA isolation medium. Values are means ± S.E. for three experiments.

	Adenine nucleotides (nmol/mg protein)	Respiration (nmol $\frac{1}{2}O_2$/min/mg protein) State 3	Uncoupled	Cytochrome oxidase (nmol/min/mg protein)
Vehicle	7.83 ± 0.61	75.0 ± 6.8	105.0 ± 3.3	1079 ± 145
Glucagon	10.54 ± 0.21[a]	85.7 ± 5.2[b]	115.2 ± 5.2[b]	1179 ± 146[b]

[a] $p < .015$, or [b] n.s. for glucagon compared to vehicle using unpaired t test (two-tailed).

All of the mitochondria preparations, from whole tissues and from hepatocytes, showed excellent respiratory control (RCR was at least 5-10) and ADP/O ratios of at least 2.5 for glutamate plus malate. No differences in these measurements were noted as a result of glucagon treatment.

DISCUSSION

Glucagon treatment of liver cells *in vivo* or *in vitro* caused a significant increase (averaging about 22-35%) in the mitochondrial ATP+ADP+AMP content. This increase was seen to some degree in every experiment, regardless of the isolation method or medium used. Differences in respiratory rates were of marginal significance, suggesting that there was no overt evidence for stabilization of mitochondria due to glucagon in these experiments (1, 2). Nevertheless, we considered whether the higher adenine nucleotide content after glucagon might have been due to a slower rate of outward leakage during isolation. This did not seem likely because the adenine nucleotide content of isolated mitochondria is in general quite stable through numerous washes and even extensive manipulations in the simple media typically used for isolation (9). Also, we have previously shown that liver mitochondria can be induced to take up or release adenine nucleotides *in vitro* only under very specific conditions (9). In the present experiments, respiratory control was similar between the experimental groups. Thus, the permeability barrier to adenine nucleotides seemed to be intact, and there was no evidence for a rate of leakage that could account for the difference correlated with glucagon treatment.

We concluded that the increase in mitochondrial adenine nucleotide content was a real and reproducible result of glucagon treatment. That it was observed in isolated cells as well as *in vivo* suggested it was a direct effect of glucagon at the cellular level. There are other physiological situations in which a stimulus is known to cause the subcellular redistribution of adenine nucleotides. For example, in the mammalian liver there is a rapid accumulation of adenine nucleotides into the mitochondria right after birth, resulting in a 3-4 fold increase (11, 12). A transport mechanism has been described by which net changes in the ATP+ADP+AMP pool size can occur (9, 13, 14).

The observed increase in the matrix adenine nucleotide content after glucagon is important from two points of view. First, intramitochondrial adenine nucleotides are known to stabilize the membrane integrity of isolated heart mitochondria (15). A similar phenomenon might now be proposed to play a role in the increased membrane stabilization sometimes noted in liver mitochondria after glucagon treatment (1). Second, and more interesting, is the possibility that the increased ATP+ADP+AMP pool size directly contributes to the regulation of some metabolic functions known to be increased after glucagon. Pathways with an adenine nucleotide dependent step localized to the matrix compartment are possible candidates for such regulation. The rates of pyruvate carboxylation (7), citrulline synthesis (6), and adenine nucleotide translocase (11, 16) are sensitive to changes in the matrix adenine pool size *in vitro*. Methods for increasing the adenine nucleotide content of isolated mitochondria *in vitro* are now well-defined (14), and could be used to simulate this effect of glucagon. This will allow further evaluation of a specific role for adenine nucleotides in mediating the effects of glucagon on particular mitochondrial functions.

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