GUANOSINE-DIPHOSPHATE CAUSING CHANGES IN THE PHOSPHORYLATION PATTERN OF ADENINE NUCLEOTIDES IN MITOCHONDRIA FROM BROWN ADIPOSE TISSUE

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1. Introduction

Oxidative phosphorylation of mitochondria isolated from brown adipose tissue (BAT) was found to be more or less loosely coupled in the sense of a decreased or even missing ADP dependent respiratory control and reduced P/O ratios [1, 2]. The "tightness" of the coupling of mitochondrial respiration and phosphorylation appears to be correlated to the thermogenetic activity of the tissue [1, 3, 4]. Respiratory control and ATP production of BAT mitochondria can be restored *in vitro* by incubating the mitochondria in a medium containing 2% bovine serum albumin [5] or certain nucleoside di- and triphosphates, especially guanosine-di-phosphate [1, 3, 6].

Attempting to analyse this recoupling mechanism in more detail we determined the intramitochondrial adenine nucleotide levels in BAT mitochondria from newborn guinea pigs during respiratory experiments under the influence of serum albumin, guanosine-diphosphate and other nucleotides. The relative amounts of adenosine phosphates should indicate the energy state of the mitochondria; recoupling of the oxidative phosphorylation stated earlier in polarographic experiments [1, 6] should be reflected in a rise of the ATP/ADP ratio in the mitochondria.

Enzymes:

Hexokinase = ATP:D-hexose 6-phosphotransferase (EC 2.7.1.1).

Pyruvate kinase = ATP:pyruvate phosphotransferase (EC 2.7.1.40).

Myokinase = ATP:AMP phosphotransferase (EC 2.7.4.3).

2. Materials and methods

Mitochondria from BAT of newborn guinea pigs were prepared as described previously [7]. The mitochondrial protein was determined by a modified biuret method [1].

The intramitochondrial adenine nucleotides were determined by radioactivity measurements. For this purpose the mitochondrial adenine nucleotides were labelled by incubating the stock suspension (10 mg protein/ml) for 30 min at 2° with 4 μM [^{14}C]ADP (specific activity 500 μCi/μmole). After careful washing (3 times) the mitochondria (1 mg protein/ml) were aerobically incubated in the presence of 3 mM α-oxoglutarate, 2 mM EDTA, 2 mM $MgCl_2$, 5 mM potassium phosphate, 0.25 M sucrose and 10 mM tri-ethanolamine·HCl buffer, pH 7.1 at 25°. At varying times samples were taken from the reaction mixture and deproteinized in 0.6 M $HClO_4$. In the neutralized extract the adenine nucleotides were separated by small column ion exchange chromatography [8, 9], and the radioactivity was measured by liquid scintillation [9]. It was checked by enzymatic determination of ATP with hexokinase

[10] that the mitochondrial adenine nucleotides were equally labelled, and the specific activity of the internal adenine nucleotides was evaluated from these measurements.

The amount of adenine nucleotides that leaked out from the mitochondria into the medium during the experiment was controlled by separating the organelles from the medium by silicone layer filtering centrifugation [11]. The leakage under the used experimental conditions was found to be less than 8% of the total mitochondrial adenine nucleotide pool.

Enzymes and nucleotides as well as α-oxoglutarate were obtained from Boehringer Mannheim GmbH (Mannheim, Germany), bovine serum albumin from Serva Entwicklungslabor (Heidelberg, Germany); other chemicals were from Merck (Darmstadt, Germany); ^{14}C-labelled adenine nucleotides were obtained from Amersham Buchler GmbH (Braunschweig, Germany).

3. Results and discussion

BAT mitochondria from newborn guinea-pigs when isolated conventionally appear to be in a state of low energy if the intramitochondrial ATP/ADP ratio is taken as a criterion for the energy state of the mitochondria. Usually 60–80% of the mitochondrial adenine nucleotides are present as AMP, the ATP/ADP ratio being below 0.5 (see figs. 1 and 2).

Fig. 1 demonstrates typical changes of the adenosine phosphate levels in BAT mitochondria oxidizing α-oxoglutarate at 25° with no ADP present in the medium. If no serum albumin is added to the reaction mixture (experiment on the left side) the ATP/ADP ratio remains low; this result is in accord with the largely uncoupled oxidative phosphorylation of BAT mitochondria which has been confirmed in polarographic experiments under similar experimental conditions [12, 13]. The remarkable phosphorylation of AMP is very likely to be caused by GTP produced in substrate level phosphorylation [14]: as reported earlier [1] BAT mitochondria from newborn guinea pigs oxidizing α-oxoglutarate exhibit P/O ratios of about 1.0 thus indicating a fully coupled substrate level phosphorylation.

If BAT mitochondria oxidize α-oxoglutarate in

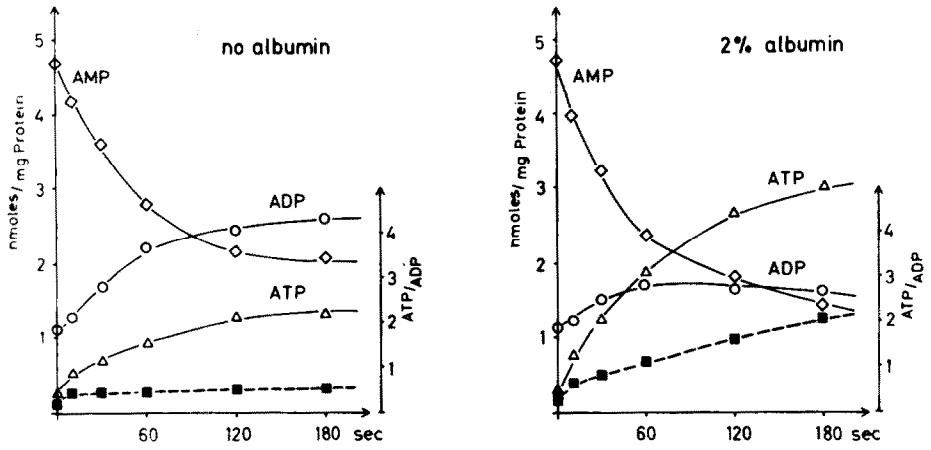

Fig. 1. Adenosine phosphates and ATP/ADP ratio (■——■——■) in isolated BAT mitochondria from newborn guinea pigs during respiration with α-oxoglutarate; no albumin and 2% albumin, resp., were present in the medium; in neither experiment was ADP added. Experimental conditions and reaction medium are described in Material and methods.

Table 1
Influence of nucleotides on the ATP/ADP ratio in BAT mitochondria from newborn guinea pigs.

Addition (mM)	–	GDP		GTP		UDP		CTP	
		0.1	2	0.1	2	0.1	2	0.1	2
ATP/ADP	0.82	2.90	3.21	0.98	3.31	0.72	2.69	0.91	2.92

ATP/ADP ratios were determined after 60 sec of respiration at 25°; substrate was α-oxoglutarate; concentrations of nucleotides present in the reaction mixture as indicated; the medium contained no albumin. Experimental conditions are described in Materials and methods.

the presence of 2% serum albumin (fig. 1, experiment on the right) the ATP level as well as the ATP/ADP ratio increases constantly. This rise of the energy state of BAT mitochondria accords with the influence of serum albumin on the mitochondrial oxygen consumption which has been observed earlier [5]: during the first minutes of incubation in the presence of albumin the extremely high oxidation of α-oxoglutarate is reduced and ADP dependent respiratory control can be demonstrated.

As demonstrated in fig. 2 addition of 100 μM GDP to the reaction mixture causes a very rapid change of the mitochondrial ATP and ADP levels; the ATP/ADP ratio rises extensively. The AMP level is relatively little influenced by GDP. The experiment shows that recoupling of BAT mitochondria by GDP is not necessarily subject to the presence of albumin in the reaction medium although albumin increases the GDP effect additionally.

This effect is quite specific for GDP as shown from the results of table 1. With other nucleoside di- and tri-phosphates, 20 times higher concentrations are needed in order to obtain a similar rise of the ATP/ADP ratio.

The rapid increase of the ATP/ADP ratio in the mitochondria caused by low concentrations of GDP corresponds to the results which have been obtained in polarographic experiments [6, 15]: 5–10 sec after the addition of 100 μM GDP to the reaction mixture the loosely coupled oxidative phosphorylation of BAT mitochondria appears to be fully restored. Other nucleoside di- and tri-phosphates must be used in 10–20 times higher concentrations (1–2 mM) to yield a similar recoupling activity.

It appears from the results that the phosphorylation pattern of the internal adenine nucleotides is a very sensitive and fast reacting indicator for the

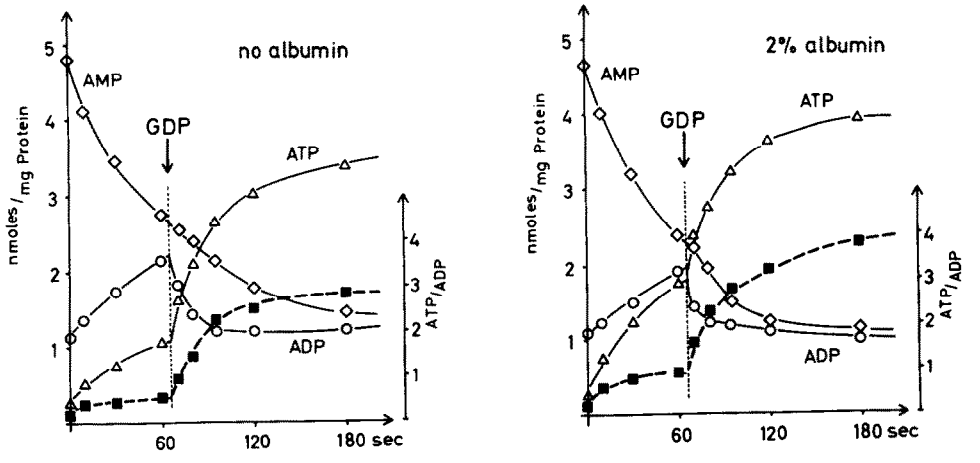

Fig. 2. Changes in the mitochondrial adenosine phosphate pool and ATP/ADP ratio (■—■—■) of isolated BAT mitochondria from newborn guinea pigs during respiration with α-oxoglutarate before and after the addition of 100 μM GDP to the reaction mixture. Experimental details were as in fig. 1.

coupling state of the respiratory chain phosphorylation in BAT mitochondria. Measurement of this phosphorylating pattern may prove as a valuable tool for elucidating the remarkable effect of GDP in recoupling the oxidative phosphorylation in brown fat mitochondria.

References

[1] J. Rafael, D. Klaas and H.-J. Hohorst, Hoppe Seyler's Z. Physiol. Chem. 349 (1968) 1711.
[2] J.I. Pedersen and M.J. Grav, European J. Biochem. 25 (1972) 75.
[3] H.J. Grav, J.I. Pedersen and E.N. Christiansen, European J. Biochem. 12 (1970) 11.
[4] M.T. Andersen, E.N. Christiansen, M.J. Grav and J.I. Pedersen, Acta Physiol. Scand. 80 (1970) 1.
[5] H.-J. Hohorst and D. Stratmann, Abstr. IV. FEBS Meeting, Oslo, 1967.
[6] H.-J. Hohorst and J. Rafael, Hoppe-Seyler's Z. Physiol. Chem. 349 (1968) 268.
[7] J. Rafael, M. Hüsch, D. Stratmann and H.-J. Hohorst, Hoppe-Seyler's Z. Physiol. Chem. 351 (1970) 1513.
[8] E. Pfaff, Doctor's thesis, Philips-Universität, Marburg 1965.
[9] H.W. Heldt, M. Klingenberg and M. Milovancev, European J. Biochem., in print.
[10] H.-J. Hohorst, F.H. Kreutz and Th. Bücher, Biochem Z. 332 (1959) 18.
[11] M. Klingenberg and E. Pfaff, Methods Enzymol. 10 (1967) 680.
[12] O. Lindberg, J. DePierre, E. Rylander and B.A. Afzelius, J. Cell Biol. 34 (1967) 293.
[13] G.A. Trusler, J.E. McBirnie, F.G. Pearson, A.G. Gornall and W.G. Bigelow, Surg. Forum 4 (1953) 72.
[14] H.W. Heldt and K. Schwalbach, European J. Biochem. 1 (1967) 199.
[15] J. Rafael, D. Klaas and H.-J. Hohorst, Abstr. V. FEBS Meeting, Prague, 1968.