CONTROL OF ATP TRANSPORT ACROSS THE MITOCHONDRIAL MEMBRANE IN ISOLATED RAT-LIVER CELLS

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1. Introduction

One of the main functions of mitochondria is to provide ATP for energy-requiring reactions in the mitochondria or in the cytosol. The adenine nucleotide translocator, which is responsible for the transport of ATP and ADP across the mitochondrial membrane, must play a key role in directing the distribution of ATP between the two cellular compartments. Its properties have been extensively studied during the last decade (for reviews, see refs [1–9]). It is specific for ATP and ADP, and is inhibited by atractyloside, carboxyatractyloside and bongkrekic acid. In energized mitochondria, the exchange of internal ATP for external ADP is much greater than in the reverse direction. The K_m for external ADP is low (1–12 μM) and external ATP inhibits ADP uptake. It is generally accepted that the transport of adenine nucleotides across the mitochondrial membrane is the rate-limiting step in oxidative phosphorylation in isolated mitochondria.

Several lines of evidence suggest that these properties are exhibited not only in isolated mitochondria, but also in the intact cell. Firstly, in the perfused liver, gluconeogenesis from alanine is inhibited by atractyloside in a concentration-dependent manner [10]. Secondly, fractionation experiments with intact livers [11] or isolated intact liver cells [12,13] have shown that the ATP/ADP ratio is higher in the cytosol than in the mitochondria, in agreement with the results obtained with isolated mitochondria [14–16]. Thirdly, the addition of atractyloside to isolated intact liver cells leads to a decrease in the cytosolic and an increase in the mitochondrial ATP/ADP ratio [12].

Thus, the adenine nucleotide translocator appears to be well-suited to act as a control point in metabolic regulation in the intact cell. Indeed, the recent demonstration that long-chain acyl-CoA esters are powerful inhibitors of the adenine nucleotide translocator in isolated mitochondria [17–24] has led to the speculation that these compounds may act as modulators of the translocator in vivo (see ref. [25] and [8,9] for reviews).

We have studied the control of ATP transport across the mitochondrial membrane during gluconeogenesis and urea synthesis in isolated intact rat-liver cells. The results reported in this paper show that the adenine nucleotide translocator is a rate-limiting step for the utilization in the cytosol of ATP generated in the mitochondria. However, although the addition of oleate leads to an increase in the level of long-chain acyl-CoA in the cells, only a slight inhibitory effect on the provision of mitochondrial ATP for ATP-utilizing reactions in the cytosol was observed.

2. Methods and materials

2.1. *Isolation of rat-liver cells*

Liver parenchymal cells were isolated from 24 h fasted male rats (Sprague-Dawley) weighing 180–220 g, according to the procedure of Berry and Friend [26], as modified by Johnson et al. [27], except that

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hyaluronidase was omitted. The perfusion technique used was that described by Williamson et al. [28].

2.2. *Incubation conditions and sampling*

The cells (usually 2.5–3.5 mg dry weight/ml) were suspended in Krebs-Ringer bicarbonate (pH 7.4) containing 2% or 4% (w/v) dialysed defatted bovine serum albumin. The incubations were carried out in 25 ml plastic Erlenmeyer flasks, which were gassed with 95% O_2–5% CO_2, stoppered, and shaken at 37°C. The incubation volume was 3 ml, or in the digitonin fractionation experiments, 5 ml. For the determination of total amounts of metabolites in the incubation mixture, samples were removed at 20 min intervals and added to $HClO_4$ (final concentration, 3.8% or 5.4%). After removing the denatured protein by centrifugation, the pH of the supernatant was adjusted to about 6.5 with 2 M KOH plus 0.3 M morpholinopropane sulphonic acid.

For the determination of metabolites in the mitochondrial and cytosolic fractions, the digitonin fractionation technique [12] was applied, with the modifications described in [29].

2.3. *Assays*

Metabolites were determined in the neutralized protein-free perchloric acid extracts using standard fluorimetric or spectrophotometric enzymic assays [30,31]. Extracellular lactate dehydrogenase was determined by measuring the activity of the enzyme [30] in the supernatant obtained after centrifuging a sample of the incubation mixture. Total lactate dehydrogenase was measured after lysing the cells with Lubrol WR (0.3 mg/mg dry weight of cells).

2.4. *Biochemicals and enzymes*

Collagenase was obtained from Worthington Biochemical Corporation, Freehold, NJ, USA, bovine serum albumin (Fraction V) from Sigma Chemical Comp., St. Louis, Mo., USA, oleate from Calbiochem, Luzern, Switzerland, and other enzymes and biochemicals from Boehringer Mannheim G.m.b.H., Mannheim, Germany.

Atractyloside was a generous gift from Professor V. Sprio, Italy. The bovine serum albumin was defatted by the method of Chen [32] and dialysed against Krebs-Ringer bicarbonate buffer (pH 7.4).

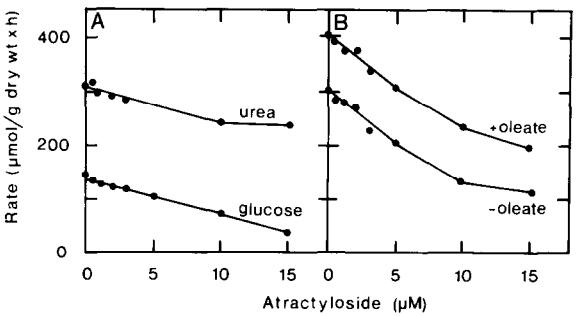

Fig.1. Effect of atractyloside on glucose and urea formation from alanine and on glucose formation from lactate + pyruvate in isolated rat-liver cells. (A) Glucose and urea formation from alanine. (B) Glucose formation from lactate + pyruvate. In A, 10 mM alanine was initially present and in B, 10 mM lactate, 2 mM pyruvate and (where indicated) 2 mM oleate. Rates were calculated from the means of the changes between the 20 and 60 min periods. The incubation mixture contained 2.5 mg dry weight of cells/ml.

3. Results

When glucose is synthesized from lactate plus pyruvate or from alanine, and when urea is synthesized from alanine, ATP is required both in the mitochondria and in the cytosol. Figure 1 shows that atractyloside inhibits both synthetic processes and that the extent of the inhibition is a linear function of the atractyloside concentration between 0 and 5–10 μM atractyloside*. With alanine as substrate, glucose synthesis was inhibited to a greater extent than urea formation (fig.1A). Similar results have been obtained in the perfused liver [10]. The relative insensitivity of urea synthesis to atractyloside may be explained by the relatively low K_m of argininosuccinate synthase for ATP (150 μM [33]).

The percentage inhibition of glucose formation from lactate plus pyruvate was greater in the absence of oleate than in its presence (fig.1B). As expected, oleate stimulated gluconeogenesis, presumably by activating pyruvate carboxylase via an increased level of acetyl-CoA (fig.1B; see [34]). It is significant that the stimulation occurred not only in the absence of atractyloside but also in its presence (fig.1B).

* It should be pointed out that atractyloside may partly be bound to albumin, so that the effective concentration in the incubation medium may be lowered

In order to circumvent the complication of pyruvate carboxylase activation, dihydroxyacetone was used as a substrate for gluconeogenesis (cf. [35]). Dihydroxyacetone can be converted either to glucose, during which ATP is utilized in the cytosol (reaction 1) or to lactate, during which ATP is synthesized in the cytosol (reaction 2).

2 Dihydroxyacetone + 2 ATP $\longrightarrow$ glucose + 2 ADP + 2 P_i (1)

2 Dihydroxyacetone + 2 ADP + 2 P_i $\longrightarrow$ 2 lactate + 2 ATP (2)

Sum: 4 Dihydroxyacetone $\longrightarrow$ glucose + 2 lactate (3)

Thus the ATP required for reaction 1 can be supplied either by reaction 2 (in which case there is a conversion of 4 molecules of dihydroxyacetone to one of glucose and two of lactate (reaction 3) or by mitochondrial oxidative phosphorylation. The ratio lactate/glucose is a measure of the relative contribution of ATP synthesized in the cytosol and in the mitochondria to glucose synthesis (cf. [35]). The effect of oleate and atractyloside on glucose formation from dihydroxyacetone is shown in fig.2A. There was some stimulation by oleate at concentrations of up to 3 mM, but at higher concentrations inhibition occurred.

Atractyloside inhibited glucose formation (fig.2A, cf. fig.1). The linear increase in the lactate/glucose ratio as a function of atractyloside concentration (fig.2B) shows that the transport of ATP is a rate-limiting step for the utilization in the cytosol of ATP generated in the mitochondria with this substrate as well.

The lactate/glucose ratio was decreased by 1–3 mM oleate both in the absence and in the presence of atractyloside (fig.2b; see also fig.3). The β-hydroxybutyrate/acetoacetate ratio, a measure of the redox state of mitochondrial NAD, was increased by oleate at concentrations of up to 3 mM (fig.3). The very low redox state observed at 4–5 mM oleate is indicative of the uncoupling effect of high concentrations of long-chain fatty acids [36–38]. Concomitantly, at these higher concentrations the lactate/glucose ratio was increased.

Although the content of long-chain acyl-CoA esters was progressively increased by the addition of

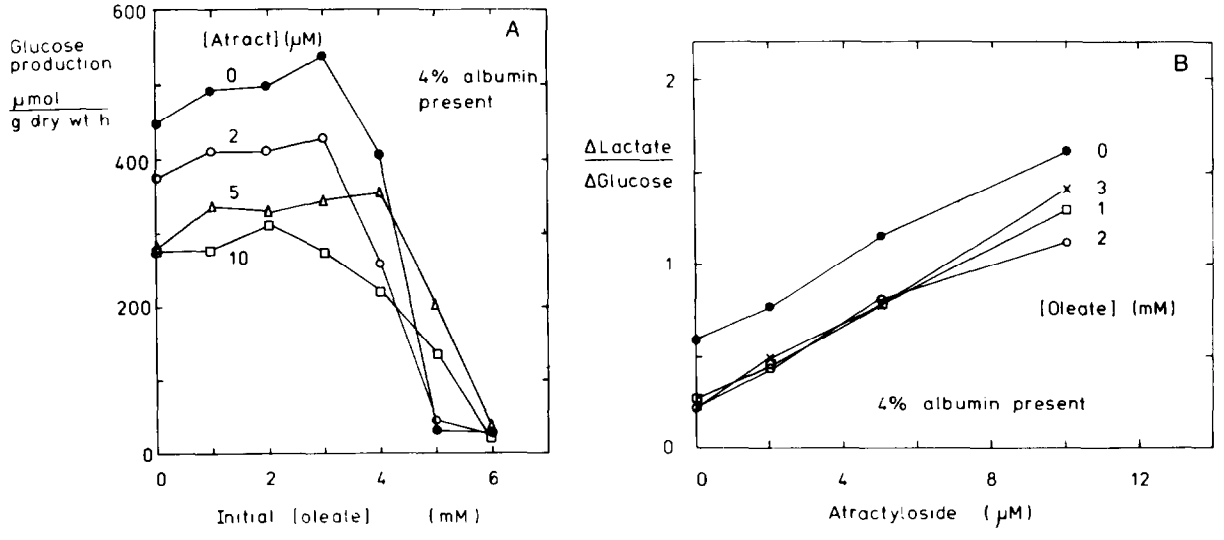

Fig.2. Effect of oleate and atractyloside on glucose formation from dihydroxyacetone (A), and on the lactate/glucose ratio (B), in isolated rat-liver cells. Initial concentration of dihydroxyacetone, 10 mM. Samples were taken at 60 min. The different symbols represent varying concentrations of atractyloside (A), or oleate (B), as indicated in the figure. The incubation mixture contained 2.9 mg dry weight of cells/ml.

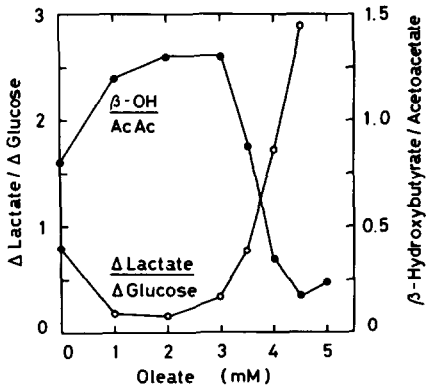

Fig.3. Effect of oleate on the Δlactate/Δglucose and β-hydroxybutyrate/acetoacetate ratios in rat-liver cells incubated with dihydroxyacetone. Initial concentration of dihydroxyacetone, 10 mM. Samples were taken at 40 min. (●——●) β-hydroxybutyrate/acetoacetate, (○——○) Δlactate/Δglucose. The incubation mixture contained 2.3 mg dry weight of cells/ml.

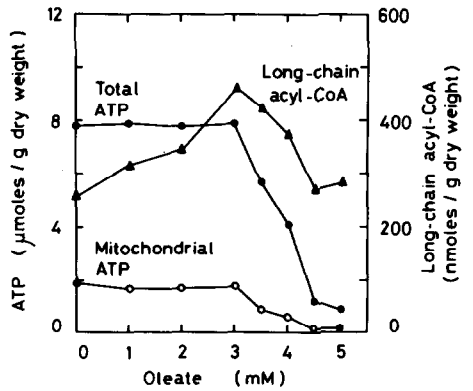

Fig.4. Effect of oleate on total ATP, mitochondrial ATP and long-chain acyl-CoA in rat-liver cells incubated with dihydroxyacetone. Experiment of fig.3. (●——●) total ATP, (○——○) mitochondrial ATP, (▲——▲) long-chain acyl-CoA.

up to 3 mM oleate, there was no effect on the total ATP or on the mitochondrial ATP content (fig.4). At higher (uncoupling) concentrations, oleate decreased both total ATP and mitochondrial ATP (cf. fig.4).

4. Discussion

The results of the experiments with atractyloside indicate that the adenine nucleotide translocator is a rate-limiting step for processes in which ATP formed in the mitochondria is utilized in the cytosol. This implies that the translocator could act as a regulatory step in cell metabolism.

Although long-chain acyl-CoA esters drastically inhibit adenine nucleotide transport in isolated mitochondria, no clear evidence could be obtained for a similar, drastic inhibition in intact liver cells. In the experiment of figs 3 and 4, the addition of 1 or 2 mM oleate to cells metabolizing dihydroxyacetone led to an increase in long-chain acyl-CoA, without having any effect on mitochondrial ATP or total ATP. At 4 or 5 mM oleate, uncoupling occurred. At the intermediate concentration of oleate (3 mM), the levels of mitochondrial and total ATP and the β-hydroxybutyrate/acetoacetate ratio were the same as at 2 mM oleate, but there was a slight increase in the lactate/glucose ratio.

From equations 1–3, it can be seen that the minimum flux of ATP from mitochondria to cytosol during glucose synthesis from dihydroxyacetone is given by the relation:

$$\text{Minimum flux} = 2\,(\Delta\text{glucose}) - \Delta\text{lactate}$$

In the presence of 0, 1, 2 and 3 mM oleate, the values for the minimum flux in the experiment of figs 3 and 4 were 470, 910, 920 and 715 μmol/g dry weight/h, respectively. Thus, in the presence of 3 mM oleate, the flux of ATP was inhibited by about 20% as compared to the flux at lower oleate concentrations. A similar small inhibition has been observed by Geelen et al. [25].

The absence of a marked inhibitory effect of oleate on ATP-requiring processes in the cytosol under conditions where atractyloside does inhibit, together with the observation that oleate does not enhance the inhibitory effect of atractyloside, lead us to conclude that inhibition by long-chain acyl-CoA esters of adenine nucleotide transport is of no physiological significance in the intact liver parenchymal cell. Although a slight inhibition of glucose synthesis can be observed at a high concentration of oleate, the possibility can not be excluded that the inhibition is due, not to inhibition of adenine nucleotide transport, but to the onset of uncoupling. Inhibition of adenine nucleotide transport should have been

accompanied by an increase in the β-hydroxybutyrate/acetoacetate ratio; this was not observed (fig.3).

Finally, the question arises of why the dramatic inhibitory effects of long-chain acyl-CoA esters on adenine nucleotide transport observed in isolated mitochondria are not seen in the intact cell. A possible explanation is that binding of long-chain acyl-CoA esters to intracellular proteins occurs in the intact cell, thus preventing inhibition.

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